Evaluation of the Potential of *Cannabis sativa L.* (Industrial Hemp) as a Substrate for Biorefinery

By

Washma Aimen

Department of Microbiology

Faculty of Biological Sciences

Quaid-I-Azam University

Islamabad, Pakistan

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بسم الله الرحمن الرحيم

DECLARATION

The information and material contained in this report is my original work. I have not recently presented any piece of this research somewhere else for any degree.

Washma Aimen

DEDICATION

In memory of my father who taxed himself over the years for my education and academic development, to my doting mother who is my role model for being an independent and determined person, and my loving siblings whose support and love always encouraged me to work harder in my field of interest.

Certificate

This thesis submitted by **Washma Aimen** is accepted in its present form by the Department of Microbiology, Quaid-I-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: _____

(Dr. Malik Badshah)

External Examiner: _____

(Dr. Fazal Adnan)

Chairperson: _____

(Prof. Dr. Naeem Ali)

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List of abbreviations

ATCC: American type culture collection

AV: acid value

BSR: Base catalyzed reaction

C/N: carbon to nitrogen ratio

CSSSO: *Cannabis sativa* seed oil

DMSO: dimethyl sulfoxide

DPPH: 2, 2-diphenyl 1-picrylhydrazyl

EV: ester value

FAME: fatty acid methyl esters

FFA: free fatty acids

FTIR: Fourier transform infrared radiation

GC-MS: gas chromatography coupled with mass spectrometry

GHS: Greenhouse gases

g: Gram

Kg: kilogram

L: liter

m: meter

M: molar

MDR: multidrug resistant

ME: methanolic extract

MFC: minimum fungicidal concentration

MHA: Mueller Hinton agar

MIC: minimum inhibitory concentration

mL: milliliter

OD: optical density

PM: Particulate matter

rpm: revolution per minute

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

TAG: triacyl glycerides

TGs: triglycerides

w/v: weight by volume

v/v: volume by volume

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Abstract

The growing human population and fossil fuel consumption are causing energy resource depletion and a health crisis, with drug resistance being a major threat. Sustainable development is crucial in these rapidly changing times. The use of biorefineries is one of the key tactics for reaching sustainable goals. These establishments concentrate on generating bioenergy and other value-added products from renewable biological resources such as biomass. In the current study ***Cannabis sativa*** also known as industrial hemp was used for biorefinery purpose. The oil from the seeds was used to generate biodiesel, while extracts from the plant's seed cake, was used to check its potential in medications with antibacterial qualities. Phytochemical screening of *C. sativa* seed oil and de-oiled seed cake extracts was carried out using qualitative phytochemical analyses. Its phytochemical properties are due to a variety of beneficial substances, including flavonoids, saponins, steroids/terpenes, resins/balsams, alkaloids, glycosides, tannins, and phenols, which were all detected in it. The antifungal potential of extracts of *Cannabis sativa* pressed seed cake and seed oil were examined against five selected strains: *Candida albicans, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Curvularia lunata.*. All fungal strains wee susceptible to the extracts. N-Hexane showed the highest antifungal activity. Mic assay carried out on microtiter plates. Show thatmst of the MIC concentrations were in range of 3-0.3 mg/mL and for synergistic assay 0.03 mg/mL of combined extract and Nilstat is required to kill 80% of the microbes. The bacterial strains were Gram-negative, Multi Drug Resistant, MDR, human pathogens (*Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella)* strains were used. All extracts were positive for antibacterial activity, but the antibacterial drug used Tetracycline showed the minimum activity indicating these strains are somewhat resistant to this drug. Oil also exhibited minimum activity. Methanolic extracts were most efficient against MDR strains. The tetracycline combined with extracts gave antagonistic effect. MIC assay indicated that extracts minimum inhibitory concentration was 30mg/mL, whereas the combined assay showed these extracts and drug has antagonistic effect. Pathogenic extracts showed little to moderate activity against extracts and oil . in synergistic assay when combined with Nilstat they gave additive effect.

Among the extracts methanolic extract showed activity in all strains. DPPH assay indicated the highest antioxidant potential is exhibited by oil, 71% whereas methanol showed least antioxidant potential of 38%. Brine shrimp assay carried out to determine cytotoxic effect indicates only methanolic and n-hexane extract s showed cytotoxic effect. Chemical transesterification was used to evaluate the viability of producing biodiesel from the oil from *Eruca sativa* seeds. At standard reaction conditions yield obtained was 60% The results showed that the acid value, free fatty acid content, saponification value, ester value, and % glycerin was, 1.33, 0.746, 194.12, 192.79, and 10.53 respectively. After optimization of parameters, the highest volumetric yield of biodiesel 84% was recorded with conditions: temperature 50 degrees, oil to methanol ratio 1:9, catalyst concentration 1%, RPM 600 and reaction time 90 minutes. The whole cell approach, with oil: methanol, 37°C temperature, n-hexane solvent, 48-hour time, yielded 63% biodiesel. Hence, the results of the current study demonstrate that the *Cannabis sativa* plant is an excellent feedstock for biorefineries.

Chapter-01

INTRODUCTION

In addition to the challenge of the upcoming "cosmic era," the planet currently faces environmental, health, and energy issues. The technologies being used for these problems are connected. A significant portion of the world's energy carriers and materials are produced by fossil fuel refineries. Most of our daily life work activities from cooking to transportation all is depending on fossil fuels. However, due to human consumption these energy resources are deteriorating day by day such that our future generations will have shortage of these non-renewable resources and will face severe energy crisis(Asif et al., 2022). Furthermore, the major problem arises when these fossil fuels cause environmental hazards because their burning results in CO2 accumulation that is a greenhouse gas. Excessive production of greenhouse gases leads to serious environmental issues such as global warming. Health crises have different aspects, use of artificially prepared drugs for disease prevention along with the overuse and abuse of drugs have led to drug resistance of microorganisms. Moreover, the pharmaceutical industries are run on fossil fuel energies contributing to global warming. The need of the hour is to develop alternate energy resources that are renewable, sustainable and effective with cheaper rates than renewable energy resources (Wang et al., 2019).

Sources of energy that can be recycled or replenished to create fuel are known as renewable sources. Sustainable energy systems include solar, tidal, hydrothermal, geothermal or energy from biomass. Most of renewable resources mentioned above require expensive infrastructure and some have low efficiency and are highly expensive which is quite impossible in developing country like Pakistan. So, the most important renewable energy source is biomass as its cheaper than the rest and its availability is superfluous. Biomass can be changed into various vitality rich energy structures such as it can be used for production of bioenergy, biofuels and biochemicals. They do release greenhouse gases but in very low quantities and are safe to use. As they are environmentally friendly and degradable, using biomass as a renewable feedstock in biorefinery will reduce large mass of waste.(Nunes et al., 2020)

The potential to substitute biomass for fossil fuels as the principal natural resource for the production of fuel and chemicals is what drives the establishment of biorefinery complexes. A processing facility for conversion of biomass into a variety of valuable

products is referred to as a "biorefinery" in general. Through the application of multiple conversion processes in biorefineries, nearly all forms of biomass feedstocks can be transformed into various classes of biofuels and biochemicals. (Amoah et al., 2019)

All organic materials that are not fossilized and have energy content are considered biomass. This includes: the flora, fauna existing in water or on land, also known as virgin biomass, waste such as municipal waste (sewage and waste). (Ciolkosz & Wallace, 2011)Ash, starches, proteins, lignin, water, the hemicellulose (a heteropolymer of various hexose plus pentose sugars), and other substances make up the majority of biomass. Cellulose, a polymer of glucose, is another important component of biomass. Technology-based solid, liquid, or gaseous fuels produced from naturally occurring biomass resources with some processing are known as biofuels or biomass fuels. Specifically, the energy generated from biomass fuels is referred to as bioenergy. Biofuels are classified into three different generations (Mat Aron et al., 2020). First generation or conventional biofuels are the ones that are prepared at large scale commercial level and the feed stock includes edible crops such as sugarcane for bioethanol or vegetable oil for biodiesel, and conventional production methods like anaerobic digestion for biogas. The second generation includes non-edible or lignocellulosic feedstock whereas third generation includes fuel production from algal or microalgal biomass or other novel fuels like furanics. The ethical debate “food vs fuel debate “is one of the major drawbacks of first-generation feed stocks. The trade-off between food and fuel relates to the possibility of using farms or crops for the development of biofuels at the expense of the food supply. Increased demand for biofuel production may lead towards increased food price inflation posing a threat to global food security and it also causes the destruction of habitats as the pressure to convert land use to agriculture increases. These consequences can be mitigated by second generation fuels(Carriquiry et al., 2011).

The second generation of feedstocks, which are primarily non-edible and lignocellulosic in nature, are thought to be perfect for the biorefinery because they do not immediately impact the food supply and chain (Naik et al., 2010). In this study feedstock used is ***Cannabis sativa. Cannabis sativa L*** is a herbaceous plant commonly known by its spiky

leaves type appearance and the thin flowers. It is the native plant of the Asia and now is cultivated in the different cities of Europe and also in different area of China, Japan Canada and the United States(Mechoulam, 2019). The word **sativa** refers to "things that are cultivated." This plant can be used for biorefinery purpose as the oil extracted from seeds can be used for biodiesel production as the main energy component and it also contains chemical compounds which are used for medicinal purposes (Afif & Biradar, 2019).

Both industrialized and developing nations are plagued by a wide number of contagious diseases that increase mortality and morbidity. The use of antibiotics may change or disrupt the gut flora, which could facilitate the colonization and spread of illnesses. Overuse, abuse, and inappropriate prescription reduce the efficacy of antibiotics. Antibiotic overuse is the most typical cause of antimicrobial resistance, AMR, it develops spontaneously. Antibiotic resistance has increased as a result of the excessive use of antifungals and antibiotics. AMR poses a risk to humans at any stage of life. Antibiotic resistance has led to a rise in the use of naturally occurring substances such as probiotics, prebiotics, essential oils, organic acids, and medicinal plants and their byproducts as antimicrobial alternatives.

Plants and medicinal herbs have a significant role in modern medicine. Herbal plant essential oils, phytocompounds, extracts of particular plant parts (stem, flowers, seeds, etc.), nutritional and health supplements are used to treat illnesses ranging from common to uncommon infectious and non-infectious conditions (Anand et al., 2019). Secondary metabolites are substances that are made by plants. These secondary metabolites frequently have bioactive properties and resemble drugs and metabolites, which has several benefits for the development of anti-infective pharmaceuticals. Antibiotics are currently manufactured by plants (microbes, yeasts, or fungi) in great majority. Higher plants primarily create antimicrobial chemicals as part of their defense strategy against pathogens, which is a process of cellular metabolism (Aftab, 2019).

Cannabis sativa had been grown and cultivated for the thousands of years and used for industrial, recreation, food and medicinal purposes. Tetrahydrocannabinol (THC) is the

primary psychotropic component of cannabis. In addition to THC, significant levels of cannabidiol (CBD), are also produced that is a non-psychoactive cannabinoid that has recently been demonstrated to counteract the effects of THC on the neurological system (Pagano et al., 2022). Cannabidiol possesses various pharmacological properties which are mostly present when psychoactive properties of THC are absent. Both the CBD and THC are preserved in their glandular form and the concentration of CBD are much greater than THC in most of the plant fibers and different oil varieties of hemp. CBD is important because of its different properties which include antimicrobial, anti-convulsive and anti-epileptic. There are more than 500 recognized chemical components, around 100 of which are aryl-substituted meroterpenes known as cannabinoids. There are eight different classes of the compound which include the carbohydrates, nitrogen compounds, fatty acids, terpenes organic acids, amino acids, hydrocarbons and nitrogen compounds. The benefits which are reported include the anticancer anti-inflammatory and the anti-thrombotic actions (Echeverry et al., 2021).

Different compounds which have the antimicrobial activity are flavonoids peptides phenols Alkaloids tannins that can be recovered from the Cannabis sativa plant. Since most of the compounds of the cannabis plant have the antimicrobial activity and some have the activity in the synergetic manner different extracts of the plant is explored having the antimicrobial activity against different types of the bacterial and fungal strains. Methanol, crude aqueous and hexane extracted from the cannabis sativa have the antimicrobial activity (Radwan et al., 2021). The oil from the seeds and also other organic solvent extracted from the cannabis plant have the antimicrobial activity. Extracts prepared from cannabis plant seed cake are tested for antibacterial activity using the diffusion method. Alteration of the membrane permeability is one of the proposed models through which cannabis performed its action. It performs its action by the disruption of the membrane or by the depolarization of the cytoplasmic membrane. The seed cake is used to determine the antimicrobial activity whereas the extracted oil is used for biodiesel production (Afif & Biradar, 2019).

The mono-alkyl esters of plant or animal lipids are what are referred to as biodiesel, an environmentally beneficial alternative to traditional petroleum diesel fuel. Alcohols like

methanol or ethanol are trans esterified to create it. Biodiesel has a variety of important technical advantages over petroleum diesel, including intrinsic lubricity, low toxicity, being made from renewable and domestic feedstocks, having an enhanced flash point for biodegradation, having a low sulfur content, and having less exhaust pollution. Biodiesel in pure form is not used alone, we can make different blends with various other diesel engines. It is commonly blended with petroleum (normally less than 10%). Transesterification process requires three main reagents: an alcohol, **Triacylglycerides**, TAG, (animal and vegetable oil consists of long chain fatty acids bound to glycerol) and an acidic or basic catalyst. Fatty acid alkyl esters, which are essentially biodiesel, are created when the TAG units interact with alcohol with the assistance of a catalyst and at a high temperature (Mathew et al., 2021). There are numerous ways to conduct transesterification like the conventional batch technique, heterogeneous catalysts, supercritical procedures, ultrasonic and microwave approaches. Chemical and biological transesterification processes are most cost-effective methods for biodiesel production (Bhatia et al., 2021). In chemical transesterification monohydric alcohols are used whereas in biological processes we can use lipase producing microorganisms or directly use the lipase catalyst. The molar ratio between oil and a methanol temperature, duration, catalyst concentration, agitation speed, kind of alcohol, and catalyst being utilized are some of the characteristics that can be modified to increase the efficiency of the transesterification process (Chozhavendhan et al., 2020).

The hemp plant, ***Cannabis sativa***, produces seeds that are used to make hemp seed oil. Hemp has less Tetrahydrocannabinol, the psychoactive substance that gives marijuana its infamously mind-altering effects, than marijuana. Hemp seed oil is widely used in many different industries like food, medicine, and now biofuel. Compared to fossil fuels, hemp plants can be harvested quite fast. Moreover, it emits fewer harmful pollutants like sulfur and particulate matter, which helps to minimize greenhouse gas emissions. It also has a smaller carbon footprint. Additionally, hemp is renowned for its adaptability and quick growth, making sustainable source for the manufacturing of biodiesel. In comparison to certain other crops used to produce biofuel, it is more environmentally friendly because it can be grown in different climatic conditions and

needs little water and pesticides (Sorrentino, 2021). Biodiesel production from hemp is cost effective as compared to fossil fuels production and can help boost the economy. Liquid biofuels must be delivered to a refuelling station via a complex infrastructure. Depending on the kind of biomasses employed, the generation of the feedstock requires energy, water, and capital inputs. Therefore, some of the social effects it has are local employment, consumer choice, greater health, and job prospects.

Because the seeds of *Cannabis. sativa* can be used to produce fuels like biogas and biodiesel as well as other products with value added components, its usage in biorefineries will be crucial for the development of the economy, society, and environment. It is possible to make biodiesel and perform antibacterial actions with the help of oil extracted from the seeds that are part of the C. sativa plant. The pressed seed cake is used to perform anti-microbial activities and can also be used for biogas production, however, the microbial communities in the anaerobic digester may be inhibited by the antibacterial phytochemicals found in pressed cake (Setti et al., 2020).

Aim and Objectives of the Study

Aim

Aim of this study is to determine the phytochemical potential of Methanol, n-Hexane, Aqueous extracts of *Cannabis sativa* (hemp) seedcake and biodiesel production by transesterification.

Objectives

The objectives of this study are:

- Identify *Cannabis sativa* seeds, extract oil and calculate its yield.
- Prepare Extract to determine the antimicrobial potential against selected microbial strains.
- Determine the radical scavenging and cytotoxicity activity of prepared extracts.
- FTIR and Phytochemical analysis of hemp seed oil and extracts.
- Evaluate the biodiesel production efficacy using *C. sativa* oil through chemical and biological trans-esterification reaction.

Chapter 02

Literature Review

The shortage of energy and health issues have been two major problems the globe has been dealing with recently. The increasing demand for energy on a worldwide scale and the decreasing quantity of conventional fossil fuel stocks are the causes of the current energy crisis (Pierrehumbert, 2019). On the contrary, the current health issue is a result of a number of causes, including lifestyle changes, environmental deterioration, and restricted access to medical facilities. Both of these issues have a profound impact on people, communities, and the Earth as a whole. The production of energy throughout the world is highly dependent on fossil fuels including natural gas, coal, and oil. In addition to being scarce, these resources worsen air pollution and the release of greenhouse gases, which both contribute to climate change. Fossil fuel combustion results in the emission of dangerous pollutants into the atmosphere, which can cause early death, heart problems, and respiratory illnesses. ecological health and biodiversity are also impacted by ecological deterioration. Drug resistance is currently one of the biggest health threats the world is facing (Morrison & Zembower, 2020). The misuse and overuse of drugs, as well as the use of drugs produced chemically for the treatment of disease, are the main causes of drug resistance in microorganisms. Antibiotic-resistant bacteria, referred to as "super bugs," have emerged as a serious issue, posing a threat to the effectiveness of current medications, and complicating the treatment of diseases. Antibiotic resistance is largely caused by the improper and excessive application of antibiotics both in medicinal and agricultural settings. The application of antibiotic in livestock husbandry and patients who don't finish their prescribed antibiotic treatments both hasten the emergence of resistant bacteria. Healthcare expenditures have greatly increased as a result of the growth in illnesses that are resistant to antibiotics and the lengthening of hospital stays and treatment. Healthcare budgets are under pressure due to the demand for more thorough diagnostic testing and the introduction of newer, more expensive antibiotics (Peters et al., 2019). Additionally, the pharmaceutical companies use fossil fuels to run, which contributes to global warming.

The creation of alternative energy sources that are cost-effective, sustainable, and renewable is urgently needed. In addition to reducing emissions, renewable energy sources have a number of other advantages. The limited influence that renewable energy

has on the environment is one of its most important benefits. These sources produce minimal to zero greenhouse gas emissions, in contrast to fossil fuels, which helps to lessen the consequences of climate change. solar power, tidal, hydrothermal, geothermal, and biomass-based systems are examples of systems that utilize renewable energy sources. Most of the aforementioned renewable energy sources necessitate costly infrastructure to operate, and some of them are both extremely expensive and inefficient, making them nearly unreachable in a developing country like Pakistan. Due to its affordability and availability, biomass is among the most major environmentally friendly power sources. Any organic material that is a renewable source of energy is referred to as biomass. Everything from crop stalk and husks to chipped wood and sawdust are included in this, as well as wastes from forestry. It is possible to classify biomass as municipal solid trash and crops developed specifically for their energy content, such as switchgrass and willow. The sustainable energy landscape must include biomass. In addition to other intermittent renewable energy sources like solar and wind, biomass offers a steady source of power. By employing biomass as a feedstock that is renewable in biorefineries, a significant amount of trash will be reduced because these materials are environmentally beneficial and biodegradable. The term "biorefinery" refers to a facility that combines several methods for converting biomass to create a variety of products and energy. Similar to oil refineries, but with an emphasis on sustainable and renewable feedstocks, biorefineries aim to emulate the idea of oil refineries. The term "biomass" describes organic substances that can be converted into energy, including plants, agricultural waste, and animal waste. Traditional fossil fuels can be replaced with biofuels, which are produced from biomass. Along with other things, they can be used to generate electricity and for transportation (Ramasar et al., 2022).

2.1 Classification of Biofuels

In general, biofuels come in three generations. The initial generations of biofuels, also referred to as traditional biofuels, are those that are generated on large scales in commercial companies using crops that are edible as substrate, such as sugar cane for the bioethanol or oil from vegetables for biodiesel, as well as conventional production methods like anaerobic digestion of biomass for biogas. The 3rd generation utilizes algae-

derived or micro algae biomass to generate fuel in addition to various cutting-edge fuels like furanics, while the 2nd generation employs inedible or lignocellulosic feedstock. The moral "food vs. fuel debate" is one of the primary problems with first-generation feed stocks.

Table2.1: Classification of biofuels

Biofuels	**First generation/ conventional biofuels**	**Second and third generation /Advanced Biofuels**
Bio ethanol	Ethanol from starch, sugar crops	Ethanol from lignin cellulosic material LCM Bioethanol from micro and macro algae
Diesel	Biodiesel from transesterification of vegetable oils	Biodiesel from micro-algae
Bio methane	Biogas by anaerobic digestion of waste	Bio- syngas (bio methane by thermal process)
Bio Hydrogen	---	Gasification with reforming of biogas (biomethane)

The trade-off among food and fuel involves the potential utilization of fields or agricultural products for the generation of biofuel at the price of the food supply (Ghosh et al., 2023). Renewable resources are utilized to create second-generation feedstocks, also known as modern or non-food feedstocks, which are used to make biofuels, biobased chemical compounds, and other bioproducts. Second-generation feedstocks are derived from inedible plant materials, agricultural byproducts, and waste materials, as opposed to

first-generation feedstocks, which largely utilize crops that are edible like corn, wheat, and sugarcane.

It is possible to employ C. sativa as a second generations feed stock in biorefineries. The plant's extracts, such as those from its leaves, flowers, stems, and seed cakes, are used to make drugs with antibacterial properties while the oil from the seeds is utilized to make biodiesel(de Medeiros Dantas et al., 2023).

2.2. *Cannabis sativa* As a feedstock for Biodiesel production

Hemp (*Cannabis sativa L.*), a crop used for its fiber, grows well in many different climates throughout the world. Industrial hemp is known scientifically as Cannabis sativa L. Top 10 countries that produce seeds are France, China, Chile, Pakistan, Russia, Ukraine, Romania, Iran, Hungry & Turkey. The majority of hemp seed is produced in Europe (61.3%), followed by Asia (37.5%), and the American continent produces 1.2% of the total (Qu et al., 2020). When grown under certain conditions, hemp grows swiftly, enabling the production of an adequate quantity of hemp seed.

Fig 2.1: *Cannabis sativa* plant leaf

2.2.1 History

Since ancient times, cannabis had been utilized to produce a vast array of products, involving not only marijuana flowers however also hemp stalk and seeds. Cannabis is one of the earliest crops that humans have ever grown. Perhaps 12,000 years have passed since it was domesticated. At least 2000 BCE saw the domestication of cannabis, which was then likely transported to other parts of Asian countries, the countries of the Middle East and Eastern Europe. Both evidence for the use of cannabis as a hallucinogen and evidence for the use of hemp fiber and seeds indicate that cannabis was cultivated as both an oilseed and fiber crop in prehistoric China, Korea, and Europe(Xie et al., 2023). Its origins in nature and/or primary domestication have previously been attributed to Southeast Asia and Central Asia and are most likely to have been essential in the development of C. sativa. Additionally, the changes that took place following the Pleistocene ice age demonstrated the manner in which C. sativa followed the evolution of the initial human communities. Complex plaited basketry made of C. sativa was made using a number of techniques; in the Czech Paleolithic site, it appears that these techniques were particularly prevalent. According to Jiang et al. (2006), It may be the world's oldest archaeological evidence of C. sativa use. Furthermore, it's possible that C. sativa was produced twelve thousand years ago for a variety of uses, including the production of ancient textiles and cordage, according to a variety of Neolithic artifacts discovered in Taiwan. It has been recognized as the earliest known material used in fishing nets and is being used as one today. The first known farmed fiber plant, in instance, has been said to be this one. In the 1980s, Luna L. E. saw the assessment of medicinal herbs by a crew of the shamans from Iquitos, Peru. They used an ayahuasca-based concoction that could cause hallucinations during shamanic rites. Because shamans could use these plants to treat illnesses in their villages despite fasting and humming particular songs, they were revered as "teachers" in those cultures. Other scientists from all across the world have endorsed this "plant-teacher" hypothesis, which may assist in explaining why C. sativa was used in ancient human practices of faith. Numerous religions have regarded C. sativa as a holy plant throughout the ages. The sacred books of Asian nations actually refer to it being a plant with supernatural abilities that is used in

sacred ceremonies. Cannabis sativa blossoms and extracts are used to facilitate meditation and spirit communication in tantric Buddhist traditions of Tibet and India as well as in Hinduism. A Buddhist myth states that Siddhartha Gautama had only the right to consume a specific C. sativa preparation called "bhang" over the course during his 6-year penance. The Americas had never seen C. sativa before the arrival and colonization of the very first European settlers. According to André et al. (2016), C. sativa was primarily used during this period for the durability and strength of its fibers. In fact, the English and Spanish colonies in the region of the Americas were principally responsible for the introduction of botanical kinds suitable for textile manufacturing(Malabadi et al., 2023).

Moreover, while being sold in large quantities in Europe through the Modern Age, C. sativa was only intended for industrial use. Additionally, *C. sativa* seed and flowers' potential for use as medicines was disputed in a number of herbalist essays of the time. Thanks to the travelogues that were published by researchers, ships chiefs, affluent tourists, pastors, and traders who traveled to African nations and the East Indies in the decades that followed, European inhabitants became aware of a range of uses for *C. sativa*.

2.2.2 Taxonomy

C. Sativa Linnaeus, C. indica Lamarck, and *C. ruderalis Janisch* were proposed by Schulte and Anderson as three potential species of cannabis in terms of taxonomy. A historical geographic split among "Eurasian" *C. sativa*, "South Asian-African" *C. indica*, and "Middle Asian" C. ruderalis was found by genetic-taxonomic analysis of allozyme frequency in a number of genes. Especially in plant species used to extract D9-THC, genetic variants have been observed, and as a result of man's breeding pressure, these variations have dramatically increased over the past 50 years. Some academics assert that the debate over C. sativa's taxonomy is still raging today. In particular, the C. sativa different species will be the subject of this review. C. sativa is a yearly member of the Cannabinaceae family that is dioecious, seldom monoecious, and has towering stems that

Depending on the climate and genetic variability, they can grow to a maximum length of 5 m (Charlier, n.d.).

