Biodiversity of Entozoic Fauna of *Odontotermes obesus* and *Heterotermes indicola* with the Screening of Phytochemicals as a Potential Source of Termite Control

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بسم الله الرحمن الرحيم

DECLARATION

I hereby declare that the work presented in the following thesis "Biodiversity of Entozoic Fauna of *Odontotermes obesus* and *Heterotermes indicola* with the Screening of Phytochemicals as a Potential Source of Termite Control" is my own effort, except where otherwise acknowledged, and that the thesis is my own composition and no part has been copied from any other source. Also, it is declared that the present work has not been submitted to any other university.

Asma Ashraf

Dedicated

to

My Parents

Acknowledgment

*I am highly grateful to **Allah Almighty** for His countless blessings throughout my life and helping me to achieve my goals and giving me the strength in the completion of this project. May Allah guide me on the right path (Ameen). Peace and blessings are upon the **Holy Prophet Hazrat Muhammad (PBUH)**, the most perfect among all human beings ever born and Who guided his followers to seek knowledge from cradle to grave.*

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Asma Ashraf

Contents

List of Abbreviations

AX	Axostyle
BAC	Bacteroidales
BL	Bell
C.I	Confidence interval
C.V	Coefficient of variability
CHR	Chromatin
CNTRBLP	Centroblepharoplast
CP	Cap
FLG	Flagella
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography mass spectroscopy
GRV	Groove
LC	Lethal concentration
Min.	Minutes
Ml	Milli litter
N	Number of samples
Nm	Nano meter
NU	Nucleus
O.R	Observed range
ODP	Observed dead population
P.NU.ZN	Pre-nuclear zone
PCR	Polymerase chain reaction
PLSM ECT	Ectoplasm
PLSM END	Endoplasm
Ppm	Parts per million
PRPL	Protoplasm
PRPL GRV	Protoplasmic groove
PRPLSM BG	Protoplasmic bulge

S.D	Standard deviation
S.E	Standard error
SPR BND	Spiral bands
STR CL	Stereocilia
TP	Total population
UV-Vis	Ultraviolet-visible spectroscopy
W PART	Wood particles
$\overline{X}$	Mean
µm	Microns

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GENERAL ABSTRACT

The subterranean termites, *Odontotermes obesus* and *Heterotermes indicola* are highly infesting wood pests causing an economic loss of billions of dollars throughout the world, especially in the tropical and subtropical regions. The *O. obesus* is a higher termite and harbors bacteria as symbionts in the hindgut which aids in wood digestion along with its cellulases secreted by gut wall and provides a mechanism for recycling of nitrates, acetates, etc. whereas *H. indicola* is a lower termite which harbors entozoic flagellates along with bacteria. Both of these termite genera are commonly found throughout Pakistan especially in the temperature moderate areas and cause serious damage to woods, wooden materials, and forest. There are certain woods and trees which are not attacked by termites as they have certain chemicals which are either termite repellent or toxic. The present study is focused to isolate and identify such chemical/s which could be a potential source for preparing termiticides either targeting directly to insect host or via its gut symbionts. For this purpose two termite species '*O. obesus*' and '*H. indicola*' were collected from the vicinity of Quaid-i-Azam University Islamabad, Pakistan and identified based on 36 morphological characters *i.e.* length and width of the body, thorax, abdomen, head, mandibles, pronotum, postmentum, antenna (scape, pedicle, flagellum) and legs. Protozoan fauna residing in the hindgut of "*H. indicola*" was also identified morphologically based on length and width of the body, the diameter of the nucleus, length of flagella, length and width of the bulge, length of axostyle and centroblepharoplast. Resultant values of both (termites and protozoa) were analyzed using "*Student t-test*" to investigate significant differences among various parameters using Minitab version 18. The largest and most abundant protozoan species, *Pseudotrichonympha grassii* was further confirmed by molecular analysis.

Bacterial fauna isolated from the hindgut of *O. obesus* and *H. indicola* was characterized based on morphology, Gram staining, biochemical identification, amplification and sequencing of 16S rRNA gene. For the evaluation of plants having significant mortality effects on termites and their gut symbionts, eight plants were selected including *Carica papaya, Eucalyptus camaldulensis, Osmium basilicum, Grevillea robusta, Eucalyptus globulus, Pongamia pinnata, Mentha longifolia,* and *Melia azedarach* for screening as anti-termite chemicals. Crude leaves extracts of plants were prepared in ethanol by

Soxhlet extraction apparatus and evaluated for their toxicity against termites and their gut endosymbionts (protozoa and bacteria) at subsequent concentrations *i.e.* 100, 500, 1000, 1500 and 2000ppm to find LC50 in no-choice bioassay for termiticidal and protozocidal activity and agar well diffusion method for antibacterial activity. Leaves extract of the most effective plant was fractionated through column and thin layer chromatography in different solvents (n-hexane, ethyl acetate, and methanol). Twenty-one fractions were collected and applied for termiticidal, protozocidal and antibacterial activities at the concentration range of 100-2000ppm. The effective plant and their active fractions were further characterized by preliminary phytochemical tests, Ultraviolet-visible spectroscopy (UV-Vis), Fourier transforms infrared spectroscopy (FTIR) and Gas chromatography coupled mass spectroscopy (GC-MS).

Morphometric measurements of termites showed that majority of external characters of soldier cast were not significantly different from each other. However, slight differences were observed in the measurements of different parts of flagellum (pedicle, scape) and legs (trochanter, tarsus, tibia, and claw) which represent the adaptive traits that help an insect to adapt themselves to the changing environmental conditions.

Nine species of flagellates belonging to three genera *i.e. Holomastigotes, Holomastigotoides,* and *Pseudotrichonympha* were identified. These include *Holomastigotes metchnikowi, Holomastigotes campanula* and *Holomastigotes annandalei, Holomastigotoides kempi, Holomastigotoides hemigynum, Holomastigotoides Koidzumi, Holomastigotoides hartmanni, Holomastigotoides metchnikowi,* and *Pseudotrichonympha grassii*. The largest and the most abundant species *P. grassii* was further identified by amplifying SSU rRNA which indicated that Pakistani isolate was phylogenetically most closely related to the Japanese isolate.

Bacterial fauna isolated from termite hindgut was also characterized by morphological, biochemical and molecular analysis that confirmed the presence of five bacterial genera from each termite species. The species *Bacillus cereus, Escherichia coli, Lysinibacillus fusiformis* were common to both termites where as *Lysinibacillus xylanilyticus* and *Lysinibacillus macrolides* were present only in *O. obesus* while *Bacillus subtilis* and *Shigella sonnei* in *H. indicola* only.

Ethanolic leaves extract of all plant species exhibited termiticidal activities but *G. robusta* showed maximum mortality of 94% (LC50=1391.01ppm) and 90% (LC50=1607.95ppm) against *O. obesus* and *H. indicola* respectively. This extract also caused 95% mortality (LC50=502.64ppm) of hindgut flagellates of *H. indicola*. Similarly, *G. robusta* extracts also showed antibacterial activity against all the isolated bacteria from both termite species however maximum zone of inhibition appears against *L. fusiformis* was shown isolate *O. obesus* (27.50±1.02mm) and *H. indicola* (24.54±1.32mm).

Column and thin layer chromatography were used for sepration of *G. robusta* leaves extract and separated 21 fractions (named as 2-22). Among all the 21 isolated fractions; 4,5,7,10 and 14 were found to be lethal for termites either directly or via symbionts (protozoa and bacteria) however fraction '5' was common having termiticidal, protozocidal and antibacterial potentials. LC50 of fraction 5 against *O. obesus* was 1342.81ppm, for *H. indicola* 1420.05ppm and for protozoa was 578.34ppm. Similarly fractions 5 was most active against bacteria of both termite species. The minimum inhibitory concentration of fraction 5 against the bacterial isolate of *O. obesus* was 100, 500 and 100ppm against isolates 1, 3 and 5 while 100ppm against the first 4 isolates and 500ppm against isolate 5 of *H. indicola*.

Being the most effective, ethanolic extract of *G. robusta* was characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis. The UV-Vis spectroscopic analysis was performed at a wavelength of 200 to 800nm and two absorption peaks were recorded at 208.94 and 282.97nm having absorption of 2.33 and 0.811 respectively. The FTIR analysis was performed for the identification of different functional groups based on characteristic peak values and the spectrum was recorded from 400 to 4000cm-1. Results confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether and halo compounds. The GC-MS analysis revealed the presence of 15 phytochemicals with a high percentage of coumarin (30.64%), 7-hydroxy, methyl hexofuranoside (27.63%) and 9,12,15-octadecatrienoic acid (Z, Z, Z) (8.97%).

Phytochemical screening of lethal fractions also showed presence of flavonoids, tannins, sterols, quinones, alkaloids, terpenoids and carbohydrates. The UV-Vis spectroscopic analysis revealed different absorption peaks with a common peak of all fractions at

665.50nm having different absorbance. The FTIR analysis of bioactive fractions (4,5,7,10 and 14) revealed the presence of alkanes, alkynes, aldehydes, amines, ether, amino acids, thiols, cyanides, nitriles, alcohols, carboxylic acids, thiocyanates, thiols, and azides. The GC-MS analysis confirmed the presence of some common chemical compounds including 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester, beta-sitosterol, 1,2-benzenedicarboxylic acid, diisodectyl ester, phytol, 1,2-benzenedicarboxylic acid, diisooctyl, hexadecanoic acid methyl ester, n-hexadecanoic acid, octadecanoic acid, and p-arbutin. However, the compound 3,7,11,15-tetramethyl-2-hexadecane-1-ol were commonly found in all the active fractions along with other identified compounds. These natural phytochemicals having termiticidal, protozocidal and antibacterial potential being eco-friendly, can be used in termiticides preparation to control termite infestation and save a huge economic loss.

GENERAL INTRODUCTION

Termites are small eusocial insects consisting of approximately 3000 described species (Krishna *et al.,* 2103) exhibiting a pronounced colonial system having soldiers, workers and reproductive cast (king and queen) present in the natural environment (Klass *et al.,* 2008). Reproductives are produced at a specific period in the year and can be winged or wingless. Nonproductive members *i.e.* workers have the foraging activity and eggs hatching while soldiers have to defend the colony. A termite colony members share shelter water and food among each other and live in cooperation. They may live in the colony nest that is subterranean or above the ground level and feed on majority of food sources including not only soil rich with humus, timber, rotten and decaying woods (Bignell and Eggleton, 2000) but also feeds on algae, fungi, lichens and members of their own colony sporadically or particularly (Hinze *et al.,* 2002). Many social insects like aphids, ants, and termites produce winged individuals that mainly function in migration and propagation of the species. Many studies have been conducted on swarming activities of termites and reported that swarming is highly correlated with temperature and rainfall (Akhtar and Shahid, 1990).

Termites can digest the most abundant biomolecules on the earth "lignocellulose" as a food source. They target efficiently on various man-made products, wooden materials, timber, crops and use all food resources that have cellulose (Akutse and Owusu, 2012). They play an important role in nutrients decomposition and recycling (Bignell, 2006; Evans *et al.,* 2011) leading to efficient breakdown of cellulose and hemicellulose; thus are highly important ecologically and referred as ecosystem engineers (Wood, 1986; Sugimoto *et al.,* 2000; Ohkuma, 2008; Eggleton, 2010; Husseneder, 2010; Watanabe and Tokuda, 2010; Palin *et al.,* 2011). Tunneling activities of termites help in soil aeration, changing the soil structure fertility, richness and recycling of atmospheric gases and water thus significantly contributing in maintaining global ecosystem (Eggleton *et al.,* 1999). Members of the family Termitidae develop fungi in nest combs that grow into mushrooms which are used as food in Africa. They are also a protein source for lizards, anteaters, birds, humans, and frogs. The soil of termites mound is

extensively used by women and kids for dietary and many other purposes (Sileshi *et al.*, 2009).

Despite the economic importance, termites are serious pests of trees in agriculture, timber in forestry and wooden household materials (Hickin, 1971). In buildings, termites attack wooden structures, papers, furnishings, and garments in sub-humid areas. Consumption of this wood cellulose results in massive damage (Ibrahim and Adebote, 2012) and the extent of loss covers both storages of commercial wooden resources as well as internal household materials (Abe *et al*., 2000). The lignocellulose digestion depends not only on the composition of the hindgut as well as the symbiotic relationship between hindgut microbial fauna with their host (Hassan *et al.*, 2017). Termites mostly rely on nitrogen-deficient food resources, so this hindgut microbial community also helps them to withstand such harsh conditions (Pranesh and Harini, 2015).

1.1: Geography and biodiversity of termites

Termites are cosmopolitan having the ability to adapt to any habitat. They are present on all the continents except Antarctica having the richest diversity in tropical and subtropical areas (Brune, 2014). They show a variety of behaviors from nest construction (Noirot, 1992) to food source and diverse symbiotic association from intestinal protists (in lower termites) to fungus cultivation (in higher termites) (Bignell and Eggleton, 2000). In subtropical and tropical areas of the world, termites constitute 10% animal biomass which may increase up to 95% when only soil insects are concerned (Donovan *et al.*, 2007; Bourguignon *et al.*, 2016). They are present 45^{O} North and South latitude, also occurring in the temperate zone of the world (Marini and Mantovani, 2002) and major decomposers in lowland tropical ecosystem. The most important ecosystems are closed-canopy rainforest having greater termites diversity with 90% members belonging to family Termitidae (Eggleton *et al.*, 1999). The numbers of recorded termite species in the Oriental region are 1059, revealing a rich fauna of termites in this region (Ahmad and Akhtar, 2002). Certain environmental factors like temperature, moisture content, energy availability, soil type, nitrogen balance, methane, and carbon dioxide are reported to

influence termites distribution pattern in tropical forests and Savannas globally (Eggleton and Tayasu, 2001; Davis *et al.,* 2016). Termites diversity is low in temperate and subtropical part of the world; but still performs a major role in forest ecosystem; however, they are more concentrated in the tropical zone of the world (Eggleton, 2000).

Termites diversity and distribution pattern in a specific area is related to the effective control measures and termiticides being used for the management of infestation caused by them (Acda, 2013). The ecological impact of termites can be determined when their biomass exceeds from mammalian herbivores and number often exceed from 6,000 members per square meter (Lee and Wood, 1971). Many termite species have been reported from different countries as a serious threat to buildings and accommodations. According to the United Nations Environment Program (UNEP), among 3,000 described species, the highest termites diversity was reported from Africa having 1,000 species. The second country rich with termites fauna is China with 440 species (Mahapatro and Chatterjee, 2018). In Asia, 435 species are reported from tropical and subtropical regions (Mahapatro and Chatterjee, 2017). Reported termite species from South America are 400 which are relatively high in number from North America and Europe inhabiting 50 and 10 termite species respectively. In Australia, 360 endemic termite species are documented so far. Reported species from Iran are 16 (Ravan, 2010), from Thailand are 13 (Rouland-Lefevre, 2010) and several subterranean termite species from Taiwan, Singapore, and Malaysia (Lee, 2002; Lee *et al.,* 2007; Yeoh and Lee, 2007).

Of 3,000 described termite species, 53 has been reported from Pakistan so far and only 13 species have been identified as a serious economic pest in both rural and urban settings (Naeem and Shafaqat, 2013). These are abundantly present throughout the country, predominantly in Khyber Pakhtunkhwa (Saljoqi *et al.,* 2012), including both wood dwellers as well as soil inhabitants (Badshah *et al.,* 2005). Important species of Pakistan includes *Coptotermes heimi, Heterotermes indicola, Microtermes unicolor* and *Microtermes obesi* in NWFP and Punjab and *Anacanthotermes vegans* in Balochistan as a serious pest (Afzal *et al.,* 2017). Three genera including *Coptotermes, Heterotermes* and *Odontotermes* are known as serious commercial pests targeting crops, trees and wooden structures (Salihah *et al.,* 1994) while *Microtermes obesi, Microtermes unicolor,*

Microtermes mycophagus, Eremotermes paradoxalis and *Odontotermes obesus* are significant pest of agroecosystems of Pakistan (Ahmed *et al.,* 2004). The distribution pattern of termites mostly varies with the type of vegetation of the area (Pearce, 1997). They are present in the soil but their abundance increase with the increase in the dry period up to the optimum temperature (Aslam *et al.,* 1994). The abrupt alteration in precipitation pattern and temperature fluctuations affect termites habitat and distribution positively (Su and Scheffrahn, 2000; Evans *et al.,* 2013) helping them to disperse and flourish in the areas that were not inhabited by termites previously (Lal, 2004; Peterson, 2010; Lee and Chon, 2011; Guerreiro *et al.,* 2014).

One of the survey conducted by Manzoor *et al.* (2010) from Punjab, Pakistan reported 11 termite species including *Heterotermes indicola, Coptotermes heimi, Odontotermes gurdaspurensis, Odontotermes horai, Odontotermes assmuthi, Odontotermes obesus, Odontotermes obesus, Bifiditermes beesoni, Microtermes pakistanicus, Microtermes unicolor, Microtermes obesi* and *Microtermes mycophagus* are more prevalent in all the forests. Among collected samples *M. obesi* was the most abundant (19%) while *B. beesoni* was the least (1.5%). The *M. obesi* was more prevalent in the forest of Bhagat, Chichawatni, Kamalia, Jallo forst and Changa Manga while *O. guptai* and *M. mycophagus* were dominant species of Attock and Daphar (Gujrat) forests. Manzoor and Mir (2010) reported that the genus *Microtermes* and *H. indicola* were available throughout the year while the genus *Odontotermes* was more prevalent from May-July.

1.2: Classification of termites

Seven families of termites have been described, viz; Hodotermitidae, Rhinotermitidae, Termopsidae, Kalotermitidae, Semitermitidae, Mastotermitidae and Termitidae (Noirot, 1992; Abe *et al.,* 2000). Members of Termitidae family are known as higher termites, because their symbiotic flagellates fauna was eliminated from hindgut during evolution and depend upon microbial biota for complete degradation of plant biomass (Noirat, 1992). It is the largest family of termites having 8 subfamilies, 250 genera, and more than 2000 species. The remaining six families are lower termites

because they are unable to digest cellulose and hemicellulose without the help of symbionts (protozoa and bacteria) along with the archaea and fungi present in their hindgut (Cleveland, 1925). Higher termites feed on a variety of food sources like grasses, leaves, roots, manure, and humus (partially digested material) which are completely digested by archaea, bacteria, and fungi residing in their hindgut (Wood, 1986).

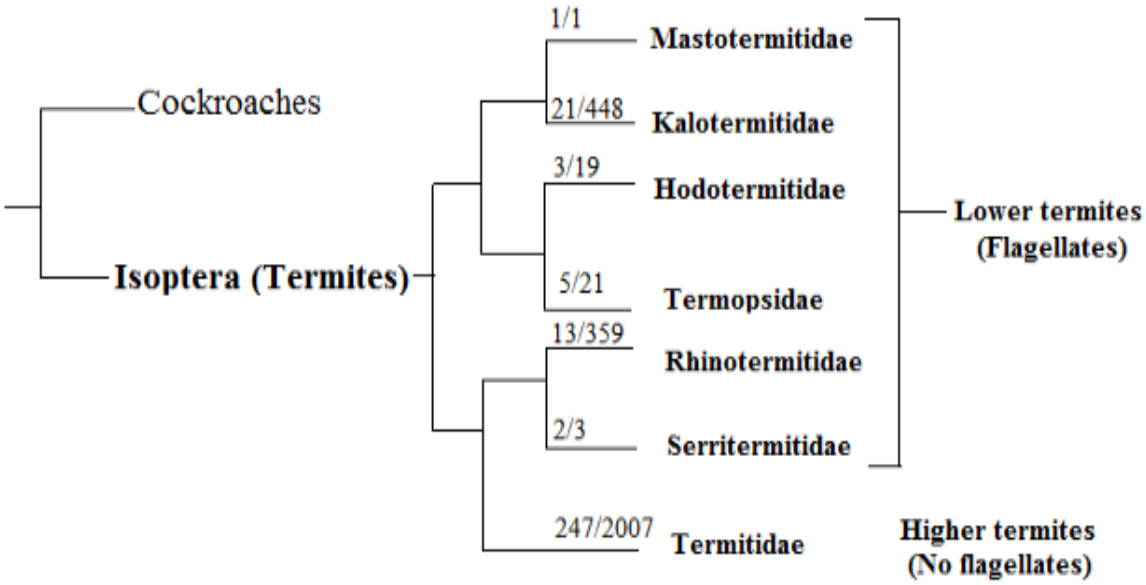


Figure 1.1: Schematic representation of termites phylogeny showing a close association with cockroaches. Numbers represents the genera and species of the families.

1.3: Endosymbionts of the termites

Termites harbor a wide variety of endosymbionts including bacteria, protists, and archaea in a distant portion of their hindgut termed as paunch (Duarte *et al.,* 2017). In this symbiotic association, termites provide mechanical maceration of wood fiber and endogenous cellulases while endosymbiotic fauna (protozoa and bacteria) assist in phagocytosis and digestion of wood particles (Brune, 2014). Lingo-cellulolytic digestion process accomplished by the combined activity of termites and their hindgut endosymbionts has led to numerous metatranscriptomic and metagenomic studies investigating the effects of diverse food types on these holobionts (Peterson *et al.,* 2015; Duarte *et al.,* 2017). Community structure and species composition of termites hindgut

protozoa are influenced by the level of carbon sourced food and extractive from plants (Mannesmann, 1972; Tanaka *et al.,* 2006).

Based on the morphology, more than 430 species of flagellates have been described from approximately 200 lower termite species and wood-feeding cockroaches *Cryptocerus* (Yamin, 1979b). More than that has been reported through molecular analysis using a variety of genetic markers (Keeling *et al.,* 1998; Ohtoko *et al.,* 2000; Gerbod *et al.,* 2004; Graber and Breznak, 2004; Hampl *et al.,* 2007). These protozoan flagellates have a strong symbiotic association with their host which emphasize on their synergistic effect of cellulase in termites metabolic pathways (Tokuda and Watanabe, 2007). One of the significant functions of protozoa is the synthesis of molecular hydrogen; a vital intermediate metabolite of termites hindgut. Parabasalid flagellates have hydrogenosomes (anaerobic energy and hydrogen-producing organelle), which produce a small amount of acetate, hydrogen and carbon dioxide (Yamin 1981; Odelson and Brenzak, 1985). Some protozoan species are highly crucial for the survival of termites (Hu *et al.*, 2011). It has also been reported previously that *Reticulitermes falvipes* died soon after the elimination of protozoa from its hindgut which are important in cellulose digestion and termites physiology (Hu *et al.,* 2011).

Protozoan symbionts of lower termites have been classified into two taxa; Parabasalian and Oxmonadean. Both taxa are considered as a eukaryotic supergroup (Excavata) having a large variety of flagellates (Adl *et al.,* 2005). In spite of that, their phylogenetic association is still controversial due to the lack of sufficient phylogenetic samples (Simpson *et al.,* 2006). Parabasalian flagellates lack mitochondria and have parabasal body and hydrogenosomes. Parabasalia are further divided into two classes based on parabasal bodies, flagella and other cytoskeleton structures (i) Trichomonadea having one group of 4-6 flagella near the interior end, and (ii) Hypermastigea having various clusters of flagella on the parabasal body (Brugerolle and Radek, 2006). Hypermastigea is more diverse and polyphyletic than Parabasalia (Ohkuma *et al.,* 2005). Oxymonades also known as preaxostyla phylum are mitochondriate flagellates, few and often differentiated based on having large axostyle (Table 1.1).

Table 1.1: Classification of symbiotic protozoa in lower termites (Adl *et al.*, 2005).

Taxa	**Sub Class**	**Genus**
Parabasalids	Trichomonadida	*Monocercomonas, Trichomitopsis, Trichomonas*
	Cristamonadida	*Devescocina, Joenia, Foaina, Metacoronympha, Calonympha,*
	Spirotrichonymphida	*Spirotrichonympha, Holomastigotes, Holomastigotides, Microjoenia,*
	Trichonymphida	*Staurojoenina, Eucomonympha, Trichonympha, Hoplonympha*
Oxmonadean	Oxymonadida	*Oxymonas, Dinenympha, Streblomstix, Pyrsonympha*

Many aerobic, anaerobic and facultative bacteria have been reported around the world and proved that these isolated bacterial species significantly aids in the digestion of hemicellulosic and cellulosic material along with the association of protozoa and termites. Commonly facultative anaerobic bacteria species have been characterized based on molecular analysis from the punch of different termites (Adams and Boopathy, 2005). Bacteria in hindgut are not only the symbionts of termites, but they are also ecto and endosymbionts of flagellates associated with the lineages of *Bacteroidales* and *Spirochaetes* (Noda *et al.,* 2006). According to an estimate, about 90% of bacterial species are symbionts of flagellates in *Mastotermes darwiniensis* (Ohkuma and Brune, 2010). The bacterial symbionts are protists host specific and this host specificity leads to the cospeciation of *Bacteroidales* with *Pseudotrichonympha* and *Endomicrobia* with *Trichonympha* (Noda *et al.,* 2007).

Prokaryotes residing in termites hindgut exhibit a promising activity by maintaining the physiochemical equilibrium within the gut (Ohkuma and Brune, 2010; Xie *et al*., 2012). These bacterial endosymbionts process the byproduct of protists by methanogenesis, acetogenesis and nitrogen-fixing process. Methanogenic bacteria (abundantly present in termites hindgut) utilize hydrogen and carbon dioxide to form methane and reduce hydrogen concentration. However, in lower termites acetogenesis dominate the methanogenesis due to the production of acetate directly from flagellates. *Spirochetes* sp. shows reductive acetogenesis due to the expression of the gene encoding

a key enzyme for reductive acetogenesis *i.e.* formayltetrahydrofolate synthase (Pester and Brune, 2006). *Bacteroidales* sp. are actively involved in polysaccharides degradation and consumption of hydrogen produced by protists thus enhancing their capacity for fermentation (Inoue *et al.,* 2008). *Endomicrobia* provides nitrogenous compounds to the protists host by playing an important role in nitrogen fixation (Hongoh *et al.,* 2008). Amount of nitrogen significantly affects cellulose breakdown in termites hindgut, so these endosymbionts of flagellate protists excrete nitrogenous waste as uric acid during metabolism (Upadhyaya *et al.,* 2012).

1.4: Economic impact

Termites have a major role in maintaining the diverse ecological system in the world by reutilizing wood and plant material (33% in tropics), modifying soil structure, altering soil conditions and productivity by increasing organic matter in the soil (Sugimoto *et al.,* 2000). They also provide food for other animals (Lee and Wood, 1971). Among the 3000 termites pest species, only 97 (3.2%) are considered serious pests (Austin *et al.,* 2002). They mostly target crops, cultivated fruits, ornamental plants, forest nurseries and man-made artificial wooden structures like building furnishers, bridges, good stores, railway sleepers and poles (Donovan *et al.,* 2007; Rouland-Lefevre, 2010). Huge economic loss in Pakistan is caused by fungus-growing termites as they mostly attack agriculture (Ahmed *et al.,* 2011). Major factors that are involved in termites infestation and heavy loss are feeding preferences of termites, their colony size and nesting behavior (Rouland-Lefevre, 2010).

Approximately US $22-40 billion are spent on the damage caused by termites worldwide annually (Buczkowski and Bertelsmeier, 2017; Mahapatro and Chatterjee, 2017; Rust and Su, 2012). In Southeast Asia, the cost of termites damage is approximately 400 million US dollars per year (Lee *et al.,* 2007). Globally subterranean termites cause 70% construction damage and 90% of economic loss (Kuswanto *et al.,* 2015). Approximately US $1000 millions are spent annually on the loss caused by the termites, its maintenance and renovation process in the United States (Pimentel, 2005).

Subterranean termites are considered as most invasive termites causing 80% economic loss (Nobre and Nunes, 2007; Rust and Su, 2012) while only 20% damage is

contributed by dry wood termites (Su and Scheffrahn, 2000). More than $1000 million has been spent by the pest industry in the United States (Pimentel *et al.,* 2005; Bourguignon *et al.,* 2016), $313 million in Europe and more than $95 million in Australia (www.chem.unep.ch/pops/termites). In China, the economic loss is about 290 million US dollars per year with 90% of buildings infestation (Zhong and Liug, 2002). In Japan, this economic loss is about 800 million US dollars (www.chem.unep.ch/pops/termites). Similarly $10 billion in Malaysia with 10% commercial buildings, 20% industrial and 70% residential damage (Lee, 2002), 42% building damage in Brazil (Gallardo*et al.,* 2002) and 53% in Spain (Gaju *et al,* 2002). As termites are considered as a devastating pest of crops, they are described to have more than 25% loss in kernels from African countries (Umeh and Ivbijaro, 1999) and 20-25% loss in maize crops from India (Joshi, 2005). However, in developing countries like Pakistan, the cost of control measures for termites' infestation and the damage is compensated because it is greater than the loss caused by them. A detailed overview of total economic loss caused by termites worldwide has been summarized in table 2.

Table 1.2: Economic loss in terms of structural damage caused by termites worldwide.

Country	Economic loss of termite damage /year (US $)	Reference
Worldwide	40 billion	Buczkowski and Bertelsmeier, 2017; Mahapatro and Chatterjee, 2017
Australia	Nearly 1billion	Scholz *et al,* 2010. www.chem.unep.ch/pops/termites
China	290 million	Zhong and Liu, 2002
Europe	313 million	www.chem.unep.ch/pops/termites
India	44 million	Rajagopal, 2002; Joshi, 2005
Japan	800 million-1 billion	Takahashi and Yoshimura, 2002 www.chem.unep.ch/pops/termites
Malaysia	10 million	Lee, 2002; Yeoh and Lee, 2007
New Orleans (France)	300 million	Suszkiw, 1998
Philippines	Millions of American dollars	Yudin, 2002; Acda, 2013
Taiwan	3-4 million	Li *et al.*, 2011
United state of America	1000-1500 million	Ibrahim and Adebote, 2012 www.chem.unep.ch/pops/termites

1.5: Termite control

To control termites infestation and damage, various synthetic chemical preservatives are applied on non-durable woods, which are cause ecological toxicity to both humans and animals; hence limiting their indoor applications (Ward *et al.,* 2009; Tascioglu *et al.,* 2013). Various synthetic chemicals like persistent organic pollutants (POPs) are commonly used for their control. These insecticides also have certain other shortcomings like they have a pungent smell (Jamil *et al.,* 2005). Another synthetic chemical cyclodiene is used for termites control in fields but it causes environmental toxicity, destroy non-target species and are costly (Sim *et al.,* 1998). Similarly, monochrotophos and dimethoate cause genotoxicity while chlordane is a carcinogenic agent (Jamil *et al.,* 2005). Polycholinated biphenyls and organochlorine persists in carpets during fumigation in homes and acts as a causative agent of leukemia in kids, toxic for the reproductive and immune system. The excessive use of these chemicals is developing resistance in insects (Kamble *et al.,* 1992; Mulrooney *et al.,* 2006). Due to their long term persistence and toxicity, their use is strictly prohibited by the government.

Biological control involves the use of biologically active products from natural resources for termites control. These bioactive compounds are derived from medicinal plants. According to World Health Organization (WHO) a medicinal plant can has pharmaceutical and therapeutic potentials and its derived organic compounds can be a precursor of novel drugs (Sofwara, 1982). Being ecofriendly, biological control method is getting more attention for the development of bioactive agents that replace synthetic compounds for the control of insect pest species as these natural products are easily degradable and not toxic for non targeted organisms (Sosan *et al.,* 2001; Moretti *et al.,* 2002; Cetin and Yanikoglu, 2006).

Almost 250,000 species of plants are reported worldwide so far; out of these 80,000 have medicinal values while 5,000 have strong therapeutic potential (Thomas *et al.,* 1998). In Pakistan 52 plants species belonging to 25 angiosperms families are considered to have medicinal values (Varaldo, 2002). In Southern Pakistan 27 plants species are reported with phytomedicinal properties (Qureshi and Khan, 2001), 41 plants

species belonging to 29 families from Chapursan valley Gilgit (Wazir *et al.*, 2004), 135 genera from 66 families are reported from Karakorum region (Afzal *et al.*, 2009). In Azad Jammu and Kashmir (Pounch valley) 68 plants species from 44 families have been reported (Khan *et al.*, 2012). Various studies were conducted on medicinal plants for the discovery of new phytochemical compounds (Schmidt, 2004; Gosbell, 2003).

Naturally occurring plants extracts, wood/plant oils and tannins have been used as an alternative strategy for termites control due to their less toxic nature (Fatima and Morrell, 2015; Gonzalez-Laredo *et al.*, 2015). Plant-derived insecticides are naturally occurring compounds in roots, leaves, heartwood and bark that do not only provide structural integrity but act as a defensive agent against xylophagous insects (Verma *et al.*, 2009; Kirker *et al.*, 2013; Adedeji *et al.*, 2017). These compounds usually act as antifeedant, repellent or toxins for the termites (Hassan *et al.*, 2017).

Recently the preferable and practical approach is to replace synthetic insecticides with bio-pesticides (Logan *et al.*, 1990). Naturally occurring bioactive compounds in plants are a rich source of chemical constituents that have different termicidal activities (Ibrahim *et al.*, 2009; Zhu *et al.*, 2003) and can be used in the agricultural system to control insect pest infestation with low menace. Many medicinal plants have effective alternate of termite control agents (Cheng *et al.*, 2007) that have been used against insects and insect born diseases (Sofowora, 1993). These natural insecticides alter insect behavior by inducing growth retardation activities (Breuer and Schmidt, 1955), feeding inhibition (Wheeler *et al.*, 2001), toxicity (Hiremath *et al.*, 1997) and suppression of calling behavior (Saxena and Mani, 1987). in number of These phytochemicals are more advantageous as they are target specific and ecofriendly (Scott *et al.*, 2004).

Many plants have been applied in laboratory and field conditions and found that they are a great reservoir of chemical compounds having termiticidal properties and can be used as an alternative method to control termites infestation instead of synthetic compounds (Sbeghen *et al.*, 2002). Plants including *Taiwania cryptomerioides* (Chang *et al.*, 2001), *Calotropis procera* (Singh *et al.*, 2002), lemongrass, *Acassia* leaves, *Eucalyptus globulus*, vetiver oil, clove bud, *Eucalyptus citroda*, cedarwood (Zhu *et al.*,

2001), *Rosmarinus officinalis, Cinammomum camphora*, *Coleus amboinicus*, isoborneol and *Cumbopogon wintrianusjowitt* (Singh *et al.,* 2004). Several experiments were conducted on crude plants extracts, such as *Lysitoma seemnii, Tabebina guaycan, Pseudotusuga menziesii, Diospyros sylvatica* (Ganapaty *et al.,* 2004), *Euphorbia kansuii* and *Curcuma aromatic* (Shi *et al.,* 2008). These extracts discourage feeding behavior and reduced the termites' survival rate. Phytochemical compounds like terpenoids, flavonoids, and saponins cause repellency in termites and ultimately kill them by interfering with their hindgut microbiota (Boue and Raina, 2003).

Leaves, seeds and wood extracts of *Withania somnifera, Grevillea robusta, Croton tigilium, Salvadora oleiodes, Adhatoda vasica, Tephrosian pupurea*, and *Hygrophilia auriculata* were tested against foraging and tunneling behavior of subterranean and higher termites (Ahmed *et al.,* 2006). Abbas *et al.* (2013) assessed the antitermitic activity of ethanolic leaves extracts of 15 medicinal plants against higher termites and their bioactive constituents were identified by phytochemical characterization. These plants species include *Croton tigilium, Nigella sativum, Ricinus communis, Allium sativum, Moringa oleifera, Capsicum frutescens, Mentha sp., Osmium basilicum, Azadirachta indica, Cichorium intybus, Melia azedarach* and *Plantago ovate.*

Instead of applying crude plants extracts, their active chemical compounds were also found to exhibit termites growth reducing activities (Kinyanjui *et al.,* 2000) and evaluated on field termites for their toxicity, repellency and antifeedant behaviors (Blaske and Hertel, 2001; Blaske *et al.,* 2003). A number of these biologically active compounds were isolated from different parts of plants like sesquiterpenes (Arihara *et al.,* 2004), flavonoids (Boue and Rania, 2003), thiophenes (Fokialakis *et al.,* 2006) and were reported to have tremendous termiticidal potential (Kinyanjui *et al.,* 2000).

Various chemical compounds are used synergistically in combination with rudimentary plants extracts to form poison baits to enhance their insecticidal potential and then studied extensively for feeding behavior (Su and Scheffrahn, 2000), reproductive and burrowing activities of termites (Cornelius and Lax, 2005). Certain chitin synthesis inhibitors such as diflubenzuron, hexaflumuron and chlorfluazuron in the

form of filter paper disks and Summon disks are also applied to study foraging and aggregation behavior in *Coptotermes species* and were found significantly effective (Rojas and Morales, 2001).

A wide range of antibiotics like tetracycline, ampicilline, and urea were also used against termites and revealed the reduced number of symbiotic protozoa and increased death rate in the population of *Reticulitermes flavipes* and *Reticulitermes virginicus* (Waller, 1996). Similarly, the protozoan population was also highly affected by the application of Vetiver oil on *Coptotermes formosanus* (Maistrello *et al.,* 2003). Another control method for termites involves the use of entomopathogenic fungi. The commonly used species are *Beauveria bassiana* Vuillemin and *Metarhizium anisopliae* Sorokin green muscardine fungus. However, the majority of the research work is conducted on fungal microbes of *M. anisopliae* in field and laboratory conditions for control of termites (Ramakrishnan *et al.,* 1999). Fungal control of termites is also preferred because of its virulence and target specificity, less expensive because of robust conidial growth and environmental friendly (Zoberi and Grace, 1990; Milner and staples 1996).

As medicinal plants are easily approachable and inexpensive, their bioactive phytochemicals can be investigated as antitermitic agents and evaluated as antifeedant, pharmacological and as antibiotic for antimicrobial activity (Elango *et al.*, 2012). Now a days, there is an increased demand to sort out such novel control strategies that should remain active even after insecticidal resistance against them (Achs and Malaney, 2002).

Objectives

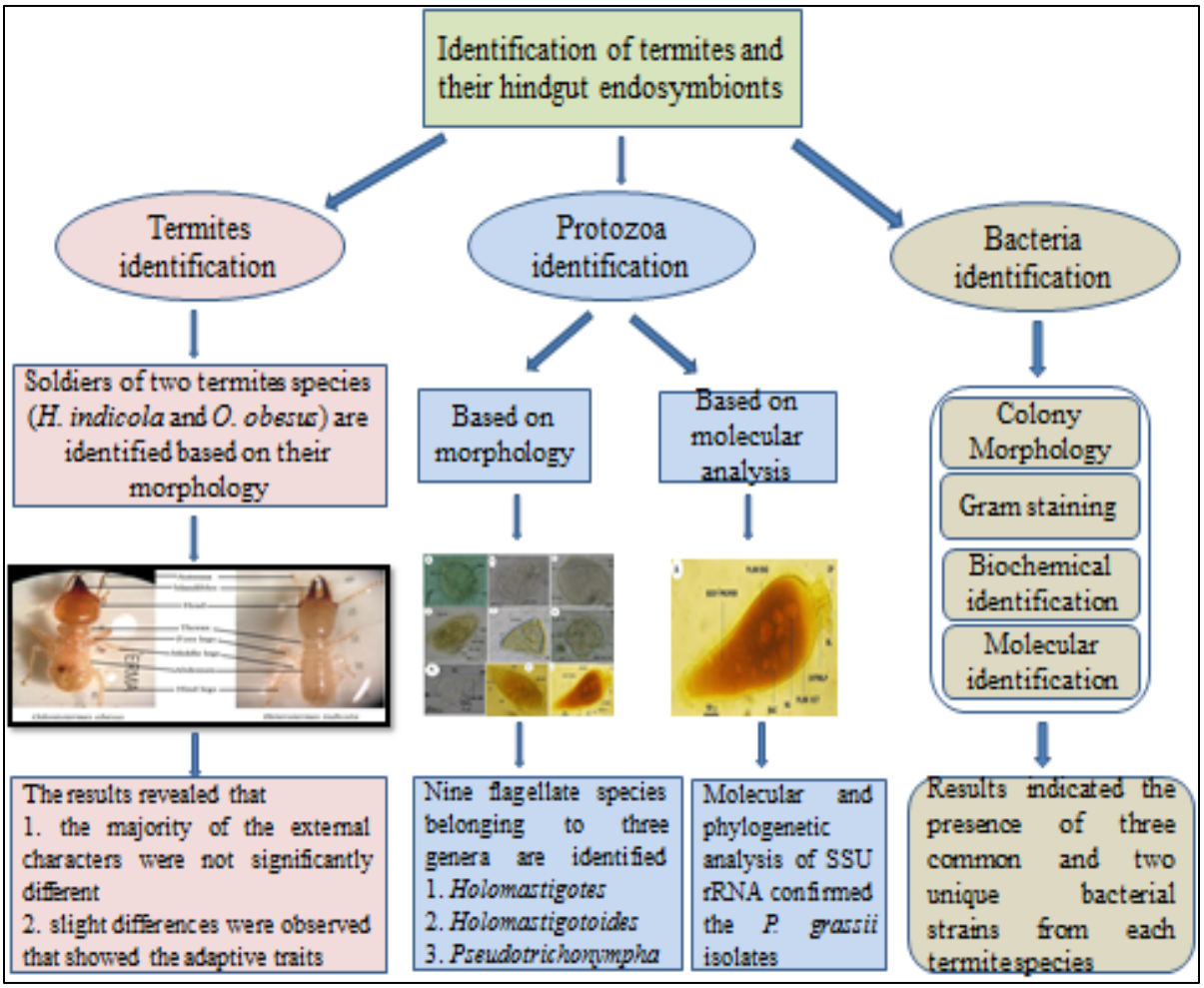
- Morphological identification of termites
- Morphological and molecular identification of termites hindgut endosymbionts (protozoa and bacteria).
- Basic screening of different plants extract by conducting antitermitic, antiprotozoan and antibacterial bioassays
- Column and thin layer chromatography of most effective plant for isolation of bioactive fractions
- Antitermitic, antiprotozoan and antibacterial assays of isolated fractions to sort out bioactive compounds.
- Phytochemical characterization of bioactive fractions using preliminary phytochemical test, VU-Vis spectroscopy, FTIR and GC-MS analysis.

ABSTRACT

Termites cause a serious menace to wood structures all over the world. They rely mostly on entozoic fauna residing in their hindgut for the digestion of cellulosic and hemicellulosic materials. Identification through morphometric measurements is the primary basis of taxonomy; it may vary with environmental factors. The present investigation is based upon the morphometric identification of two termite species '*Odontotermes obesus*' and '*Heterotermes indicola*' collected from the vicinity of Quaid-i-Azam University Islamabad, Pakistan. Thirty-six morphological characters *i.e.* length and width of the body, thorax, abdomen, head, mandibles, pronotum, postmentum, antenna (scape, pedicle, flagellum), legs and their parts were measured. The present study also investigated the diversity profile of flagellate protists isolated from the hindgut of a lower termite "*H. indicola*". Different morphological characteristics were examined including length and width of the body and bulge, the diameter of the nucleus and length of axostyle, flagella and centroblepharoplast and statistically analyzed by "*Student t-test*" using the Minitab version 18. The largest and most abundant species *Pseudotrichonympha grassii* was further identified by molecular studies using SSU rRNA. Similarly, bacterial fauna isolated from the hindgut of *O. obesus* and *H. indicola* was also characterized based on morphology, and molecular analysis of 16S rRNA. Morphometeric measurements indicated that most of the external body features of the soldier cast were not significantly different from each other. However, slight differences were observed in the measurements of different parts of flagellum (pedicle, scape) and legs (trochanter, tarsus, tibia, and claw) that show the adaptive traits which support an insect to adapt according to the environmental stress. Nine flagellate species belonging to three genera *i.e. Holomastigotes, Holomastigotoides* and *Pseudotrichonympha* were recorded based on morphology. The observed species were *Holomastigotes metchnikowi, Holomastigotes campanula, Holomastigotoides hemigynum, Holomastigotes annandalei, Holomastigotoides hartmanni, Holomastigotoides kempi, Holomastigotoides Koidzumi, Holomastigotoides metchnikowi,* and *Pseudotrichonympha grassii*. Molecular and phylogenetic analysis of SSU rRNA confirmed that the *P. grassii* isolates observed in the present investigation were found to be more closely associated with Japanese isolate

phylogenetically. Similarly, in the present study, the morphological, biochemical and molecular analysis confirmed the presence of five bacteria from each termite species experimented. The *Bacillus cereus, Escherichia coli, Lysinibacillus fusiformis* were common to both termites whereas *Lysinibacillus xylanilyticus* and *Lysinibacillus macrolides* were found in *O. obesus* only and *H. indicola* harbor *Bacillus subtilis* and *Shigella sonnei* in addition to common three ones. The biodiversity of the termites and their entozoic fauna (Protozoa and Bacteria) is important for biological control of termites as well as for isolation and culturing of hindgut endosymbionts for the production of cellulases, an important industrial enzyme.

Graphical abstarct



INTRODUCTION

Termites hindgut microbiota harbors a variety of symbiotic microorganisms including archaea, bacteria and eukarya (Radek *et al.,* 2018) which not only form a mutualistic relationship with each other but also with their termite host (Ohkuma and Kudo, 1996). The microbiota makes about 40% of total termites weight (600,000 numbers/gut) and resides freely in the punch or attached to the gut wall of their host (Bignell and Eggleton, 2000). They help in digestion of cellulosic material (Ohkuma, 2003; Hongoh, 2011) and provide an additional defense mechanism to their host, assist in energy metabolism, nitrogen and vitamin supply (Salem *et al.,* 2014; Peterson *et al.,* 2015; Zheng *et al.,* 2015). The ecological success of termites is mainly dependent upon the existence of these microorganisms in their hindgut (Slaytor and Chappell, 1994). Termites also transfer their gut microbes among each other through trophallaxis or coprophagy (Bignell and Eggelton, 2000).

The effective breakdown of lignocellulose in termite hindgut is carried out by the synergistic activity of microbial fauna (flagellates) of termite gut along with the wide diversity of cellulolytic enzymes (Tokuda and Watanabe, 2007). Such a group of termites is named as lower termites whereas those depending solely upon their gut wall cellulases for the digestion of wood, although they harbor bacteria and archaea, are called higher termites. The presence of these microbes shows that they are the source of nitrogen, carbon, and energy for their host. About 74-99% lignocellulose and 65-87% hemicellulose digestion is accomplished by termites. These organic materials are mainly composed of lignin, hemicellulose and cellulose, which are initially converted into simple sugars and then finally into ethanol by the action of the microbial community present in termites hindgut (Ohkuma, 2003).

2.1: Termites identification

As termites have a wide range of behavioral diversity, they are considered as an important group to understand the evolution of eusocial behavior in diploid organisms. Termites biodiversity is much more concentrated in subtropical and tropical areas (Eggleton, 2000). According to the United Nation Environment Program (UNEP); among

3000 described species, more than 1000 have been reported from Africa. About 1059 species have been reported from the oriental region having diverse termites fauna (Ahmed and Akhtar, 2002). In Pakistan, 53 termite species have been reported (Akhtar, 1983) which have a wide distribution in different areas of Punjab, Balochistan and Khyber Pakhtunkhwa.

In the study of external morphology, body measurements form a very technical tool especially for the identification of species. However, the consistency of the measurements depends upon the extent of unevenness among the parameters of species between defined localities (Manzoor and Akhtar, 2006). It helps us to trace evolutionary changes and adaptations of species according to their environmental fluctuations (Arif *et al.,* 2012). Some studies on morphometric variation in termites are available (Ahmad *et al.,* 1949; Akhtar and Anwar, 1991; Sheikh *et al.,* 2005, Manzoor and Akhtar, 2006; Manzoor, 2009; Manzoor, 2010). Therefore, statistics and facts on all features associated with termites, including morphometry of external anatomy, are needed to discover in demand to the effective control and prevention of the damage caused by them.

2.1.1: *Odontotermes obesus*

Genus *Odontotermes* is confined to Indomalayan and Ethiopian region. Emerson (1971) reported 93 species of *Odontotermes* genus with 62 species inhabited in the Indomalayan region. Thakur (1981) documented 195 species, out of which 107 are present in the Ethiopian region and 88 in the Oriental region. According to Chhotani (1997), 176 species are present having 91 reported species from the Oriental region and 85 in the Ethiopian region. Indian white termite, *Odontotermes obesus* (Rambur), has a wide range of distribution in Pakistan, India, and Bangladesh (Akhtar and Anwar, 1975; Chhotani, 1979). It is highly damaging termite species; lives in huge mud mounds, frequently feed on wood and cause serious damage to it. Major economic loss is caused by damaging floor timber, fuel timber, wooden cabinets and railway tracks (Akhtar and Anwar, 1991). In Pakistan crops, especially green foliage crops are commonly attacked by fungus-growing termites (*Odontotermes sp.* and *Microtermes sp.*). *Odontotermes obesus* and *Microtermes obesi* attack wheat crops and cause severe damage to it.

Sunflowers and wheat are badly affected by *O. obesus* because it is a very serious pest of both of these.

2.1.2: *Heterotermes indicola*

Genus *Heterotermes* lies at the top three economically important pests of family Rhinotermitidae (Baker and Bellamy, 2006; Griebenow and Eaton, 2016). This genus has a worldwide distribution having 30 existing species globally (Krishna *et al.,* 2013) however, only 17 species are serious building infesting pests (Su and Scheffrahn, 2000). Phylogenetically genus *Heterotermes* is closely related to the eastern subterranean termite *Reticulitermes flavipes* Kollar (Griebenow, 2017). *Heterotermes indicola* (Wasmann), a wood destroying termite is commonly prevalent in Pakistan, Afghanistan, and India (Maiti, 2006). In Pakistan, it is more prevalent in Punjab, Balochistan and Khyber Pakhtunkhwa (Ahmad, 1962). These subterranean termites remain active throughout the year (Manzoor and Mir, 2010) and despite causing alarming damage to wooden materials, it has also been found to infest every cellulose-containing material like clothing, papers and stored products (Mahapatro and Kumar, 2013; Hassan *et al.,* 2017) thus producing a good deal of structural damage (Mahapatro and Chatterjee, 2017). It is considered as one of the serious pests of agricultural and urban areas (Manzoor *et al.,* 2010). *Heterotermes indicola* attacks timber and other wooden materials moving through mud galleries.

2.2: Protozoa biodiversity

Heterotermes indicola (Blattodea: Rhinotermitidae) is a lower termite which harbors entozoic flagellates responsible for cellulose digestion acquired from the wood through their enzyme machinery-cellulases (Hongoh, 2011) thus causing considerable damage to forests and wooden materials throughout the world except for Antarctica. In previous studies, 434 species of protist flagellates belonging to three orders: Trichomonadida, Hypermastigida, and Oxymonadida have been identified from the hindgut of different termite species (Inoue *et al.,* 2000).

From the last few decades, molecular methods have been considered as a reliable technique for species identification and tracing evolutionary histories of termites hindgut

flagellates (Yang and Rannala, 2012). Two species of genus *Pseudotrichonympha* (*P. hertwegi* and *P. pauliatana*) from the hindgut of *Coptotermes testaceus* and *Heterotermes tenuis* were described based on morphology and phylogeny by Saldarriaga *et al.* (2011). Noda *et al.* (2007) also described the phylogenetic analysis of 15 species of *Pseudotrichonympha* isolated from different species of termites and proposed that only a single species of *Pseudotrichonympha* exist in a termite hindgut at a time. This fact made the evolutionary history of *Pseudotrichonympha* more important not only because of host specificity as well as a host of bacterial endosymbionts. The knowledge about the protozoan diversity in termites hindgut will be more helpful for controlling termites by targeting their protozoa.

2.3: Bacterial biodiversity

Bacterial flora present in termites hindgut is not only the symbionts of termites but also live as ecto and endosymbiont of flagellates (Noda *et al.,* 2006). Although these bacteria are not directly involved in lignocellulose digestion, they are also crucial for the survival of termites. In *Mastotermes darwiniensis*, approximately 90% of bacteria were found to be associated with flagellates as symbionts (Berchtold *et al*., 1999). Major functions of bacteria in termite hindgut were associated with methanogenesis, acetogenesis, and nitrogen fixation process (Upadhyaya *et al.*, 2012). These endomicrobes have a major role in cellulase synthesis that helps in cellulose digestion by breaking down into propionate, butyrate, and acetate. They also aid in nitrogen fixation (Mathew *et al.*, 2012) and uric acid recycling (Thong-On *et al.*, 2009). Such small insects like termites cause huge damage to the wooden materials with the assistance of these endomicrobes.

All methanogenic bacteria belonging to the genus *Methanobrevibactor* in termites convert H_2 and CO_2 to form methane and reduce H_2 concentration (Brune, 2014). During acetogenesis, acetate is directly produced by flagellates however *Spirochaetes* identified from *Reticulitermes santonensis* and *Cryptotermes secundus* also shows reductive acetogenesis. So in wood-feeding termites (lower termites) acetogenesis plays an important role and dominates over methanogenesis (Pester and Brune, 2006).

Furthermore, endosymbiotic *Bacteriodales* rapidly consume H_2 produced by protists and help in polysaccharides degradation by enhancing the capability of protists for fermentation (Inoue *et al*., 2005). Nitrogen fixating bacteria provide nitrogenous compounds to their protist host in termite gut (Hongoh *et al*., 2008). Amount of nitrogen is very critical in hindgut content because it indirectly affects cellulose degradation, so endosymbionts of flagellates help to maintain nitrogen content by disposing of nitrogenous waste as uric acid during metabolic activities (Upadhyaya *et al*., 2012).

Bacterial fauna in termite hindgut consists of both obligate and facultative anaerobes. Soil feeding termites (*O. obesus*) do not harbor anaerobic bacteria probably because they are present in soil rich in organic matter having plenty of nitrogenous compounds (Thong-On *et al.,* 2009). However wood feeding lower termites mainly depends upon protozoan community for nitrogen fixation and only single report is available about the presence of anaerobic bacterial species (*Desulfovibrio termitidis*) in *H. indicola* (Trinkerl *et al.,* 1990). For the survival of facultative anaerobes, oxygen penetrates the gut through the spiracles via the tracheal system (Shelton and Appel, 2000). To find out the metabolic and physiological features of the bacterial community in termites hindgut, species identification is very crucial (Breznak, 2000). Sequence analysis results of endosymbionts genome revealed the fact that they provide different amino acids and co-factors to their host termites (Hongoh *et al*., 2008). The 16S rRNA gene analysis of the microbial community in termite hindgut was found to be helpful in the evaluation of molecular diversity without the cultivation of these microbes (Kohler *et al*., 2012; Schauer *et al*., 2012). These molecular studies revealed that high bacterial diversity is present in termite hindgut as well as termite specific bacterial lineages (Hongoh *et al*., 2003). Furthermore, the knowledge about genes involved in reductive acetogenesis, fermentation, lignocellulose digestion, and nitrogen fixation is also elaborated (Tarter *et al*., 2009; Matteotti *et al*., 2011).

As termites are very diverse, so the knowledge about the evolutionary relationship between termites and their endosymbionts is insufficient. Here the main aim of the present study was to contribute towards taxonomic knowledge about termites by studying the variations in morphological features of soldier cast of both termite species (*O. obesus*

and *H. indicola*) along with diversity profile of symbiotic protozoan species. Most abundant species, *P. grassii* from the hindgut of *H. indicola* was further characterized by phylogenetic analysis. Bacterial fauna residing in hindgut of both termite species was also characterized by morphological, biochemical and molecular characteristics. Moreover to control the population rate of termites and to protect the huge wood damage and economic loss, it is effective to eliminate the population of gut symbionts (protozoa and bacteria) which play an important role in cellulose digestion of termite and other metabolic activities.

MATERIALS AND METHODS

2.1: Study area and collection of termites

Population samples of two termite species *i.e. H. indicola* and *O. obesus* were collected from dead wooden logs and mud mounds using a collection trap unit as described by Sornnuwat *et al.* (1996) with some modifications from the vicinity of Quaid-i-Azam University, Islamabad (33.729388^{o}N, 73.093146^{o} W) (figure 2.1). The selection was based upon (i) from same locality, (ii) to avoid environmental and geographic influence and (iii) easily approachable. Alive specimens were kept in Petri dishes having moistened filter paper at 27±2°C for a week before experiment and identified using keys described by Akhtar (1983). Samples were also preserved in 70% ethanol for morphometric analysis.

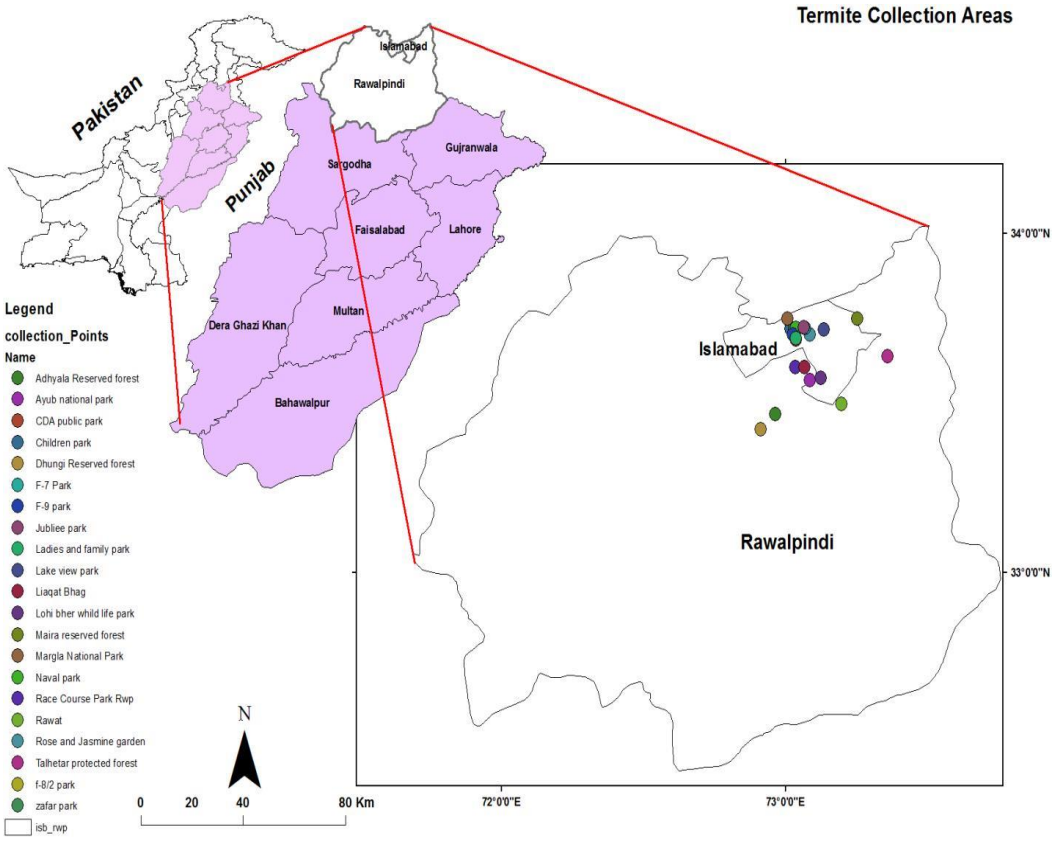


Figure 2.1: Termites collection sites from Islamabad, Pakistan.

2.2: Morphological identification of termites

The soldier cast of two termite species (*O. obesus* and *H. indicola*) was identified by morphometric analysis (Roonwal, 1970). Specimens from preserved samples were picked up randomly and observed under a stereoscopic binocular microscope with a built-in magnification changer. The external morphology of different body parts including 36 parameters of (1) body (2) head (3) thorax (4) abdomen (5) antenna (6) and legs was measured with the help of calibrated ocular micrometer. Photographs of these variables were also taken with the help of Olympus binocular attached camera at 90X magnification. Data of external morphometric variables were analyzed by applying the "*Student t-test*" in Minitab version 18 resulted in mean, standard deviation, standard error, confidence interval and analysis of variance which determine the variations among different parameters.

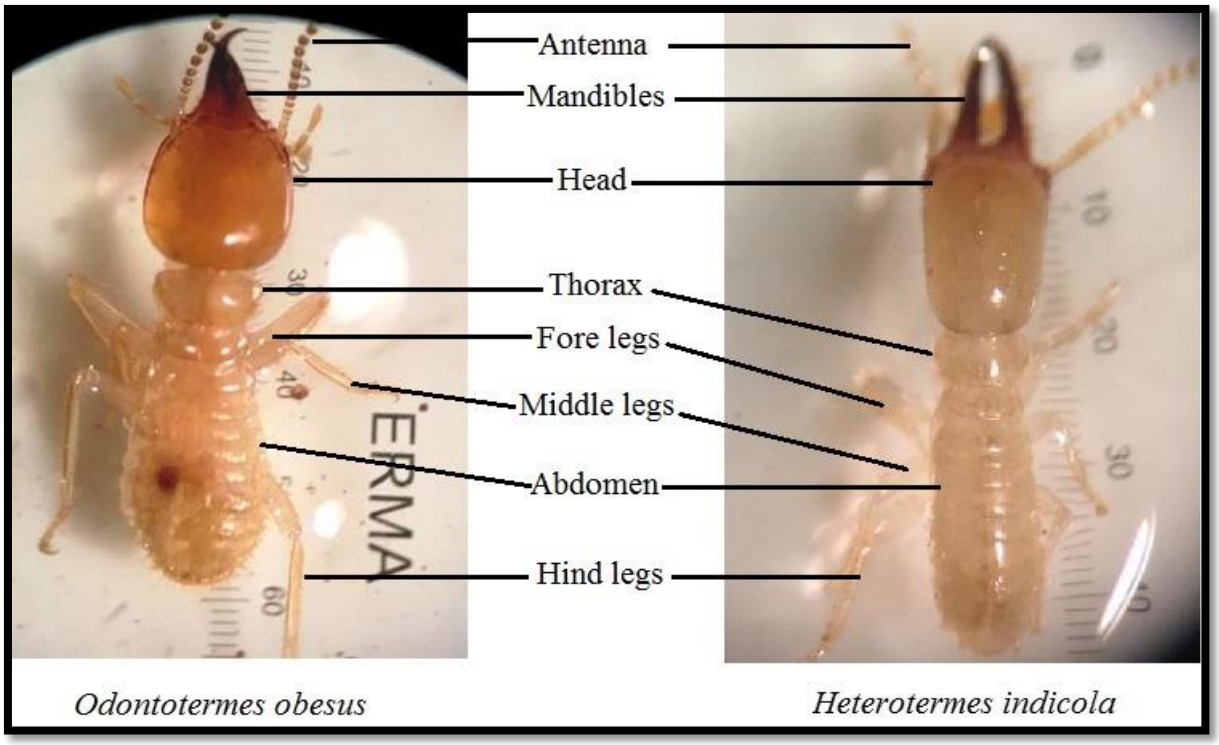


Figure 2.2: Different body parts of *O. obesus* and *H. indicola.*

2.3: Protozoa identification

2.3.1: Permanent slide preparation of protozoa

Permanent and temporary slides of protozoa were prepared on small square coverslips smeared with fresh albumen. The hindgut contents of termites were opened into 0.02% normal saline solution and protozoa smear was left to semi-dry. They were then fixed in Shaudin's fixative (1:1 aqueous solution of concentrated mercuric chloride with absolute alcohol added with one drop of glacial acetic acid in 100ml of solution), the smear was passed through 70, 50 and 30% alcohol for hydration and stained with haematoxilin then dehydrated gradually by passing through different grades of alcohol (30, 50, 70, 90, 95 and 100%). The coverslip containing smear was inverted over glass slide and mounted by Canada balsam/PDX. For comparison, temporary slides were prepared by a drop of normal saline tinged with neutral red and flagellates were observed under the oil immersion lens at 40 and 100X magnification.

2.3.2: Light microscopy and morphometry

The hindgut contents of termites were opened in a drop of 0.02% normal saline and tinged with grams iodine solution. Flagellates were observed under Optika trinocular digital microscope (Optika B-350, Italy) at 40 and 100X magnification using an oil immersion lens. Different morphological characteristics including body length and width, the diameter of the nucleus, length of flagella, length and width of the bulge, length of axostyle and centroblepharoplast were measured from hematoxylin stained slides and species were identified following de Mello (1919, 1927), Saleem (1952) and Kudo (1966). Images and videos were captured in real-time with a 12 megapixels digital CCD camera attached with a trinocular Optika microscope (Optika B-350, Italy).

2.3.3: Single-cell isolation of *P. grassii*

The gut of a termite worker was opened in 5µl of filter-sterilized buffer (0.1M NaC1, 10mM Na_3PO_4, pH 6.9) in the cavity slide. The gut contents were diluted a minimum of four times by adding 4ml of a buffer each time and 1ml of the final diluted contents was used for observations. The flagellates were examined under a digital biological microscope. Individual *P. grassii* cells, identified based on morphology, were picked with a micropipette and collected in PCR tubes.

2.3.4: PCR amplification of SSU rRNA gene

The cells were directly used as a template in a PCR to amplify their nearly full-length SSU rRNA gene. The PCR was performed on five cells. An initial PCR was done using a set of forward and reverse primers (Table 2.1). The reaction mixture consisted of template cells, dNTP mix (0.2mM), forward and reverse primers (1µM each), PCR buffer (1X), *Ex-Taq* DNA polymerase (2.5U) and $MgCl_2$ (1.5mM). Amplification consisted of 35 cycles of 1min. at 92°C, 1min. at 50°C and 1.5min. at 72°C. Agarose gel electrophoresis of the reaction revealed no bands. Therefore, using the first reaction contents as a template, a nested PCR was performed. The 25µl of PCR products was added with the same constituents as used for primary PCR by the second set of primers for nested PCR (Table 2.1).

Table 2.1: Set of primers used for primary and nested PCR

Primer Name		**Sequence**	**Reference**
Primary PCR	Eukl8	5'-TGAGGATCCMGGTTGATYCTGCC-3'	Ohkuma *et al.* 2000
	Euk1627	5'-CCGAAGCTTACGGGCGGTGTGTRC-3'	
Nested PCR	Har-F	5'-GCGCTACCTGGTTGATCCTGCC-3'	Harper *et al.* 2009
	Har-R	5'-TGATCCTTCTGCAGGTTCACCTAC-3'	

2.3.5: Agarose gel electrophoresis, purification and sequencing of PCR products

Amplified PCR products were visualized using 1.5% agarose gel. Samples were loaded stained with ethidium bromide. Bands were observed under UV light and the images were taken with Dolphin gel documentation camera (Wealtec, USA). The pieces of gel containing DNA band were excised from the gel and subjected to DNA purification using Gene JET Gel Extraction Kit (Cat No. K0691, MBI Fermentas). The pGEM-T-Easy vector (Cat No. A1360, Promega) was used to clone the purified products and transformed into DH5α *E. coli* cells. This was followed by plasmid DNA extraction and DNA sequencing of the cloned PCR product.

2.3.6: Phylogenetic analysis

For phylogenetic analysis, our indigenous *P. grassii* taxon was compared with that of isolated from the other localities of the world. The sequence alignment and data matrix construction were done in the software Geneious® version 6.1 (Biomatters Ltd., New Zealand). The neighbor-joining analysis was performed using the DNA evolution model (Tamura and Nei, 1993) in the Geneious build algorithm. Wagner parsimony (Farris, 1970) and Maximum likelihood (Guindon and Gascuel, 2003) analysis were carried out using PAUP (Swofford, 2002) Geneious plugin. Bayesian analysis was done using MrBayes (Huelsenbeck and Ronquist, 2001) Geneious plugin. Finally, based on the above analysis a consensus evolutionary tree was generated.

2.4: Bacterial identification

2.4.1: Dissection and inoculum preparation

Exterior surface of about 100 termite workers was washed with distilled water and gut content was removed with the fine tip of sterilized forceps in 0.08% normal saline solution. A volume of 1ml of gut content was serially diluted to get discreet bacterial colonies. Cultures were prepared on nutrient agar plates by incubating at 37^{o}C for 24 hours and transferred to fresh media plate after every week to get pure colonies (Adams, 2004). Pure cultures were preserved in nutrient agar slants at 4^{o}C for further investigation.

2.4.2: Bacterial identification

Isolated bacteria were identified and characterized by the following methods.

- Colony morphology
- Gram staining
- Biochemical identification
- Molecular identification

2.4.2.1: Colony morphology

The following characteristics were observed for the morphological identification of bacteria based on visual differences in bacterial colonies.

- **Size** small, large, moderate, pinpoint
- **Shape** rhizoid, circular, irregular
- **Pigmentation** color of the colony
- **Elevation** convex, raised, flat
- **Margin** filamentous, entire, serrate, lobate, undulate
- **Opacity** translucent, opaque
- **Appearance** dull, shiny

2.4.2.2: Gram staining

Gram staining is a technique used to discriminate between gram-negative and gram-positive bacteria based on the differences in their cell wall composition (Moyes *et al.*, 2009).

2.4.2.3: Biochemical identification

Biochemical characterization of isolated bacterial cultures was performed by Bergey's Manual of Determinative Bacteriology (9^{th} Edition) (Holt *et al.*, 1994) and following biochemical tests were performed.

- Triple sugar iron test for lactose/ glucose fermentation.
- Indole and H_2S test
- Citrate Utilization test
- Nitrate Reduction test
- Catalase test
- Urease test
- Methyl red vogas proskauer test

2.4.2.3.1: Triple sugar iron test for lactose/glucose fermentation

Triple sugar iron (TSI) agar supplemented with 5% NaCl was used to prepare the slants for glucose/lactose fermentation test. The stab-streak inoculation method was used for isolates inoculation and incubated at 37^oC for 24 hours for color change. The yellow color of the slant indicated acid production and pink-red color indicated an alkaline

reaction. The reaction of the slope in slants was due to lactose fermentation while the butt reaction was due to glucose fermentation.

2.4.2.3.2: Indole and H_2S test

Following reagents were used to check the Indole production

a) Kovac's reagent (Indole)

- Hydrochloric acid ----------50ml
- Amyl Alcohol ----------150ml
- p-dimethylamine benzaldehyde-------10g

Agar deep tubes supplemented with 5% NaCl were prepared for indole production. The stab-streak inoculation method was used for isolates inoculation and incubated at 37^{o}C for 24 hours. Indole presence was detected by adding Kovac's reagent. The appearance of the cherry red layer indicated the presence of indole. The H_2S production might be associated with reduction *i.e.* hydrogenation of organic sulpher which is the part of amino acids. The H_2S is colorless generally, however ferrous ammonium sulphate present in the medium served as an indicator, forming an insoluble black ferrous sulphate precipitate by combining with the gas indicating the presence of H_2S production.

2.4.2.3.3: Citrate utilization test

Citrate utilization test was used to determine the ability of bacteria to ferment citrate as a source of carbon. Simmons's Citrate agar slants supplemented with 5% NaCl were inoculated and incubated at 37^{o}C for 24 hours. Positive results indicated the appearance of the blue color of slants.

2.4.2.3.4: Nitrate reduction test

This test was used to determine the ability of bacteria to reduce nitrates (NO_3) into nitrites (NO_2). Nitrate reduction broth was used as a medium for nitrate reduction test and other reagents were prepared as follows.

a) Solution A

- Acetic acid (5M) 30% ---------- 1000ml
- Sulphanilic acid ---------- 8.0g

b) Solution B

- Acetic acid (5M) ---------- 1000ml
- Alpha-naphthyl amine ---------- 5.0g

Nitrate reduction broth supplemented with 5% NaCl was used for isolates inoculation and incubated at 37^{o}C for 24 hours. An immediate cherry red color production after the addition of solution A and solution B indicated a positive test. A small amount of Zinc dust was also added for confirmation of cherry red color production.

2.4.2.3.5: Catalase test

A small colony of the bacterial isolate was transferred on a sterilized glass slide. A drop of H_2O_2 was poured on the colony. Production of bubbles on the slide indicated the positive results while absence confirmed negative catalase test.

2.4.2.3.6: Urease test

Autoclaved and cooled 5ml urea broth (0.2/5 g/ml) was added by a sterilized syringe filter with bacterial isolates and incubated at 37^{o}C for 48 hours. The appearance of light pink color indicated positive results while no color change showed negative results.

2.4.2.3.7: Methyl red vogas proskauer test

The following reagents were used for the MR-VP test.

a) MR-VP broth

- Distilled water ---------- 1000ml
- Peptone ---------- 7.0g
- Dextrose ---------- 5.0g
- Potassium sulphate ---------- 5.0g

b) Methyl red indicator

- Distilled water ---------- 2000ml
- Ethanol 95% ---------- 300ml
- Methyl red ---------- 0.1g

c) Barrit's reagent:

Solution A and solution B was prepared for Barrit's reagent.

Solution A

- Ethanol ---------- 100ml
- Alpha-naphthol ---------- 5.0g

Solution B

- Distilled water ---------- 100ml
- Potassium Hydroxide ---------- 40g.

MR-VP broth was prepared, autoclaved and dispensed 5ml in each test tube. Test tubes were then inoculated and incubated at 37^{o}C for 24 hours. After incubation isolates were divided into two parts and transferred into fresh test tubes. In the first test tube, methyl red was added and color change was observed. The bright red color indicated positive results while no color change was observed in the case of a negative test. In the second test tube, 1ml of potassium hydroxide added with 3ml of alpha-naphthol and shaked. The color change appeared within 2-5 minutes. The appearance of a pink color indicated a positive reaction.

2.4.2.4: Molecular identification

2.4.2.4.1: DNA extraction

Bacterial DNA was extracted using the CTAB method (Jara *et al.*, 2008). A sufficient amount of pure bacterial isolates was mixed with 300µl of TE buffer in an Eppendorf tube and centrifuged for 1 minute at 5000rpm. The 20µl of 10% SDS and 3µl of proteinase k was added and incubated for 1 hour at 37^{o}C. 70µl of each 5M NaCl and CTAB was added in the Eppendorf tube, vortexed and incubated for 30 minutes at 65^{o}C. An equal volume of chloroform/isoamyl alcohol (600µl) was added, vortexed and centrifuged at 10,000rpm for 5 minutes. The supernatant was transferred to a new tube and phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and centrifuged for 5 minutes at 10,000rpm. The above step was repeated 2-3 times, the supernatant was transferred to the new tube and an equal volume of isopropanol was added. Tubes were shaken and incubated at 4^{o}C for the whole night. Centrifugation was performed at

10,000rpm for 5 minutes, the supernatant was discarded and 70% chilled ethanol was added. Centrifuged for 5 minutes at 10,000rpm to wash the DNA. The supernatant was discarded and the pellet was air-dried and re-dissolved in 80µl of 1XTE buffer and stored at -20˚C for future use.

2.4.2.4.2: PCR amplification of bacterial 16S rRNA gene

PCR amplification of the 16S rRNA gene was performed by the following set of primers UNR-5'-GGACTACCAGGGTATCTAAT-3' and UI-5'-CCAGCAGCCGCGGTAATACG-3' (Barghouthi, 2011). PCR reaction was conducted using Thermocycler (96 universal Gradient Peq Star Lab Peq UK) having a reaction mixture of 50µl, comprises PCR water (33.3µl), Taq buffer (5µl), $MgCl_2$ (4µl), dNTPs (1µl), forward and reverse primer (2µl), Taq DNA polymerase (0.7µl) and DNA template (2µl). The reaction was conducted using the following cyclic conditions (Table 2.2).

Table 2.2: PCR cyclic conditions used for the amplification of the 16S rRNA gene.

1 Cycle	**35 Cycle**			**1 Cycle**	
Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Hold
95 ^{o}C for 4 min.	95 ^{o}C for 1 min.	60 ^{o}C for 2 min.	72 ^{o}C for 2 min.	72 ^{o}C for 10 min.	4 ^{o}C

2.4.2.4.3: Agarose gel electrophoresis, purification and sequencing of PCR product

The amplified PCR product was visualized using 1.5% agarose gel. Samples were loaded stained with ethidium bromide. Bands were observed under UV light and the image was taken with Dolphin gel documentation camera (Wealtec, USA). The gene jet purification kit (Invitrogen) was used for separation of particular PCR product according to the manufacturer's protocol. Amplified PCR products were sequenced according to the protocol provided by ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction Kit v. 3 (Applied Biosystems) using ABI 373A automated sequencer.

RESULTS

2.1: Termites identification

2.1.1: Morphometric measurements of different body parts of *O. obesus* in millimeter

The full body length of *O. obesus* varies from 5.71-6.12 having the mean value of 5.97±0.02 and coefficient of variability 4.35. Length of the prothorax, mesothorax, and metathorax varied between 0.33-0.49, 0.33-0.44 and 0.27-0.44 having a mean value of 0.41±0.27, 0.38±0.02 and 0.38±0.23 respectively. The coefficient of variability was 1.78, 2.93 and 2.43. The length of the abdomen was in the range of 2.10-2.33. The mean value was 2.20±0.86 with a coefficient of variability 2.43. Length from head to mandible tip was 2.22-2.49 with mean value 2.45±0.44 and coefficient of variability 3.72 (Table 2.3).

The length and width of the head varied between 1.16-1.38, 1.12-1.27 with mean value 1.29±0.92, 1.18±0.44 and coefficient of variability 1.27, 2.43 respectively. Similarly, length and width of pronotum varied between 1.00-1.27, 0.49-0.64 with mean value 1.13±0.54, 0.57±0.44 and coefficient of variability 2.30, 4.92 respectively. The length and width of the postmentum were in the range of 1.11-1.33, 0.94-1.05 having mean value 1.22±0.17, 0.94±0.28 and coefficient of variability 3.43, 5.98 respectively. Length of left and right mandible was 0.88-0.99, 0.88-0.99 with mean value 0.93±0.44, 0.96±0.16, and coefficient of variability 2.39, 5.21 respectively (Table 2.3).

The length of the tooth on left mandible was 0.35-0.44 with mean value 0.38±0.28 and the coefficient of variability was 2.66. Different segments of antenna *i.e.* scape, pedicle and flagellum have a length in the range of 0.22-0.33, 0.05-0.08 and 1.83-1.99 with mean values 0.27±0.28, 0.07±0.16 and 1.91±0.20 having a coefficient of variability 5.20, 2.00 and 3.76 respectively. Different segments of front leg *i.e.* coxa, trochanter, femur, tibia tarsus and claw has length in the range of 0.61-0.72, 0.27-0.38, 0.83-0.99, 0.77-0.94, 0.33-0.44 and 0.11-0.16 respectively having mean values of 0.66±0.28, 0.33±0.28, 0.96±0.44, 0.94±0.28, 0.38±0.28 and 0.16±0.28 with coefficient of variability 1.92, 6.93, 4.39, 2.88, 4.66 and 1.73 respectively (Table 2.3).

Similarly, different segments of the middle leg *i.e.* coxa, trochanter, femur, tibia tarsus, and claw has the length in the range of 0.61-0.72, 0.22-0.33, 0.88-1.00, 0.88-0.99,

0.27-0.38 and 0.08-0.16 respectively having mean values of 0.55±0.28, 0.27±0.28, 0.94±0.28, 0.93±0.28, 0.34±0.20 and 0.14±0.16 with coefficient of variability 3.86, 5.20, 5.98, 3.54, 2.75 and 2.00 respectively. Length of different segments of hind leg *i.e.* coxa, trochanter, femur, tibia tarsus and claw varied between 0.44-0.55, 0.16-0.22, 0.88-0.99, 0.94-1.05, 0.33-0.44 and 0.11-0.22 respectively having mean values of 0.53±0.44, 0.20±0.44, 0.92±0.44, 1.02±0.39, 0.38±0.28 and 0.16±0.28 with coefficient of variability 3.69, 2.54, 1.84, 2.95, 4.66 and 1.73 respectively (Table 2.3). The Comparative analysis of morphometric measurements of *O. obesus* with previous reports is given in table 2.4.

Table 2.3: Morphometric measurements of *O. obesus* in millimeter

Parameter (P<0.05) (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**95% CI**	**CV**
Full body length	11	5.71-6.21	5.97	0.67	0.02	5.27-6.48	4.35
Length of prothorax	11	0.33-0.49	0.41	0.62	0.27	0.25-0.46	1.78
Length of mesothorax	11	0.33-0.44	0.38	0.02	0.47	0.19-0.47	2.93
Length of metathorax	11	0.27-0.44	0.38	0.40	0.23	0.68-0.49	2.43
Length of abdomen	11	2.10-2.33	2.20	0.50	0.86	1.80-2.63	2.43
Length from head to mandible tip	11	2.22-2.49	2.45	0.76	0.44	2.06-2.67	3.72
Length of head	11	1.16-1.38	1.29	0.60	0.92	0.85-1.72	1.27
Width of head	11	1.12-1.27	1.18	0.76	0.44	0.79-1.39	2.43
Length of pronotum	11	1.00-1.27	1.13	0.76	0.54	0.91-1.33	2.30
Width of pronotum	11	0.49-0.64	0.57	0.76	0.44	0.30-0.72	4.92
Length of postmentum	11	1.11-1.33	1.22	0.02	0.17	0.64-1.72	3.43
Widthof postmentum	11	0.94-1.05	0.94	0.50	0.28	0.80-1.08	5.98
Length of right mandible	11	0.88-0.99	0.93	0.76	0.44	0.75-1.17	2.39
Length of left mandible	11	0.88-1.05	0.96	0.28	0.16	0.90-1.06	5.21
Length of the tooth on left mandible	11	0.35-0.44	0.38	0.50	0.28	0.24-0.85	2.66
Length of antenna							
(i) Scape	11	0.22-0.33	0.27	0.50	0.28	0.13-0.41	5.20
(ii) Pedicle	11	0.05-0.08	0.07	0.28	0.16	0.03-0.10	2.00
(iii) Flagellum	11	1.83-1.99	1.91	0.08	0.20	1.34-2.46	3.76
Length of front legs							
(i) Coxa	11	0.61-0.72	0.66	0.50	0.28	0.52-0.80	1.92
(ii) Trochanter	11	0.27-0.38	0.33	0.50	0.28	0.19-0.47	6.93
(iii) Femur	11	0.83-0.99	0.96	0.76	0.44	0.75-1.17	4.39
(iv) Tibia	11	0.77-0.94	0.94	0.50	0.28	0.80-1.08	2.88
(v) Tarsus	11	0.33-0.44	0.38	0.50	0.28	0.24-0.52	4.66
(vi) Claw	11	0.11-0.16	0.16	0.50	0.28	0.06-0.30	1.73

Length of middle legs							
(i) Coxa	11	0.61-0.72	0.55	0.50	0.28	0.41-0.69	3.86
(ii) Trochanter	11	0.22-0.33	0.27	0.50	0.28	0.13-0.35	5.20
(iii) Femur	11	0.88-1.00	0.94	0.50	0.28	0.80-0.10	5.98
(iv) Tibia	11	0.88-0.99	0.93	0.50	0.28	0.80-1.08	3.54
(v) Tarsus	11	0.27-0.16	0.34	0.36	0.20	0.24-0.42	2.75
(vi) Claw	11	0.08-0.16	0.14	0.28	0.16	0.07-0.22	2.00
Length of hind legs							
(i) Coxa	11	0.44-0.55	0.53	0.76	0.44	0.32-0.74	3.69
(ii) Trochanter	11	0.16-0.22	0.20	0.56	0.44	0.02-0.35	2.54
(iii) Femur	11	0.88-0.99	0.92	0.76	0.44	0.71-1.13	1.84
(iv) Tibia	11	0.94-1.05	1.02	0.68	0.39	0.83-1.21	2.95
(v) Tarsus	11	0.33-0.44	0.38	0.50	0.28	0.24-0.52	4.66
(vi) Claw	11	0.11-0.22	0.16	0.50	0.28	0.02-0.30	1.73

N Number of samples; O.R Observed range; $\overline{X}$ Mean; S.D Standard Deviation; S.E Standard Error; C.I Confidence interval; C.V Coefficient of variability

Table 2.4: Comparative analysis of present morphometric measurements of *O. obesus* with previous reports.

Sr. No.	Body parameters	Present study (mm)	Vidyashree *et al.* (2018)	Manzoor and Akhtar (2006)
1	Head length	1.29	1.23	-
2	Head width	1.18	1.14	1.27
3	Mandible length	0.96	0.76	0.88 (0.87-0.90)
4	Tooth length	0.38	0.28	0.32
5	Length of Pronotum	1.13	0.50	0.58 (0.57-0.60)
6	Width of Pronotum	0.57	0.86	0.93 (0.92-0.95)
7	Length of Postmentum	1.22	0.74	0.78 (0.75-0.82)
8	Width of Postmentum	0.94	0.48	0.50

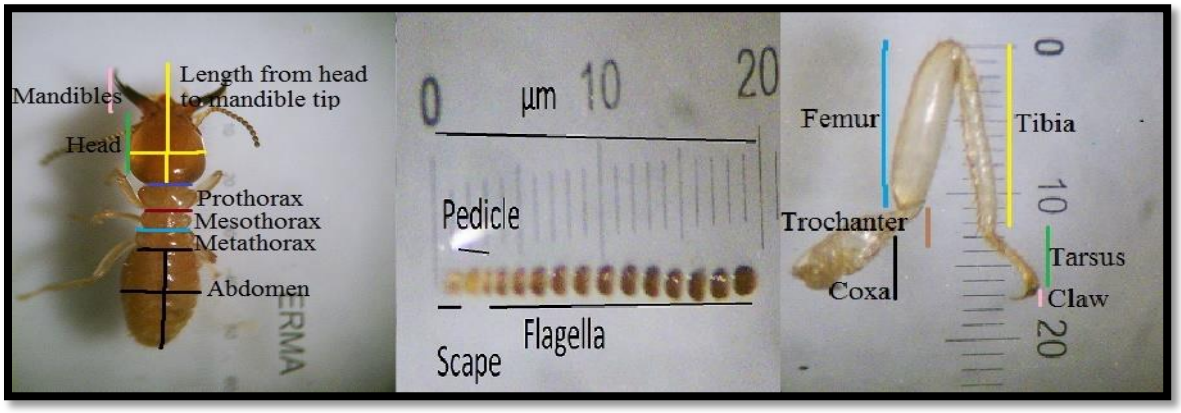


Figure 2.3: Morphometric measurements of different body parts of *O. obesus*

2.1.2: Morphometric measurements of different body parts of *H. indicola* in millimeter

The full body length of *H. indicola* varies from 5.57-7.10 having the mean value of 6.31±2.47 and coefficient of variability 5.60. Length of the prothorax, mesothorax, and metathorax varied between 0.33-0.49, 0.33-0.45 and 0.26-0.43 having mean value 0.44±0.30, 0.42±0.54 and 0.34±0.32 respectively. Their coefficient of variability was 3.76, 3.63 and 2.90. The length of the abdomen was in the range of 2.22-2.98. The mean value was 2.68±0.52 with a coefficient of variability 5.83. Length from head to mandile tip was 1.99-2.92 with mean value 2.68±0.72 and coefficient of variability 2.39 (Table 2.5).

The length and width of head varied between 1.09-1.90, 0.98-1.35 with mean value 1.55±1.10, 1.14±0.57 and coefficient of variability 3.54, 2.72 respectively. Similarly, the length and width of pronotum varied between 0.69-0.85, 0.44-0.67 with mean value 0.76±0.47, 0.55±0.51, and coefficient of variability 1.29, 3.27 respectively. The length and width of the postmentum were in the range of 1.32-1.55, 0.74-0.91 having mean value 1.50±1.05, 0.81±0.98, and coefficient of variability 4.82, 5.64 respectively. Length of left and right mandible was 0.82-1.17, 0.77-1.12 with mean value 1.08±0.78, 1.00±0.04, and coefficient of variability 4.91, 5.21 respectively (Table 2.5).

The length of the tooth on left mandible was 0.32-0.39 with mean value 0.33±0.57 and the coefficient of variability was 3.53. Different segments of antenna *i.e.* scape, pedicle and flagellum have the length in the range of 0.17-0.33, 0.04-0.10 and 1.38-1.78 with mean values 0.30±0.02, 0.09±0.09 and 1.58±1.07 having a coefficient of variability 2.40, 3.20 and 4.95 respectively. Different segments of the front leg *i.e.* coxa, trochanter, femur, tibia tarsus and claw has the length in the range of 0.59-0.72, 0.24-0.35, 0.88-1.00, 0.88-1.02, 0.25-0.38 and 0.08-0.16 respectively having mean values of 0.57±0.32, 0.29±0.39, 0.94±0.28, 0.93±0.30, 0.33±0.41 and 0.13±0.18 with the coefficient of variability 2.71, 3.20, 2.43, 2.06, 5.45 and 4.62 respectively (Table 2.5).

Similarly different segments of middle leg *i.e.* coxa, trochanter, femur, tibia tarsus, and claw has length in the range of 0.55-0.71, 0.29-0.37, 0.83-0.99, 0.81-0.98, 0.32-0.41 and 0.10-0.17 respectively having mean values of 0.55±0.27, 0.33±0.31,

0.91±0.43, 0.87±0.30, 0.34±0.30 and 0.13±0.26 with coefficient of variability 2.02, 3.54, 2.26, 2.39, 3.66 and 4.90 respectively. Length of different segments of hind leg *i.e.* coxa, trochanter, femur, tibia tarsus and claw varied between 0.41-0.44, 0.13-0.24, 0.79-0.88, 0.77-0.91, 0.32-0.44 and 0.11-0.23 respectively having mean values of 0.42±0.39, 0.19±0.45, 0.83±0.42, 0.82±0.39, 0.37±0.30 and 0.16±0.28 with coefficient of variability 4.69, 2.40, 5.34, 2.30, 5.90 and 3.89 respectively (Table 2.5). The comparative analysis of morphometric measurements of *H. indicola* with previous reports is given in table 2.6.

Table 2.5: Morphometric measurements of *H. indicola* in millimeter

Parameter (P<0.05) (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**95% CI**	**CV**
Full body length	11	5.57-7.10	6.31	0.81	0.47	5.38-7.25	5.60
Length of prothorax	11	0.33-0.49	0.44	0.26	0.30	0.24-0.52	3.76
Length of mesothorax	11	0.33-0.45	0.42	0.10	0.54	0.24-0.48	3.63
Length of metathorax	11	0.26-0.43	0.34	0.02	0.32	0.24-0.45	2.90
Length of abdomen	11	2.22-2.98	2.68	0.09	0.52	1.92-3.15	5.83
Length from head to mandible tip	11	1.99-2.92	2.60	0.67	0.72	1.87-3.03	2.39
Length of head	11	1.09-1.90	1.55	0.30	0.10	0.96-2.04	3.54
Width of head	11	0.98-1.35	1.14	0.43	0.57	0.86-1.48	2.72
Length of pronotum	11	0.69-0.85	0.76	0.70	0.47	0.54-0.98	1.29
Width of pronotum	11	0.44-0.67	0.55	0.32	0.51	0.27-0.78	3.27
Length of postmentum	11	1.32-1.55	1.50	0.20	0.05	0.82-1.85	4.82
Width of postmentum	11	0.74-0.91	0.81	0.45	0.98	0.55-1.02	5.64
Length of right mandible	11	0.82-1.17	1.08	0.90	0.78	0.66-1.30	4.91
Length of left mandible	11	0.77-1.12	1.00	0.82	0.04	0.70-1.21	5.21
Length of the tooth on left mandible	11	0.32-0.39	0.33	0.69	0.57	0.29-0.60	3.53
Length of antenna							
(i) Scape	11	0.17-0.33	0.30	0.84	0.02	0.11-0.37	2.40
(ii) Pedicle	11	0.04-0.10	0.09	0.50	0.09	0.03-0.19	3.20
(iii) Flagellum	11	1.38-1.78	1.58	0.48	0.07	1.14-2.29	4.95
Length of front legs							
(i) Coxa	11	0.59-0.72	0.57	0.98	0.32	0.41-0.79	2.71
(ii) Trochanter	11	0.24-0.35	0.29	0.57	0.39	0.13-0.35	3.20
(iii) Femur	11	0.88-1.00	0.94	0.98	0.28	0.78-1.08	2.43
(iv) Tibia	11	0.88-1.02	0.93	0.02	0.30	0.83-1.08	2.06
(v) Tarsus	11	0.25-0.38	0.33	0.98	0.41	0.15-0.43	5.45
(vi) Claw	11	0.08-0.16	0.13	0.45	0.18	1.01-2.20	4.62
Length of middle legs							
(i) Coxa	11	0.55-0.71	0.55	0.45	0.27	0.39-0.88	2.02

(ii) Trochanter	11	0.29-0.37	0.33	0.50	0.31	0.21-0.45	3.54
(iii) Femur	11	0.83-0.99	0.91	0.67	0.43	0.75-1.17	2.26
(iv) Tibia	11	0.81-0.98	0.87	0.55	0.30	0.80-0.10	2.39
(v) Tarsus	11	0.32-0.41	0.34	0.50	0.30	0.24-0.52	3.66
(vi) Claw	11	0.10-0.17	0.13	0.47	0.26	0.06-0.30	4.90
Length of hind legs							
(i) Coxa	11	0.41-0.44	0.42	0.66	0.39	0.31-0.66	4.69
(ii) Trochanter	11	0.13-0.24	0.19	0.52	0.45	0.03-0.33	2.40
(iii) Femur	11	0.79-0.88	0.83	0.67	0.42	0.65-1.02	5.34
(iv) Tibia	11	0.77-0.91	0.82	0.48	0.39	0.69-1.15	2.30
(v) Tarsus	11	0.32-0.44	0.37	0.50	0.30	0.17-0.58	5.90
(vi) Claw	11	0.11-0.23	0.16	0.58	0.28	0.04-0.33	3.89

Table 2.6: Comparative analysis of present morphometric measurements of *H. indicola* with previous reports.

Sr. No.	Body parameters	Present study (mm)	Mahapatro and Kumar (2013)	Poovoli and Rajmohna (2013)
1	Full body length	6.31	4.47 (4.05-4.89)	-
2	Head length with mandibles	2.60	-	1.72 (1.64-1.81)
3	Head width	1.14	0.95 (0.91-1.00)	0.83
4	Length of left Mandible	1.00	0.86 (0.80-0.93)	-
5	Length of right Mandible	1.08	0.84 (0.79-0.90)	-
6	Length of Pronotum	0.76	0.50 (0.49-0.52)	-
7	Width of Pronotum	0.55	0.80 (0.75-0.85)	0.63
8	Length of Postmentum	1.50	1.30 (1.16-1.44)	-
9	Width of Postmentum	0.81	0.42 (0.38-0.47)	-

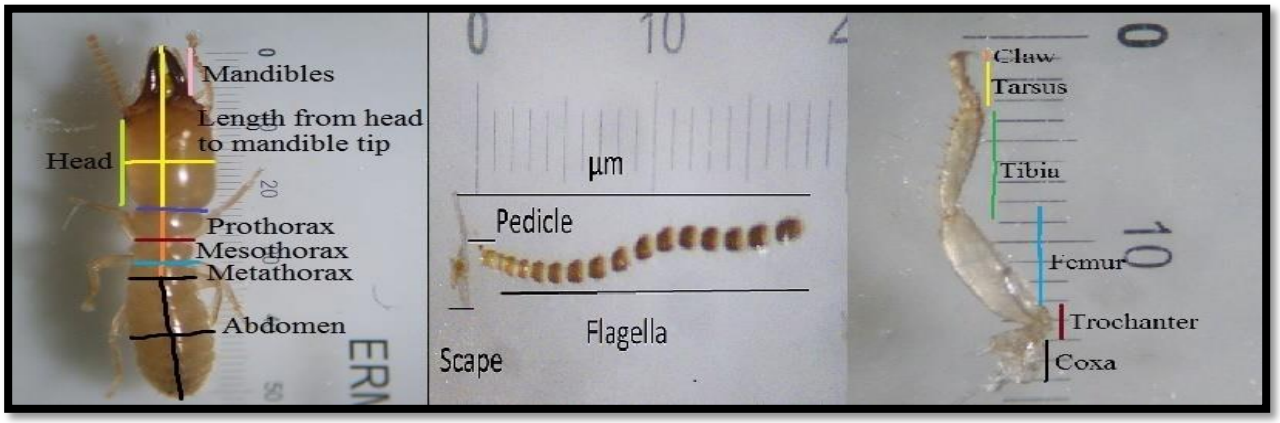


Figure 2.4: Morphometric measurements of different body parts of *H. indicola*

2.2: Protozoa biodiversity

Nine species of protozoan symbionts were identified up to species level following Adl *et al.* (2005). These species belonged to three genera *i.e. Holomastigotoides, Holomastigotes*, and *Pseudotrichonympha*.

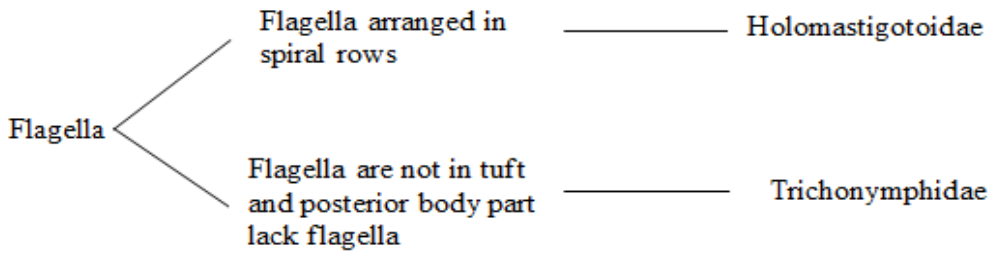
2.2.1: Taxonomic classification of flagellates isolated from *H. indicola*

Class Mastigophora (Diesing): Member of class Mastigophora usually has one to several flagella; the nucleus is vesicular with endosomes.

Sub class Zoomastigia: These may be free-living or parasitic and their body lacks chromatophores. Ensysment is usually common.

Order Hypermastigida (Grassii and Foa): Flagellates belonging to the order Hypermastigida have numerous flagella and complex cytoplasmic organization.

KEY TO FAMILY



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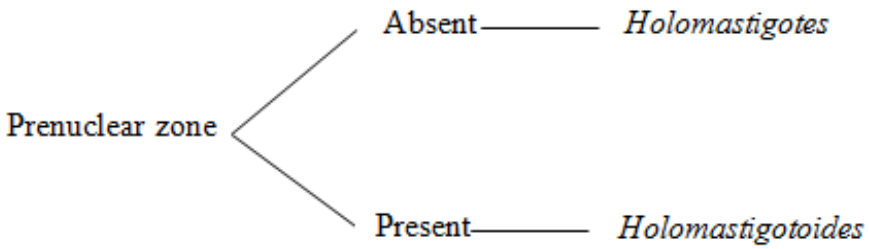


2.2.2: Genus *Holomastigotes*

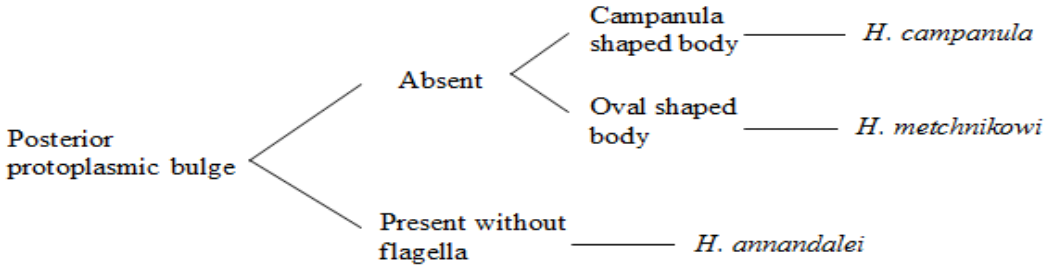
Taxonomic classification

Phylum	Parabasalia Honigberg 1973
Class	Spirotrichonymphea Grassii 1952
Order	Hypermastigida Grassii & Foa
Family	Holomastigotoidea Janick
Genus	*Holomastigotes* Grassii 1882

Genus *Holomastigotes* was described by Grassii in 1882 for the 1st time, latter on by de Mello in 1927, Kudo in 1947 and Salem in 1952.

Diagnosis: Nucleus not surrounded by dense protoplasm; basal granules are deep in the cytoplasm and form deeply stained spiral bands which cover most of the body; flagella arise from basal granules; bulge may or may not be present posteriorly; a long axostyle present; body covered with periplast.

KEY TO SPECIES



2.2.2.1: *Holomastigotes campanula*

Diagnosis: Body campanula shaped; broadly round at the anterior end; truncated posteriorly; body covered with flagella in the form of spiral bands; prenuclear zone absent; axostyle long, arises near the nucleus and extend towards the posterior end (figure 2.5).

Morphometeric description (n=22)

Body length and width of *H. campanula* varied in the range of 118.00-239.00 and 9.09-41.00µm having mean values of 157.92±0.69 and 20.18±2.20µm respectively. The observed range of nucleus diameter was 2.90-9.45µm with an average value of 5.02±0.24µm. Variation in flagella length was 2.00-5.00µm having a mean value of 2.34±0.13µm (Table 2.7).

Table 2.7: Morphometric measurements in different body parts of *H. campanula*.

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	118.0-239.0	157.92	3.23	0.69	6.49-9.35
Body width	9.09-41.0	20.18	10.32	2.20	15.61-24.76
Nucleus diameter	2.90-9.45	5.02	1.14	0.24	4.51-5.53
Flagella length	2.00-5.00	2.34	0.64	0.13	2.05-5.62

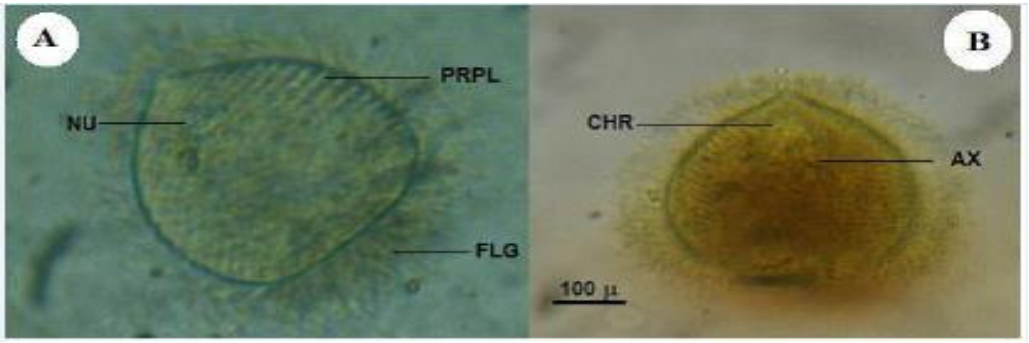


Figure 2.5: Different microscopic views of *H. campanula* at 100X; NU (Nucleus); PRPL (Protoplasm); CHR (Chromatin); AX (Axostyle); FLG (Flagella).

2.2.2.2: *Holomastigotes metchnikowi*

Diagnosis: Cylindrical body; rounded interiorly; no protoplasmic bulged; Nucleus at the anterior end; chromatin material at the periphery; spirally arranged flagella are present on all over the body; cytoplasm differentiated into ectoplasm and endoplasm (figure 2.6).

Morphometeric description (n=22)

The observed range in body length and width varied between 16.38-142.00 and 13.00-64.26µm with mean values of 81.68±9.26 and 49.20±3.38µm respectively. Variations in nucleus diameter were in the range of 3.45-14.00µm having a mean value of 9.06±0.50µm. Similarly, flagella length varied from 2.36-13.00µm with the mean value of 7.92±0.69µm (Table 2.8).

Table 2.8: Morphometric measurements in different body parts of *H. metchnikowi*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	16.38-142.0	81.68	43.45	9.26	62.42-100.95
Body width	13.00-64.26	49.20	15.85	3.38	42.17-56.22
Nucleus diameter	3.45-14.00	9.06	2.38	0.50	8.00-10.11
Flagella length	2.36-13.00	7.92	3.23	0.69	6.49-9.35

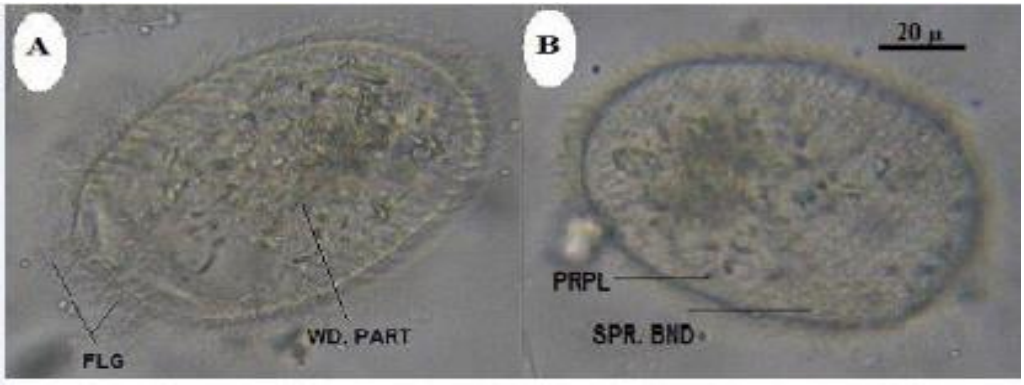


Figure 2.6: Different microscopic views of *H. metchnikowi* at 100X; PRPL (Protoplasm); SPR BND (Spiral bands); FLG (Flagella); W PART (Wood particles).

1.2.2.3: *Holomastigotes annandalei*

Diagnosis: Body oval-shaped with broadly round anterior end; nucleus anterior in position; chromatin in the form of 2 to 3 patches; axostyle present; flagella of uniform size cover all the body in the form of dextral spiral bands; protoplasmic groove is present at posterior end; No distinction of ectoplasm or endoplasm (figure 2.7).

Morphometeric description (n=15)

The variation in the observed range of body length was 14.78-62.21µm having a mean value of 36.25±6.7µm. Body width variations were from 7.65-58.00µm with the mean values of 29.25±6.25µm. Nucleus diameter was varied from 3.10-15.39um having a mean value of 3.62±0.49µm. The observed range in flagella length was 1.76-6.35µm with a mean value of 3.50±0.50µm. Bulge length and width varied between 3.40-7.07 and 7.89-22.07µm having a mean value of 7.38±1.29 and 20.38±4.42µm respectively (Table 2.9).

Table 2.9: Morphometric measurements in different body parts of *H. annandalei*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	14.78-62.21	36.25	19.06	6.7	20.31-52.19
Body width	7.65-58.00	29.25	17.68	6.25	14.47-44.03
Nucleus diameter	3.10-15.39	3.62	1.40	0.49	2.44-4.80
Flagella length	1.73-6.35	3.50	1.43	0.50	2.29-4.70
Bulge length	3.40-7.07	7.38	3.66	1.29	4.31-10.44
Bulge width	7.89-22.07	20.38	12.49	4.42	9.93-30.82

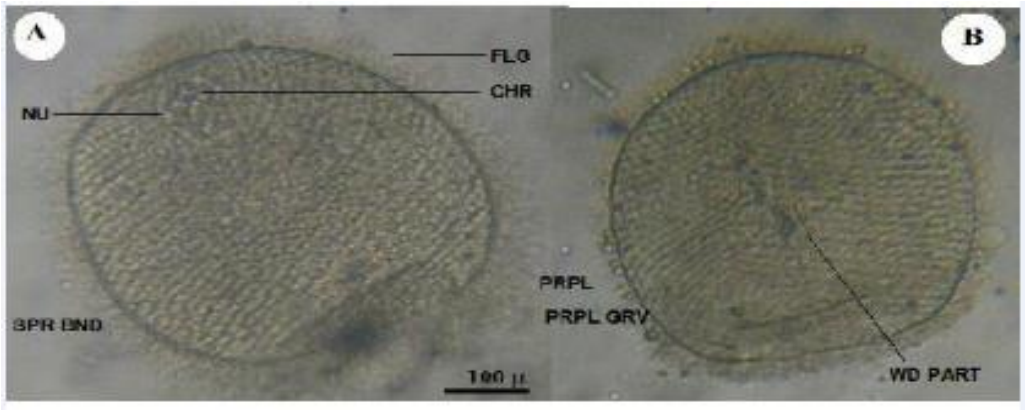


Figure 2.7: Different microscopic views of *H. annandalei* at 100X; NU (Nucleus); PRPL (Protoplasm); PRPL GRV (Protoplasmic groove); SPR BND (Spiral bands); CHR (Chromatin); FLG (Flagella); W PART (Wood particles).

2.2.3: *Genus Holomastigotoides*

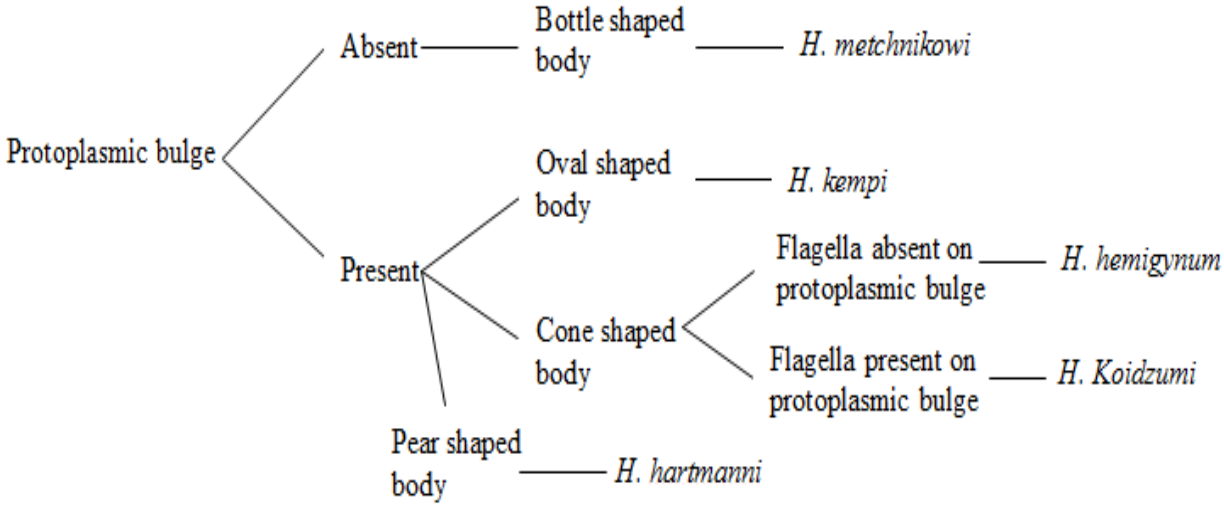
Taxonomic classification

Phylum	ParabasaliaHonigberg 1973
Class	Spirotrichonymphea Grassii 1952
Order	Spirotrichonymphida Grassii 1952
Family	Holomastigotoididae Grassii 1917
Genus	*Holomastigotoides* Grassi & Foà 1911

Holomastigotoides was described by Grassii and Foa in 1911 for the 1st time, latter on by de Mello in 1927, Kudo in 1947 and Saleem in 1952.

Diagnosis: Prenuclear zone present, consists of dense cytoplasm; Basal granules from deeply stained spiral bands that lie deep in the cytoplasm and cover most of the body; flagella arise from basal granules extend from anterior to posterior end; bulge may or may not be present at the posteriorly, may be smooth or covered with flagella; long axostyle present; body covered with periplast.

KEY TO SPECIES



2.2.3.1: *Holomastigotoides metchnikowi*

Diagnosis: Body bottle-shaped; body breadth 1/3rd-1/4th of the length and is uniform till the neck; protoplasmic bulge absent; distinguishable cytoplasm into ecto and endoplasm; flagella arises from basal granules in cytoplasm, arranged in spiral rows; nucleus lies slightly anteriorly to the middle of the body; chromatin material accumulated at the periphery of nucleus; axostyle present and projects downward from the nucleus (figure 2.8).

Morphometeric description (n=24)

The observed range in body length and width was varied between 20.04-692.00 and 19.84-618.00µm with the mean values of 122.4±11.4 and 123.8±10.6µm respectively. Variations in nucleus diameter were in the range of 4.05-83.00µm having a mean value of 20.25±2.8µm. Similarly, flagella length varied from 2.00-71.38µm with the mean value of 15.07±2.5µm (Table 2.10).

Table 2.10: Morphometric measurements in different body parts of *H. metchnikowi*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	20.04-692.0	122.4	34.8	11.4	36.7-189.8
Body width	19.84-618.0	123.8	40.8	10.6	38.3-199.8
Nucleus diameter	4.05-83.00	20.25	4.4	2.8	7.67-18.46
Flagella length	2.00-71.38	15.07	7.5	2.5	6.13-14.45

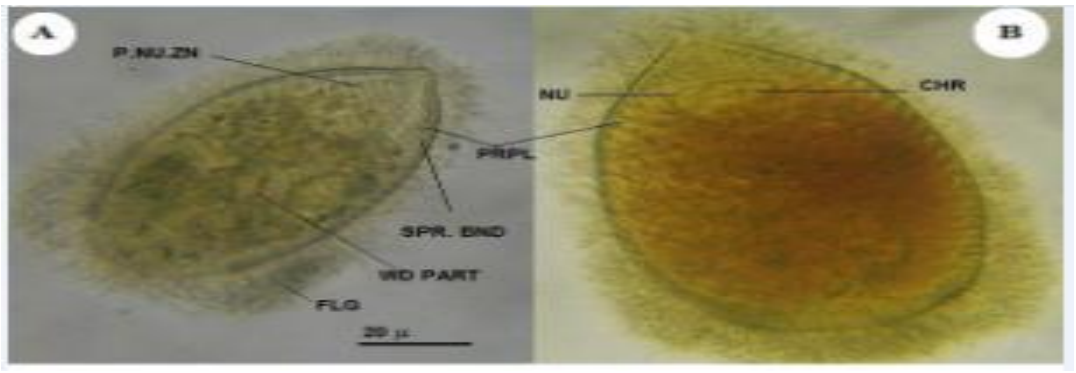


Figure 2.8: Different microscopic views of *H. metchnikowi* at 100X; NU (Nucleus); PRPL (Protoplasm); SPR BND (Spiral bands); CHR (Chromatin); FLG (Flagella); W PART (Wood particles). P.NU.ZN (Pre-nuclear zone).

2.2.3.2: *Holomastigotoides koidzumi*

Diagnosis: Cone-shaped body at the anterior end; truncated at the posterior end; nucleus round, lies anteriorly; no differentiation of cytoplasm into ectoplasm and endoplasm; distinct prenuclear zone arranged in spiral rows; long flagellar bands are wrapped around the body three time; axostyle is surrounded by nucleus which extends to posterior (figure 2.9).

Morphometeric description (n=20)

Body length and width of *H. Koidzumi* varied in the range of 21.23-72.00 and 18.88-54.00µm having mean values of 49.65±4.18 and 28.96±2.76µm respectively. The observed range in nucleus diameter was 2.50-10.08µm with an average value of 6.23±0.39µm. Variation in flagella length was 1.98-8.20µm having a mean value of 3.70±0.31µm. Axostyle length was varied between 3.00-12.00µm with an average value of 6.95±0.77µm (Table 2.11).

Table 2.11: Morphometric measurements in different body parts of *H. Koidzumi*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	21.23-72.00	49.65	18.70	4.18	40.90-58.40
Body width	18.88-54.00	28.96	12.36	2.76	23.17-34.74
Nucleus diameter	2.50-10.08	6.23	1.74	0.39	5.41-7.05
Flagella length	1.98-8.20	3.70	1.40	0.31	3.04-4.35
Axostyle length	3.00-12.00	6.95	3.45	0.77	5.33-8.57

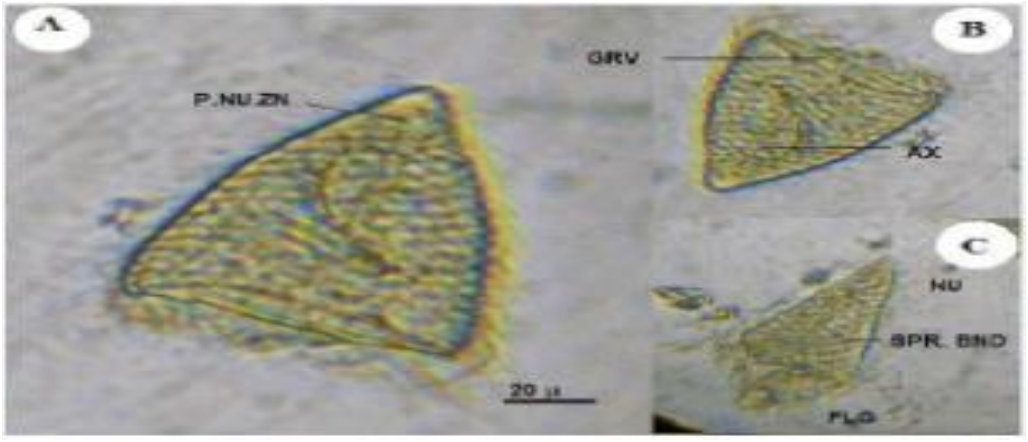


Figure 2.9: Different microscopic views of *H. koidzumi* at 100X; NU (Nucleus); SPR BND (Spiral bands); AX (Axostyle); FLG (Flagella); P.NU.ZN (Pre-nuclear zone); GRV (Groove).

2.2.3.3: *Holomastigotoides kempi*

Diagnosis: Oval-shaped body with its anterior part slightly drawn out forming a nipple shape; no differentiation of cytoplasm; broad protoplasmic bulge is present at posterior end of body; flagella cover whole the body; nucleus round in shape; distinct nuclear membrane present; chromatin material arranged in the form of groups or strands; 2 to 3 nucleoli generally visible, their number varies with individuals; axostyle posterior to the nucleus and protrudes downwards; prenuclear zone present (figure 2.10).

Morphometeric description (n=25)

The variation in the observed range of body length was 17.45-132.00 having a mean value of 65.46±8.58µm. Body width variations were from 11.9-108.00µm with the mean values of 51.95±7.56µm. Nucleus diameter was varied from 8.38-15.00µm having a mean value of 10.45±0.48µm. The observed range in flagella length was 4.76-96.00µm with a mean value of 39.86±6.31µm. Bulge length and width varied between 4.76-62.70 and 9.52-96.00µm having a mean value of 32.64±4.50 and 39.61±6.13µm respectively (Table 2.12).

Table 2.12: Morphometric measurements in different body parts of *H. kempi*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	17.45-132.0	65.46	15.46	8.58	47.36-83.56
Body width	11.9-108.0	51.95	12.65	7.56	36.00-67.89
Nucleus diameter	8.38-15.00	10.45	2.05	0.48	9.43-11.47
Flagella length	4.76-96.00	39.86	26.76	6.31	26.56-51.17
Bulge length	4.76-62.70	32.64	19.07	4.50	23.15-42.12
Bulge width	9.52-96.00	39.61	25.99	6.13	26.68-52.53

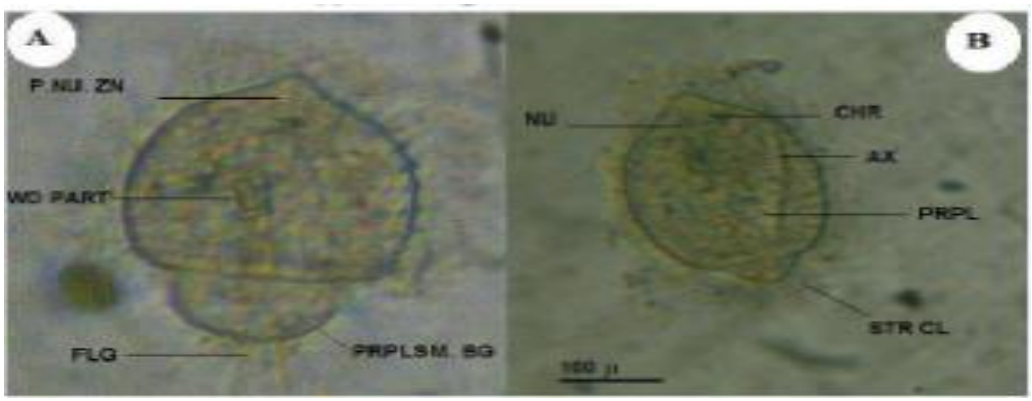


Figure 2.10: Different microscopic views of *H. kempi* at 100X; NU (Nucleus); PRPL (Protoplasm); PRPL GRV (Protoplasmic groove); SPR BND (Spiral bands); CHR (Chromatin); AX (Axostyle); FLG (Flagella); W PART (Wood particles). P.NU.ZN (Pre-nuclear zone); STR CL (Stereocilia); PRPLSM BG (Protoplasmic bulge).

2.2.3.4: *Holomastigotoides hemigynum*

Diagnosis: Body cone-shaped; no differentiation of protoplasm into ecto and endoplasm; round nucleus has 1 to 2 nucleoli and covered by a nuclear membrane; chromatin granules arranged in 2 to 3 groups; axostyle projects downwards into the naked protoplasmic bulge from the posterior end of the nucleus (figure 2.11).

Morphometeric description (n=18)

The observed range in body length and width was varied between 14.29-43.00 and 9.81-24.09µm with the mean values of 26.91±2.33 and 16.86±0.691µm respectively. Variations in nucleus diameter were in the range of 2.96-6.04µm having a mean value of 3.45±0.22µm. Similarly, flagella length varied from 7.14-11.9µm with the mean value of 9.33±0.33µm (Table 2.13).

Table 2.13: Morphometric measurements in different body parts of *H. hemigynum*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	14.29-43.00	26.91	9.87	2.33	28.87-66.92
Body width	9.81-24.09	16.86	3.89	0.91	19.02-44.54
Nucleus diameter	2.96-6.04	3.45	0.94	0.22	2.20-3.91
Flagella length	7.14-11.9	9.33	1.43	0.33	8.62-10.04

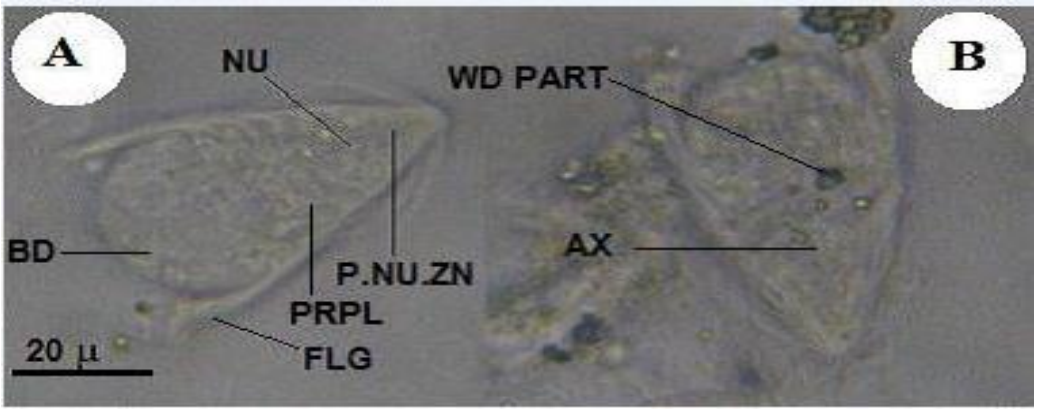


Figure 2.11: Different microscopic views of *H. hemigynum* at 100X; NU (Nucleus); PRPL (Protoplasm); AX (Axostyle); FLG (Flagella); W PART (Wood particles); P. NU.ZN (Pre-nuclear zone).

2.2.3.5: *Holomastigotoides hartmanni*

Diagnosis: Body pear-shaped, slightly pointed and elongated anteriorly; body covered with periplast that gives permanent shape to the body; posterior part of the body without periplast, no spiral bands are present; stereocilia are attached; the stereocilia are different from cilia due to their attachment pattern and are lacks basal granules; flagella arise from basal granules that lie in the cytoplasm; nucleus lies near anterior end; have 1to3 nucleoli (figure 2.12).

Morphometeric description (n=12)

Body length and width of *H. hartmanni* varied in the range of 15.08-74.26 and 9.28-22.00µm having mean values of 47.90±8.05 and 16.82±1.85µm respectively. The observed range in nucleus diameter was 3.00-23.8µm with an average value of 3.50±6.55µm. Variation in flagella length was 2.19-8.53µm having a mean value of5.31±0.76µm (Table 2.14).

Table 2.14: Morphometric measurements in different body parts of *H. hartmanni*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	15.08-74.26	47.90	22.76	8.05	28.87-66.92
Body width	9.28-22.00	16.82	5.23	1.85	12.44-21.19
Nucleus diameter	3.00-23.8	3.50	1.55	0.55	2.20-4.80
Flagella length	2.19-8.53	5.31	2.17	0.76	3.49-7.12

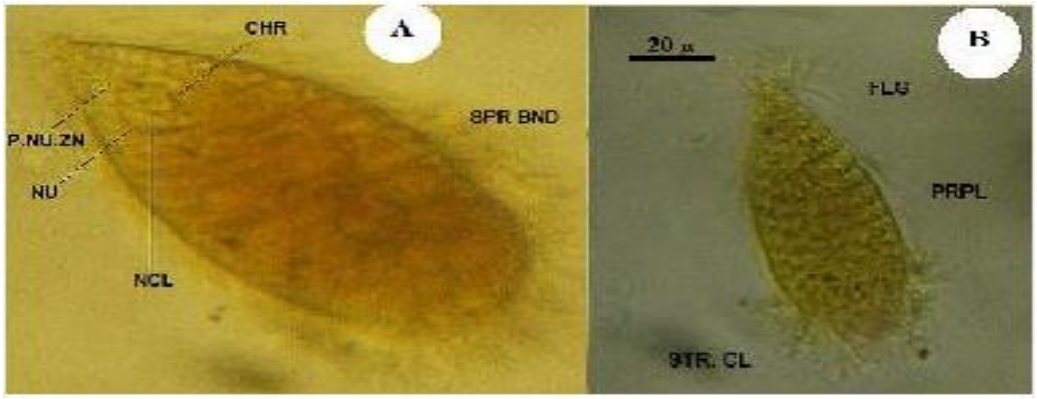


Figure 2.12: Different microscopic views of *H. hartmani* at 100X; NU (Nucleus); PRPL (Protoplasm); SPR BND (Spiral bands); CHR (Chromatin); FLG (Flagella); STR CL (Stereocilia); P. NU.ZN (Pre-nuclear zone).

1.2.4: Genus *Pseudotrichonympha*

Taxonomic classification

Phylum Parabasalia Honigberg 1973

Class Trichonymphea Poche 1913

Order Trichonymphida Poche 1913

Family Teranymphidae Koidzumi 1921

Genus *Pseudotrichonympha* Grassi & Foa 1911

The genus *Pseudotrichonympha* is described by Grassii in 1917 for the first time as *Trichonympha*, then as *Pseudotrichonympha* by de Mello in 1927, Saleem in 1952 and Kudo in 1966.

Diagnosis: Spindle-shaped body divided into two distinct parts; bell and body proper; bell at the anterior end of the centroblepharoplast is barrel-shaped and covered by a cap;

spiral rows of flagella present on the whole body; cytoplasm divided into ectoplasm and endoplasm.

1.2.4.1: *Pseudotrichonympha grassii*

Diagnosis: Spindle-shaped body; divided into head, neck and body proper; flagella arranged in dextral spiral rows completely cover the body; **head** made up of a bell surrounded by transparent hyaline cap; its upper margin lies near to the bell and the lower margin folded and lack flagella; **neck** is also known as centroblepharoplast, anteriorly it is tube-like and has varying thickness; centroblepharoplast is made up of basal granules, composed of basal granules placed near to each other, having the appearance of single structure; neck flagella long, active, in the form of three series and perform jerky movements; **flagella of Series 1** arise from the bell; their length increase from top to bottom and showed strong jerky movements. Flagella arising from centroblepharoplast are known as **flagella of series 2**, longer than the flagella of series 1; **Flagella of series 3** arise from the posterior region of the neck, are larger than the other two series and perform very active jerky movements during locomotion; **nucleus** round or oval in shape; has two nucleoli; chromatin material is evenly distributed or scattered randomly; no definite position of nucleus; it can be present at anterior end, posterior end or at the middle of the body (figure 2.13).

Morphometeric description (n=22)

Body length and width of varied in the range of 9.04-689.00 and 15.30-610.05um having mean values of 184.90±7.90 and 158.7±7.34µm respectively. The observed range in nucleus diameter was 2.38-7.70µm with an average value of 5.07±0.28µm. Variation in flagella length was 3.57-8.40µm having a mean value of 5.44±0.35µm. Centroblepharoplast length was varied between 3.23-8.00µm with average value 4.13±0.16µm (Table 2.15).

Table 2.15: Morphometric measurements in different body parts of *P. grassii*

Parameters (µm)	O.R	$\overline{X}$	S.D	S.E	95% CI
Body length	9.04-689.0	184.9	39.70	7.90	89.7-250.1
Body width	15.30-610.05	158.7	34.36	7.34	76.1-187.3
Nucleus diameter	2.38-7.70	5.07	1.35	0.28	4.47-5.67
Flagella length	3.57-8.40	5.44	1.67	0.35	4.75-6.24
Length of Centroblepharoplast	3.23-8.00	4.13	0.774	0.16	3.79-4.48

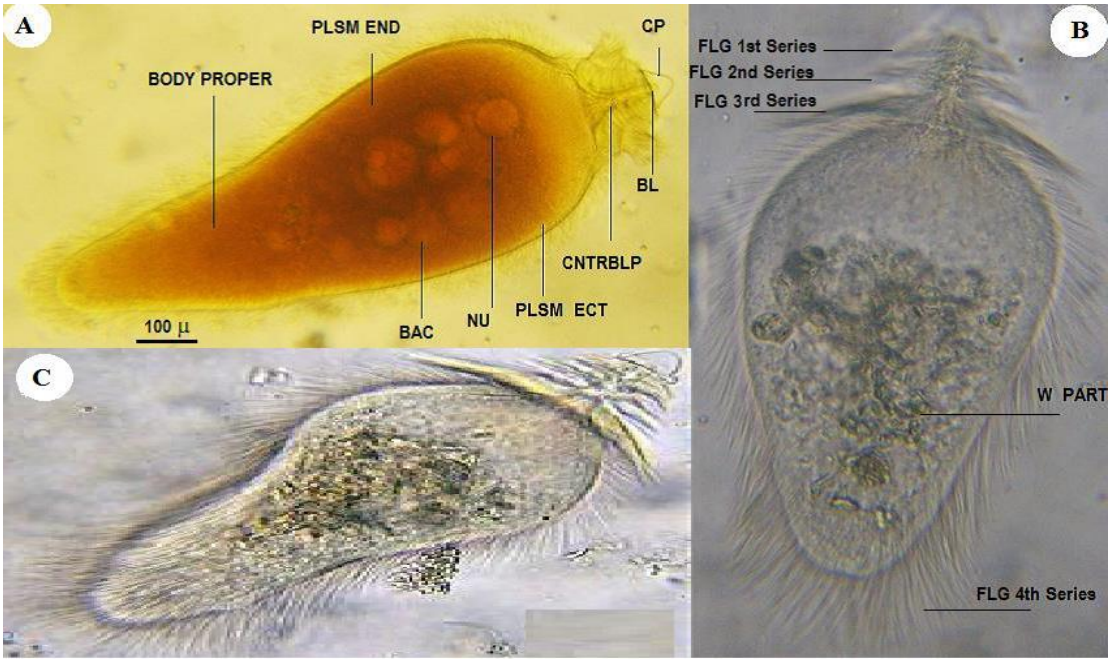


Figure 2.13: Different microscopic views of *P. grassii* at 100X; CP (Cap); BL (Bell.); CNTRBLP (Centroblepharoplast); PLSM ECT (Ectoplasm); PLSM END (Endoplasm); W PART (Wood particles); NU (Nucleus); BAC (Bacteroidales); FLG (Flagella).

Table 2.16: Synonym, type host and additional host of the representative species reported from literature

Name of species	Synonym	Type host	Add Host
Holomatigotes campanula	*Leidya campanula* (De Mello, 1927)	*Heterotermes indicola* (Portergaise)	*Heterotermes indicola* (Islamabad)
	Holomastigotiodes campanula (De Mello, 1927)		
	Holomastigotes campanula (Saleem, 1952)		
Holomatigotes metchnikowi	*Leidya metchnikowi* (Franca, 1919)	*Heterotermes indicola* (Portergaise)	*Heterotermes indicola* (Islamabad)
	Holomastigotoides gigas (De Mello, 1927)		
Holomatigotes annandalei		*Heterotermes indicola* (Lahore)	*Heterotermes indicola* (Islamabad)
Holomastigotoides metchnikowi	*Leidya metchnikowi* (Franca, 1919)	*Coptotermes heimi*	*Heterotermes indicola* (Islamabad)
	Holomastigotoides metchnikowi (De Mello, 1927)		
Holomastigotoides Kempi	*Leidya kempi*(De Mello, 1919)	-	*Heterotermes indicola* (Islamabad))
Holomastigotoides hemigynum	*Holomastigotoides hemigynum* (Grassii, 1917)	*Coptotermes lecteus* (Australia)	*Lecuotermes indicola* (Brazil)
	Holomastigotoides hemigynum (De Mello, 1927)		*Heterotermes indicola* (Portergaise)
	Holomastigotoides hemigynum (Saleem, 1952)		*Coptotermes heimi* (Lahore)
	Leidya annadalei (De Mello, 1919)		*Heterotermes indicola* (Islamabad)
Holomastigotoides hartmanni	*Holomastigotoides hartmanni* (Koidzumi, 1921)	*Heterotermes indicola* (Portergaise)	*Heterotermes indicola* (Islamabad)
	Holomastigotoides H. hartmanni (De Mello, 1927)	*Heterotermes indicola* (Lahore)	
	Holomastigotoides hartmanni (Saleem, 1952)		
Pseudotrichonympha grassii	-	*Coptotermes formosanus*	*Heterotermes indicola*
		Coptotermes heimi	

1.2.5: PCR amplification of SSU rRNA

Two sets of primers (Table 2.1) were used for the amplification of the SSU rRNA gene. The amplification was confirmed by performing gel electrophoresis assay and the results showed a DNA band of 1.4 KB (figure 2.14).