2.2.3Conditions of the Climate:

Geographically, Cannabis sativa is widely dispersed and flourishes in a range of conditions. When addressing the environmental limits of Cannabis sativa, the domesticated plant is usually brought up because it has more constrained tolerance than its wild-growing relative (Small, 2015). The sativa variety of cannabis favors open areas and requires full sunlight to grow. Cultivated plants need enough water for their roots, but their shoots can withstand hot, dry circumstances. It has been discovered throughout Europe that primitive plants are much more tolerant of drought than cultivars. Though the plants were small and in sandy soils in Illinois, as reported by Bazzaz and Haney (1970), wildly hemp appeared to be able to withstand dry circumstances in North America. However, roots will reportedly penetrate loose-textured soils more deeply, providing access to deep sources of water. The apparent scarcity of weedy hemp throughout western North America has been attributed to the region's relative aridity. Although it prefers moist soils, C. sativa does not tolerate waterlogging. Even more so in the first 6 weeks following sowing, consistent rainfall is highly beneficial. The sativa strain of cannabis does not do well in freezing temperatures. Cultivated plants thrive in a range of 14 to 27 degrees Celsius. Although mature plants can withstand minor frosts (falling to minus five to -6°C), they cannot withstand strong frosts or prolonged periods of cold temperatures. Although seedlings can withstand brief exposures to temperatures as low as -8 to -10°C, cultivar seeds normally do not germinate properly until the ground is warm (likely at least 10°C). Plants that grow naturally are more wind tolerant than most cultivars because of their smaller stature, woodier, and bendable stems. In Eurasia, the variety of climate and altitudes where the plant naturally flourishes is much greater than it is in North America. While it grows at heights of numerous thousand meters in the Himalayas, ruderal hemp only thrives at a few 100 meters in Canada (Cherney & Small, 2016).

2.2.4 Substratum:

Natural C. sativa is an easy-to-remove nitrophile that thrives in manure-rich substrates. In specimens from the herbarium that were gathered in Canada, the existence of manure is commonly mentioned. When feces from wild animals is added to soil that was previously fertilized by grazing cattle, wild hemp thrives in low places and ravines in Russia. The humus in manure is essential for maintaining the moisture that hemp needs in addition to providing (Small et al., 2003)nutrients. Despite the plants' small size, weedy hemp was harvested in the United States in sandy conditions with little nitrogen. C. sativa is quite adaptable, like many weeds, and will thrive in soil that has an abundance of nutrients since it will grow there much faster .

2.2.5 Communities where the species can be found:

In disturbed pastures, vacant lots, farmyards, waste areas, and occasionally fallow fields, C. sativa flourishes as a weed. The edges of farmed fields, bridges walls, lowland drainage tributaries, fence rows, railroad tracks, creeks, and open woods are some places where it might be discovered. The species typically only colonizes these areas after the soil was recently disturbed, and it shows up to be very poorly adapted to infiltrating established perennial in nature plant stands Flood waters in the US may aid in the dispersal of the seeds. Uncultivated land, native pastures, deserted fields, cultivated pastures, and the borders of cultivated fields were all found to have wild hemp (De Prato et al., 2022).

2.2.6 Bioactive substances

More than 538 chemical constituents of cannabis have been identified, of which 100 are aryl substituted meroterpenes called cannabinoids. The lipids, aromatic compounds, hydrocarbons, the amino acids, and nitrogen-containing compounds are only a few of the eighteen different chemical types of molecules that exist (Walker et al., 2021). Delta-9-tetrahydrocannabinol (THC), a cannabinols that produces psychoactive effects whose high lipophilicity allows it to penetrate the barrier between the blood and the brain, and cannabidiol, a no psychoactive cannabinoid, are the two most significant marijuana-based

components in cannabis (CBD). Female inflorescences of the cannabis plant have the highest concentrations of THC (Freeman et al., 2021). In accordance with their THC level, the following three cannabis strains are categorized: In order to produce fiber and edible oil, hemp, or chemotype III, which possesses a lower Tetrahydrocannabinol ratio (below 1), has been employed. The psychotropic chemotype I, which is utilized to produce narcotics like marijuana and hashish, has an increased Tetrahydrocannabinol ratio (above 1). A non-psychoactive or low activity chemotype II, which has an average THC/CBD ratio (near to 1). Cannabinoid acids such as such as cannabidiolic acids, cannabigerolic acids, and theirs decarboxylated equivalents, cannabidiol and cannabigerol, make up a majority of the cannabinoids found in fiber-type cannabis. Cannabichromene, THC breakdown byproducts cannabinoid acid, as well as canna bichrome Nic acid are included with smaller levels of each in hemp (Pattnaik et al., 2022).

2.3. Bioavailability of Cannabis sativa:

Leading Active Substances Peak plasma concentrations of THC or CBD are reached swiftly, around Three and 10 minutes after inhalation, and these are typically higher than those reached after ingesting cannabis. THC is typically 10–30% systemically bioavailable after inhalation, but CBD is typically 31% systemically bioavailable.

2.3.1 *Cannabis sativa's* bioactive compounds' primary modes for functioning

Endocannabinoid signaling system:

The receptors for CB1 and CB2, the endocannabinoid membrane transporters, N-arachidonoyl ethanolamine (anandamide), 2-arachidonoylglycerol, and the enzymes accountable for their synthesis and metabolism, as well as the endocannabinoid system is composed of CB1 associated enzyme 1a, which controls the signaling transduction of receptors called CB1.

Following significant investigation into THC's impacts, CB1's receptor has been cloned in 1990, and is now well known. It was named anandamide in 1992 when a major natural CB 1 ligand was found. 1993 saw the successful cloning of the peripherals or CB2

cannabinoid receptor from macrophage and the spleen. Later on, scientists found further parts of the endocannabinoid signaling system. The endocannabinoid system controls many bodily functions, including those related to pain, emotion, appetite, thermogenesis, or heat metabolic processes, sleep, movements, stress response, and addiction. Additionally, it influences memory, the development of new neurons, immunological and inflammatory responses, and the differentiation of fetal cells. They regulate key intracellular transmission of signals pathways, such as calcium networks, D-type potassium networks, the protein kinase signaling pathway, and the activity of the cyclic adenosine monophosphate (Lu & Mackie, 2021).

2.4 Medicinal importance

The first recorded usage of C. sativa for therapeutic purposes dates back to the creation of the very first Chinese pharmacopoeia by Emperor Chen Nung, who is known as the "the father" of Chinese agricultural and the "king" of the nation. According to this ancient source, C. sativa was suggested as a remedy for fatigue, rheumatism, and malaria. Furthermore, C. sativa seeds' proteins and vegetable oils were extensively used by Chinese physicians. Doctors recommend C. sativa seeds for inflammatory conditions like eczema and psoriasis because they are high in -linoleic acid (Pagano et al., 2022). The Egyptian Ebers Papyrus and Assyrian clay tablets, both of which date to circa 3000 B.C., both provide extensive record of the utilization of Cannabis sativa for therapeutic purposes. C. sativa was utilized by ancient Egyptian women to reduce pain and lift their spirits.

Galen, a renowned Roman doctor in both antiquity and medieval times, offered several remarks on C. sativa. The Roman elite had a custom of finishing luncheon with a cannabis's dessert, which he specifically discussed. Chronic THC use can exacerbate psychotic symptoms and lead to feelings of sorrow, anxiety, and decreased motivation (De Faria et al., 2021). THC also has the ability to suddenly raise the pressure in the blood and heart rate in a manner that depends on the dose. Cannabidiol generally goes down well and has less major negative consequences than the THC molecule. However, a small number of studies have noted drug-drug interactions, constipation, tiredness,

nausea, somnolence, and hepatic disorders. Due to the possibility of adverse effects, cannabis therapy shouldn't be used on people who have severe mental breakdowns, heart-related, kidney-related, or hepatic disorders. CBD has been shown in numerous studies to have analgesic, anti-inflammatory, neuroprotective effects, antiemetic, and anticonvulsant(Ghelani, 2023) .

2.5 Cannabinoids roles in diseases:

In treatment strategies for numerous illnesses, several C. sativa preparations—both natural and synthetic—are used. Because the endocannabinoid system is so prevalent in the brain and other peripheral locations, it is safe to say that its activation or inhibition regulates a number of pathological processes.

2.5.1. Colitis:

Intestinal problems like diarrhea, gastroenteritis, and gastrointestinal pain have all been treated with various C. sativa preparations. Gut inflammation can be lessened by Phyto cannabinoids. According to various papers, some extracted C. sativa components, such as CBC, CBD, and CBG, have been demonstrated to have advantageous effects in clinical cases of intestinal inflammation following a gastrointestinal shock(Chda et al., 2023) .

2.5.2. Spasticity:

Over eighty-five percent of those with Multiple Sclerosis experience some degree of stiffness. The current medications employed for treating stiffness brought on by multiple sclerosis include baclofen, tizanidine, anxiety medications, and the chemical botulinum toxin. Since they aren't always completely effective, side effects may limit how often or how much of these treatments can be taken. This has led to an increase in C. sativa utilization among MS patients worldwide. Numerous studies have really found that cannabis might lessen MS-related spasticity. Nabiximols, oral cannabis extracts, and synthesized tetrahydrocannabinol have all been proven to be helpful in reducing MS patients' reported signs of spasticity and pain when other drugs haven't worked as well (Withanarachchie et al., 2023).

2.5.3. Vomit and nauseating

Long recognized is C. sativa's capacity to diminish or stop gastrointestinal distress brought on by a range of illnesses. This led to in-depth investigation, which demonstrated the critical function cannabis derivatives play in the management of vomiting and dizziness. Numerous products derived from the cannabis sativa plant are available to alleviate symptoms of vomiting and nausea because the immune system that generates endocannabinoid is spread in the colon. The FDA, which stands for the Food and Drug Administration, approved the use of nabilone, an artificial version of THC, in 1985 to treat chemotherapy-related vomit and nausea that did not get better after being treated with traditional antiemetics (dopamine receptors -2 (D2) antagonist) (Khurshid et al., 2021).

2.5.4. Anorexia

By interfering with a variety of cerebral and peripheral regulatory pathways that control the distribution of calories, cannabinoids help to manage the body's mass in general. Because anorexic and bulimic people have greater amounts of CB1 receptors expressed in the frontal part of the brain, inferior temporal lobe, and insulate, the decoding of interceptives, gustatory, and reward-related behaviors could be disrupted (Bourdy & Befort, 2023).

2.5.5. Anxiety

Anxiogenic or sedative effects of hemp may exist, according to recent study. In contrast to CBD, which operates on the cerebellar and paralimbic parts of the brain to have anxiety-reducing characteristics in both animals and humans, D9-THC has a tranquilizer effect in small doses but is anxiogenic in high doses. The D9-THC and CBD present in C. sativa, both of which have unique psychotropic effects, are to blame for this. Cannabis has a relaxing effect, which may help to explain why so many people with anxiety disorders use marijuana as "self-medication"(Stella, 2023).

2.5.6. Epilepsy:

Cannabinoids may be used to treat epilepsy, according to preclinical studies. Both preliminary and clinical studies have been conducted to examine the epileptic properties of Cannabidiol and cannabidivarin, the propyl derivative of Cannabinoids. For the first time, the FDA (Food and Drug Administration) approved the medication EPIDIOLEX, that includes Cannabidiol produced by hemp, for the relief of Lennox-Gas taut syndrome, also known as (LG syndrome) and the medical condition of Dravet, 2 devastating and unusual kinds of epilepsy (Morano et al., 2020).

2.5.7. Alzheimer's disease:

Recently, endocannabinoids have drawn interest as a major target for the treatment of Several aging neurological disorders and cognitive impairment. With several research revealing contradictory outcomes in young and adult cannabis users, both at high as well as low dosages, cannabis's impacts upon memories and Alzheimer's disease in particular has been the subject of intense discussion in recent years. However, it seems that the majority of people concur that adults who use cannabis in moderation may avoid or even treat AD (Viana et al., 2022).

2.6 Antibiotic Resistance:

Microorganisms that are resistant to antibiotics are a major threat on a global scale. According to Martens & Demain (2017), infections caused by bacteria account for seventeen millions fatalities each year, making infections the 2nd biggest cause of mortality. An unprecedented number of microorganisms are resistant to one or more types of antibiotics. So far, only 15 antibiotic classes that target different bacterial cell structures have been able to sidestep a resistance mechanism. There are currently no options to assist minimize antimicrobial resistance because so few new medicines have been investigated and approved in recent decades . In such a situation, alternative approaches must be investigated to mitigate the threat of resistant bacterial strains. Most plant and phytochemical extracts have demonstrated antibacterial activity against infections, including bacteria that have been clinically proven to be resistant strains, and

have historically been used as an important component of natural and good for human health products. Animal models of infection demonstrated antibacterial's impacts of plants isolates in specific instances. Additionally, synergistic advantages may be attained when extractions from plants or their hyperactivated components are coupled with antibiotic to avoid resistance from bacterial agents(Andrei et al., 2019)

2.7 Cannabis and its antibacterial activity

Cannabis has long been known to possess antibacterial qualities capable of combating a wide range of hazardous bacteria as well as some fungus. THC and CBD are the primary antimicrobial components. Numerous studies, however, have revealed that this effect is also present in plant extracts or essential oils. Numerous antimicrobial compounds found in C. sativa, including alkaloids, flavonoids, peptides, tannins, and phenols (Rios et al., 2016). As a result, a variety of chemicals contained in cannabis extracts may have additive or synergistic antibacterial actions. Cannabis seed cake extracts were tested against different bacteria, and the results show that the extracts strongly inhibit both Gram positive and Gram-negative bacteria. Although specific cannabinoids can be detected in cannabis leaves, CBD and 9-THC have gotten the most attention, many of the 480 compounds currently identified in cannabis plants have not been tested for their ability to fight microorganisms. Some of these chemicals may be more effective antibacterial agents than others. Furthermore, it is unknown how the elements of essential oils interact with one another. Oil components' synergistic and antagonistic qualities are extremely likely to exist and are what cause the oils' varied activities (Karas et al., 2020).

2.7.1. Antibacterial Mechanism of Action

Although being short of an effective mode of administration for curing infections caused by bacteria, recent advancements in the realm of cannabinoid have been developed. Permeation of membranes is one of the potential mechanisms for the actions of cannabis compounds. The terpenes limonene has the potential to impair the strength of cells and wall construction, which could cause various cell constituents to seep out. It has been shown that CBG can penetrate the cytoplasmic membrane of gram-positive bacteria. CBG could carry out gram-positive bacteria's functions since gram-negative bacteria's

inner membrane was permeable. The microbial cell membranes and nucleoid were altered by CBCA, according to a microscopic analysis of its capacity to promote the development of B. subtilis. Studies in vitro revealed that CBD depolarized and ruptured the membrane of S. aureus while also depolarizing it. The interaction between CBD and bacitracin can result in a variety of cell division mistakes as well as anomalies of the cell membrane. According to theory, the aberrations resulted from a shortage of genes that regulate cell division. Cannabinoids are also useful for altering cell communication because they stop bacteria from releasing membranes vesicles. The medicine might also lessen a quorum sensing (QS) system's ability to detect and respond to bacteria's stimuli. It also improved Vibrio Harvey i's ability to swim. According to the findings of these studies, CBD may be particularly efficient at rupturing bacterial membranes (Saleemi et al., 2022).

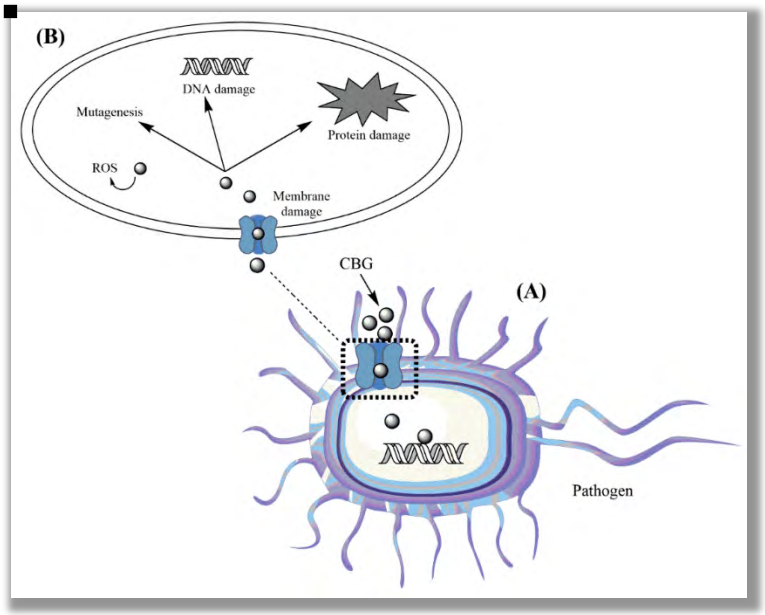


Fig2.2: Antibacterial Mechanism of action of Cannabinoids

Source: https://www.mdpi.com/1424-8247/15/10/1228

2.7.2. Antifungal Effects of *Cannabis sativa*

Terpenes, cannabinoids, flavonoids, and phenols, which have potent antifungal activities and may be useful in stopping the growth of A. flavipes, are abundant in C. sativa. A

fundamental component of Cannabis sativa, cannabinoids, is widely used in pharmacological research and the creation of medications to treat human ailments. Aspergillus niger, Aspergillus flavus, Candida albicans, and Fpxysporum species were tested against various extracts from hemp seed cake in a recent study that is similar to our work. The main modes of action of antifungal medications include disruption of cytoplasmic and spindle microtubule activity, ergosterol depletion or binding, and squalene (terbinafine) buildup. Hemp seed oil and extracts aid in the treatment of infections brought on by fungi due to its antifungal properties (Berardo et al., 2024).

2.8 Cannabis Essential oils and terpenes

Terpenoids make up the majority of essential oils, which are aromatic, oily liquids that are obtained from plants. The biological action of EOs, particularly their antibacterial activity, is provided by terpenoids. A fluorescent protein is called Eos. Additionally, cannabis EOs may include trace levels of certain cannabinoids that have synergistic effects, including CBD, CBC, and cannabidivarin (CBDV)(Mancianti & Ebani, 2020). Beta-caryophyllene, caryophyllene oxide, myrcene, limonene, alpha-pinene, and beta-pinene are terpene molecules with antibacterial characteristics. Several pathogen microorganisms have been shown to be slightly vulnerable to the moderate antibacterial effects of some Cannabis sativa essential oils, including Acinetobacter calcoaceticus, Bacillus subtilis, E. coli, Y. enterocolitica, M. luteus, and Staphylococcus aureus (Moo et al., 2020). Nissen et al. (2010) evaluated the EOs isolated from the flowering stage of three distinct cultivars of Cannabis sativa in terms of their antibacterial potency and terpenoid profile (Appendino et al., 2008). Eliminating Staphylococcus aureus biofilms was another skill the essential oil displayed. thus, demonstrating a marginal bactericidal activity against Listeria monocytogenes. The ability of L. monocytogenes to move, invade, and form biofilms was also impacted, which had an impact on the bacteria's virulence features (Nafis et al., 2019)

2.8.1. *Cannabis sativa L* seed oil

Hemp is a substitute oilseed plant that yields 3-5 mm brown seeds of various colors. In addition to its various advantages, the plant Cannabis sativa L. has the potential to serve

as the basis for the creation of energy (Kraszkiewicz et al., 2019). It may, for instance, be grown successfully in a range of climes. Agriculture inputs including lessened water, pesticide, and fertilizer requirements help to lower the cost of running a hemp plantation and simplify hemp cultivation. As a result, archeological studies have revealed that this plant has existed for around 8000 years. In addition to 20 to 30% carbs, 20 to 25% protein, 10-15% fiber, potassium, magnesium, calcium, minerals, phosphorus, zinc, iron, and sulphur, hemp seeds often include 25 to 35% oil. According to some research, hemp seed oil may contain as much as 51.06%. Numerous plants are grown as crops for industrial purposes all throughout the world. Numerous commercial applications, industrial designs, and even genetic optimization studies have been conducted over the years for each of these plant seeds. The growing of hemp is, however, virtually forbidden in many countries, making it challenging to conduct research.

Hemp seed biodiesel is another exciting study subject in this field. Cannabis sativa L seed oil is one essential component for producing biodiesel (Crini et al., 2020).Only a few studies have examined esterification of hemp seed oil with methanol and KOH for generation of biodiesel, though. In reality, as far as we are aware, there isn't a study in the literature right now that deals with the Taguchi technique's The creation of biofuel from oil requires the improvement of reaction conditions from Cannabis sativa L. seed oil. Therefore, according to the results of our experimental study, seed oil from Cannabis sativa L. is a substantial prospective raw material for the production of biodiesel.

2.9 Biodiesel:

Biodiesel is utilized in a manner similar to that of mineral diesel fuel and is chemically composed of a lipid methyl esters. By trans esterifying monohydric alcohols like methanol or ethanol with vegetable or animal fats and oils, the chemical industry creates biodiesel. The biofuel that has so far contributed the most to providing the transport sector in the European Union is biodiesel. There was widespread social agreement to introduce and increase the supply of biodiesel toward the end of the 20th century since it was seen as sustainable and environmentally benign. Growing demand over time has resulted in international trade in biodiesel, which was in part related to the growth of

agricultural land, such as through slash-and-burn practices. The availability of raw materials that are sustainable, do not compete with the production of food and feed, or do not cause the extinction of species will determine whether a broad use is socially acceptable (Mishra & Goswami, 2018).

2.9.1 Properties of Biodiesel:

Biodiesel is a yellow to dark brown liquid with a high boiling point up to 200 ^{O}C and low vapour pressure i.e., less than mm of Hg at 22 ^{O}C that is scarcely miscible with water, depending on the raw material used to make it. It has less sulphur than mineral diesel and doesn't have any benzene or other aromatics. Due to its greater flash point than diesel fuel, biodiesel is not a dangerous substance**.** The flash point is above 130 °C and is therefore significantly higher than that of regular diesel. It has distillation range of 195-325 ^{o} C. Biodiesel typically has a stable reactivity, a mild musty and soapy odour, and is insoluble in water. Biodiesel is non-volatile or less volatile in nature, renewable, non-toxic, biodegradable, less flammable, and highly lubricating, causes less pollutants, and is free of aromatics and sulphates. It is an **effective fuel** as a result. Additionally, studies claim that biofuels can cut GHG by up to 65%. These characteristics of biodiesel make it the ideal alternative fuel and allow for its widespread usage in many nations, particularly in ecologically sensitive areas (Chuah et al., 2021).

2.9.2. Feedstocks used for Biodiesel production:

Figure 2.3 illustrates the classification of possible materials for biodiesel production into five categories: fossil fuels, 1st generation edible oil-producing agricultural products, 2nd generation inedible agricultural products, 3rd generation waste products, 4th generation algae-based agricultural products, and 5th generation crops that have undergone genetic modification (ACS Omega 2021). The two primary categories of biomass are feedstocks of the first and second generations. Recognized 1st-generation raw materials that can be used to make biofuels and other products include consumable agricultural products like sunflower, cereal grains, grain, soybeans, wheat, and rapeseed. The most popular first-generation biofuels are ethanol from biomass, bio diesel, and biogas from strach. Bioethanol is obtained through the fermentation of a variety of feedstocks that contain

carbs/ sugars that can be brewed. The manufacturing of bioethanol uses the sugar cane, sugar beets, and plants that produce starch, including grain and corn (Sales et al., 2022).

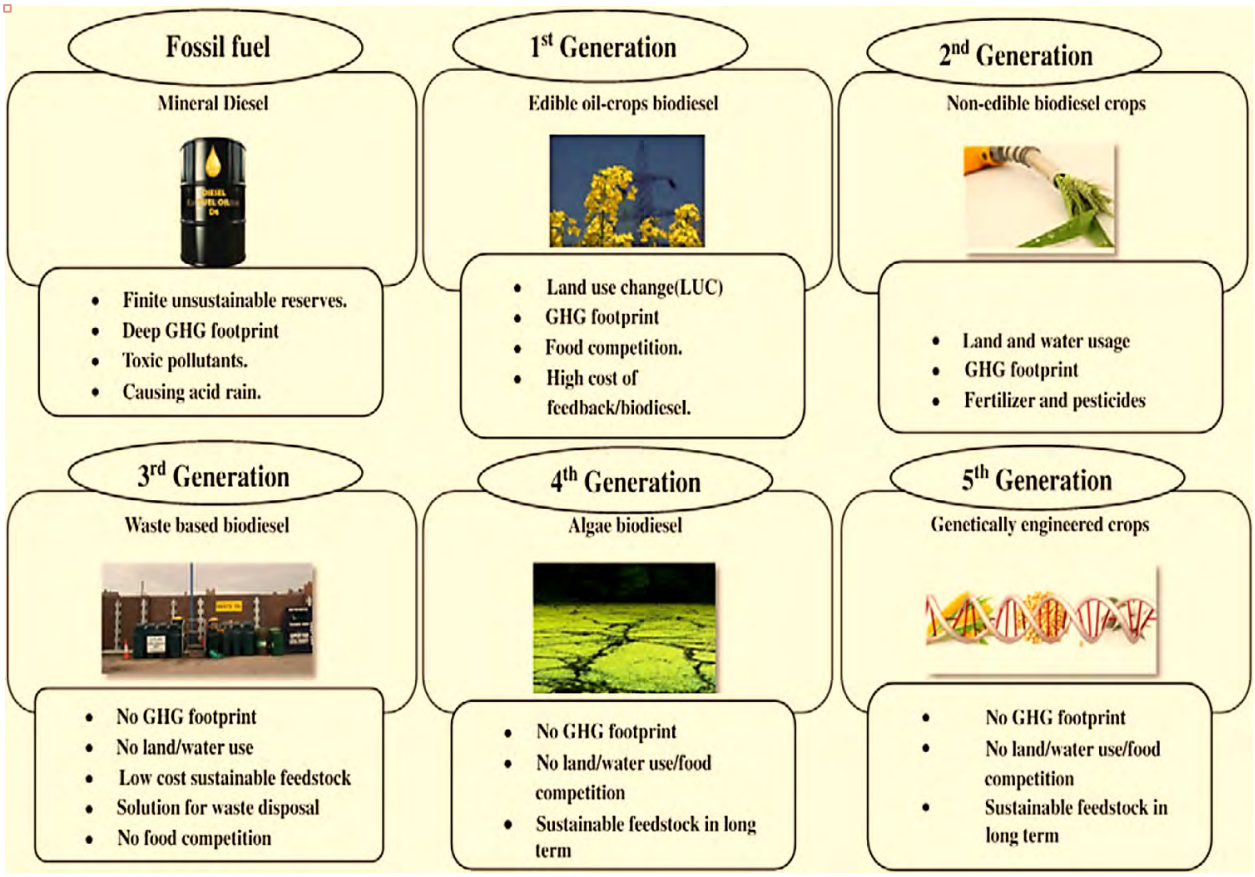


Fig 2.3: Classification of feedstocks for biodiesel synthesis

One of the most widely used biofuels is biodiesel, which is made from plants that are based on edible fats and oils such soybean, sunflower, rapeseed, and palm. About 31.6 billion litres of biodiesel were produced globally in 2015. The EU, USA, and Brazil are the three economies that produce the most biodiesel, with annual production of Thirteen, five, and four billion Liters of the gasoline, correspondingly (Chowdhury et al., 2019).