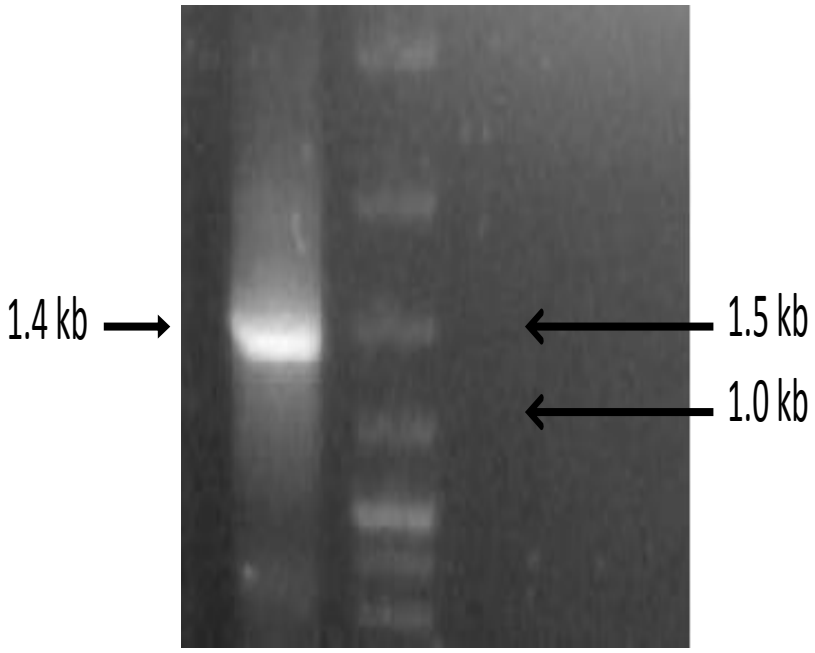


Figure 2.14: PCR amplified product of SSU rRNA of *P. grassii*

2.2.5.1: Phylogenetic analysis of *P. grassii*

BLAST analysis of sequenced PCR product confirmed that it was the SSU rRNA gene of *P. grassii*. The phylogenetic analysis confirmed that *P. grassii* isolate of the present investigation is more closely related to the Japanese isolate. (Table 2.17 and figure 2.15). The accession JX649947 was obtained after the submission of sequences in NCBI Gene Bank.

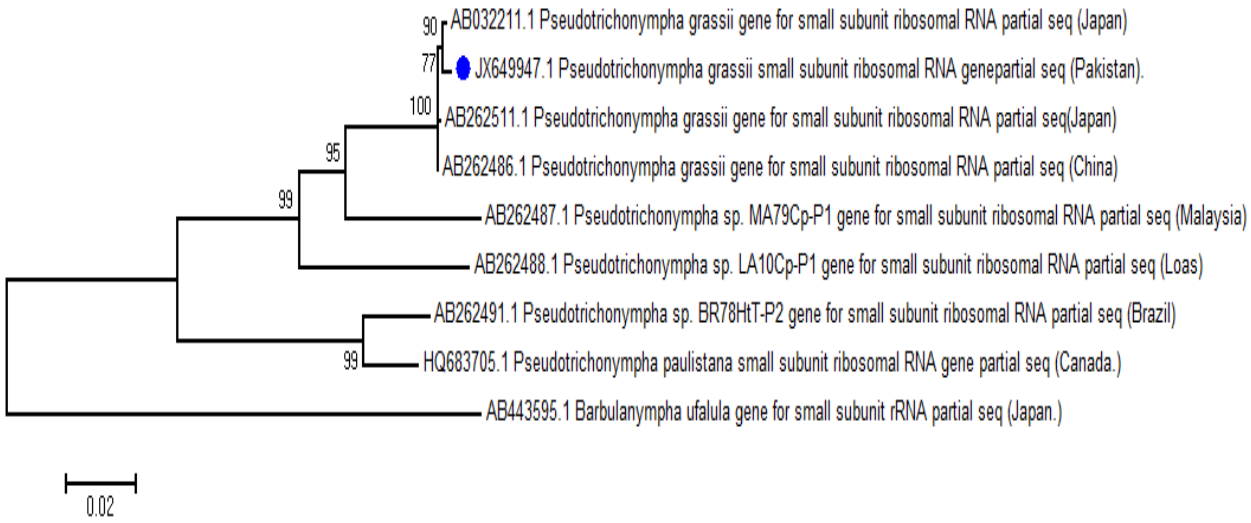


Figure 2.15: Molecular phylogenetic tree of *P. grassii*

Table 2.17: Accession number of *Pseudotrichonympha grassii* from NCBI used for constructing a phylogenetic tree

Taxon		**NCBI Gene bank Accession**	**Country of Origin**
In group	*Pseudotrichonympha grassii*	JX649947	Pakistan
	Pseudotrichonympha grassii	AB262511	Japan
	Pseudotrichonympha grassii	AB262486	China
	Pseudotrichonympha grassii	AB032211	Japan
	Pseudotrichonympha sp.	AB262487	Malaysia
	Pseudotrichonympha sp.	AB262488	Laos
	Pseudotrichonympha sp.	AB262491	Brazil
	Pseudotrichonympha paulistana	HQ683705	Canada
Outgroup	*Barbulanympha ufalula*	AB443595	Japan

2.3: Bacterial biodiversity

2.3.1: Colony morphology

The colony morphology of pure cultures isolated from the hindgut of *O. obesus* and *H. indicola* was observed under a magnifying glass. Morphological characteristics including shape, margin, elevation, size, appearance, pigmentation, and opacity of the colony were noted. The results of morphological identification are given in table 2.18 and 2.19.

Table 2.18: Morphological properties of bacterial cultures isolated from the hindgut of *O. obesus*

Morphological characters	**Bacterial isolates**				
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
Shape	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex
Size	Small	Small	Small	Small	Small
Appearance	Dull	Dull	Shiny	Dull	Shiny
Pigmentation	None	None	None	None	None
Opacity	Opaque	Translucent	Opaque	Opaque	Opaque

Table 2.19: Morphological properties of bacterial cultures isolated from the hindgut of *H. indicola*

Morphological characters	**Bacterial isolates**				
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
Shape	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex
Size	Small	Puncitiform	Small	Small	Puncitiform
Appearance	Dull	Dull	Dull	Dull	Shiny
Pigmentation	None	None	None	None	None
Opacity	Opaque	Translucent	Opaque	Opaque	Opaque

2.3.2: Gram staining

Gram staining was performed on pure bacterial isolates of both termite species to discriminate between gram-negative and gram-positive bacteria. The results of Gram staining are elaborated in table 2.18 and 2.19.

2.3.3: Biochemical identification

The biochemical characterization of bacterial isolates was carried out by different biochemical tests according to Bergey's Manual of Determinative Bacteriology (9^{th} Edition) (Holt *et al.,* 1994). The results of biochemical identification are given in table 2.20 and 2.21.

Table 2.20: Gram staining and biochemical identification of bacterial isolate from the hindgut of *O. obesus*

Characteristics and test	**Bacterial isolates**				
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
Gram staining					
Gram reaction	+ve	+ve	–ve	+ve	+ve
Shape	Rods	Rods	Rods	Rods	Rods
Motility	Motile	Motile	Motile	Motile	Motile
Biochemical test					
Methyl red	-	-	+	-	-
Voges-Proskauer	+	+	-	-	+
Urease	+	+	-	-	+
Nitrate reduction	-	+	+	-	-
Citrate utilization	+	+	-	-	+
Catalase	+	+	+	+	+
H_2S production	-	-	-	-	-
Indole	-	-	+	-	-
Glucose	-	+	+	-	-
Lactose	-	-	+	-	-

Table 2.21: Gram staining and biochemical identification of bacterial isolate from the hindgut of *H. indicola*

Characteristics and test	**Bacterial isolates**				
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
Gram staining					
Gram reaction	+ve	–ve	–ve	+ve	+ve
Shape	Rods	Rods	Rods	Rods	Rods
Motility	Motile	Motile	Motile	Motile	Motile
Biochemical test					
Methyl red	-	+	+	-	-
Voges-Proskauer	+	-	-	+	+
Urease	-	-	-	+	+
Nitrate reduction	+	+	+	+	-
Citrate utilization	+	-	-	+	+
Catalase	+	+	+	+	+
H_2S production	-	-	-	-	-
Indole	-	+	+	-	-
Glucose	+	+	+	+	-
Lactose	+	+	+	-	-

2.3.4: Molecular identification

2.3.4.1: PCR amplification of 16S rRNA gene of bacterial isolates from hindgut of *O. obesus* and *H. indicola*

The amplification of the 16S rRNA gene was confirmed by performing gel electrophoresis assay and results revealed that bacterial strains isolated from the hindgut of *O. obesus* and *H. indicola* had a nucleotides sequence of 193bp (figure 2.16).

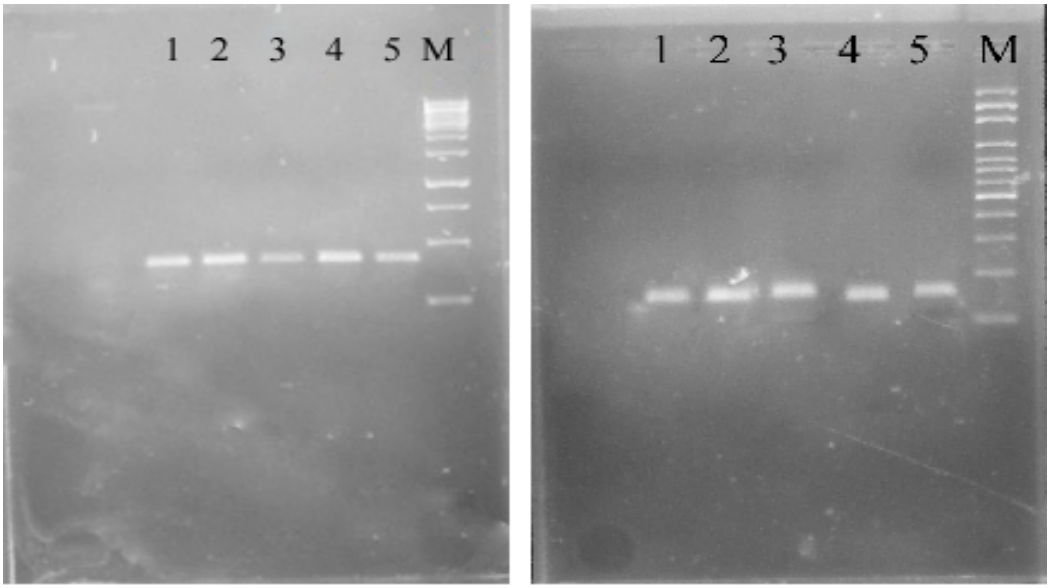


Figure 2.16: PCR amplification of the 16S rRNA gene of (a) *O. obesus* (b) *H. indicola*. Lane Labeled Numerically, 1,2,3,4 and 5: samples (193bp DNA bands), Lane M: 100bp DNA Ladder Marker.

2.3.4.2: Phylogenetic analysis

The NCBI (http://blast.ncbi.nlm.nih.gov/) blast search results for these isolated samples showed the highest sequence similarity with the different bacteria represented in tables 1.22 and 1.23. Moreover, the neighbor-joining tree based on the 16S rRNA gene sequence was constructed to show the relationship between different isolates. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013). These results also confirmed the results of NCBI-BLAST for bacterial isolates, the phylogenetic tree based on the 16S rRNA gene sequence showed the relationship between the different isolates and the representative species of the family Enterobacteriaceae and Bacillaceae (figure 2.17 and 2.18).

Table 2.22: Accession number of different bacterial species from NCBI used for constructing a phylogenetic tree of isolates 1-5 from the hindgut of *O. obesus*

Sample ID	Species name	Similarity index (%)	Country	Accession number
Sample 1	*L. fusiformis*	98.84%	Pakistan	MH718361.1
	L. fusiformis	98.84%	India	MH014941.1
Sample 2	*B. cereus*	96.55%	India	MH997476.1
	B. cereus	96.55%	Iraq	MK418732.1
	B. cereus	96.55%	Egypt	KJ524498.1
	B. cereus	96.52%	Pakistan	KM409709.1
Sample 3	*E. coli*	99.19%	Nigeria	MK332576.1
	E. coli	99.19%	Turkey	KY655118.1
	E. coli	98.79%	Egypt	MF802731.1
	E. coli	98.79%	Korea	FJ789743.1
Sample 4	*L. macrolides*	99.22%	India	KP299182.1
	L macrolides	98.83%	Iran	MK100923.1
	L. macrolides	98.83%	China	MF662483.1
Sample 5	*L. xylanilyticus*	98.80%	Turkey	KY078784.1
	L. xylanilyticus	98.40%	India	MF188191.1
	L. xylanilyticus	89.40%	USA	KF772240.1
	L. xylanilyticus	98.92%	China	MG984078.1

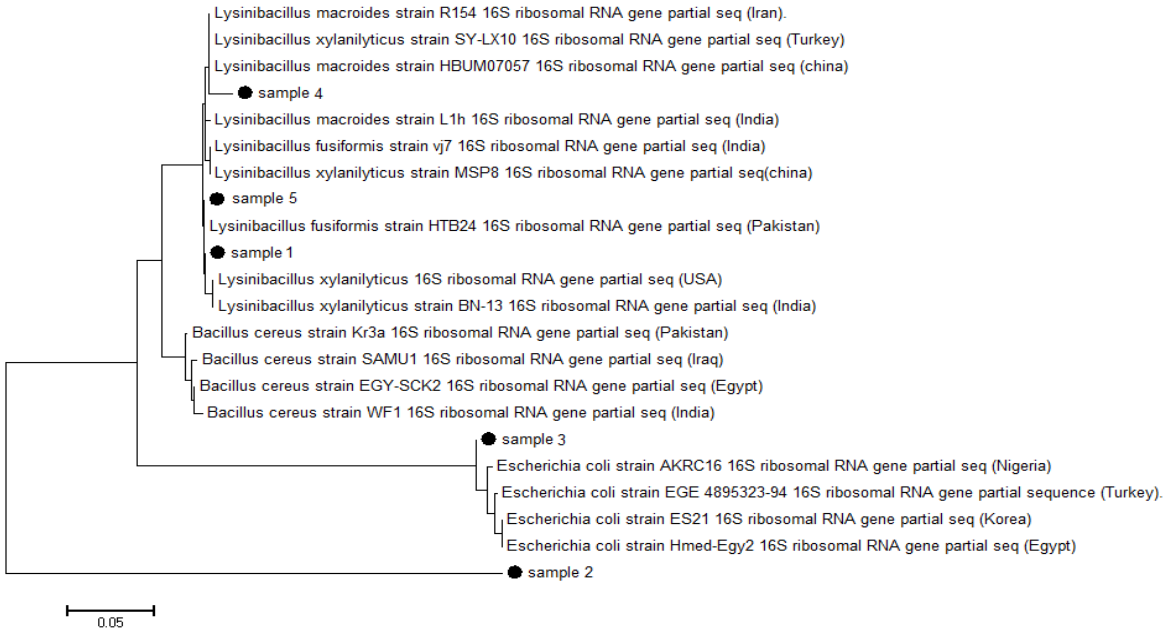


Figure 2.17: Molecular phylogenetic tree of bacterial isolates (1-5) from the hindgut of *O. obesus*

Table 2.23: Accession number of different bacterial species from NCBI used for constructing a phylogenetic tree of isolate 1-5 from the hindgut of *H. indicola*

Sample ID	Species name	Similarity index (%)	Country	Accession number
Sample 1	*B. subtilis*	99.24	China	AY913755.1
	B.subtilis	99.61	India	FJ867920.1
	B.subtilis	99.23	China	MH040963.1
	B.subtilis	99.23	Nigeria	MK712481.1
Sample 2	*S. sonnei*	100	India	MK185059.1
Sample 3	*E.coli*	100	China	MK621269.1
	E.coli	100	Iraq	MK182251.1
Sample 4	*B. cereus*	90.55	Mexico	JQ832674.1
	B. cereus	90.05	India	MH779830.1
	B. cereus	89.37	South Africa	KY344855.1
Sample 5	*L. fusiformis*	97.75	USA	DQ339667.1
	L. fusiformis	97.93	Nigeria	MG881862.1
	L. fusiformis	97.93	Brazil	MK165399.1
	L. fusiformis	97.93	Thiland	MH210864.1

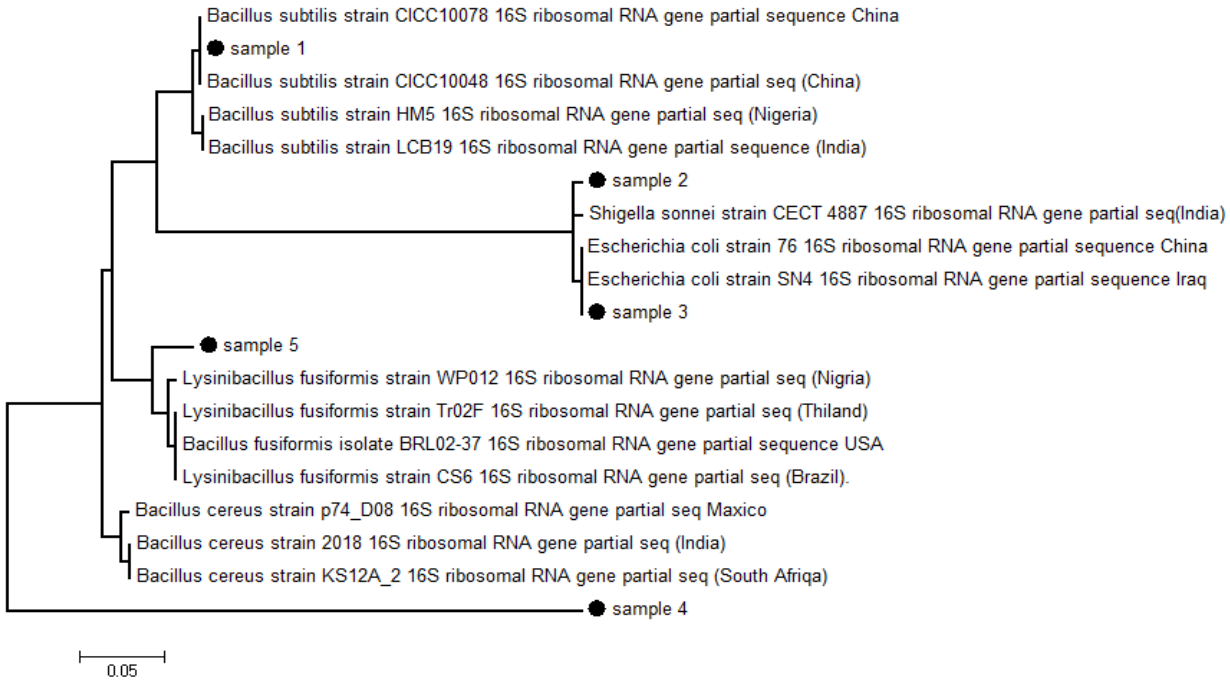


Figure 2.18: Molecular phylogenetic tree of bacterial isolates (1-5) from the hindgut of *H. indicola*

DISCUSSION

Termite's identification and classification depend upon the variation in their morphological characteristics. The present investigation was carried out for the morphological identification of two termite species (*O. obesus* and *H. indicola*). Among all the collected samples, no significant variations were observed however slight variations were present in tooth length, antenna, and legs. These variations show adaptive traits in different environments. According to Arif *et al.* (2012) termites use the antenna as a sensing organ to find out environmental changes and their prey while leg modifications are due to their foraging and movement activities in search of shelter and food. Morphometric variations also help to trace out geographical distribution of termites (Sheikh *et al.*, 2005).

The results of the present findings are comparable with the study conducted by Manzoor and Akhtar (2006) and Vidyashree *et al.* (2018) on *O. obesus* and Mahapatro and Kumar (2013) and Poovoli and Rajmohna (2013) on *H. indicola* (Table 1.4 and 1.6). The size of different parameters in the present study was found to be larger than previous reports. The possible reasons for variations in morphometric measurements may be due to the difference in their chronological age or involvement of different environmental factors such as time of sample collection, humidity, temperature and feeding habit that help them to adapt according to environmental stress in particular area. Similar studies were also conducted by Manzoor (2009) on *Odontotermes matangensis*, Manzoor (2010) on *Odontotermes takensis* and Manzoor and Akhtar (2006) on *Odontotermes parvidens* from different localities of Lahore. However, very little information is available about *O. obesus* and *H. indicola* in the described area of Pakistan. The variations among morphological features provide sufficient taxonomic knowledge about these species.

In the present studies, the nine species of flagellates belonging to three genera viz, *Holomastigotes* (*H. campanula, H. annandalei,* and *H. metchnikowi*), *Holomastigotoides* (*H. hemigynum, H. hartmanni, H. kempi, H. koidzumi,* and *H. metchnikowi)* and *Pseudo trichonympha* (*P. grassii*) were identified from *H. indicola* morphologically. In addition, *P. grassii* was identified on a molecular basis analyzed phylogenetically. Previously it

was reported that *P. grassii* is the most abundant and largest species in the hindgut of *H. indicola* among all species of protists. These findings are supported by the study conducted by Noda *et al.* (2005). Phylogenetic analysis confirmed that *P. grassii* isolate from Pakistan is associated with Japanese isolate and fall in Asian clade.

It is suggested that in termites hindgut wood digestion largely depends upon this comparatively large flagellate as *P. grassii* is found to be replete with wood particles. The *P. grassii* digests lignocellulose in the wood which is the major component of cellulose and provides digested food material to the termites. Thus *P. grassii* dominates over the significance of other protozoan symbionts as it may alone be sufficient to fulfill the nutritional requirements of the termites. Hence, protozocidal drugs against *P. grassii* could be a good choice for controlling termites and infestation caused by them. The genus *Pseudotrichonympha* is present exclusively in all members of Rhinotermitidae except *Reticulitermes*, more closely associated with *Heterotermes* and *Coptotermes* (Lo *et al.,* 2004; Inward *et al.,* 2007). The symbiotic protozoa have a strong relationship with their host (termites) and have the ability to transfer to new hosts within the colony by protocdeal trophallaxis (Inoue *et al.,* 2000) and from mother colony to newly established colony by alates. Proctodeal trophallaxis phenomenon usually refers to the food transfer and other body fluids through the anus to mouth feeding among the member of the colony. This idea illustrates the linkage of the flagellate assemblage in termite hindgut with the host phylogeny (Kirby, 1937; Grasse and Noirot, 1959; Honigberg, 1970; Kitade, 2004). This concept is explained by Noda *et al.* (2007) reporting that the largest parabasalian flagellate, *Pseudotrichonyympha* is present in all Rhinotermitid hosts. However progressive reduction was also observed in flagellate diversity during termites evolution that is; a cockroach genus (*Cryptocercus*) harbors twenty-five species while in most Rhinotermitids host the number is reduced to three. The acquisition of novel flagellate species has also been reported during evolution (Radek *et al.,* 2018).

Different combination of symbiotic protozoa is present in different genera of termites. Even termites within similar locality have different protozoan fauna (Kitade, 2004). However, it was also observed that similar flagellates fauna is present in highly unrelated termite genera (Kitade and Matsumoto, 1998). According to Noda *et al.* (2007)

this unusual behavior can be explained based on the horizontal transfer of intestinal fauna in *Reticulitermes*that that has been replaced with the member of *Hodotermopsis* or its close relatives. If this hypothesis is true then *Psuedotrichonympha* spp. or other protozoa might be present in *Reticulitermes* that are common in other Rhinotermitids (Saldarriaga *et al.,* 2011).

Termites hindgut microbiota carries out different physiological functions *i.e.* degradation of hemicellulose, cellulose, methanogenesis, hydrogenises, acetogenesis, nitrogen fixation and sulphate reduction (Adams and Boopathy, 2005). The breakdown of these biopolymers into the simplest sugars and then into ethanol is accomplished by these bioreactors of termites hindgut (Konig *et al.,* 2006). Scientists investigated different species of symbiotic microbiota and revealed their promising role in cellulose digestion. Several studies are available on the identification and isolation of hindgut bacteria of different termites e.g. *Methanobrevibacter* genus belongs to lower termites while *Methanomicrococcus* is associated with higher termites (Brauman *et al.,* 2001; Friedrich *et al.,* 2001). Similarly, 16S rRNA clones analysis of gut fauna of *Reticulitermes speratus* showed the presence of Spirochetes, Clostridius bacterial strain and Proteo-bacteria (Ohkuma *et al.,* 2000). The presence of these bacteria suggests unique lineages of bacteria residing in termites hindgut. Nakajima *et al.* (2006) also investigated that symbiotic microbiota associated with the gut wall is different from the bacteria present at the other places of the gut. The present study has been conducted for isolation and characterization of bacterial fauna from the hindgut of *O. obesus* and *H. indicola*. The absence of protozoan fauna from the hindgut of higher termite species makes them more interesting subject towards the cellulose-containing food digestion process.

Prem Anand *et al.* (2010) isolated some gram-positive and gram negative bacteria from gut of *Bombyx mori* including *Bacillus circulans Proteus vulgaris, Escherichia coli, Serratia liquefaciens, Pseudomonas aeruginosa, Pseudomonas flourescens, Enterobacter sp., Erwinia sp.* and *Aeromonas sp.* which carry out xylanolytic and cellulolytic activity. They are also involved in the digestion of pectin and lignin-containing compounds. *Bacteroides, Parabacteroides, Dysgonomonas,* and phylotypes of genus *Alistipes* are abundantly present in *Odontotermes* than *Microtermes sp. Bacteroides* abundance in

Odontotermes sp. is a key finding in other fungus-growing termites, promoting their significant role in termite hindgut (Hongoh *et al.,* 2008). These bacteria secrete glycosyl hydrolases involved in sugar metabolism and polysaccharides breakdown (Sonnenburg *et al.,* 2010; Xu *et al.,* 2003). In the present study, bacterial isolates belonging to different families Enterobacteriaceae and Bacillaceae were investigated from peripheral hindgut contents of *O. obesus* and *H. indicola* which play a significant role in maintaining the internal hindgut environment healthy by secreting different enzymes. The presence of these microbes helps in nitrate fixing from the food chain and acts as a resistant barrier against microbial pathogens to protect microbial fauna of termite hindgut. *Bacillus subtilis* has been reported previously as aerobic, gram-positive, rod-shaped bacteria which help in nitrate fixation, inhibit the fungal pathogens and have resistant against them (Phae *et al.,* 1990). Yuki *et al.* (2015) investigated that bacteria belonging to genus *Bacillus*produce alkaline enzymes like cellulase, hemicellulase, protease, and amylases.

Different aerobic, anaerobic and facultative bacteria have been isolated from termites belonging to *Staphylococcus, Streptococcus,* Enterobacteriaceae, *Bacteroides*, and Bacillaceae (Wolber *et al.,* 1986). Similar results were reported from the present study. All the bacterial isolates from the hindgut of *O. obesus* (higher termite) and *H. indicola* (lower termite) belong to family Enterobacteriaceae and Bacillaceae. As termites feed on a variety of food sources having cellulose and hemicellulose major components of their diet which may contribute to the presence of plant-associated bacteria in their hindgut. Diverse bacterial species belonging to different genera like *Bacillus, Pseudomonas, Delftia, Serratia, Cedecea, Viridbacillus*, and *Lysinibacillus* have been isolated from bryophytes of natural raised swamp ecosystem. *Serratia, Bacillus*, and *Pseudomonas* were found frequently with plants suppressing the growth of bacterial and fungal pathogens e.g. *Erwinia sp.* and *Alernaria alternaria* (Szentes *et al.,* 2013). The presence of genus *Lysinibacillus* has also been confirmed from the hindgut of *O. obesus* and *H. indicola* in the present study suggesting the feeding habit of termites on various food sources.

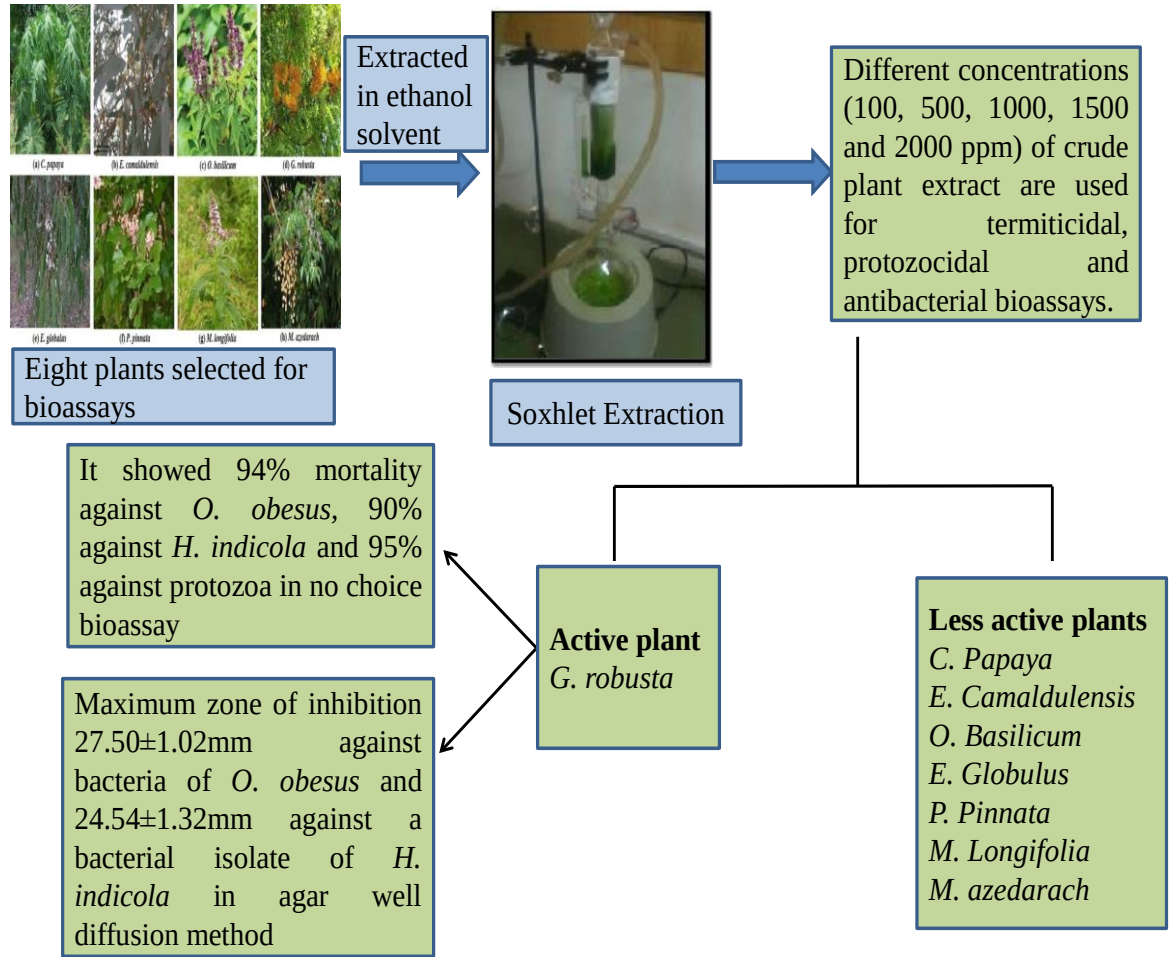
The endosymbiotic fauna present in the hindgut of termites assists in cellulose digestion and enables these small insects to cause millions of damage to wooden

materials. Thus by reviewing morphology and characteristics of symbiotic fauna (protozoa and bacteria), residing in the hindgut of *O. obesus* and *H. indicola*, it was proposed that cellular digestion of hemicelluloses is possible due to the symbiotic association of protozoan flagellates and bacteria. The termite's gut flagellates have a key role in the survival of termites. Using protozocides instead of insecticides may be more environmentally friendly as the low concentration of protozocides will be required for killing their symbiotic flagellates (Qureshi *et al.*, 2012). It is also concluded from the present study that termite hindgut (*O. obesus* and *H. indicola*) harbors the bacteria belonging to the families Enterobacteriaceae and Bacillaceae. The isolated bacteria are related to their feeding habitats but their function is somewhat similar in all termite species including acetogenesis and cellulase production thus providing the ability to digest wood. As these termites are detritivorous, feeding on plants, litter, soil and other substances which may contribute to the varying degree of microbial fauna in the hindgut.

ABSTRACT

Termites harbor a great diversity of endosymbionts in their hindgut that plays a vital role in hemicellulose digestion. For control of termites, ethanolic leaves extracts of eight medicinal plants including *Carica papaya* Linn. *Eucalyptus camaldulensis* Dehn, *Ocimum basilicum* Linn., *Grevillea robusta* Cunn., *Eucalyptus globulus* Labill, *Pongamia pinnata* Linn., *Mentha longifolia* Linn. and *Melia azedarach* Linn. were used against *H. indicola* and *O. obesus* in the present study in concentration range of 100-2000ppm. No-choice bioassay was used for antitermitic and antiprotozoan activities while ager well diffusion method for antibacterial activity. The *G. robusta* showed maximum mortality of 94% (LC_{50} = 1391.01ppm) and 90% (LC_{50} = 1607.95ppm) of *O. obesus* and *H. indicola* respectively among all the tested plants. Similarly, the effect of these plants was also assessed on the protozoa population of termite hindgut after every 48 hours of 16 days experiment using Neubauer's chamber. All plants cause significant ($P<0.05$) reduction in total protozoa population with the highest activity of *G. robusta* that causes the removal of 95% (LC_{50}=502.64ppm) of hindgut flagellates while *M. longifolia* (LC_{50}=3093.47) and *M. azedarach* (LC_{50}=2475.97) were found least effective and have a non-significant effect on protozoa population. Results from agar well diffusion method for evaluation of the antibacterial activity of medicinal plants extract against bacterial isolates from the hindgut of both termite species showed that *G. robusta* leaves extract to possess strongest antibacterial activity with the maximum zone of inhibition 27.50±1.02mm against bacteria of *O. obesus* and 24.54±1.32mm against a bacterial isolate of *H. indicola*. From the results, it is concluded that among the tested plants, leaves extract of *G. robusta* have considerable termiticidal, protozocidal and antimicrobial potential and it can provide a renewable and affordable source of natural bioactive compounds.

Graphical abstract



INTRODUCTION

Termites are called "silent destroyer" as they ruin the houses and wooden materials without showing external damage at initial stages. Approximately US $ 22-40 billion are spent annually on the damage caused by termites worldwide (Su, 2002; Rust and Su, 2012; Buczkowski and Bertelsmeier, 2017). In Southeast Asia, the cost of termite damage is approximately 400 million dollars per year (Lee *et al.*, 2007). Globally 90% of economic loss and 70% of construction damage is caused by subterranean termites *Coptotermes formosanus* Shiraki and *Coptotermes gestroi* Wasmann (Kuswanto *et al.*, 2015). Approximately $1000 million are spent annually on the loss caused by the termites, its maintenance and the renovation process in the United States (Pimentel *et al.*, 2005). The expenditures required for termites control are generally less in developed countries however they are not economically viable in the areas where pest population density is high (Robinson, 1996).

To control termite infestation various chemical insecticides like carbamates, organochlorine, synthetic pyrethroids, chlordane, Bifenthrin, Fipronil and Termidor are used which have some serious shortcomings as they are very expensive, damaging to the natural environment and excessive use of these pesticides is developing resistance against termite (Boue and Rania, 2003; Hu, 2005). These insecticides directly affect human health causing some serious disorders like birth defects in infants, cancer and immune system deformities (Nigam and Bhatt, 2001). Therefore, alternate methods for pest control are the ultimate requirement that should be least toxic and environment-friendly.

Therefore, there is a need to explore natural products for identifying new pesticides or termiticides being biodegradable and environment-friendly. Bioactive compounds from different plants can be used to replace synthetic insecticides being cheaper, target-specific and easily biodegradable to nontoxic products (Belmain *et al.*, 2001). These are safer for the environment, can be used in integrated pest management and a good substitute for the development of safer insect control agents (Moreira *et al.*, 2007). The current study is based upon the use of *Caria papaya, Eucalyptus camaldulensis, Ocimum basilicum, Grevillea robusta, Eucalyptus globulus, Pongamia*

pinnata, Mentha longifolia, and *Melia azedarach* leaves extracts for evaluating their termiticidal, protozocidal and antibacterial properties based upon their insecticidal, antifungal and antibacterial effects.

Carica papaya belongs to the family Caricaceae is a native plant of Mexico and northern South America. It grows generally 5-10m long as a single stem and has spirally arranged leaves at the top of the tree trunk. The *C. papaya* is a medicinally important plant and widely used for pest control as a whole plant as well as their seeds, leaves, and fruits. The *C. papaya* leaves extract exhibits antifungal, antibacterial, wound healing and antitumor potential (Baskaran *et al.,* 2012).

Genus *Eucalyptus* belongs to the family Myrtaceae and has more than 700 species. Members of the genus *Eucalyptus* are native to Australia and are widely used as an antiseptic, insect repellent, flavoring and fragrance agent as well as in pharmaceutical industry (Salari *et al.,* 2006). Antitermitic activity of *E. citriodora* oil has been reported against higher termites and *H. indicola* (Manzoor *et al.,* 2012). The antibacterial activity of *E. camaldulensis* and *E. globulus* against some bacterial strains has been evaluated by Ghalem and Mohamed (2008). Leaves extracts of *E. globulus* showed their growth inhibitory effect against *E. coli* and *S. aureus* strains. The antibacterial activity was found to increase with an increase in concentration, minimum at 25% and maximum activity at 100% concentration. Its oil extracts were also reported to have promising antimicrobial activity (Cimanga *et al.,* 2002; Takarada *et al.,* 2004). Antitermitic activities of *E. camaldulensis* leaves oil extract has been reported against *Coptotermes formosanus* in Thailand by both contact method and fumigation. Results showed that *E. camaldulensis* oil extracts exhibit tremendous termiticidal potential (Siramon *et al.,* 2009).

Grevillea robusta is widely used as an ornamental plant, belongs to genus *Grevillea* and family Proteaceae. The genus *Grevillea* has 190 species and has a wide distribution in Australia and eastern Malaya. The *G. robusta* leaves extract in different solvents including methanol, chloroform, hexane, carbon tetrachloride, and water was evaluated for their cytotoxic, thrombolytic, antimicrobial and membrane stabilizing

activities (Ullah *et al.*, 2014). Termiticidal activity of *G. robusta* leaf extract has been evaluated by Afzal *et al.* (2019).

Member of genus *Ocimum* belongs to family Labiatae. This genus contains 60 species; many of them are of medicinal importance with *O. basilicum* as more important. Their extracts in different solvents were reported to have strong antioxidant and antibacterial activities (Jirovetz *et al*., 2003; Sacchetti *et al*., 2004). Extracts of *O. basilicum* in methanol, ethanol, chloroform, hexane, and acetone were used against different bacterial strains and showed antibacterial and antimicrobial activities (Adiguzel *et al.*, 2005; Kaya *et al*., 2008). Leaves extracts of *O. basilicum* were tested against adult mosquitoes in the form of aerosol and mosquito coils with pronouncing activity (Umerie *et al.*, 1998).

Melia azedarach belongs to family Meliaceae. It is a deciduous plant and native to Australia and Indomalaya. The genus *Melia* has four other species which are semi-evergreen and distributed from Southeast Asia to Northern Australia. The member of this family contains phytochemicals exhibiting development modifying properties, antifeedant effects and insecticidal activities (Nikoletta and Filippo, 2010).

Mentha longifolia commonly called horsemint belongs to the family Lamiaceae. It is an herbaceous perennial plant with characteristic peppermint aroma. Genus *Mentha* is native to Europe excluding Britain and Ireland. Traditionally *M. longifolia* has been used for the treatment of various ailments like respiratory, menstrual, gastrointestinal, infectious and inflammatory disorders all over the world (Farzaei *et al.*, 2017). *Mentha* spp. has also used to cure bronchitis, flatulence, ulcerative colitis, nausea and liver disorders (Gulluce *et al.*, 2007). Fumigant toxicity of *Mentha arviensis* leaves extracts on *Coptotermes heimi*, *Heterotermes indicola* and their gut flagellates has also been evaluated previously (Qureshi, 2014).

Pongamia pinnata is commonly known as Karanj belongs to the genus *Pongamia* and family Fabaceae. This plant is commonly present along the seashores and waterways having its roots in fresh or saltwater. It is the plant of a humid and sub-tropic environment. Extracts of *P. pinnata* has been evaluated for larvicidal activity in methanol

and hydroalcoholic extracts against *Aedes aegypti*, *Anopheles stephesi* and *Culex quinquefasciatus* (Kolli *et al.,* 2013), antioxidant and antimicrobial properties (Sajid *et al.,* 2012). *Pongamia pinnata* was also used in the treatment of various diseases and ailments of the human being in traditional and folk medicines (Chopade *et al.,* 2008).

On the basis of these properties the present study was designed to assess the termiticidal potential of eight medicinal plants ethanolic leaves extract like *E. camaldulensis*, *O. basilicum*, *G. robusta*, *C. papaya*, *E. globulus*, *M. longifolia*, *P. pinnata*, and *M. azedarach* to identify the most effective plant. Then this plant will be subjected to isolation and identification of the bioactive compounds present in them. So that the natural plants' products could be a good substitute for synthetic insecticides to prevent different health hazards.

MATERIALS AND METHODS

3.1: Sample collection

Workers of two termite species (*H. indicola* and *O. obesus*) were collected from dead wooden logs and mud mounds respectively by collection trap units (Sornnuwat *et al*., 1996) with some modifications from Quaid-i-Azam University Islamabad, Pakistan and identified with the help of taxonomic keys (Akhtar, 1983). Live specimens were kept in Petri dishes with soaked cotton plug in the laboratory at temperature 27±1^{o}C and relative humidity 80±5%. They were fed on Whatman filter paper (No. 1) for one week before the experiment to remove debris and other wood particles.

3.2: Plants selected for bioassays

Plants selected for antitermitic, antiprotozoan and antibacterial activity are listed in the table and figure 3.1.

Table 3.1: Plants selected for antitermitic, antiprotozoan and antibacterial bioassays and their biological properties.

Sr. No.	Botanical name	Common name	Family	Biological properties	References
1	***C. papaya***	Papita	Caricaceae	Antifungal, antibacterial, wound healing and antitumor potential	Baskaran *et al.* 2012
2	***E. camaldulensis***	Safaida	Myrtaceae	Antitermitic and antibacterial activity, fumigant properties and growth inhibitory effects	Cimanga *et al.* 2002; Takarada *et al.* 2004; Ghalem and Mohamed 2008; Siramon *et al.* 2009; Manzoor *et al.* 2012
3	***O. basilicum***	Niazbo	Labiatae	Antibacterial, antitermitic, larvicidal and antimicrobial activities	Umerie *et al.* 1998; Adiguzel *et al.* 2005; Kaya *et al.* 2008; Abbas *et al.* 2013.

4	***G. robusta***	Bahekar	Proteaceae	Antitermitic, cytotoxic, thrombolytic, antimicrobial and membrane stabilizing activities	Ullah *et al.* 2014; Afzal *et al.* 2019
5	***E. globulus***	Safaida	Myrtaceae	Antimicrobial, antitermitic and antibacterial activity, fumigant properties and growth inhibitory effects	Cimanga *et al.* 2002; Takarada *et al.* 2004; Ghalem and Mohamed 2008; Siramon *et al.* 2009; Manzoor *et al.* 2012
6	***P. pinnata***	Sukchain	Leguminosae	Larvicidal activity, antioxidant and antimicrobial properties, used in the treatment of various diseases in traditional and folk medicines	Chopade *et al.* 2008; Sajid *et al.* 2012; Kolli *et al.* 2013.
7	***M. longifolia***	Podina	Mentheae	Menstrual, gastrointestinal, and inflammatory disorders, bronchitis, flatulence, ulcerative colitis, nausea and liver disorders.	Gulluce *et al.* 2007; Qureshi 2014; Farzaei *et al.* 2017.
8	***M. azedarach***	Dhraik	Meliaceae	Development modifying properties, antitermitic, antifeedant effects and insecticidal activities.	Nikoletta and Filippo, 2010; Abbas *et al.* 2013.

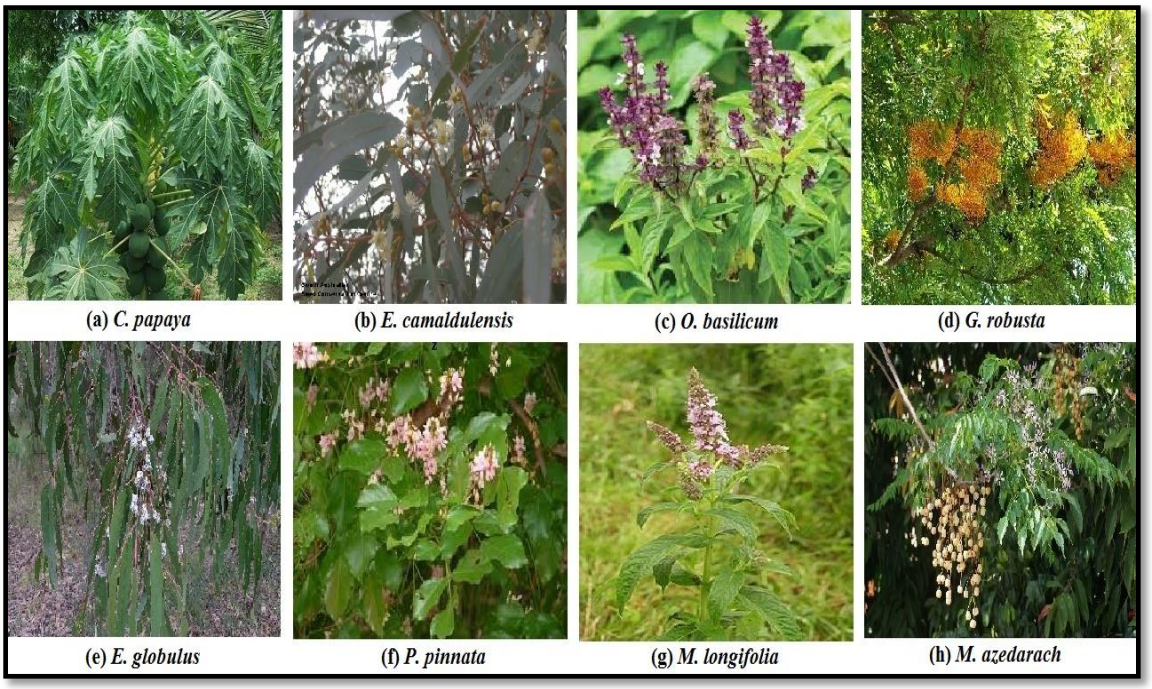


Figure 3.1: Plants selected for antitermitic, antiprotozoan and antibacterial bioassays.

3.3: Preparation of plant extracts

The leaves of each plant experimented were washed, shade dried at the room temperature (27-37^{o}C) and powdered mechanically with an electric blender (Daigger Scientific®, USA). Thirty grams of plant powder were subjected to obtain extract in 250ml of ethanol solvent by the Soxhlet apparatus at 40-60^{o}C (two cycles per hour) for 8 hours. The extract was concentrated by evaporating the solvent with a rotary vacuum evaporator (R-300, Rotavapor®, Germany). The stock solution was prepared by dissolving 1g of crude extract in 100ml of distilled water and different concentrations (100, 500, 1000, 1500 and 2000ppm) were prepared by applying formula $C_1V_1{=}C_2V_2$.

$$V1 = \frac{Required\ ppm \times Required\ Volume}{Stock\ Solution}$$

Where,

C_1: Concentration of stock solution; C_2: required concentration; V_2: required volume and V_1: volume to be removed.

3.4: Bioassays or experimental design

3.4.1: Termiticidal bioassay

The no-choice bioassays were used to evaluate the toxic potential of plant extracts (Elango *et al.,* 2012). Filter papers (Whatman No. 1, 2.5×2.5 inches) were pre-weighed and treated with 1.5ml of each plant extract (100, 500, 1000, 1500 and 2000ppm), air-dried and kept in Petri dishes (3×3 inches). The experiment was conducted in triplicates and control was treated with water only. Fifty termite workers of each species were placed in each Petri dish. A few drops of distilled water were periodically added to keep up the moisture contents. The Petri dishes were kept in a dark environment at 27±1^{o}C and 80±5 RH. Percentage mortality was calculated against each plant extract at every dose treatment after every 24 hours for sixteen days by applying formula.

$$\%\, Mortality = ODP \div TP \times 100$$

Where,

ODP: Observed dead population of workers; TP: Total population of workers

3.4.2: Protozoa counting

Protozoa count was made for evaluating the toxic potential of each plant extract against the hindgut flagellate population (Hassan *et al.,* 2017). After every twenty-four hours, two termite workers were removed and their hindgut contents were opened in 250µl of 0.2% saline solution using sterilized forceps and needle. The gut content of two termites was homogenized in sterilized micro-pestle and used as a single sample for each treatment. Protozoa number was calculated by loading 25µl of resulting gut content onto Neubauer's chamber (Bright-Line ^{TM} Sigma, USA) under a digital trinocular microscope (Optika® B-500, Italia). Counts were made by calculating the mean number of protozoa in four square of each chamber and results were compared with control for determining a decrease in the protozoa population (Lewis and Forschler, 2010).

$$X_{=} \frac{\left(No.\ of\ cell\ counted \times Volume\ of\ saline \frac{solution}{sample}\right)}{\left(Volume\ of\ Neubauer's\ c\square amber \times No. \frac{termites}{sampe}\right)}$$

3.4.3: Antibacterial bioassay

The antibacterial activity of different plant extract was assessed by agar well diffusion method on different bacterial strains isolated from the hindgut of *O. obesus* and *H. indicola*. Nutrient agar plates were prepared and 6 wells were pinched using sterile 5mm cork borer. Using a sterile cotton swab, the bacterial inoculum was uniformly spread on sterile agar plates. The 150µl of each plant extract (100, 500, 1000, 1500 and 2000ppm) was poured into the wells while using water as negative control and left for diffusion at room temperature. Plates were then incubated for 24 hours at 37˚C±1˚C. After 24 hours of incubation antibacterial activity of plants, the extract was evaluated and the zone of inhibition was measured in mm (Balouiri *et al.*, 2016).

3.5: Statistical analysis

Mean mortality of termites and protozoa for each plant extract and concentration was compared and statistically significant differences were determined by one-way ANOVA with Tukey's multiple comparison tests using "R language". Results with P value less than 0.05 were considered to be significant and less than 0.005 was considered strongly significant. Stars (*, **, ***) denote statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. LC_{50} value of each plant extract against termites and their gut protozoa was calculated by applying “Probit analysis” using SPSS version 19.

RESULTS

3.1: Antitermitic activity of plants extract against *O. obesus*

Antitermitic activity of leaves extract of eight plants in ethanol solvent against *O. obesus* was evaluated by no choice bioassay. Five different concentrations (100, 500, 1000, 1500 and 2000 ppm) were used in sixteen days experiment and the mean survival of termites was calculated. All concentrations were found to have significant effect on termite survival as compared to control ($F\ (df)$ = 289.0 (5); $P < 0.05$) in time-dependent manner, that also differed significantly from each other ($F\ (df)$ = 216.0 (16); $P < 0.05$). Similarly all plants showed differential antitermitic activity in dose and time-dependent manner ($F\ (df)$ = 209.6 (8); $P < 0.05$). The survival of *O. obesus* in controlled conditions is represented in figure 3.2 while the effect of days, concentrations and plants on mean survival of termites is represented in figure 3.3. Among the tested plants, *G. robusta* was found most effective with significant ($P<0.05$) reduction in survival of termites (94 %) at the end of the experiment. Similarly *O. basilicum*, *E. camaldulensis* and *E. globulus* also significantly ($P<0.05$) reduced termite population with 76, 60 and 40 % mortality respectively. However, *M. azedarach*, *C. papaya*, *M. longifolia*, and *P. pinnata* have a non-significant effect on termite survival with less than 30 % reduction in the termite population (figure 3.4). Level of significance of all plants and their LC_{50} values are represented in table 3.2 and 3.3.

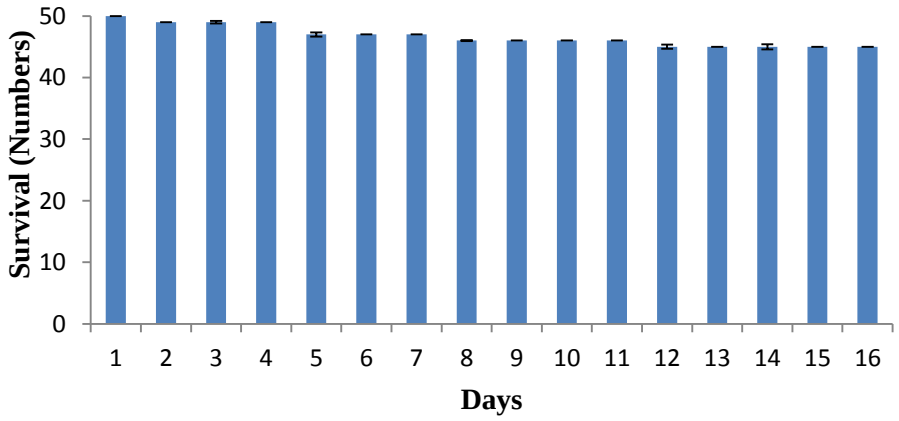


Figure 3.2: Survival of *O. obesus* in controlled conditions.

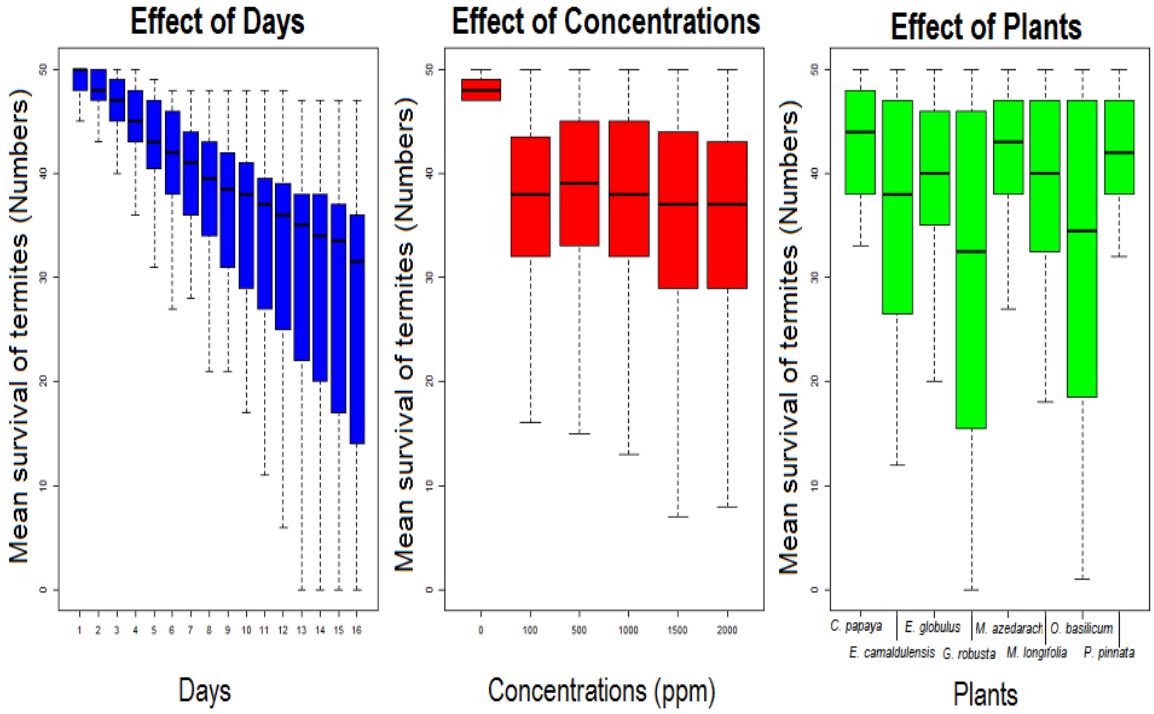


Figure 3.3: Box plot representing the effect of days, concentrations and plants on the mean survival of *O. obesus.*

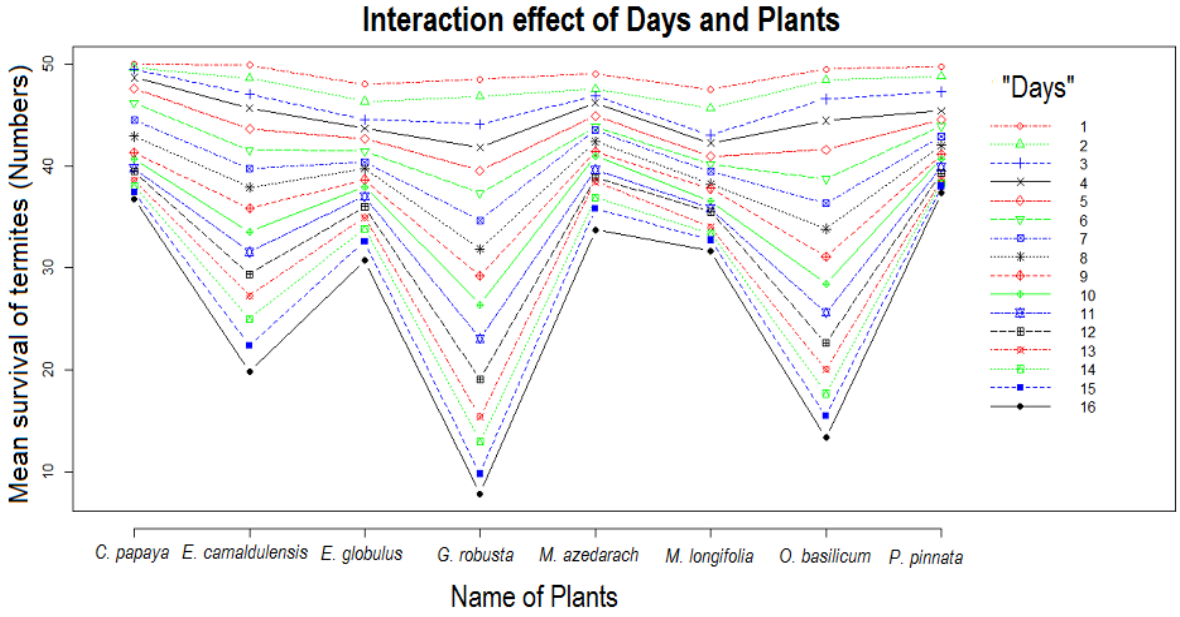


Figure 3.4: Interaction plot representing the interaction effect of plants extract with days on mean survival of *O. obesus.*

3.2: Antitermitic activity of plants extract against *H. indicola*

The activity of the above mentioned eight plant leaves extract was also evaluated against the mean survival of *H. indicola.* An almost similar response was observed in this termite species as in *O. obesus*. All concentrations were found to have significant effect on termite survival as compared to control (*F (df)* = 343.0 (5); P < 0.05) in time-dependent manner, that also differed significantly from each other (*F (df)* = 261.6 (16); P < 0.05). Similarly all plants showed differential antitermitic activity in dose and time-dependent manner (*F (df)* = 234.8 (8); P < 0.05). The survival of *H. indicola* in controlled conditions is represented in figure 3.5 while the effect of days, concentrations and plants on mean survival of termites is represented in figure 3.6. Among all plants used, *G. robusta* showed significant (p<0.05) decrease in the termite population with 50% termite death after the 10th day of the experiment. About 90% of termite mortality was recorded at the end of the experiment. Similarly, *E. camaldulensis, O. basilicum, E. globulus*, and *M. azedarach* were also found to have a significant (p<0.05) effect on termite survival and reduced their population up to 70, 65, 59 and 54% respectively. However, *C. papaya, M. longifolia*, and *P. pinnata* have a non-significant effect on termite survival with less than 30% reduction in the termite population (figure 3.7). Level of significance of all plants and their LC_{50} values are represented in table 3.2 and 3.3.

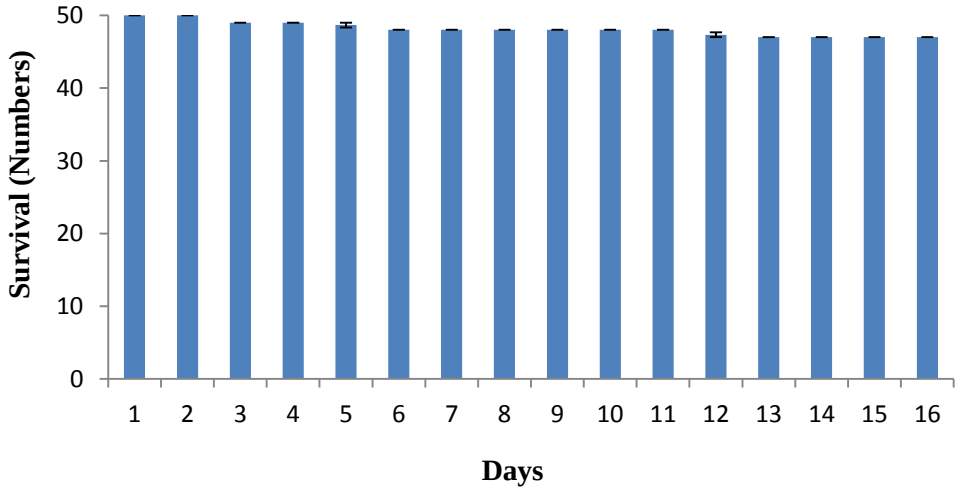


Figure 3.5: Survival of *H. indicola* in controlled conditions.

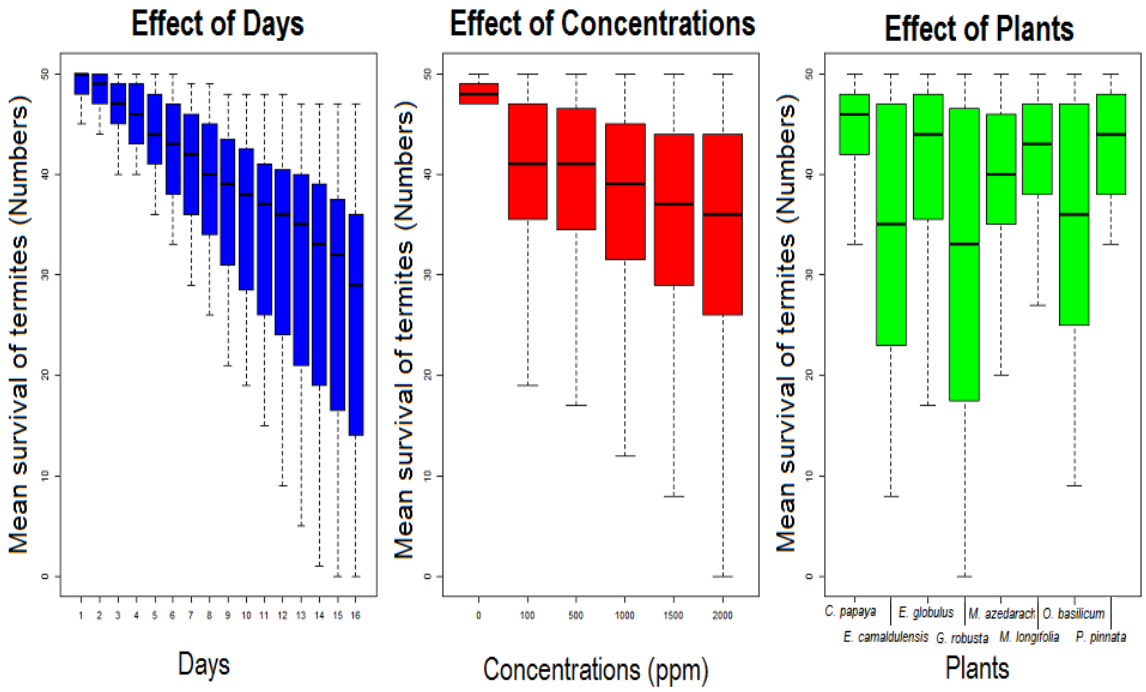


Figure 3.6: Box plot representing the effect of days, concentrations and plants on the mean survival of *H. indicola.*

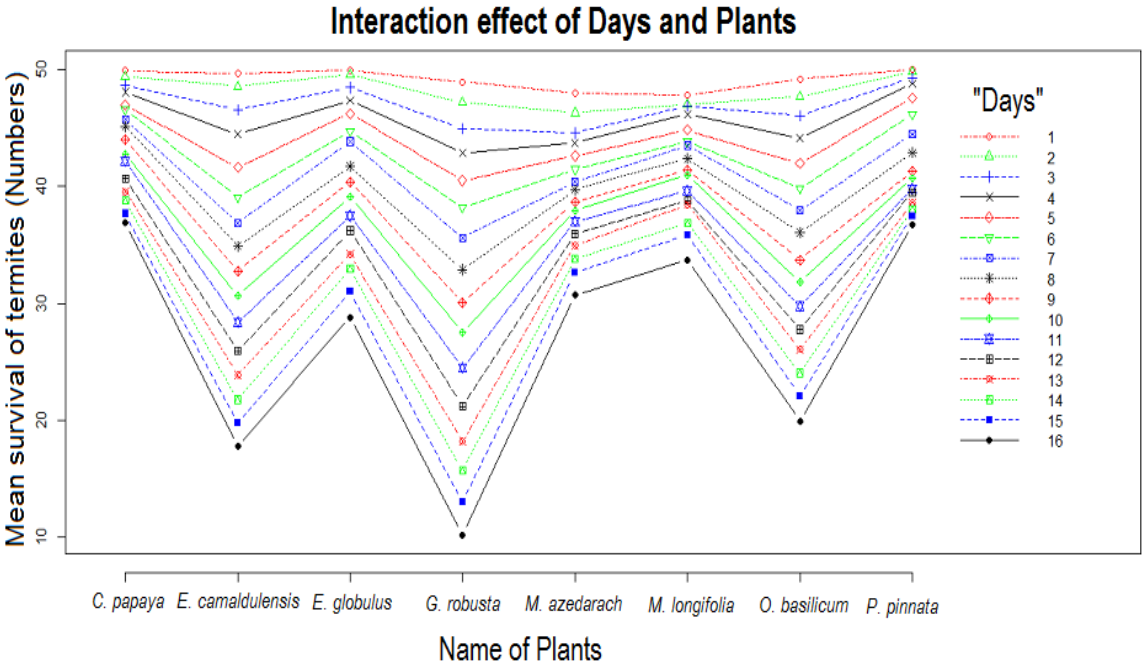


Figure 3.7: Interaction plot representing the interaction effect of plants extract with days on mean survival of *H. indicola.*

3.3: Protozocidal activity of plants extract

Protozocidal activity of above mentioned eight plants were also calculated against the mean survival of the protozoa population isolated from the hindgut of *H. indicola*. All concentrations were found to have significant effect on protozoa survival as compared to control ($F\ (df)$ = 553.5 (5); $P < 0.05$) in time-dependent manner, that also differed significantly from each other ($F\ (df)$ = 177.2 (7); $P < 0.05$). Similarly all plants showed differential protozocidal activity in dose and time-dependent manner ($F\ (df)$ = 141.3 (8); $P < 0.05$). The survival of protozoa in controlled conditions is represented in figure 3.8 while the effect of days, concentration and plants on mean survival of the protozoa population is represented in figure 3.9. Among all the plants *G. robusta*, *E. camaldulensis* and *O. basilicum* were found most effective plants and have significant ($P<0.05$) effect on survival of protozoa with 95, 78 and 74% reduction in the population at the end of the experiment. Similarly *C. papaya*, *E. globulus*, and *P. pinnata* also significantly ($P<0.05$) reduced protozoa population with 66, 56 and 46% mortality respectively. However, *M. longifolia* and *M. azedarach* have a non-significant effect on the protozoa population (figure 3.10). Level of significance of all plants and their LC_{50} values are represented in table 3.2 and 3.3.