2.9.3. Potential Feedstocks' Suitability for Biodiesel's Production:

The selection of the process to use for biodiesel synthesis, together with the feedstock, is one of the most crucial factors to consider. The feedstock pays for 75 percent of the price of making biodiesel. The oil content is determined by the type of feedstock itself. Figure

2.2 displays the breakdown of the overall costs for the manufacturing of biodiesel process (Cheah et al., 2020).

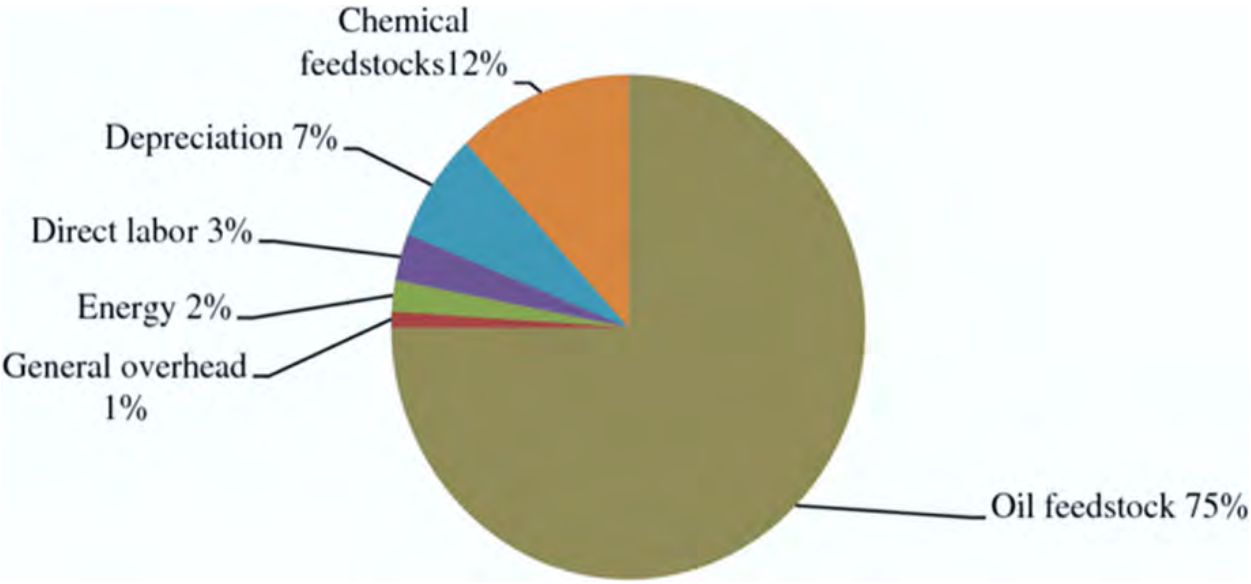


Fig. 2.4 Biodiesel synthesis cost breakdown overall (*ACS Omega* 2021)

Because of this, generating biodiesel can be done at a much lower cost by using cheaper feedstocks.

The biggest advantages of using consumable materials for biological refinery applications are their higher fat and sugar material, lower recalcitrance, and simple transformation into biofuels and other products. Edible feedstocks have attracted interest from all across the globe, but their feasibility is impeded by problems which includes competitiveness for food supplies and the requirement for obtaining property for bio fuels and other goods, which raises the price of food. This competition generates moral, social, and political concerns regarding the way it should be used.

A new process has been developed to create biofuels from 2nd generation of inedible raw materials, which are viewed as potential choices for the construction of biorefineries. Several lignin sources of energy, biomass made from wood, waste from agriculture leftovers, grassland biomass, municipality garbage, residues of forests, and animal fats

are examples of non-edible feedstocks. The nature of second generation feedstocks is abundant and diverse, and they can be exploited using a range of technologies to generate biofuels. Several techniques, including thermochemical, pyrolysis by flash, and enzymatic ones, are used to make synfuel or other gas or liquid biofuels. The effectiveness, efficiency, and environmental performance of the renewables and value-added chemicals produced from second-generation feedstocks are superior to those produced using generation feedstocks in various ways (Singh et al., 2020).

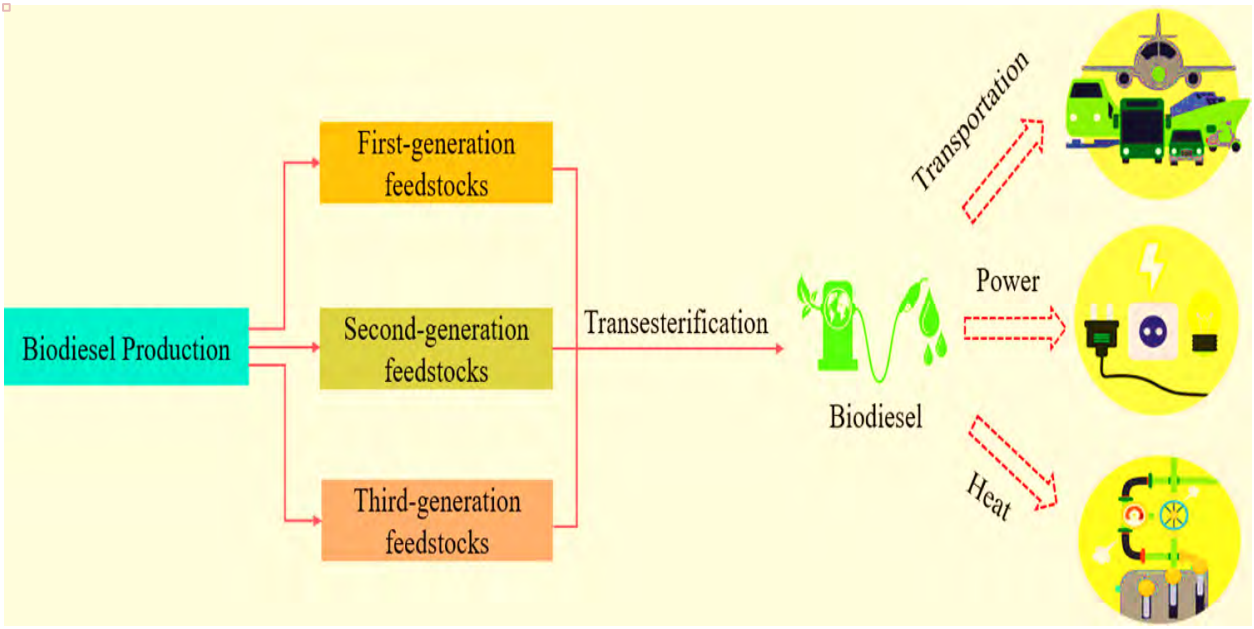


Fig. 2.5: Biodiesel's production feedstocks (*ACS Omega* 2021)

2.10 Biodiesel production:

Longer chained alkyl fatty acid esters, also referred to as biodiesel, can be produced by combining triglyceride with alcohols (ethanol or methanol) under the control of a catalyst. Biodiesels are non-toxic, almost sulphur-free, recyclable, and non-aromatic fuels that are good for the environment. These three repeatable phases make up the basic transesterification process: Triglyceride to diglyceride conversion, diglyceride to monoglyceride conversion, and glycerin synthesis are all examples of triglyceride to glyceride conversion.

Oil + alcohol → biodiesel + glycerin

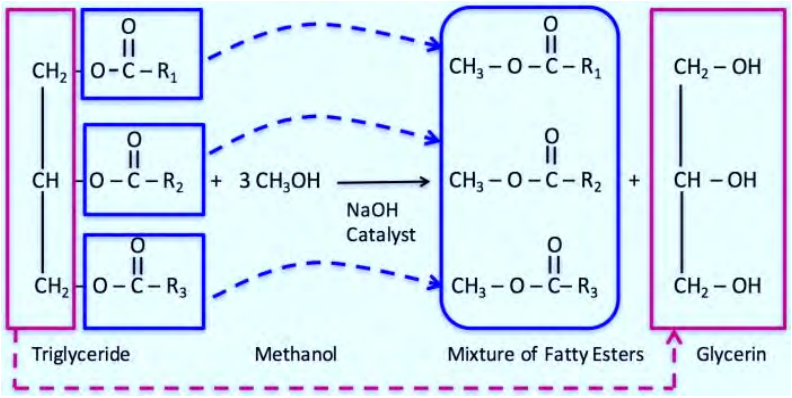


Fig. 2.6: Transesterification Process for biodiesel production

The above mentioned steps leads to biodiesel esters (methyl esters) production. The triglycerides and an alcohols ratio are kept 1:3. But its constraint to have high alcohol value in comparison of oil, so that continue reaction can be made in forward direction and due to high volatile nature of the alcohols it is sufficient for the reaction to be completed (Vasudevan & Briggs, 2008). Commercially, a large numbers of alcohols that have been available for biodiesel production, but for better results methanol and ethanol have received utmost importance due to its cost-effectiveness and easily soluble. The catalyst are being used for many reaction that make reactions rate fast and yield increase biodiesel production. There are two types of catalyst i.e. alkaline and acidic. The alkaline catalyst are cost effective, efficient, heat stable and require low temperature and pressure for reaction to proceed in comparison to acid catalyst. However, utilizing alkaline catalyst has one disadvantage: it can interact with fatty acids that are free to produce soap, which could lower the amount of catalyst used and, in turn, impair the effectiveness of the procedure. For effective outcomes in chemical reactions, sodium hydroxide (NaOH) and the potassium hydroxides (KOH) are already widely utilized as base alkaline catalysts. Potassium hydroxide alkaline catalyst are more expensive as compared to sodium hydroxide catalyst. However, for biodiesel production mostly potassium hydroxide is being used as these are more efficient and require minimum time for the completion of reaction (Abbaszaadeh et al., 2012). The alkaline transesterification is mainly used for the creation of bio-diesel from non-consumable feedstocks. So, when the free fatty acids

have lower value than 1 0r 2%, the transesterification process is smooth and without any obstacle. For cannabis sativa which have low FFA content, so alkaline transesterification reaction should be used.

Apart from the chemical used in the transesterification, microbial cells and their enzymes are also used for transesterification. These microbial produce enzymes like lipase which are involved in the reaction. The transesterification process is utilized to turn lipids into biodiesel, and the triacylglycerol (TAG) acyl hydrolase lipase enzyme is used in this process. These substances were alpha and beta hydrolase-related enzymes. According to studies by, aspartic acid, serine, and histidine AA are present on their active site where hydrogen bonds are present. After amylases and proteases, these enzymes are listed third on the list of biotechnological uses that have a significant commercial and industrial impact. These enzymes play role in the breakdown of triglyceride into the fatty acids and the generate glycerol at the water-oil interface, that can be reverse in a liquid and non-liquid solution, as they are involved in the metabolism of fats and oils. The production of biodiesel by lipase enzymes are more production, eco-friendly and energy saving as compared to biodiesel produced by chemical methods of transesterification (Ghaly et al., 2010).The fungal and bacterial lipases are abundantly used for the efficient production of biodiesel. So, as compared to chemical method, microbial lipases are more economical and efficient for biodiesel production.

2.10.1 Factors affecting transesterification reaction:

The efficiency of the biodiesel process depends on a variety of variables like the molar proportion of alcohol to oil, the reaction time it takes for the reaction to complete, the concentration of the catalyst, and agitation.

Molar ratio:

To produce biodiesel, the molecular proportion of triglyceride to alcohol is essential. Although in practice more alcohol is needed to advance the reaction and reduce the chance of alcohol evaporation, the stoichiometric proportion of ethanol to triglycerides for the production of biodiesel is 3:1. According to past studies, the optimal ethanol to oil

molar proportion for the production of biodiesel was frequently determined to be 6:1. If the molecular ratio of ethanol to oil is greater than the optimal ratio (6:1), the amount produced is not improved; rather, the process expenses are increased. Increased ethanol to oil mol ratios—up to 15:1—are necessary, especially for acid catalysts, to make up for high content of FFA (Musa, 2016).

Time to respond:

The rate at which triglycerides are transformed into esters is also influenced by the reaction time. Fats and oils combine and spread alcohols; the reaction begins slowly. Later, the reaction's rate rises until the optimal reaction time when it hits its peak. Reports state that the biodiesel output peaks at reaction durations under 90 minutes and that adding more time after this point had no beneficial effect on the esters yield. Furthermore, going above the ideal reaction time range decreases product yield, which ultimately causes esters to be lost and FFA to form to produce greater amounts of soaps (Kasim & Harvey, 2011).

Temperature of the reaction:

The transesterification reaction's temperature influences how much biodiesel is produced. As temperature goes up, so does response time and viscosity of oil. If the temperature of the reaction is raised above its optimal range, soap will be produced. This is not recommended since alcohol will quickly evaporate at temperatures greater than its boiling point. the optimal temperature for producing biodiesel is usually around 50 and 60 °C based on the kind of oil utilized (Hoque et al., 2011).

Concentration of the catalyst:

Ester’s yield can be increased in large part by increasing the catalyst concentration. For the manufacture of biodiesel to be effective, more catalyst must be used. The development of soap will arise from increasing the catalyst past its optimal level, which will result in the loss of esters and greater process costs. According to a number of studies, the ideal catalyst concentration for a higher biodiesel output is 1.5percent (Hossain & Mazen, 2010).

2.10.2 Fuelling vehicles with biodiesel:

The use of biodiesel as a substitute for Petro-diesel is made possible by the fact that it shares many characteristics with the latter and may be utilized in diesel-powered vehicles without requiring any modifications. As a result, using bio-diesel as a vehicle fuel increases: Energy security, enhances the quality of the air & surroundings, and offers safety benefits.

Because biodiesel increases fuel lubricity, it also increases the cetane content of gasolines. A greater cetane number results in a quicker and smoother engine start-up. Diesel engines depend on the fuel's lubricity to avoid premature deterioration on moving parts. A higher level of lubricity prevents further wear by reducing resistance between the moving elements. The ability to increase lubricity of the fuel at blend levels below as one percent is one of biodiesel's main benefits. (the US Dept. of Energy) (Ajala et al., 2015).

Chapter-03

Methodology

Materials and Methods:

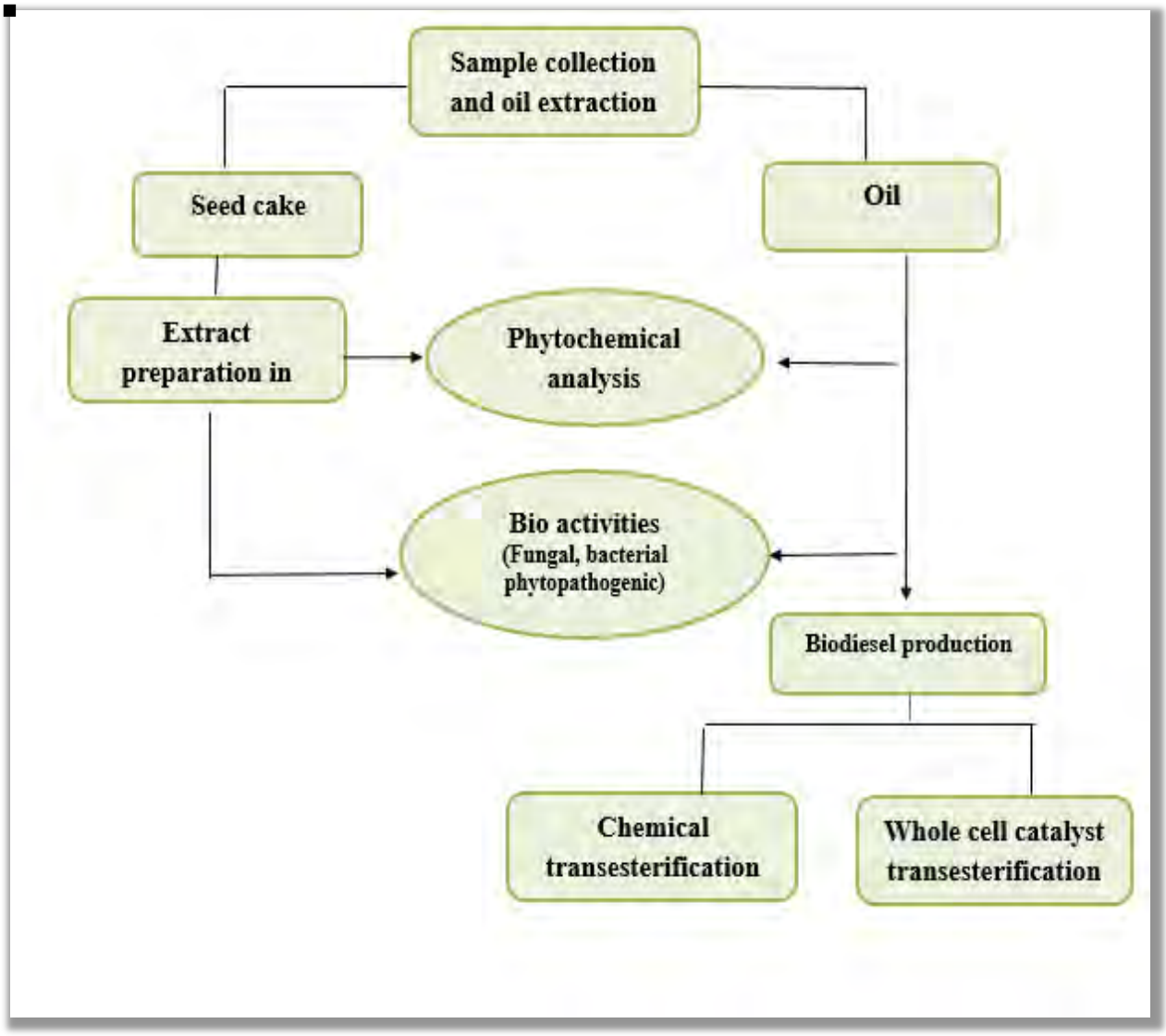


Fig 3.1: Flowsheet showing methodology for the current study

3.1 *Cannabis sativa* seed Oil Extraction:

Cannabis sativa seeds were purchased from the local market of **Orakzai agency.** Seeds were identified from the **Herbarium of Pakistan,** Quaid-e Azam university Islamabad. They were then subjected to an oil expeller in **Pakistan Counsil of Scientific and Industrial Research, PCSIR,** Peshawar. Total of 5 kg seeds were used and oil extracted from them was almost 632g (0.632Kg) and the remaining sed cake almost weighed about 3.8 kg. The de-oiled seed cake was kept at 4 °C in sterile zipper bags after oil was extracted, and the oil was kept in the dark for later use.

Figure 3.2: Seeds of *Cannabis sativa* L

3.2 Oil Yield:

Oil from seeds was extracted in laboratory in PCSIR and was filtered to get rid of solid contaminants. After being heated at 105 °C for a few hours to eliminate the moisture, the crude oil was then kept in the dark for later use. The following formula was used to determine the oil yield.

Oil yield (%) = (Oil extracted in Liters / Total weight of seeds in kg) × 100

3.3 Extract Preparation:

Extracts from the seed cake were prepared in three solvents: methanol, n-hexane and aqueous (distilled water). Following steps were taken to prepare de-oiled *Cannabis sativa* plant seed extracts:

The de-oiled seed cake underwent a pre-treatment in a grinder to turn it completely into powder. In 250 mL of each solvent (methanol, aqueous and n-hexane) 50 g powdered de-oiled seed cake of *C. sativa* was dissolved. Solvents were placed in incubator at 37°C for 12 days at 100 rpm in a shaking incubator. Whatman filter paper was used to filter the solution, and then air dry the filtrate at room temperature. Respective yields for the extracts from chloroform, ethyl acetate, methyl acetate, methanol, and n-hexane were 10.8, 10.6, 9.4, and 8.5%. Each extract was dissolved in DMSO. DMSO is added in order to liquify the extracts. Filtering was done on the *C. sativa* seed extracts using sterile syringe filters (0.2 m pore size). In order to check the sterility of extracts, each extract was spread and incubated onto Muller-Hinton Agar, MHA, and plates were incubated at 37 °C.

3.4 FTIR (Fourier transform infrared analysis):

FTIR spectroscopy (Bruker Tensor 27) with range of 400-4000 cm-1 was used to investigate de-oiled *C. sativa* seed cake extracts and seed oil.

3.5 Phytochemical screening

Using qualitative phytochemical analysis, the extracts of de-oiled seed cake and seed oil from *C. sativa* were screened for phytochemical content (Haq et al., 2021).

3.5.1 Qualitative phytochemical screening

By using the procedures outlined below, phytochemicals such as flavonoids, steroids, alkaloids, saponins, glycosides, resins/balsams, tannins, and phenols were qualitatively tested in *C. sativa* seed oil and de-oiled seed cake extracts.

3.5.1.1 Alkaloids:

Two milliliters (2ml) of 10% aqueous HCl acid were added to 2ml of extract and stirred. Using a few drops of Mayer's reagent, one milliliter (1 ml) of the filtrate was treated. Wagner's reagent was used to treat one milliliter of the filtrate with a few drops of the reagent because the appearance of a creamy precipitate indicated the presence of alkaloids in the extract. Alkaloids were also detected in the extract by a reddish-brown precipitate.

3.5.1.2 Flavonoids:

Using sodium hydroxide, a three milliliter (3ml) portion of the filtrate was turned alkaline (NaOH). The appearance of a yellow color suggested the potential existence of flavonoid molecules**.**

3.5.1.3 Tannins and phenols:

Drop by drop, 5% ferric chloride solution was added to 2-3 ml of the extract. The existence of tannins is indicated by a precipitate that is dark green in color.

3.5.1.4 Resins:

90 percent ethanol was combined with two milliliters (2ml) of extract. The combination was given two drops of an alcoholic ferric chloride solution. Dark green tint was seen.

3.5.1.5 Glycosides:

For the purpose of determining the presence of glycosides, 2.5 mL of seed cake extract or oil was combined in 5 mL of 50% H2SO4 and boiled for 15 minutes in boiling water. After cooling the mixture , 10% NaOH solution was used to neutralize it. 10 mL of A,B Fehling's solutions (1:1) was added and boiled for 5 minutes The presence of glycosides was detected by the dense brick red precipitate formation.

3.5.1.6 Steroids:

The extract and seed oil were dissolved in 5ml of chloroform using 5 grammes (5g) of the material. To create the lower layer, two milliliters of concentrated sulfuric acid were carefully applied. The presence of a steroidal ring is indicated by a reddish-brown tint at the interface.

3.5.1.7 Saponins:

2.5 ml of Fehling solution A and B was added in 2.5 ml of extract. Bluish green ppts indicates the presence of saponins.

3.6 Bioactivities of seed oil of *Cannabis sativa* and the de-oiled seed cake Extracts

3.6.1 Antifungal Activities:

Five clinical fungal strains were used to determine the anti-fungal properties of the de-oiled seed cake of *C. sativa*. These opportunistic pathogenic fungal strains were: *Candida albicans, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Curvularia lunata*. Activities were checked at diluted extracts in DMSO (500mL), pure concentrated extracts and then synergistic effect was checked by combining concentrated extracts with Nilstat antifungal.

Following procedure was used for the bioactivity:

All five strains were grown on Sabouraud Dextrose Agar, SDA, growth medium and were incubated at 30 °C for 72 hrs. The strains were routinely refreshed after each 10-15 days and stored at 4°C. Well diffusion method was used to determine the bioactivity(Magaldi et al., 2004). The standardized inoculum of each strain was swabbed onto the corresponding plates containing Sabouraud dextrose agar (SBA) growth medium using sterile cotton swabs. The hardened growth medium in the plates was drilled into with an 8 mm diameter sterile copper borer. (Autoclaved 1ml tips can be used to make wells on plates). Label each well in correspondence with the extracts. 100 μL of *C. sativa* seed oil

and de-oiled seed extracts were placed in their respective wells. For positive control Nilstat, a common antifungal drug was used, whereas for negative control DMSO was used. To allow for treatment diffusion, the inoculation petri plates were maintained at room temperature for one hour prior to fungal development. The plates were incubated at 30°C for 48 hours after that the **zones of inhibition (ZOI)** was measured surrounding the wells. Same procedure was repeated for concentrated extracts. For synergistic effect Nilstat was combined with the concentrated extracts (50µL+ 50µL extract and Nilstat) and positive negative control were not checked. % increase in ZOI were calculated. First calculate the mean of individual values of ZOI of concentrated extract and Nilstat. Calculate % by using following formula: (Madaras-Kelly et al., 1996)

% = (New value – old value/ old value) 100

New value = ZOI of combined extract and Nilstat

Old value = Mean ZOI of individua extract and Nilstat

3.6.1.1 MFC (Minimum Fungicidal Concentration):

For determination of minimum fungicidal concentrations (MFCs) microtiter plates containing 96 wells (Hernandes et al., 2013) were used. Fill each well with 100 µL of different dilutions of *C. sativa* seed oil or de-oiled seed cake extracts. Each fungal strain's spore suspension (in Sabouraud dextrose broth) was dispensed in separate wells containing 100 µL of different dilutions and incubated at 30 °C for 48 hours. As a positive control, Nilstat was utilized and for negative control, DMSO was utilized. For each strain, the percent growth was calculated by following formula:

$$\mathbf{MFC\ (\%) = 100\ [C_{ab} - T_{ab}/\ C_{ab}]}$$

ab = Absorbance

C = Control

T= Test sample

The minimum inhibitory concentration (MIC) is the lowest concentration at which the turbidity of the extract or seed oil was reduced by 80% as compared to the negative control. For the determination of MFCs, 20 µL of the extracts or seed oil from the wells with 80% reduction were spread on MHA plates and incubated at 30 °C for 48 hours.

3.6.2 Antibacterial Activities

3.6.2.1 Bacterial Culture Maintenance:

The bacterial strains that were **Gram-negative, Multi Drug Resistant, MDR,** human pathogens were selected. Gram negative (***Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella)*** strains were used**.** Every strain was cultured, maintained, and sub cultured on fresh media at regular intervals on nutrient agar media and kept at 4°C.

- **<u>It must be noted that all this work must be done in Biosafety cabinets, BSC</u>**,

3.6.2.2 Bacterial Suspension Preparation:

Bacterial suspensions of strains were made and stored by following method:

250 ml nutrient broth was made and autoclaved. The media was poured equally in 3 flasks of 30 ml. The media was inoculated with selected bacterial strains and was placed in a shaking incubator at 37^{o} C for 24 hours. After the incubation the cultured media was poured in falcon tubes and weighed. After equalizing the weight of each falcon tube, they were centrifuged at 4000 rpm for 5 minutes at 4^{o} C. The centrifugation causes the formation of supernatant and pallets at the bottom of the tubes. The supernatant was discarded and 20 ml N saline solution was added in each tube. Again, weighed and centrifuge for 5 minutes at 4 ^{o} C and 4000 rpm. Supernatant was again discarded and 20 ml N saline solution was added in each tube. The suspension was stored safely at 4^{o} C.