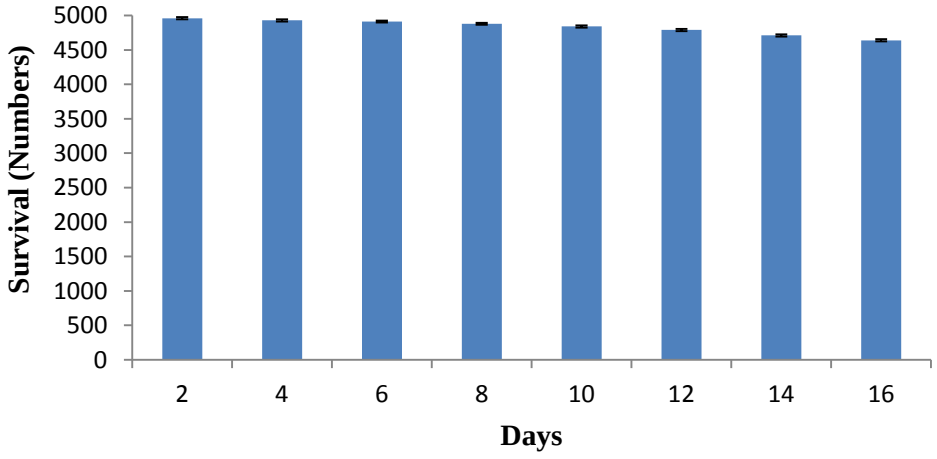


Figure 3.8: survival of the Protozoa population in the hindgut of *H. indicola* in controlled conditions.

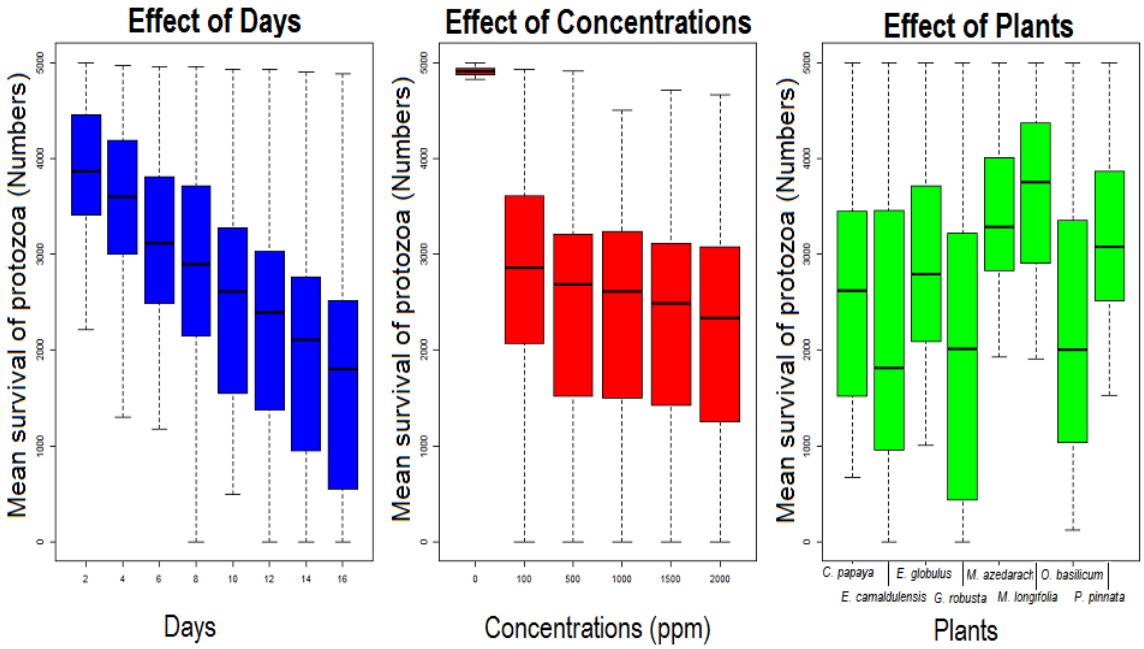


Figure 3.9: Box plot representing the effect of days, concentration and plants on the mean survival of protozoa isolated from the hindgut of *H. indicola.*

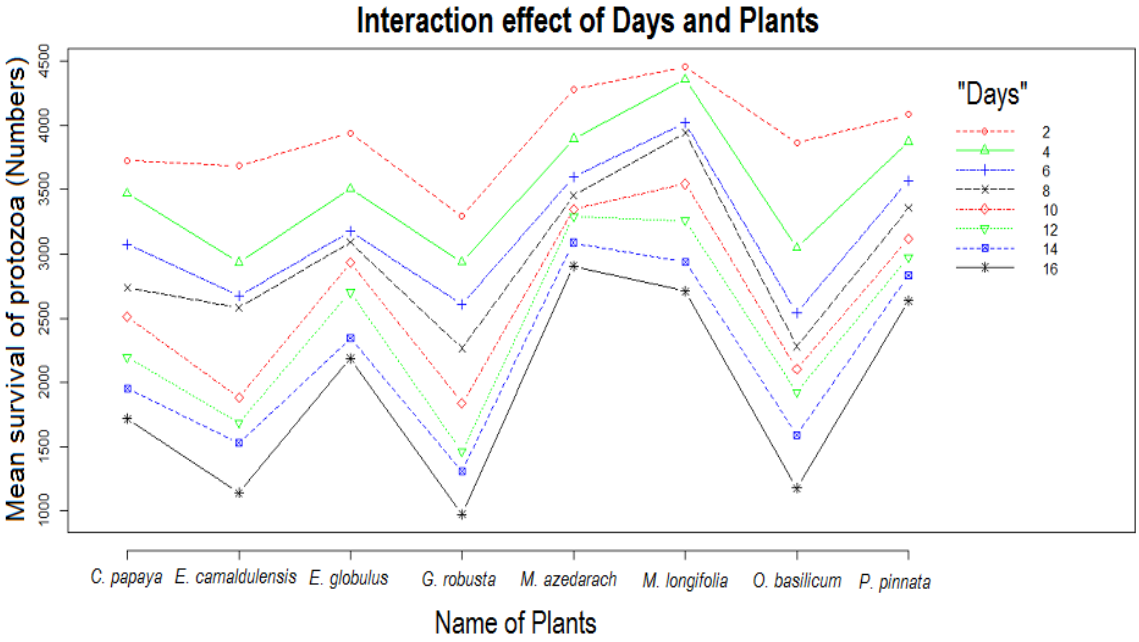


Figure 3.10: Interaction plot representing the interaction effect of plants extract with days on mean survival of protozoa isolated from the hindgut of *H. indicola.*

Table 3.2: Significance level of plants used for antitermitic activity against *O. obesus*, *H. indicola*, and its hindgut Protozoa.

Plants Name	Level of Significance		
	O. obesus	***H. indicola***	**Protozocidal**
C. papaya	Non-Significant	Non-Significant	Significant***
E. camaldulensis	Significant**	Significant**	Significant***
E. globulus	Significant*	Significant*	Significant**
G. robusta	Significant***	Significant***	Significant***
M. azedarach	Non-Significant	Significant*	Non-Significant
M. longifolia	Non-Significant	Non-Significant	Significant*
O. basilicum	Significant***	Significant**	Significant***
P. pinnata	Non-Significant	Non-Significant	Non-Significant

Table 3.3: LC_{50} values of plants used for antitermitic activity against *O. obesus*, *H. indicola*, and its hindgut Protozoa.

Plants Name	**LC_{50} (ppm)** **Upper confidence limit - Lower confidence limit**		
	O. obesus	***H. indicola***	**Protozocidal**
C. papaya	6329.7 (7478.7-5519.8)	3546.8 (3776.7-3350.4)	1046.2 (1052.6-1039.8)
E. camaldulensis	2973.6 (3231.4-2762.9)	2214 (2351.3-2097.0)	594.70 (601.77-588.29)
E. globulus	3148 (3375.3-2957.1)	2754.8 (2889.6-2635.8)	1373.2 (1380.4-1366.1)
G. robusta	1391.0 (1455.0-1331.8)	1607 (1686.0-1537.4)	502.64 (506.97-497.10)
M. azedarach	5308.7 (6093.7-4726.9)	3154.9 (3383.5-2962.5)	2475.9 (2498.8-2453.8)
M. longifolia	9408.1 (14283.4-7124)	5395.0 (6220.8-4788.1)	3093.4 (3129.3-3058.6)
O. basilicum	1926 (2039.5-1827.6)	2515.1 (6220.8-4788.1)	683.60 (688.63-678.56)
P. pinnata	5468.7 (6293.1-4860.3)	6240.5 (7348.1-5455.1)	2080.2 (2096.9-2063.8)

3.4: Antibacterial activity of medicinal plants

3.4.1: Potential of plants extracts against bacteria isolated from the hindgut of *O. obesus*

The potential of eight tropical plant extracts in ethanol solvent was tested against bacterial isolates from the hindgut of *O. obesus*. All plants showed differential antibacterial activity at all concentrations (100, 500, 1000, 1500 and 2000). *Pongamia pinnata* and *C. papaya* were found least effective plants having activity against two isolates only at higher concentration *i.e.* 1500 and 2000 ppm. *Pongamia pinnata* showed a zone of inhibition against *B. cereus* (4.3±0.03, 7.8±0.67mm) and *L. macrolides* (8.6±0.45, 9.5±0.89mm). Similarly, *C. papaya* also has a zone of inhibition against *B. cereus* (9.8±0.05, 12.0±0.45mm) and *E. coli* (5.7±0.06, 9.3±0.57mm). The minimum inhibitory concentration (MIC) of both plants was 1500ppm. *Melia azedarach* exhibit zone of inhibition against *L. fusiformis* at all above-mentioned concentrations *i.e.* 3.50±0.09, 4.90±0.57, 4.90±0.02, 5.30±0.03 and 6.90±0.88mm respectively having the minimum inhibitory concentration of 100ppm. However it was active against *L. macrolides* and *L. xylanilyticus* only at 1500 and 2000ppm having the zone of inhibition 8.06±0.92, 11.40±1.22mm and 7.06±0.31, 9.56±0.43mm respectively with MIC of 1500ppm (Table 3.4).

Mentha longifolia was found active against *B. cereus, L. macrolides* and *L. xylanilyticus* at higher concentrations (1000, 1500 and 2000ppm) having MIC of 1000ppm for *B. cereus* and 1500ppm for *L. macrolides* and *L. xylanilyticus*. *Eucalyptus globulus* showed activity against *B. cereus* (MIC=100ppm) and *E. coli* (MIC=100ppm) at all concentrations while active against *L. macrolides* only at 1500 and 2000ppm having the zone of inhibition 11.50±1.32 and 14.40±1.53mm respectively with minimum inhibitor concentration of 1500ppm. *Eucalyptus globulus* has the highest zone of inhibition (21.40±0.95mm) at 2000ppm against *E. coli* and lowest zone of inhibition (11.33±0.16mm) against *B. cereus* at 100ppm (Table 3.5). The minimum inhibitory concentration of *E. globulus* was 100ppm for *B. cereus* and *E. coli* while 1500ppm for *L. macrolides*. However, it does not have any growth inhibitory effect on *L. fusiformis* and *L. xylanilyticus* (Table 3.4).

The third most effective plant was *E. camaldulensis* having activity against all isolates having the minimum inhibitory concentration of 100ppm except *E. coli.* Its maximum zone of inhibition was 24.83±1.08mm at 2000ppm against *L. macrolides* and the minimum zone of inhibition was 5.00±0.65mm at 100ppm against *L. fusiformis*. *Osmium basilicum* was second most active plant at all concentration except 100ppm against *B. cereus, E. coli, L. macrolides*, and *L. xylanilyticus* while against *L. fusiformis* it was active only at 1500 and 2000ppm having the zone of inhibition 5.65±1.21 and 9.31±1.87mm respectively. Its maximum zone of inhibition was recorded against *L. macrolides* (14.89±1.32, 20.16±2.12, 22.89±0.87 and 23.33±1.18mm) at all active concentrations as compared to the rest of the isolates. The minimum inhibitory concentration of *O. basilicum* was 500ppm for all tested organisms except *L. fusiformis* (MIC=1500ppm). *Grevillea robusta* was the most effective among all the tested plants having activity against all isolates at all concentrations having a minimum inhibitory concentration of 100ppm against all bacterial isolates (Table 3.4). Its maximum zone of inhibition was recorded at 1500 and 2000ppm of against *L. macrolides* and *L. xylanilyticus i.e.* 23.33±1.56, 25.26±3.01mm and 26.33±0.87, 27.50±1.02mm respectively. Similarly, its lowest zones of inhibition were recorded against *L. fusiformis* at all above-mentioned concentrations having values 5.4±0.03, 8.52±1.74, 12.26±1.36, 13.50±1.20 and 15.00±1.44mm respectively (Table 3.5).

Table 3.4: Minimum inhibitory concentration of plants extract against five bacterial isolates from the hindgut of *O. obesus*.

Plants	**Minimum inhibitory concentration (ppm)**				
	L. fusiformis	***B. cereus***	***E. coli***	***L. macrolides***	***L. xylanilyticus***
P. pinnata	-	1500	-	1500	-
E. camaldulensis	100	100	-	100	100
G. robusta	100	100	100	100	100
M. azedarach	100	-	-	1500	1500
M. longifolia	-	1500	-	1500	1500
O. basilicum	1500	100	500	500	500
E. globulus	-	100	100	1500	-
C. papaya	-	1500	1500	-	-

Table 3.5: Zone of inhibition (mm) measured against five bacterial isolates from the hindgut of *O. obesus*.

Bacterial isolates	**Concentration (ppm)**	**Zone of inhibition (mm ± S.E)**							
		P. pinnata	***E. camaldulensis***	***G. robusta***	***M. azedarach***	***M. longifolia***	***O. basilicum***	***E. globulus***	***C. papaya***
L. fusiformis	**100**	00±00	5.00±0.65	5.4±0.03	3.50±0.09	00±00	00±00	00±00	00±00
	500	00±00	7.82±0.23	8.52±1.74	4.90±0.57	00±00	00±00	00±00	00±00
	1000	00±00	9.07±0.42	12.26±1.36	4.90±0.02	00±00	00±00	00±00	00±00
	1500	00±00	12.00±0.76	13.50±1.20	5.30±0.03	00±00	5.65±1.21	00±00	00±00
	2000	00±00	13.66±1.20	15.00±1.44	6.90±0.88	00±00	9.31±1.87	00±00	00±00
B. cereus	**100**	00±00	5.82±0.02	10.00±0.86	00±00	00±00	00±00	11.33±0.16	00±00
	500	00±00	9.91±0.64	17.66±2.94	00±00	00±00	13.52±1.72	15.50±1.45	00±00
	1000	00±00	10.6±0.06	18.16±0.72	00±00	4.3±0.02	16.28±2.76	16.83±2.30	00±00
	1500	4.3±0.03	11.00±0.02	18.63±3.75	00±00	5.0±0.48	17.83±1.54	18.50±0.76	9.8±0.05
	2000	7.8±0.67	12.50±1.04	19.63±3.73	00±00	8.5±0.23	17.53±0.32	20.16±2.16	12.0±0.45
E. coli	**100**	00±00	00±00	13.00±0.57	00±00	00±00	00±00	15.56±1.16	00±00
	500	00±00	00±00	15.43±1.05	00±00	00±00	12.5±0.54	17.20±0.86	00±00
	1000	00±00	00±00	16.00±2.40	00±00	00±00	17.24±1.76	18.91±1.01	00±00
	1500	00±00	00±00	18.06±1.31	00±00	00±00	19.10±2.21	21.34±1.32	5.7±0.06
	2000	00±00	00±00	20.66±0.72	00±00	00±00	20.76±0.60	21.40±0.95	9.3±0.57
L. macrolides	**100**	00±00	8.55±1.76	12.75±0.47	00±00	00±00	00±00	00±00	00±00
	500	00±00	12.16±0.12	18.54±1.87	00±00	00±00	14.89±1.32	00±00	00±00
	1000	00±00	17.16±1.45	22.80±0.50	00±00	00±00	20.16±2.12	00±00	00±00
	1500	8.6±0.45	21.55±2.25	23.33±1.56	8.06±0.92	8.3±0.05	22.89±0.87	11.50±1.32	00±00
	2000	9.5±0.89	24.83±1.08	25.26±3.01	11.40±1.22	11.0±0.02	23.33±1.18	14.40±1.53	00±00
L. xylanilyticus	**100**	00±00	10.45±0.36	9.50±0.76	00±00	00±00	00±00	00±00	00±00
	500	00±00	14.687±2.01	16.16±2.61	00±00	00±00	14.36±0.47	00±00	00±00
	1000	00±00	16.54±0.57	25.00±1.04	00±00	00±00	19.66±1.92	00±00	00±00
	1500	00±00	19.96±1.30	26.33±0.87	7.06±0.31	10.5±0.97	19.16±1.16	00±00	00±00
	2000	00±00	22.54±1.32	27.50±1.02	9.56±0.43	12.16±1.01	21.66±2.68	00±00	00±00

3.4.2: Potential of plants extracts against bacteria isolated from the hindgut of *H. indicola*

The potential of plant extract was also evaluated against bacterial isolates of *H. indicola.* All plants showed differential antibacterial activity at all concentrations (100, 500. 1000, 1500 and 2000ppm). *Pongamia pinnata* was least effective among all tested plants and have the zone of inhibition against isolate *B. subtilis* at 2000ppm and *L. fusiformis* at 1500 and 2000ppm. Second least effective plant was *C. papaya* that has the zone of inhibition against *B. subtilis* at 1500 and 2000ppm (9.0±0.32mm, 10.3±0.08mm) and against *S. sonnei* at all concentrations with the zone of inhibition 2.8±0.01, 5.0±0.01, 5.0±0.57, 11.3±0.08 and 11.8±0.02mm respectively. Similarly, *M. azedarach, M. longifolia,* and *E. globulus* were also active against only two isolates. *Melia azedarach* was active against *B. subtilis* (MIC=500ppm) and *L. fusiformis* (MIC=100ppm) at above all mentioned concentrations having the zone of inhibition 00±00, 6.83±0.72, 9.93±1.05, 12.00±0.76, 15.66±1.20mm and 5.30±0.57, 7.09±0.12, 11.83±1.48, 15.06±0.31, 19.90±0.57mm respectively (Table 3.7).

Mentha longifolia has activity only against *B. subtilis* and *B. cereus* at all concentrations except 100ppm with the zone of inhibition 2.9±0.35, 4.9±0.57, 8.0±0.12, 10.3±0.08mm and 4.57±0.54, 5.80±2.30, 7.16±0.72, 7.50±0.57mm respectively. The minimum inhibitory concentration of *M. longifolia* was 500ppm for both bacterial strains. *Eucalyptus globulus* was also effective against *B. subtilis* (MIC=1000ppm) and *E. coli* (MIC=100ppm). It showed a zone of inhibition at 1000ppm (11.83±1.48mm), 1500ppm (14.06±1.31mm) and 2000ppm (18.90±0.57) against *B. subtilis*. While it was found active at all above-mentioned concentrations against *E. coli* having the zone of inhibition 5.50±0.98, 6.67±2.12, 7.72±0.06, 9.3±0.57 and 12.30±0.01 respectively (Table 3.7).

Osmium basilicum showed antibacterial activity against *B. subtilis*, *E. coli* and *B. cereus*. The highest zone of inhibition (22.10±0.18mm) of *O. basilicum* was reported against *B. cereus* at 2000ppm and the lowest zone of inhibition (9.30±1.01mm) was against *B. subtilis* at 1500ppm. The minimum inhibitory concentration of *O. basilicum* against *B. subtilis*, *E. coli* and *B. cereus* was 1000, 1500 and 100ppm respectively (Table

3.6). The second most effective plant was *E. camaldulensis* against all isolates except *E. coli* at all above-mentioned concentrations. It was found least effective at 100ppm having no zone of inhibition against all isolates except *B. cereus* (7.67±0.56mm) while antibacterial activity was increased with increase in concentration having the highest zone of inhibition at 2000ppm with 19.54±0.57, 20.73±0.88, 22.75±0.56 and 21.66±2.68mm against *B. subtilis*, *S. sonnei*, *B. cereus*, and *L. fusiformis* respectively. Its minimum inhibitory concentration was 100ppm for *B. cereus*, 500ppm for *S. sonnei* and *L. fusiformis* while 1500ppm for *B. subtilis*. *Grevillea robusta* was found most effective among tested plants and showed zone of inhibition against all isolates at all concentrations except *B. subtilis* at 100 and 500ppm where no zone of inhibition was recorded (Table 3.7). The minimum inhibitory concentration of *G. robusta* was 100ppm for all bacterial strains except *B. subtilis* having MIC of 1000ppm (Table 3.6).

Table 3.6: Minimum inhibitory concentration of plants extract against five bacterial isolates from the hindgut of *H. indicola*.

Plants	**Minimum inhibitory concentration (ppm)**				
	B. subtilis	***S. sonnei***	***E. coli***	***B. cereus***	***L. fusiformis***
P. pinnata	2000	-	-	2000	1500
E. camaldulensis	1500	500	-	100	500
G. robusta	1000	100	100	100	100
M. azedarach	500	-	-	-	100
M. longifolia	500	-	-	500	-
O. basilicum	1000	-	1500	100	-
E. globulus	1000	-	100	-	-
C. papaya	1500	100	-	-	-

Table 3.7: Zone of inhibition (mm) measured against five bacterial isolates from the hindgut of *H. indicola*.

Bacterial isolates	**Concentration (ppm)**	**Zone of inhibition (mm ± S.E)**							
		P. Pinnata	***E. camaldulensis***	***G. robusta***	***M. azedarach***	***M. longifolia***	***O. basilicum***	***E. globulus***	***C. papaya***
B. subtilis	**100**	00±00	00±00	00±00	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	6.83±0.72	2.9±0.35	00±00	00±00	00±00
	1000	00±00	00±00	10.06±0.74	9.93±1.05	4.9±0.57	9.30±1.01	11.83±1.48	00±00
	1500	00±00	16.83±0.64	12.75±0.47	12.00±0.76	8.0±0.12	11.26±1.07	14.06±1.31	9.0±0.32
	2000	4.5±1.16	19.54±0.57	14.50±0.76	15.66±1.20	10.3±0.08	13.46±1.03	18.90±0.57	10.3±0.08
S. sonnei	**100**	00±00	00±00	13±0.86	00±00	00±00	00±00	00±00	2.8±0.01
	500	00±00	11.06±0.92	18.16±1.30	00±00	00±00	00±00	00±00	5.0±0.01
	1000	00±00	13.40±1.22	21.66±1.16	00±00	00±00	00±00	00±00	5.0±0.57
	1500	00±00	18.06±0.80	22.16±1.30	00±00	00±00	00±00	00±00	11.3±0.08
	2000	00±00	20.73±0.88	23.16±1.16	00±00	00±00	00±00	00±00	11.8±0.02
E. coli	**100**	00±00	00±00	10.83±0.72	00±00	00±00	00±00	5.50±0.98	00±00
	500	00±00	00±00	17.83±0.92	00±00	00±00	00±00	6.67±2.12	00±00
	1000	00±00	00±00	19.33±1.48	00±00	00±00	00±00	7.72±0.06	00±00
	1500	00±00	00±00	19.80±1.74	00±00	00±00	11.50±1.32	9.3±0.57	00±00
	2000	00±00	00±00	21.93±1.88	00±00	00±00	14.40±1.53	12.30±0.01	00±00
B. cereus	**100**	00±00	7.67±0.56	11.33±1.20	00±00	00±00	10.16±0.72	00±00	00±00
	500	00±00	10.29±0.12	16.83±3.72	00±00	4.57±0.54	13.54±0.54	00±00	00±00
	1000	00±00	15.16±2.65	17.50±0.76	00±00	5.80±2.30	18.36±0.92	00±00	00±00
	1500	00±00	19.34±1.18	17.83±1.64	00±00	7.16±0.72	20.66±1.87	00±00	00±00
	2000	6.3±0.02	22.75±0.56	19.83±1.64	00±00	7.50±0.57	22.10±0.18	00±00	00±00
L. fusiformis	**100**	00±00	00±00	15.50±2.36	5.30±0.57	00±00	00±00	00±00	00±00
	500	00±00	14±1.32	18.33±1.16	7.09±0.12	00±00	00±00	00±00	00±00
	1000	00±00	20.16±2.12	19.16±1.16	11.83±1.48	00±00	00±00	00±00	00±00
	1500	7.65±2.15	19.16±1.16	21.16±1.30	15.06±0.31	00±00	00±00	00±00	00±00
	2000	9.33±0.57	21.66±2.68	24.54±1.32	19.90±0.57	00±00	00±00	00±00	00±00

DISCUSSION

Termites are wood-feeding (lower termites) and soil feeding, fungus growing (higher termites) insects that usually built nests and construct tunnels in the ground, humid areas and wooden material (Ahmad *et al*., 2006). They are an important pest of wood and standing crops worldwide and cause a 12% loss in crop production due to lack of plantation knowledge in Pakistan (Satter and Saliha, 2001). Their ability to digest wooden material is always carried out with the help of symbiotic microbiota (protozoa and bacteria) residing in their hindgut.

For the control of termites, synthetic insecticides have been used from the last few decades that have resulted in insecticidal resistance and human health disorders and environmental issues. So the replacement of synthetic insecticides with bio-pesticides is strongly required (Venkateswara *et al*., 2005) as they are a rich source of bioactive compounds and less toxic (Scott *et al.,* 2004). In the past, many studies have been conducted to evaluate the toxic effect of plants on termites and their hindgut endosymbionts (Manzoor, 2010).

Abbas *et al.* (2013) reported the antitermitic activity of fifteen medicinal plants including *Ricinus communis, Croton tiglium, Ocimum basilicum, Psoralea corylifola, Nigella sativum, Mentha sp., Cichorium intybus, Peganum harmala, Foeniculum vulgare, Allium sativum,, Plantago ovate, Moringa oleifera, Capsicum frutescens, Melia azedarach* and *Azadirachta indica* against *O. obesus* and found that *O. basilicum, P. harmala*, and *F. vulgare* have potential to be used as antitermitic agents. These plants have many bioactive constituents that can be used against termites. In previous studies essential oils of some plants including clove bud, cedarwood, *Cassia fistula* leaves vetiver grass lemongrass and *Eucalyptus* oil have been reported to exhibit significant toxicity against Formonsanus subterranean termite (Zhu *et al.,* 2001).

In the present study antitermitic, the antiprotozoan and antibacterial activity of ethanolic leaves extracts of eight medicinal plants including *C. papaya, E. camaldulensis, E. globulus, G. robusta, M. azedarach, M. longifolia, O. basilicum,* and *P. pinnata* have been assessed against *H. indicola* and *O. obesus*. For antitermitic and antiprotozoan no-

choice bioassays were conducted b using treated filter papers with different concentrations (100, 500, 1000, 1500 and 2000ppm) of plant extract and results were compared with control which showed significant mortality in both termite species at different dose levels. Among tested plants, *G. robusta* was found to have promising activity and cause significant ($P<0.05$) mortality in the termite population. About 50 % termite death was recorded on the 10^{th} day of experiment leading to 90 and 94% mortality at the end of the experiment in both termite species *i.e. H. indicola* and *O. obesus*

E. camaldulensis and *O. basilicum* were also reported to have significant ($P<0.05$) effect on termite survival and reduce up to 70 and 65% *H. indicola* population but they differ non-significantly from each other ($P=0.378$). However, in the case of *O. obesus*, *O. basilicum* was found to be more effective than *E. camaldulensis* and cause 76 and 60% mortality in the termite population. Similarly, *E. globulus* and *M. azedarach* were also active significantly ($P<0.05$) against termites but have non-significant ($P=0.20$) differences among each other. The results of the present study are comparable with a recent study conducted by Hassan *et al.,* (2018) that showed that the population of *Reticulitermes flavipus* reduced significantly when exposed to the extracts of *Morus alba* and found that percentage mortality of termite was concentration-dependent. The activity profile of all tested plants against *H. indicola* is as follows; *G. robusta* > *E. camaldulensis* > *O. basilicum* > *E. globulus* > *M. azedarach* > *M. longifolia* > *C. papaya* > *P. pinnata.* Similarly, the activity profile of all tested plants against *O. obesus* is as follows; *G. robusta* > *O. basilicum* > *E. camaldulensis* > *E. globulus* > *M. longifolia* > *M. azedarach* > *C. papaya* > *P. pinnata.*

Protozocidal activity by feeding on extract-treated filter papers was also investigated against the endosymbiotic flagellate population of *H. indicola*. In previous studies, the toxic potential of some natural products against hindgut flagellates has been reported. One of the study conducted by Doolittle and his coworkers (2007) used bioactive compounds from neem extract, gleditschia from *Gleditschia triacanthas* and capsaicin from cayenne pepper (*Capsicum sp.*) against endosymbiotic fauna of Formosanus subterranean termites and evaluated that neembokil was more significant to decrease the population of *Pseudotrichonympha grassii* and *spirochaete* genus.

Similarly, the comparative effect of synthetic disodium octaborate tetrahydrate with natural *vetiver* oil and *nootkatone* on endosymbionts to *C. formosanus* was conducted in the laboratory trails for 12 months by Maistrello *et al.,* (2003). In choice and no-choice bioassays it was revealed that *nootkatone* and *vetiver* oil had significant ($P \leq 0.05$) effect on termite hindgut flagellates than disodium octaborate tetrahydrate.

In the present study leaves extract of eight medicinal plants was evaluated against protozoan fauna of *H. indicola* and found that all plants have differential protozocidal activity in a dose-dependent manner with an increase in time. Among all the plants, *G. robusta*, *O. basilicum* and *E. camaldulensis* showed high activity profile and cause significant ($P<0.05$) elimination of protozoan fauna from termite hindgut. However, they have non-significant differences among each other *i.e. G. robusta* and *E. camaldulensis* ($P=0.204$), *G. robusta* and *O. basilicum* ($P=0.030$), *E. camaldulensis* and *O. basilicum* ($P=0.996$). These results are compatible with the study conducted by Jones *et al.* (1983). They concluded that a reduction in the protozoa population was directly related to the filter paper area consumption treated with plant extract.

Similarly, *C. papaya*, *E. globulus* and *M. longifolia* infected protozoan population significantly ($P<0.05$) and cause a decline in their survival up to 66, 56 and 46% respectively, however, the termite survival rate was high in those experimental units where filter papers were treated with extract of these plants. The present findings are comparable with the study conducted by Hassan *et al.* (2018) on protozoan fauna of *Reticulitermes flavipus*. They reported that heartwood extracts of *Morus alba* at its highest concentration (10mg/ml) reduced protozoan population up to 50% but the survival rate of termites was 7%. So it is recommended that termite mortality is not only the result of the decline in protozoan population, however many other physiological phenomenon may also affect their survival while continuous exposure to plant extract (Raje *et al.,* 2015). Similarly, in the present study, the decline in the protozoan population was higher in treated termites but their survival rate was also high, suggesting that the plant extract might have some other mode of action which causes termite mortality. Our results are also supported by another study where the protozoa population was declined totally in termite hindgut when exposed to toxic compounds

that eventually lead to termite death (Mannesmann, 1972b). The activity profile of all tested plants against protozoa was as follows; *G. robusta* > *E. camaldulensis* > *O. basilicum* > *C. papaya* > *E. globulus* > *M. longifolia* > *P. pinnata* > *M. azedarach*

Numerous anaerobic, aerobic and facultative bacteria from the termite hindgut have been isolated and investigated form the last few decades and found that these bacteria have a significant role in lignocellulose digestion in association with protozoan fauna. Various species of facultative anaerobic bacteria from the hindgut of termites have been characterized at the molecular level to highlight their role in cellulose digestion (Adams and Boopathy, 2005). However slight investigations have been carried out to control termites by disrupting their bacteria (Waller, 1996), as these bacteria are of higher significance from an evolutionary point of view (Ohkuma *et al.,* 2001).

In the present study, the antibacterial activity of crude ethanolic leaves extracts of eight medicinal plants was assessed against bacterial isolates from the hindgut of two termite species (*O. obesus* and *H. indicola*). All plants exhibit differential antibacterial activity at all concentrations (100, 500, 1000, 1500 and 2000ppm), which increased with an increase in dose level. The difference in antibacterial activity of ethanolic leaves extract of these plants is might be due to the difference in their bioactive antibacterial compounds. The results of the present study are supported by Rabe & Van-Staden (1997) who reported the different response of gram positive and negative bacteria against plants extracts due to difference in thickness of their peptidoglycane layer.

Grevillea robusta, E. camaldulensis, and *O. basilicum* were found to have maximum antibacterial activity against almost all bacterial isolates of both species at all concentrations. The most promising activity was observed at higher dose levels *i.e.* 1500 and 2000ppm. The maximum zone of inhibition of *G. robusta* was 24.54±1.32mm and 27.50±1.02mm against *L. fusiformis* isolated from *H. indicola* and *O. obesus* respectively. Similarly, maximum inhibition of *E. camaldulensis* and *O. basilicum* were 22.75±0.50, 22.10±0.18 and 24.83±1.08, 22.33±1.18mm respectively against isolate 4 of both species. However, remaining plants were least effective and showed activity against

one or two isolates at higher concentrations. The results of the present findings are supported by Ghalem and Mohamed (2008). They reported that ethanolic leaves extracts of *E. camaldulensis* were a more potent antibacterial agent as compared to the *E. globulus* against *S. aureus.* Similar findings were also reported by Akin *et al.* (2010) that crude leaves extract of *E. camaldulensis* in ethanol has strong antibacterial activity against *B. subtilis, S. epidermidis, E. coli, S. marcescens,* and *S. aureus. Ocimum basilicum* is one of the most important plants among 60 species of *Ocimum* (Jirovetz *et al.,* 2003; Sacchetti *et al.,* 2004). Methanol, ethanol, chloroform, acetone and hexane extract of *O. basilicum* were used against *Shigella sp., S. aureus, L. monocytogenes,* and *P. aeruginosa.* Results showed that *O. basilicum* has promising antimicrobial activities against tested pathogens (Adiguzel *et al.,* 2005; Kaya *et al.,* 2008).

However contradictory results were observed against leaf extracts of *C. papaya* and *E. globulus* in the present study as they were found as least effective against bacterial isolates. Whereas antitumor, antibacterial, antifungal and wound healing activities of *C. papaya* were assessed by Baskaran *et al.* (2012). Alabi *et al.* (2012) and Baskaran *et al.* (2012) conducted antibacterial and antifungal activities of *C. papaya* leaves extract and found that antibacterial activities are more prominent then antifungal activities. However, the present results are very different from the previous studies as *C. papaya* exhibits very little antibacterial activity against isolate 2 of *O. obesus* at 2000ppm with the zone of inhibition 12.0±0.45mm. Similar results were observed for *E. globulus* that was not more effective in present studies but previously reported to have prominent antibacterial activities.

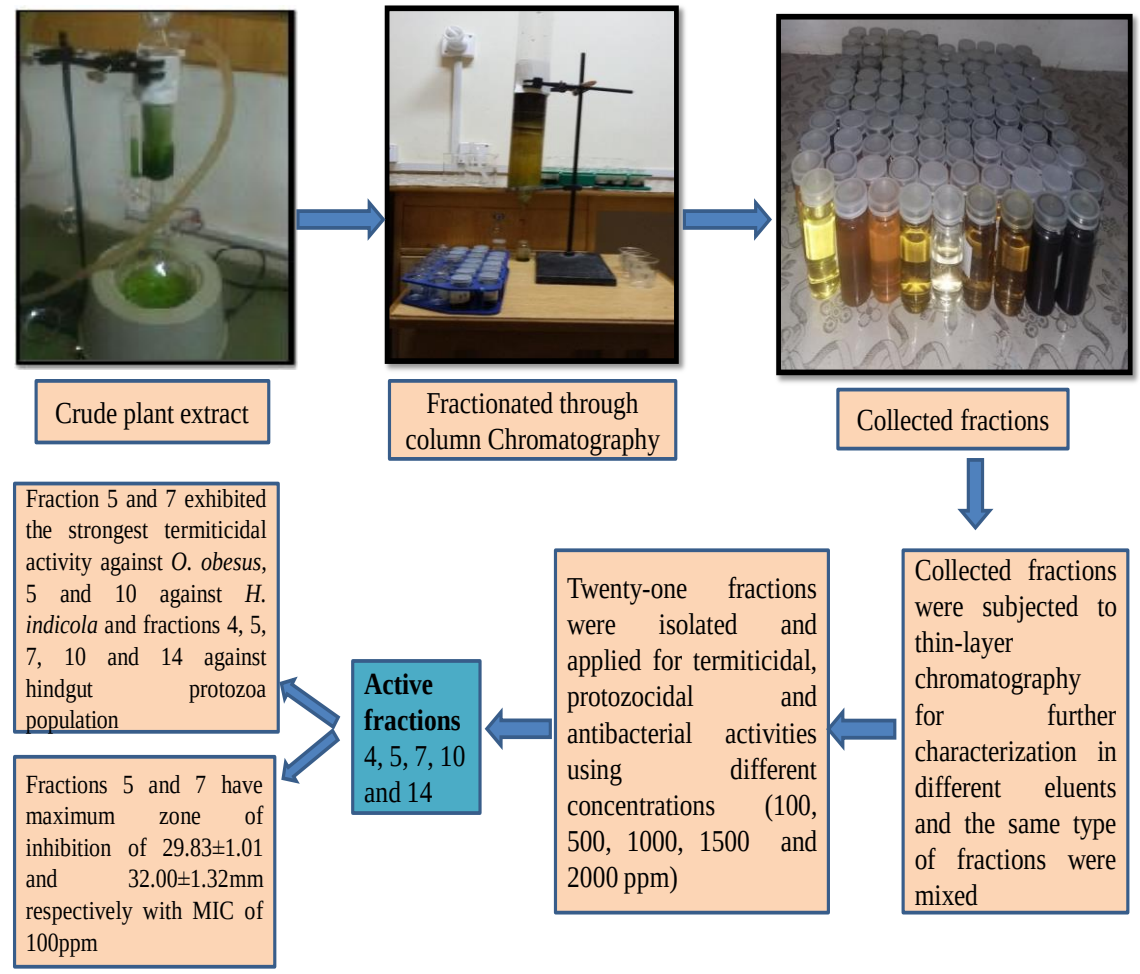
The effectiveness of these plants extract may be due to their hydrophobic nature which helps them to penetrate deep into bacterial cells, destroying their cellular organelles releasing essential molecules leading to bacterial cell death (Daroui-Mokaddem *et al.,* 2010). These preliminary studies indicate the effectiveness of *G. robusta, E. camaldulensis,* and *O. basilicum* could be used to control termites by targeting their gut bacteria.

It is concluded from the present study that plant extract could be an effective alternative for synthetic insecticides for the control of termites by targeting their hindgut endosymbionts. As bioactive insecticides, these plants contain phytochemical compounds that could be used in a refined form to synthesize such bioactive preservative that can be used to protect infestation and damage caused by termites. Moreover, these plants are easily available in local areas of Pakistan and affordable. Further studies are required for isolation, identification and characterization of bioactive compounds from *G. robusta* leaves extract by column chromatography and their characterization by GC-MS analysis for their application in industries.

ABSTRACT

Chromatographic techniques have significantly contributed to the discovery of novel compounds of biomedical and pharmaceutical importance. It opened a new gateway for isolation of pure secondary metabolites from crude plant extracts. Leaves and roots of *Grevillea robusta* Cunn. (Proteaceae) contains certain allelochemicals that inhibit the growth of surrounding plants. In the present study, the crude leaves extract of *G. robusta* was fractionated by column chromatography and further characterized by thin-layer chromatography using different solvents such as n-hexane, ethyl acetate and methanol for isolation of bioactive secondary metabolite. Twenty-one fractions were isolated and applied for termiticidal, protozocidal and antibacterial activities against *O. obesus*, *H. indicola* and their gut symbionts (protozoa and bacteria). Different concentrations (100, 500, 1000, 1500 and 2000ppm) were used in a no-choice bioassay using filter paper as a substrate. Among all the fractions used, 5 and 7 exhibited the strongest termiticidal activity against *O. obesus* having LC_{50} value of 1342.81 and 1273.18ppm respectively. Similarly, most effective fractions against *H. indicola* were 5 and 10 with an LC_{50} value of 1420.05 and 2214.77ppm respectively. Results of protozocidal bioassay showed that fractions 4, 5, 7, 10 and 14 were most active causing 78, 80, 82, 75 and 79% decline in hindgut protozoa population having LC_{50} value of 710.88, 578.34, 625.78, 536.85 and 793.01ppm respectively. The antibacterial activity results revealed that fractions 5 and 7 exhibited the strongest growth inhibitory effect having a maximum zone of inhibition 29.83±1.01 and 32.00±1.32mm respectively with the minimum inhibitory concentration of 100ppm against tested strains. The present study, it is concluded that out of twenty-one isolated fractions, 5 was found to most effective against all tested organism *i.e.* termites, protozoa and bacteria.

Graphical abstract



INTRODUCTION

Plant allelochemicals are natural compounds present in wood that unlike cellulose and lignin do not provide structural integrity but play an important role in defense against the attack of wood-eating insects (Maistrello *et al.,* 2003). These defensive compounds are added in the diet of termites in the form of extracts and forced to the gut by feeding that harms symbiotic fauna (protozoa and bacteria) of termite hindgut. The toxicity of these botanical extracts is related to reduce the feeding rate of the host on different food sources leading to the complete loss of symbionts ultimately causing termite death (Hassan *et al.,* 2018).

Medicinal plants are getting more attention in recent years as they are a rich source of a variety of bioactive secondary metabolites such as quinine, vincristine, morphine, codeine, digoxin, and atropine which are safe, cheaper and more efficient (Abdulhamid *et al*., 2017). These are chemically diverse compounds that are widely used in different pharmacological activities. Different phytochemicals belonging to various chemical classes were found to have an inhibitory effect on all types of microorganisms. Crude plant extracts derived from any part of the plant are believed to be biologically more effective than isolated compounds due to their synergistic effect (Yadav and Agarwala, 2011).

Although synthetic insecticides are a more powerful control agent for infestation caused by termite they have created a lot of problems related to human health, ecological imbalance, insect resistance and harm to the mammals (Potter and Hillery, 2002). Chemical insecticides especially persistent organochlorines are not only increasing resistance in targeted pests but also creating secondary pest outbreak and contamination of the global environment (Singh and Saratchandra, 2005; Chouvenc *et al.*, 2011). In developing countries wood preservation is mostly attained by chemical preservatives including borates and chromate copper arsenate that are highly toxic to human beings and environment; limiting indoor applications of synthetic insecticides (Ward *et al.,* 2009; Tascioglu *et al.,* 2013). Therefore is a strong urge for the development of biologically active phytochemicals, which may be expected to be less hazardous to the human health

and other non targeted organisms by minimizing their accumulation in the environment (Rahuman *et al.,* 2008). Therefore crude plant extract is subjected to different chromatographic techniques like column chromatography and thin-layer chromatography for isolation and purification of bioactive phytochemicals to synthesize valuable anticancer, antibiotic, insecticidal and herbicidal products (Ahmed *et al.,* 2016).

Column chromatography is one of the analytical techniques used for isolation and quantification of individual bioactive compound from a mixture of compounds (Church, 2005). It is commonly used for the preparation of synthetic chemicals from a very small amount to kilograms on a large scale. Column chromatography is advantageous over other analytical techniques because it is comparatively less expensive and the stationary phase used during the process is easy to dispose of which avoids cross-contamination and its degradation during the recycling process. Thin-layer chromatography is also an analytical technique used for the identification of compounds and to determine their purity (Abdulhamid *et al*., 2017).

Grevillea robusta Cunn. is commonly known as Australian silver oak belongs to family Proteaceae, which is an evergreen fast-growing tree. It is a native tree of Australia and in other subtropical and rainforest environment where average rainfall is more than 1,000mm per year (Ullah *et al*., 2014). *G. robusta* is a deciduous plant ranging from medium-sized to large trees 12-25 meter tall and can reach up to 40 meters in length. Flowering mostly occurs in October to November and the tree starts flowering when it is about 6 years old. Due to its height, attractive shape, and beautiful flowering, it is used as an ornamental plant in parks and Gardens (Orwa *et al*., 2009).

From the last few decades, serious efforts have been made for isolation and identification of bioactive compounds from medicinal plants and their biological properties were determined. A large number of these compounds are now being synthesized in the laboratory for commercial use in the pharmaceutical industry (Salihu and Usman, 2015). Extracts from different plant parts reported for termiticidal and protozocidal properties could be a good substitute for synthetic chemicals for termite control. Moreover, these naturally occurring compounds are a unique approach for the

development of new wood preservatives (Salem *et al.*, 2016). Therefore the main aim of this study was isolation and identification of bioactive fractions from crude plant extract by column chromatography and TLC. Furthermore the application of isolated fraction in the laboratory for evaluation of antitermitic, antiprotozoan and antibacterial activity.

MATERIALS AND METHODS

4.1: Preparation of crude plant extract

The powdered leaves (65gm) of *G. robusta* were extracted in 400ml of ethanol solvent by the Soxhlet extraction apparatus at 60^{o}C for 8-10 hours (6 cycles per hour). Detailed methodology is given in chapter 2. After extraction, the extract was evaporated using a vacuum rotary evaporator (R-300, Rotavapor®, Germany) to get crude plant extract which was used further for column and thin-layer chromatography.

4.2: Fractions isolation through column chromatography

4.2.1: Column packing

For isolation of different fractions from plant extract, a column (28×3 inches) was prepared with silica gel (0.063-0.200mm) in dry powder form and kept for overnight. The column was run with 300ml of n-hexane for settling down (figure 4.1).

Figure 4.1: Column used for isolation of fractions from the crude extract of *G. robusta.*

4.2.2: Loading of sample and column treatment

The crude ethanolic extract of *G. robusta* (15gm) was poured to the silica gel open chromatographic column and the step gradient technique was used to run the column in three different solvents *i.e.* n-hexane, ethyl acetate, and methanol in increasing order of their polarity. Various ratios of n-hexane and ethyl acetate (pure n-hexane, 20:80, 40:60, 80:20 and pure ethyl acetate) then ethyl acetate and methanol (pure ethyl acetate, 20:80, 40:60, 80:20 and pure methanol) were used for isolation of fractions at different rates.

4.3: Thin layer chromatography

Fractions initially collected from column chromatography were subjected to thin-layer chromatography (TLC) for further characterization in different eluents and the same type of fractions were mixed. Solvents were evaporated and crude fractions were used for the preparation of the stock solution.

4.4: Preparation of stock solution and dilutions

The stock solution of isolated fractions was prepared by dissolving 1g of crude extract in 100ml of distilled water and different dilutions (100, 500, 1000, 1500 and 2000ppm) were prepared using the formula $C_1V_{1=}C_2V_2$

$$V1 = \frac{Required\ ppm \times Required\ Volume}{Stock\ Solution}$$

4.5: Bioassays or experimental design of isolated fractions

4.5.1: Termiticidal bioassay

The no-choice bioassay was designed to evaluate the toxic potential of plant extract against *O. obesus* and *H. indicola* (Elango *et al*., 2012). Detailed methodology is given in chapter 3.

4.5.2: Protozoa counting

Protozoa count was made by the method described by Hassan *et al.* (2017) for evaluating the toxic potential of each fraction against the hindgut flagellate population. Detailed methodology is given in chapter 3.

4.5.3: Antibacterial bioassay

The antibacterial activity of different plant extract was assessed using agar well diffusion method on different bacterial strains isolated from the hindgut of *O. obesus* and *H. indicola* described by Balouiri *et al.* (2016). Detailed methodology is given in chapter 3.

4.6: Statistical analysis

Mean mortality of termites and protozoa for each plant extract and concentration was compared and statistically significant differences were determined by one-way ANOVA with Tukey's multiple comparison tests using "R language". Results with P value less than 0.05 were considered to be significant and less than 0.005 was considered strongly significant. Stars (*, **, ***) denote statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. LC_{50} value of each plant extract against termites and their gut protozoa was calculated by applying "Probit analysis" using SPSS software version 19.

RESULTS

4.1: Fractions collected through column chromatography

Initially, 172 fractions (20ml) were collected in fraction collecting tubes through column chromatography having silica gel column, which was further characterized by thin-layer chromatography (TLC) in different eluents (hexane, ethyl acetate, methanol) and the same type of fractions was mixed. Twenty-two samples were collected after TLC (figure 4.2). Fraction 1 was simple hexane because it didn't reveal the presence of any brand on the TLC plate.

Figure 4.2: Fractions collected through column chromatography

4.2: Antitermitic activity of fractions isolated from *G. Robusta*

4.2.1: Antitermitic activity against *O. obesus:*

Antitermitic activity of different fractions was investigated by no-choice bioassay against *O. obesus.* Twenty-one (2-22) fractions were used in sixteen days experiment and the mean survival of termites was calculated using different concentrations (100, 500, 1000, 1500 and 2000ppm). All concentrations were found to have significant effect on termite survival as compared to control ($F\ (df)$ = 591.5 (5); $P < 0.05$) in time-dependent manner, that also differ significantly from each other ($F\ (df)$ = 409.6 (16); $P < 0.05$). Similarly, all fractions showed differential antitermitic activity in dose and time-dependent manner ($F\ (df)$ = 244.7 (21); $P < 0.05$). The survival of termites in controlled conditions is represented in figure 4.3 while the effect of days, concentrations and

fractions on mean survival of termites is represented in figure 4.4. Fraction 5 and 7 were found to be most effective ($p<0.05$) having a 50% decline in termite population after one week with 90 and 78% decrease respectively at the end of the experiment. Fraction 7 caused an 18% decline after 24 hours of the experiment. Fraction 4, 8 and 10 also significantly ($p<0.05$) reduce termite survival up to 66, 60 and 68% respectively. Similarly, observed termite mortality due to the toxic effect of fraction 3, 6, 11, 13, 14 and 16 was 42, 34, 38, 36, 46 and 38% respectively. While fraction 2, 9, 12, 15, 17, 18, 19, 20, 21 and 22 were found to be least effective with non significant ($p>0.05$) decrease in termite population (figure 4.5). The level of significance of all fractions and their LC_{50} values are represented in table 4.1 and 4.2.

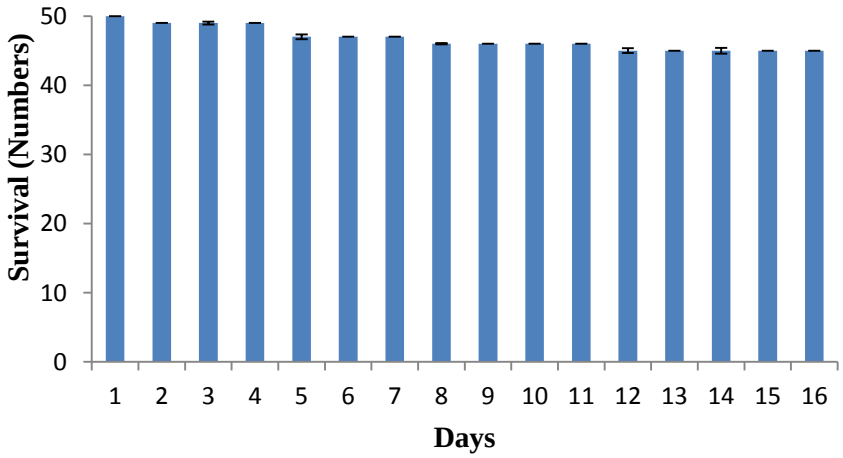


Figure 4.3: Survival of *O. obesus* in controlled conditions.

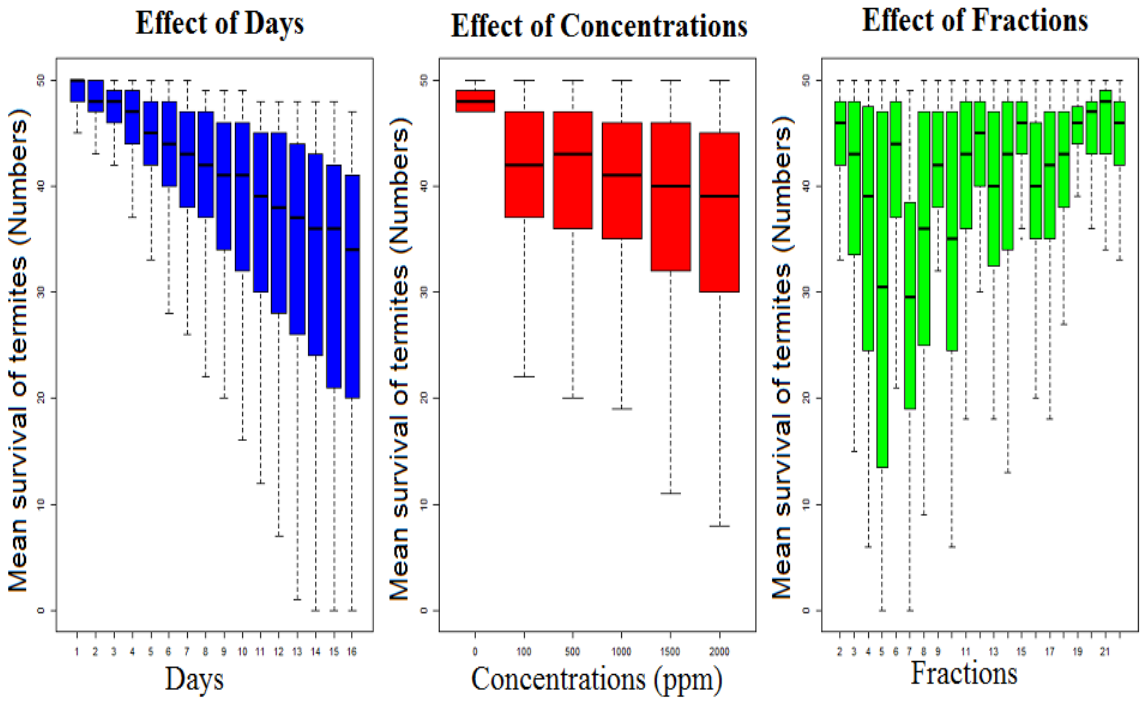


Figure 4.4: Box plot showing the effect of days, concentrations and fractions on the survival of *O. obesus.*

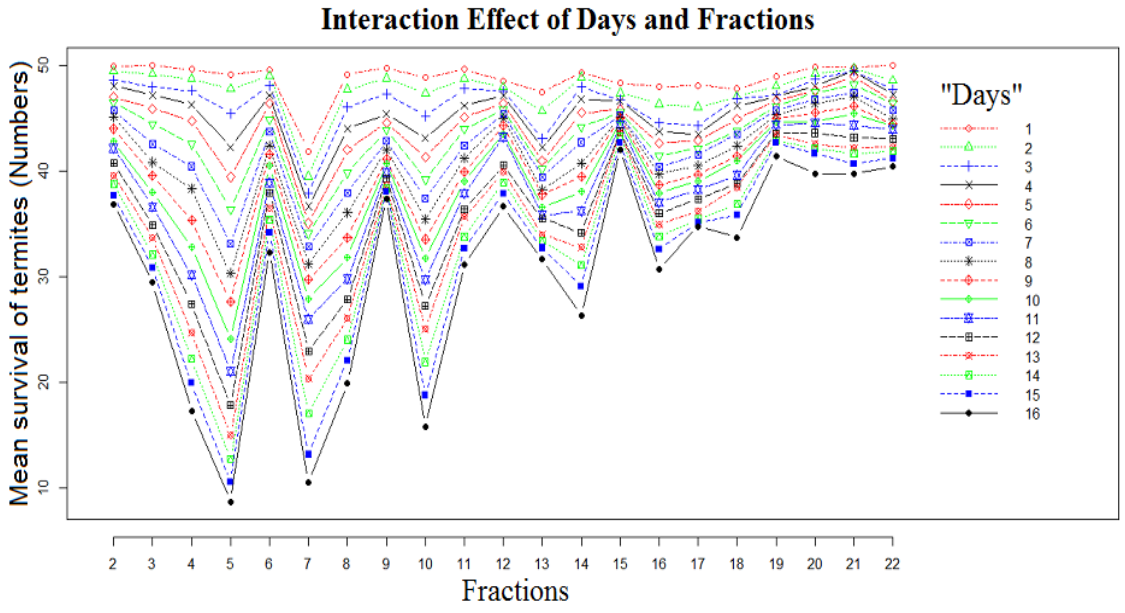


Figure 4.5: Interaction effect of days and fractions on the survival of *O. obesus.*

4.2.2: Antitermitic activity against *H. indicola*

Antitermitic activity of isolated fractions was also investigated against *H. indicola* by no-choice bioassay. Twenty-one (2-22) fractions were used in different concentrations (100, 500, 1000, 1500 and 2000ppm) in sixteen days experiment and the mean survival of termites was calculated. All concentrations were found to have significant effect on termite survival as compared to control ($F\ (df)$ = 516.9 (5); $P < 0.05$) in time-dependent manner, that also differ significantly from each other ($F\ (df)$ = 363.7 (16); $P < 0.05$). Similarly all fractions showed differential antitermitic activity in dose and time-dependent manner ($F\ (df)$ = 138.3 (21); $P < 0.05$). The survival of termites in controlled conditions is represented in figure 4.6 while effect of days, concentrations and fractions on mean survival of termites is represented in figure 4.7. Fraction 5 was found to be most effective that significantly ($p<0.05$) decrease the termite population with 92% mortality at the end of the experiment. The second most active fraction was 10 having a 64% decline while fraction 4, 7 and 14 showed 44, 50 and 40% decrease in termite population respectively. However, remaining all fractions were found to be least toxic with non-significant ($p>0.05$) decrease in termite population (figure 4.8). The level of significance of all fractions and their LC_{50} values are represented in table 4.1 and 4.2.

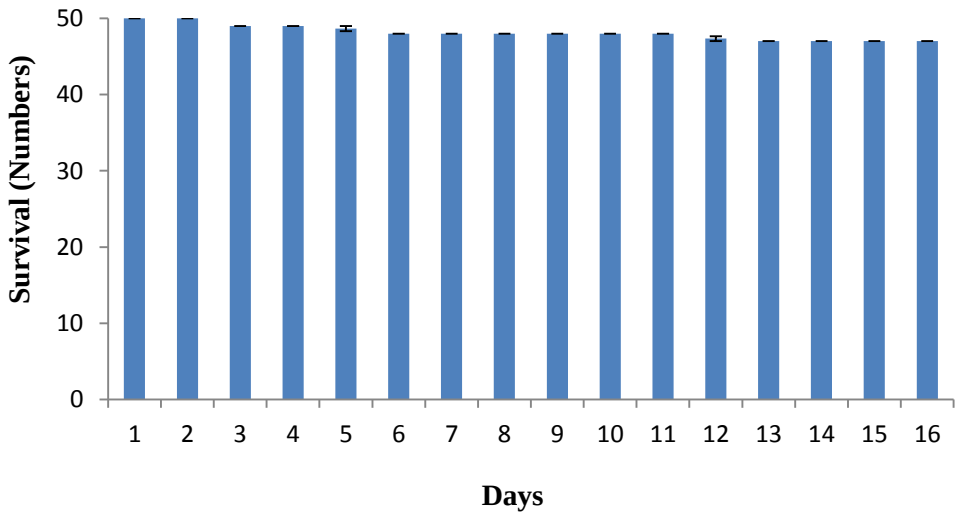


Figure 4.6: Survival of *H. indicola* in controlled conditions.

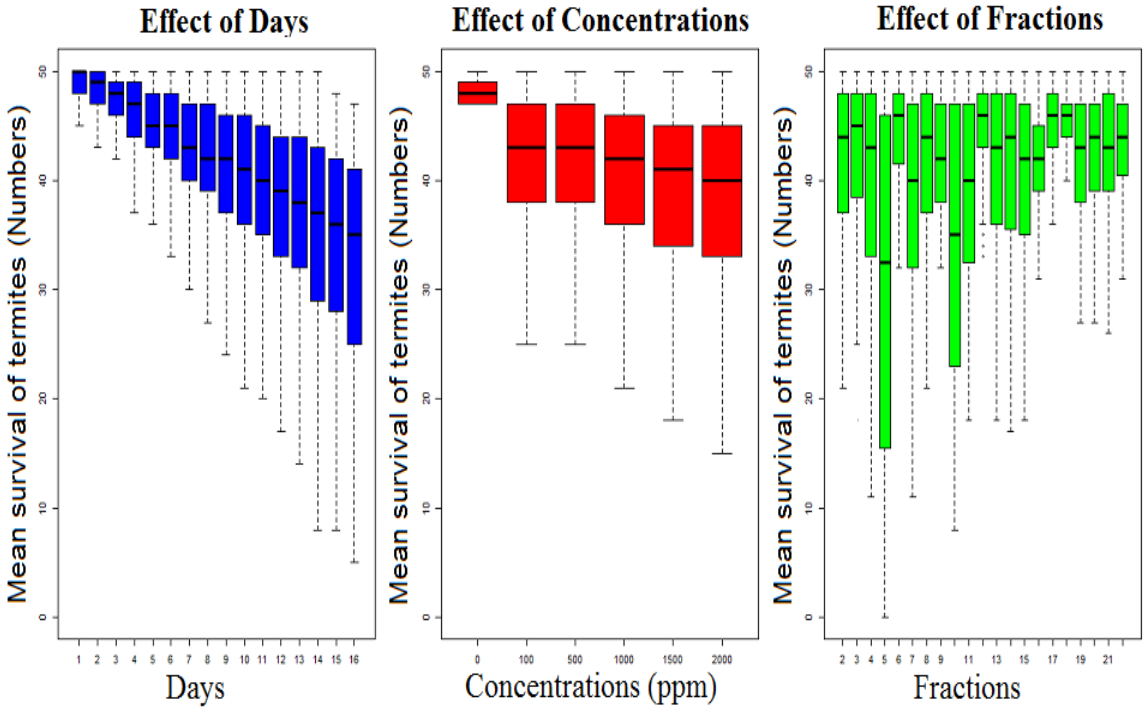


Figure 4.7: Box plot showing the effect of days, concentrations and fractions on the survival of *H. indicola.*

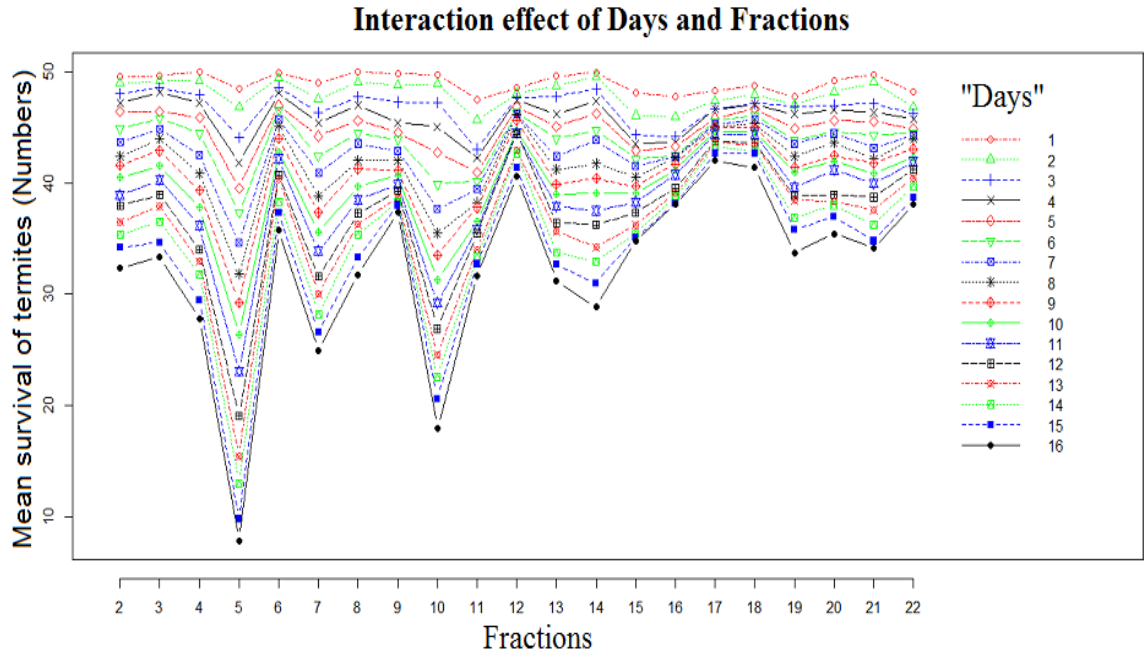


Figure 4.8: Interaction effect of days and fractions on the survival of *H. indicola.*

4.2.3: Anti-protozoan activity

Protozocidal activity of the above-mentioned fractions was calculated against the mean survival of the protozoa population from *H. indicola* hindgut. All the fractions exhibited varying degree of protozocidal activity and after two days of feeding on treated filter paper, flagellates population was reduced against each concentration. All concentrations were found to have significant effect on protozoa survival as compared to control ($F\ (df)$ = 2558.1 (5); $P < 0.05$) in time-dependent manner, that also differ significantly from each other ($F\ (df)$ = 991.3 (16); $P < 0.05$). Similarly all fractions showed differential protozocidal activity in dose and time-dependent manner ($F\ (df)$ = 154.9 (21); $P < 0.05$). The survival of protozoa in controlled conditions is represented in figure 4.9 while the effect of days, concentrations and fractions on mean survival of the protozoa population is represented in figure 4.10. All fractions affected the protozoa population in termite hindgut significantly ($p<0.05$) and have dose-dependent activity with increasing time. After 16 days of the experiment, fractions 5 and 7 were found to be most effective with 80 and 82% removal of symbiotic flagellates. Similarly fraction 4, 8, 10, 13 and 14 also cause decline in protozoa population significantly ($p<0.05$) with 78, 74, 75, 74 and 79% respectively. Fraction 9 was found least effective having a 40% decline in the protozoa population (figure 4.11). The level of significance of all fractions and their LC_{50} values are represented in table 4.1 and 4.2.