3.6.2.3 Antibacterial Assay:

Using the agar well diffusion method, the antibacterial potential of *cannabis sativa* seed oil and de-oiled seed extracts was assessed. Using sterile cotton swabs, the standardized inocula were swabbed onto the respective plates that contained Mueller Hinton agar (MHA) growth media. The plates' solidified growing media was bored into with an 8 mm diameter sterile copper borer. (you can use the blue tips to make wells) *C. sativa* seed oil

and de-oiled seed extracts each 100 µL were poured into each well after it had been correctly labelled. As a positive control, tetracycline was used. DMSO served as the negative control. The inoculation petri plates were kept at room temperature for an hour prior to the bacterial growth to allow for treatment diffusion. The plates were incubated for 24 hours at 37°C before the zones of inhibition (ZOI) around the wells were measured. For synergistic effect 50µL of extracts and antibiotic was added in the wells.

3.6.2.4 MIC (Minimum Inhibitory Concentration):

For determination of minimum inhibitory concentrations (MICs) microtiter plates containing 96 wells were used. Dilutions of extracts were made, up to 10^{-4} dilutions of each extract were made. 100 µL of each strain was run as a control. Fill each well with 100 µL of different dilutions of *C. sativa* seed oil or de-oiled seed cake extracts. Each MDR bacterial strain's suspension (in Sabouraud dextrose broth) was dispensed in separate wells containing 100 µL of different and incubated at 37 °C for 24 hours. Absorbance was taken at 600 nm. As a positive control, Tetracycline was utilized and for negative control, DMSO was utilized. For each strain, the percent growth was calculated by following formula:

- **MIC (%) = 100 [C_{ab} - T_{ab}/ C_{ab}]**

- ab = Absorbance
- C = Control
- T= test sample

The minimum inhibitory concentration (MIC) is the lowest concentration at which the turbidity of the extract or seed oil was reduced by 80% as compared to the negative control. For the determination of MICs, 20 µL of the extracts or seed oil from the wells with 80% reduction were spread on MHA plates and incubated at 37 °C for 24 hours.

3.6.3 Anti Phytopathogenic Activities:

three clinical fungal strains were used to determine the anti-fungal properties of the de-oiled seed cake of *C. sativa*. These opportunistic pathogenic fungal strains were:, *Aspergillus flavus, Fusarium* and *Pencillium*. Activities were checked at pure

concentrated extracts and then synergistic effect was checked by combining concentrated extracts with Nilstat antifungal.

Following procedure was used for the bioactivity:

All three strains were grown on Sabouraud Dextrose Agar, SDA, growth medium and were incubated at 30 °C for 72 hrs. The strains were routinely refreshed after each 10-15 days and stored at 4°C. Well diffusion method was used to determine the bioactivity(Magaldi et al., 2004). The standardized inoculum of each strain was swabbed onto the corresponding plates containing Sabouraud dextrose agar (SBA) growth medium using sterile cotton swabs. The hardened growth medium in the plates was drilled into with an 8 mm diameter sterile copper borer. (Autoclaved 1ml tips can be used to make wells on plates). Label each well in correspondence with the extracts. 100 µL of *C. sativa* seed oil and de-oiled seed extracts were placed in their respective wells. For positive control Nilstat, a common antifungal drug was used, whereas for negative control DMSO was used. To allow for treatment diffusion, the inoculation petri plates were maintained at room temperature for one hour prior to fungal development. The plates were incubated at 30°C for 48 hours after that the **zones of inhibition (ZOI)** was measured surrounding the wells. For synergistic effect Nilstat was combined with the concentrated extracts (50µL+ 50µL extract and Nilstat) and positive negative control were not checked. % increase in ZOI were calculated. First calculate the mean of individual values of ZOI of concentrated extract and Nilstat. Calculate % by using following formula: (Madaras-Kelly et al., 1996)

% = (New value – old value/ old value) 100

New value = ZOI of combined extract and Nilstat

Old value = Mean ZOI of individua extract and Nilstat

3.6.3.1 MIC (Minimum Inhibitory Concentration):

For determination of minimum concentrations microtiter plates containing 96 wells (Hernandes et al., 2013) were used. Fill each well with 100 µL of different dilutions of *C. sativa* seed oil or de-oiled seed cake extracts. Each fungal strain's spore suspension (in

Sabouraud dextrose broth) was dispensed in separate wells containing 100 µL of different dilutions and incubated at 30 °C for 48 hours. As a positive control, Nilstat was utilized and for negative control, DMSO was utilized. For each strain, the percent growth was calculated by following formula:

$$\mathbf{MFC\ (\%) = 100\ [C_{ab} - T_{ab}/\ C_{ab}]}$$

ab = Absorbance

C = Control

T= Test sample

The minimum inhibitory concentration (MIC) is the lowest concentration at which the turbidity of the extract or seed oil was reduced by 80% as compared to the negative control. For the determination of MFCs, 20 µL of the extracts or seed oil from the wells with 80% reduction were spread on MHA plates and incubated at 30 °C for 48 hours.

3.7 2, 2-diphenyl 1-picrylhydrazyl (DPPH) free radical scavenging assay:

The ability of *Cannabis sativa* seed oil and de-oiled seed cake extracts to scavenge free radicals against DPPH (Sharma & Bhat, 2009) was tested. 100 µL of each diluted extract or seed oil was combined with 100 µL of DPPH methanolic solution. 4 different Dilutions of extracts were made (300, 30,3,0.3,0.03 mg/ml) For 30 minutes, the mixture was incubated at room temperature in the dark.(e.g. in a cupboard ,etc.) After incubation, the samples' absorbance was measured at 517nm using a microplate reader. The shift in color to yellow also verified the antioxidant action. As a negative control, DMSO was utilized and ascorbic acid as positive control. The following equation was used to compute the percentage of radical scavenging.

$$\mathbf{DPPH\ Radical\ scavenging\ (\%) = 100\ [C_{ab} - T_{ab}/\ C_{ab}]}$$

ab = Absorbance C = Control T= test sample

- Subtract the obtained percentage from the DMSO anti-oxidant percentage.

3.8 Cytotoxicity with brine shrimp

The brine shrimp (Artemia salina) were used to test the cytotoxic effects(Chavez et al., 1997) of hemp seed oil and de-oiled seed extracts. Artificial sea water solution was made by dissolving 38 g of fake sea salt in 1 L of distilled water, filtering through Whatman paper, and sterilizing in an autoclave. In sterilized artificial sea water near a light source at 37 °C for 24 hours, approximately 1 g of Artemia salina cysts (eggs) were hatched. To a 2 mL artificial salt solution containing 10 active nauplii, 100 L of seed oil or de-oiled seed cake extract were added separately. The volume was then increased to 5 mL by adding additional artificial sea salt water in test vials. The range of the extracts' or seed oil's final concentrations in vials was 0.03-2 mg/mL. These vials underwent a 24-hour incubation period at 37 °C. Vincristine and DMSO were employed as positive and negative controls, respectively. Dead shrimps were counted after a 24-hour incubation period, and the LD50 was determined. The concentration at which 50% of nauplii are killed is known as the LD50. Three duplicates of each reaction were run through each step. The following formula was used to compute the % mortality of nauplii.

Death rate (%) = ((CN - ST) CN) x 100

The (ST) stands for the test sample, while (CN) is the negative control.

3.9 Biodiesel Production from *Cannabis sativa* /Industrial grade hemp seed oil

3.9.1 Physicochemical properties of oil and biodiesel

3.9.1.1 Acid Value and percent free fatty acid (%FFA)

The amount of potassium hydroxide needed, in milligrams (mg), to neutralize the free acids present in 1 g of the substance (oil), is expressed as the acid value (AV). It frequently tracks the conversion of free fatty acids from triacylglycerol, which is

detrimental to the quality of many lipids and oils. Following procedure was carried out to determine the AV and FFA (Haq et al., 2021) of raw oil:

5.0 g of *Cannabis sativa* oil is poured in dried 50 ml flask. Then 25ml of absolute ethanol and 2-3 drops of phenolphthalein were added. The mixture was heated at 65 ^{o} C in control temperature water bath with gentle shaking. The mixture was then cool down and titrated against 0.1 N KOH solution until permanent pink color appears that is its end point. Amount of KOH solution was calculated.

The acid value (AV) and free fatty acid (%FFA) was calculated as:

AV= (ml of KOH) x N x 56 / Weight of sample= mg of KOH

% Free Fatty Acid x FFA = AV x 0.503

N = Normality of KOH

3.9.1.2 Saponification Value

The milligrams of potassium hydroxide needed to neutralize the free acids and saponify the esters in 1 g of oil are known as the "saponification value." It is a measurement of the triacylglycerols' average molecular weight in a sample. Following procedure was carried out to determine saponification value:

Weighted 2 g of hemp seed oil was placed to a 100 ml flask along with 25 ml of a 0.5 N alcoholic potassium hydroxide, KOH, solution. To make alcoholic KOH, 15g of potassium hydroxide were dissolved in 10 ml of water, and the final amount was increased to 500 ml using 95% ethanol. For 24 hours, the solution was stored, followed by filtering through paper. After that, the flask was connected to the reflux condenser and left in a water bath at control temperature for an hour while being sometimes shaken. Three drops of phenolphthalein indicator were added to the solution after the first hour while it was still hot, and once the indicator had been added, the solution was titrated against 0.5 N hydrochloric acid until a permanent color disappeared, which is the end point. The Blank underwent the same process, but without the inclusion of the oil sample and X ml of the 0.5 N HCL at the conclusion, denoted by B.

The saponification value was calculated as:

SP NO. = 56.1 (B-S) x N of HCL / Gram of sample

B: ml of HCL required by Blank

S: ml of HCL required by Sample.

3.9.1.3 Ester value and % glycerin

The ester value is the quantity, measured in milligrams of potassium hydroxide, needed to react one gramme of oil or fat with glycerin (glycerol/glycerin). Using a formula, it was determined from the acid value (AV) and the saponification value (SV).

Ester Value (EV) = Saponification value (SV) – Acid Value (AV)

% glycerin = Ester Value x 0.054664

3.9.2 Chemical Transesterification of *Cannabis sativa* seed oil

3.9.2.1 Alkaline transesterification

As the FFA content of *Cannabis sativa* oil, CSO, was less than 3 %, alkaline catalyzed transesterification will be carried out. The first reaction was carried out on standard conditions as described by Berchmans and Hirata (2008) and Veljković et al., (2006) (Berchmans & Hirata, 2008).

Apparatus

For biodiesel production, Soxhlet extractor is used for condensation and extraction of alcohol being used. A round bottom flask is attached to it in which your reaction mixture containing oil, methanol and catalyst. This whole equipment is placed in a water bath with adjusted rpm and temperature.

If Soxhlet apparatus is unavailable we can design a water bath system on a hot plate. A water bath is made by using a beaker containing enough water to partially submerge the flask containing the reaction mixture.

Procedure:

The oil was first preheated to become completely homogenized. (Oil to methanol ratio used is 1:6). 2% potassium hydroxide (based on the weight of the oil) was dissolved in methanol (molar ratio: 6 based on the weight of the oil), and the reaction mixture was thermostated to the desire temperature and then added to the flask containing preheated oil. The reaction temperature was set to 60 ° C, the stirring speed to 600 rpm, and the reaction time to 2 hours. The reaction mixture was placed into a separating funnel after a two-hour reaction was completed, and it was given 24 hours to separate into its several layers. *for ratio keep in mind it's molar ratio not volumetric. Calculate Mass of oil by using mola rmass formula and then density formula for volume of each liquid.

3.9.2.2 Purification of Biodiesel

Alcohol separation:

Excess methanol must be removed from the biodiesel sample in order to purify the produced biodiesel. We can remove methanol/ ethanol either by using the distillation flask or by simply evaporating the alcohol.

- For simple evaporation place your sample on hot plate and adjust temperature at 65^0 C which is the boiling point of methanol (78^0C for ethanol). But keep in mind the boiling point of your oil as well. *C.S* seed oil boiling point is 365^0F.
- We can remove alcohol by distillation, biodiesel was added to the distillation apparatus's round bottom flask, which had its temperature set to 65 ° C, the boiling point of methanol. After the distillation tube's opposite side's flask's methanol collecting stopped, the distillation equipment was halted. A quantity of pure biodiesel was recorded; it was kept in glass vials with screw caps and in the dark.

Alkali removal:

By neutralizing and washing with acidified distilled water, KOH was removed. For this:

100 ml of hot distilled water was mixed with 1 ml of concentrated H_2SO_4. The trans-esterified mixture was then added to the acidified distilled water, which was then centrifuged for 10 minutes at 3000 rpm to separate the K_2SO_4 that had produced. This process was repeated twice to ensure that no catalyst was still present. To separate water from the finished product, the catalyst-free product was once more centrifuged at 3000 rpm for ten minutes. To remove any remaining moisture, anhydrous Na_2SO_4 was applied to the water-free product.

3.9.2.3 Biodiesel Yield

There was a noticeable amount of fatty acids methyl esters (FAMEs) in the transesterified mixture. The following equation was used to determine FAMES yield:

FAME yield (%) = (weight of FAME in grams / total weight of oil in grams) × 100

3.9.3 Optimization of reaction Parameters for Optimized Biodiesel Production

Using the approach of Patil and Deng (2009) with minor adjustments, parameters controlling the alkaline transesterification reaction were optimized. (Patil & Deng, 2009) To evaluate the ideal oil to methanol molar ratio, catalyst quantity, and time at 600 rpm and 60 °C, a base catalyzed transesterification reaction setup was used. In the batch setting after preheating the oil to the correct temperature, the catalyst and methanol were added to the oil and agitated for two hours at 600 rpm. The reactions were carried out at various catalyst concentrations, oil to methanol molar ratios as well as reaction times. Following the confirmation of the ideal parameters, the impact of various temperatures was also assessed at the ideal molar ratio, amount of KOH, and reaction time. Following table shows different parameters and their values used for optimization of biodiesel yield.

Table3.1: Parameters for optimization of alkali based transesterification process

Parameters			
Reaction time (min)	**60**	**90**	**120**
Oil: Methanol ratio	**1:6**	**1:9**	**1:12**
Catalyst Concentration (%)	**1**	**1.5**	**2**
Agitation (rpm)	**300**	**600**	**900**
Temperature ^{0}C	**50**	**55**	**60**

3.9.4 Biodiesel production using whole cell catalyst:

Already identified Q5 strain *Bacillus subtilis* C17(KU681037) was used for biodiesel production. The lipase activity, methanol sensitivity was already determined for our strain.

3.9.4.1 Oil toxicity

C. sativa seeds have antibacterial properties so the toxicity of seed oil was assessed against the chosen bacterial strain in order to ascertain its antibacterial properties. Olive oil was used as reference oil. Media for oil toxicity was prepared. (peptone 2%, kh_2po_4 o..1%, NaCl 0.25 %, $MgSO_4$ 0.04%, Ca 0.04% oil 2%, and tween 20 1-2 drops). Strain was individually introduced to flasks with a 5% newly enriched inocula, and then incubated in a shaking incubator set at 150 rpm at 37 °C. The unvaccinated well served as a reference control. The OD at 650 nm for each strain was measured.

3.9.4.2 Whole cell catalyst preparation

Lipase producing bacterial strain was selected for biodiesel production using whole cell approach. Initially, the selected lipase producing strains were refreshed in Luria broth (LB) with composition: Tryptone, 1% w/v; NaCl, 0.5% w/v and yeast extract, 0.5% w/v 100 mL volume of Luria broth was poured in sterile flask. Media was inoculated with strains and incubated in shaking incubator at 37degree C at 150 rpm for 48 hrs. The media was centrifuged at 4000 rpm for 15 min and the cell pellet was used for biodiesel production.

3.9.4.3 Biodiesel synthesis

Oil was added in sterile flask (methanol: oil is 1:9). 1 mL of the whole cell pellet was added in the flask and the mixture was homogenized to form aa protective layer for cells. Stepwise addition of methanol was done in order to avoid enzyme inactivation. 300 L n-hexane was added as an emulsifier. Flasks were incubated for 48 hrs. at 37 CCC at 150 rpm. Mixture was shifted n a separating funnel to form complete layers. FAME was calculated by the same formula.

3.9.5 Optimization of Biodiesel Production using Whole Cell Catalyst using Plackett-Burman Design:

The Plackett-Burman design was utilized to optimize response parameters using Stat-Ease Design Expert Software version 7.0. The concentration of the catalyst, temperature, agitation, oil to methanol ratio, and reaction duration were the five parameters that were selected for optimization and entered into the design program. After then, 15 runs of the design were created.

3.9.6 Biodiesel Analysis or FAME Analysis

Fatty acid methyl ester formed were analyzed as:

3.9.6.1 Fourier Transform Infrared Spectrometer

The methyl esters produced were identified and examined by FTIR utilizing a Bruker Tensor27 FTIR spectrophotometer and Opus65 software equipped ZnSe ATR. *Cannabis sativa* oil and biodiesel samples totaling 5 microliters each were fed into sample injectors, where scans were carried out between 400 and 4000 cm^{-1}. The average was shown in the form of a spectrum with various peak ranges.

3.9.6.2 Gas Chromatography Mass Spectrophotometer (GC-MS):

The GC operates under the premise that a heated mixture will split into discrete substances. It recognizes traces of substances that are present in the samples. By comparing the retention times of known unknowns with the mass spectra of known substances, typically electron ionization (EI) mass spectra with known standards, the qualitative GC-MS study is carried out. The GCMS-QP2010 Ultra Gas Chromatograph Mass Spectrometer was used to analyze the methyl esters generated by alkali catalysis. A DB-5MS Agilent capillary column underwent separation (30m x 0.25mm, 0.25μm of film thickness). Helium was used as the carrier gas, and the flow rate was 1.5 mL/min. The column temperature was programmed to rise at a rate of 10 C/min from 50 to 300 degrees centigrade. Both the injector and the detector were set to a temperature of 250^0C. Utilizing split mode, a sample with a volume of 0.2 μL in CHCl3 was injected. With the electron impact (EI) method of ionization, the mass spectrometer was programmed to scan in the m/z 50–550 with the electron impact mode of ionization.

Chapter-04

RESULTS

4.1 Oil Yield:

Oil yield (%) = (Oil extracted in Liters / Total weight of seeds in kg) × 100

=0.632L/ (4.966Kg)100

Through mechanical extraction 13% oil was obtained.

4.2. Extracts yield:

After the extract preparation, its yield was calculated and listed in the Table 4.1.

Table 4.1. Extracts and their respective yields

Extracts	**Yield (%)**
Aqueous	4..26%
Methanol	5.59%
n-hexane	4.5%

Figure 4.1: Filtration process during preparation of extracts from de-oiled seed cake.

4.3. Phytochemical analysis of Hemp seed extracts and oil

To interpret the chemical composition of all extracts and oil of hemp seeds, a few conventional experiments were performed. Different qualitative tests were performed to elucidate the major phytochemical components of the oil and pressed cake extracts. This

conventional phytochemical revealed the presence of several phytochemicals such as tannins, flavonoids, steroids, glycosides, resins and saponins in oil and pressed seed cake. Number of phytochemicals pressed in seed cake and oil are listed in Table 4.2.

Table 4.2. Number of phytochemicals present in different extracts.

Extracts	**Alkaloids**		**Resins**	**Flavonoid**	**Glycosides**	**Steroids**	**Saponin**	**Tannins/ Phenol**
	Mayer's test	**Wagner's test**						
Aqueous	-	+	-	+	-	+	-	-
Methanol	-	+	+	-	+	+	+	+
n-hexane	+	+	-	+	-	+	-	-
Oil	-	-	-	+	-	-	+	+

+: Present in extract/Oil, -: Absent in extract/Oil

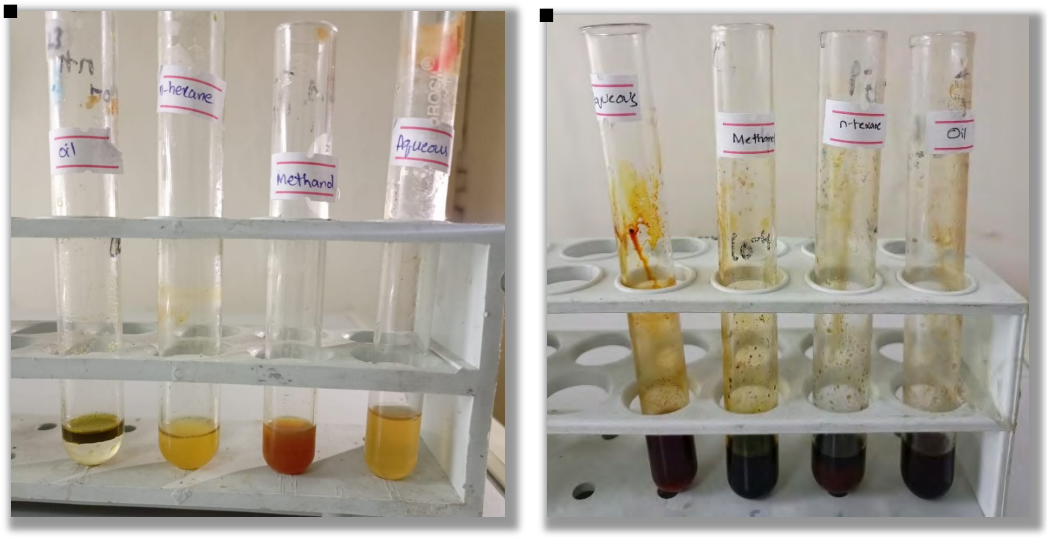


Fig 4.2 a) Mayer's test b) Wagner's test

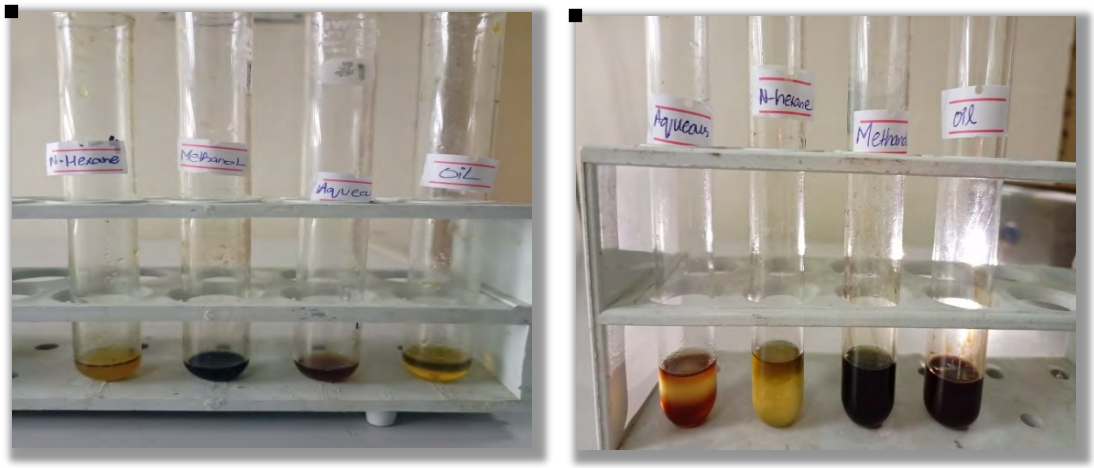


Fig 4.3 a) Steroids test b) Flavonoids test

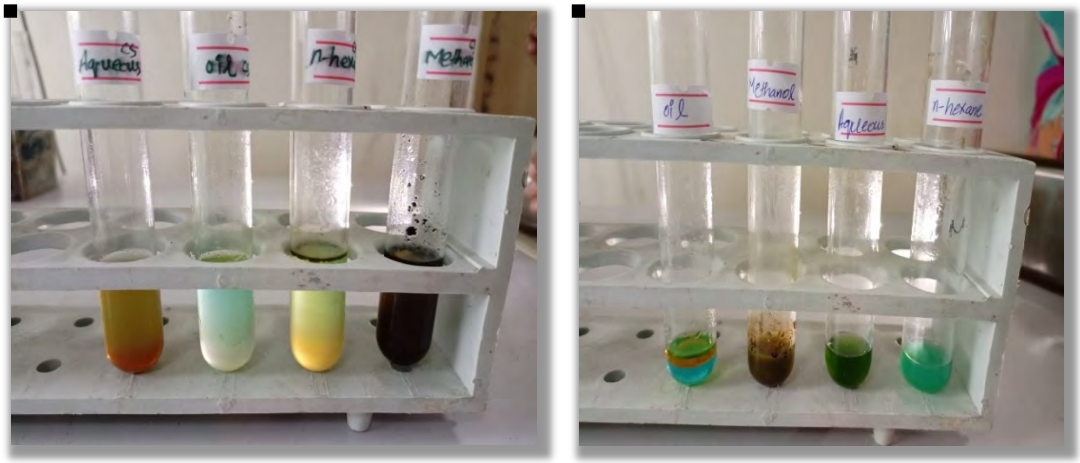


Fig 4.4 a) Glycosides test b) Saponins test

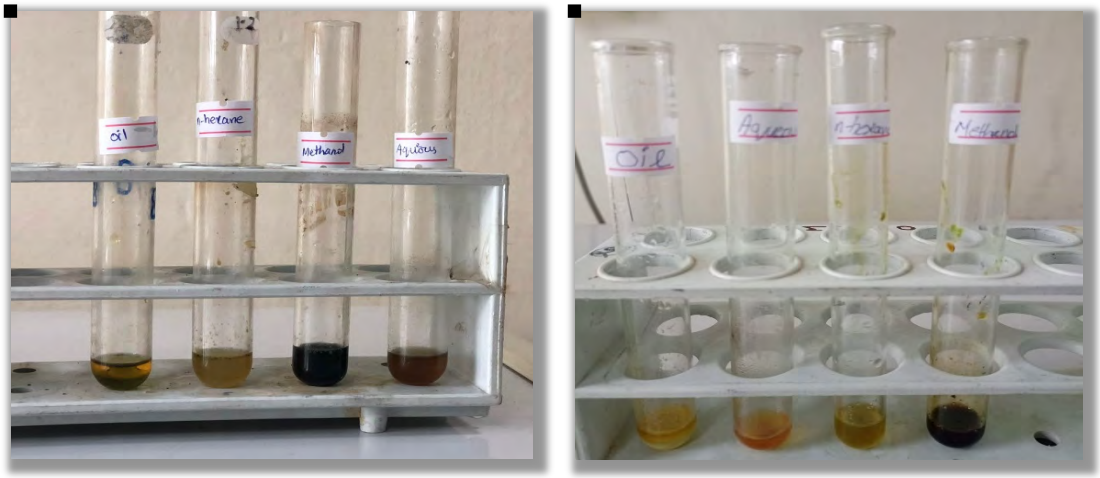


Fig 4.5 a) Resins test b) Tannins test

4.3.1 FTIR analysis of *C. sativa* de-oiled seed extracts and oil:

In addition to conventional qualitative phytochemical assays, characterization of the extracts and oil was also performed by its analysis through FTIR spectra. FTIR spectroscopy is an analytical technique which is generally used to indicate the presence of different functional groups in different type of materials (Organic, inorganic or polymeric). FTIR spectra of extracts and oil indicates that broad range of different compounds like amide linkages, ester and ether linkages, carbon-carbon, carbon-hydrogen bonds, aromatic functional groups and carbonyl linkages are present that correspond to the presence of cellulose, lignin and hemicellulose components. The corresponding functional groups present in extracts and oil are listed below in Table.

Methanol

Fig. 4.6. FTIR absorption spectrum obtained for Hemp seed methanol extract in the range of 4000-400 cm^{-1}.

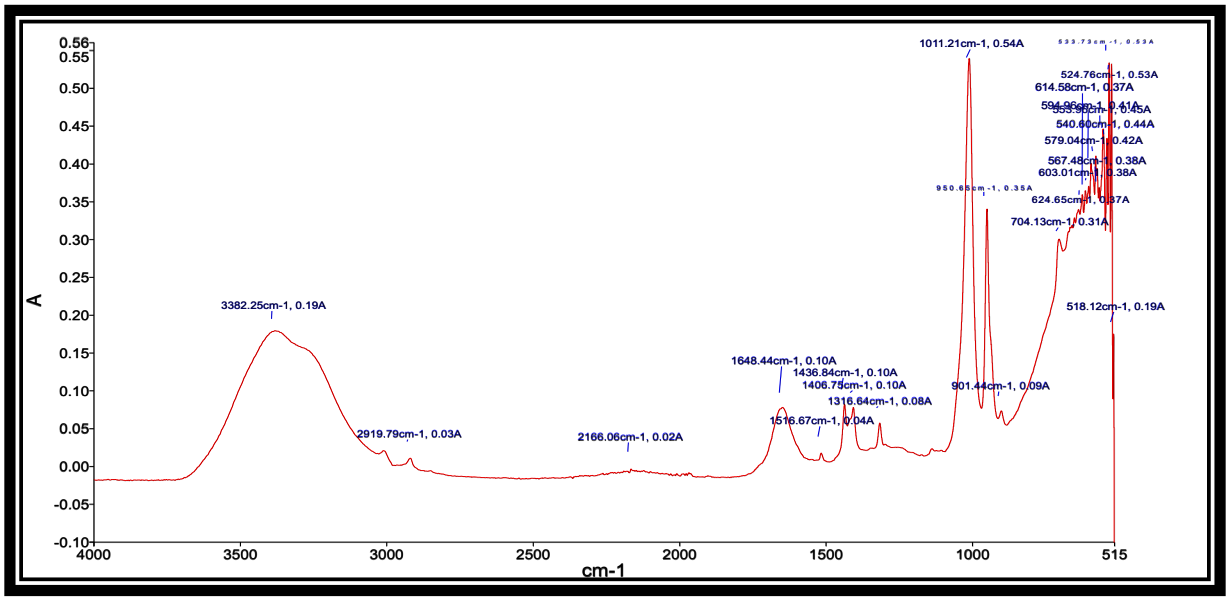


Table 4.3. FTIR stretches with corresponding functional groups present in methanolic extract of Hemp pressed seed cake

Band positions (cm^{-1})	**Inferences of FTIR spectrum**
3482	Carboxylic acid OH stretch, N-H stretch, Alcohol OH stretch
2919	-C-H stretch
2166	Alkyne stretches
1648	C=O, C=N, C=C stretches
1436	CH_3, CH_2
1406	CH_3, CH_2
1316	CH_3
1011	C-OH
950	C-OH
704, 614	CH out of plane bending (Carbohydrate)

N. Hexane

Fig. 4.7. FTIR absorption spectrum obtained for Hemp n-hexane extract in the range of 4000-400 cm^{-1}.

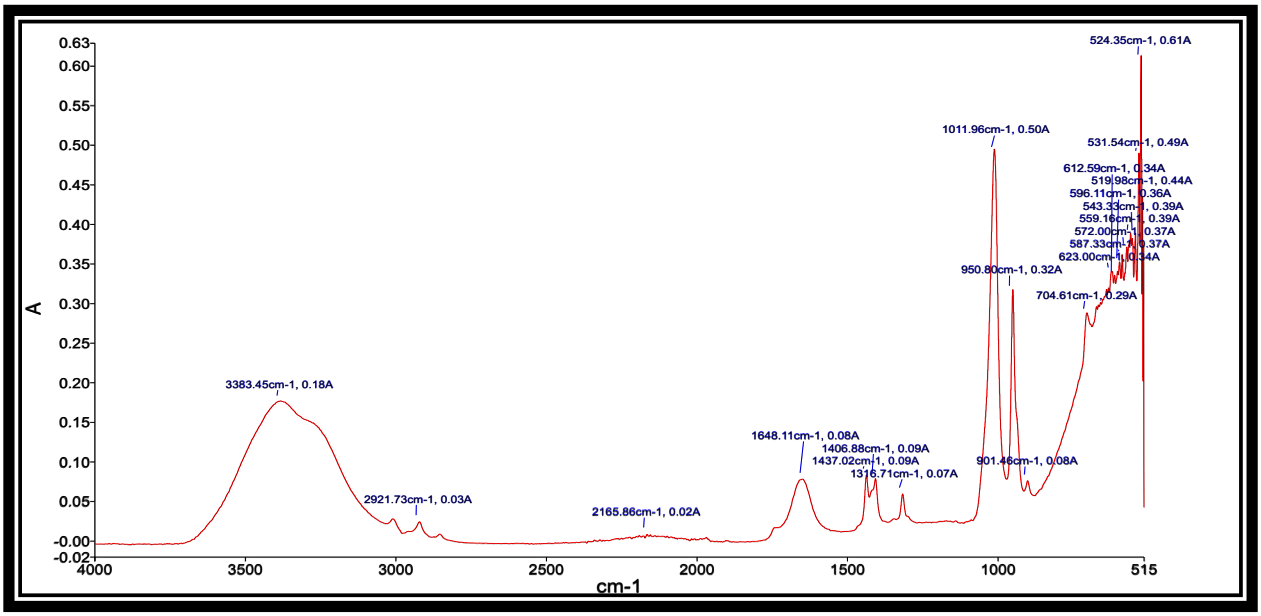


Table 4.4. FTIR stretches with corresponding functional groups present in n-hexane extract of Hemp pressed seed cake

Band positions (cm^{-1})	**Inferences of FTIR spectrum**
3383	Carboxylic acid OH stretch, N-H stretch, Alcohol OH stretch
2921	-C-H stretch
2165	Alkyne stretches
2009	C=C asymmetric stretches
1648	C=C (medium intensity alkene stretches)
1437	CH_3, CH_2
1408	CH3
1318	CH_3, NO_2 stretches
1011	C-OH
950	C-OH
704, 623	CH out of plane bending (Carbohydrate)

Aqueous

Fig. 4.8. FTIR absorption spectrum obtained for Hemp Aqueous extract in the range of 4000-400 cm^{-1}.

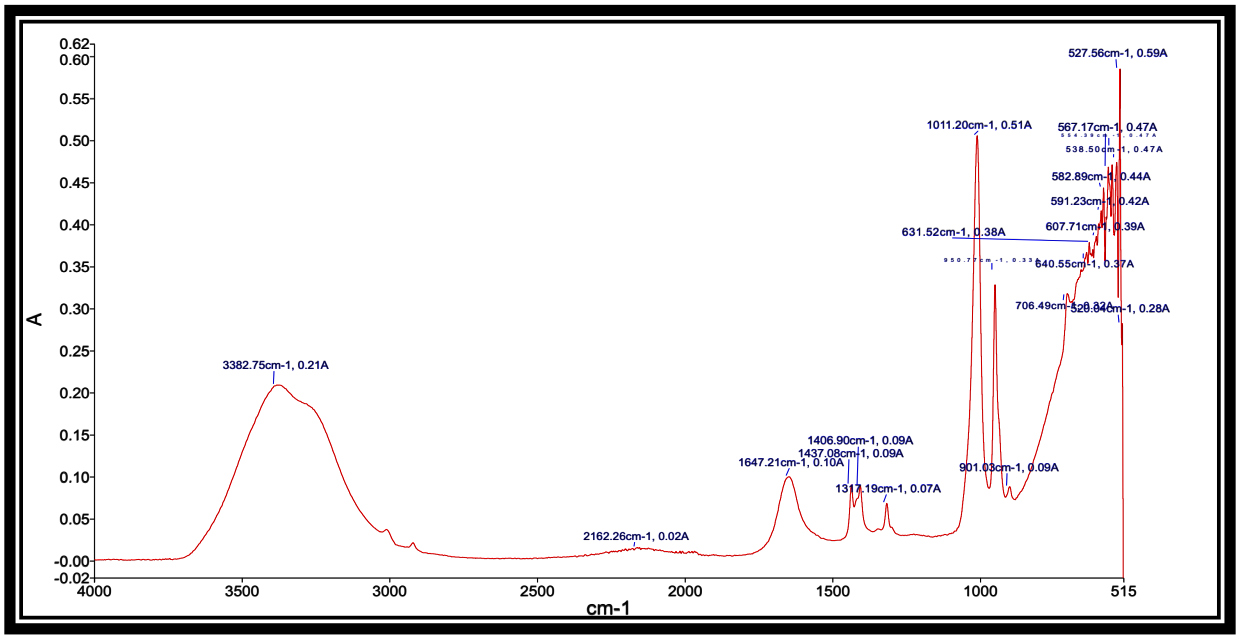


Table 4.5. FTIR stretches with corresponding functional groups present in Aqueous extract of *C. sativa* pressed seed cake

Band positions (cm^{-1})	**Inferences of FTIR spectrum**
3382	Carboxylic acid OH stretch, N-H stretch, Alcohol OH stretch
2162	Alkyne stretches
1647	C=O, C=N, C=C stretches
1437	CH_3, CH_2
1406	CH_3, CH_2
1319	CH_3
1011	C-OH
901	C-OH
706, 641, 607	CH out of plane bending (Carbohydrate)

***Cannabis sativa* seed oil**

Fig 4.9: *FTIR spectra of crude Hemp seed oil*

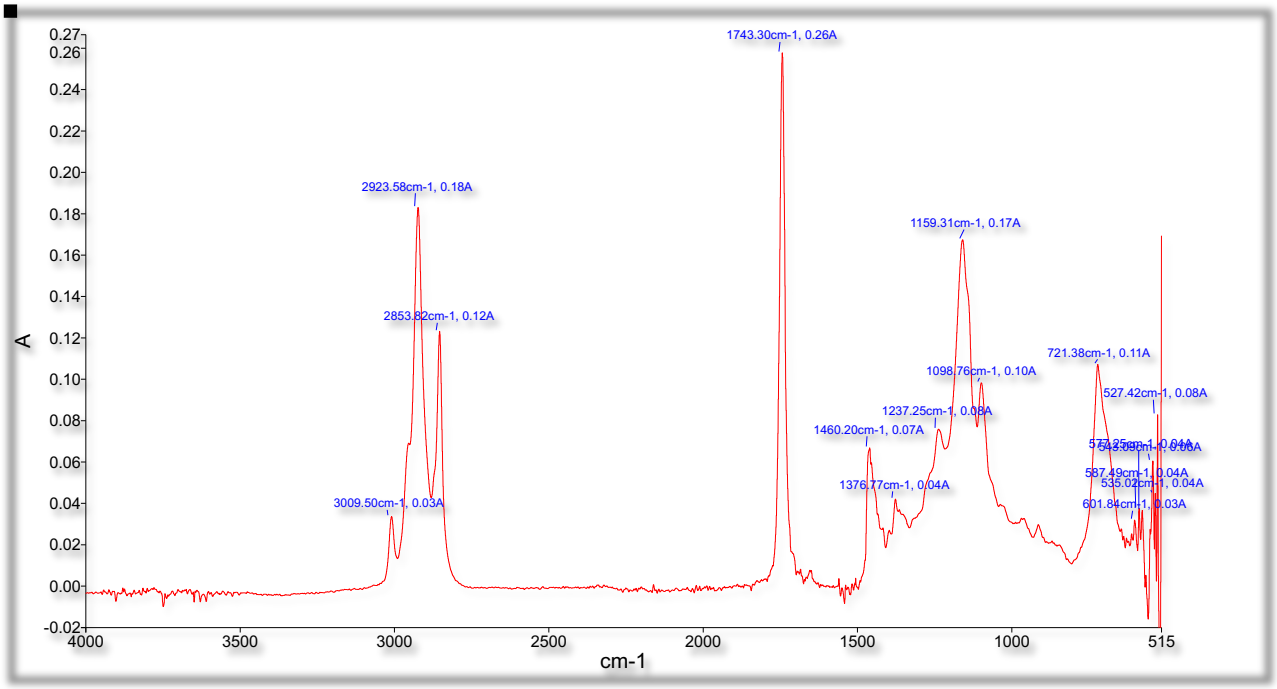


Table: 4.6 FTIR stretches with corresponding functional groups present in *Cannabis sativa* oil

Band positions (cm^{-1})	**Inferences of FTIR spectrum**
3009	Carboxylic acid OH stretch, N-H stretch, Alcohol OH stretch
2923,2853	-C-H stretch
2162	Alkyne stretches
1743	C=O Ketone stretches
1460	CH_3, CH_2
1376	CH_3
1237	CH3
1098	C-OH
721	CH out of plane bending (Carbohydrate)

4.4 Bioactivities of seed oil of *Cannabis sativa* and the de-oiled seed cake Extracts

4.4.1 Antifungal Activity

In the current study, extracts of de-oiled pressed seed cake and oil was used to check their antifungal activity. For this purpose, agar well diffusion method was used. Fungal suspension was spread over the agar plate and 100 microliter extract was added in the wells and its activity was measured in millimeter. Crude extracts of pressed seed cake and Oil showed promising results on the inhibition of fungal growth. The antifungal potential of extracts of *C.sativa* pressed seed cake and seed oil were examined against five selected strains: *Candida albicans, Apergillus flavus, Apergillus niger, Fusarium oxysporum* and *Curvularia lunata.* Both the concentrated form of extracts and their dilutions made in DMSO were checked. Furthermore, the synergistic effect of the antibiotic Nilstat along with the concentrated extracts was also determined.

4.4.1.1 Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Fusarium oxysporum:*

Fusarium oxysporum is a huge species complex of plant and human diseases that target a wide range of species in a host-specific way. In individuals with weakened immune systems, F. oxysporum is an opportunistic pathogen. Concentrated n-hexane extract showed highest activity against ***Fusarium oxysporum*** with zone of inhibition 29mm. synergistic effect was positive with all extracts. N hexane extract showed high synergistic effect with an increase of 46% in the ZOI. Nilstat as a positive control showed zone of inhibition 31mm and DMSO showed no activity. Concentrated extracts showed higher activity than the diluted ones and also synergistic effect was enhanced.

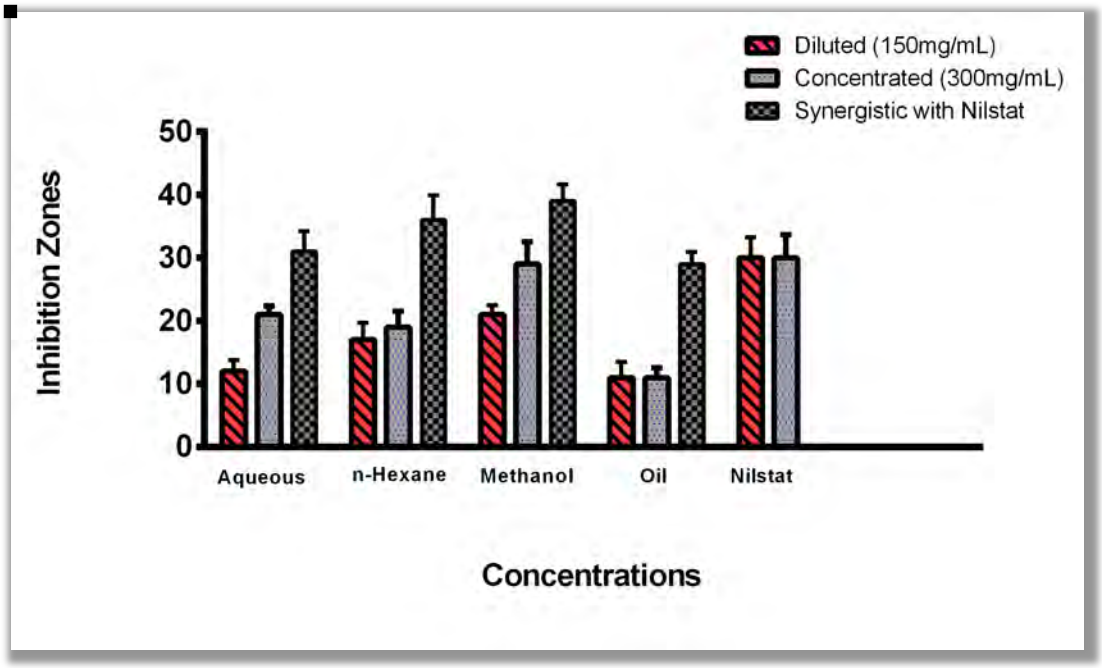
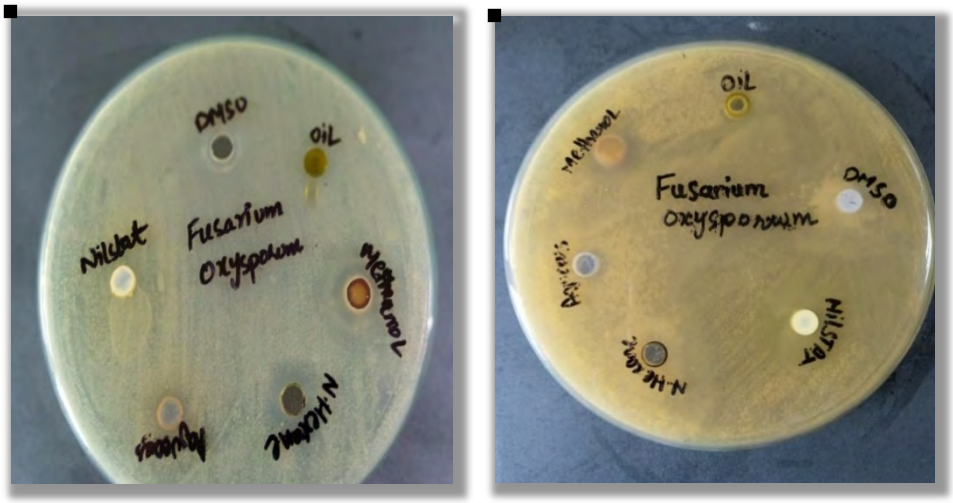


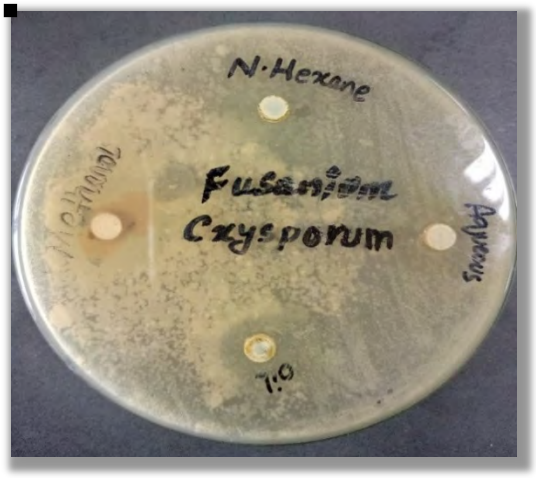
FIG 4.10: Graph showing the Zone of inhibition of different extracts against *Fusarium oxysporum*

Table 4.7: Zone of inhibition of different extracts against *Fusarium oxysporum* and %increase in synergistic assay

Fusarium oxysporum				
Extracts	Diluted (500mL)	Concentrated	Synergistic effect	
			Concentrated extracts with Nilstat	% increase in ZOI
Aqueous	12	21	31	21
n-Hexane	17	19	36	46
Methanol	21	29	39	32
Oil	11	11	29	41
Nilstat	30	30	-	-
DMSO	0	0	-	-



a) **b)**



c)

FIG 4.11: The Zone of inhibition of different extracts against *Fusarium oxysporum*.
a) Diluted concentration (150mg/mL) **b)** at concentrated 300mg/Ml **c)** synergistic assay

4.4.1.2 Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Candida albicans:*

Candida albicans is an opportunistic human fungal pathogen. *C. albicans*, despite being a typical part of our gut flora, has the capacity to colonize practically every human tissue and organ, causing dangerous, invasive infections. Methanol extract showed highest activity against *Candida albicans* with ZOI 17 and 20 mm in diluted and concentrated extracts respectively. Seed oil showed zero activity so during synergistic assay Nilstat showed more than 100% increase in ZOI indicating oil may have reacted with Nilstat and have more antifungal properties as compared to alone activity of oil. In synergistic effect n hexane showed higher % increase in ZOI. Nilstat as a positive control showed zone of inhibition 30mm and DMSO showed no activity.

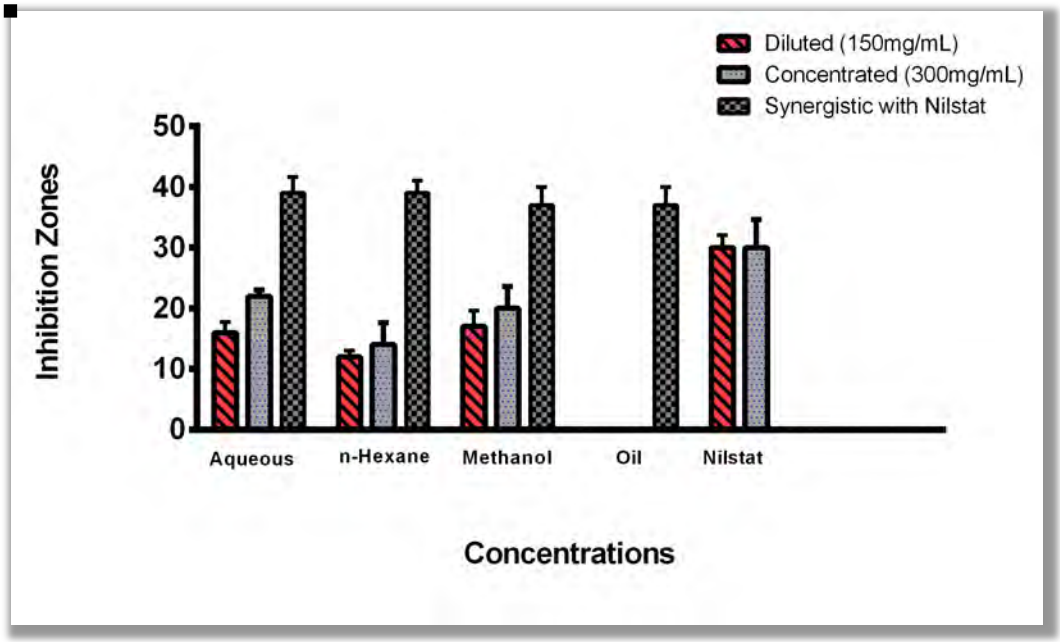
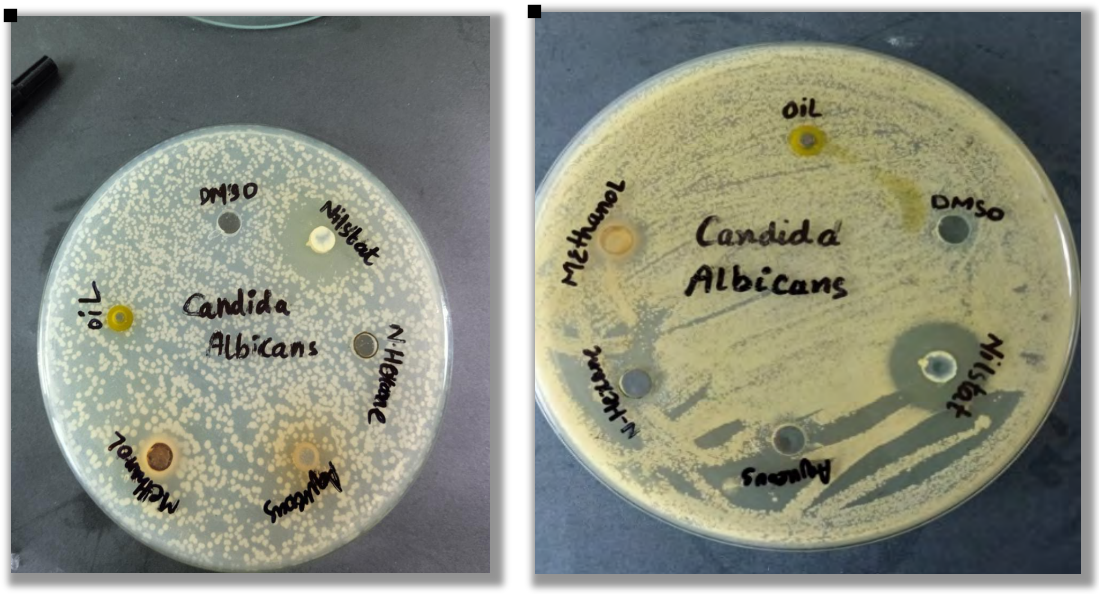


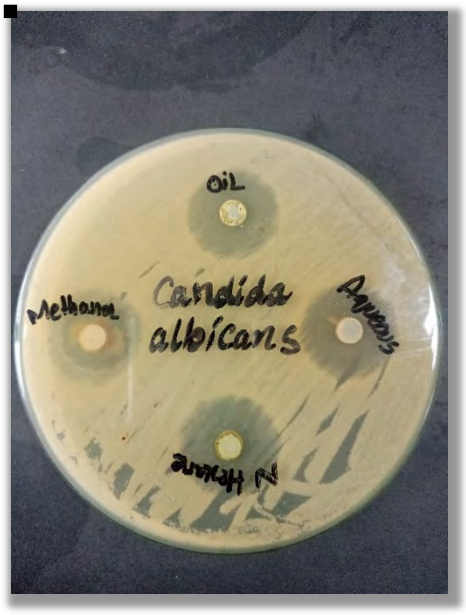
FIG4.12: Graph showing the Zone of inhibition of different extracts against *Candida albicans*

Table 4.8: Zone of inhibition of different extracts against *Candida albicans* and %increase in synergistic assay

Candida albicans				
Extracts	Diluted (500mL)	Concentrated	Synergistic effect	
			Concentrated extracts with Nilstat	% increase in ZOI
Aqueous	16	22	39	50
n-Hexane	12	14	39	77
Methanol	17	20	37	48
Oil	0	0	37	146
Nilstat	30	30	-	-
DMSO	0	0	-	



a) **b)**



c)

FIG 4.13: the Zone of inhibition of different extracts against *Candida albicans*

a) diluted concentration (150mg/mL) b) at concentrated 300mg/mL c) synergistic assay

4.4.1.3 Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Aspergillus flavus:*

Aspergillus flavus is an opportunistic pathogen that causes both invasive and non-invasive aspergillosis in humans, animals, and insects. In humans, it also produces allergic responses. Methanol and Aqueous/ extracts showed highest activity against ***Aspergillus flavus*** with zone of inhibition 30 and 33mm both in diluted and concentrated extracts . Hemp oil showed ZOI 11mm. in synergistic assay % increase in ZOI was highest in n hexane. Nilstat as a positive control showed zone of inhibition 33mm and DMSO showed no activity.