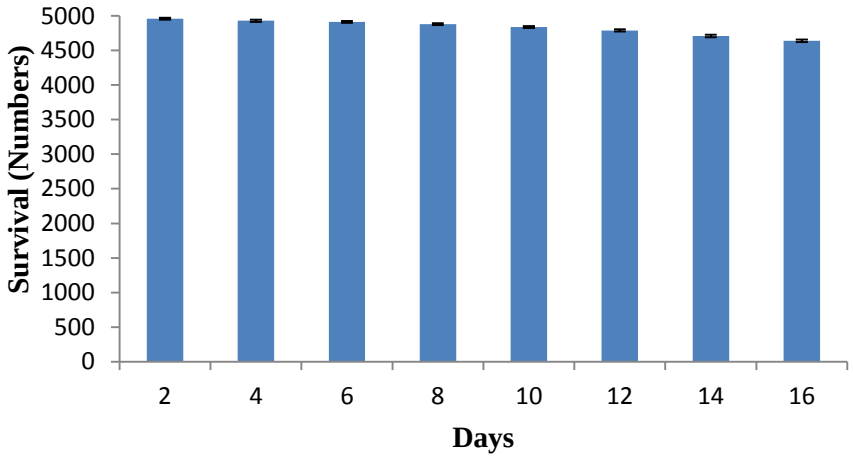


Figure 4.9: survival of the Protozoa population in the hindgut of *H. indicola* in controlled conditions.

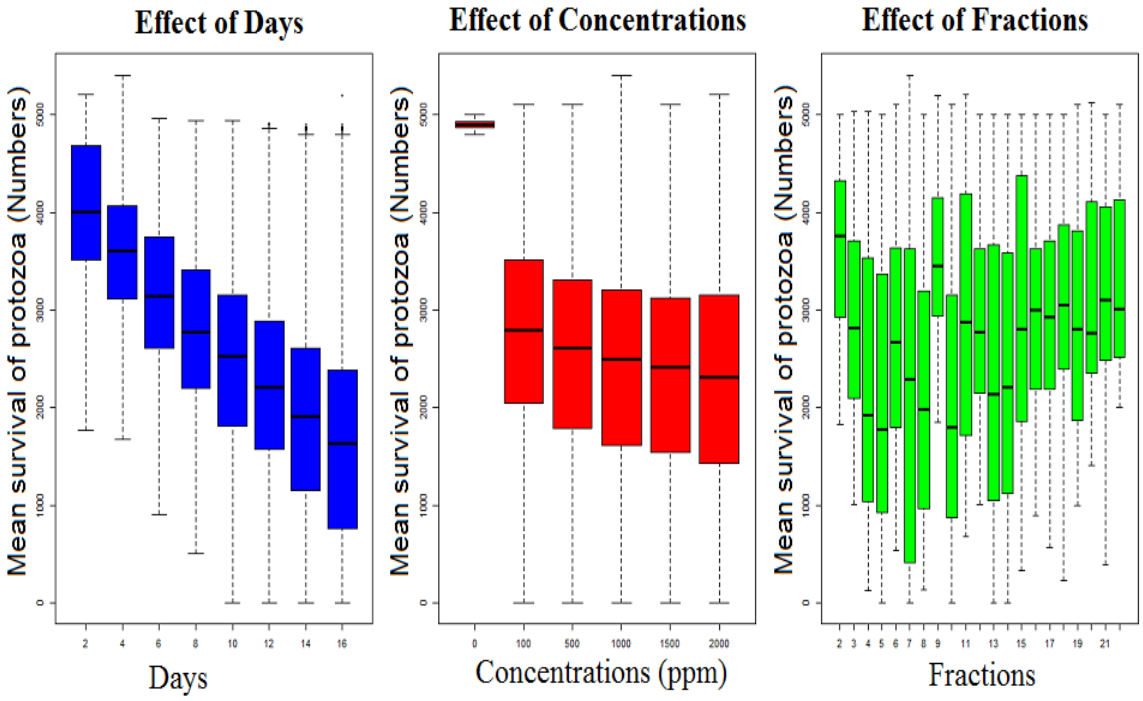


Figure 4.10: Box plot showing the effect of days, concentrations and fractions on the survival of the Protozoa population in the hindgut of *H. indicola.*

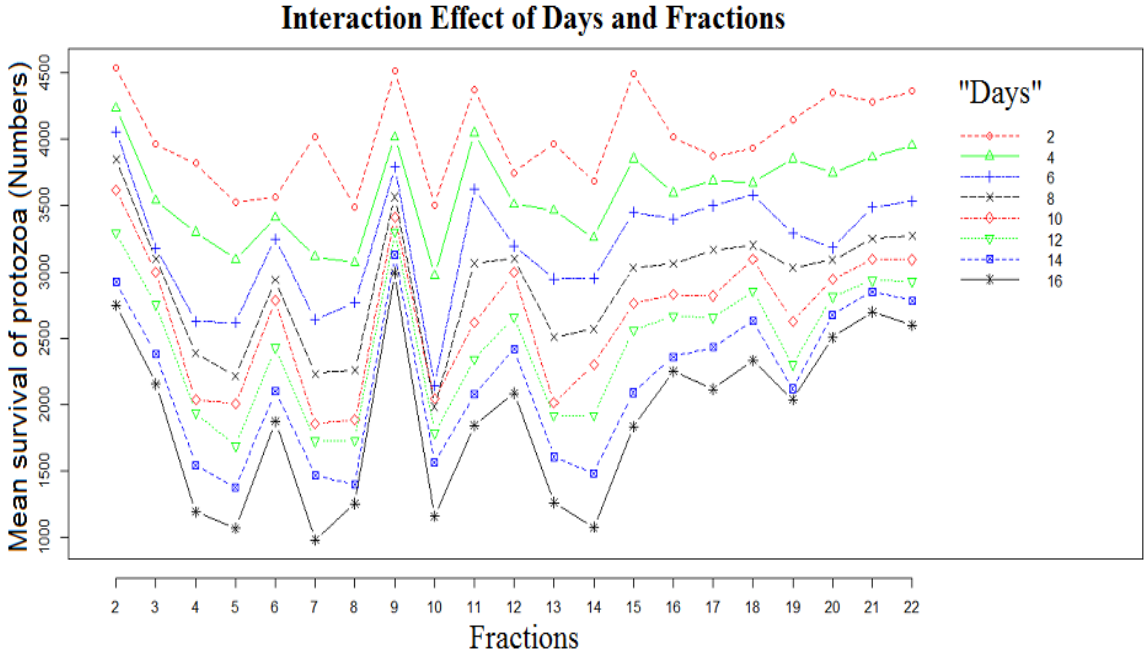


Figure 4.11: Interaction effect of days and fractions on the survival of the Protozoa population in the hindgut of *H. indicola.*

Table 4.1: Significance level of fractions used for antitermitic activity against *O. obesus*, *H. indicola*, and its hindgut Protozoa

Number of Fractions	**Level of Significance**		
	O. obesus	***H. indicola***	**Protozocidal**
2	Non-Significant	Non-Significant	Significant
3	Significant*	Non-Significant	Significant*
4	Significant**	Significant*	Significant***
5	Significant***	Significant***	Significant***
6	Significant*	Non-Significant	Significant*
7	Significant***	Significant*	Significant***
8	Significant**	Non-Significant	Significant**
9	Non-Significant	Non-Significant	Non-Significant
10	Significant**	Significant**	Significant**
11	Significant*	Non-Significant	Significant*
12	Non-Significant	Non-Significant	Significant*
13	Significant*	Non-Significant	Significant**
14	Significant*	Significant*	Significant***
15	Non-Significant	Non-Significant	Significant*
16	Significant*	Non-Significant	Significant*
17	Non-Significant	Non-Significant	Significant*
18	Non-Significant	Non-Significant	Significant*
19	Non-Significant	Non-Significant	Significant*
20	Non-Significant	Non-Significant	Significant*
21	Non-Significant	Non-Significant	Significant*
22	Non-Significant	Non-Significant	Significant*

Table 4.2: LC_{50} values of different fractions used for antitermitic activity against *O. obesus*, *H. indicola*, and its hindgut Protozoa.

Number of Fractions	LC_{50} (ppm) Upper confidence limit - Lower confidence limit		
	O. obesus	***H. indicola***	**Protozocidal**
2	3535.8 (3763.6-3341.1)	3409.4 (3645.9-3209.1)	2087.3 (3111.8-3063.2)
3	2952.9 (3127.4-2802.0)	3141.5 (3316.7-2989.2)	1434.0 (1439.8-1428.3)
4	2672.6 (2874.2-2504.1)	2728.0 (2869.1-2604.1)	710.88 (714.86-706.90)
5	1342.8 (1405.4-1284.7)	1420.0 (1488.3-1351.5)	578.34 (582.45-574.15)
6	3409.4 (3645.9-3209.1)	3524.0 (3750.5-3330.3)	1168.9 (1173.6-1164.2)
7	1273.1(1328.4-1221.2)	2624.3 (2793.7-2507.8)	625.78 (629.34-622.22)
8	2411.6 (2569.8-2276.5)	4381.1 (4867.0-3998.9)	519.32 (524.89-513.72)
9	5468.7 (6293.1-4860.7)	5468.7 (6293.1-4860.3)	2991.6 (3016.2-2967.6)
10	2221.5 (2360.4-2101.9)	2214.7 (2351.3-2097.0)	536.88 (540.53-533.21)
11	3423.0 (3681.6-3206.6)	9408.1 (14283.4-7124.0)	1445.3 (1451.3-1439.3)
12	6502.5 (7689.4-5666.5)	7358.6 (8183.1-5260.7)	1480.7 (1487.8-1473.6)
13	9408.1 (14283.4-7124.0)	3423.0 (3681.6-3206.6)	816.94 (821.50-812.39)
14	2432.1 (2532.0-2342.3)	2754.8(2889.6-2635.8)	793.01 (797.44-788.58)
15	6634.3 (6734.2-6534.0)	3724.7 (4061.2-3450.5)	1521.4 (1514.5-1528.4)
16	3148.3 (3375.3-2953.1)	6569.4 (7952.1-5636.0)	1596.3 (1604.3-1588.3)
17	3724.7 (4061.2-3450.5)	8634.3 (10213.8-6016.8)	1665.2 (1572.6-1557.9)
18	5763.3 (6181.4-4767.6)	6040.0 (6958.6-5362.7)	1819.5 (1829.2-1710.0)
19	5976.7 (6870.1-5315.3)	5367.3 (6181.4-4767.6)	1502.2 (1510.4-1494.1)
20	7003.7 (8324.1-6081.4)	4179.3 (4573.9-3859.8)	1864.2 (1874.5-1853.9)
21	5643.8 (6395.6-5073.1)	3513.8 (3768.2-3299.6)	2104.3 (2116.5-2092.3)
22	8486.4 (10689.3-7090.5)	6532.6 (7768.7-5670.7)	2273.0 (2288.4-2257.9)

4.4: Antibacterial activity

4.4.1: Antibacterial activity against bacterial isolates of *O. obesus*

When these fractions were tested against bacterial isolates from the hindgut of *O. obesus* at the above-mentioned concentrations (100, 500, 1000, 1500 and 2000ppm), differential antibacterial activity was observed. Out of 21 (2-22) fractions only 3, 5, 7, 8, 9, 10, 11, 13, 14 and 16 showed antibacterial activity against some isolates while the rest of fractions exhibit no zone of inhibition. The activity of faction 3 was reported to be zero against *B. cereus, E. coli* and *L. macrolides* at all concentrations; however observed zone of inhibition against *L. fusiformis* was 00±00, 9.16±1.30, 21.13±0.73, 23.5±1.00 and 24.9±1.06 respectively. While zone of inhibition against *L. xylanilyticus* was 14.73±0.81, 19.60±0.95, 21.66±2.33, 21.83±0.60 and 24.80±0.62 respectively at all mentioned concentrations. Their minimum inhibitory concentration is represented in table 4.4.

Fraction 5 was 2nd most effective fraction and showed antibacterial activity against three isolates (*L. fusiformis*, *E. coli,* and *L. xylanilyticus*). The reported zone of inhibition against *L. fusiformis* was 9.80±0.66, 13.16±0.60, 23.20±0.73, 26.63±0.68 and 29.83±1.01mm respectively at all concentrations. Against *E. coli*, it did not show any activity at 100ppm however activity was increased with increasing concentration with the maximum at 2000ppm with the zone of inhibition 22.16±1.01mm. Observed zone of inhibition against *L. xylanilyticus* was 10.83±0.72, 17.83±0.92, 19.33±1.48, 19.80±1.74 and 21.93±1.88mm respectively at all above-mentioned concentration. Fraction 7 was most effective and exhibited the strongest antibacterial activity against *B. cereus, E. coli*, and *L. xylanilyticus*. Observed zone of inhibition against *B. cereus* was 12.00±0.86, 19.00±1.80, 28.66±1.48, 29.83±0.92 and 32.00±1.32mm respectively at all concentrations while zone of inhibition against *E. coli* was 15.66±1.01, 19.16±0.72, 21.83±1.30, 23.00±1.60 and 24.66±1.48mm and against *L. xylanilyticus* was 13±0.86, 18.16±1.30, 21.66±1.16, 22.16±1.30 and 23.16±1.16 respectively (Table 4.3) with minimum inhibitory concentration is represented in table 4.4.

Fraction 8 showed activity only at higher concentrations *i.e.* 1500 and 2000ppm against four isolates with the maximum zone of inhibition against *B. cereus* (15.66±1.20mm) and (18.30±0.88mm) while minimum against *E. coli* (6.70±0.88) and

(7.93±0.74mm) at 1500 and 2000ppm respectively. Fraction 9 exhibited activity against *L. xylanilyticus* only with the maximum zone of inhibition at 2000ppm. Fraction 10 was active only against *B. cereus* with the zone of inhibition 00±00, 6.83±0.72, 9.93±1.05, 13.10±0.66 and 14.63±0.78 respectively. Similarly, fraction 13 was active against *L. fusiformis* with increasing activity with an increase in concentration. Fraction 14 gave reliable results against *B. cereus* and *E. coli* isolate at all concentrations but mostly zero activity at all concentrations' with one exception of 1000ppm against *L. fusiformis*. The isolate 4 (*L. macrolides*) was susceptible only at 1500 and 2000ppm with the zone of inhibition represented in table 4.3. Fraction 16 was inactive against all isolates except for 1500 and 2000ppm against *E. coli*. Their minimum inhibitory concentration is represented in table 4.4.

Table 4.3: Zone of inhibition (mm) measured against five bacterial isolates from the hindgut of *O. obesus*.

Number of fractions	Concentration (ppm)	Zone of inhibition (mm)				
		L. fusiformis	***B. Cereus***	***E. Coli***	***L. macrolides***	***L. xylanilyticus***
3	100	00±00	00±00	00±00	00±00	14.73±0.81
	500	9.16±1.30	00±00	00±00	00±00	19.60±0.95
	1000	21.13±0.73	00±00	00±00	00±00	21.66±2.33
	1500	23.5±1.00	00±00	00±00	00±00	21.83±0.60
	2000	24.9±1.06	00±00	00±00	00±00	24.80±0.62
5	100	9.80±0.66	00±00	00±00	00±00	10.83±0.72
	500	13.16±0.60	00±00	12.80±0.75	00±00	17.83±0.92
	1000	23.20±0.73	00±00	15.90±0.86	00±00	19.33±1.48
	1500	26.63±0.68	00±00	18.76±0.64	00±00	19.80±1.74
	2000	29.83±1.01	00±00	22.16±1.01	00±00	21.93±1.88
7	100	00±00	12.00±0.86	15.66±1.01	00±00	13±0.86
	500	00±00	19.00±1.80	19.16±0.72	00±00	18.16±1.30
	1000	00±00	28.66±1.48	21.83±1.30	00±00	21.66±1.16
	1500	00±00	29.83±0.92	23.00±1.60	00±00	22.16±1.30
	2000	00±00	32.00±1.32	24.66±1.48	00±00	23.16±1.16
8	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	00±00	00±00
	1000	00±00	12.10±0.73	00±00	00±00	00±00
	1500	9.50±0.86	15.66±1.20	6.70±0.88	00±00	8.70±0.88
	2000	13.00±2.08	18.30±0.88	7.93±0.74	00±00	11.23±1.40
	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	00±00	7.53±1.03

9	1000	00±00	00±00	00±00	00±00	8.80±0.62
	1500	00±00	00±00	00±00	00±00	11.66±1.16
	2000	00±00	00±00	00±00	00±00	13.66±1.64
10	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	6.83±0.72	00±00	00±00
	1000	00±00	00±00	9.93±1.05	00±00	00±00
	1500	00±00	00±00	13.10±0.66	00±00	00±00
	2000	00±00	00±00	14.63±0.78	00±00	00±00
11	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	00±00	9.30±1.01
	1000	00±00	00±00	00±00	00±00	11.26±1.07
	1500	00±00	00±00	00±00	00±00	13.46±1.03
	2000	00±00	00±00	00±00	00±00	15.30±1.01
13	100	11.50±1.44	00±00	00±00	00±00	00±00
	500	12.73±0.99	00±00	00±00	00±00	00±00
	1000	16.16±1.01	00±00	00±00	00±00	00±00
	1500	19.16±1.01	00±00	00±00	00±00	00±00
	2000	22.83±1.30	00±00	00±00	00±00	00±00
14	100	00±00	11.33±1.16	15.83±1.16	00±00	00±00
	500	00±00	21.50±1.80	17.50±0.86	00±00	00±00
	1000	13.06±0.69	22.83±1.30	18.66±1.01	00±00	00±00
	1500	00±00	24.50±0.76	23.50±1.32	11.50±1.32	00±00
	2000	00±00	25.16±1.16	25.40±0.95	14.40±1.53	00±00
16	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	00±00	00±00
	1000	00±00	00±00	00±00	00±00	00±00
	1500	00±00	00±00	12.33±1.30	00±00	00±00
	2000	00±00	00±00	17.66±1.30	00±00	00±00

Table 4.4: Minimum inhibitory concentration of effective fractions against five bacterial isolates from the hindgut of *O. obesus*.

Bacterial isolates	**Minimum inhibitory concentration (ppm)**									
	3	**5**	**7**	**8**	**9**	**10**	**11**	**13**	**14**	**16**
L. fusiformis	500	100	-	500	-	-	-	100	-	-
B. Cereus	-	-	100	1000	-	-	-	-	100	-
E. Coli	-	500	100	1500	-	500	-	-	100	1500
L. macroides	-	-	-	-	-	-	-	-	1500	-
L. xylanilyticus	100	100	100	1500	500	-	500	-	-	-

4.4.2: Antibacterial activity against bacterial isolates of *H. indicola*

Among twenty-two fractions used, seven (3, 5, 7, 8, 10, 14 and 16) showed differential antibacterial activity against all isolates while the rest of the fraction showed no zone of inhibition. Fraction 3 did not show any activity against the first four isolates, however, the recorded zone of inhibition against *L. fusiformis* was 10.20±1.15, 13.33±1.01, 14.73±0.67, 15.12±0.97 and 16.16±1.01mm respectively with the minimum inhibitory concentration of 100ppm. Somehow close results were noted in fraction 16, with zero zones of inhibition in all isolates at all concentrations except two *i.e.* 500ppm was effective against *E. coli* and 2000ppm against *L. fusiformis* (Table 4.6).

The third least active fraction was eight having no activity against all isolates at 100 and 500ppm. At 1000ppm zone of inhibition in only *L. fusiformis* was recorded (11.83±1.48mm). Two of the fractions (5 and 14) give some astonishing results, with no activity against *S. sonnei* and good results against the rest of isolates. Their minimum inhibitory concentrations are mentioned in table 4.6. The strongest activity was noted in the case of fraction 7 in which nearly all isolates were found susceptible. At 100ppm the zone of inhibition recorded in all 5 isolates was 00±00, 10.00±0.86, 12.26±1.36, 12.00±1.44 and 00±00 respectively at 500ppm the values noted were 10.06±0.74, 17.66±2.94, 15.00±1.44, 20.66±1.87 and 16.16±2.61 respectively. Similarly, in the rest of concentrations, this fraction showed zone of inhibition in all isolates with the minimum of 12.75±0.47mm and maximum of 25.83±1.87mm of respectively with the minimum inhibitory concentration of 500ppm against *B. subtilis* and *L. fusiformis* while 100ppm against isolate *S. sonnei, E. coli* and *B. cereus* respectively (Table 4.6).

The 2nd active fraction was fraction 5 which is active at against four isolates except *S. sonnei* with the zone of inhibition represented in table 3.5. In overall view, it can be assessed the weakest zone of inhibitions (5.50±0.98mm and 6.67±2.12mm) were noted in fraction 16, followed by fraction 3 with the highest zone of inhibition (16.16±1.01mm) but still fraction 16 is better than fraction 3 because it showed its activity against two strains while fraction 3 activity was noted only against *L. fusiformis*. The 3rd less active fraction is 10 which showed its activity against 3 isolates (*B. subtilis*,

E. coli and *B. cereus*). Fraction 8 gave reliable results against all isolates at 1500 and 2000ppm but mostly zero activity at lower concentrations' with one exception of 1000ppm against *L. fusiformis*. The minimum inhibitory concentration of all isolates is represented in table 4.6.

Table 4.5: Zone of inhibition (mm) measured against five bacterial isolates from the hindgut of *H. indicola*.

Number of fractions	Concentration (ppm)	Zone of inhibition (mm)				
		B. subtilis	***S. sonnei***	***E. coli***	***B. cereus***	***L. fusiformis***
3	100	00±00	00±00	00±00	00±00	10.20±1.15
	500	00±00	00±00	00±00	00±00	13.33±1.01
	1000	00±00	00±00	00±00	00±00	14.73±0.67
	1500	00±00	00±00	00±00	00±00	15.12±0.97
	2000	00±00	00±00	00±00	00±00	16.16,1.01
5	100	11.33±1.20	00±00	15.50±2.36	10.55±0.86	00±00
	500	16.83±3.72	00±00	18.33±1.16	14.16±2.12	9.50±0.76
	1000	17.50±0.76	00±00	19.16±1.16	19.16±1.74	13.83±0.92
	1500	17.83±1.64	00±00	21.16±1.30	21.55±1.25	18.55±1.32
	2000	19.83±1.64	00±00	24.54±1.32	24.83±1.16	21.50±1.32
7	100	00±00	10.00±0.86	12.26±1.36	12.00±1.44	00±00
	500	10.06±0.74	17.66±2.94	15.00±1.44	20.66±1.87	16.16±2.61
	1000	12.75±0.47	18.16±0.72	17.00±1.60	25.80±4.50	25.00±1.04
	1500	14.50±0.76	18.63±3.75	18.06±1.31	23.33±1.01	25.83±1.87
	2000	15.43±1.05	19.63±3.73	20.66±0.72	26.16±1.01	25.50±2.02
8	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	00±00	00±00	00±00
	1000	00±00	00±00	00±00	00±00	11.83±1.48
	1500	18.06±0.80	12.00±0.76	00±00	11.06±0.92	23.06±2.31
	2000	20.73±0.88	15.66±1.20	22.96±1.04	13.40±1.22	26.90±2.57
10	100	00±00	00±00	00±00	00±00	00±00
	500	13.00±1.04	00±00	18.33±1.64	11.66±1.30	00±00
	1000	18.00±1.32	00±00	16.50±1.04	18.16±1.30	00±00
	1500	18.66±1.01	00±00	20.66±0.72	20.16±0.72	00±00
	2000	19.16±1.87	00±00	27.16±1.16	19.5±1.89	00±00
14	100	00±00	00±00	00±00	10.16±0.72	00±00
	500	17.83±1.16	00±00	17.5±1.44	17.66±1.16	14±1.32
	1000	19.50±0.86	00±00	18.00±0.76	22.66±1.92	20.16±2.12
	1500	21.33±1.01	00±00	19.66±1.01	22.66±1.87	19.16±1.16
	2000	24.16±1.64	00±00	20.50±1.80	22.83±2.18	21.66±2.68
16	100	00±00	00±00	00±00	00±00	00±00
	500	00±00	00±00	6.67±2.12	00±00	00±00
	1000	00±00	00±00	00±00	00±00	00±00
	1500	00±00	00±00	00±00	00±00	00±00
	2000	00±00	00±00	00±00	00±00	5.50±0.98

Table 4.6: Minimum inhibitory concentration of effective fractions against five bacterial isolates from the hindgut of *H. indicola*.

Bacterial isolates	**Minimum inhibitory concentration (ppm)**						
	3	**5**	**7**	**8**	**10**	**14**	**16**
B. subtilis	-	100	500	1500	500	500	-
S. sonnei	-	100	100	1500	-		-
E. coli	-	100	100	2000	500	500	-
B. cereus	-	100	100	1500	500	100	2000
L. fusiformis	100	500	500	1000	-	500	-

DISCUSSION

Phytochemical compounds are well known for their antifeedant, repellent and antitermitic activities against different insect pest species (Almedia *et al.,* 2015). The presence of these phytochemicals may activate lingering behavior and feeding deterrence in termites making them unable to maintain their intestinal fauna (protozoa and bacteria) active and alive (Maistrello *et al.,* 2003). Termite control by targeting their gut symbionts is a method that has shown some success (Adams, 2004; Doolittle *et al.,* 2007).

Most of the bioactive compounds having termiticidal properties have not yet been identified. An active component Azadirachtin, isolated from neem oil has been reported as a week feeding deterrent to *Coptotermes formosanus*, however limonoids; another active component of the neem oil showed strong antifeedant activity to *Reticulitermes separatus* (Maistrello *et al.,* 2003). Abbaszadeh *et al.* (2014) reported the effect of bioactive fractions (clerodin, 15-methoxy14, 15-dihydroclerodin and 15-hydroxy-14, 15-dihyroclerodin) isolated from methanolic leaves extract of *Clerodendron infortunatum* L. against highly polyphagous pest *Helicoverpa armigera*, the cotton bollworm. In no-choice bioassay clerodin and 15-methoxy14,15-dihydroclerodin showed significantly higher antifeedant activity compared to the key ingredients in commercial pesticides. In another study conducted by Ahmed *et al.* (2016) revealed that the crude extract of *Nepeta leavigata, Rhynchosia reniformis* and *Nepeta leavigata* and their bioactive fractions exhibit moderate to strong insecticidal activities. *n*-butanol fraction isolated *Nepeta kurramensis* showed 89% mortality against *Tribolium castaneum* followed by the strongest insecticidal activity with 89% mortality against *Tribolium castaneum* followed by 88% mortality in the case of *Nepeta leanigata*. Similar studies have been conducted by Almeida *et al.* (2015). They evaluated the toxicity of phytochemical compounds derived from six medicinal plants including *Schinus teribinthifolius, Pittosporum undulatum, Lippia sidoides, Lippia adracilis, Mentha arvensis* and *Croton eajucara* against *Heteotermes sulcatus* by no-choice bioassay and reported that the bioactive compounds; limonene, carvacrol, and thymol isolated from these plants caused 100% mortality in the termite population.

In the present study, *G. robusta* was selected as most effective among initially used eight plants having strong antitermitic, antiprotozoan and antibacterial activity. Different fractions of *G. robusta* were isolated by column chromatography and applied against termite, their hindgut symbionts (protozoa and bacteria) in no-choice bioassays. Treated filter papers with different concentrations (100, 500, 1000, 1500 and 2000ppm) of each fraction were used as the substrate for termites. Different fractions exhibit differential activity against both termite species and protozoa in a dose-dependent manner when compared with control. Fraction 5 was found to be most effective among all used fraction (2-22) and significantly ($P<0.05$) reduced termite and protozoa population having 90% (LC_{50}=1342.81ppm) mortality in *O. obesus*, 92% (LC_{50}=1420.05ppm) in *H. indicola* and 80% (LC_{50}=578.34ppm) in symbiotic protozoa population. Similar results were reported by Maistrello *et al.* (2003). They evaluated the effect of vetiver oil, nootkatone and disodium octaborate tetrahydride on the protozoa population of *Coptotemes formasanus* and found that nootkatone and vetiver oil significantly ($P<0.05$) reduce protozoa population which may be due to the feeding deterrent activities of this compounds. These results are also comparable with the study conducted by Hassan *et al.* (2017). They reported the force-feeding effect of heartwood extract of *Cedrus deodara, Pinus roxburghii, Tectona grandis* and *Dalbergia sissoo* on feeding and mortality rate of two termite species (*H. indicola* and *R. flavipes*) and their hindgut protozoa population. All plant extract rapidly decreases the protozoan number in termite hindgut ultimately leading to the death of termites workers. Protozoa decline in both termite species was dose-dependent; highest at the concentration of 10mg/ml.

Fraction 7 and 8 were found significant against *O. obesus* having 78 and 60% mortality with LC_{50} value of 1342.81 and 2411.60ppm respectively while against *H. indicola* observed termite mortality was 50% in fraction 7 (LC_{50}=2642.34ppm). Whereas fraction 8 was found to be non-significant against *H. indicola* with a higher LC_{50} value of 4381.14ppm. However, fraction 9 was found to be least effective having higher LC_{50} values against all tested organisms (*O. obesus, H. indicola,* and protozoa) with the non-significant decline in their survival. Similarly fraction 2, 12, 15, 17, 18, 19, 20, 21 and 22 were non significant ($P>0.05$) against both termite species (*O. obesus*, *H. indicola*)

having LC_{50} of 3535.82, 6502.53, 6634.30, 3724.75, 5763.31, 5976.73, 7003.75, 5643.80, 8486.41ppm and 3409.49, 7358.61, 3724.75, 12634.3, 6040.05, 5367.31, 4179.34, 3513.87, 6532.66ppm respectively but all these fractions were found to have significant effect on protozoa survival with LC_{50} of 3087.34, 1480.701521.48, 1565.26, 1819.58, 1502.25, 1864.21, 2104.32 and 2273.07ppm respectively. The differential decrease in the protozoa population may be due to the alteration in gut microenvironment induce by the toxicity of bioactive compounds at the cellular level (Maistrello *et al.,* 2003). Different concentrations of extracts directly affect the protozoa population before their host ultimately leading to the death of the host. This correlation between protozoa and termite host suggests that the decline in the protozoa population was the primary cause of termite mortality. However toxicity of different components from plant extract on termite host cannot be ignored (Qureshi *et al.,* 2016). The protozocidal properties of fractions isolated from *G. robusta* are comparable with the study conducted by Qureshi *et al.* (2012). They reported the toxicity of the heartwood extract of *E. camaldulensis* and *D. sissoo* on the hindgut protozoa population of *H. indicola* and observed complete elimination suggesting that different components from the heartwood extract of these plants could be the potential protozocidal agent. Tumerone from turmeric extract was proved highly toxic and induce termite mortality by targeting their nervous system and respiratory disruption (Raje *et al.,* 2015).

Different studies revealed that bioactive phytochemicals have a growth inhibitory effect *in-vitro* on the pathogenic bacterial strain, as plants are a rich source of different secondary metabolites such as alkaloids, flavonoids, tannins, and terpenoids (Prescott *et al.,* 2002). In the present antibacterial activity of fractions isolated from *G. robusta* was evaluated against bacteria isolated from the hindgut of *H. indicola* and *O. obesus.* Antibacterial activity of all fractions eluted in different solvents, susceptibility and zone of inhibition of bacterial isolates varied among different strains used in the present study. According to Prescott *et al.* (2002), the toxicity of an agent varies greatly depending upon the nature of the target species. The diameter of the zone of inhibition is determined by the rate of diffusion of antimicrobial agents, the population density of tested microorganism and their growth rate. These findings support the results of the present

study. Differential antibacterial activity was observed with an increase in concentration from 100 to 2000ppm. Seven fractions (3, 5, 7, 8, 10, 14 and 16) have growth inhibitory effect against bacterial isolates of *H. indicola* and ten fractions (3, 5, 7, 8, 9, 10, 11, 13, 14 and 16) against bacteria of *O. obesus.* Mbahi *et al.* (2018) reported the same results that antibacterial activity increased with an increase in concentration despite the presence of the bioactive component that is responsible for antibacterial activities.

Fraction 7 was most active against bacteria of both termite species (*H. indicola* and *O. obesus*). In the case of *O. obesus* maximum zone of inhibition (32.00±1.32mm) of fraction 7 was observed against *B. cereus* having the minimum inhibitory concentration of 100ppm against all affected isolates *i.e. B. cereus, E. coli,* and *L. xylanilyticus.* Similarly, in the case of *H. indicola* maximum zone of inhibition was observed against all isolates having the minimum inhibitory concentration of 100ppm against *S. sonnei, E. coli* and *B. cereus* while 500ppm against *B. subtilis* and *L. fusiformis*. According to Dahiru and Obidoa, (2008) two possible reasons account for the higher antibacterial activity of this fraction. It might be due to the presence of a biologically active compound or stronger extraction capacity of eluent used for fractionation of crude plant extract which has yielded a greater number of active phytoconstituent responsible for antimicrobial activity. The results of the present study are also supported by Mbahi *et al.* (2018). They reported the antibacterial activity of *Ziziphus mauritiana* methanolic extract and its bioactive fractions on *Staphylococcus aureus*, *Bacillus subtillius*, *Salmonella typhi*, and *Escherichia coli*. Among four collected fractions (A-D); faction C showed strong antibacterial activity against all tested strains. They concluded that the isolation and fractionations process concentrated the bioactive phytochemicals in fraction C.

The 2nd and 3rd most active fractions against *H. indicola* bacterial isolate were 5 and 14 having antibacterial activity against four isolates at all concentrations (100, 500, 1000, 1500 and 2000ppm). Fraction 3 has the least antibacterial activity with the zone of inhibition only against *L. fusiformis*. Similarly, fraction 8, 10 and 16 also have the least antibacterial activity at higher concentrations having the highest zone of inhibition. In the case of *O. obesus,* the 2nd most active fraction was 5 having the zone of inhibition against *L. fusiformis, E. coli* and *L. xylanilyticus* with the minimum inhibitory concentration of

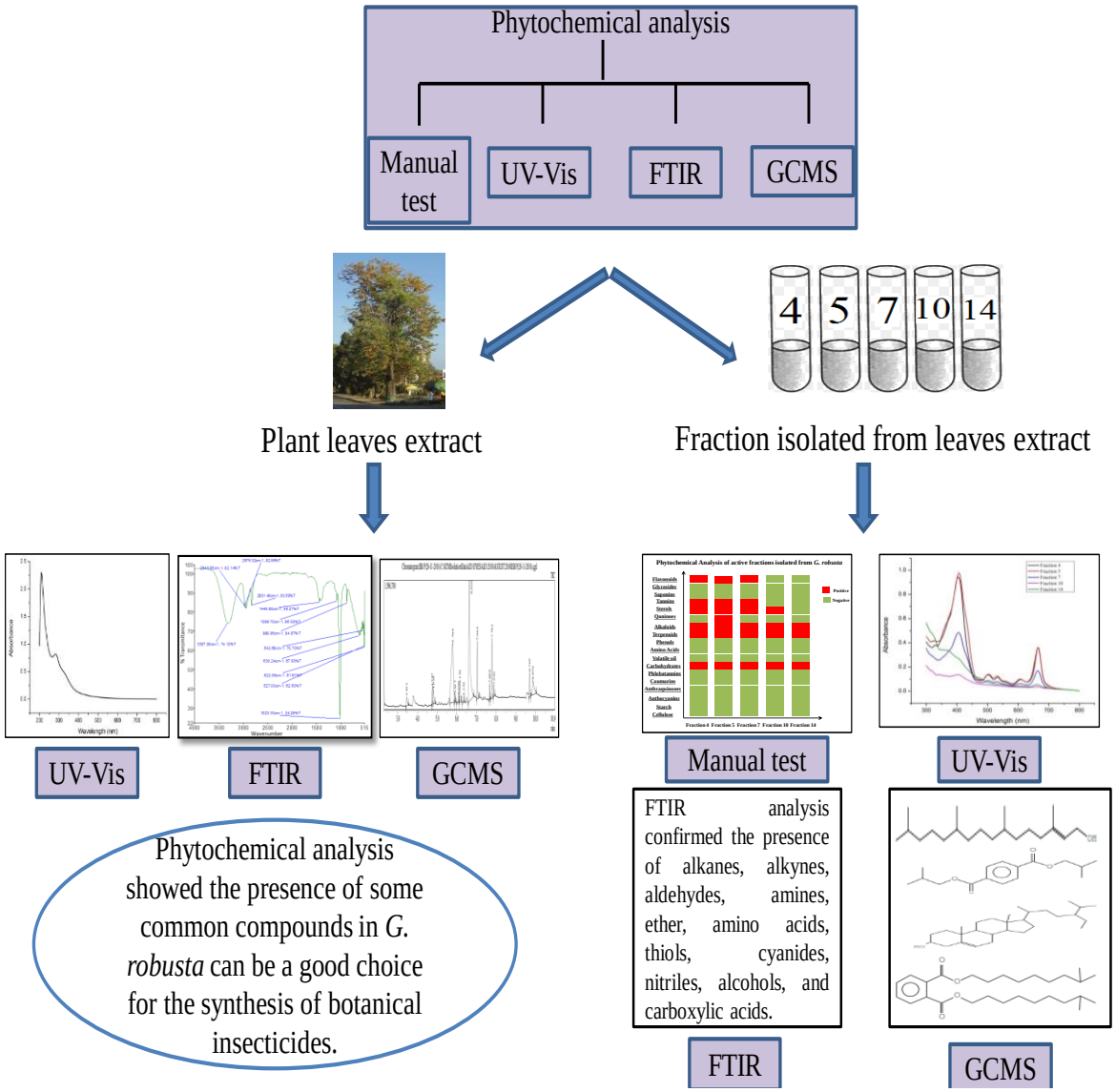
100, 500 and 100ppm respectively. Fraction 8 was active against 4 isolates with the highest minimum inhibitory concentration of 1500ppm. Similarly, fractions 9, 10, 11, 13 and 16 were only effective against single isolate. Similar findings were also reported by Mansouri *et al.* (2001) while evaluating the antimicrobial activity of fractionated constituents and crude extract of *Myrtus communis.* Growth inhibitory effect of *Jasminum mesnyi* and its fractions on different Gram-positive and Gram-negative bacteria were reported by Verma *et al.* (2018) and found that Diethyl ether fraction was more active with the highest zone of inhibition against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* at 250µg.

The results of the present study conclude that bioactive fractions from crude leaves extract of *G. robusta* possess a wide range of phytochemical compounds and showed promising termiticidal properties with a significant decline in the population of hindgut symbionts (protozoa and bacteria) during no-choice bioassay. These findings can help to constitute an effective alternate of harmful chemical insecticides that persist in the environment and cause environmental toxicity for other non-targeted organisms. Further investigations are required for isolation and purification of the pure bioactive compound that could be used in the development of cost-effective natural wood preservatives for insect pest control with the minimum side effect.

ABSTRACT

Phytochemical characterization by different spectroscopic techniques provides a considerable knowledge about the extent and nature of the chemical substances present in extracts. Being most effective, the extract of *G. robusta* was characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis. Two absorption peaks were recorded by UV-Vis spectroscopy at 208.94 and 282.97nm having absorption of 2.33 and 0.811 respectively. FTIR analysis confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether and halo compounds. GC-MS analysis revealed the presence of fifteen phytochemicals with a high percentage of Coumarin (30.64%), 7-hydroxy, Methyl hexofuranoside (27.63%) and 9,12,15-Octadecatrienoic acid (Z,Z,Z) (8.97%). Similarly, basic phytochemical screening of bioactive fractions (4, 5, 7, 10 and 14) showed the presence of flavonoids, tannins, sterols, quinones, alkaloids, terpenoids, and carbohydrates. The UV-Vis spectroscopic analysis of bioactive fractions was performed at 200 to 800nm and different absorption peaks were recorded at different wavelengths with a common peak of all fractions at 665.50nm having different absorbance. The FTIR analysis was performed for the identification of different functional groups based on characteristic peak values and the spectrum was recorded from 400-4000cm^{-1}. The FTIR analysis of fractions (4, 5, 7, 10 and 14) confirmed the presence of alkanes, alkynes, aldehydes, amines, ether, amino acids, thiol, cyanide, nitriles, alcohol, carboxylic acids, Thiocyanate, Thiols, and Azides. The GC-MS analysis revealed the presence of some common chemical compounds including 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, beta-Sitosterol, 1,2-Benzenedicarboxylic acid, diisodectyl ester, Phytol, 1,2-Benzenedicarboxylic acid, diisooctyl, Hexadecanoic acid methyl ester, n-hexadecanoic acid, Octadecanoic acid, and p-Arbutin. The presence of these phytochemicals in bioactive fractions indicates that *G. robusta* can be a good choice for the synthesis of botanical insecticides.

Graphical abstrcat



INTRODUCTION

Plants are a potential source of synthesizing a wide variety of lower molecular weight organic compounds having unique and complex molecular structures. However, the medicinal activities of plants are due to these secondary metabolites (Savithramma *et al.,* 2011) and play a significant role in the formulation of crude drugs (Kalimuthu and Prabakaran, 2013). They have also been reported to have interesting biological activities and are used in pharmaceutical industries, as dyes, fragrance, flavoring agent and insecticides (Jayapriya and Shoba, 2015).

Phytochemical screening is considered to be an effective technique in the discovery of such compounds in plant extracts having potential uses in ethnopharmacology. Chemical features of different secondary metabolites isolated from crude plant extract vary significantly among plant species. Plants derived phytochemicals are part of phytomedicines since immemorial time. These chemicals can be extracted from different parts of plants like flowers, roots, leaves, seeds, bark, and stem (Yadav and Agarwala, 2011). Phytochemical characterization (VU-Vis, FTIR and GC-MS) provides a good tool for qualitative analysis of different secondary metabolites.

The spectroscopic analysis involves the interaction between light and matter. Ultraviolet and visible spectroscopy involves the electromagnetic radiations range from 190-800nm. This absorption spectroscopy is divided into two regions *i.e.* ultraviolet (190-400nm) and visible (400-800nm). It is also termed as electronic spectroscopy because after the absorption of radiation by molecules leads to the transition between different energy levels. The information based on UV-Vis spectroscopy gives valuable knowledge about any structure when combined with the spectral data provided by IR and NMR (Kumar, 2008).

Fourier transform infrared spectroscopy use electromagnetic radiations from the infrared (IR) region for identification and determination of functional groups and molecular structures. The IR spectrometers use optical instruments for focusing and scattering electromagnetic radiations from the infrared region that passes through the sample and absorbance is measured against a specific reference beam (Stuart *et al.,*

1996). At ambient temperatures, organic molecules in a compound are constantly moving and produce vibrations. Each bond within the molecule has characteristic bending and stretching properties. When infrared radiations (400-4000cm^{-}) are allowed to pass through the organic sample, various are absorbed and transformed into vibrational energy while some of the radiations that do not interact with the sample are passed without any absorption. The % transmittance of a compound or a sample plotted against frequency is termed as an infrared spectrum (Kumar, 2008).

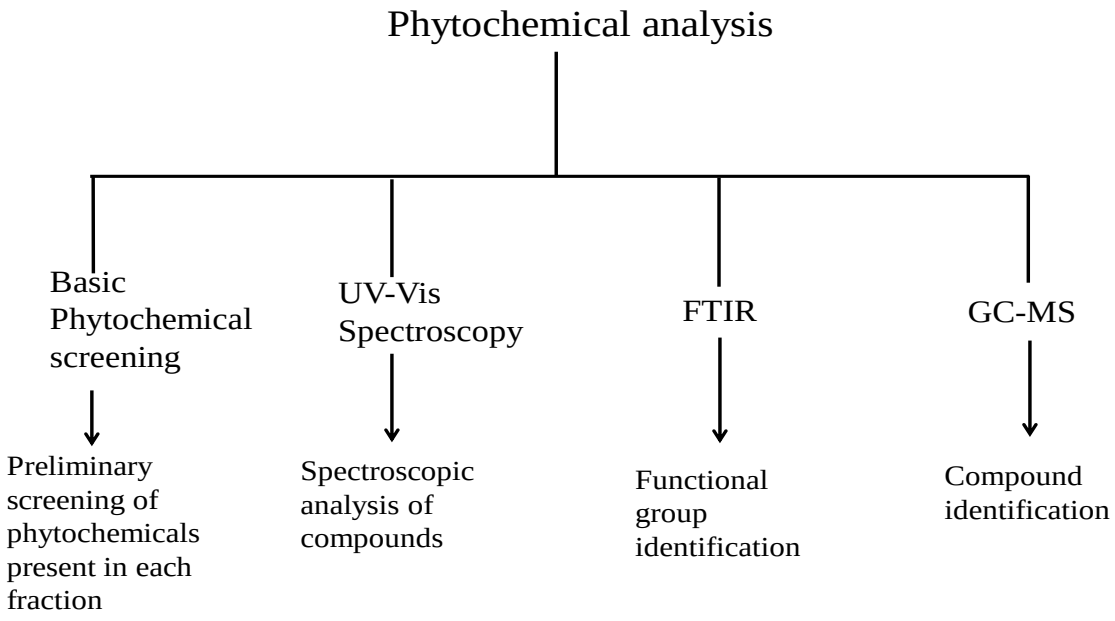
Gas chromatography coupled mass spectrometry (GC-MS) is an analytical technique that unites the properties of gas chromatography with mass spectrometry for identification of different compounds present in a sample. Gas chromatography isolates the chemical compounds without their identification. Similarly, mass spectrometry provides comprehensive knowledge about the structure of the compounds that helps in their exact identification but it cannot separate these compounds. Therefore GC-MS is a combination of both analytical techniques that are used for the analysis of complex biochemical and organic compounds (Skoog *et al.*, 2017). The spectrum of the isolated compound is recorded using mass spectrometer which characterizes the compounds depending upon their mass to charge ratio (Hussain and Maqbool, 2014).

Phytochemical extraction and characterization from the green plants have opened a new gateway to some high activity profile drugs. For the synthesis of complex chemical compounds from these bioactive substances requires more pronounced knowledge about their chemical constituents (Vaghasiya *et al.*, 2011). In the present study, qualitative phytochemical analysis of crude leaves extract of *G. robusta* and its bioactive fraction was carried out by manual phytochemical tests, VU-Vis Spectroscopy, FTIR and GC-MS analysis.

MATERIALS AND METHODS

5.1: Phytochemical analysis

The qualitative phytochemical analysis of most effective plant (*G. robusta)* and its bioactive fractions (4, 5, 7, 10 and 14) was conducted by different phytochemical tests.



5.1.1: Basic phytochemical screening

The qualitative phytochemical analysis of effective fractions (4, 5, 7, 10 and 14) isolated from *G. robusta* was conducted by different phytochemical tests. A summary of phytochemical screening is given in table 5.1.

5.1.2: Ultraviolet-visible spectroscopy (UV-Vis)

The ethanolic leaves extract of the most effective plant and its bioactive fractions (4, 5, 7, 10 and 14) were examined by VU-Vis spectroscopic analysis for its spectral profile. The extract was centrifuged for 10 minutes at 3000rpm and filtered by Whatman No. 1 filter paper. The sample was diluted ten times with ethanol. The spectra were obtained by scanning the extract from 200 to 800nm using spectrophotometer (UV-1601, Shimadzu) and characteristic spectral peaks were recorded (Tripathy and Middha, 2016).

5.1.3: Fourier transform infrared spectroscopy (FTIR)

Functional groups in ethanolic leaves extract of the most effective plant and its bioactive fractions (4, 5, 7, 10 and 14) were detected by FTIR analysis and the characteristic peak values were detected and recorded in the infrared region from 400-4000cm^{-1} (Sahu and Saxena, 2014). The samples were prepared by mixing 2mg of leaves extract in 100mg of KBr (FTIR grade). The mixture was compressed to prepare 3mm (diameter) salt discs which were loaded in FTIR spectroscope (Shimadzu ETIR spectrophotometer) and scanned to record infrared spectra at different wavelengths (400-4000cm^{-1}).

5.1.4: Gas chromatograph coupled mass spectroscopy (GC-MS)

Ethanolic leaves extract of the most effective plant and its bioactive fractions (4, 5, 7, 10 and 14) was further analyzed by gas chromatography coupled with a mass spectrophotometer (GC-2010, Shimadzu). The chromatographic system was installed with an auto-injector AOC-20i, autosampler AOC20s, and mass selective detector QP2010. The fused silica capillary column (DB-5Ms, 0.25mm×30m×0.25µm) having a maximum temperature capacity of 325^{o}C was used for screening. Column's initial temperature was 100^{o}C and maintained at this temperature for 0.5 minutes. The temperature of the oven was increased to 280^{o}C (24^{o}C/min) and maintained for 3 minutes. Injector temperature was 250^{o}C with a flow rate of Helium as1.0ml/min. The chromatograph was operated in split injection mode with an injection volume of the 1.0µl and split ratio of 10:1. The ionization voltage was 70eV and the mass spectral scan range was 50-350m/z.

5.1.5: Identification of phytoconstituents

National Institute of Standards and Technology (NIST) database having more than 62000 patterns was used for the interpretation of the mass spectrum. The comparison between spectra of known and unknown compounds, from the NIST library, helps in the interpretation of the mass spectrum and the determination of names, chemical formula, chemical structures and molecular weights of identified compounds (Rukshana *et al.*, 2017).

Table 5.1: Summary of different phytochemical tests conducted for the analysis of bioactive fractions.

Sr. #	Compound tested	Name of test	Reagents added	Results	References
1	Flavonoids	Alkaline reagent test	1ml of plant extract 1ml of 2N NaOH	Yellow color	Onwukaeme *et al.* 2007
		Ferric chloride test	1ml of plant extract Few drops of $FeCl_3$	Blackish Red precipitates	Harborne, 1984
2	Glycosides	Keller Killani test	1ml of plant extract 1ml of glacial acetic acid 2 drops of $FeCl_3$ and few drops of H_2SO_4	Reddish-brown color ring	Parekh and Chanda, 2007
3	Saponins	Frothing/Foam test	2ml of distilled water was added with 2ml of plant extract and shaken for 15 minutes.	1cm or thicker layer of foam	Parekh and Chanda, 2007
4	Tannins	Ferric chloride test	1ml of plant extract 2ml of 5% $FeCl_3$	Greenish black or dark blue color	Kumar *et al.* 2007
5	Sterols	Salkowski's test	2ml of plant extract +5ml of chloroform 1ml of concentrated H_2SO_4 was added along the wall of test tubes	The appearance of reddish-brown color in the lower layer	Harborne, 1984
6	Quinones	----------	1ml of plant extract 1ml of concentrated H_2SO_4	Red color	Harborne, 1984
7	Alkaloids	Mayer test	2ml of plant extract 2ml of concentrated HCl followed by the addition of Mayer reagent	Green color or formation of white precipitates	Harborne, 1984
		Hager test	1ml of plant extract 1ml of diluted HCL 1ml of Hager reagent	Yellow ppt.	Harborne, 1984
		Wagner's test	1ml of plant extract 1ml of Wagner's reagent	Reddish-brown ppt.	Chanda *et al.* 2006
8	Terpenoids	Salkowski's test	5ml of plant extract 2ml of chloroform 3ml of concentrated H_2SO_4	The red-brown color at the interface	Edeoga *et al.* 2005

9	Phenols	Ellagic acid test	1ml of plant extract Few drops of 5% glacial acetic acid Few drops of $NaNO_2$	Muddy brown color	Harborne, 1984
10	Amino acids and protein	Ninhydrin test	1ml of plant extract Few drops of ninhydrin reagent	Purple or pink in color	Harborne, 1984
		Xanthoproteic test	1ml of plant extract 1ml of concentrated H_2SO_4	White precipitates	Harborne, 1984
11	Volatile oil	----------	2ml of plant extract 0.11ml of diluted NaOH A small quantity of dilute HCl	The appearance of white precipitates	(Dahiru *et al.* 2006)
12	Carbohydrates	Molisch's test	1ml of plant extract Few drops of distilled water Few drops of Molish reagent and 1ml of concentrated H_2SO_4	The appearance of brown ring	Harborne, 1984
		Fehling's test	1ml of plant extract 5-8 drops of Fehling's solution Heat on the water bath for half an hour	Brick red ppt.	Akinyemi *et al.*, 2005
13	Phlobatannins	Precipitates test	2ml of plant extract+2ml of 1% HCl Heat the solution	Red precipitates	Edeoga *et al.* 2005
14	Coumarins	----------	2ml of plant extract 3ml of 10% NaOH	Yellow color	Sofowora, 1993
15	Anthocyanins	NaoH Test	1ml of plant extract 2ml of NaOH	Bluish-green color	Harborne, 1984

RESULTS

5.1: Characterization of the most effective plant

5.1.1: Ultraviolet-visible spectroscopy (UV-Vis)

Qualitative UV-Vis spectroscopic analysis of *G. robusta* leaves extract in ethanol solvent showed a spectrum profile at 200 to 800nm due to the sharpness of peaks and proper baseline. The peak spectra of *G. robusta* showed characteristic peaks at 208.94 and 282.97nm with the absorption of 2.33 and 0.811 respectively (Table 5.2 and figure 5.1).

Table 5.2: UV-Vis spectrum values of ethanolic leaves extract of *G. robusta*.

Sr. No.	Wavelength (nm)	Absorbance
1	08.94	2.33
2	282.97	0.811

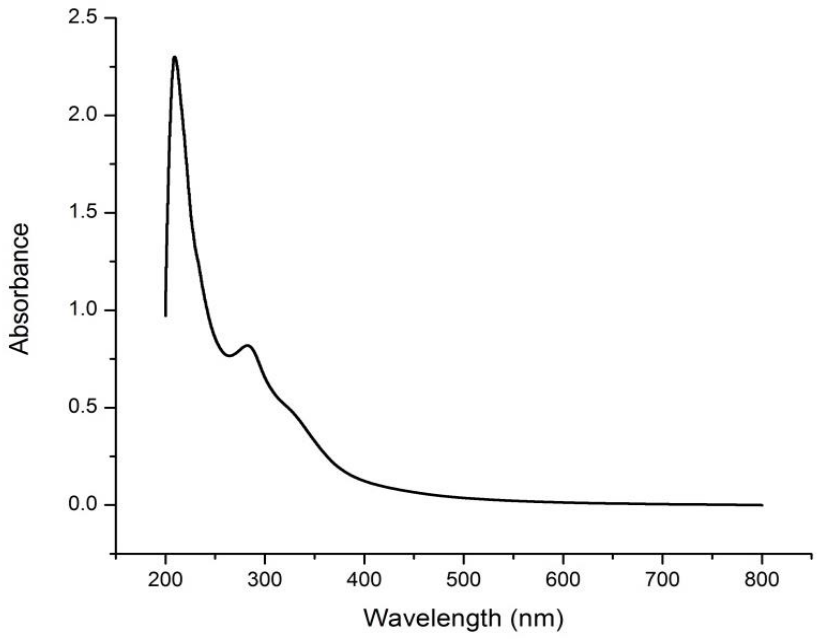


Figure 5.1: The UV-Vis spectra of ethanolic leaves extract of *G. robusta*

5.1.2: Fourier transform infrared spectroscopy (FTIR)

Functional groups of active compounds in ethanolic leaves extract of *G. robusta* were identified by FTIR analysis. Each peak in the spectrum represents the specific peak value corresponding to the specific functional group. The absorbance was noted from 400 to 4000cm^{-1}. The results of FTIR analysis confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether and halo compounds. The absorption peak at 3307.00cm^{-1} represents N-H stretch/O-H stretch (Amines/Alcohols). The absorbance at the wavelength of 2974.32cm^{-1} exhibit C-H stretch (alkanes) and at 1488.88cm^{-1} C-H bending (alkanes) while absorption at 2943.98cm^{-1} confirmed the presence of C≡N (nitriles). The peak at the wavelength of 2831.48cm^{-1} showed C-H stretch (aldehydes). The characteristic peaks at the wavelength of 1088.70cm^{-1}, 1023.33cm^{-1} and 880.88cm^{-1} showed C-O stretch (Ethers/Alcohols), C-N stretch (Amines) and C-H bending (1,2,4 trisubstituted compounds) respectively. The absorbance between 630.24-522.55cm^{-1} revealed C-X stretch which confirmed the presence of halo compounds (Table 5.3 and figure 5.2).

Table 5.3: FTIR spectral peak values and functional groups obtained from ethanolic leaves extract of *G. robusta.*

Peak #	Peak Value (cm^{-1})	Type of stretching	Functional group
1	3307.00	N-H stretch/O-H stretch	Amine/Alcohol
2	2976.32	C-H stretch	Alkane
3	2943.98	C≡N	Nitriles
4	2831.48	C-H stretch	Aldehydes
5	1488.88	C-H bending	Alkane
6	1088.70	C-O stretch	Ether/Alcohol
7	1023.33	C-N stretch	Amines
8	880.88	C-H bending	1,2,4 trisubstituted
9	630.24	C-X stretch	Halo compound
10	542.66	C-X stretch	Halo compound
11	527.03	C-X stretch	Halo compound
12	522.55	C-X stretch	Halo compound

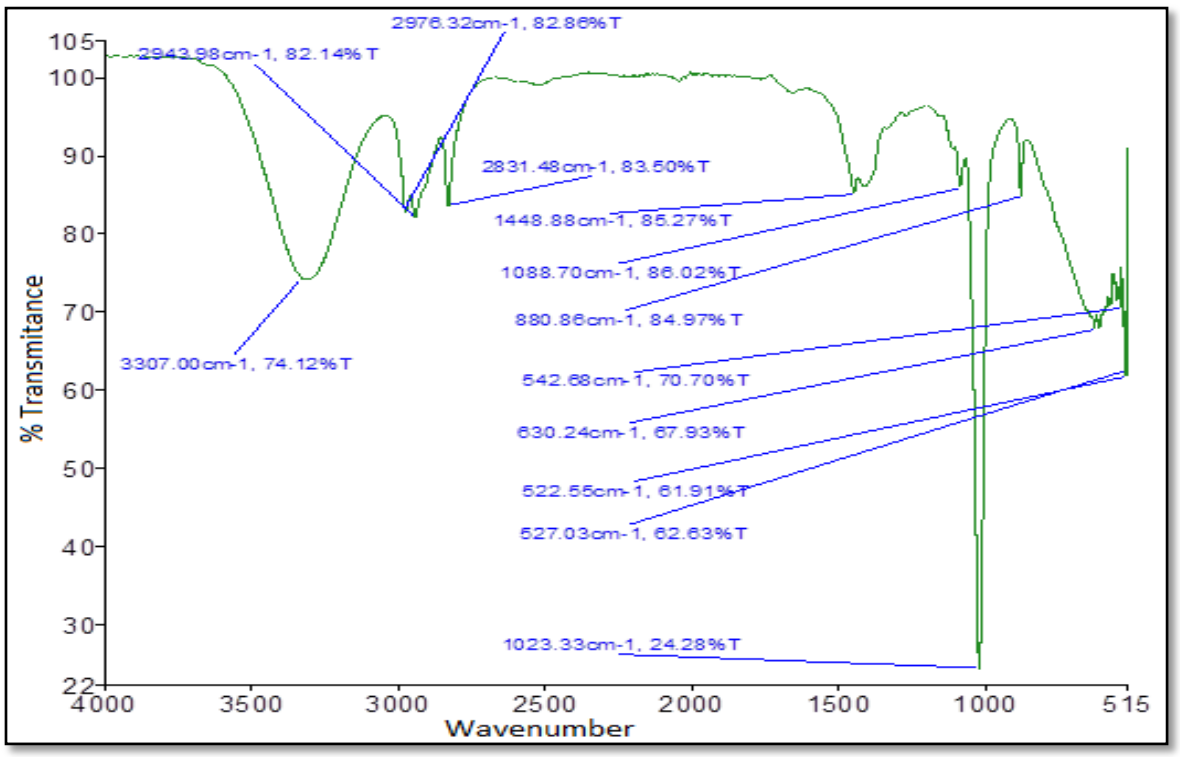


Figure 5.2: FTIR spectrum of ethanolic leaves extract of *G. robusta.*

5.1.3: Gas chromatograph coupled mass spectroscopy (GC-MS)

Gas Chromatograph coupled Mass Spectroscopy (GC-MS) of ethanolic leaves extracts of *G. robusta* indicated fifteen peaks which revealed the presence of fifteen phytochemical compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound names, retention time, area percentage, molecular weights and a molecular formula of identified components are listed in table 5.4 and figure 5.3. Coumarin, 7-hydroxy (30.64%), Methyl hexofuranoside (27.63%) and 9,12,15- Octadecatrienoic acid(Z,Z,Z) (8.97%), are reported as a major compounds in ethanolic leaves extract of *G. robusta* while 2,3-dihydrobenzofuran (1.93%), 3-Hydroxy-2-methylbenzaldehyde (3.07%), 2-Coumaranone (2.55%), 1,2,3,4,5-Cyclohexanepentol (1.77%), 5-Hydroxy-1-tetralone (0.69%), Beta-d-Mannofuranoside, methyl (3.34%), 5,5,8a-Trimethyldecalin-1-one (0.73%), n-Hexadecanoic acid (6.30%), Phytol (1.37%), 9,12,15- Octadecatrienoic acid (2.16%), Bis(2-ethylexyl)phthalate (3.08%) and 4-Hexyloxyphenol (5.77%) are present as minor constituents in *G. robusta* leaves extract.

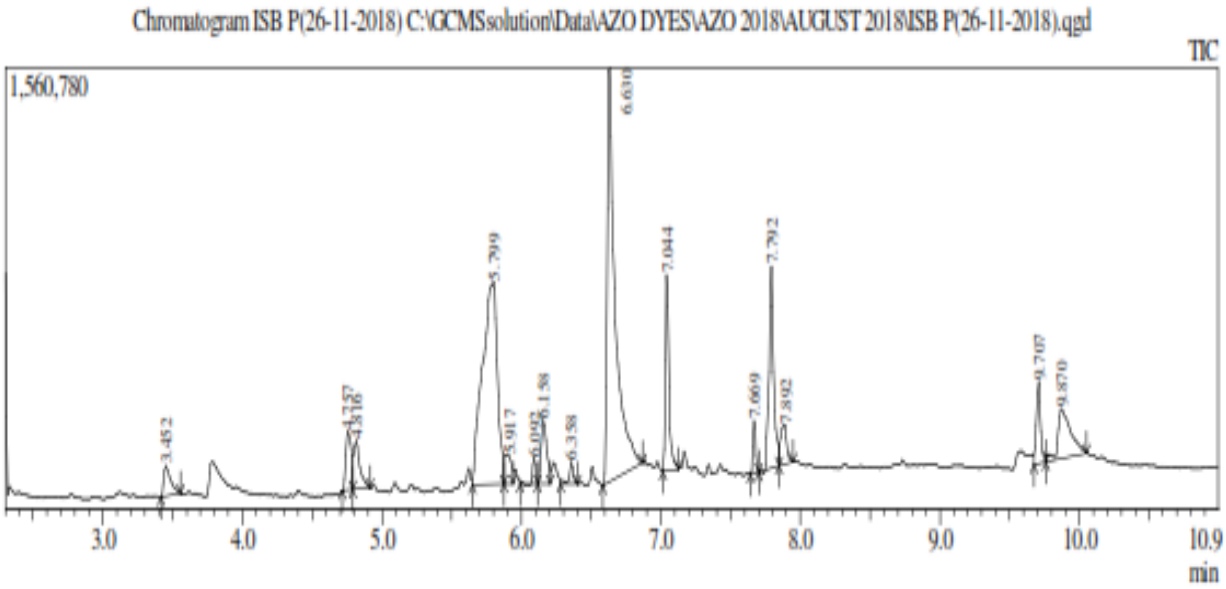
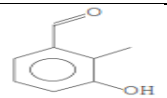
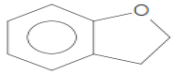
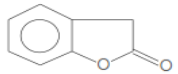
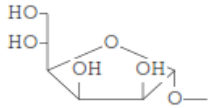
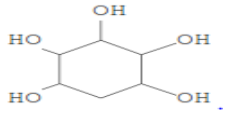
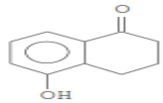
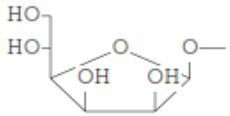
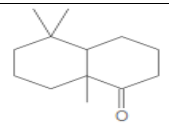
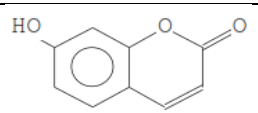
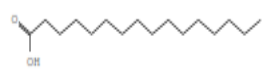
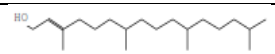
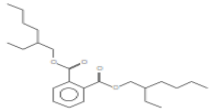
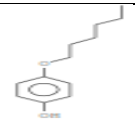


Figure 5.3: GC-MS chromatogram of ethanolic leaves extract of *G. robusta.*

Table 5.4: Phytocomponents identified in ethanolic leaves extract of *G. robusta* by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	3.452	1.93	C_8H_8O	120	2,3-dihydrobenzofuran	
2	4.757	3.07	$C_8H_8O_2$	136	3-Hydroxy-2-methylbenzaldehyde	
3	4.816	2.55	$C_8H_6O_2$	134	2-Coumaranone	
4	5.799	27.63	$C_7H_{14}O_6$	194	Methyl hexofuranoside	
5	5.917	1.77	$C_9H_{14}O_2$	154	1,2,3,4,5-Cyclohexanepentol	
6	6.092	0.69	$C_{11}H_{14}O_2$	162	5-Hydroxy-1-tetralone	
7	6.158	3.34	$C_7H_{14}O_6$	194	Beta-d-Mannofuranoside, methyl	

8	6.358	0.73	$C_{14}H_{22}O_3$	238	5,5,8a-Trimethyldecalin-1-one	
9	6.630	30.64	$C_9H_6O_3$	162	Coumarin, 7-hydroxy	
10	7.044	6.30	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
11	7.669	1.37	$C_{20}H_{40}O$	296	Phytol	
12	7.792	8.97	$C_{18}H_{30}O_2$	278	9,12,15- Octadecatrienoic acid(Z,Z,Z)	
13	7.892	2.16	$C_{21}H_{36}O_4$	352	9,12,15- Octadecatrienoic acid	
14	9.707	3.08	$C_{16}H_{22}O_4$	278	Bis(2-ethylexyl)phthalate	
15	9.870	5.77	$C_{10}H_{14}O_2$	166	4-Hexyloxyphenol	

5.2: Phytochemical screening of bioactive fractions

Preliminary phytochemical analysis of bioactive fractions 4, 5, 7, 10 and 14 showed the presence of different secondary metabolites such as flavonoids, tannins, sterols, quinones, alkaloids, terpenoids, and carbohydrates. Alkaloids, terpenoids, and carbohydrates were present in all fractions, sterols were absent in fraction 14 while present in the other four fractions. Flavonoids and tannins were present in fraction 4, 5 and 7 while quinones were only in fraction 5 (Table 5.5 and figure 5.4).