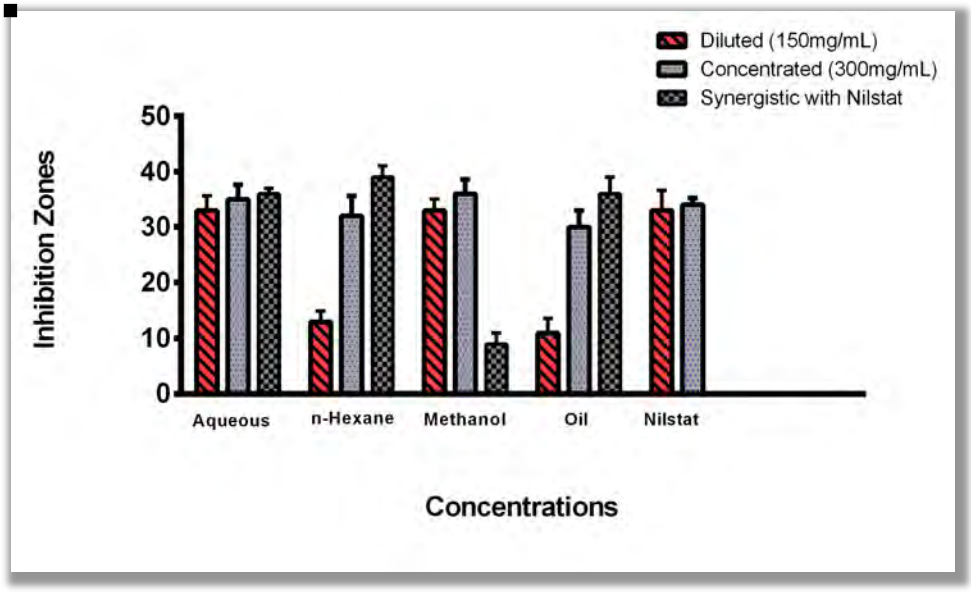
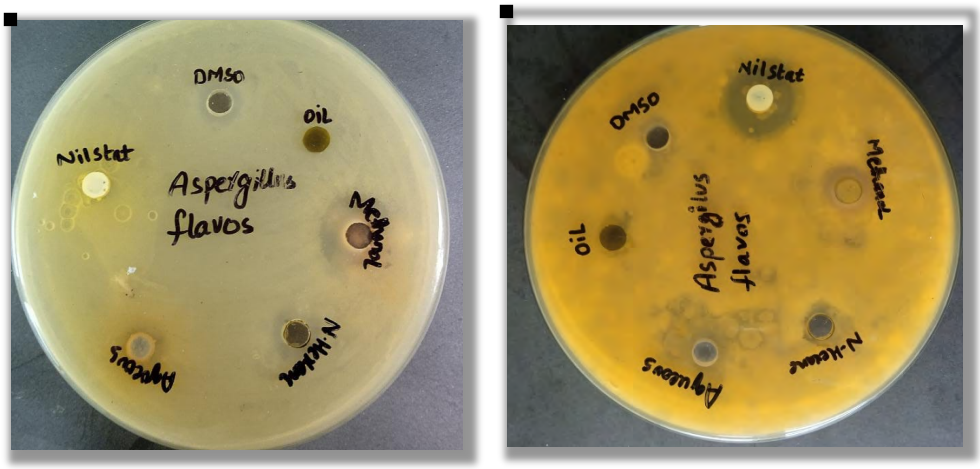


FIG 4.14: Graph showing the Zone of inhibition of different extracts against *Aspergillus fllavus*

Table 4.9: Zone of inhibition of different extracts against *Aspergillus fllavus* and %increase in synergistic assay

Aspergillus fllavus				
Extracts	Diluted (500mL)	Concentrated	Synergistic effect	
			Concentrated extracts with Nilstat	% increase in ZOI
Aqueous	30	33	35	6
n-Hexane	10	13	32	39
Methanol	30	33	36	9
Oil	11	11	30	36
Nilstat	33	33	--	
DMSO	0	0	-	



a) **b)**

c)

FIG4.15: The Zone of inhibition of different extracts against *Aspergillus fllavus*

a) diluted concentration (150mg/mL) b) at concentrated 300mg/mL c) synergistic assay

4.4.1.4 Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Aspergillus niger:*

Aspergillus niger is an opportunistic pathogen that may be found in a number of indoor and outdoor situations. *A. niger* spores are easily aerosolized and have the potential to be accumulated in bronchioles of the human respiratory system. Methanol extracts showed highest activities against *Aspergillus niger* with ZOI 30mm and 33mm in diluted and concentrated extracts respectively. Hemp oil showed no activity. in synergistic assay highest % increase in ZOI was in n-hexane. Aquoeuss extract had negative effect when combined with the antifungal drug and showed 11% derease in ZOI Nilstat as a positive control showed zone of inhibition 50mm and DMSO showed no activity.

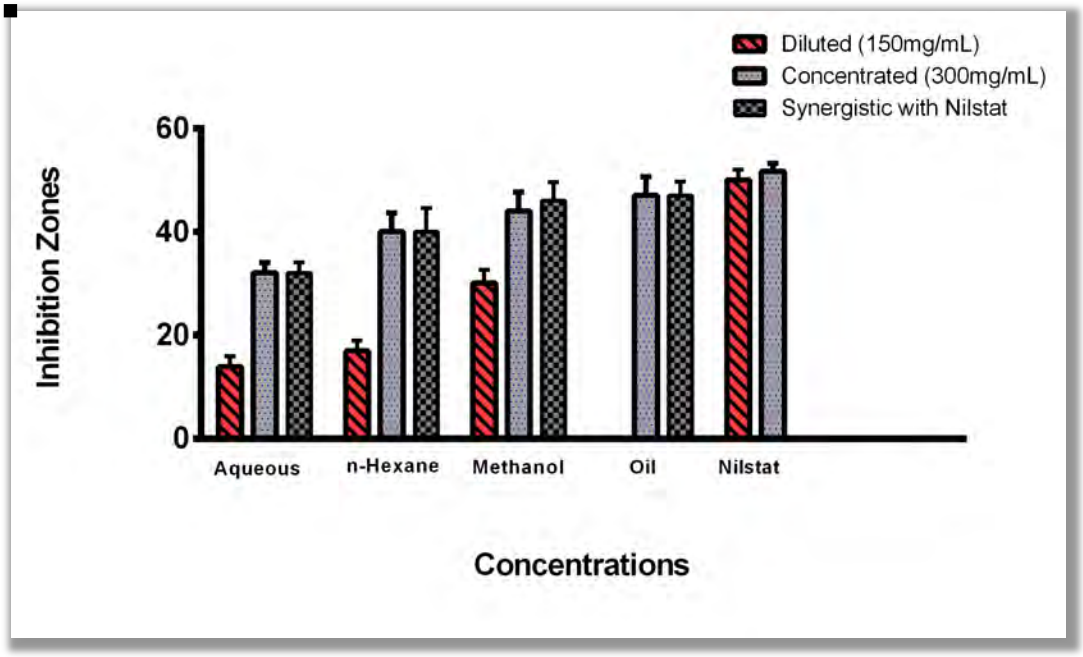
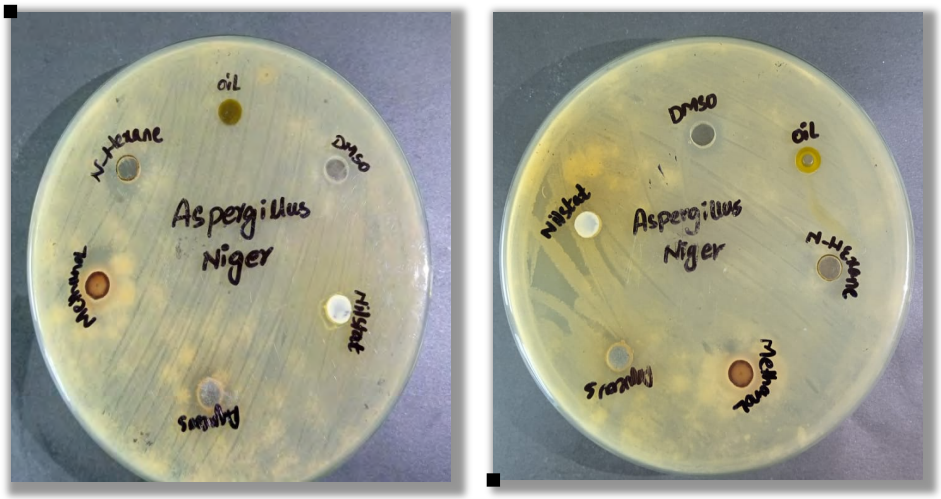


FIG4.16: Graph showing the Zone of inhibition of different extracts against *Aspergillus niger*

Table4.10: Zone of inhibition of different extracts against *Aspergillus niger* and %increase in synergistic assay

Aspergillus niger				
Extracts	Diluted (500mL)	Concentrated	Synergistic effect	
			Concentrated extracts with Nilstat	% increase in ZOI
Aqueous	14	22	32	Negative effect
n-Hexane	17	18	40	23
Methanol	30	33	46	10
Oil	0	0	47	88
Nilstat	50	50	-	
DMSO	0	0	-	



a) **b)**

c)

FIG 4.17: The Zone of inhibition of different extracts against *Aspergillus niger*

a) diluted concentration (150mg/mL) **b)** at concentrated 300mg/mL **c)** synergistic assay

4.4.1.5 Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Curvularia lunata:*

Curvularia lunata is a fungal plant pathogen that can infect people and animals. methanol extracts showed highest activity against *Curvularia lunata* with ZOI 12mm, 18mm, 36mm in diluted, concentrated and synergistic assay respectively. Seed oil showed zero activity so during synergistic assay Nilstat showed more than 100% increase in ZOI indicating oil may have reacted with Nilstat and have more antifungal properties as compared to alone activity of oil. In synergistic assay highest % increase in ZOI was in Aqueous extract. Nilstat as a positive control showed zone of inhibition 32mm and DMSO showed no activity.

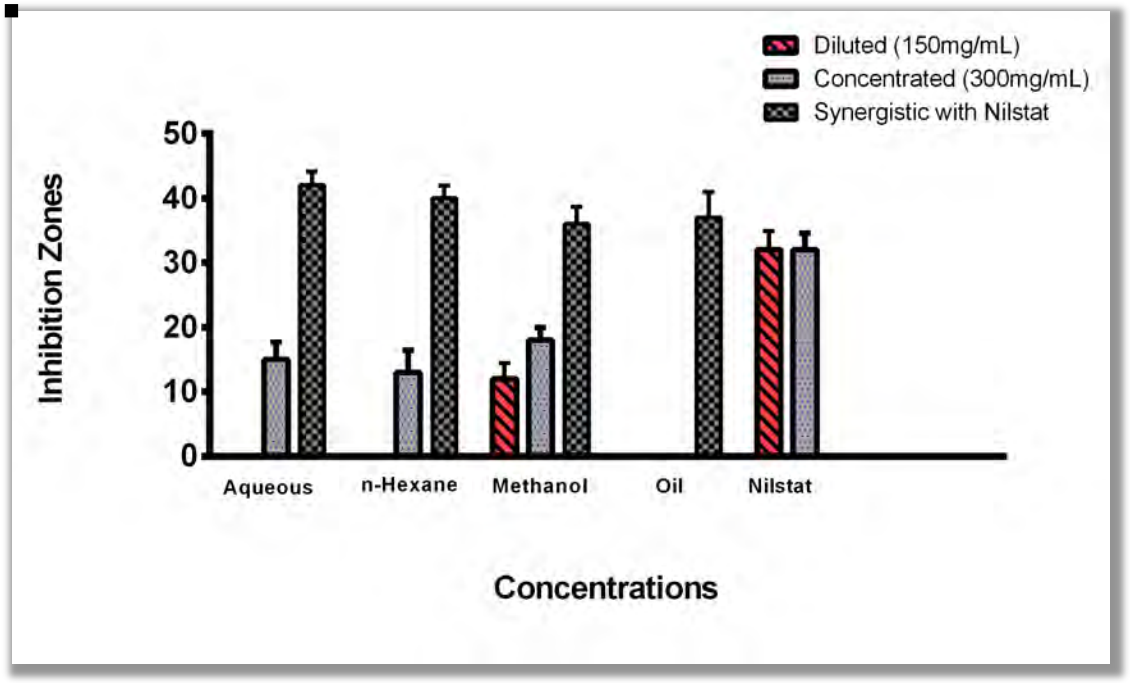
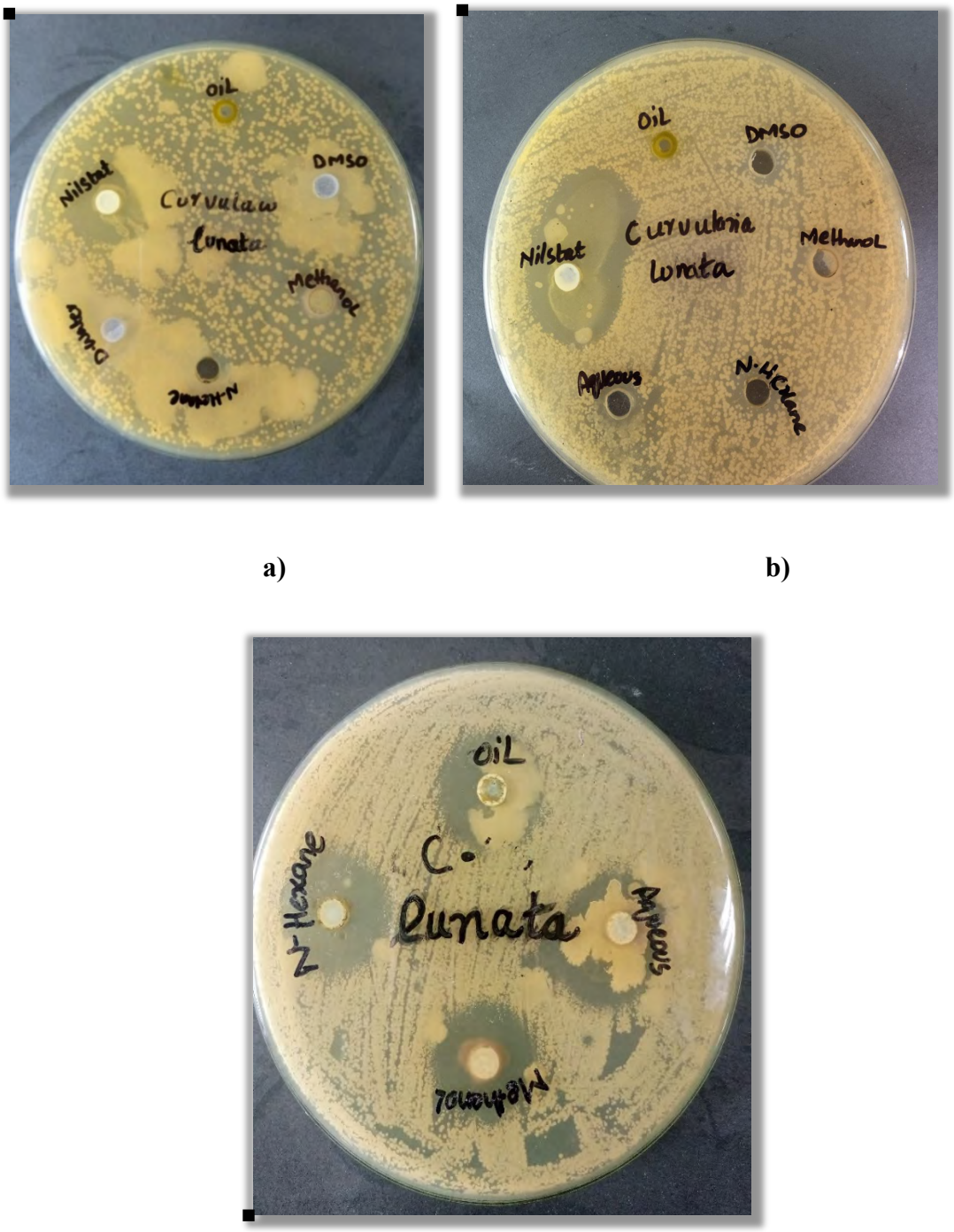


FIG 4.18: Graph showing the Zone of inhibition of different extracts against *Curvularia lunata.*

Table 4.11: Zone of inhibition of different extracts against *Curvularia lunata* and %increase in synergistic assay

Curvularia lunata				
Extracts	Diluted (500mL)	Concentrated	Synergistic effect	
			Concentrated extracts with Nilstat	% increase in ZOI
Aqueous	0	15	42	78
n-Hexane	0	13	40	29
Methanol	12	18	36	44
kjOil	0	0	37	131
Nilstat	32	32	-	
DMSO	0	0	-	



a) **b)**

c)

FIG4.19: the Zone of inhibition of different extracts against *Curvularia lunata*

a) diluted concentration (150mg/mL) **b)** at concentrated 300mg/mL **c)** synergistic assay

If we compare the synergistic assay data for all strains and their extracts it indicates that the highest activity was shown by n- hexane and methanol extract against the fungal strains. Oil is showing highest activity although it has minimum susceptibility when used alone indicating that oil and the antifungal drug may have additive effect.

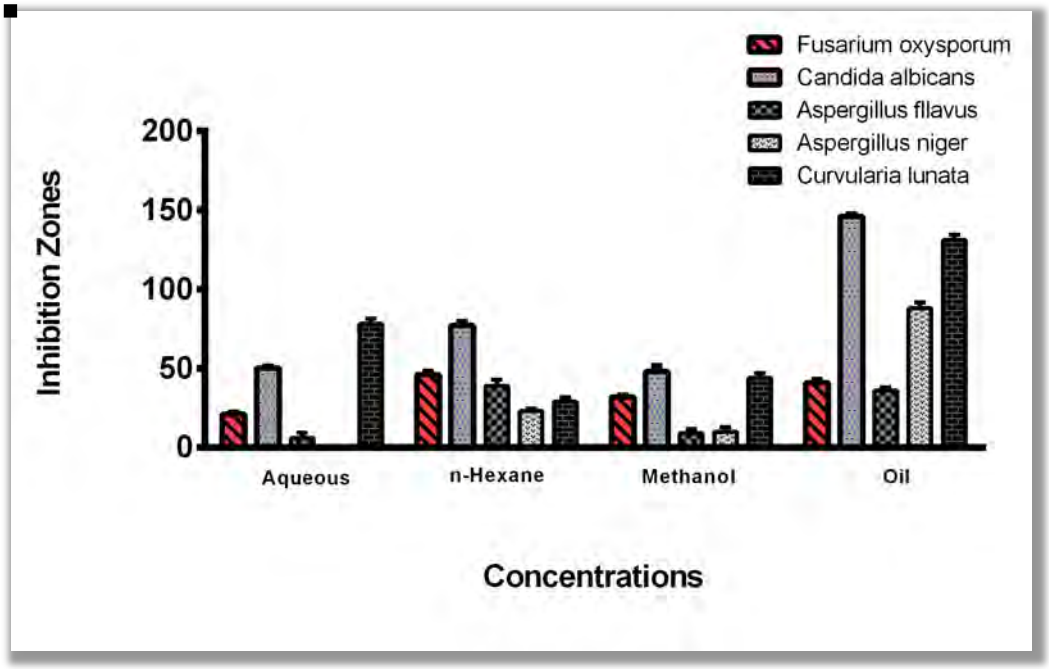


FIG4.20: Graph showing the % increase in zone of inhibition of different extracts against five different fungal strains in synergistic assay

4.4.1.6 MFC (Minimum Inhibitory Concentration):

For determination of minimum inhibitory concentrations (MICs) microtiter plates containing 96 wells were used. Results indicate that each strain has different MICs. For the extracts MIC values range in 0.03-3 mg/mL. For synergistic assay vales range in 0.03-30 which indicates some extracts has additive or synergistic effect while some are indifferent when combined.

Table 4.12: Minimum Inhibitory concentration for Fungal Strains

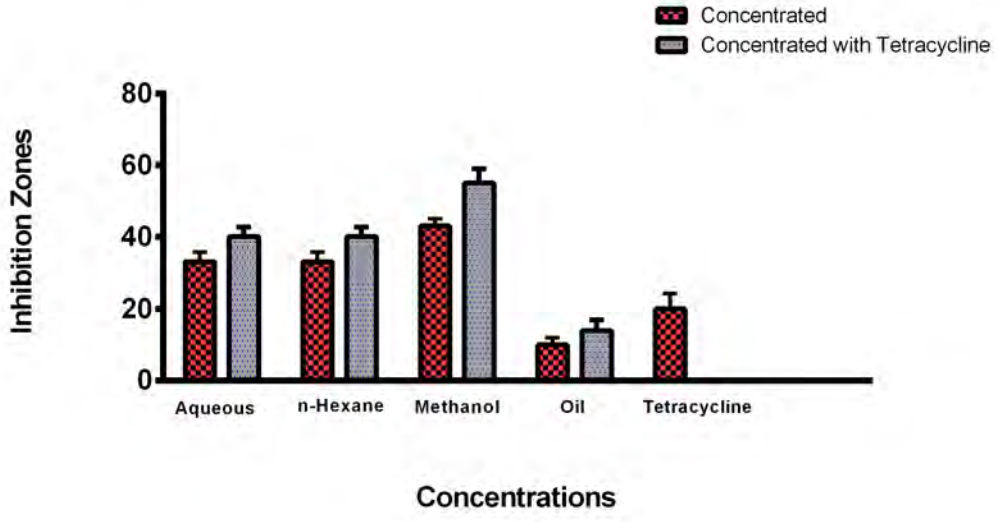
MIC (mg/mL)										
Strains	***Fusarium oxysporum***		***Candida albicans***		***Aspergillus flavus***		***Aspergillus niger***		***Curvularia lunata***	
Extracts	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***
Aqueous	3	0.3	3	0.03	0.03	0.03	3	0.3	30	0.03
Methanolic	0.3	0.03	0.3	0.03	0.3	0.03	0.3	0.03	3	0.03
n-Hexane	3	0.03	30	0.03	30	0.3	3	0.03	30	0.03
Oil	30	0.3	0	0.03	30	0.3	0	0.03	0	0.3

4.4.2 Antibacterial Assay:

In the current study, extracts of de-oiled pressed seed cake and oil was used to check their antibacterial activity against MDR gram-negative bacterial strains. For this purpose, agar well diffusion method was used. Bacterial suspension was spread over the agar plate and 100 microliter extract was added in the wells and its activity was measured in millimeter. Een some extracts had high activity than tetracycline methanol and n hexane showed good activity against MDR bacterial strains. Synergistic assay showed that the antibacterial drug and extracts have antagonistic effect. Tetracycline, a broad-spectrum antibiotic, was used as a positive control while DMSO was used as negative control.

4.4.2.1 Antibacterial Activity of extracts of de-oiled pressed seed cake and oil against *Klebsiella pneumonia:*

Klebsiella pneumonia a Gram-negative bacterium was most susceptible to the methanolic extract which showed ZOI of 43mm. n-hexane and aqueous extracts show good activity against *Klebsiella pneumonia* with ZOI 32mm, DMSO was used as a negative control and Tetracycline showed the high activity. In synergistic all three extracts showed high



susceptibility.

FIG 4.21: Graph showing the Zone of inhibition of different extracts against *Klebsiella pneumonia*

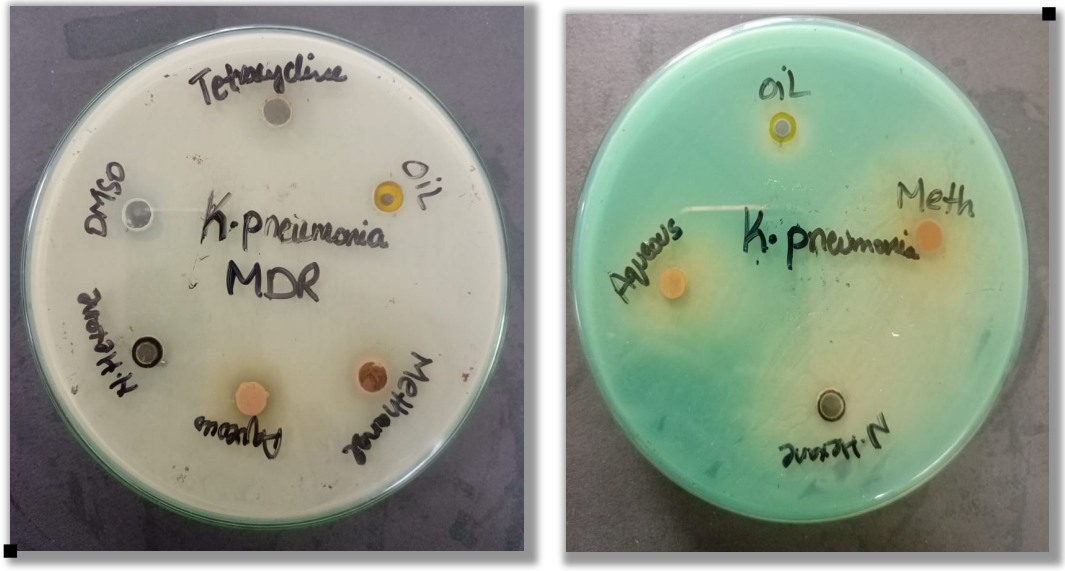


FIG4.22: the Zone of inhibition of different extracts against *Klebsiella pneumonia* a) at concentrated 300mg/mL b) synergistic assay

4.4.2.2 Antibacterial Activity of extracts of de-oiled pressed seed cake and oil against *Salmonella*

Salmonella a Gram-negative bacterium was most susceptible to the methanolic extract which showed ZOI of 25mm. n-hexane and aqueous extracts show good activity with ZOI 19mm and aqueous 22mm, DMSO was used as a negative control and Tetracycline showed moderate 16 mm zone activity. In synergistic assay only methanol showed susceptibility. Other extracts did not show ay ZOI which indicates the two must have antagonistic effect.

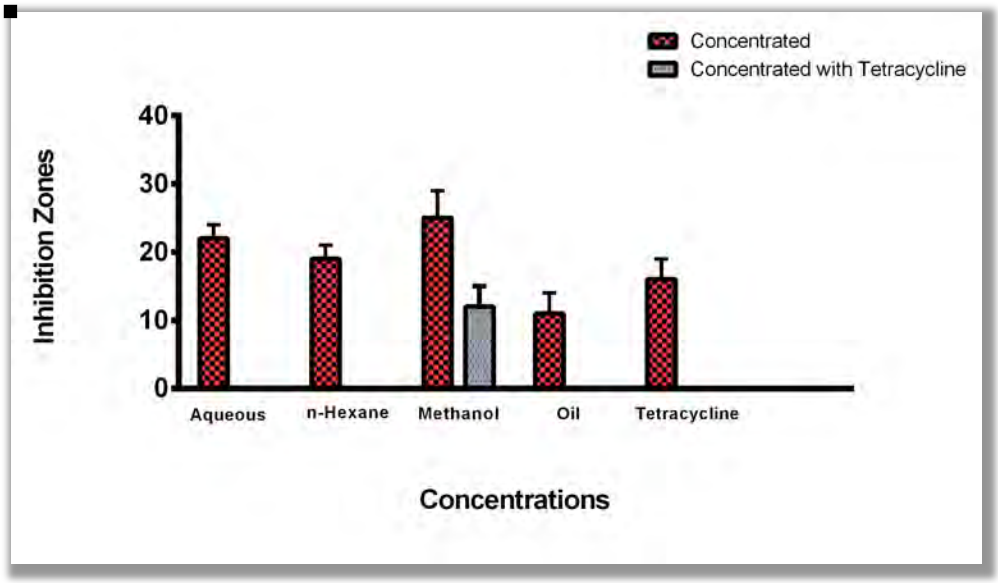


FIG 4.23: Graph showing the Zone of inhibition of different extracts against *Salmonella*

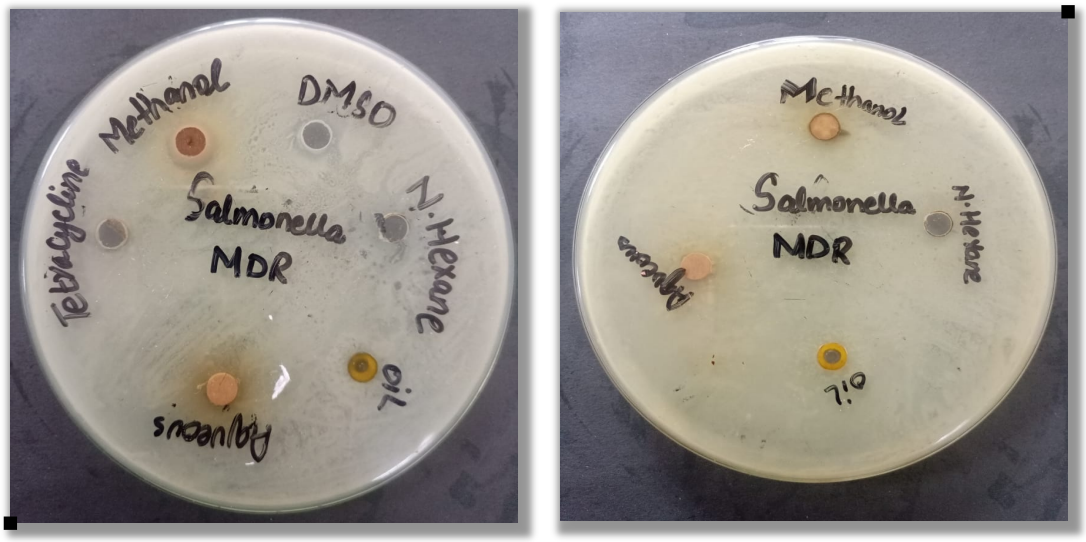


FIG4.24: the Zone of inhibition of different extracts against *Salmonella*

a) at concentrated 300mg/mL **b)** synergistic assay

4.4.2.3 Antibacterial Activity of extracts of de-oiled pressed seed cake and oil against *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa a Gram-negative bacterium was most susceptible to the n-hexane extract which showed ZOI of 20mm. n-hexane and aqueous extracts show 18mm ZOI and methane had 12 mm ZOI. DMSO was used as a negative control and Tetracycline showed 13 mm ZOI. In synergistic assay only methanol showed positive effect here as other extracts had antagonistic effect.

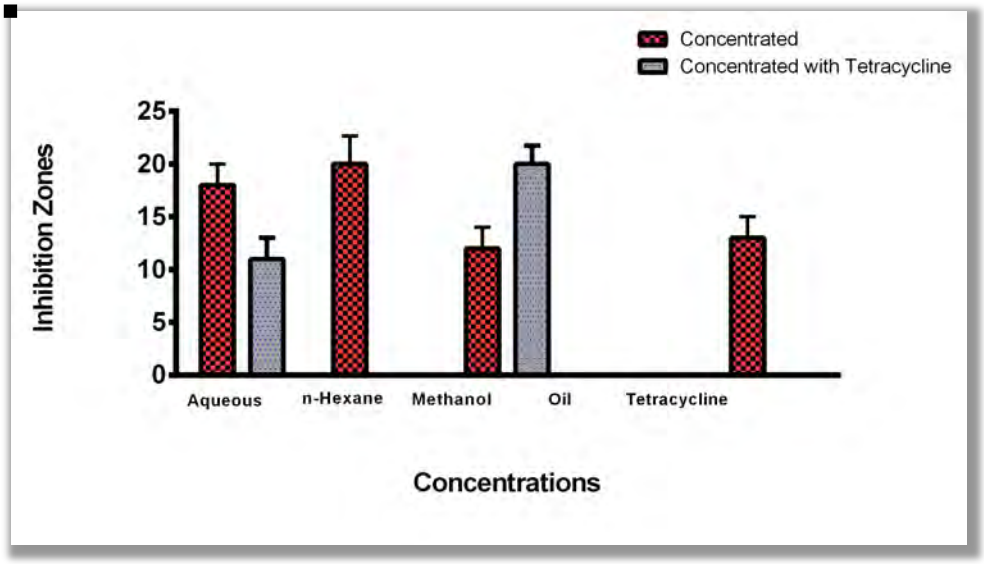


FIG 4.25: Graph showing the Zone of inhibition of different extracts against *Pseudomonas aeruginosa*

FIG4.26: the Zone of inhibition of different extracts against *Pseudomonas aeruginosa*
a) at concentrated 300mg/mL **b)** synergistic assay

The synergistic assay data for all strains and their extracts indicates that only has synergistic effect whereas rest of the two strains has antagonistic effect when tetracycline is combined with extracts.

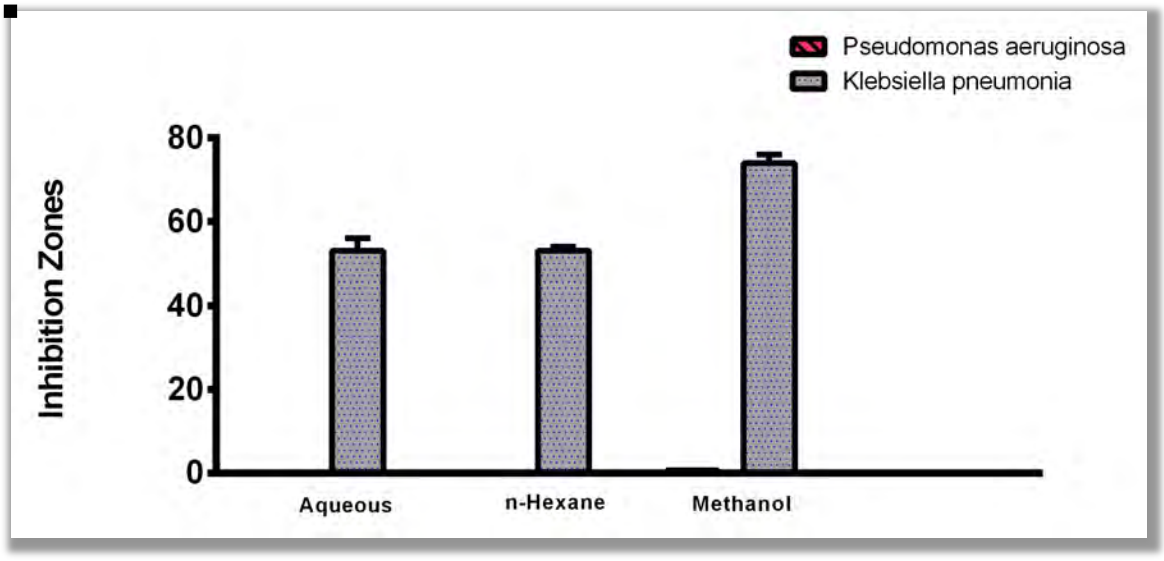


FIG4.27: Graph showing the % increase in zone of inhibition of different extracts against three different bacterial strains in synergistic assay

4.4.2.3.4 MIC

MC was tested against Gram-negative, Multi Drug Resistant, MDR, human pathogens (*Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella)* strains. Results indicate that Mic for aqueous, n-hexane and methanolic extract is 0.03—3 mg/mL. For oil MIC is30 mg/mL which indicates that oil has less antimicrobial property as compared to extracts. For synergistic assay only *Klebsiella pneumonia* has showed activity and Mic value for all three extracts is 0.03 mg/mL whereas for oil 30mg/mL is required.

Table4.13: Minimum Inhibitory concentration for multi drug resistant bacteria Strains

MIC (mg/mL)						
Strains	***Pseudomonas aeruginosa***		***K. pneumonia***		***Salmonella***	
Extracts	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***	***Concentrated***	***Synergistic assay***
Aqueous	3	Antagonistic effect (AE)	0.3	0.03	3	AE
Methanolic	0.3	3	0.03	0.03	3	AE
n-Hexane	3	AE	0.3	0.03	0.3	AE
Oil	30	AE	30	30	30	AE

AE *Antagonistic effect

4.4.3 Antipathogenic Assay:

Pathogenic extracts showed little to moderate activity against extracts and oil . in synergistic assay when combined with Nilstat they gave additive effect. Among the extracts methanolic extract showed activity in all strains.

4.4.3.1 Antipathogenic activity of extracts of de-oiled pressed seed cake and seed oil against *Fusarium:*

Fusarium is a huge species complex of plant and human diseases that target a wide range of species in a host-specific way. Concentrated methanolic extract showed highest activity against ***Fusarium***. synergistic effect was positive with all extracts. N hexane extract showed high synergistic effect with an increase of 46% in the ZOI. Concentrated extracts showed higher activity than the diluted ones and also synergistic effect was enhanced.

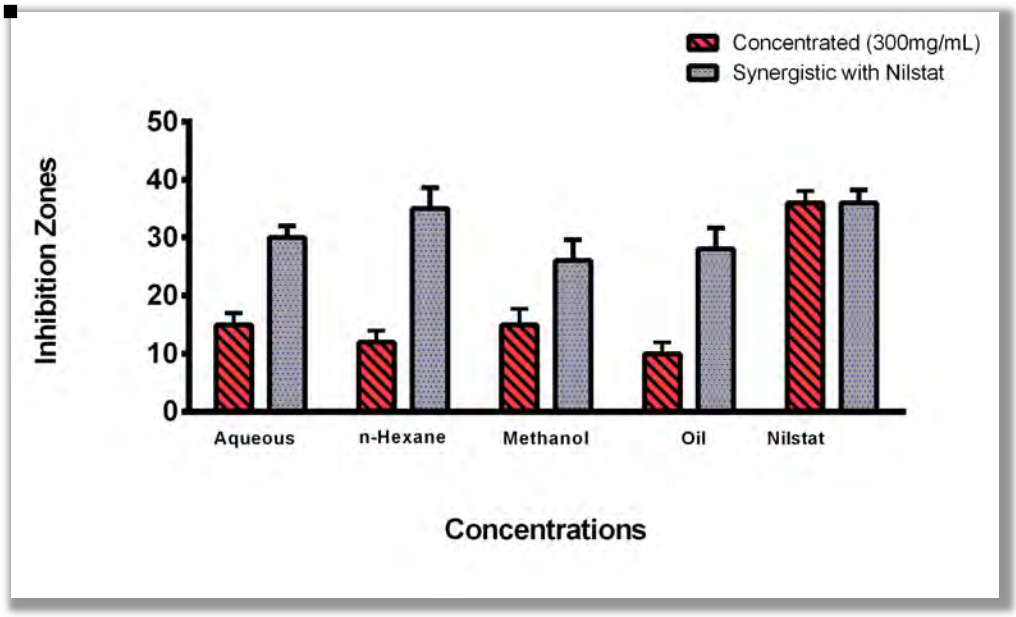


FIG 4.28: Graph showing the Zone of inhibition of different extracts against ***Fusarium***

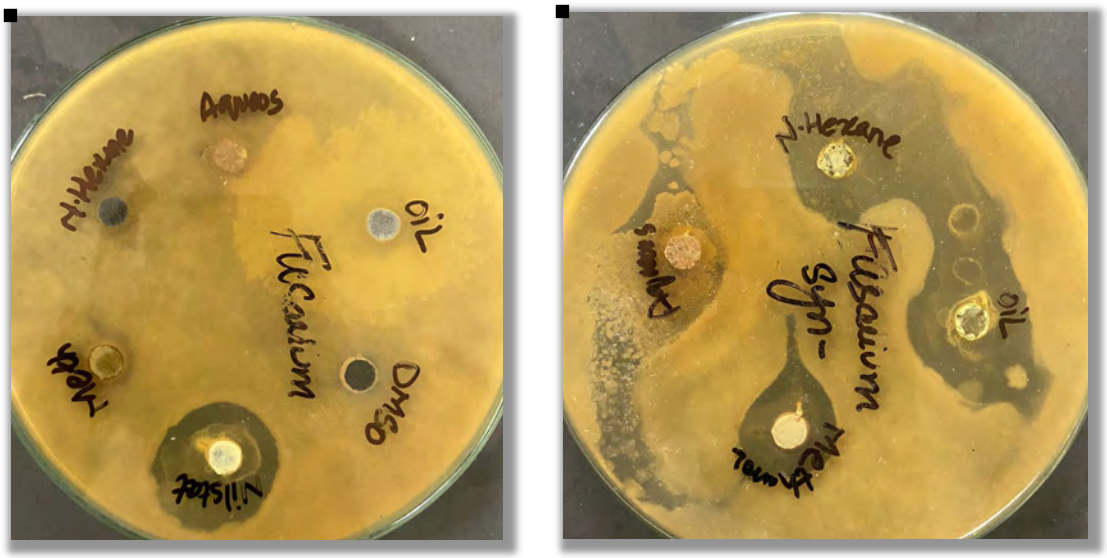


FIG 4.29: *The Zone of inhibition of different extracts against Fusarium a) at concentration 300mg/mL b) synergistic assay*

4.4.3.2. Antifungal activity of extracts of de-oiled pressed seed cake and seed oil against *Aspergillus flavus:*

Aspergillus flavus is a huge species complex of plant that targets a wide range of species in a host-specific way. The concentrated methanolic and n hexane showed moderate activity but aqueous and oil does not have antifungal. synergistic effect was positive with all extracts. Oil and aqueous extract showed high synergistic effect.

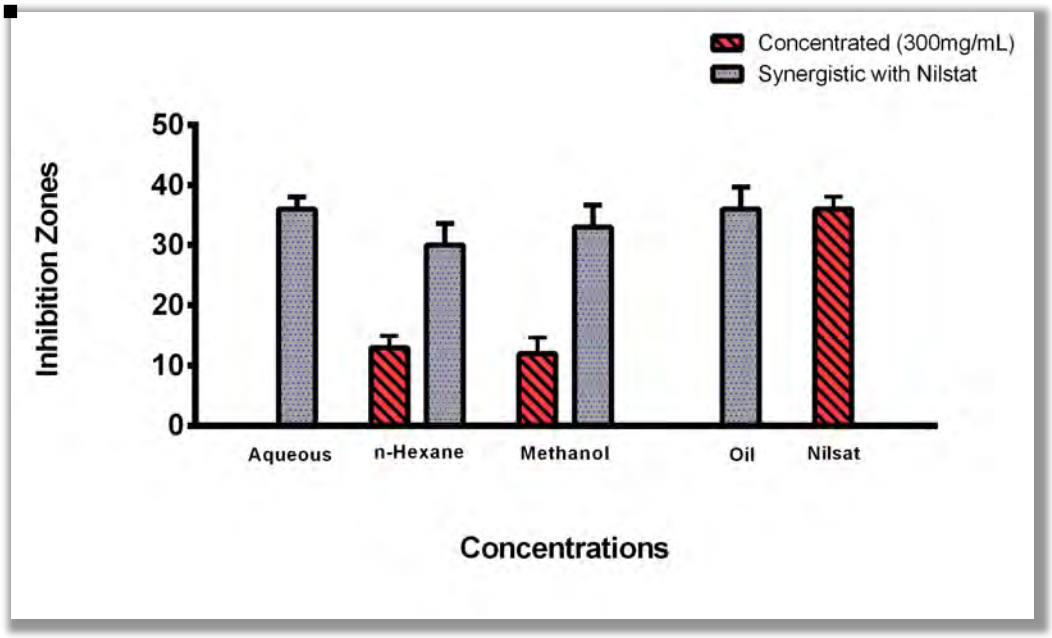


FIG 4.30: Graph showing the Zone of inhibition of different extracts against *Aspergillus flavus*

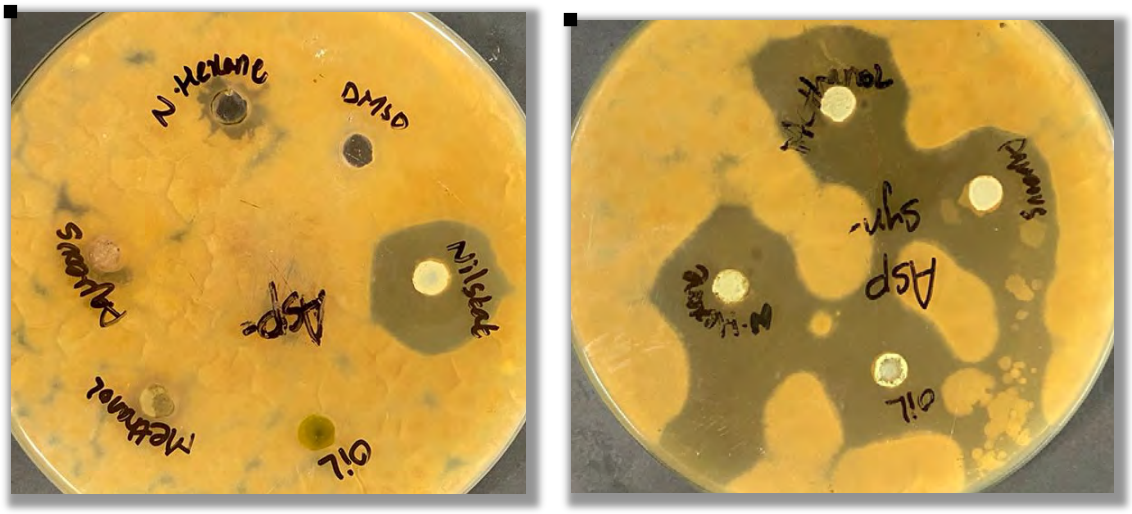


FIG 4.31: The Zone of inhibition of different extracts against *Aspergillus flavus* a) at concentration 300mg/mL b) synergistic assay

4.4.3.3. Antipathogenic activity of extracts of de-oiled pressed seed cake and seed oil against *Penicillium chrysogenum:*

Penicillium chrysogenumum is a huge species complex of plant and human diseases that target a wide range of species in a host-specific way. Concentrated methanolic and aqueous showed low activity. synergistic effect was positive with all extracts. Concentrated extracts showed low but synergistic effect was enhanced.

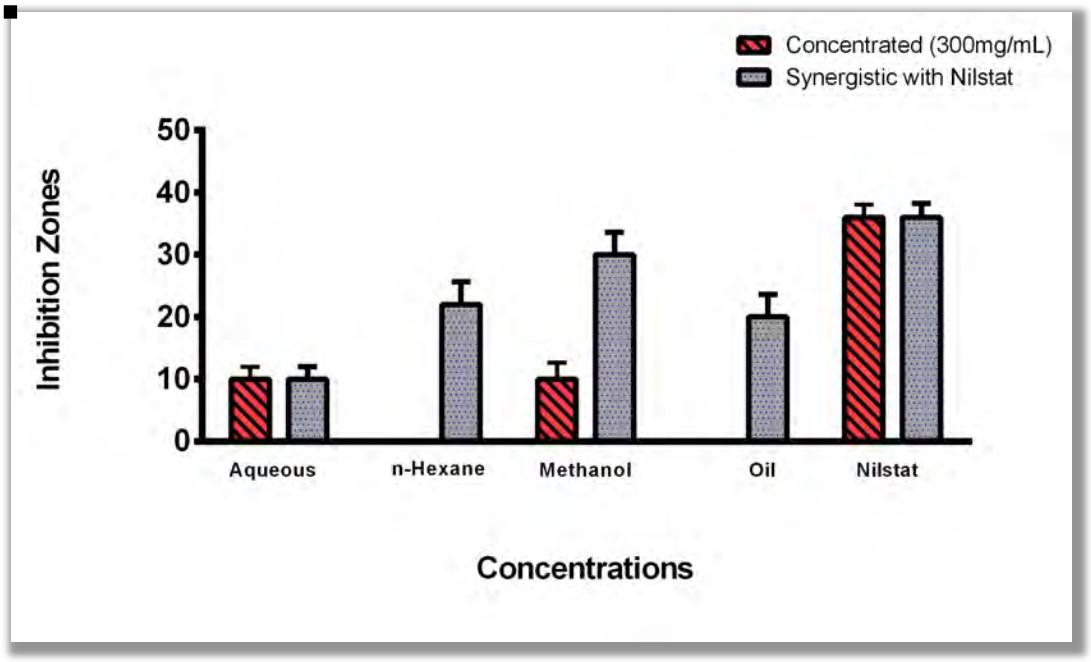


FIG 4.32: Graph showing the Zone of inhibition of different extracts against ***Penicillium chrysogenum***

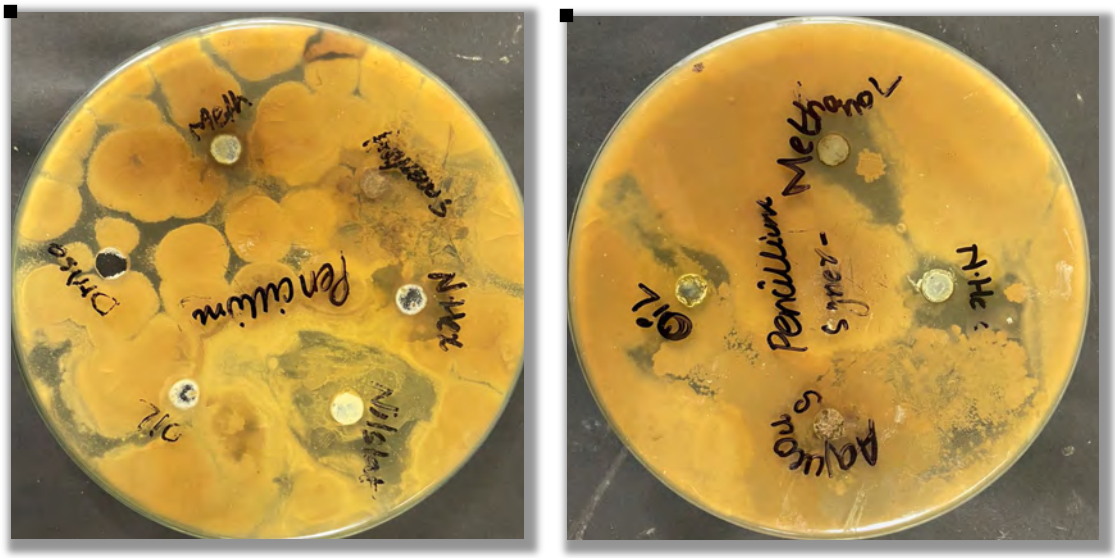


FIG 4.33:*The Zone of inhibition of different extracts against Penicillium chrysogenum a) at concentration 300mg/mL b) synergistic assay*

The synergistic assay graph reveals that *Aspergillus flavus* is most susceptible to these extracts and if we talk about extracts methanol and extracts show activity in most extracts but for synergistic assay *Aspergillus flavus* shows good results.

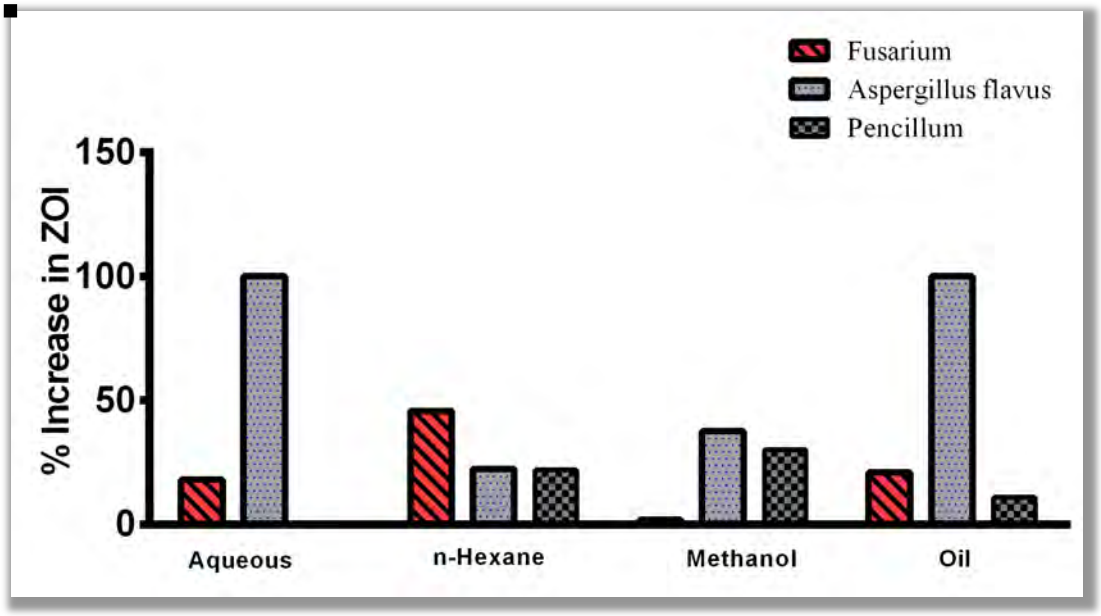


FIG 4.34: Graph showing the % increase in zone of inhibition of different extracts against three different phytopathogenic strains in synergistic assay

4.4.3.4 MIC

MC was tested against these phytopathognic strains. Results indicate that Mic for aqueous, n-hexane and methanolic extract 30-300 mg/mL. For oil MIC is300 mg/mL which indicates that oil has less antimicrobial property as compared to extracts. For synergistic assay Mic value for all three extracts is 0.3 -3 mg/mL.