Table 5.5: Preliminary phytochemical screening of bioactive fractions

Name of compound	**Fraction 4**	**Fraction 5**	**Fraction 7**	**Fraction 10**	**Fraction 14**
Flavonoids	+	+	+	-	-
Glycosides	-	-	-	-	-
Saponin	-	-	-	-	-
Tannins	+	+	+	-	-
Sterols	+	+	+	+	-
Quinones	-	+	-	-	-
Alkaloids	+	+	+	+	+
Terpenoids	+	+	+	+	+
Phenols	-	-	-	-	-
Amino Acids	+	+	+	+	+
Volatile oil	-	-	-	-	-
Carbohydrate	+	+	+	+	+
Phlobatannins	-	-	-	-	-
Coumarins	+	+	+	-	-
Anthocyanins	-	-	-	-	-

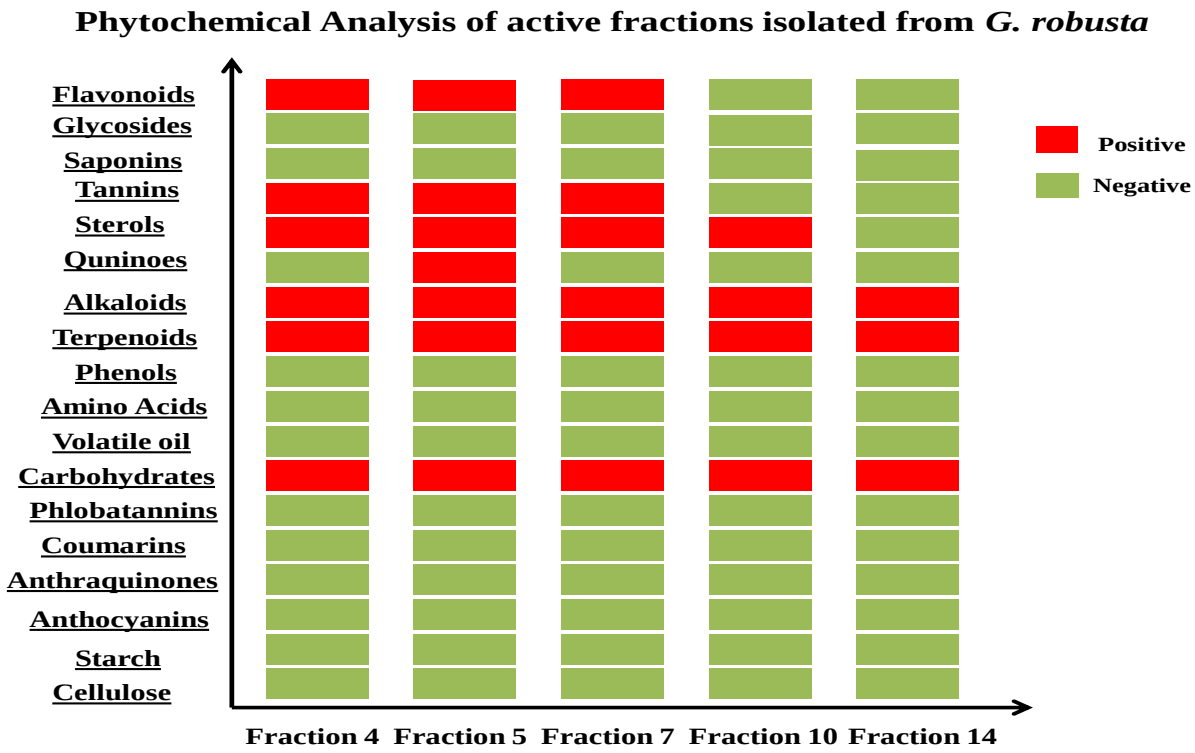


Figure 5.4: Preliminary phytochemical screening of bioactive fractions

5.3: UV-Vis Spectroscopic analysis

Qualitative UV-Vis spectroscopic analysis of active fractions (4, 5, 7, 10 and14) showed a spectrum profile at 300 to 800nm due to the sharpness of peaks and proper baseline. The peak spectra of fraction 4 showed characteristic peaks at 665.50, 607.00, 532.50 503.50, 400.00 and 326.00nm with the absorption of 0.365, 0.097, 0.116, 0.137, 0.952 and 0.389 respectively. In case of fractions 5 observed peaks were at 665.00, 607.50, 532.50 503.50, 407.00 and 326.50nm with absorption of 0.362, 0.100, 0.126, 0.141, 1.000 and 0.440 respectively. Peaks of fraction 7 were at 665.00, 607.50, 532.50, 503.50 and 407.50nm with absorption spectra of 0.172, 0.062, 0.079, 0.088 and 0.488 respectively. The observed peaks of fraction 10 were at 661.50, 403.00 and 344.50nm with the absorption of 0.038, 0.139 and 0.158 respectively while fraction 14 peaks were at 662.00, 369.50 and 353.50 having an absorbance of 0.054, 0.349 and 0.351 respectively (Table 5.6-5.10 and figure 5.5).

Table 5.6: UV-Vis spectrum values of fraction 4

Sr. No.	Wavelength (nm)	Absorbance
1	665.50	0.365
2	607.00	0.097
3	532.50	0.116
4	503.50	0.137
5	400.00	0.952
6	326.00	0.389

Table 5.7: UV-Vis spectrum values of fraction 5

Sr. No.	Wavelength (nm)	Absorbance
1	665.00	0.362
2	607.50	0.100
3	532.50	0.126
4	503.50	0.141
5	407.00	1.000
6	326.50	0.440

Table 5.8: UV-Vis spectrum values of fraction 7

Sr. No.	Wavelength (nm)	Absorbance
1	665.00	0.172
2	607.50	0.062
3	532.50	0.077
4	503.50	0.088
5	407.50	0.488

Table 5.9: UV-Vis spectrum values of fraction 10

Sr. No.	Wavelength (nm)	Absorbance
1	665.50	0.038
2	403.00	0.139
3	344.50	0.158

Table 5.10: UV-Vis spectrum values of fraction 14

Sr. No.	Wavelength (nm)	Absorbance
1	665.00	0.054
2	369.50	0.349
3	353.50	0.351

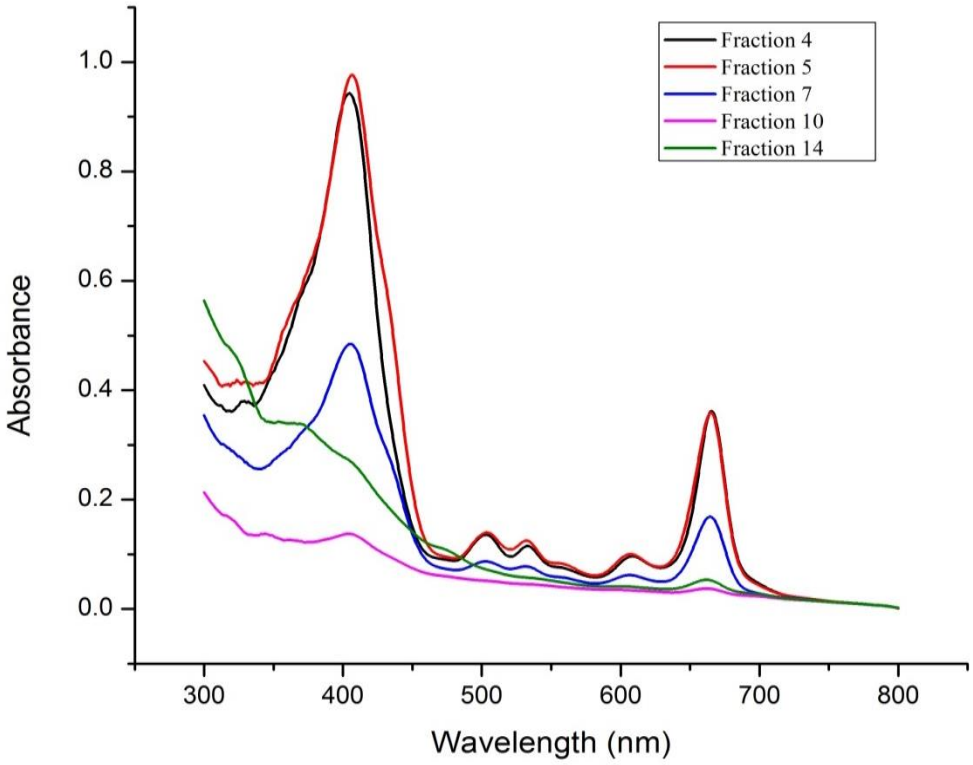


Figure 5.5: UV-Vis spectra of bioactive fractions (4, 5, 7, 10 and14)

5.4: Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis of bioactive fractions (4, 5, 6, 7 10 and 14) in ethanol solvent helps in the identification of functional groups of active compounds present in each fraction. Each peak in spectrum represents the specific peak value corresponding to the specific functional groups. The absorbance was noted from 400 to 4000cm^{-1} wavelength. The results of FTIR analysis confirmed the presence of alkanes, alkynes, aldehydes, amines, ether, amino acids, thiols, cyanides, nitriles, alcohols, and carboxylic acids. In all fractions (4, 5, 7, 10 and 14), the absorbance between wavelength 2865-2984cm^{-1} confirmed the presence of alkanes. The absorption peak at 2821-2841cm^{-1} represents ether while absorption at 2782-2833cm^{-1} represents methylamino. The absorption range of aldehydes was 2723-2775cm^{-1} and peak at 2445cm^{-1} exhibit aliphatic cyanides. The absorbance between 2298-2398cm^{-1} revealed the presence of amino acids and at 2272-2277cm^{-1} was Isocyanate. The absorption peak of nitriles and isothiocyanate was between 2225-2235cm^{-1} and 2017-2117cm^{-1} respectively.

Thiocyanate was observed only in fraction 4 (2144.99cm^{-1}), 7 (2150.46) and 14 (2157.08). Terminal alkynes were present in fraction 4 and 5 having absorbance at 2122.44cm^{-1} and 2107.04-2135.42cm^{-1} respectively. Medial alkynes were only in fraction 7 with an absorption peak of 2195.01cm^{-1}. Thiols were absent in fraction 4 while in remaining fraction absorption was between 2543.97-2586.09cm^{-1}. Carboxylic acids with O-H stretch have maximum absorption at 2625.06-2689.97cm^{-1} in fraction 5 while with N-H stretch at 2371.64cm^{-1} and 2374.35cm^{-1} in fraction 5 and 10 respectively. Alcohols with O-H stretch in fraction 7 were present in range 2608.69-2689.97cm^{-1}, in fraction 10 and 14 the peak was at 2612.79-2669.81cm^{-1} and 2661.82cm^{-1} respectively. Azides were present only in fraction 10 at the absorption peak of 2158.14cm^{-1} (Table 5.11-5.15 and figure 5.6-5.10).

Table 5.11: FTIR spectral peak values and functional groups of fraction 4

Peak #	Peak value (cm^{-1})	Type of stretching	Functional group
1	2984.27	C-H	Methyl stretch
2	2957.37	C-H	Methyl stretch
3	2928.51	C-H	Methylene stretch
4	2887.07	C-H	Methylene stretch
5	2865.94	C-H	Methylene stretch
6	2841.25	O-CH_3	Methoxy, methyl ether
7	2810.57	N- CH_3	Methyl amino
8	2782.88	N- CH_3	Methyl amino
9	2756.59	C-H stretch	Aldehydes
10	2723.93	C-H stretch	Aldehydes
11	2509.73	Unknown	-
12	2445.75	-	Aliphatic cyanide/nitriles
13	2386.59	N-H stretch	Amino acids
14	2333.37	N-H stretch	Amino acids
15	2298.10	N-H stretch	Amino acids
16	2272.24	-N=C=O	Isocyanate
17	2225.65	C≡N	Medial alkynes/Nitriles
18	2144.99	S-C=N	Thiocyanate
19	2122.44	C≡H	Terminal alkanes
20	2056.60	N=C=S	Isothiocyanate
21	2027.76	N=C=S	Isothiocyanate

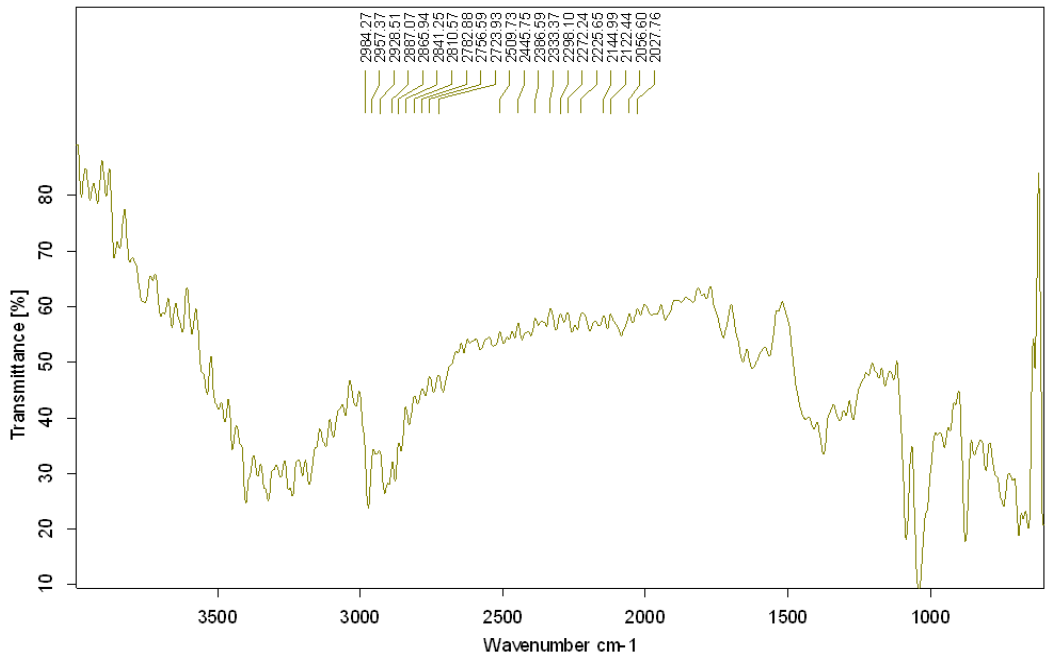


Figure 5.6: FTIR spectrum of fraction 4

Table 5.12: FTIR spectral peak values and functional groups of fraction 5

Peak #	Peak value (cm^{-1})	Type of stretching	Functional group
1	2954.20	C-H	Methyl stretch
2	2933.10	C-H	Methylene stretch
3	2911.88	C-H	Alkanes
4	2832.34	O-CH_3	Methoxy, Methyl Ether
5	2755.67	C-H stretch	Aldehydes
6	2733.99	C-H stretch	Aldehydes
7	2689.97	O-H	Hydrogen bonded carboxylic acids
8	2684.42	O-H	Hydrogen bonded carboxylic acids
9	2625.06	O-H	Hydrogen bonded carboxylic acids
10	2586.09	S-H stretch	Thiols
11	2565.81	S-H stretch	Thiols
12	2541.10	Unknown	-
13	2425.88	Unknown	-
14	2402.69	Unknown	-
15	2371.64	N-H stretch	Carboxylic acids
16	2323.03	N-H stretch	Amino acids
17	2271.08	-N=C=O	Isocyanate
18	2230.03	C≡C	Medial alkynes
19	2191.82	C≡C	Medial alkynes
20	2135.42	C≡C	Terminal alkynes
21	2107.04	C≡C	Terminal alkynes
22	2049.97	N=C=S	Isothiocyanate
23	2018.11	N=C=S	Isothiocyanate

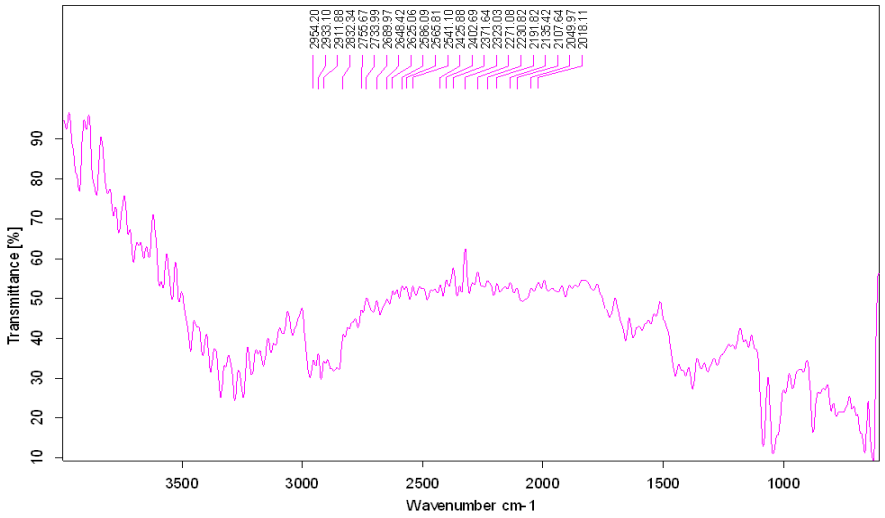


Figure 5.7: FTIR spectrum of fraction 5

Table 5.13: FTIR spectral peak values and functional groups of fraction 7

Peak #	Peak value (cm^{-1})	Type of stretching	Functional group
1	2956.95	C-H	Methyl stretch
2	2946.30	C≡N	Nitriles
3	2894.20	C-H	Methylene stretch
4	2869.34	C-H	Methyl stretch
5	2821.87	O-CH_3	Methoxy, methyl ether
6	2799.66	N- CH_3	Methyl amino
7	2767.85	C-H stretch	Aldehydes
8	2744.81	C-H stretch	Aldehydes
9	2723.99	C-H stretch	Aldehydes
10	2689.90	OH stretch	Alcohols
11	2608.69	OH stretch	Alcohols
12	2569.71	S-H stretch	Thiols
13	2536.78	Unknown	-
14	2477.52	Unknown	-
15	2441.73	Unknown	-
16	2411.55	Unknown	-
17	2367.57	N-H stretching	Amino acids
18	2345.54	N-H stretching	Amino acids
19	2322.86	Unknown	-
20	2277.55	N=C=O	Isocyanate
21	2235.03	C≡N	Nitriles
22	2195.01	C≡C	Medial alkynes
23	2150.46	S-C≡N	Thiocyanate
24	2017.09	N=C=S	Isothiocyanate

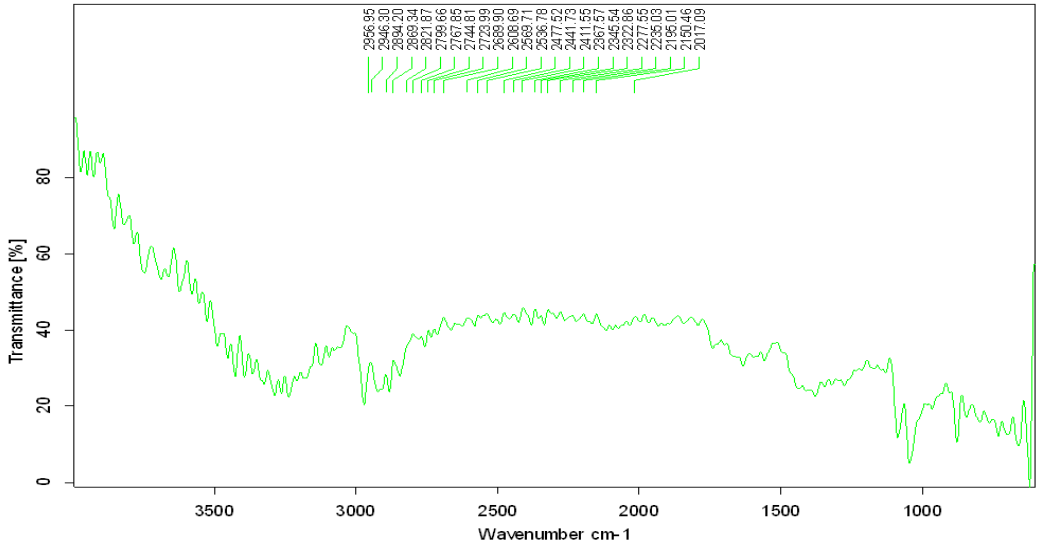


Figure 5.8: FTIR spectrum of fraction 7

Table 5.14: FTIR spectral peak values and functional groups of fraction 10

Peak #	Peak value (cm^{-1})	Type of stretching	Functional group
1	2942.48	C≡N	Nitriles
2	2916.61	C-H	Methylene stretch
3	2833.03	O-CH_3	Methoxy, methyl ether
4	2795.80	N-CH_3	Methyl amino
5	2775.29	C-H stretch	Aldehydes
6	2753.77	C-H stretch	Aldehydes
7	2669.81	O-H stretch	Alcohols
8	2648.81	O-H stretch	Alcohols
9	2612.79	O-H stretch	Alcohols
10	2559.95	S-H stretch	Thiols
11	2475.67	Unknown	-
12	2451.33	Unknown	-
13	2394.48	N-H stretch	Amino acids
14	2374.35	N-H stretch	Carboxylic acids
15	2354.37	N-H stretch	Amino acids
16	2306.83	Unknown	-
17	2277.02	N=C=O	Isocyanate
18	2225.17	C≡N	Nitriles
19	2158.14	N=N=N	Azides
20	2061.12	N=C=S	Isothiocyanate
21	2033.11	N=C=S	Isothiocyanate

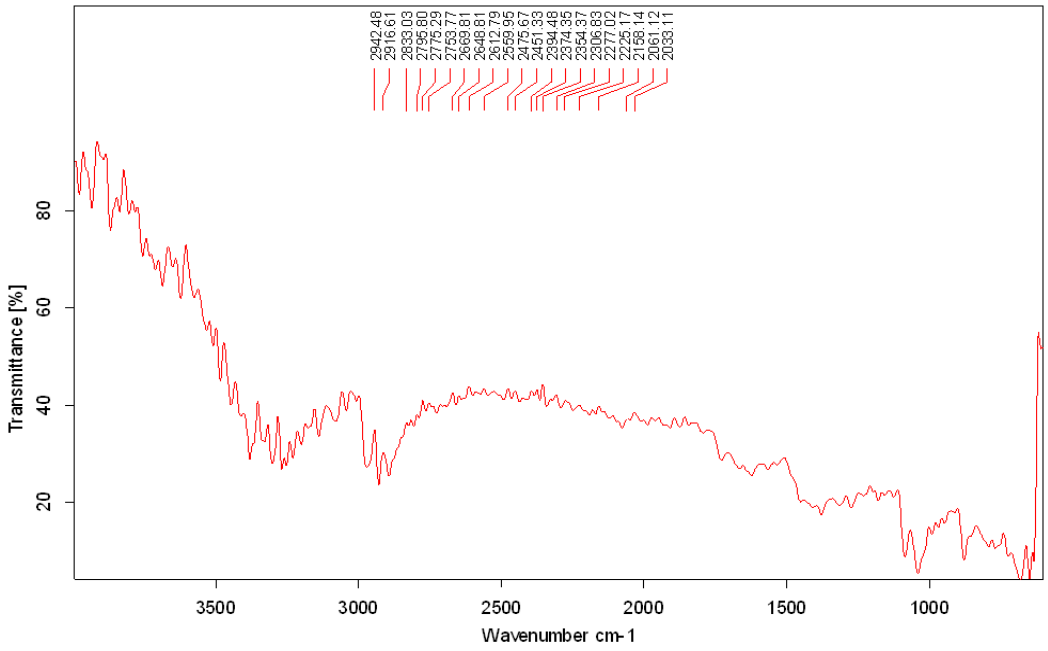


Figure 5.9: FTIR spectrum of fraction 10

Table 5.15: FTIR spectral peak values and functional groups of fraction 14

Peak #	Peak value (cm^{-1})	Type of stretching	Functional group
1	2958.50	C-H	Methyl stretch
2	2926.58	C-H	Methylene stretch
3	2906.36	C-H	Methyl stretch
4	2864.18	C-H	Methyl stretch
5	2833.48	O-CH_3	Methoxy, methyl ether
6	2812.44	N-CH_3	Methyl amino
7	2782.52	N-CH_3	Methyl amino
8	2730.88	C-H stretch	Aldehydes
9	2688.36	Unknown	-
10	2661.82	O-H stretch	Alcohols
11	2543.97	S-H stretch	Thiols
12	2419.86	Unknown	-
13	2398.83	N-H stretching	Amino acids
14	2369.62	N-H stretching	Amino acids
15	2334.90	N-H stretching	Amino acids
16	2310.79	Unknown	-
17	2276.97	N=C=O	Isocyanate
18	2226.73	C≡N	Nitriles
19	2157.08	S-C≡N	Thiocyanate
20	2117.22	N=C=S	Isothiocyanate
21	2064.98	N=C=S	Isothiocyanate
22	2033.10	N=C=S	Isothiocyanate

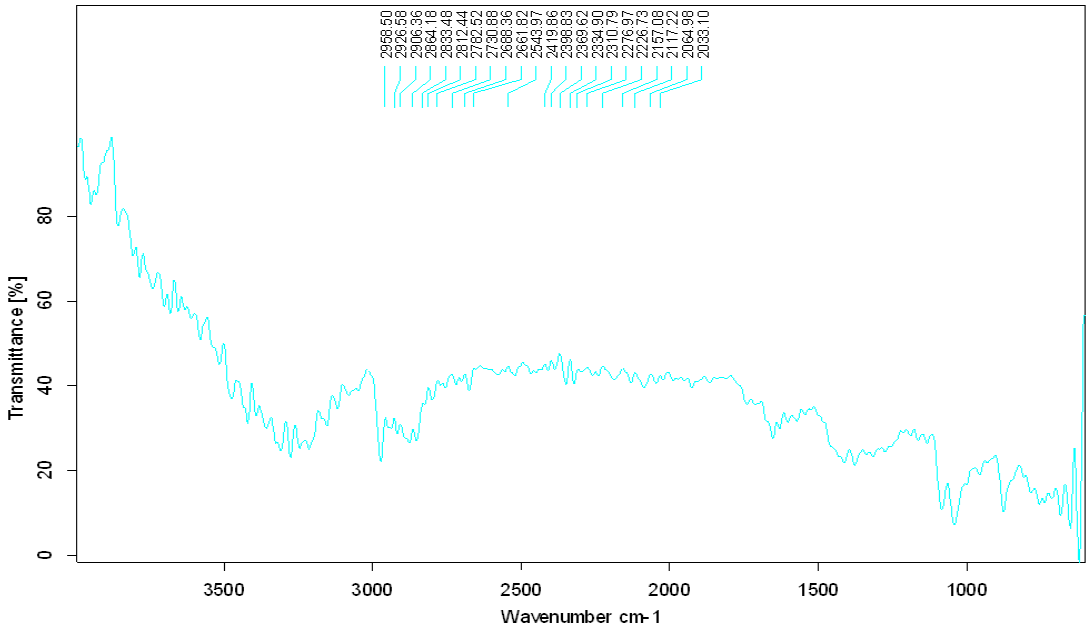


Figure 5.10: FTIR spectrum of fraction 14

5.5: Gas chromatograph coupled mass spectroscopy (GC-MS)

5.5.1: GC-MS analysis of fraction 4

The GC-MS chromatography of fraction 4 isolated from ethanolic leaves extract of *G. robusta* showed ten peaks which confirmed the presence of ten bioactive compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound names, retention time, area percentage, molecular weights and a molecular formula of identified components are listed in table 5.16. The major bioactive compounds in fraction 4 are Vitamin E (48.69%) and Coumarin, 7-hydroxy (11.12%) while 3,7,11,15-Tetramethyl-2-hexadecane-1-ol (3.86%), 1,2-Benzenedicarboxylic acid, diundecyl ester (5.71%), Phytol (5.74%), 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (4.11%), Dihexadecyl phosphate (4.07%), p-Arbutin (7.56%), 1,2-Benzenedicarboxlic acid, diisodecyl ester (6.47%) and 9-Hexacosanol (2.67%) are present in minor quantity. The GC-MS chromatogram of fraction 4 is represented in figure 5.11.

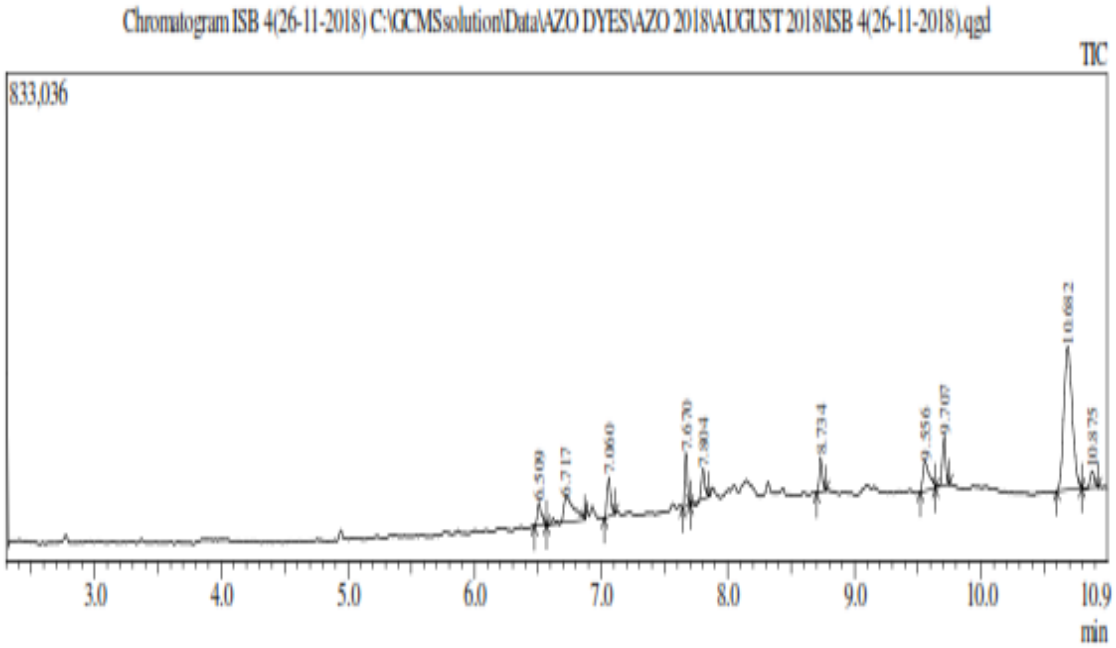
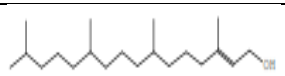
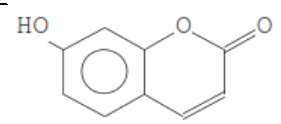
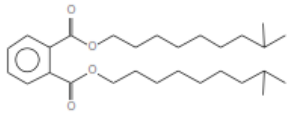
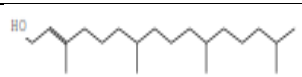
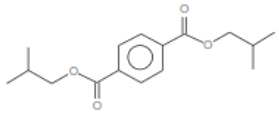
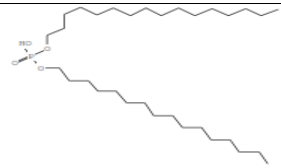
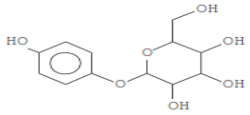
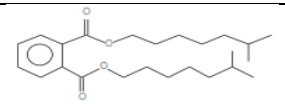
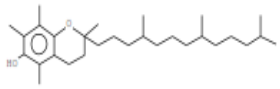


Figure 5.11: GC-MS chromatogram of fraction 4

Table 5.16: Phytocomponents identified in fraction 4 by GC-MS peak report

Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	6.509	3.86	$C_{20}H_{40}O$	296	3,7,11,15-Tetramethyl-2-hexadecane-1-ol	
2	6.717	11.12	$C_9H_6O_3$	162	Coumarin, 7-hydroxy	
3	7.060	5.71	$C_{28}H_{46}O_4$	446	1,2-Benzenedicarboxylic acid, diisodecyl ester	
4	7.670	5.74	$C_{20}H_{40}O$	296	Phytol	
5	7.808	4.11	$C_{16}H_{22}O_4$	278	1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	
6	8.374	4.07	$C_{32}H_{67}O_4P$	546	Dihexadecyl phosphate	
7	9.556	7.56	$C_{12}H_{16}O_7$	272	p-Arbutin	

8	9.707	6.47	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxlic acid, diisooctyl ester	
9	10.682	48.69	$C_{29}H_{50}O_2$	430	Vitamin E	
10	10.875	2.67	$C_{26}H_{52}$	364	9-Hexacosanol	

5.5.2: GC-MS analysis of fraction 5

The GC-MS chromatogram of fraction 5 isolated from ethanolic leaves extract of *G. robusta* revealed ten peaks at different retention times and confirm the presence of ten compounds (Table 5.17). beta-Sitosterol and Vitamin E are the major components of fraction 5 having an area percentage of 52.29% and 15.48% respectively. Other bioactive compounds present include 3,7,11,15-Tetramethyl-2-hexadecane-1-ol (5.50%), Hexadecanoic acid, methyl ester (2.25%), 1,2-Benzenedicarboxylic acid, diundecyl ester (2.88%), Phytol (3.88%), 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (3.23%), Octadecanoic acid, 5-hydroxy-delta lactone (2.89%), p-Arbutin(5.49%) and 1,2-Benzenedicarboxlic acid, diisodecyl ester (6.11%). The GC-MS chromatogram of fraction 5 is represented in figure 5.12.

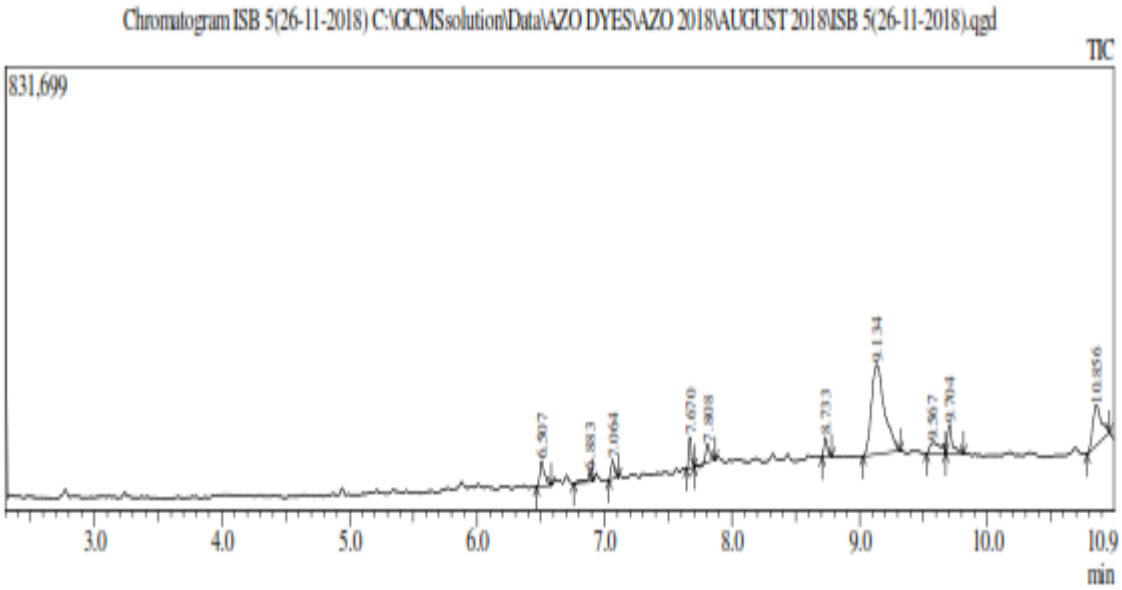
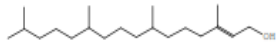
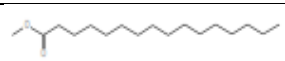
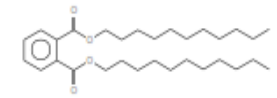
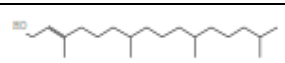
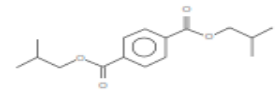
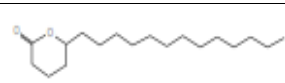
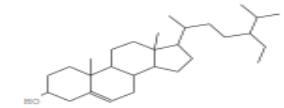
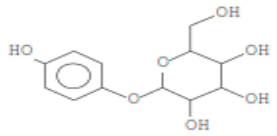
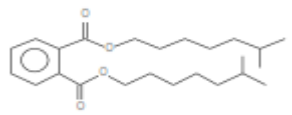
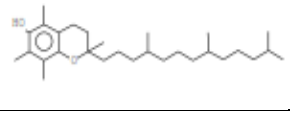


Figure 5.12: GC-MS chromatogram of fraction 5.

Table 5.17: Phytocomponents identified in fraction 5 by GC-MS peak report

Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	6.507	5.50	$C_{20}H_{40}O$	296	3,7,11,15-Tetramethyl-2-hexadecane-1-ol	
2	6.883	2.25	$C_{17}H_{32}O_2$	270	Hexadecanoic acid, methyl ester	
3	7.064	2.88	$C_{28}H_{46}O_4$	446	1,2-Benzenedicarboxylic acid, diundecyl ester	
4	7.667	3.88	$C_{20}H_{40}O$	296	Phytol	
5	7.808	3.23	$C_{16}H_{22}O_4$	278	1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	
6	8.733	2.89	$C_{18}H_{34}O_2$	282	Octadecanoic acid, 5-hydroxy-delta lactone	
7	9.134	52.29	$C_{29}H_{50}O$	414	beta.-Sitosterol	
8	9.567	5.49	$C_{12}H_{16}O_7$	272	p-Arbutin	

9	9.704	6.11	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxlic acid, diisodecyl ester	
10	10.856	15.48	$C_{26}H_{52}$	430	Vitamin E	

5.5.3: GC-MS analysis of fraction 7

The GC-MS chromatogram of fraction 7 isolated from ethanolic leaves extract of *G. robusta* revealed ten peaks at different retention times and confirm the presence of ten compounds (Table 5.18). beta-Sitosterol was the most abundant component having an area percentage of 52.10%. 2,6,6-Trimethyl-2-hydroxycyclohexylidene acetic acid lactone (2.16%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (6.59%), Hexadecanoic acid, methyl ester (2.03%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (6.95%), Phytol (5.13%), 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (6.44%), Octadecanoic acid, 5-hydroxy-delta lactone (3.51%), 1,2-Benzenedicarboxlic acid, diisodecyl ester (7.54%) and 9-Octadecenoic acid (Z)-tetradecyl ester (7.56%) are the minor component. The GC-MS chromatogram of fraction 7 is represented in figure 5.13.

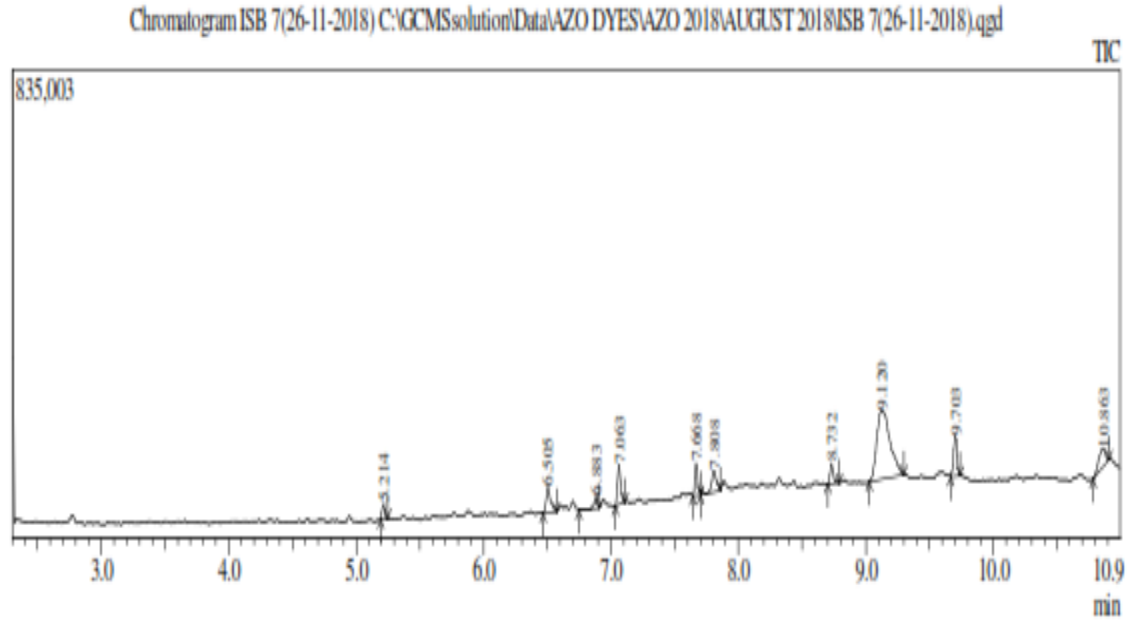
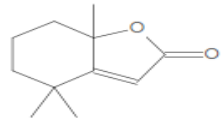
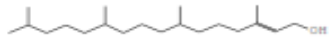
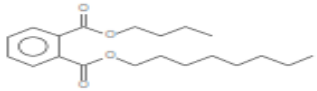
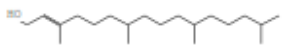
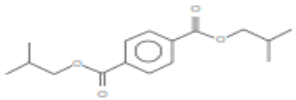
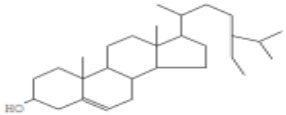
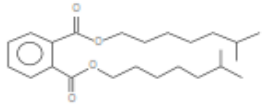
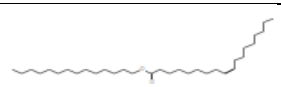


Figure 5.13: GC-MS chromatogram of fraction 7

Table 5.18: Phytocomponents identified in fraction 7 by GC-MS peak report

Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	5.214	2.16	$C_{11}H_{16}O_2$	180	2,6,6-Trimethyl-2-hydroxycyclohexylidene acetic acid lactone	
2	6.505	6.59	$C_{20}H_{40}O$	296	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	
3	6.883	2.03	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester	
4	7.063	6.95	$C_{20}H_{30}O_4$	334	1,2-Benzenedicarboxylic acid, butyl octyl ester	
5	7.688	5.13	$C_{20}H_{40}O$	296	Phytol	
6	7.808	6.44	$C_{16}H_{22}O_4$	278	1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	
7	8.732	3.51	$C_{18}H_{34}O_2$	282	Octadecanoic acid, 5-hydroxy-delta lactone	
8	9.120	52.10	$C_{29}H_{50}O$	414	beta-Sitosterol	
9	9.703	7.54	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxlic acid, diisodecyl ester	

10	10.863	7.56	$C_{24}H_{38}O_4$	478	9-Octadecenoic acid (Z)-tetradecyl ester	

5.5.4: GC-MS analysis of fraction 10

The nine peaks were reported in the GC-MS chromatogram of fraction 10 isolated from ethanolic leaves extract of *G. robusta*. Four major components were reported including delta.4-Sitosterol-3-one, n-Hexadecanoic acid, beta.-Sitosterol and 3-Tetradecynoic acid having an area percentage of 29.18%, 22.86%, 22.34%, and 13.96% respectively. Five minor components include 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.75%), Hexadecanoic acid, methyl ester (0.80%), Octadecenoic acid (4.90%),1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (1.06%) and 1,2-Benzenedicarboxylic acid, diisooctyl ester (3.25%) (Table 5.19). The GC-MS chromatogram of fraction 10 is represented in figure 5.14.

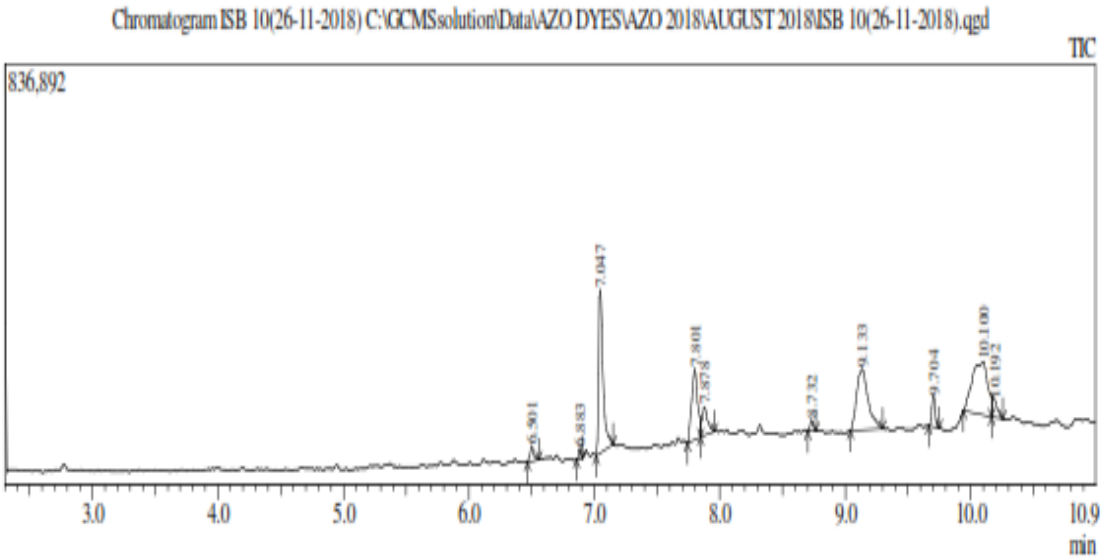
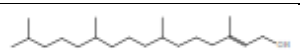
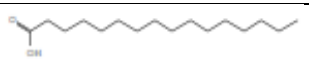
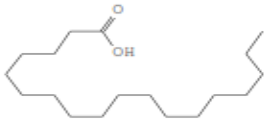
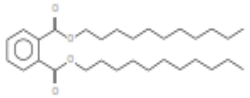
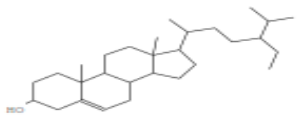
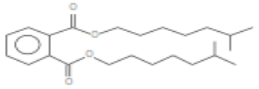
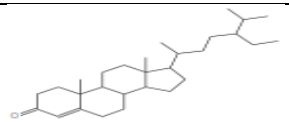


Figure 5.14: GC-MS chromatogram of fraction 10

Table 5.19: Phytocomponents identified in fraction 10 by GC-MS peak report

Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	6.501	1.75	$C_{20}H_{40}O$	296	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	
2	6.883	0.80	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester	
3	7.047	22.86	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
4	7.801	13.96	$C_{14}H_{24}O_2$	224	3-Tetradecynoic acid	
5	7.878	4.90	$C_{18}H_{36}O_2$	248	Octadecenoic acid	
6	8.372	1.06	$C_{16}H_{22}O_4$	278	1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	
7	9.133	22.34	$C_{29}H_{50}O$	414	beta.-Sitosterol	
8	9.704	3.25	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxylic acid, diisooctyl ester	

9	10.100	29.18	$C_{29}H_{48}O$	412	delta.4-Sitosterol-3-one	

5.5.5: GC-MS analysis of fraction 14

The GC-MS chromatogram of fraction 14 consist of seven peaks having 9,12,15-Octadecatrienoic acid (48.82%) and n-Hexadecanoic acid (32.17%) as major components while 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (0.68%), 9-Hexadecanoic acid (0.84%), Octadecenoic acid (9.86%), beta.-Sitosterol (6.00%) and 1,2-Benzenedicarboxylic acid, diisooctyl ester (1.62%) as the minor components (Table 5.20). The GC-MS chromatogram of fraction 14 is represented in figure 4.15. Reported biological activities of phytochemical compounds isolated from bioactive fractions (4, 5, 7, 10 and 14) are represented in table 5.21.

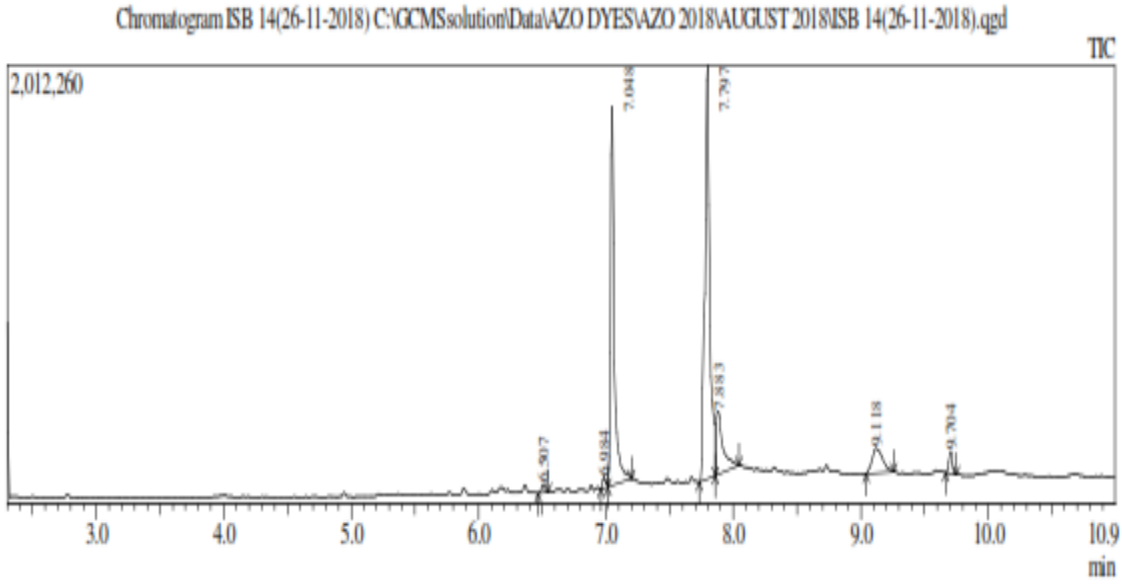


Figure 5.15: GC-MS chromatogram of fraction 14

Table 5.20: Phytocomponents identified in fraction 14 by GC-MS peak report

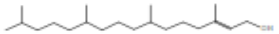
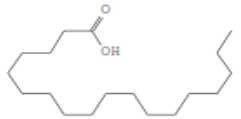
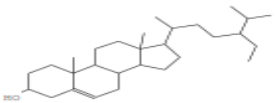
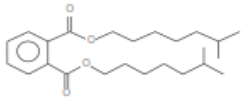
Peak #	Retention time	Area %age	Molecular formula	Molecular weight	Compound name	Structure
1	6.507	0.68	$C_{20}H_{40}O$	296	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	
2	6.984	0.84	$C_{16}H_{30}O_2$	254	9-Hexadecanoic acid	
3	7.048	32.17	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
4	7.797	48.82	$C_{18}H_{30}O_2$	278	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	
5	7.883	9.86	$C_{18}H_{36}O_2$	248	Octadecenoic acid	
6	9.118	6.00	$C_{29}H_{50}O$	414	beta.-Sitosterol	
7	9.704	1.62	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxylic acid, diisooctyl ester	

Table 5.21: Biological activities of phytochemical compounds isolated from bioactive fractions (4, 5, 7, 10 and 14) of *G. robusta* ethanolic leaves extract

Sr. No.	Compound name	Compound nature	Biological activity	Reference
1	3,7,11,15-Tetramethyl-2-hexadecane-1-ol	Terpene alcohol	Antimicrobial, anti-cancerous, anti-inflammatory, antioxidant	Sermakkani and Thangapandian, 2012; Prabakaran and Mani 2017.
2	Phytol	Diterpene	anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive activities	Sermakkani and Thangapandian, 2012; Casuga *et al.* 2106.
3	1,2-Benzenedicarboxlic acid, diisooctyl ester	Plasticizer compound	Antimicrobial and antifouling	Sermakkani and Thangapandian, 2012
4	Vitamin E		Cytotoxic and anticancerous	Casuga *et al.* 2106.
5	n-Hexadecanoic acid	Fatty acid/Palmitic acid	Hypocholesterolemic, antioxidant, nematicides, lubricant, pesticide, flavor, haemolytic	Sermakkani and Thangapandian, 2012
6	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	Fatty acid/ Linolenic acid	Insectifuge, anti-inflammatory, hypocholesterolemic, cancer-preventive, nematicide, hepatoprotective	Sermakkani and Thangapandian, 2012; Prabakaran and Mani 2017.
7	Octadecenoic acid	Stearic acid	No activity reported	Sermakkani and Thangapandian, 2012.
8	beta.-Sitosterol	Sterols	Anti-inflammatory, antioxidant, anti-cancerous	Casuga *et al.* 2106.
9	Hexadecanoic acid, methyl ester	Fatty acid ester	Pesticide, Nematicide, hypocholesterolemic, antioxidant, flavor, 5-alpha-reductase inhibitor, antibacterial, antiallergic	Sermakkani and Thangapandian, 2012; Rukshana *et al.* 2017; Prabakaran and Mani 2017.

DISCUSSION

Extraction and analysis of natural bioactive compounds from the medicinal plants help in quality control of herbal medicines and their use to secure human being and other animals from the attack of natural toxins (Gomathi *et al.,* 2015). Plants are a rich source of many phytochemicals that are used in different industries as a part of many herbal medicines from crude plant extract. Therefore in the present study characterization of the most effective plant (*G. robusta*) was carried out by VU-Vis Spectroscopy, FTIR and GC-MS analysis. The UV-Vis spectroscopic analysis of *G. robusta* leaves extract was performed at a wavelength of 200 to 800nm and maximum absorption was observed at 208.94nm (2.33) and 282.97 (0.811). Similar absorption peaks were observed by Rani *et al.* (2016) in leaf extract of *Meizotropis pellita*. The nearest absorption spectra were also reported by Rajeshkumar and Jayaprakash (2016) on selected red seaweed (*Acanthophora specifera*). The results of FTIR analysis confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether, 1,2,4 trisubstituted and halo compounds at the wavelength of 3307.00, 2976.32, 2943.98, 2831.48, 1088.70, 1023.33, 880.88, 630.24, 542.66, 527.03, 522.55nm respectively. The presence of these functional groups in plant extract has been confirmed previously (Ashokkumar and Ramaswamy, 2014; Packialakshmi and Naziya, 2014; Nithyadevi and Sivakumar, 2015).

The GC-MS analysis of *G. robusta* leaves extracts characterized fifteen phytochemicals by comparing their mass spectrum with the NIST library. Among the identified bioactive compounds n-Hexadecanoic acid have been reported previously to have antimicrobial, antioxidant, antifibrinolytic and hemolytic activity (Gomathi *et al.,* 2015). Similarly n-Hexadecanoic acid, Phytol and 9,12,15- Octadecatrienoic acid(Z,Z,Z) characterized in *G. robusta* leaves extract has also been identified by Sermakkani and Thangapandian (2012) in methanolic leaves extract of *Cassia italica* and reported their biological activities. n-Hexadecanoic acid (Palmitic acid) has an antiandrogenic, antioxidant, hypocholesterolemic, flavoring agent, nematicides, lubricant, and pesticidal activities. Phytol belongs to Diterpenes and has anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive activities (Al-Tameme *et al.,* 2015; Sermakkani and

Thangapandian, 2012). 9,12,15- Octadecatrienoic acid (Z,Z,Z) is a fatty acid ester compound and has hepatoprotective, anti-inflammatory, insectifuge antihistaminic, 5-alpha-reductase inhibitor, antiandrogenic, pesticidal and cancer preventive activities (Sermakkani and Thangapandian, 2012; Kadhim *et al.*, 2016). 2,3-Dihydrobenzofuran has been reported in *Origanum vulgare* seeds that have antimicrobial and anti-inflammatory activities (Al-Tameme *et al.*, 2015). In previous studies, Macrocyclic phenols, cinnamic acid derivatives, and alkylresorcinols have been reported in *G. robusta* (Chuang *et al.*, 2011).

Preliminary phytochemical screening of bioactive fractions (4, 5, 7, 10 and 14) isolated from ethanolic leaves extract of *G. robusta* was carried out by different tests which confirms the presence of various secondary metabolites such as flavonoids, tannins, sterols, quinones, alkaloids, terpenoids, and carbohydrates. Alkaloids, terpenoids, and carbohydrates were present commonly in all fractions while sterols were absent in fraction 14 while present in the other four fractions. Flavonoids and tannins were present in fraction 4, 5 and 7 while quinones were only in fraction 5. Similar phytochemicals are reported by Kalimuthu and Prabakaran (2013) in phytochemical screening of *Ceropegia pusilla*, Jabin and Nasreen (2016) in leaves extract of *Lawsonia inermis, Punica granatum, Syzygium cumini, Capsicum annum, Jatropha curcas*, *Murraya koenigii* and Aylooa *et al.* (2008) in extracts of *Magnifera indica*, *Vernonia amygdalina*, *Carica papaya*, and *Psidium guajava*. Phytochemical analysis of different parts of medicinal plants including *Ipomea aquatic* (leaves), *Ricinus communis* (roots), *Bryophyllum pinnatum* (leaves), *Tinospora cordifolia* (leaves) *Xanthium strumarium* (leaves), *Terminalia bellerica* (leaves), *Tinospora cordifolia* (stem) and *Oldenlandia corymbosa* (whole plant) was conducted by Yadav and Agarwala (2011) and confirmed the presence of tannins, saponins, steroids, terpenoids, glycosides, flavonoids and alkaloids as in accordance with results reported in the present study.

Secondary metabolite such as flavonoids, tannins and aromatic compounds in plants serves as a defense mechanism against microorganisms. Presence of saponins, alkaloids, tannins, flavonoids, polysteroles, phenolic compounds and terpenoids in plant extract to make them useful for antiplasmodic, bacteriocidal and analgesic activities

(Nithyadevi and Sivakumar, 2015). Flavonoids are a class of polyphenolic compounds that act as anti-inflammatory and anti-microbial agent by inhibiting the growth of antibiotic-resistant microbes (Kalimuthu and Prabakaran, 2013). Flavonoids also have antioxidant activities providing inflammatory actions, they are free radical scavengers, highly water-soluble which help to prevent oxidative cell damage and act as anti-cancerous agents (Njoku and Akumefula, 2007). Alkaloids are toxic to many organisms and have a bitter taste (Gupta *et al.*, 2010). Tannins are complex biological molecules produced by most of the plants as a protective substance. Tannins are frequently used as a tanning agent and have antimicrobial, antidiarrheal, astringent, antioxidant and anti-inflammatory activities (Killedar and More, 2010). Terpenoids are volatile compounds and have antibacterial, antifungal, anti-cancerous and cytotoxic activities (Rajeswari *et al.,* 2011). They are also used as a defensive agent against different insects, insects vectored fungi and pathogenic endophytic fungi (Langenheim, 1994).

The UV-Vis spectroscopic analysis of bioactive fractions was performed at 200 to 800nm and a common absorption peak was reported at 665.50 in all fractions having different absorption spectra. Jain *et al.* 2016 reported similar absorption spectra at 665nm and the nearest remaining peaks wavelength of *the Mentha spicata* plant extract. Nearest absorption peaks were observed by Rani *et al.* (2016) in leaf extract of *Meizotropis pellita* at 660nm and by Rajeshkumar and Jayaprakash (2016) at 669.12nm on selected red seaweed (*Acanthophora specifera*). UV absorption spectra of fraction 4, 5 and 7 were almost similar having similar absorption peaks. Rajeshkumar and Jayaprakash (2016) reported that the absorption spectra between 300-350nm give information about the nature of flavonoids. Absorption spectra in the range of these wavelengths also reported in the present study which confirm the presence of flavonoids and its derivatives.

The FTIR analysis of bioactive fractions (4, 5, 7, 10 and 14) isolated from *G. robusta* was carried out using Shimadzu ETIR spectrophotometer and absorption spectra were recorded from 400 to 4000cm^{-1}. The results of FTIR analysis confirmed the presence of alkanes, alkynes, aldehydes, amines, ether, amino acids, thiol, cyanide, nitriles, alcohol and carboxylic acids in all the fractions however Thiocyanate was observed only in fraction 4. Terminal alkynes were present in fraction 4 and 5 while

medial alkynes were only in fraction 7. Thiols were absent in fraction 4 and Azides were present only in fraction 10. Ashokkumar and Ramaswamy (2014) performed in FTIR analysis of *Senna auriculata, Phyllanthus amarus, Solanum torvum* and *Phyllanthus maderaspatensis* in different solvents and confirmed the presence of alkanes, alkenes, alkyl halides, alcohols, phenols, aldehydes, amides, amines, carboxylic acids, ketones, esters and ethers in methanolic extract. Similar functional groups are also reported in the present study in ethanolic leaves extract of *G. robusta*. Packialakshmi and Naziya (2014), Nithyadevi and Sivakumar (2015) and Sithara *et al.* (2017) also reported these functional groups in different medicinal plants extract. Among all the functional groups observed in bioactive fractions, OH groups were found more commonly. As OH group has the hydrogen binding capacity, so its presence probably indicates the higher potential of ethanolic leaves extract of *G. robusta* towards growth inhibitory activities against microorganisms (Ashokkumar and Ramaswamy, 2014).

The GC-MS analysis is a powerful tool that provides more precise information about the qualitative analysis of plant extract. The Mass spectrum provides information about the nature and structure of active compounds while the height of different peaks represents the relative concentration of each component (Prabakaran and Mani, 2017). In the present study, GC-MS analysis of bioactive fractions isolated from ethanolic leaf extracts of *G. robusta* was carried out by gas chromatography coupled with mass spectrophotometer (GC-2010, Shimadzu). Compound name, retention time, area percentage, molecular weight and a molecular formula of identified components compared with the NIST library are listed in table 5.13-5.17. Results of GC-MS analysis revealed that 3,7,11,15-Tetramethyl-2-hexadecen-1-ol is commonly present in fractions (4, 5, 7, 10 and 14). 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester is present in all fractions except 14. Similarly, beta-Sitosterol is also reported from all except fraction 4. 1,2-Benzenedicarboxylic acid, diisodectyl ester, and Phytol was present in fraction 4, 5 and 7. 1,2-Benzenedicarboxylic acid, diisooctyl ester was reported from fractions 4,10 and 14. The hexadecanoic acid methyl ester is present in fraction 5, 7 and 10. n-hexadecanoic acid and Octadecanoic acid are present in fraction 10 and 14. p-Arbutin is present in fraction 4 and 5.

Abirami and Rajendran (2012) reported n-Hexadecanoic acid, 3,7,11,15-Tetramethyl-2-hexadecen and Octadecanoic acid in methanolic leaves extract of *Vernonia cinerea*. Among the identified bioactive compounds n-Hexadecanoic acid has been reported previously to have antimicrobial, antioxidant, antifibrinolytic, and hemolytic activity (Gomathi *et al.,* 2015). Similarly, n-Hexadecanoic acid, Hexadecanoic acid methyl ester, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1,2-Benzenedicarboxylic acid diisooctyl ester, Phytol and Octadecanoic acid characterized in bioactive fractions have also been identified by Sermakkani and Thangapandian 2012 in methanolic leaves extract of *Cassia italica* and reported their biological activities. n-Hexadecanoic acid (Palmitic acid) have antiandrogenic, antioxidant, hypocholesterolemic, flavoring agent, nematicides, lubricant, and pesticidal activities. Hexadecanoic acid methyl ester has a hypocholesterolemic, flavoring agent, antioxidant and pesticidal properties. 3,7,11,15-Tetramethyl-2-hexadecen-1-ol has antimicrobial activities and 1,2-Benzenedicarboxylic acid diisooctyl ester has antifouling and antimicrobial activities.

Phytol belongs to Diterpenes and has anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive activities while Octadecanoic acid has no reported activity yet (Sermakkani and Thangapandian 2012; Al-Tameme *et al.,* 2015). Singaravadivel and Santhanaraj (2016) reported that phytol has excellent immunostimulant properties. Similarly, antibacterial activities of phytol against *Mycobacterium tuberculosis* and *Staphylococcus aureus* were reported by Inou *et al.* (2005) and Saikia *et al.* (2013). Casuga *et al.* (2016) reported beta-Sitosterol in methanolic leaves extract, phytol in methanol and ethyl acetate leaves extract and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol in ethyl acetate extract of *Broussonetia luzonica*. Prabakaran and Mani (2017) reported n-Hexadecanoic acid, Hexadecanoic acid methyl ester, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, Hexadecanoic acid methyl ester, and 9,12,15-Octadecatrienoic acid, (Z,Z,Z) in leaves extract of *Eichhomia crassipes*. Similar compounds were also reported by Jayapria and Shoba (2015) in the methanolic leaves extract of *Justicia adhatoda*. Verma *et al.* (2013) reported Hexadecanoic acid methyl ester in ethanolic leaves extract and 1,2-Benzenedicarboxylic acid diisooctyl ester in chloroform extract of *Calotropis procera*. All these reports are consistent with the findings of the present study.

From the present study, it can be concluded that ethanol has a strong capacity for isolation of several bioactive components from *G. robusta* leaves extract that is responsible for many biological activities. Identification of various functional groups and biologically active phytocomponents (n-Hexadecanoic acid, Hexadecanoic acid methyl ester, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1,2-Benzenedicarboxylic acid diisooctyl ester, Phytol and Octadecanoic acid, etc.) suggest that leaves extract of *G. robusta* have certain antimicrobial and pesticidal activities. These biologically active constituents can be further eluted and might be utilized for the formulation of traditional pesticides which can be used for the control of different insect pests.

GENERAL DISCUSSION

Termites are highly eusocial insects that are cosmopolitan having their diversity highly concentrated in tropical and subtropical areas (Eggleton, 2000). Morphological measurements are a key factor for species identification, taxonomy, and classification, helping us to locate species adaptation according to their environment ultimately leading to evolutionary changes (Arif *et al.,* 2012). However few reports are available about the morphometric analysis of *O. obesus* (Manzoor and Akhtar, 2006; Manzoor and Akhtar, 2006; Manzoor, 2009; Manzoor, 2010) and *H. indicola* (Chhotani and Das, 1979; Poovoli and Rajmohana, 2013; Mahapatro and Kumar, 2013).

In the present study soldier cast of two termite species, *O. obesus* and *H. indicola* was identified based on morphological measurements. Thirty-six parameters including length and width of the body, thorax, abdomen, head, mandibles, pronotum, postmentum, antenna (scape, pedicle, flagellum), legs and their parts were observed and analyzed by "*Student t-test*". Most of the body features showed overlapping values and are not significantly different among the samples. However certain adaptive traits were observed in few morphological characters of both species like tooth, antenna, and legs. According to Arif *et al.* (2012), these variations are due to environmental stress because termites use the antenna as a sensor tool for detecting their prey and leg for traveling in search of food and shelter. Similarly, Sheikh *et al.* (2005) conducted a study on morphometric measurements of a fungus growing termite (*Microtermes mycophagous*) from different regions of Pakistan to understand its geographic range.

Molecular techniques are considered as a good tool for species identification (Yang and Rannala, 2012). Previously, termite gut flagellates have been classified by phylogenetic analysis and 434 species were recorded from the hindgut of different termite species that belongs to three different orders. These are Trichomonadida, Hypermastigida, and Oxymonadida (Inoue *et al.,* 2000). In the present study, nine species of flagellates belonging to the three genera like *Holomastigotes, Holomastigotoides,* and *Pseudotrichonympha* were identified based on morphological characteristics from the hindgut of *H. indicola*. These species include *Holomastigotes campanula,*

Holomastigotes annandalei, Holomastigotes metchnikowi, Holomastigotoides hemigynum, Holomastigotoides hartmanni, Holomastigotoides kempi, Holomastigotoides Koidzumi, Holomastigotoides metchnikowi, and *Pseudotrichonympha grassii*. Being most abundant the *P. grassii* was further confirmed based on molecular analysis and compared with the studies conducted previously (Noda *et al.,* 2005). Phylogenetic analysis revealed that *P. grassii* isolated from the hindgut of Pakistani *H. indicola* was more closely related to the Japanese isolate. Noda *et al.* (2007) reported that *P. grassii,* the largest parabasalian flagellate is present in all genera of family Rhinotermitidae except the genus *Reticulitermes*. This evidence also supports the presence of *P. grassii* isolate in the hindgut of *H. indicola* collected from Islamabad, Pakistan.

The hindgut microbiota of termites releases cellulases that aids in productions of acetate, propionate, and butyrate as an end product of cellulose digestion. They also keep the internal environment of hindgut at the homeostatic level by fixing nitrogen (Mathew *et al*., 2012; Zhou *et al.,* 2019) and recycling of uric acid (Thong-On *et al.,* 2009) by secreting different enzymes. In the present study, bacterial fauna from the hindgut of both termite species (*O. obesus* and *H. indicola*) was also identified based on morphological, biochemical and molecular characterization. Five pure bacterial strains were isolated from each termite species by serial dilution method and identified. The isolated bacterial strains belong to two different families; Enterobacteriaceae and Bacillaceae. The identified species were *Bacillus cereus, Escherichia coli, Lysinibacillus fusiformis, Lysinibacillus xylanilyticus, Lysinibacillus macrolides*, *Bacillus subtilis,* and *Shigella sonnei*. This study also highlights the presence of some common bacteria in higher and lower termite species *i.e. Bacillus cereus, Escherichia coli,* and *Lysinibacillus fusiformis* were found to be common in both termite species along with *Lysinibacillus xylanilyticus* and *Lysinibacillus macrolides* in *O. obesus* only whereas *Bacillus subtilis* and *Shigella sonnei* in *H. indicola* hindgut. Wolber *et al.* (1986) isolated different aerobic, anaerobic and facultative bacteria from termite hindgut belonging to the *Staphylococcus, streptococcus,* Enterobacteriaceae, *Bacteroides,* and Bacillaceae. Bacteria identified in the present study also belong to the same families. The bacterial community in termite hindgut varies depending upon their feeding habit as termite feed on a variety of food

sources like dry wood (dry and hardwood), damp wood (humid decaying logs) and humus. Numerous plants related bacteria have been identified previously from bryophytes like *Bacillus, Pseudomonas, Delftia, Serratia, Cedecea, Viridbacillus*, and *Lysinibacillus*. The genera *Bacillus* and *Lysinibacillus* have also been reported in the present study from both termite species that strongly support this evidence. Similarly, *Serratia, Bacillus*, and *Pseudomonas* were found frequently with plants suppressing the growth of bacterial and fungal pathogens e.g. *Erwinia sp.* and *Alernaria alternaria* (Szentes *et al.,* 2013).

Biodegradation of wooden material by termites is a serious problem causing a huge economic loss. Globally, about $20 billion are spent annually for the control and repair process of infestation caused by them (Su, 2002). Termites can attack a wide range of materials, ranging from paper fabrics to noncellulosic material like asphalt, lead, asbestos, metal foil and bitumen (Bultman *et al.,* 1979). Subterranean termites control is one of the key factors for reducing the extent of damage and protection of any building structure or it's constituent. The two most effective control measures are baiting and soil treatment (Su and Scheffrahn, 2002) however the commonly used strategy is soil barrier treatment (Rust and Saran, 2006). This usually involves the application of insecticide in the form of a chemical barrier to reduce the risk of termite attack (Blaske *et al.,* 2003).

The high toxicity of chemical insecticides and their undesirable ecological impacts have limited their indoor applications. One of the major concerns is the appearance of exposed insecticides residues in food and their toxicity for non targeted organisms (Arnason *et al.,* 1989; Abudulai *et al.,* 2001). However at present due to the lack of any effective alternate, these chemical insecticides are allowed for crop and soil treatment (Alshehry *et al.,* 2014). Phytophagous insects mostly relay upon the plants' volatiles to recognize and reach their host pant. Therefor plants secondary metabolites can be used as an alternative control method to repel insect pests as non-host volatile emission (Mauchline *et al.,* 2008).

Medicinal plants are getting more importance in recent years as plants are a rich source of potential drugs for therapeutic activities (Yadav and Agarwala, 2011). These

plants have some bioactive compounds like flavonoids, alkaloids, tannins, carbohydrates, steroids, and terpenoids which have a certain physiological effect on the human body (Edeoga *et al.,* 2005). These secondary metabolites are synthesized during the metabolic activities of the living organism and were found to have an inhibitory effect and also serve as a defense mechanism against insects and microorganisms (Vaghasiya *et al.,* 2011).

Serious efforts have been made from the last few decades for the development of natural ecofriendly products such as microbial sprays, insect growth regulators and botanical insecticides (Blaske and Hertal, 2001). Previously, many medicinal plant species have been recruited to evaluate their toxicity and effect of natural products on the environment. The leaf and seed extracts of *Jatropha curcas, the* bark extract of *Melia* azedarach, rhizome extract of *Curcuma longa*, leaves extract of *Nerium indicumi* and crude seed extract of *Annona* were evaluated for behavioral and tunneling activities of subterranean termites (Nisar *et al.,* 2102; Acda, 2014). *Curcuma longa* extracts were applied against *Heterotermes indicola* and were found to be more effective as termites didn't cross the barrier (Manzoor *et al.,* 2011). Similarly, the effect of crude extracts of *Azadirachta indica, Phyllanthus niruri, Andrographis paniculata* and *Leucaena leucocephala* on behavior and tunneling activities was studied against *Coptotermes gestroi* and *Globitermes sulphurues* (Bakaruddin and Ab Majid, 2019). Many investigations were carried out on crude leaves extract and observed that these plants reduce termites' survival rate by changing their feeding behavior. These plants include *Taiwania cryptomerioides* (Chang *et al.,* 2001) *Calotropis procera* (Singh *et al.,* 2002) lemongrass, *Acassia* leaves, *Eucalyptus globulus*, vetiver oil. Clove bud, *Eucalyptus citroda*, cedarwood (Zhu *et al.,* 2001), *Rosmarinus officinalis, Cinammomum camphora, Coleus amboinicus*, isoborneol and *Cumbopogon wintrianusjowitt* (Singh *et al.,* 2004) *Lysitoma seemnii, Tabebina guaycan, Pseudotusuga menziesii, Diospyros sylvatica* (Ganapaty *et al.,* 2004), *Euphorbia kansuii* and *Curcuma aromatic* (Shi *et al.,* 2008).

In the present study leaves extract of eight tropical plants *i.e. C. papaya, E. camaldulensis, E. globulus, G. robusta, M. azedarach, M. longifolia, O. basilicum,* and *P. pinnata* were investigated for their antitermitic, antiprotozoan and antibacterial against *O.*

obesus and *H. indicola.* No-choice bioassays were carried out for the evaluation of antitermitic and antiprotozoan activity. The filter papers treated with different concentrations (100, 500, 1000, 1500 and 2000ppm) of plant extract were used as a substrate for termites. Among tested plants, *G. robusta* was found to have promising activity and cause significant ($P<0.05$) mortality in the termite population leading to the 90 and 94% death at the end of the experiment in both termite species *i.e. H. indicola* and *O. obesus* respectively. Similarly, *E. camaldulensis* and *O. basilicum* were also reported to have a significant ($P<0.05$) effect on termite survival and reduce up to 70 and 65% *H. indicola* population. However, in the case of *O. obesus, O. basilicum* was found to be more effective than *E. camaldulensis* and cause 76 and 60% mortality. Similar results were observed by Hassan *et al.* (2018). They reported that the population of *Reticulitermes flavipus* reduced significantly when exposed to the extracts of *Morus alba* and found that the percentage mortality of termites was concentration-dependent.

When termites were exposed to the plants extract behavioral changes were also observed *i.e.* at the start of experiment termites remained calm without any movement for a few seconds when exposed to the treated filter papers. Slowly their normal behavior changed with some abnormal responses when compared with control as the experiment proceeded. Termite feeding was reduced; their movements become inactive and lethargic followed by signs of weakness, trembling, fell on their backs and eventually died. Similar behavior changes were observed in termites previously (Edori and Dibofori-Orji, 2016; Meshram *et al.,* 2019). Meshram *et al.* (2019) reported that these behavioral abnormalities in termites are due to the change in the physiology of sensory system and cuticle due to their exposure to the alkaloids isolated from *Epipremnum aureum* extract. Similar results were also observed by Singha *et al.* (2011) on *Microtermes obsei* when exposed to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae.*

Similarly, the leaves extract of eight plants was also evaluated against the symbiotic protozoa population of *H. indicola* hindgut. Protozoa count was made after every twenty-four hours by Neubauer's chamber and the mean number of protozoa population was calculated. The results revealed that all plants cause a reduction in protozoa survival in a dose-dependent manner with an increase in time. The highest

protozocidal activity of *G. robusta* was reported than *O. basilicum* and *E. camaldulensis* as compared to the rest of the plants used in the study. These plants significantly reduce the protozoa population and cause a pronounced decline in their survival. These results are comparable with a study conducted by Qureshi *et al.* (2012). They reported that the heartwood extract of *E. camaldulensis* and *D. sissoo* caused complete removal of the protozoa population from the hindgut of *H. indicola.* These findings are also supported by *Jones et al.* (1983), that reduction in the protozoa population is directly related to the consumption of treated filter paper. One of the studies conducted by Hassan *et al.* (2018) reported that the highest concentration (10mg/ml) of *Morus alba* extracts negatively affect the termite survival and reduces to 7%, although the protozoan survival rate was high (50%). From these findings it is suggested that host mortality is not directly related to the decline in protozoa survival, however many other physiological processes are involved that caused termite mortality after their exposure to plants extracts (Raje *et al*., 2015). However the results of the present study are contradictory to these findings as termite survival rate was high in *C. papaya, E. globulus* and *M. longifolia* treated experimental units but protozoa survival was reduced to 66, 56 and 46% respectively revealing that plant extract might have some other mode of action leading to termite death.

One of the ways to control termites is by controlling their hindgut bacteria as they have the great ability to degrade lignocellulose. This digestion process not only provides a new way of energy synthesis but also a suitable method for controlling termites by targeting their endophytic bacteria as termite get deprive of from their nutritional requirements (Zhou *et al.,* 2019). In the present study, the effect of crude ethanolic leaves extracts of these plants was also evaluated against bacteria isolated from the hindgut of *H. indicola* and *O. obesus* by agar well diffusion method. Antibacterial activity of all plant extracts varied at different concentrations (100, 500, 1000, 1500 and 2000ppm) in their increasing order being maximum at the highest concentration. The growth inhibitory effect of all plants and susceptibility of bacterial isolates varied among the tested strains in the present study. The toxic effect of any applied substance differs among different

strains depending upon the nature of targeted species. Population density and growth rate of tested strain along with the diffusion rate of antimicrobial agent significantly affect the diameter of zone of inhibition (Prescott *et al.,* 2002).

Among all the tested plants most the strongest antibacterial activity was observed by *G. robusta, E. camaldulensis,* and *O. basilicum* against almost all bacterial isolates. The most promising activity was observed at higher dose levels *i.e.* 1500 and 2000ppm. The maximum zone of inhibition of *G. robusta* was 27.50±1.02 and 24.54±1.32mm against *L. fusiformis* isolated from the hindgut of both termite species (*O. obesus* and *H. indicola*) respectively. Similarly, maximum growth inhibitory effect of *E. camaldulensis* and *O. basilicum* was observed against *L. macrolides* isolated from *O. obesus* having maximum zone of inhibition *i.e.* 24.83±1.08, 22.33±1.18mm respectively and *B. cereus* isolated from hindgut of *H. indicola* having zone of inhibition 22.75±0.50, 22.10±0.18 respectively as compared to the rest of the plants that have activity only against one or two isolates at higher concentrations. Previous studies support these results that ethanolic leaf extract of *E. camaldulensis* was a more potent antibacterial agent as compared to the *E. globulus* against *S. aureus, S. epidermidis, B. subtilis, S. maarcescens,* and *E. coli* (Ghalem and Mohamed, 2008). Similarly strong antibacterial activities of *O. basilicum* were observed against different strains of *Shigella sp., S. aureus, L. monocytogenes,* and *P. aeruginosa* (Adiguzel *et al.,* 2005; Kaya *et al.,* 2008). However contradictory results were observed against leaf extracts of *C. papaya* and *E. globulus* in the present study as they were found as least effective against bacterial isolates but previously, they were reported to have strong antimicrobial activities (Alabi *et al.,* 2012; Baskaran *et al.,* 2012). The effectiveness of these plants depends upon the hydrophobic nature that enables them to penetrate bacterial cells by destroying their cell wall, rapturing cellular organelles and releasing essential molecules leading to bacterial cell death (Daroui-Mokaddem *et al.,* 2010).

These preliminary studies indicate the effectiveness of *G. robusta* crude leaves extract as a potential termiticidal, protozocidal and antibacterial agent that could be used to control termites by targeting their hindgut symbionts (protozoa and bacteria). Therefore phytochemical characterization of *G. robusta* was carried out using VU-Vis

Spectroscopy, FTIR and GC-MS analysis to sort out its chemical composition and biological activities. The UV-Vis spectroscopic analysis was performed at a wavelength of 200 to 800nm and maximum absorption was observed at 208.94nm (2.33) and 282.97 (0.811). Similar absorption peaks were observed previously in different plants leave extract (Rani *et al.,* 2016; Rajeshkumar and Jayaprakash, 2016). The results of FTIR analysis confirmed the presence of amines, alcohol, alkanes, nitriles, aldehydes, ether, 1,2,4 trisubstituted and halo compounds reported previously (Ashokkumar and Ramaswamy, 2014; Packialakshmi and Naziya, 2014; Nithyadevi and Sivakumar, 2015). The GC-MS analysis of *G. robusta* leaves extracts revealed the presence of bioactive compounds like n-Hexadecanoic acid, Phytol, 9,12,15-Octadecatrienoic acid(Z,Z,Z) and 2,3-Dihydrobenzofuran. These compounds have been reported previously to have antimicrobial, antioxidant, antifibrinolytic, and hemolytic activity (Gomathi *et al.,* 2015) antiandrogenic, hypocholesterolemic, flavoring agent, nematicides, lubricant, pesticidal, anti-cancerous, anti-inflammatory and anti-microbial activities (Al-Tameme *et al.*, 2015; Sermakkani and Thangapandian, 2012), hepatoprotective, insectifuge antihistaminic, 5-alpha-reductase inhibitor (Kadhim *et al.*, 2016).

The phytochemical analysis of the most effective plant revealed that its leaves contain various valuable bioactive compounds that can be used as an alternative to synthetic insecticides. Therefore further studies are carried out for isolation, identification and characterization of bioactive compounds from *G. robusta* leaves extract by column chromatography and their characterization by qualitative phytochemical analysis. Phytochemical characterization from different plant parts has led to the discovery of some highly active drugs (Vaghasiya *et al.,* 2011) as they are the rich source of biologically active compounds (Yadav and Agarwala, 2011) like flavonoids, alkaloids, tannins, carbohydrates, steroids, terpenoids, quinine, vincristine, morphine, codeine, digoxin and atropine that have many physiological effect of living cells (Abdulhamid *et al*., 2017; Edeoga *et al.,* 2005). These are metabolic byproducts of plants and act as a defensive agent against insects and microorganism (Vaghasiya *et al.,* 2011) and also has a growth inhibitory effect (Yadav and Agarwala, 2011). Plants being a rich source of

natural bioactive compounds are widely used in the last few decades as an alternative of synthetic pesticides and their demand is significantly increasing as they are ecofriendly.

Chromatographic techniques are commonly used methods for characterization and quantification of individual bioactive compounds from the mixture (Church, 2005). Column chromatography has certain advantages over other analytical techniques being less expensive, avoids cross-contamination and easy disposal and degradation of stationary phase. Thin-layer chromatography is also an analytical technique used for the identification of compounds and to determine their purity (Abdulhamid *et al*., 2017). In the present study, crude ethanolic leaves extract of *G. robusta* (15gm) was fractionated by column chromatography using step gradient technique in different solvents. Collected fractions were further characterized by thin-layer chromatography (TLC) in different eluents. Twenty-one (2-22) fractions were collected and evaluated for their termiticidal, protozocidal and antibacterial activity in a no-choice bioassay using a concentration range of 100-2000ppm.

Differential activity of all used fractions was observed against both termite species (*O. obesus* and *H.* indicola) and their hindgut endosymbionts (protozoa and bacteria) when compared with control. Fraction 5 was found to be most effective among all used fraction (2-22) and significantly ($P<0.05$) reduced termite and protozoa population having 90 and 92% mortality in *O. obesus* and *H. indicola* having LC_{50} value of 1342.81 and 1420.05ppm respectively while 80% (LC_{50}=578.34ppm) decline in protozoa population was observed at the end of experiment. The results of the present study are in accordance with Meshram *et al*., (2019). They reported that alkaloid fractions (a8 and a31) isolated from *Epipremnum aureum* significantly induce repellent activities, behavioral changes and caused termite mortality in subterranean termites when compared with the standard alkaloid Nicotine and chemically synthesized termiticide Monocrotophose. The 2nd most effective fraction was 7 causing 78 and 50% mortality with LC_{50} value of 1342.81 and 2642.34ppm respectively in both termite species. Against protozoa, 82% of mortality was observed in fraction 7 treated units. These results are supported by a study conducted by Doolittle *et al*. (2007). They used bioactive compounds from neem extract, gleditschia from *Gleditschia triacanthas* and capsaicin

from cayenne pepper (*Capsicum sp.*) against endosymbiotic fauna of Formosanus subterranean termites and evaluated that neembokil was more significant to decrease the population of *P. grassii* and *spirochaete* genus. These results are also comparable with a study conducted by Maistrello *et al.* (2003). They evaluated the effect of vetiver oil, nootkatone and disodium octaborate tetrahydride on the protozoa population of *Coptotemes formasanus* and found that nootkatone and vetiver oil significantly ($P<0.05$) reduce protozoa population which may be due to the feeding deterrent activities of this compounds. Tumerone from turmeric extract was proved highly toxic and induce termite mortality by targeting their nervous system and respiratory disruption (Raje *et al.*, 2015).

Fractions 4, 10 and 14 were also observed to reduce termites and protozoa population significantly, however the remaining fractions were found to be least effective against termites and affected termites population non-significantly but all these fractions were found to have significant effect on protozoa survival with lower LC_{50} values *i.e.* 2087.34, 1480.701521.48, 1565.26, 1819.58, 1502.25, 1864.21, 2104.32 and 2273.07ppm respectively except fraction 9 that was found to be least effective having higher LC_{50} values *i.e.* 5468.71, 5468.71 and 2991.65ppm against *O. obesus*, *H. indicola* and protozoa respectively. According to Maistrello *et al.* (2003), this differential decline in protozoa population residing in termite hindgut may be due to the toxic effect of bioactive compounds that cause physiological changes in the gut microenvironment. Different concentrations of extracts directly affect the protozoa population before their host ultimately leading to the death of their host.

Similarly, in the present study, the antibacterial activity of isolated fractions was also evaluated by agar well diffusion method at above mentioned all concentrations (100, 500, 1000, 1500 and 2000ppm). Among all the used fractions, seven were active against *H. indicola* and ten were found to be active against *O. obesus* with 3, 5, 7, 8, 10, 14 and 16 were commonly active against some or all bacterial isolates of both termite species while 9, 11 and 13 were only active against *O. obesus*. Antibacterial activity of isolated bioactive compound increase with an increase in concentration despite the presence of a compound that caused bactericidal action (Mbahi *et al.*, 2018).

Fractions 5, 7 and 14 were most active against bacteria of both termite species (*H. indicola* and *O. obesus*). The minimum inhibitory concentration of fraction 5 against a bacterial isolate of *O. obesus* was 100, 500 and 100ppm against isolates 1, 3 and 5 while 100ppm against the first 4 isolates and 500ppm against isolate 5 of *H. indicola.* Similarly, in the case of *H. indicola,* the minimum inhibitory concentration of fraction 7 was 100ppm against isolates 2, 3 and 4 while 500ppm against isolates 1 and 5 while it was 100ppm against all affected isolates (2, 3, 5) of *O. obesus*. The reported minimum inhibitory concentration of fraction 14 against bacterial isolates of *O. obesus* was 100ppm against isolate 2 and 3 and 1500ppm against isolate 4. Similarly, in the case of *H. indicola,* MIC was 100ppm against isolate 4 while 500ppm against the rest of the bacteria. Dahiru and Obidoa (2008) reported two possible reasons for the higher antibacterial activity of isolated fractions. It might be due to the strong extraction capacity of solvent used for fraction isolation during column chromatography or the presence of a biologically active compound in crude plant extracts responsible for antimicrobial activity. These findings are also in accordance with Mbahi *et al.,* (2018). They reported the antibacterial activity of *Ziziphus mauritiana* methanolic extract and its bioactive fractions on *Staphylococcus aureus*, *Bacillus subtillius*, *Salmonella typhi*, and *Escherichia coli*. Among four collected fractions (A-D); faction C showed strong antibacterial activity against all tested strains. They concluded that the isolation and fractionations process concentrated the bioactive phytochemicals in fraction C. Fractions 3, 9, 10, 11, 13 and 16 and were only effective against single isolate has the least antibacterial activity with the zone of inhibition only against one or two isolates only at higher concentrations. Fraction 8 was active against 4 isolates of *O. obesus* with the highest minimum inhibitory concentration of 1500ppm while it was not active against bacterial isolates of *H. indicola.* However, the rest of the fractions has no antibacterial activity and exhibited zero zones of inhibition against test strain at all concentrations. One study supports our results conducted on the antimicrobial activity of fractionated constituents and the crude extract of *Myrtus communis* (Mansouri *et al.,* 2001). Similarly, Verma *et al.* (2018) reported that diethyl ether fraction of *Jasminum mesnyi* was more

active with the highest zone of inhibition against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* at 250µg.

The qualitative phytochemical analysis provides a good tool for the identification of different secondary metabolites in the crude extract of plants. These compounds act as a defensive mechanism against microorganisms and the presence of saponins, alkaloids, tannins, flavonoids, polysteroles, phenolic compounds, and terpenoids makes them a vulnerable choice for antiplasmodic, bacteriocidal and analgesic activities (Nithyadevi and Sivakumar, 2015). In the present investigation 5 bioactive fractions (4, 5, 7, 10 and 14) having promising antitermitic, antiprotozoan and antibacterial activities were selected and subjected to qualitative phytochemical characterization by basic phytochemical screening tests, UV-Vis spectroscopy, FTIR and GC-MS analysis.

Preliminary phytochemical characterization of bioactive fractions (4, 5, 7, 10 and 14) was carried out by different phytochemical tests according to Sasidharan *et al.* (2011) with some modifications which confirm the presence of some common metabolites *i.e.* alkaloids, terpenoids and carbohydrates in all active fractions. Sterols were present in all four fractions except 14. Flavonoids and tannins were present in fraction 4, 5 and 7 while quinones were only in fraction 5. Similar phytochemicals were reported previously in many plant extracts (Aylooa *et al.*, 2008; Yadav and Agarwala 2011; Kalimuthu and Prabakaran, 2013; Jabin and Nasreen, 2016). These secondary metabolites belong to different classes and are responsible for many biological activities like flavonoids are polyphenolic compounds that act as anti-inflammatory and anti-microbial agents (Kalimuthu and Prabakaran, 2013) antioxidant and anti-cancerous agent (Njoku and Akumefula, 2007). Alkaloids are toxic to many organisms and have insecticidal properties even at low concentration but their mechanism of action varies (Gupta *et al.*, 2010; Meshram *et al*., 2019). Tannins are complex biological molecules and have antimicrobial, antidiarrheal, astringent, antioxidant and anti-inflammatory activities (Killedar and More, 2010). Terpenoids are volatile compounds and have antibacterial, antifungal, anti-cancerous and cytotoxic activities (Rajeswari *et al.*, 2011).

The UV-Vis spectroscopic analysis of bioactive fractions was performed at 200 to 800nm and a common absorption peak was reported at 665.50 in all fractions having different absorption spectra. Jain *et al.* (2016) reported similar absorption spectra at 665nm and the nearest remaining peaks wavelength of the *Mentha spicata* plant extract. Nearest absorption peaks were observed by Rani *et al.* (2016) and Rajeshkumar and Jayaprakash (2016). According to Rajeshkumar and Jayaprakash (2016), the absorption spectra between 300-350 give information about the nature of flavonoids, similar spectra are also reported in the present study. Similarly, the FTIR analysis of bioactive fractions (4, 5, 7, 10 and 14) was performed (Shimadzu ETIR spectrophotometer) from 400 to 4000cm^{-1} and absorption spectra were recorded. The results confirmed the presence of alkanes, alkynes, aldehydes, amines, ether, amino acids, thiol, cyanide, nitriles, alcohol and carboxylic acids in all the fractions. Thiocyanate was present in fraction 4, Terminal alkynes in fraction 4 and 5 while medial alkynes in fraction 7. Thiols were present in all except fraction 4 and Azides were present only in fraction 10. Previously the presence of these compounds has been confirmed in many studies (Ashokkumar and Ramaswamy, 2014; Packialakshmi and Naziya, 2014; Nithyadevi and Sivakumar 2015; Sithara *et al.*, 2017). According to Ashokkumar and Ramaswamy (2014), OH group has the hydrogen binding capacity and the common presence of this group in all bioactive fractions indicates the higher binding capacity of *G. robusta* leaves extracts imparts growth inhibitory activities against microorganisms.

The Maas spectrum is a powerful tool that provides information about the nature and structure of active compounds (Prabakaran and Mani, 2017). Results of GC-MS analysis of bioactive fractions confirmed the presence of a common compound in all fractions *i.e.* 3,7,11,15-Tetramethyl-2-hexadecen-1-ol. 1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester is present in all fractions except 14 and beta-Sitosterol was absent in fraction 4. 1,2-Benzenedicarboxylic acid, diisodecyl ester and Phytol were present in fraction 4, 5 and 7. 1,2-Benzenedicarboxylic acid, diisooctyl ester was reported from fractions 4, 10 and 14. The hexadecanoic acid methyl ester is present in fraction 5, 7 and 10. n-hexadecanoic acid and Octadecanoic acid are present in fraction 10 and 14. p-Arbutin is present in fraction 4 and 5. The phytochemical compounds

identified in the present study are reported previously and their biological activities are elaborated (Sermakkani and Thangapandian, 2012; Al-Tamem *et al.,* 2015; Casuga *et al.,* 2016; Prabakaran and Mani, 2017). Among the identified bioactive compounds n-Hexadecanoic acid have an antimicrobial, antioxidant, antifibrinolytic, antiandrogenic, antioxidant, hypocholesterolemic, flavoring agent, nematicides, lubricant, pesticidal and hemolytic activity (Sermakkani and Thangapandian 2012; Gomathi *et al.,* 2015). Hexadecanoic acid methyl ester has a hypocholesterolemic, flavoring agent, antioxidant and pesticidal properties. 3,7,11,15-Tetramethyl-2-hexadecen-1-ol has antimicrobial, anti-cancerous, anti-inflammatory, antioxidant activities and 1,2-Benzenedicarboxylic acid diisooctyl ester have antifouling and antimicrobial activities. Biological activities of phytol are anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive (Sermakkani and Thangapandian 2012; Al-Tameme *et al.,* 2015). Among the commonly identified compounds *i.e.* n-Hexadecanoic acid, Hexadecanoic acid methyl ester, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1,2-Benzenedicarboxylic acid diisooctyl ester, Phytol and Octadecanoic acid have certain antimicrobial and pesticidal activities. However, one of the compounds, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol is present common in all bioactive fractions. These biologically active constituents can be further eluted and might be utilized for the formulation of traditional pesticides which can be used for the control of different insect pests.

Conclusion

Termites cause serious damage to wood and wooden structures with the help of entozoic fauna residing in their hindgut. From the present study, it is concluded that for the control of termites and their hindgut endosymbionts (protozoa and bacteria) phytochemicals from natural products could be an effective alternate being ecofriendly. As bioactive insecticides, these medicinal plants contain such phytochemical compounds that could be used in a refined form to synthesize bioactive preservative that can be used to protect infestation and damage caused by termites. Moreover, a symbiotic association between protozoan flagellates and bacteria is a key factor in the cellular digestion of cellulose and hemicellulose thus leading to the successful survival of their termite host. The *Pseudotrichonympha grassii* is a comparatively large flagellate in termite hindgut and digests lignocellulose; a major component of cellulose and alone can fulfill all the nutritional requirements of termites. Similarly, cellulose digestion is also accomplished by the activity of bacterial fauna of hindgut that keeps the gut environment healthy and rich with nutrients. Thus using protozocides and bactericides instead of insecticides are more eco-friendly as they are effective in low concentrations. Among all the plants used in the present study, crude leaves extract of *G. robusta* exhibited promising termiticidal, protozocidal and antibacterial activity. Being the most effective, fractions of *G. robusta* isolated through column chromatography were also subjected to their biological activities (termiticidal, protozocidal and antibacterial). All fractions (21) showed their biological activities to a varying degree with 5 most bioactive fractions *i.e.* 4, 5, 7, 10 and 10 against all the tested organisms and were found to have inhibitory effects against termites or their gut symbionts (protozoa and bacteria). However, fraction 5 was found to be effective in all the bioassays of the present study. When these fractions were qualitatively analyzed by preliminary phytochemical screening, UV-Vis spectroscopy, FTIR and GC-MS analysis showed that various bioactive compounds are present including n-Hexadecanoic acid, Hexadecanoic acid methyl ester, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1,2-Benzenedicarboxylic acid diisooctyl ester, Phytol and Octadecanoic acid. The compound 3,7,11,15-Tetramethyl-2-hexadecen-1-ol was identified from all the active fractions. It is diterpene alcohol and has reported anti oxidant, antimicrobial, anti-inflammatory and

anti-cancerous activities. This bioactive compound in *G. robusta* leaves extracts suggests that it has certain pesticidal and antimicrobial activities and can be further eluted and utilized for the formulation of traditional pesticides that can be used for the control of different insect pests. Furthermore, this plant is easily available in different areas of Pakistan, cheap and affordable. These findings can help us to constitute an effective alternate of harmful chemical insecticides that persist in the environment and cause environmental toxicity for other non-targeted organisms. The compound 3,7,11,15-Tetramethyl-2-hexadecen-1-ol is commercially available could be used in the development of cost-effective natural termiticides for insect pest control and their applications on the industrial level.

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DIVERSITY PROFILE OF PROTISTS FLAGELLATES ISOLATED FROM HINDGUT OF *Heterotermes indicola* WASMANN (BLATTODEA: RHINOTERMITIDAE) IN PAKISTAN

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Termites cause a serious menace to wood structures all over the world. They rely mostly on the entozoic fauna for the digestion of cellulosic materials. The present study is based upon the diversity of flagellates protists isolated from the gut of a lower termite, *Heterotermes indicola,* belonging to three genera i.e. *Holomastigotes* (*H. campanula, H. annandalei* and *H. metchnikowi*), *Holomastigotoides* (*H. hemigynum, H. hartmanni, H. kempi, H. koidzumi* and *H. metchnikowi)* and *Pseudotrichonympha* (*P. grassii*). The largest and most abundant species *Pseudotrichonympha grassii* was identified by molecular studies using the SSU rRNA gene, confirmed by phylogenetic analysis and compared with that of the *P. grassii* isolates reported from other parts of the world. The results showed that the *P. grassii* observed in our study was phylogenetically most closely related to the Japanese *P. grassii* isolate. The biodiversity of the entozoic flagellates is important in targeting for biological control of termites as well as for isolation and culturing of flagellates to produce cellulases, an important industrial enzyme.

Keywords: Biodiversity, *Heterotermes indicola*, entozoic flagellates, *Holomastigotes, Holomastigotoides, Pseudotrichonympha.*

INTRODUCTION

Heterotermes indicola (Blattodea: Rhinotermitidae) is a lower termite which harbor entozoic flagellates for cellulose digestion thus causing considerable damage to forests and wooden materials throughout the world except Antarctica. About 434 flagellate's species have been identified in the hindgut of different termite species which belong to one of the three orders: Trichomonadida, Hypermastigida and Oxymonadida (Inoue *et al.,* 2000). During the last two decades termite gut flagellates have been classified using molecular methods, which are considered to be good tools for species identification and for defining evolutionary inferences (Yang and Rannala, 2012). Saldarriaga *et al.* (2011) described the morphology and phylogeny of two *Pseudotrichonympha* species i.e. *P. hertwigi* from *Coptotermes testaceus* and *P. paulistana* from *Heterotermes tenuis*. Phylogenetic analysis of fifteen species of *Pseudotrichonympha* was described by Noda *et al*. (2007) from different termite species. However, he suggested that only one species of *Pseudotrichonympha* is found in a termite species at one time. Thus the evolutionary history of *Pseudotrichonympha* is important not only due to its host specificity but as a host of bacterial endosymbionts, which account for more than two third of the total bacterial population in the termite gut (Noda *et al.,* 2007).The endomicrobes found in the hindgut of termites play a major role in cellulose digestion and manufacture cellulases that break down cellulose into acetate, propionate and butyrate. They also fix nitrogen that gets incorporated into the termite's tissues, excrement and secretions (Mathew *et al*., 2012). Recycling of uric acid by the termite gut bacteria is another source of nitrogen (Thong-On *et al.,* 2012). With the aid of these endomicrobes, such small insects (termites) cause damage worth billions to woods and wooden materials. The information pertaining to protozoa, found in the termites as symbionts, will be helpful towards designing strategies for the production of cellulases and control of lower termites via the development of drugs targeting these protozoa.

Owing to their importance in the termite hindgut, information about these endomicrobes and their diversity is extremely important. The present work was aimed to investigate the diversity of flagellated protozoa with a special focus on *P. grassii*, the largest and the most abundant protozoan found in the hindgut of *H. indicola*. Moreover, to control the population rate of termites and to protect the huge wood damage and economic loss, it is necessary to eliminate the population of gut flagellates which play an important role in cellulose digestion of termites and made symbiotic relationship with each other.

MATERIALS AND METHODS

***Study area and Collection of termites*:** Workers of *H. indicola* were collected from the metropolitan areas of Islamabad (33.729388°N, 73.093146°W) (Fig. 1) Pakistan from March to May 2017. Termites were kept in Petri dishes at 30°C and fed on moistened blotting paper for a week prior to the experiment and identified by using key (Akhtar, 1983). This helped in clearing debris and wood particles from flagellates for distinct morphology.

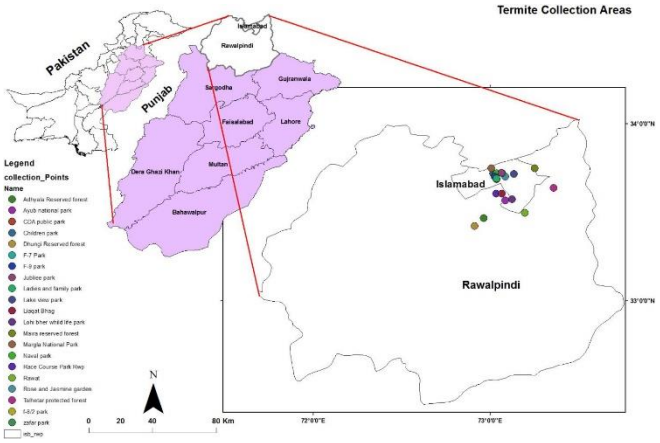


Figure 1. Termites collection sites from Islamabad, Pakistan.

***Light microscopy*:** The hindgut contents of termites were opened in a drop of 0.2% normal saline and tinged with gram iodine solution. Flagellates were observed by using Optika trinocular digital microscope (Optika B-350, Italy). Images and videos were captured in real time with a 12 megapixels digital CCD camera on a trinocular Optika microscope.

***Single cell isolation of P. grassii*:** The gut of termite workers was opened in a 5µl of filter-sterilized buffer (0.1 M NaC1, 10mM Na_3PO_4, pH 6.9) in the cavity slide. From this mixture of gut contents and the buffer, 1ml was taken on another cavity slide and diluted further by adding 4ml of the buffer. This dilution was repeated four times, 1ml of the final diluted contents was used for observations. The flagellates were examined under a digital biological microscope. Individual *P. grassii* cells, identified on the basis of morphology, were picked with a micropipette and collected in PCR tubes.

***PCR amplification and sequencing*:** The cells were directly used as a template in a PCR to amplify their nearly full length SSU rRNA gene. The PCR was performed on five cells. An initial PCR was done by using set of forward and reverse primers (Table 1). The reaction mixture consisted of template cells, dNTP mix (0.2 mM), primers (1µM each), PCR Buffer (1X), *Ex-Taq* DNA polymerase (2.5U) and $MgCl_2$ (1.5mM). Amplification was consisted of 35 cycles of 1 min at 92°C, 1 min at 50°C and1.5 min at 72°C. Agarose gel electrophoresis of the reaction revealed no bands. Therefore, using the first reaction contents as a template, a nested PCR was performed. 25µl of PCR products was added with the same constituents as used for primary PCR by using the second set of primers for nested PCR (Table 1).

The pieces of gel containing the DNA band were excised from the gel and subjected to DNA purification using the Gene JET Gel Extraction Kit (Cat No. K0691, MBI Fermentas). The pGEM-T-Easy vector (Cat No. A1360, Promega) was used to clone the purified products and transformed into DH5α *E. coli* cells. This was followed by plasmid DNA extraction and DNA sequencing of the cloned PCR product.

***Phylogenetic analysis*:** For the phylogenetic investigation, we analyzed our indigenous *P. grassii* taxon and compared it with that of isolated from the other localities of the world. The details are given in Table 4. The sequence alignment and data matrix construction were done in the software Geneious® version 6.1 (Biomatters Ltd., New Zealand). Neighbor joining analysis was performed using the DNA evolution model (Tamura and Nei, 1993) in the Geneious build algorithm. Wagner parsimony (Farris, 1970) and Maximum likelihood (Guindon and Gascuel, 2003) analysis were carried out using PAUP (Swofford, 2002) Geneious plugin. Bayesian analysis was done using MrBayes (Huelsenbeck and Ronquist, 2001) Geneious plugin. Finally, based on the above analysis a consensus evolutionary tree was generated.

RESULTS

***Flagellates biodiversity*:** The protozoan fauna isolated from hindgut of *H. indicola* were categorized according to Adl *et al.* (2005). We identified nine species of flagellates belonged to three genera *Holomastigotes*, *Holomastigotoides* and *Pseudotrichonympha*.

***Taxonomic classification of flagellates isolated from H. indicola*:**

***Class Mastigophora (Diesing)*:** Member of class Mastigophora usually have one to several flagella, the nucleus is vesicular with endosomes.

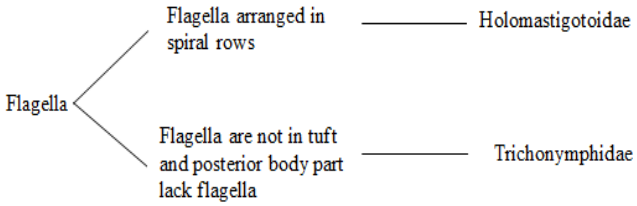
Table 1. Set of primers used for primary and nested PCR.

Primer Name		Sequence	Reference
Primary PCR	Eukl8	5'-TGAGGATCCMGGTTGATYCTGCC-3'	Ohkuma *et al. (*2000)
	Euk1627	5'-CCGAAGCTTACGGGCGGTGTGTRC-3'	
Nested PCR	Har-F	5'-GCGCTACCTGGTTGATCCTGCC-3'	Harper *et al.* (2009)
	Har-R	5'-TGATCCTTCTGCAGGTTCACCTAC-3'	

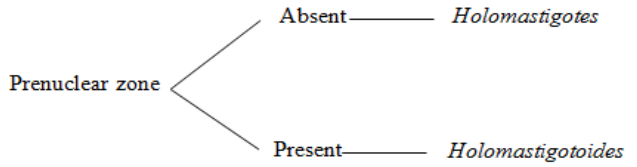
***Sub Class Zoomastigia*:** These may be free living or parasitic and their body lacks chromatophores. Ensysment is usually common.

***Order Hypermastigida (Grassii and Foa)*:** Flagellates belonging to the order Hypermastigida have numerous flagella and complex cytoplasmic organization.

KEY TO FAMILY



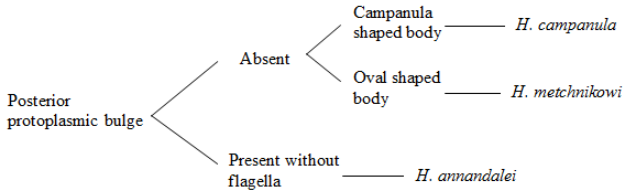
KEY TO GENERA



Genus *Holomastigotes* was described by Grassii in 1882 for the 1st time, later on by de Mello in 1927, Kudo in 1947 and Saleem in 1952.

Diagnosis: Nucleus not surrounded by dense protoplasm; basal granules are present deep in the cytoplasm and form deeply stained spiral bands which cover most of the body; flagella arise from basal granules; bulge may or may not be present at posterior end of body; a long axostyle present; body covered with periplast.

KEY TO SPECIES



***Holomastigotes campanula*:**

***Diagnosis*:** Campanula shaped body; broadly round at the anterior end; truncated posteriorly; body covered with flagella in the form of spiral bands; prenuclear zone absent; axostyle long arises near the nucleus and extend towards the posterior end (Fig. 2, Table 2 & 3).

***Holomastigotes metchnikowi*:**

***Diagnosis*:** Cylindrical body; rounded interiorly; no protoplasmic bulged; Nucleus at the anterior end; chromatin material at the periphery; spirally arranged flagella are present on all over the body; cytoplasm differentiated into ectoplasm and endoplasm (Fig. 2, Table 2 & 3).

***Holomastigotes annandalei*:**

***Diagnosis*:** Body oval shaped with broadly round anterior end; nucleus anterior in position; chromatin in the form of 2 to 3 patches; axostyle present; flagella of uniform size present

Table 2. Synonym, type host and additional host of the representative species reported from literature.

Name of species	Synonym	Type host	Add Host
Holomatigotes campanula	*Leidya campanula* (De Mello, 1927) *Holomastigotiodes campanula* (De Mello, 1927) *Holomastigotes campanula* (Saleem, 1952)	*Heterotermes indicola* (Portergaise)	*Heterotermes indicola* (Islamabad)
Holomatigotes metchnikowi	*Leidya metchnikowi* (Franca, 1919) *Holomastigotoides gigas* (De Mello, 1927)	*Heterotermes indicola* (Portergaise)	*Heterotermes indicola* (Islamabad)
Holomatigotes annandalei		*Heterotermes indicola* (Lahore)	*Heterotermes indicola* (Islamabad)
Holomastigotoides metchnikowi	*Leidya metchnikowi* (Franca, 1919) *Holomastigotoides metchnikowi* (De Mello, 1927)	*Coptotermes heimi*	*Heterotermes indicola* (Islamabad)
Holomastigotoides Kempi	*Leidya kempi* (De Mello, 1919)	-	*Heterotermes indicola* (Islamabad)
Holomastigotoides hemigynum	*Holomastigotoides hemigynum* (Grassii, 1917) *Holomastigotoides hemigynum* (De Mello, 1927) *Holomastigotoides hemigynum* (Saleem, 1952) *Leidya annadalei* (De Mello, 1919)	*Coptotermes lecteus* (Austerlia)	*Lecuotermes indicola* (Brazil) *Heterotermes indicola* (Portergaise) *Coptotermes heimi* (Lahore) *Heterotermes indicola* (Islamabad)
Holomastigotoides hartmanni	*Holomastigotoides hartmanni* (Koidzumi, 1921) *Holomastigotoides H. hartmanni* (De Mello, 1927) *Holomastigotoides hartmanni* (Saleem, 1952)	*Heterotermes indicola* (Portergaise) *Heterotermes indicola* (Lahore)	*Heterotermes indicola* (Islamabad)
Pseudotrichonympha grassii	-	*Coptotermes formosanus* *Coptotermes heimi*	*Heterotermes indicola*

Table 3. Sample T-test for the morphometric diversity of flagellates isolated from *Heterotermes indicola*.

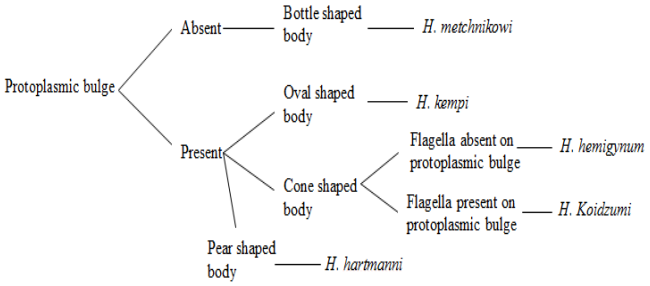
Species	Parameters	N	O.R	$\bar{x}$	S.D	S.E	95% CI
Holomastigotoides kempi	Body Length	25	17.45-132.0	65.46	15.46	8.58	47.36-83.56
	Body width	25	11.9-108.00	51.95	12.65	7.56	36.00-67.89
	Nucleus Diameter	25	8.38-15.00	10.45	2.05	0.48	9.43-11.47
	Flagella Length	25	4.76-96.00	39.86	26.76	6.31	26.56-51.17
	Bulge Length	25	4.76-62.70	32.64	19.07	4.50	23.15-42.12
	Bulge width	25	9.52-96.00	39.61	25.99	6.13	26.68-52.53
Holomastigotoides metchnikowi	Body Length	24	20.04-692.0	122.4	34.80	11.40	36.7-189.80
	Body width	24	19.84-618.0	123.8	40.80	10.60	38.3-199.80
	Nucleus Diameter	24	4.05-83.00	20.25	4.40	2.80	7.67-18.46
	Flagella Length	24	2.00-71.38	15.07	7.50	2.50	6.13-14.45
Holomastigotoides hartmanni	Body Length	12	15.08-74.26	47.90	22.76	8.05	28.87-66.92
	Body width	12	9.28-22.00	16.82	5.23	1.85	12.44-21.19
	Nucleus Diameter	12	3.00-23.80	3.50	1.55	0.55	2.20-4.800
	Flagella Length	12	2.19-8.530	5.31	2.17	0.76	3.49-7.120
Holomastigotoides hemigynum	Body Length	18	14.29-43.00	26.91	9.87	2.33	28.87-66.92
	Body width	18	9.81-24.09	16.86	3.89	0.91	19.02-44.54
	Nucleus Diameter	18	2.96-6.040	3.45	0.94	0.22	2.20-3.910
	Flagella Length	18	7.14-11.90	9.33	1.43	0.33	8.62-10.04
Holomastigotoides Koidzumi	Body Length	20	21.23-72.00	49.65	18.70	4.18	40.90-58.40
	Body width	20	18.88-54.00	28.96	12.36	2.76	23.17-34.74
	Nucleus Diameter	20	2.50-10.08	6.23	1.74	0.39	5.41-7.050
	Flagella Length	20	1.98-8.200	3.70	1.40	0.31	3.04-4.350
	Axostyle length	20	3.00-12.00	6.95	3.45	0.77	5.33-8.570
Holomastigotes annandalei	Body Length	15	14.78-62.21	36.25	19.06	6.70	20.31-52.19
	Body width	15	7.65-58.00	29.25	17.68	6.25	14.47-44.03
	Nucleus Diameter	15	3.10-15.39	3.62	1.40	0.49	2.44-4.800
	Flagella Length	15	1.73-6.350	3.50	1.43	0.50	2.29-4.700
	Bulge Length	15	3.40-7.070	7.38	3.66	1.29	4.31-10.44
	Bulge width	15	7.89-22.07	20.38	12.49	4.42	9.93-30.82
Holomastigotes metchnikowi	Body Length	22	16.38-142.0	81.68	43.45	9.26	62.42-100.9
	Body width	22	13.00-64.26	49.20	15.85	3.38	42.17-56.22
	Nucleus Diameter	22	3.45-14.00	9.06	2.38	0.50	8.00-10.11
	Flagella Length	22	2.36-13.00	7.92	3.23	0.69	6.49-9.350
Holomastigotes campanula	Body Length	22	118.0-239.00	157.92	3.23	0.69	6.49-9.350
	Body width	22	9.09-41.00	20.18	10.32	2.20	15.61-24.76
	Nucleus Diameter	22	2.90-9.450	5.02	1.14	0.24	4.51-5.530
	Flagella Length	22	2.00-5.000	2.34	0.64	0.13	2.05-5.620
Pseudotrichonympha grassii	Body Length	22	9.04-689.0	184.90	39.70	7.90	89.7-250.10
	Body width	22	15.30-610.0	158.70	34.36	7.34	76.1-187.30
	Nucleus Diameter	22	2.38-7.700	5.07	1.35	0.28	4.47-5.670
	Flagella Length	22	3.57-8.400	5.44	1.67	0.35	4.75-6.240
	Length of Centroblepharoplast	22	3.23-8.000	4.13	0.77	0.16	3.79-4.480

in the form of dextral spiral bands on all over the body; protoplasmic groove is present at posterior end; No distinction of ectoplasm or endoplasm (Fig. 2, Table 2 & 3).

***Genus Holomastigotoides*:**

Holomastigotoides was described by Grassii and Foa (1911) for the 1st time, later on by de Mello (1927), Kudo (1947) and Saleem (1952).

***Diagnosis*:** Prenuclear zone present, consists of dense cytoplasm; Basal granules found deep in the cytoplasm, in the form of deeply stained spiral bands, cover most of the body; flagella arise from basal granules extend from anterior to posterior end; bulge may or may not be present at the posterior end of body, may be smooth or covered with flagella; long axostyle present; body covered with periplast.



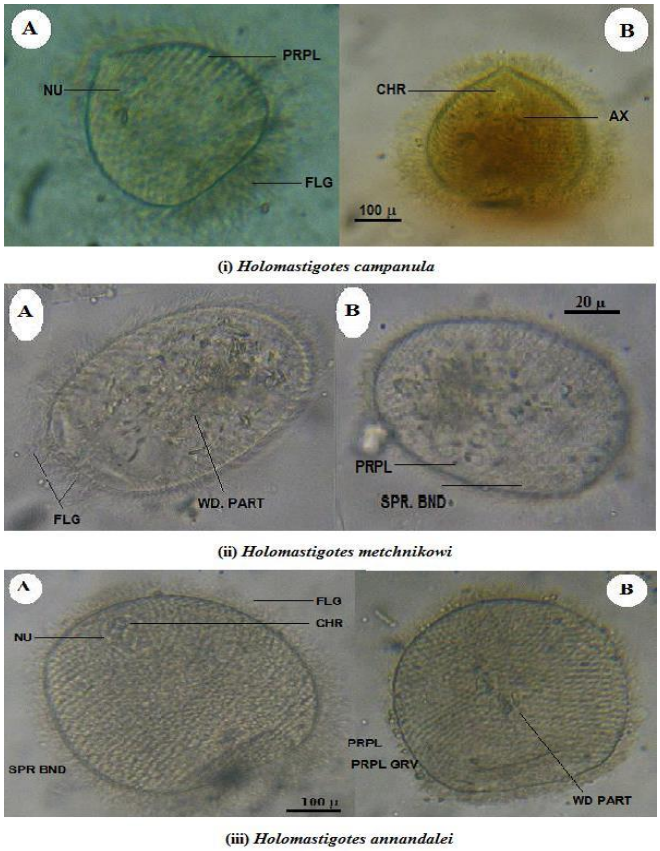


Figure 2. Different microscopic views of (i) *Holomastigotes campanula* (ii) *Holomastigotes metchnikowi* (iii) *Holomastigotes annandalei* at 100X; NU (Nucleus); PRPL (Protoplasm); PRPL GRV (Protoplasmic groove); SPR BND (Spiral bands); CHR (Chromatin); FLG (Flagella); W PART (Wood particles).

KEY TO SPECIES

***Holomastigotoides metchnikowi*:**

***Diagnosis*:** Body bottle shaped; body breadth 1/3rd to 1/4th of the entire length and is uniform till the neck; protoplasmic bulge absent; distinguishable ectoplasm and endoplasm; flagella arise from basal granules in cytoplasm and are arranged in spiral rows; nucleus lies slightly anterior to the middle of the body; chromatin material aggregated to the periphery of the nucleus; axostyle present and projects downward from the nucleus (Fig. 3, Table 2 & 3).

***Holomastigotoides koidzumi*:**

***Diagnosis*:** Body cone-shaped anteriorly; truncated posteriorly; nucleus round and anterior in position; no differentiation of cytoplasm into ectoplasm and endoplasm; prenuclear zone distinct from body wall is arranged into spiral rows; long flagella are present and flagella bands are whorled three times around the body; nucleus surrounds axostyle which extends to posterior (Fig. 3, Table 2 & 3).

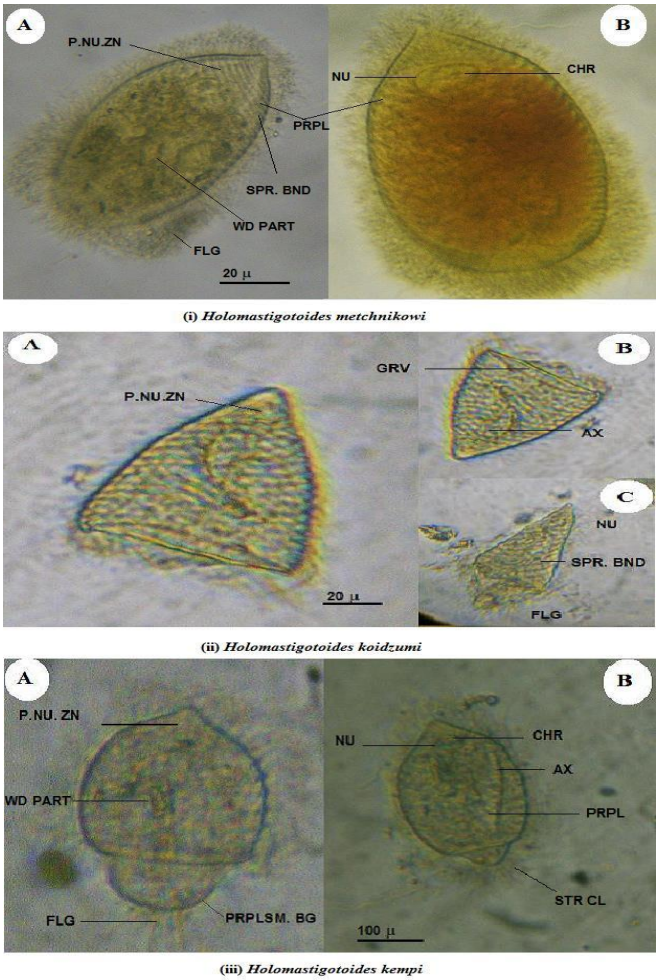
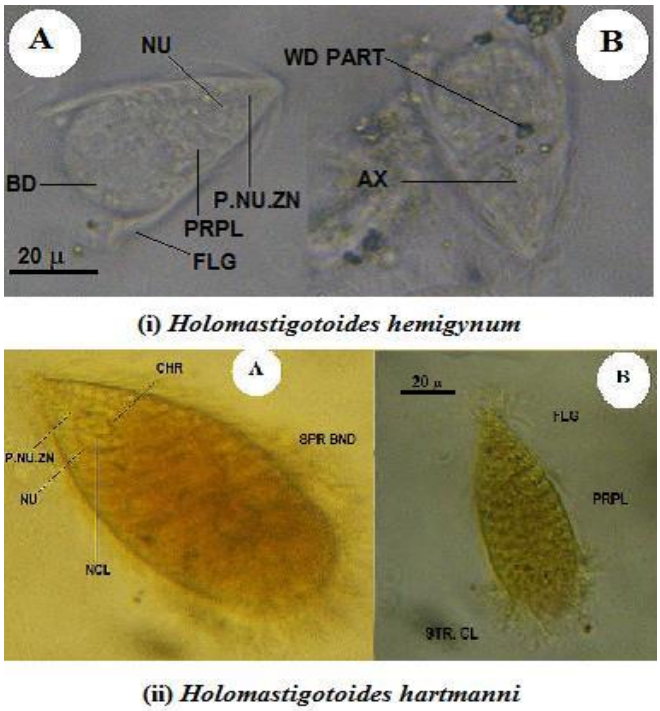


Figure 3. Different microscopic views of (i) *Holomastigotoides metchnikowi* (ii) *Holomastigotoides koidzumi* (iii) *Holomastigotoides kempi* at 100X; NU (Nucleus); PRPL (Protoplasm); PRPL GRV (Protoplasmic groove); SPR BND (Spiral bands); CHR (Chromatin); AX (Axostyle); FLG (Flagella); W PART (Wood particles). P. NU. ZN (Pre-nuclear zone); GRV (Groove); PRPLSM BG (Protoplasmic bulge).

***Holomastigotoides kempi*:**

***Diagnosis*:** Oval shaped body with its anterior part slightly drawn out forming a nipple shape; no differentiation of cytoplasm into ectoplasm and endoplasm; broad protoplasmic bulge present at posterior end of body; flagella cover whole the body; nucleus round in shape; distinct nuclear membrane present; chromatin material arranged in the form of groups or strands; 2 to 3 nucleoli generally visible, their number varies with individuals; axostyle posterior to the nucleus and protrudes downwards; prenuclear zone present (Fig. 3, Table 2 & 3).

***Holomastigotoides hemigynum*:**
***Diagnosis*:** Body cone-shaped; no differentiation of protoplasm into ecto and endoplasm; round nucleus has 1 to 2 nucleoli and covered by a nuclear membrane; chromatin granules arranged in 2 to 3 groups; axostyle projects downwards into the naked protoplasmic bulge from the posterior end of nucleus (Fig. 4, Table 2 & 3).



(i) ***Holomastigotoides hemigynum***

(ii) ***Holomastigotoides hartmanni***

Figure 4. Different microscopic views of (i) *Holomastigotoides hemigynum* and (ii) *Holomastigotoides hartmani* at 100X;
NU (Nucleus); PRPL (Protoplasm); SPR BND (Spiral bands); CHR (Chromatin); AX (Axostyle); FLG (Flagella); W PART (Wood particles) STR CL (Steriocilia); P. NU. ZN (Pre-nuclear zone).

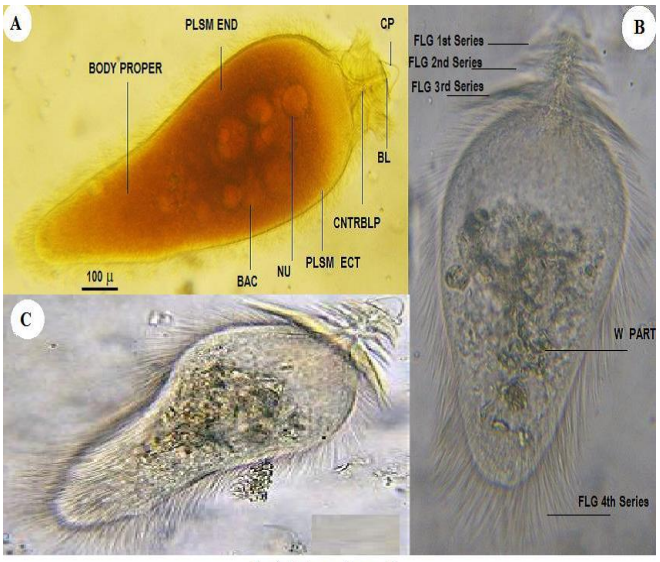
***Holomastigotoides hartmanni*:**
Diagnosis: Body pear-shaped with elongated and slightly pointed anterior part; most of the body covered with periplast which helps in permanency of body shape; posterior part of the body without periplast, no spiral bands are present; steriocilia are attached; the steriocilia are different from cilia because of their mode of attachment and are devoid of basal granules; flagella arise from basal granules that lies in the cytoplasm; nucleus lies near the anterior end; have 1to3 nucleoli (Fig. 4, Table 2 & 3).

GENUS

***Pseudotrichonympha*:** The genus *Pseudotrichonympha* is described by Grassii in 1917 for the first time as *Trichonympha*, then as *Pseudotrichonympha* by de Mello in 1927, Saleem in 1952 and Kudo in 1966.

***Diagnosis*:** spindle shaped body divided into two distinct parts; bell and body proper; bell at the anterior end of the centroblepharoplast is barrel-shaped and covered by a cap; spiral rows of flagella present on the whole body; cytoplasm divided into ectoplasm and endoplasm.

Pseudotrichonympha grassii:
Diagnosis: Spindle-shaped body; divided into head, neck and body proper; flagella arranged in the form of dextral spiral rows completely cover the body; head composed of a bell covered by a transparent hyaline cap; upper margin of the cap lies close to the bell and lower margin folded and devoid of flagella; neck also called the centroblepharoplast, tube like anteriorly and has varying thickness; centroblepharoplast composed of basal granules lying close to each other, which appear to constitute a single structure; neck flagella long, active, in the form of three series and perform jerky movements; flagella of Series 1 arise from the bell; their length increase from top to bottom and showed strong jerky movements. Flagella that root from the centroblepharoplast are referred to as *flagella of series 2* and they are longer than the flagella of series 1; Flagella of series 3 arise from the posterior region of the neck, are larger nucleus than the other two series and perform very active jerky movements during locomotion; round or oval in shape; has two nucleoli; chromatin material is evenly distributed or scattered randomly; nucleus has no definite position; it can be at the anterior end, the middle part, or the posterior end of the body (Fig. 5, Table 2 & 3).



Pseudotrichonympha grassii

Figure 5. Different microscopic views of *Pseudotrichonympha grassii* at 100X; CP (Cap); BL (Bell.); CNTRBLP (Centroblepharoplast); PLSM ECT (Ectoplasm); PLSM END (Endoplasm); W PART (Wood particles); NU (Nucleus); BAC (Bacteroidales); FLG (Flagella).

Table 4. Accession number of *Pseudotrichonympha grassii* from NCBI used for constructing a phylogenetic tree.

Taxon		NCBI Gene Bank accession	Country of origin
In group	*Pseudotrichonympha grassii*	JX649947	Pakistan
	Pseudotrichonympha grassii	AB262511	Japan
	Pseudotrichonympha grassii	AB262486	China
	Pseudotrichonympha grassii	AB032211	Japan
	Pseudotrichonympha sp.	AB262487	Malaysia
	Pseudotrichonympha sp.	AB262488	Laos
	Pseudotrichonympha sp.	AB262491	Brazil
	Pseudotrichonympha hertwigi	HQ683706	Canada
	Pseudotrichonympha paulistana	HQ683705	Canada
Out group	*Barbulanympha ufalula*	AB443595	Japan

***PCR amplification of SSU rRNA*:** Two sets of primers (Table 1) were used for amplification of the SSU rRNA gene. The amplification was confirmed by performing gel electrophoresis assay and the results revealed a DNA band of the expected size of 1.4 KB (Fig. 6).

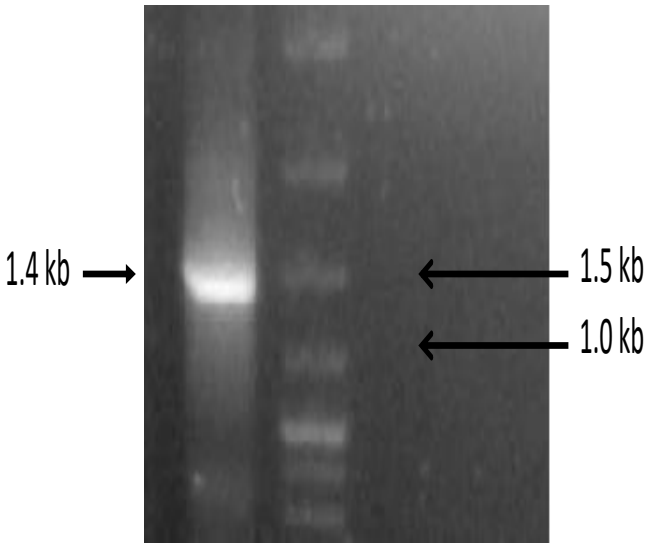


Figure 6. PCR amplified product of SSU rRNA of *P. grassii*.

***Phylogenetic analysis of P. grassii*:** BLAST analysis of the DNA sequence of the cloned PCR product performed on NCBI's website (http://blast.ncbi.nlm.nih.gov/) confirmed that the PCR product was *P. grassii* SSU rRNA gene. The phylogenetic tree shows that *P. grassii* isolated from Pakistan closely relates to the *P. grassii* isolated from Japan. Bayesian probability together with bootstrap values for Niebuhr Joining, Parsimony and Maximum Likelihood analysis strongly supported our phylogenetic tree and its clades (Fig. 7, Table 4). The accession JX649947 was obtained after the submission of sequences in Gene Bank.

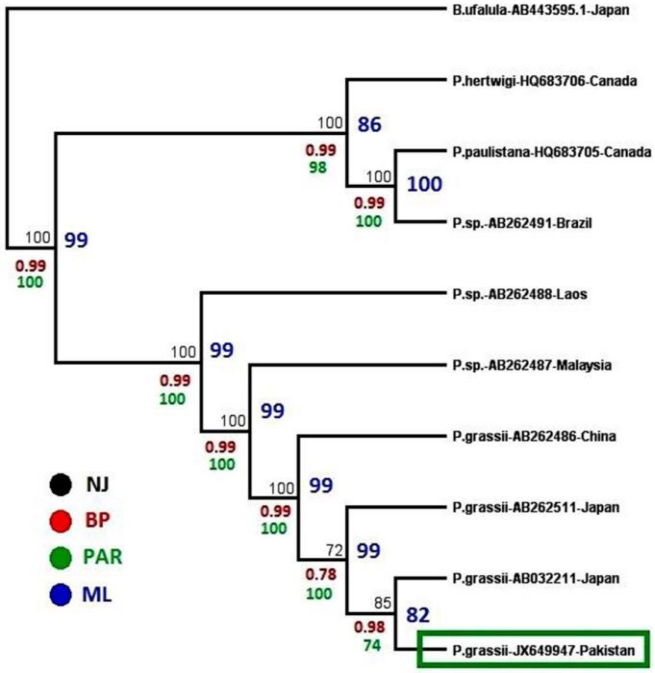


Figure 7. Molecular phylogenetic tree of *P. grassii* (NJ, Neighbor Joining; BP, Bayesian Probability; PAR, Parsimony; ML, Maximum Likelihood; for the details of taxa used.

DISCUSSION

In the present studies, the nine species of flagellates belonging to three genera viz, *Holomastigotes* (*H. campanula, H. annandalei* and *H. metchnikowi*), *Holomastigotoides* (*H. hemigynum, H. hartmanni, H. kempi, H. koidzumi* and *H. metchnikowi)* and *Pseudo trichonympha* (*P. grassii*) were identified from *H. indicola* morphologically. In addition, of *P. grassii* was identified on a molecular basis analyzed phylogenetically. Studies on the relative abundance of some species of protists in the hindgut of *H. indicola* demonstrated that *P. grassii* is the largest and the most abundant species found in *H. indicola*. The comparison of our *P. grassii* isolates

with the *P. grassii* isolate in other parts of the world, the results of our molecular phylogenetics are in agreement with those of Noda *et al.* (2005). Evolutionary tree (Fig. 7) depicts that Pakistani *P. grassii* isolate was closely related to the Japanese *P. grassii* isolate and falls in the Asian clade.

In the termite hindgut *P. grassii* is found replete with wood particles it is suggested that wood digestion largely depends upon this comparatively large flagellate. The *P. grassii* digests lignocellulose in the wood which is the major component of cellulose and provide digested food material to the termites. Thus *P. grassii* may alone be sufficient to fulfill the entire nutritional needs of the termite and dominates over the significance of other flagellate species. Therefore, antiprotozoal drugs against *P. grassii* may prove a valuable tool for termite eradication. *Pseudotrichonympha* occurs almost exclusively in Rhinotermitidae except for the genus *Reticulitermes* closely related to *Heterotermes* and *Coptotermes* (Inward *et al.,* 2007: Lo *et al.,* 2004). It is the only parabasalian symbiont identified in the genera *Termitogeton* and *Parrhinotermes* of termites (Kitade and Matsumoto, 1998). The protozoan fauna has a close relationship with their termite host and are transferred to their next host within the colony by protodeal trophallaxis (Inoue *et al.,* 2000) and from the mother colony to newly established colony by allates. Different termite genera possess different combinations of the gut protozoa. Even at a given location similarity in symbiotic fauna found in different termite genera is rarely observed (Kitade, 2004). There is a report that two unrelated genera *Reticulitermes* and *Hodotermopsis* have similar protists fauna (Kitade and Matsumoto, 1998). This unusual observation has been explained by Noda *et al.* (2007), which says that the intestinal fauna in *Reticulitermes* might have got replaced with those from close relatives or ancestors of *Hodotermopsis* through horizontal transfer. Saldarriaga *et al.* (2002) suggested if this hypothesis was true then some species of *Reticulitermes* might have in them *Pseudotrichonympha* spp. or any protozoan species that are common to other Rhinotermitids.

***Conclusion*:** The endomicrobes found in the hindgut of termites play a major role in cellulose digestion and manufacture cellulases that break down cellulose into acetate, propionate and butyrate. On the other hand, with the aid of these endomicrobes such small insects cause millions of damages to woods and wooden materials. Thus, by reviewing morphology and characteristics of symbiotic flagellates residing in the hindgut of *H. indicola* it was proposed that cellular digestion of hemicelluloses is possible due to the symbiotic association of protozoan flagellates. The termites gut flagellates have a key role in the survival of termites. By using protozoacides instead of insecticides may be more environment friendly as the low concentration of protozoacides will be required for killing their symbiotic flagellates.

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Original Research

Termiticidal and Protozocidal Potentials of Eight Tropical Plant Extracts Evaluated Against *Odontotermes obesus* Rambur (Blattodea; Termitidae) and *Heterotermes indicola* Wasmann (Blattodea; Rhinotermitidae)

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Abstract

The subterranean termites *Odontotermes obesus* and *Heterotermes indicola* are highly infesting wood pests that cause economic losses that cost billions of dollars throughout the world – especially in tropical and subtropical regions. It is highly significant to formulate insecticides from plant-based chemicals rather than synthetic ones. The eight plant species resistant to termites were selected for screening as anti-termite phytochemicals. Extracts from different parts of the plants were collected by using ethanol solvents in different ratios. These extracts were evaluated for their toxicity against termites and their gut flagellates at subsequent concentrations in the range of 100-2000 ppm to find $LC_{50.}$ Although the plants tested were found to be effective, extract from *Grevillea robusta* showed maximum mortality and reduced termite survival rate to 94% (LC_{50} = 1391.01) and 90% (LC_{50} = 1607.95) of *O. obesus* and *H. indicola* respectively. This extract also reduced the survival rate of hindgut flagellates of *H. indicola* up to 95% with an LC_{50} value of 502.64 ppm. The extract of *Grevillea robusta* was characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis. Two absorption peaks were recorded by UV-Vis spectroscopy at 208.94 and 282.97nm having absorption of 2.33 and 0.811 respectively. FTIR analysis confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether and halo compounds. GC-MS analysis revealed the presence of 15 phytochemicals with a high percentage of Coumarin (30.64%), 7-hydroxy, Methyl hexofuranoside (27.63%) and 9,12,15-Octadecatrienoic acid

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(Z,Z,Z) (8.97%). The natural phytochemicals from *G. robusta* having termiticidal and protozocidal potential being eco-friendly can replace synthetic pesticides for termite control.

Keywords: endosymbiont, termiticidal, *Grevillea robusta*, phytochemicals, spectroscopy, GC-MS

Introduction

Termites are "silent killers" as they ruin houses and wooden materials without showing external damage at initial stages. Globally, 90% of economic loss and 70% of construction damage is caused by the subterranean termites *Coptotermes formosanus* Shiraki and *Coptotermes gestroi* Wasmann [1-3]. Approximately $1 billion is spent annually on the loss caused by termites in the United States [4]. In Southeast Asia, the cost of termite damage is approximately $400 million per year [5]. Currently, a number of insecticides are available in the market, like carbamates, organochlorine, synthetic pyrethroids, chlordane, bifenthrin, fipronil and Termidor. Exposure to these synthetic insecticides causes serious health issues to humans, e.g., hyperglycemia, oxidative stress, and metabolic and nervous system disorders [6]. However, at the present time due to the lack of any effective alternative, these chemical insecticides are allowed for crop and soil treatments [7]. The urge for using naturally occurring botanical compounds as wood preservatives has increased from the last few decades [8-10]. Therefore, there is a need to explore natural products for developing pesticides or termiticides being biodegradable and environmentally friendly. Bioactive compounds from different plants that are cheaper, target-specific, biodegradable and non-toxic to humans can be used to replace synthetic insecticides [11]. Plant-derived biocides are naturally occurring compounds in roots, leaves, heartwood and bark, where they act as a defensive agent against xylophagous insects [12]. Termiticidal compounds like 2-methyl-9,10-anthracenedione, 1-methyl-3,4-dihydroisoquinoline and squalene have been reported to be present in the heartwood of *Tectona grandi*s [8]. Rasib et al. [13] reported that carboxylic acid isolated from *Magnolia grandifolia* and coniferyl alcohol from *Saraca asoca* were found to be a feeding deterrent against *Odontotermes obesus.* Similarly, a phytochemical "resorcinol" derived from *Morus alba* proved to be toxic against *Reticulitermes flavipes* [14].

Carica papaya (Caricaceae) is native to Mexico and northern South America. It is a medicinally important plant and is widely used as a whole plant as well as its seeds, leaves and fruits being used for pest control. *C. papaya* leaf extract exhibits antifungal, antibacterial, wound healing and antitumor potential [15]. The genus *Eucalyptus* (Myrtaceae) has more than 700 species native to Australia and is widely used as an antiseptic, repellent for insects, flavoring and fragrance agent, as well as in pharmaceuticals [16]. Antitermitic activity of *Eucalyptus citriodora* oil has been reported against higher termites and *Heterotermes indicola* [17] and antibacterial activity [18]. Antitermitic activities of *E. camaldulensis* leaf oil extract has been reported against *Coptotermes formosanus* in Thailand by both contact and fumigation methods [19]. The current study is based on the use of eight tropical plant leaf extracts for evaluating their termiticidal properties as they are locally available. The chemical characterization of the most effective plant was carried out by VU-Vis Spectroscopy, FTIR and GC-MS analysis. So that the natural plant products could become a good substitute for synthetic insecticides to prevent health hazards.

Materials and Methods

Termite Collection

Termite workers were collected from dead wooden logs and mud mounds using a collection of trap units prepared by plastic bottles containing wet toilet tissue rolls. Molasses was used as a phagostimulant to attract the termites. The traps were buried half underground at termite collection sites and monitored regularly. After 15 days, traps were brought to the laboratory, where active and healthy workers were separated. Live specimens were kept in Petri dishes with a soaked cotton plug in the laboratory at temperature 27±1ºC and relative humidity 80±5%. Termites were fed on No. 2 Whatman filter paper for one week, before the trail, to remove debris and other wood particles.

The plants leaves (Table 1 and Fig. 1) collected from the same vicinity were washed, shade dried at room temperature (27-37ºC) and powdered mechanically with an electric blender (Daigger Scientific, USA). Thirty grams of plant powder were subjected to obtain extract in 250 ml of ethanol solvent by using a Soxhlet apparatus at 40-60ºC (two cycles per hour) for 8 hours. The extract was concentrated by evaporating the solvent with a rotary vacuum evaporator (R-300, Rotavapor, Germany). The stock solution was prepared by dissolving 1g of crude extract in 100ml of distilled water and different concentrations of 100, 500, 1000, 1500 and 2000 ppm were prepared by applying formula $C_1V_1 = C_2V_2$.

$$V1 = \frac{Required\ ppm \times Required\ Volume}{Stock\ Solution}$$

Experimental Design

No-choice bioassay [20] was designed to evaluate the toxic potential of plant extract against two species of termites: *O. obesus* and *H. indicola*. Whatman

Table 1. Plants selected for antitermitic and antiprotozoan bioassays.

Sr. No.	Botanical name	English name	Common name	Family
1	*Carica papaya*	Papaya	Papita	Caricaceae
2	*Eucalyptus camaldulensis*	River red gum	Safaida	Myrtaceae
3	*Ocimum basilicum*	Sweet basil	Niazbo	Lamiaceae
4	*Grevillea robusta*	Silky oak	Bahekar	Proteaceae
5	*Eucalyptus globules*	Blue gum	Safaida	Myrtaceae
6	*Pongamia pinnata*	Indian beech tree	Sukchain	Leguminosae
7	*Mentha longifolia*	Horse mint	Podina	Lamiaceae
8	*Melia azedarach*	Chinaberry tree	Dhraik	Meliaceae

No. 1 filter paper (2.5"×2.5") was pre-weighed and treated with 1.5 ml of different concentrations of (100, 500, 1000, 1500 and 2000ppm) for each plant extract. Papers were then air-dried at room temperature and kept in Petri dishes (3"×3"). Fifty termite workers of each species were placed in each petri dish containing plant extract-treated filter paper. The Petri dishes were kept in a dark environment at 27±1ºC and 80±5 RH, and a few drops of distilled water were periodically added to each petri dish to keep up moisture content. All the experiments were conducted in replicates, and control was treated with distilled water only. Percentage mortality was calculated against each plant extract at every dose treatment after each 24 h for 16 days by applying the formula:

$$\% \, Mortality = ODP \div TP \times 100$$

…where ODP is observed dead population of workers and TP is total population.

Counting Protozoa

Protozoa count was made by using the method described by Hassan et al. [8] for evaluating the toxic potential of each plant extract against the hindgut flagellate population. Every 24 hours, two termite workers were removed and their hindgut contents were opened in 250 µl of 0.2% saline solution by using sterilized forceps and needle. The gut contents of two termites were homogenized in sterilized micro-pestle and used as a single sample for each treatment. Protozoa number was calculated by loading 25 µl of resulting gut content onto Neubauer's chamber (Bright-Line Sigma, USA) under a digital trinocular microscope

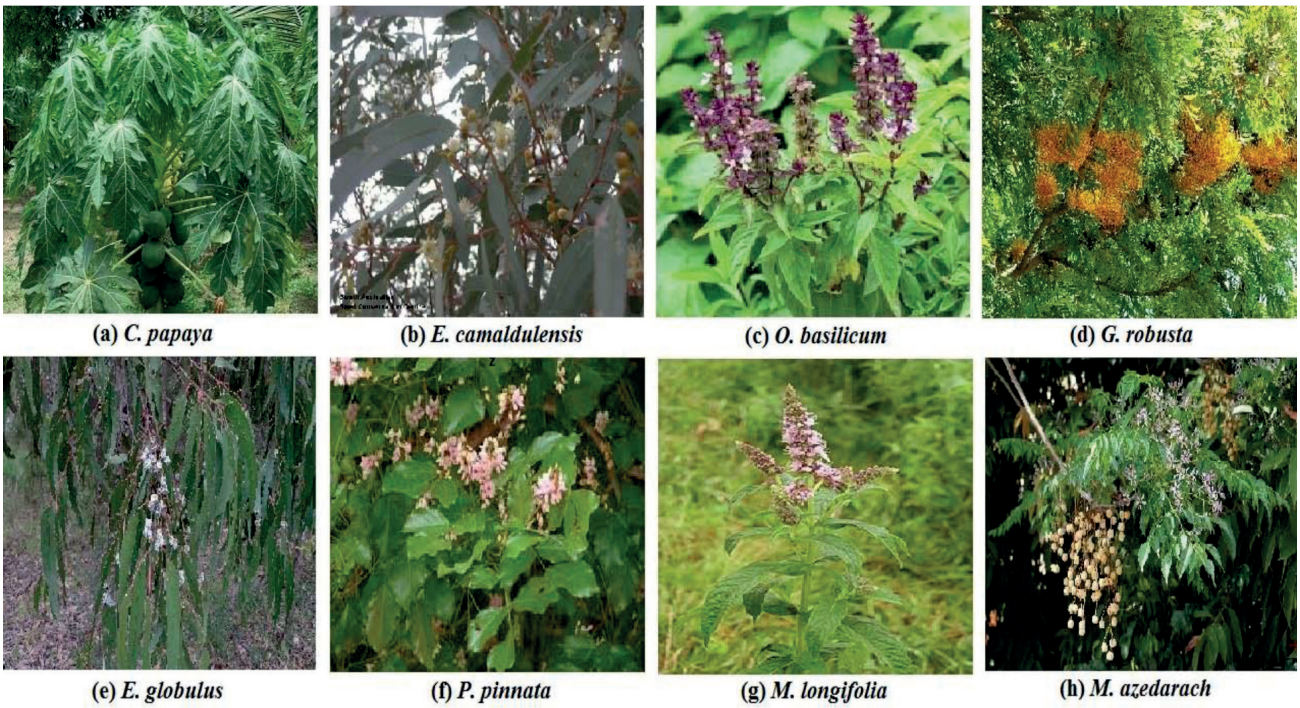


Fig. 1. Plants selected for antitermitic and antiprotozoan bioassays.

(Optika B-500, Italia). Counts were made by calculating the mean number of protozoa in four squares of each chamber according to Lewis and Forschler [21], and results were compared with control for determining a decrease in the protozoa population.

$$X = \frac{\left(No.\ of\ cell\ counted \times Volume\ of\ saline \frac{solution}{sample}\right)}{\left(Volume\ of\ Neubar's\ chamber \times No. \frac{termites}{sample}\right)}$$

Characterization of the Most Effective Plant Extract

UV-Vis Spectroscopy

The leaf extract was examined under UV-Vis spectroscopic analysis for its spectral profile. The extract was centrifuged for 10 minutes at 3000 rpm and filtered using Whatman No. 1 filter paper. The sample was then diluted 10 times with ethanol. The spectra were obtained by scanning the extract from 200 to 800 nm wavelength using a spectrophotometer (UV-1601, Shimadzu), and characteristic spectral peaks were recorded [22].

Fourier Transform Infrared Spectroscopy (FTIR)

Functional groups were detected by FTIR analysis and the characteristic peak values were recorded in the infrared region from 400-4000 cm^{-1} [23]. The sample was prepared by mixing 2 mg of leaf extract in 100 mg of KBr (FTIR grade). The mixture was then compressed to prepare 3 mm (diameter) salt discs which were loaded in FTIR spectroscopy (Shimadzu ETIR spectrophotometer) and scanned to record infrared spectra at different wavelengths (400-4000 cm^{-1}).

Gas Chromatograph-Coupled Mass Spectroscopy (GC-MS)

Leaf extract was further analyzed by using gas chromatography coupled with a mass spectrophotometer (GC-2010, Shimadzu). The chromatographic system was installed with an AOC-20i auto-injector, AOC20s autosampler and QP2010 mass selective detector. The fused silica capillary column (DB-5Ms, 0.25 mm×30 m×0.25 µm) having a maximum temperature capacity of 325ºC was used for screening. The column's initial temperature was 100ºC and maintained at this temperature for 0.5 minutes. The temperature of the oven was then increased to 280ºC (24ºC/min) and maintained for 3 minutes. Injector temperature was 250ºC with a flow rate of Helium as1.0ml/min. The chromatograph was operated in split injection mode with an injection volume of 1.0 µL and split ratio of 10:1. The ionization voltage was 70 eV and the mass spectral scan range was 50-350 m/z.

Identification of Phytoconstituents

The National Institute of Standards and Technology (NIST) database having more than 62000 patterns was used for interpreting the mass spectrum. The

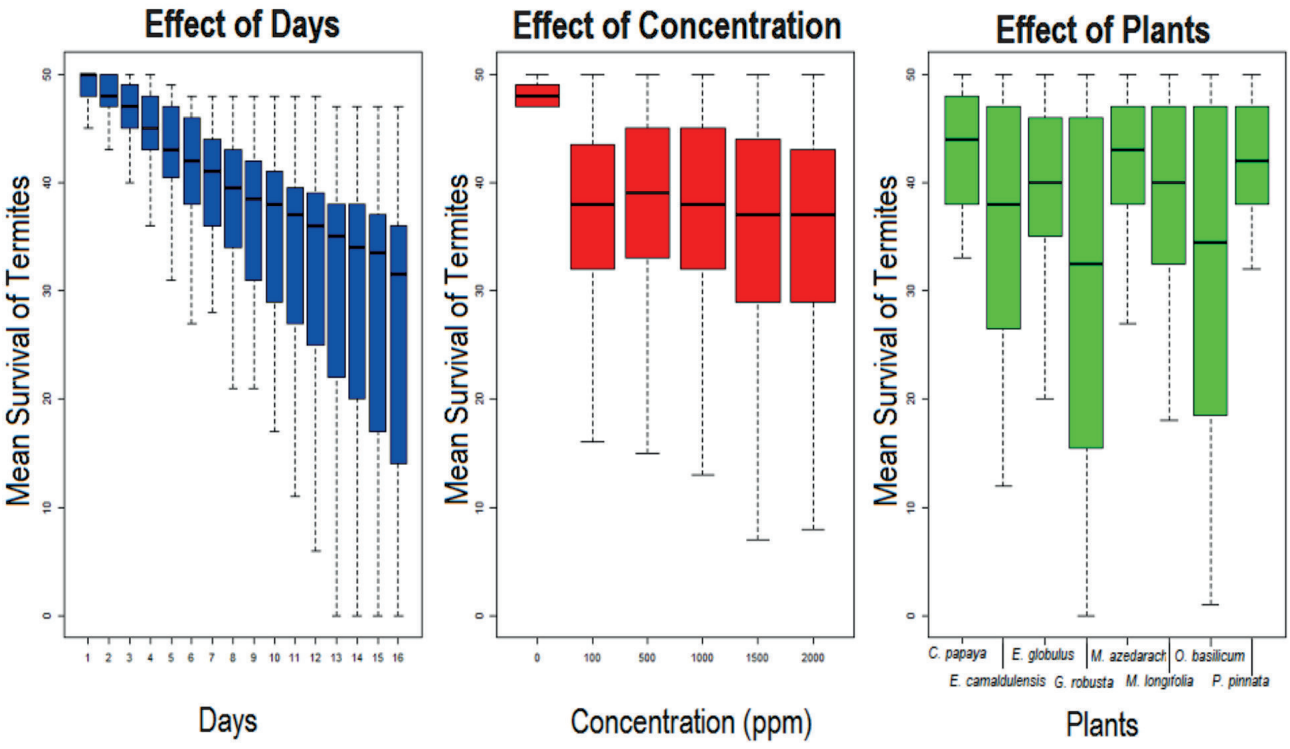


Fig. 2. Box plot representing the effect of days, concentration and plants on the mean survival of *O. obesus*.

comparison between spectra of known and unknown compounds, from the NIST library, helps in interpreting the mass spectrum and determining name, chemical formula, chemical structure and molecular weight of identified compounds [24].

Statistical Analysis

Mean mortality of termites and protozoa for each plant extract and concentration was compared, and statistically significant differences were determined using one-way ANOVA with Tukey's multiple comparison tests using "R language". Results with P value less than 0.05 were considered to be significant and less than 0.005 considered strongly significant. Stars (*, **, ***) denote statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. LC_{50} value of each plant extract against termites and their gut protozoa was calculated by applying "probit analysis" using SPSS software version 19.

Results and Discussion

Antitermitic Activity of Plant Extract Against *O. obesus* and *H. indicola*

Antitermitic activity of ethanolic leaf extract of eight plants against two termite species *(O. obesus* and *H. indicola)* was evaluated by using a no-choice bioassay. Five different concentrations (100, 500, 1000, 1500 and 2000) were used in a 16-day experiment, and the mean survival of termites was calculated. All concentrations were found to have a significant effect on termite survival against *O. obesus* as compared to control (*F (df)* = 289.0 (5); P<0.05) in a time-dependent manner, which also differed significantly from each other (*F (df)* = 216.0 (16); P<0.05). Similarly, all plants showed different antitermitic activity in dose- and time-dependent manners (*F (df)* = 209.6 (8); P<0.05). The effects of days, concentrations and plants on mean survival of termites is represented in Fig. 2. Among the tested plants, *G. robusta* was found to be most effective, with a significant (P<0.05) reduction in survival of termites (94%) at the 16th day of the experiment. Similarly, *O. basilicum, E. camaldulensis* and *E. globulus* also significantly (P<0.05) reduced termite populations with 34, 40 and 60% survival rate, respectively. However, *M. azedarach*, *C. papaya, M. longifolia* and *P. pinnata* have a non-significant effect on termite survival with less than 30% reduction in the termite population (Fig. 3).

An almost similar response was observed against the mean survival of *H. indicola* in *O. obesus*. All concentrations were found to have a significant effect on termite survival as compared to control (*F (df)* = 343.0 (5); P<0.05) in a time-dependent manner, that also differed significantly from each other (*F (df)* = 261.6 (16); P<0.05). Similarly, all plants showed differential antitermitic activity in dose- and time-dependent manners (*F (df)* = 234.8 (8); P<0.05). The effect of days, concentrations and plants on mean survival of termites is represented in Fig. 4. Among all plants used, *G. robusta* showed a significant (p<0.05) decrease in the termite population with 50% termite death after the 10th day of the experiment. About 90% of termite mortality was recorded on the 16th day of the experiment. Similarly, *E. camaldulensis, O. basilicum,*

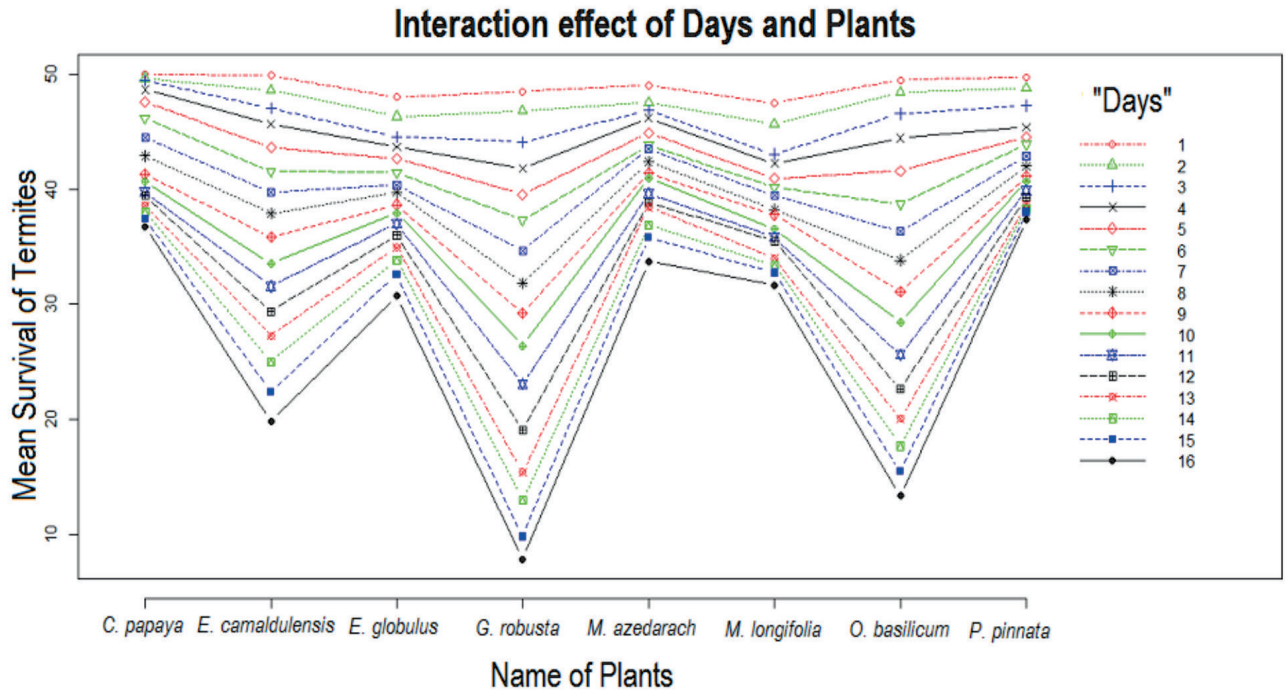


Fig. 3. Interaction plot representing the interaction effect of plant extract with days on mean survival of *O. obesus.*

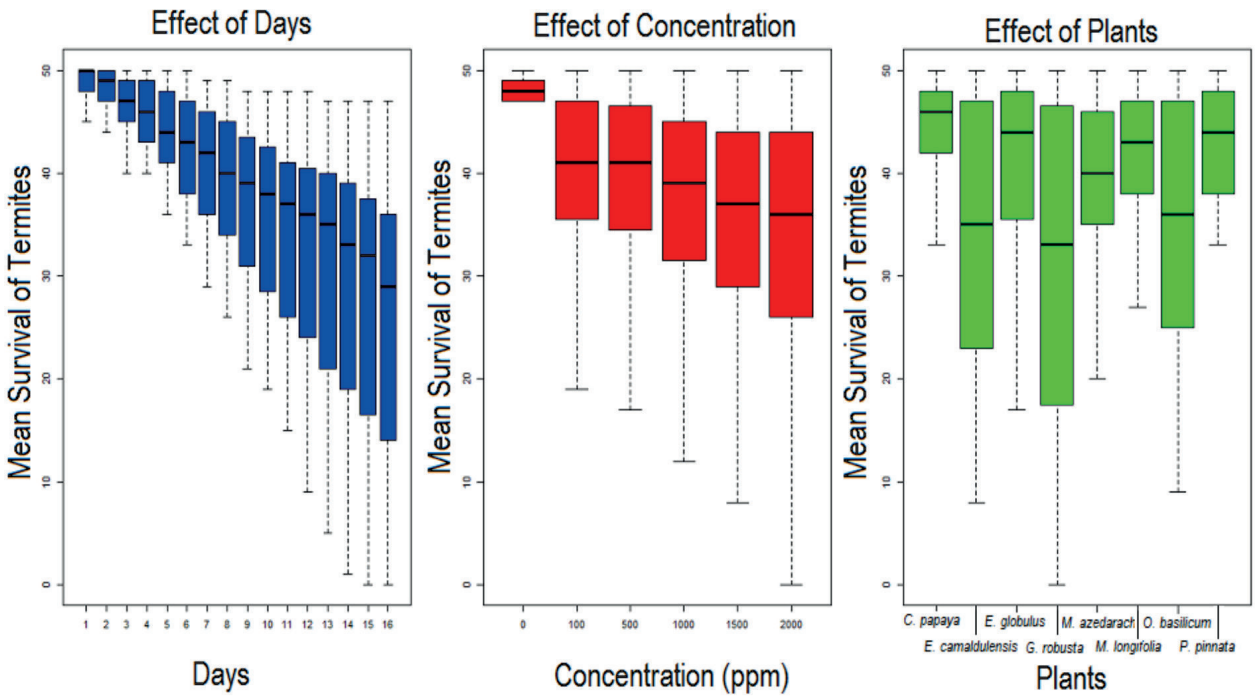


Fig. 4. Box plot representing the effect of days, concentration and plants on the mean survival of *H. indicola.*

E. globulus and *M. azedarach* were also found to have a significant ($p<0.05$) effect on termite survival and reduced their populations by up to 70, 65, 59 and 54%, respectively. However, *C. papaya, M. longifolia* and *P. pinnata* have a non-significant effect on termite survival with less than 30% reduction in the termite population (Fig. 5). Calculated LC_{50} values of all plants are represented in Table 4. The level of significance of all the plants and their comparative analysis between each other is represented in Tables 2 and 3.

For the control of termites, synthetic insecticides have been used from the last few decades that have resulted in insecticidal resistance, human health disorders, and

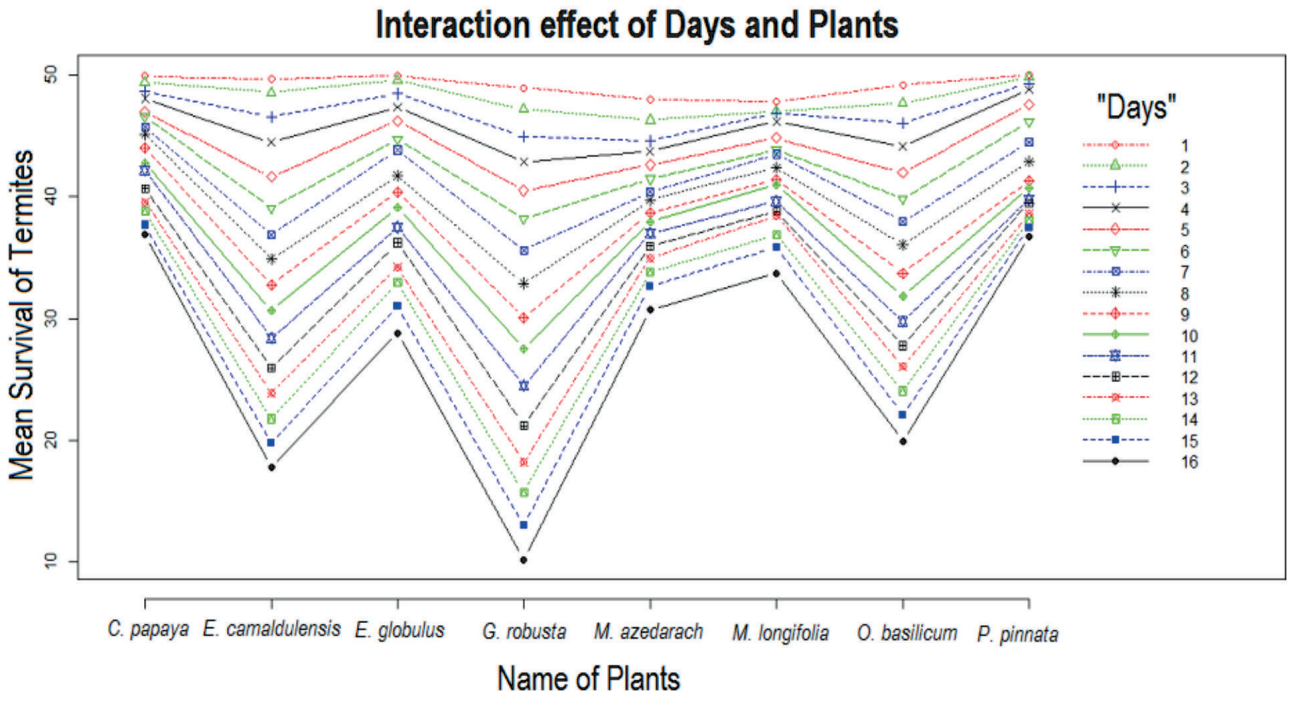


Fig. 5. Interaction plot representing the interaction effect of plants extract with days on mean survival of *H. indicola.*

Table 2. Comparative significance analysis of plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plants Name	Level of Significance		
	O. obesus	*H. indicola*	Protozocidal
C. papaya	Non-Significant	Non-Significant	Significant***
E. camaldulensis	Significant**	Significant**	Significant***
E. globulus	Significant*	Significant*	Significant**
G. robusta	Significant***	Significant***	Significant***
M. azedarach	Non-Significant	Significant*	Non-Significant
M. longifolia	Non-Significant	Non-Significant	Significant*
O. basilicum	Significant***	Significant**	Significant***
P. pinnata	Non-Significant	Non-Significant	Non-Significant

Table 3. Comparison of significance level (P<0.05) between plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plant Name	*O. obesus*		*H. indicola*		Protozocidal	
	Level of Significance	P value	Level of Significance	P value	Level of Significance	P value
C. papaya - E. camaldulensis	Significant	0.000	Significant	0.000	Significant	0.000
C. papaya - E. globulus	Significant	0.000	Significant	0.000	Significant	0.000
C. papaya - G. robusta	Significant	0.000	Significant	0.000	Significant	0.000
C. papaya - M. azedarach	Non-Significant	0.129	Significant	0.000	Significant	0.000
C. papaya - M. longifolia	Significant	0.000	Significant	0.000	Significant	0.000
C. papaya - O. basilicum	Significant	0.000	Significant	0.000	Significant	0.000
C. papaya - P. pinnata	Non-Significant	0.698	Non-Significant	0.720	Significant	0.000
E. camaldulensis - E. globulus	Significant	0.000	Significant	0.000	Significant	0.000
E. camaldulensis - G. robusta	Significant	0.000	Significant	0.000	Non-Significant	0.204
E. camaldulensis - M. azedarach	Significant	0.000	Significant	0.000	Significant	0.000
E. camaldulensis - M. longifolia	Significant	0.000	Significant	0.000	Significant	0.000
E. camaldulensis - O. basilicum	Significant	0.000	Non-Significant	0.378	Non-Significant	0.996
E. camaldulensis - P. pinnata	Significant	0.000	Significant	0.000	Significant	0.000
E. globulus - G. robusta	Significant	0.000	Significant	0.000	Significant	0.000
E. globulus - M. azedarach	Significant	0.000	Non-Significant	0.020	Significant	0.000
E. globulus - M. longifolia	Non-Significant	0.641	Non-Significant	0.317	Significant	0.000
E. globulus - O. basilicum	Significant	0.000	Significant	0.000	Significant	0.000
E. globulus - P. pinnata	Significant	0.000	Significant	0.000	Significant	0.000
G. robusta - M. azedarach	Significant	0.000	Significant	0.000	Significant	0.000
G. robusta - M. longifolia	Significant	0.000	Significant	0.000	Significant	0.000
G. robusta - O. basilicum-	Significant	0.000	Significant	0.000	Non-Significant	0.030
G. robusta - P. pinnata	Significant	0.000	Significant	0.000	Significant	0.000
M. azedarach - M. longifolia	Significant	0.000	Significant	0.000	Non-Significant	0.246
M. azedarach -O. basilicum	Significant	0.000	Significant	0.000	Significant	0.000
M. azedarach - P. pinnata	Non-Significant	0.974	Significant	0.000	Non-Significant	0.197
M. longifolia - O. basilicum	Significant	0.000	Significant	0.000	Significant	0.000
M. longifolia - P. pinnata	Significant	0.000	Non-Significant	0.027	Significant	0.000

Table 4. LC_{50} values of plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plants Name	LC_{50} (ppm) Upper confidence limit - Lower confidence limit		
	O. obesus	*H. indicola*	Protozoa
C. papaya	6329.70 (7478.71-5519.87)	3546.81 (3776.76-3350.46)	1046.24 (1052.65-1039.86)
E. camaldulensis	2973.69 (3231.45-2762.92)	2214 (2351.38-2097.04)	594.70 (601.77-588.29)
E. globulus	3148 (3375.31-2957.11)	2754.81 (2889.69-2635.82)	1373.28 (1380.49-1366.13)
G. robusta	1391.01 (1455.08-1331.88)	1607 (1686.03-1537.49)	502.64 (506.97-497.10)
M. azedarach	5308.70 (6093-78-4726.97)	3154.99 (3383.57-2962.57)	2475.97 (2498.83-2453.83)
M. longifolia	9408.11 (14283.4-7124.08)	5395.08 (6220.86-4788.18)	3093.47 (3129.35-3058.63)
O. basilicum	1926 (2039.53-1827.68)	2515.19 (6220.86-4788.18)	683.60 (688.63-678.56)
P. pinnata	5468.71 (6293.14-4860.36)	6240.51 (7348.15-5455.17)	2080.22 (2096.91-2063.87)

environmental issues. So the replacement of synthetic insecticides with bio-pesticides is strongly required as they are a rich source of bioactive compounds and less toxic [25]. In the past, many studies have been conducted to evaluate the toxic effects of plants on termites and their hindgut endosymbionts. Abbas et al. [26] reported the antitermitic activity of 15 medicinal plants, including *Ricinus communis, Croton tiglium, Ocimum basilicum, Psoralea corylifola, Nigella sativum, Mentha* sp., *Cichorium intybus, Peganum harmala, Foeniculum vulgare, Allium sativum, Plantago ovate, Moringa oleifera, Capsicum frutescens, Melia azedarach* and *Azadirachta indica* against *O. obesus,* and found that *O. basilicum, P. harmala* and *F. vulgare* have potential to be used as antitermitic agents. These plants have many bioactive constituents that can be used against termites. In previous studies, the leaf and seed extracts of *Jatropha curcas,* bark extract of *Melia azedarach*, rhizome extract of *Curcuma longa*, leaf extract of *Nerium indicumi* and crude seed extract of *Annona* were evaluated for behavioral, feeding and tunneling activities of subterranean termites [27]. Similarly the effect of crude extracts of *Azadirachta indica, Pyllanthus niruri, Andrographis paniculata* and *Leucaena leucocephala* on behavior and feeding activities was studies against *Coptotermes gestroi* and *Globitermes sulphurues* [28].

In the present study among the tested eight plant species, *G. robusta* was found to have promising activity and cause significant ($P<0.05$) reduction in termite survival. About 50% termite death was recorded on the 10th day of the experiment, with a survival rate of 6 and 10% (94 and 90% mortality) on the 16th day of the experiment in both termite species, i.e., *O. obesus* and *H. indicola.*

E. camaldulensis and *O. basilicum* were also reported to have a significant ($P<0.05$) effect on termite survival and reduce up to 70 and 65% *H. indicola* population, but they do not differ significantly from each other ($P = 0.378$). However, in the case of *O. obesus, O. basilicum* was found to be more effective than *E. camaldulensis* and caused 76 and 60% reduction in the termite population. Similarly, *E. globulus* and *M. azedarach* were also active significantly ($P<0.05$) against termites, but have non-significant ($P = 0.20$) differences among each other. The results of the present study are comparable with a recent study conducted by Hassan et al. [14] showing that the population of *Reticulitermes flavipus* was reduced significantly when exposed to the extracts of *Morus alba*, and we found that the percentage mortality of termites was concentration-dependent. The activity profile of all tested plants against *O. obesus* is as follows; *G. robusta>O. basilicum>E. camaldulensis> E. globulus>M. longifolia>M. azedarach>C. papaya> P. pinnata*. Similarly, the activity profile of all tested plants against *H. indicola* is as follows: *G. robusta> E. camaldulensis>O. basilicum>E. globulus>M. azedarach>M. longifolia>C. papaya>P. pinnata.*

Protozocidal Activity of Plant Extract

Protozocidal activity of the above-mentioned eight plants were also calculated against the mean survival of the protozoa population isolated from the hindgut of *H. indicola*. All concentrations were found to have a significant effect on protozoa survival as compared to control ($F\ (df)$ = 553.5 (5); $P<0.05$) in a time-dependent manner, which also differed significantly from each other ($F\ (df)$ = 177.2 (7); $P < 0.05$). Similarly, all plants showed differential protozocidal activity in dose- and time-dependent manners ($F\ (df)$ = 141.3 (8); $P<0.05$). The effect of days, concentration and plants on the mean survival of the protozoa population is represented in Fig. 6. Among all the plants, *G. robusta*, *E. camaldulensis* and *O. basilicum* were found to be most effective and have a significant ($P<0.05$) effect on survival of protozoa with 95, 78, and 74% reduction in the population on the 16th day of the experiment. Similarly, *C. papaya, E. globulus* and *M. longifolia* also significantly ($P<0.05$) reduced the protozoa population with 66, 56 and 46% mortality respectively. However, *M. azedarach* and *P. pinnata* have a non-significant

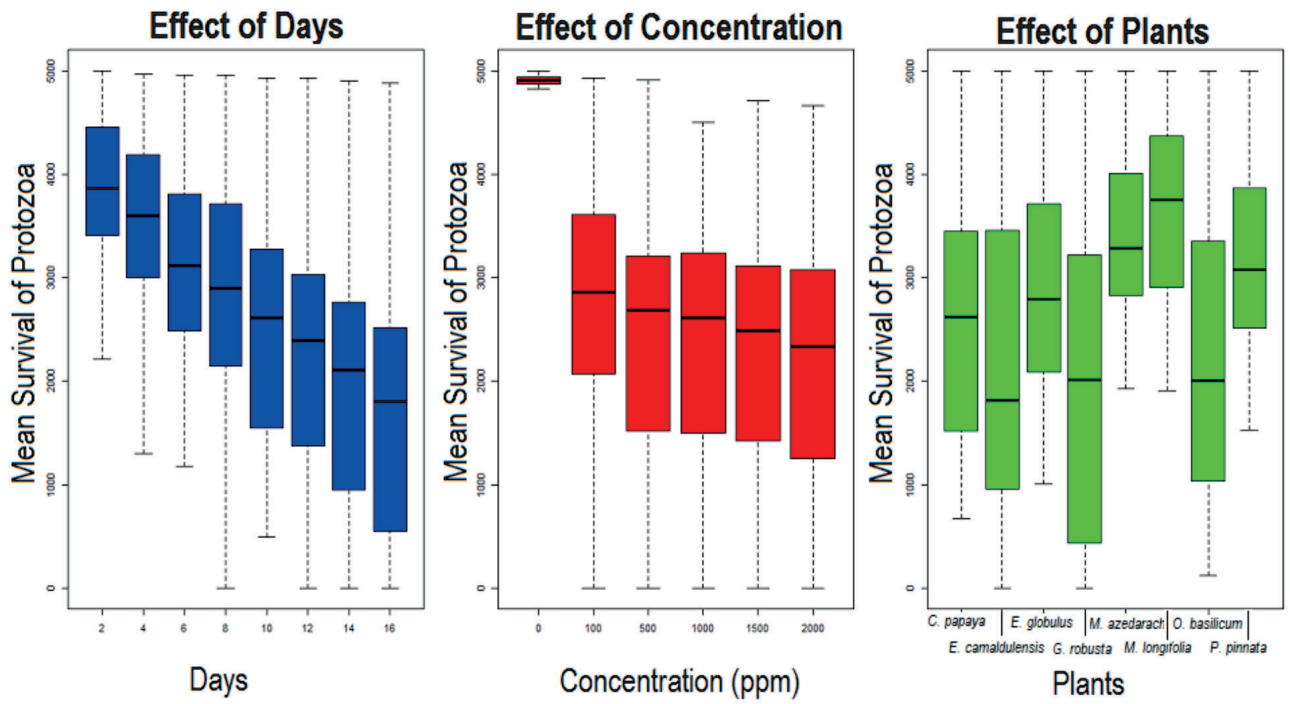


Fig. 6. Box plot representing the effect of days, concentration and plants on the mean survival of protozoa isolated from the hindgut of *H. indicola.*

effect on the protozoa population (Fig. 7). Calculated LC_{50} values of all plants are represented in Table 4. The level of significance of all the plants and their comparative analysis between each other is represented in Tables 2 and 3.

Protozocidal activity by feeding on extract-treated filter papers was also investigated against the endosymbiotic flagellate population of *H. indicola*. In previous studies, the toxic potential of some natural products against hindgut flagellates has been

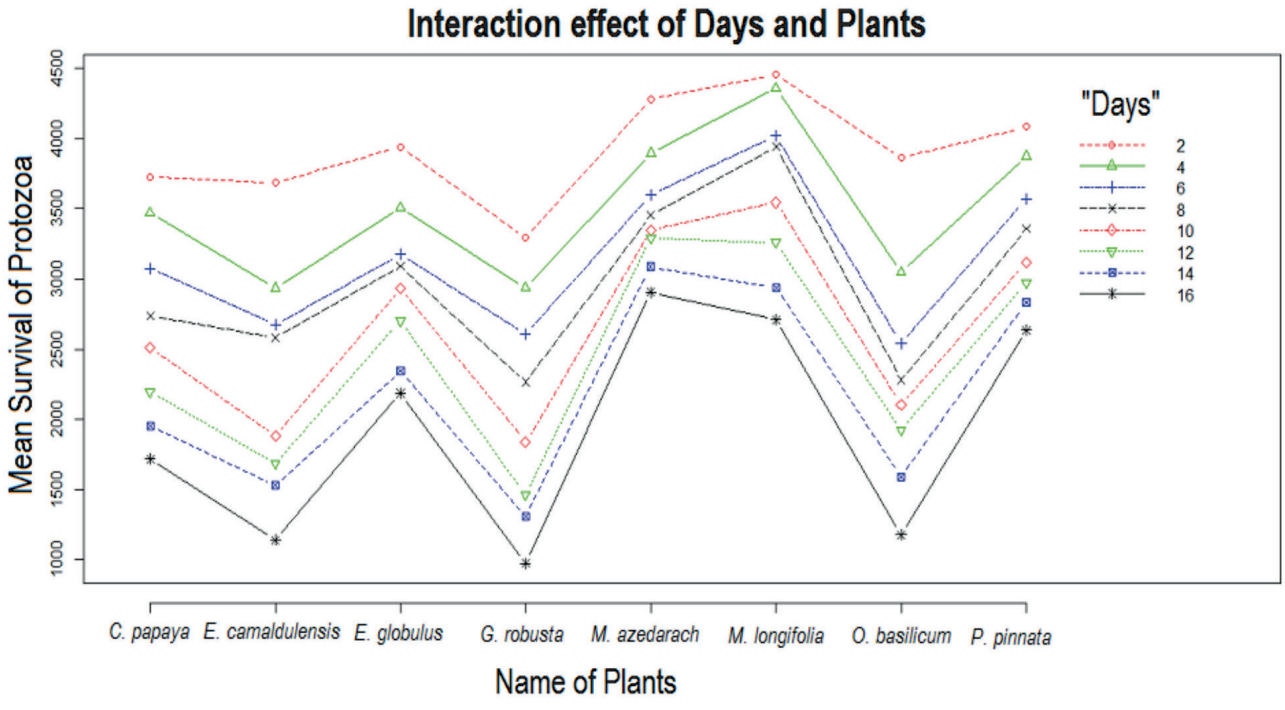


Fig. 7. Interaction plot representing the interaction effect of plant extracts with days on mean survival of protozoa isolated from the hindgut of *H. indicola.*

reported. One study, conducted by Doolittle and his coworkers [29], used bioactive compounds from neem extract, gleditschia from *Gleditschia triacanthas* and capsaicin from cayenne pepper (*Capsicum* sp.) against endosymbiotic fauna of Formosanus subterranean termites, and evaluated that neembokil was more significant for decreasing the population of *P. grassii* and *spirochaete* genus. Similarly, the comparative effect of synthetic disodium octaborate tetrahydrate with natural *vetiver* oil and *nootkatone* on endosymbionts to *C. formosanus* was conducted in the laboratory trials for 12 months [30]. In choice and no-choice bioassays it was revealed that *nootkatone* and *vetiver* oil had a more significant ($P \leq 0.05$) effect on termite hindgut flagellates than disodium octaborate tetrahydrate.

In the present study, leaf extract of eight plants was evaluated against protozoan fauna of *H. indicola* and found that all plants have differential protozocidal activity in a dose-dependent manner with an increase in time. *G. robusta, O. basilicum* and *E. camaldulensis* showed high activity profile and caused significant ($P<0.05$) elimination of protozoan fauna from termite hindgut. However, they have non-significant differences among each other, i.e., *G. robusta* and *E. camaldulensis* ($P = 0.204$), *G. robusta* and *O. basilicum* ($P = 0.030$), and *E. camaldulensis* and *O. basilicum* ($P = 0.996$). These results are compatible with a study conducted by Afzal et al. [31], who concluded that a reduction in the protozoa population was directly related to the filter paper area consumption treated with plant extract and found that after two weeks of exposure of termites to treated filter paper in no-choice bioassay at the concentration of 20mg/ml of plant leaf extract reduced the protozoa population by 62.90% as compared to the untreated negative control. Similarly, *C. papaya, E. globulus* and *M. longifolia* infected protozoan populations significantly ($P<0.05$) and caused a decline in their survival of up to 66, 56 and 46% respectively. However, the termite survival rate was high in those experimental units where filter papers were treated with extract of these plants. The present findings are comparable with the study conducted by Hassan et al. [14] on protozoan fauna of *Reticulitermes flavipus*, who reported that heartwood extracts of *Morus alba* at its highest concentration (10mg/ml) reduced the protozoan population by up to 50%, but the survival rate of termites was 7%. So while it is recommended that termite mortality is not only the result of the decline in protozoan population, many other physiological phenomenon may also affect their survival during continuous exposure to plant extract [32]. Similarly, in the present study, a decline in the protozoan population was higher in treated termites, but their survival rate was also high, suggesting that the plant extract might have some other mode of action that causes termite mortality (Figs 5 and 7). The protozoa population was declined totally in termite hindgut when exposed to toxic compounds that eventually led to termite death. The activity profile of all tested plants against protozoa was as follows: *G. robusta>E. camaldulensis>O. basilicum>C. papaya>E. globulus>M. longifolia>P. pinnata>M. azedarach.*

Characterization of Most Effective Plants

UV-Vis Spectroscopy

Qualitative UV-Vis spectroscopic analysis of *G. robusta* leaves extract in ethanol solvent showed a spectrum profile at 200 to 800nm due to the sharpness of peaks and proper baseline. The peak spectra of *G. robusta* showed characteristic peaks at 208.94 and 282.97nm with the absorption of 2.33 and 0.811 respectively (Fig. 8).

Fourier Transform Infrared Spectroscopy (FTIR)

Functional groups of active compounds in ethanolic leaf extract of *G. robusta* were identified using FTIR analysis. Each peak in the spectrum represents the specific peak value corresponding to the specific functional group. The absorbance was noted from 400 to 4000cm^{-1} wavelength. The results of FTIR analysis confirmed the presence of amines, alcohol, alkanes, nitriles, aldehyde, ether, and halo compounds (Table 5 and Fig. 9).

Gas Chromatograph-Coupled Mass Spectroscopy (GC-MS)

Gas chromatograph-coupled mass spectroscopy (GC-MS) of ethanolic leaf extracts of *G. robusta* indicated 15 peaks, which revealed the presence of 15 phytochemical compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound name, retention time, area percentage, molecular weight and a molecular formula

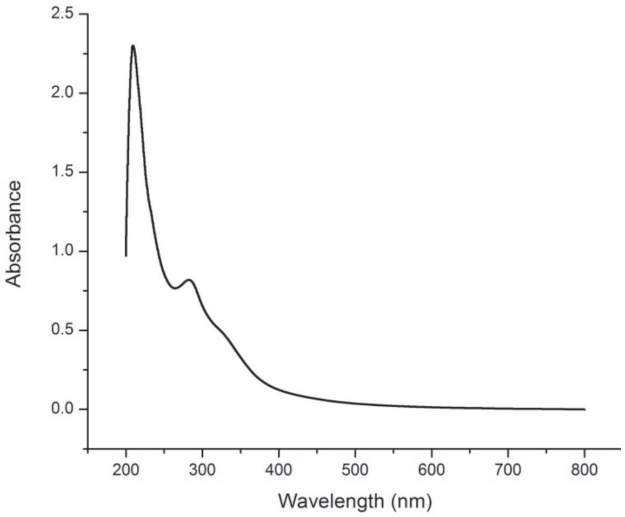


Fig. 8. UV-Vis spectra of ethanolic leaf extract of *G. robusta.*

Table 5. FTIR spectral peak values and functional groups obtained from ethanolic leaf extract of *G. robusta.*

Peak #	Peak Value	Type of stretching	Functional group
1	3307.00	N-H stretch/O-H stretch	Amine/Alcohol
2	2976.32	C-H stretch	Alkane
3	2943.98	C≡N	Nitriles
4	2831.48	C-H stretch	Aldehyde
5	1488.88	C-H bending	Alkane
6	1088.70	C-O stretch	Ether/Alcohol
7	1023.33	C-N stretch	Amines
8	880.88	C-H bending	1,2,4 trisubstituted
9	630.24	C-X stretch	Halo compound
10	542.66	C-X stretch	Halo compound
11	527.03	C-X stretch	Halo compound
12	522.55	C-X stretch	Halo compound

of identified components are listed in Table 6. The GC-MS chromatograms of identified compounds are presented in Fig. 10.

Extraction and analysis of natural bioactive compounds from the medicinal plants help in quality control of herbal medicines and their use to secure human beings and other animals from an attack of natural toxins [33]. Therefore, in the present study characterization of a most effective plant (*G. robusta*) was carried out using VU-Vis spectroscopy, FTIR and GC-MS analysis. UV-Vis spectroscopic analysis of *G. robusta* leaf extract was performed at a wavelength of 200 to 800nm, and maximum absorption was observed at 208.94nm (2.33) and 282.97 (0.811). Similar absorption peaks were observed by Rani et al. [34] in the leaf extract of *Meizotropis pellita*. The nearest absorption spectra were also reported by Rajeshkumar and Jayaprakash [35] on selected red seaweed (*Acanthophora specifera*). FTIR analysis of extract was carried out by using Shimadzu ETIR spectrophotometer, and absorption spectra were recorded from 400 to 4000 cm^{-1}. The results of FTIR analysis confirmed the presence of amines, alcohol, alkanes, nitriles, aldehyde, ether, and 1,2,4 trisubstituted and halo compounds at

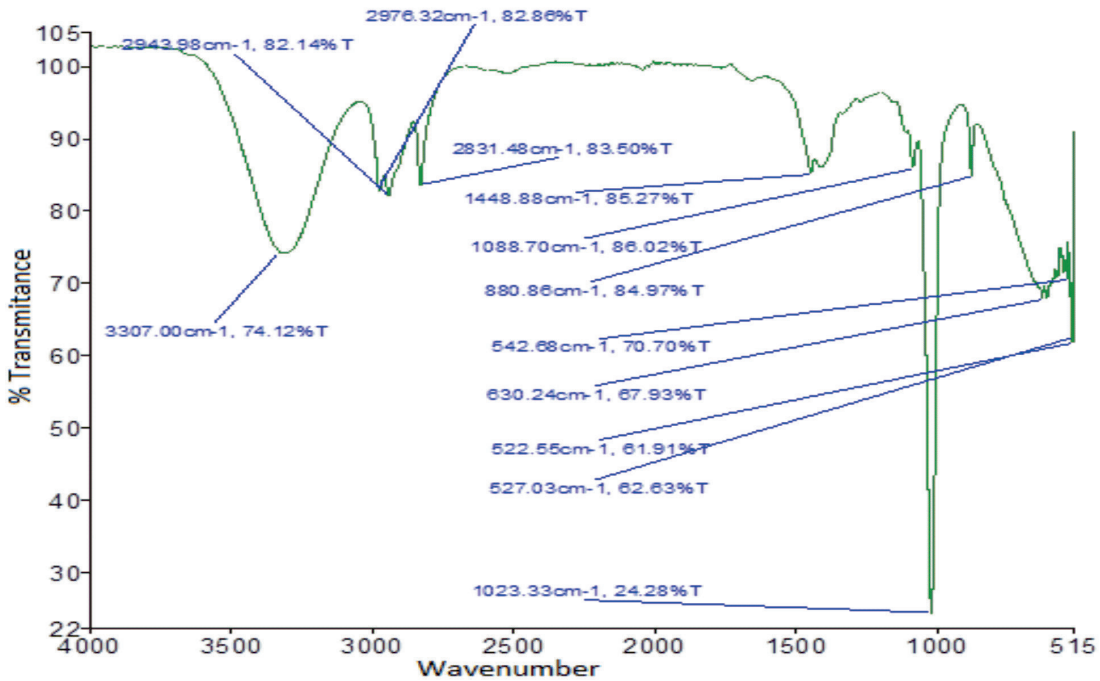


Fig. 9. FTIR spectrum of ethanolic leaf extract of *G. robusta.*

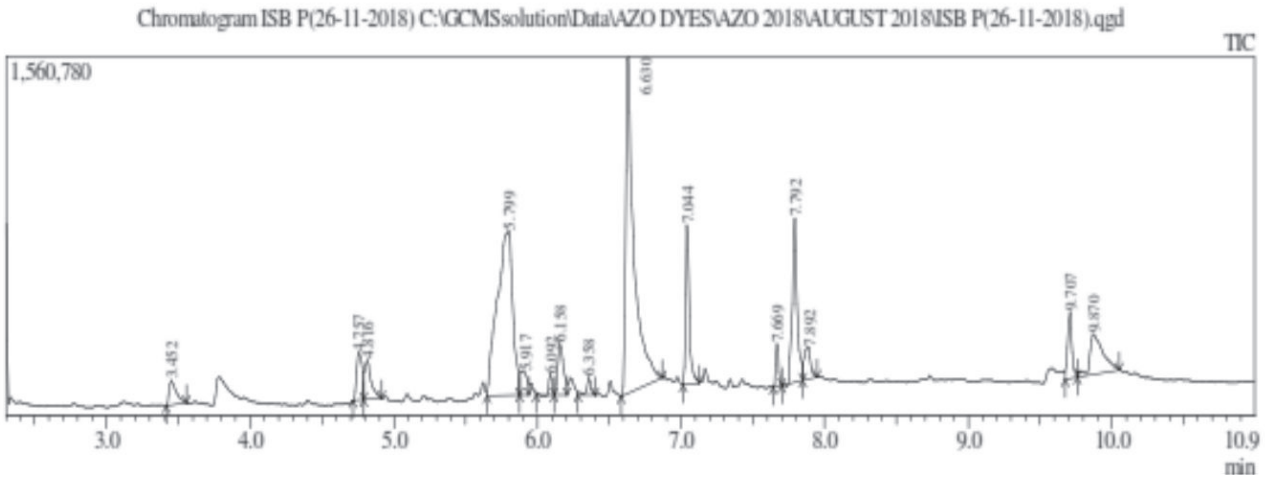


Fig. 10. GC-MS chromatogram of ethanolic leaves extract of *G. robusta.*

Table 6. Phytocomponents identified in ethanolic leaf extract of *G. robusta* by GC-MS peak report.

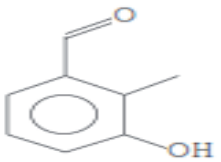
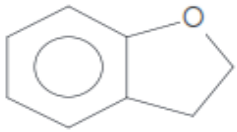
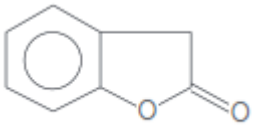
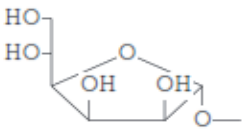
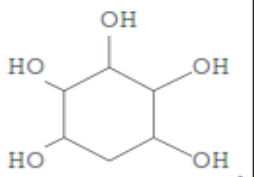
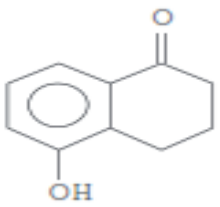
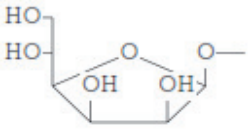
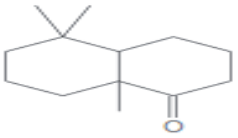
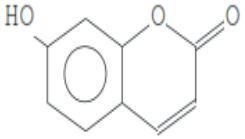
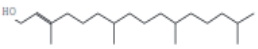
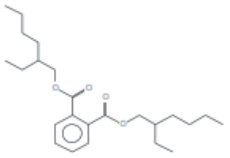
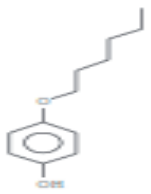
Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight	Compound name	Structure
1	3.452	1.93	C_8H_8O	120	2,3-dihydrobenzofuran	
2	4.757	3.07	$C_8H_8O_2$	136	3-Hydroxy-2-methylbenzaldehyde	
3	4.816	2.55	$C_8H_6O_2$	134	2-Coumaranone	
4	5.799	27.63	$C_7H_{14}O_6$	194	Methyl hexofuranoside	
5	5.917	1.77	$C_9H_{14}O_2$	154	1,2,3,4,5-Cyclohexanepentol	
6	6.092	0.69	$C_{11}H_{14}O_2$	162	5-Hydroxy-1-tetralone	

Table 6. Continued.

7	6.158	3.34	$C_7H_{14}O_6$	194	Beta-d-Mannofuranoside, methyl	
8	6.358	0.73	$C_{14}H_{22}O_3$	238	5,5,8a-Trimethyldecalin-1-one	
9	6.630	30.64	$C_9H_6O_3$	162	Coumarin, 7-hydroxy	
10	7.044	6.30	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
11	7.669	1.37	$C_{20}H_{40}O$	296	Phytol	
12	7.792	8.97	$C_{18}H_{30}O_2$	278	9,12,15- Octadecatrienoic acid(Z,Z,Z)	
13	7.892	2.16	$C_{21}H_{36}O_4$	352	9,12,15- Octadecatrienoic acid	
14	9.707	3.08	$C_{16}H_{22}O_4$	278	Bis(2-ethylexyl)phthalate	
15	9.870	5.77	$C_{10}H_{14}O_2$	166	4-Hexyloxyphenol	

the wavelength of 3307.00, 2976.32, 2943.98, 2831.48, 1088.70, 1023.33, 880.88, 630.24, 542.66, 527.03, 522.55 respectively. The presence of these functional groups in plant extracts has been confirmed by many studies previously [36, 37].

The GC-MS analysis of *G. robusta* leaf extracts characterized 15 phytochemicals by comparing their mass spectrum with the NIST library. Among the identified bioactive compounds, n-Hexadecanoic acid has been reported to have antimicrobial, antioxidant, antifibrinolytic, and hemolytic activity [38]. Similarly, n-Hexadecanoic acid, Phytol and 9,12,15- Octadecatrienoic acid(Z,Z,Z) characterized in *G. robusta* leaf extract has also been identified by Sermakkani and Thangapandian [39] in methanolic leaf extract of *Cassia italica* and reported their biological activities. n-Hexadecanoic acid (Palmitic acid) has an antiandrogenic, antioxidant, hypocholesterolemic, flavoring agent, nematicides, lubricant and pesticidal activities. Phytol belongs to Diterpenes and has anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive activities [39, 40]. 9,12,15- Octadecatrienoic acid (Z,Z,Z) is a fatty acid ester compound and has hepatoprotective, anti-inflammatory, insectifuge antihistaminic, 5-alpha-reductase inhibiting, antiandrogenic, pesticidal and cancer preventive activities [39, 41]. 2,3-Dihydrobenzofuran has been reported in *Origanum vulgare* seeds that have antimicrobial and anti-inflammatory activities [40]. In previous studies macrocyclic phenols, cinnamic acid

derivatives and alkylresorcinols have been reported in *G. robusta* [42].

Conclusions

We have concluded from the present study that plant extracts could be an effective alternative for synthetic insecticides for the control of termites by targeting their hindgut endosymbionts. As bioactive insecticides, these plants contain phytochemical compounds that could be used in a refined form to synthesize bioactive preservatives that can be used to prevent infestation and damage caused by termites. Moreover, these plants are easily available and affordable in local areas of Pakistan. The phytochemical analysis of the most effective plant revealed that its leaves contain various valuable bioactive compounds that can be used as an alternative to synthetic insecticides. Further studies are required for isolation, identification and characterization of bioactive compounds from *G. robusta* leaves extract by column chromatography and their characterization by GCMS analysis for their application in industries.

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Conflict of Interest

All the authors have approved the manuscript and declare no conflict of interest.

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