Table4.14: Minimum Inhibitory concentration for Phytopathogenic Strains

MIC (mg/mL)						
Strains	***Fusarium***		***A. flavus***		***Penicillium***	
Extracts	**Concentrated**	**Synergistic assay**	**Concentrated**	**Synergistic assay**	**Concentrated**	**Synergistic assay**
Aqueous	**30**	**0.3**	**300**	**0.3**	**300**	**AE**
Methanolic	**300**	**0.3**	**30**	**0.3**	**300**	**3**
n-Hexane	**30**	**3**	**30**	**0.3**	**300**	**0.3**
Oil	**300**	**3**	**300**	**0.3**	**300**	**3**

4.4.4 2, 2-diphenyl 1-picrylhydrazyl (DPPH) free radical scavenging assay:

DPPH is done to evaluate the radical scavenging activity of the extracts. Our extracts: methanolic, n-hexane, oil and aqueous were used. Each extract had 4 different dilutions made 9 each dilution was 10 times diluted from the previous dilution starting from the stock solution. (300, 30, 3, 0.3, 0.03 mg/mL).

**Table 4.15:* Extracts and their antioxidant activity at different concentrations

	Extracts and their anti-oxidant activity %				
Dilutions mg/mL	**Methanol**	**Aqueous**	**n-hexane**	**Oil**	**Ascorbic acid + control**
300	38	41	50	71	94
30	34	33	45	53	93
3	28	28	44	52	92.9
0.3	18	16	38	41	92.4
0.03	0.9	4.9	33	39	92

4.4.5 Cytotoxicity with brine shrimp

Table 4.16*:* Extracts/Oil and their Cytotoxic activity at different concentrations

Extracts and Oil	Dilutions (mg/ml)	Time	
		24 hours	48 hours
Aqueous	0.3	alive	alive
	0.03	alive	alive
n-Hexane	0.3	alive	7 alive
	0.03	alive	9 alive
Methanol	0.3	8 alive	6 alive
	0.03	alive	8 alive
Oil	300	alive	alive
	30	alive	alive
Positive control Vincristine		dead	dead
Negative control		alive	alive

4.5 Biodiesel Production from *Cannabis sativa* /Industrial grade hemp seed oil

4.5.1 Determination of Acid value, FFA content and Saponification value of *Cannabis sativa* oil:

For determination of each value, specific experiments were performed in triplicates and their mean value was listed in table 4.2.

Table 4.17. Determination of physicochemical parameter of Hemp Oil:

Experiment for each parameter was performed in triplets and average value was calculated

Parameter	Experimental value	Mean value
Acid value	**1.0**	**1.33**
	1.5	
	1.5	
FFA (%)	**0.56**	**0.746**
	0.84	
	0.84	
Saponification value	**189.57**	**194.12**
	197.43	
	195.37	
Ester value	**188.57**	**192.79**
	195.93	
	193.87	
% Glycerin	**10.30**	**10.53**
	10.71	
	10.59	

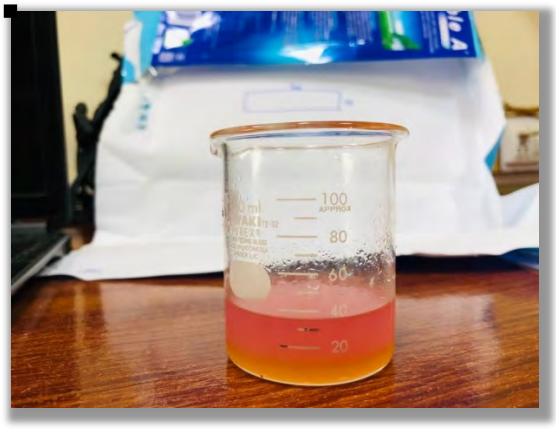


Fig4.35: Permanent pink color after Titration with KOH for FFA content

a b

Fig 4.36: (a) blank run for saponification number, (b) pink color disappears after titration

4.5.2 Biodiesel production from *Cannabis sativa* oil:

C. sativa oil was used to check its conversion into biodiesel through chemical transesterification process. Initially, reaction was conducted at standard conditions; Temperature (60°C), Oil to Methanol ratio (1:6), Catalyst concentration (2%), RPM (600) and reaction time was 120 mins. FAME production yield was 60%.

Fig 4.37 Layers formed after transesterification reaction

4.5.3 Optimization of Parameters for Biodiesel production:

Temperature, catalyst concentration, agitation, oil to methanol ratio, and reaction time were all optimized for *C. sativa* oil biodiesel synthesis. Experiments were carried out under different parameters, and the percentage volumetric yield was recorded. Each parameter was run on the optimized condition of the previous parameter.

4.5.3.1 Effect of Different Temperatures on Methyl Ester Yield

Effect of temperature on biodiesel yield was calculated by using different temperature ranges. Experiment was conducted at 50, 55 and 60 degrees Celsius respectively. Other parameters such as oil: methanol, catalyst concentration, agitation and tie were kept as

per the standard conditions. Highest yield was obtained at 50 degrees almost 65%, 62% at 55 degrees ad 60 % at 60 degrees.

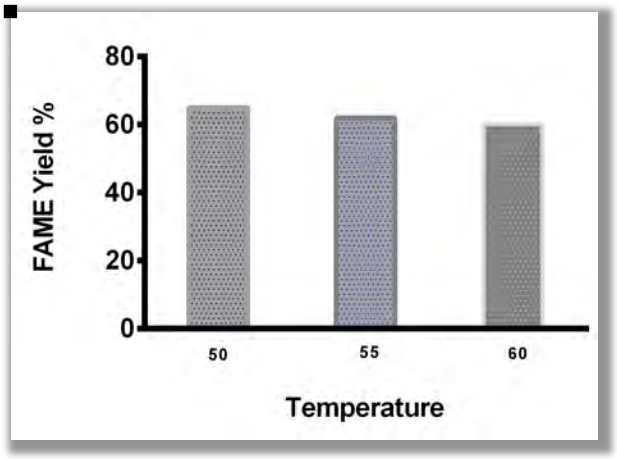


Fig: 4.38 Graph showing FAME yield obtained at different temperatures

4.5.3.2 Effect of different reaction time on Methyl Ester Yield

Effect of time on biodiesel production was studied by using different time ranges. Maximum yield was obtained at 90 minutes, 71 %at the standard time 2 hrs. (120 min) yield was 65% and only 60% yield was obtained at 60 minutes.

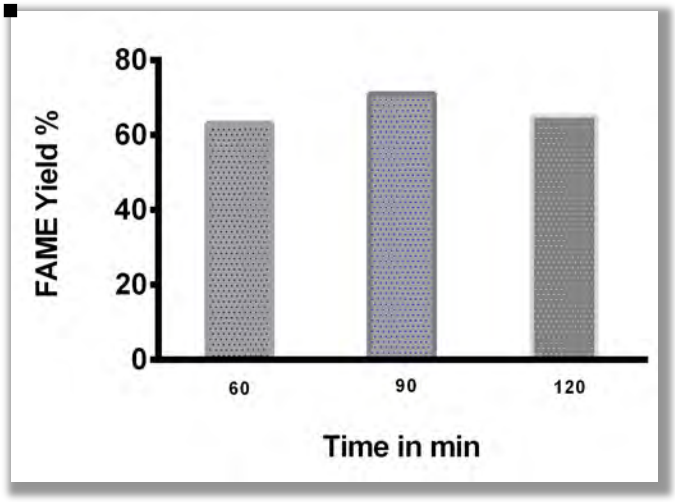


Fig:4.92 Graph showing FAME yield obtained at different Reaction time

4.5.3.3 Effect of Different Catalyst concentration on Methyl Ester Yield

Effect of potassium hydroxide used as catalyst for transesterification was studied by using various concentrations; 1%, 1.5% and 2%. Maximum yield was obtained at 1% catalyst 71 %, 66% at 1.5% catalyst and 60% at 2 % catalyst. Temperature was set at 50 degrees and rests of the parameters were at standard conditions.

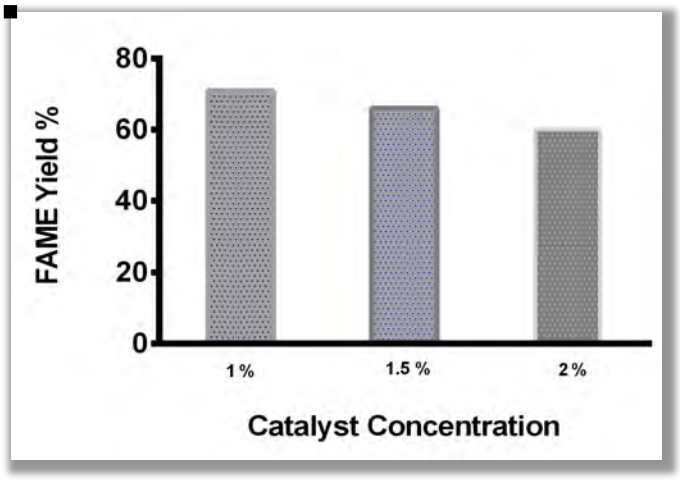


Fig 4.40 Graph showing FAME yield obtained at different Catalyst concentration

4.5.3.4 Effect of Different Molar Ratios on Methyl Ester Yield

Molar ratio for biodiesel production was optimized using different ratios.; 1:6,1:9 and 1:12. Maximum yield was obtained at 1:9 that is 84%. 75% was obtained at 1:6 and 80 % yield was obtained at 1;12 molar ratio.

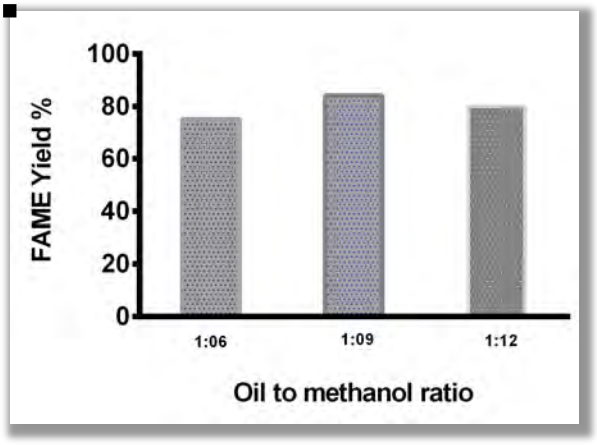


Fig 4.41Graph showing FAME yield obtained at different Molar Ratios

4.5.3.5 Effect of Different Agitation on Methyl Ester Yield

The rate of FAME production depends upon the mass- transfer limitations. Agitation speed effect was studied using different speed ranges: 300, 600 and 900 rpm. Yield was obtained at 600 rpm almost 84%, indicating optimization of agitation does not have an effect on biodiesel yield.

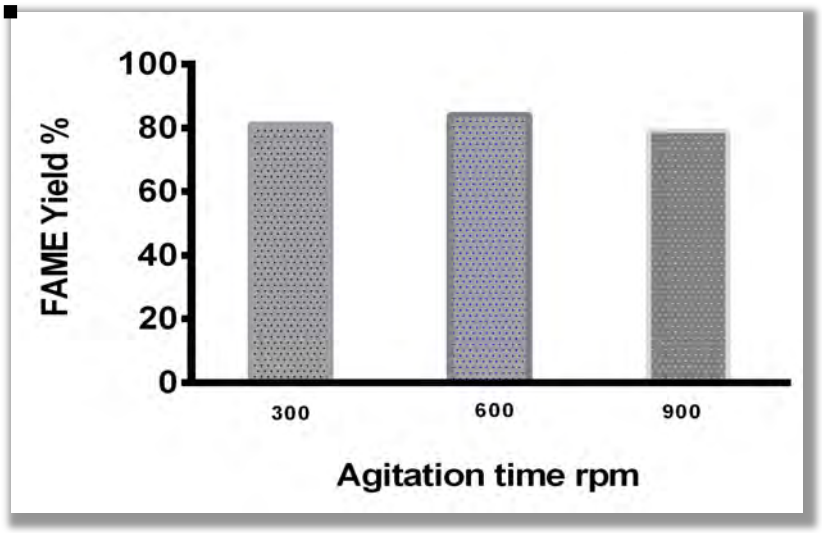


Fig: 4.42 Graph showing FAME yield obtained at different Agitation speed

So, the optimized condition are 1:6 oil to methanol ratio, 1% catalyst, 90 min reaction time, temperature 50 degrees and agitation time 600 rpm and the yield obtained a these conditions is 85%.

4.6 FTIR analysis of FAME produced by alkali catalyzed trans-esterification of Hemp seed oil:

FTIR analysis of the biodiesel was performed to confirm the FAME presence in the reaction mixture. One major and two minor stretches of ester bands were observed at 1741.11, 1195.89 and 1033.61 confirmed the presence of FAME in the reaction content. Peak at 1196 and 1113.7 corresponds to the C-O stretches for esters and these stretches are only present in the FAME confirming the biodiesel production from hemp oil.

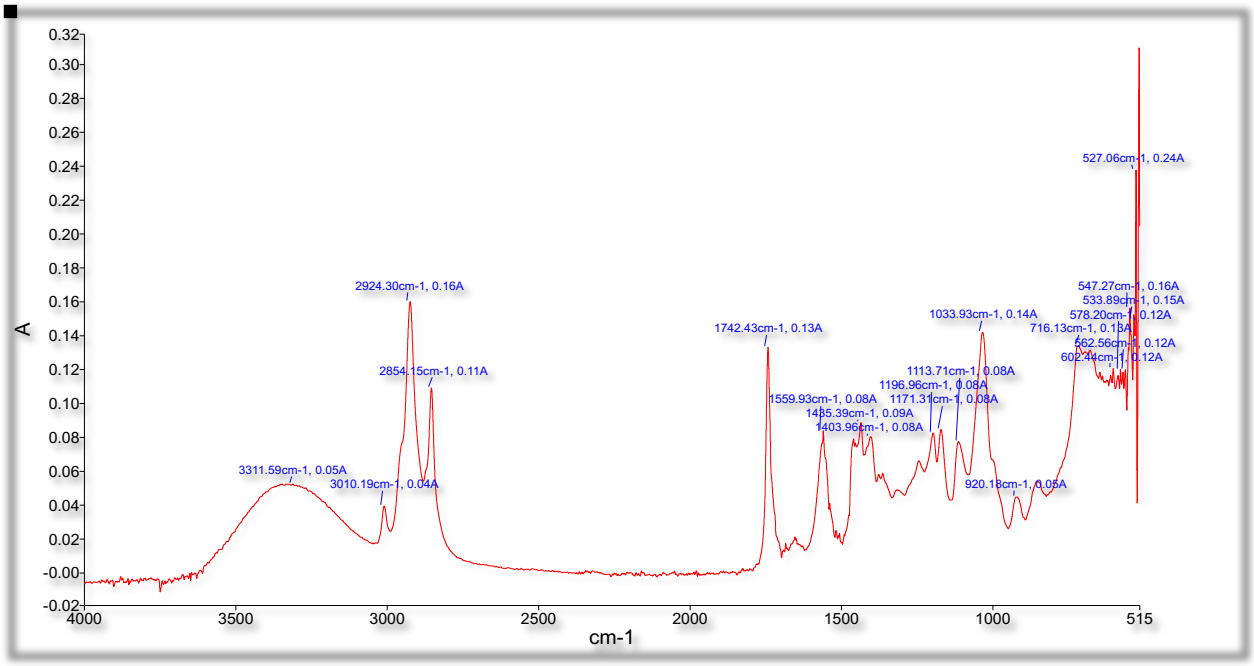


Fig4.43: FTIR spectra of FAME produced by alkaline trans-esterification of Hemp seed oil

4.7 Biodiesel production using whole cell Catalyst.

47.1 Oil Toxicity:

Hemp seed oil has reported antimicrobial activity so oil toxicity test was run for our selected strain, *Bacillus subtilis* C17(KU681037). Cooking oil and olive oil were used as reference oils. Selected strain showed efficient growth activity and highest growth activity occurred at 24 hrs. there was no growth inhibition in all three oils that indicates the strain produces lipase in these oils.

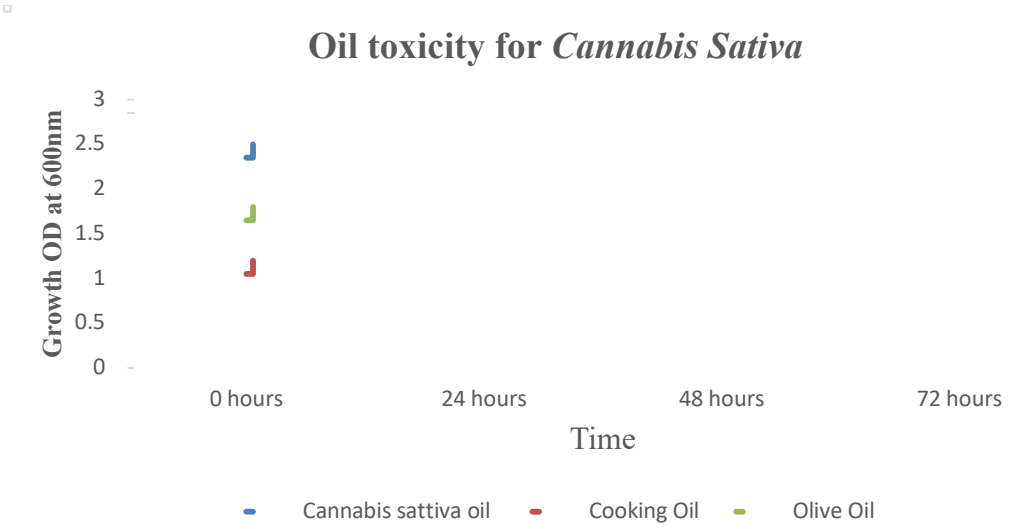


Fig:4.44 Graph showing oil toxicity of crude Hemp seed oil, cooking oil and olive oil

For whole cell approach parameters used were: oil: methanol 1:9, temperature 37 degrees, solvent used is n hexane and time is 48 hrs. Biodiesel yield obtained from whole cell catalyst approach is 68%. This indicates that biological production technique is more efficient and sustainable than chemical transesterification.

4.8 Optimization of Biodiesel Production using Whole Cell Catalyst using Plackett-Burman Design:

Discussion

The world is facing two major energy and health crises, interrelated. Energy shortages due to increased fossil fuel consumption are causing health problems, while ecological decline affects biodiversity. Drug resistance is a major health risk, caused by drug abuse and overuse. To address these issues, quick action is needed to achieve Sustainable Development Goals. Plants, including their parts, have long been used to combat drug resistance. A large range of secondary metabolites are also present in plants. The seed possesses stimulating and rubefacient qualities and can be used to overcome drug resistance. Besides drug production oil extracted from plants can be used for energy production. Industrial hemp, *Cannabis sativa*, is the product that researchers are most interested in because of its wealth of nutritional advantages, and bioactive potential. It is being researched for its potential in pharmaceutical and energy sector. Pakistan offers ideal conditions for C. sativa cultivation.

5.1 Phytochemical analysis of Hemp seed extracts and oil:

According to research on the phytochemical makeup of *Cannabis sativa* seeds and their chemical makeup, the seeds included to 20 to 30% carbs, 20 to 25% protein, 10-15% fiber, potassium, magnesium, calcium, minerals, phosphorus, zinc, iron, and sulphur, hemp seeds often include 25 to 35% oil. According to some research, hemp seed oil may contain as much as 51.06% oil content. However, the oil content in the current investigation was discovered to be 13 percent. Numerous factors, including seed ripeness and plant watering, climatic and soil conditions have an impact on the oil content. The presence of 20 to 25% protein suggests that *C. sativa* is a superior option for a feed supplement. On the other hand, hemp seed extract contains vital secondary metabolites such flavonoids, phenols, alkaloids, saponins and tannins that are frequently found in conventional medicines and utilized as therapies for a number of illnesses. In literature seed cake extracts have been reported for different bioactive substances. Different compounds were found in different extracts such that one compound was present in one extract and absent in the other (De Vita et al., 2022). Further GCMS assays can help us clarify and identify these compounds. Hemp seeds have substantial amounts of bioactive

compounds called cannabinoids and they have antimicrobial activity, which might be employed in the development of medications for human and animal health.

5.2 Antifungal and Anti pathogenic Activities

Five clinical fungal strains were used to determine the anti-fungal properties of the de-oiled seed cake of *C. sativa*. These opportunistic pathogenic fungal strains were: *Candida albicans, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Curvularia lunata*. Activities were checked at diluted and pure concentrated extracts made in DMSO. For diluted 150mg/ml of the extracts and for concentrated 300mg/ml stock solution was used. All fungal strains wee susceptible to the extracts. Hexane showed the highest antifungal activity followed by methanolic and aqueous extracts. *Fusarium oxysporum* and , *Candida albicans* had maximum susceptibility to hexane. *Aspergillus niger* had negative effect during synergistic assay indicating that extract may have reacted with Nilstat and canceled each other's effect. *C. lunata* showed highest activity n aqueous extract. Oil is giving the highest percentage one cause its only Nilstat that's giving the zones further reason can be given that oil may have an additive effect when combined with Nilstat. to Oil gave least activity showing that oil may lack the cannabinoids and other bioactive substances to kill the fungus cells. In literature data for *A. niger* and *C. albicans* have been reported and it shows susceptibility to methanolic extracts and resistance to the aqueous and n hexane extracts (Isahq et al., 2015) where as leaf extracts they showed ZOI of 2-3 mm at 0.003 mg/ML(Lone & Lone, 2012) . Synergistic assay was done with concentrated extracts and Nilstat which was used as positive control. Oil in combined assay gave synergistic effect indicating that bioactive compounds may have reacted with antifungal drug and gave positive results.977 Minimal inhibitory concentration was found using Mic assay on microtiter plates. Most of the MIC concentrations were in range of 3-0.3 mg/mL and for synergistic assay 0.03 mg/mL of combined extract and Nilstat is required to kill 80% of the microbes.

Antipathogenic Assay:

Extracts showed little to moderate activity against *Aspergillus flavus, Pencillium and Fusarium strains.* in synergistic assay when combined with Nilstat they gave additive effect. Among the extracts methanolic extract showed activity in all strains. The results

show that theses trains are not much susceptible to these extracts. Inn literature work is not being done on phytopathogenic strains however as the cannabinoids ae mostly present in flowers as higher ratio their extracts can show higher activity against these strains.

5.3 Antibacterial Assay Against MDR strains

The current study examined the hemp oil's antibacterial qualities against MDR bacterial strains, which are a growing concern among hospitalized patients. The bacterial strains were **Gram-negative, Multi Drug Resistant, MDR,** human pathogens (***Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella)*** strains were used **.** These are all capable of causing blood infections such as urinary tract infections (UTI), respiratory tract infections, dysentery-like diarrhea, wounds, burns, and other blood infections. The current study looked at the fact that hemp oil has less activity than its extracts against tested microbes and can be used to treat bacterial infections. All extracts were positive for antibacterial activity, but the antibacterial drug used Tetracycline showed the minimum activity indicating these strains are somewhat resistant to this drug. Oil also exhibited minimum susceptibility. Methanolic extracts were most efficient against MDR strains. In literature ***Pseudomonas*** aeruginosa has been reported to show ZOI of about 12-15 mm(Lone & Lone, 2012) and ***Klebsiella*** pneumonia has reported activity in methanolic and n hexane extracts and were resistant in aqueous extracts (Isahq et al., 2015). The values in our current study are higher than those reported and also leaf extracts has the highest antimicrobial activity as compared to the seed extracts (Lone & Lone, 2012). During synergistic assay strains showed antagonistic effect. Tetracycline and extracts cancelled the effect of each other and bacterial lawn was observed on plates. Only in *Klebsiella pneumonia* the synergistic assay showed positive effect. The tetracycline combined with extracts gave antagonistic effect. MIC was done with microtiter plate and indicated that extracts minimum inhibitory concentration was 30mg/mL, whereas the combined assay showed these extracts and drug has antagonistic effect.

The results of the current study demonstrate that *C. sativa* seed extracts have antibacterial properties but has antagonistic effect in synergistic assay maybe when combined with some other antibiotic it gives positive results so, further work can be done to enhance the synergistic effect of these extracts. So hemp seed cake extracts are a potential competitor

in the pharmaceutical and in order to find bioactive compounds that could be used as potent antimicrobials for therapeutic purposes as an alternative drug, more research is needed.

5.4 2: 2-diphenyl 1-picrylhydrazyl (DPPH) free radical scavenging assay and Cytoxicity assay

DPPH is done to evaluate the radical scavenging activity of the extracts. Our extracts: methanolic, n hexane , oil and aqueous were used. Each extract had 4 different dilutions made 9 each dilution was 10 times diluted from the previous dilution starting from the stock solution. (300,30,3 ,0.3,0.03 mg/mL).

Brine shrimp assay indicates that N-hexane and methanolic extracts were only showing cytotoxicity. These extracts can be used for treating cancerous cells or tumor cells.

5.5 Biodiesel Production from *Cannabis sativa*

Cannabis sativa oil had acid value 1..33 and an almost 1% (0.746%) FFA level, therefore alkali-based trans-esterification was carried out. Literature has reported acid value of about ~1 (Li et al., 2010). On alkali basis, the trans-esterification of *C. sativa* oil depends on the molar ratio, catalyst concentration, and reaction temperature. On the standard conditions : oil to methanol ratio 1:6, temperature 60 degrees, agitation 600 rpm and catalyst concentration as 2% the oil yield obtained was 60 %. Literature has reported about 90% conversion as well values may vary due to number of factors such as climate, soil etc. Further optimization can be done in order to obtain high yield (Li et al., 2010).

5.6 Optimization of Parameters for Biodiesel production:

Manual optimization of each parameter was done separately. First temperature was optimized and each parameter was run on the optimized condition of the previous parameter. Highest volumetric yield of biodiesel 84% was recorded with conditions: temperature 50 degrees, oil to methanol ratio 1:9, catalyst concentration 1%, RPM 600 and reaction time 90 minutes. In literature little work has been done on optimization of biodiesel produced from hemp seed oil. Almost 85%yeild was obtained (Rashid et al., 2016)and 90% conversion has also been reported (Li et al., 2010).

5.6.1 Effect of reaction temperature

In order to study the effect of temperature for transesterification of hemp seed oil experiments were carried out on 3 different temperatures, 50^0c, 55^0c, 60^0c. Other reaction conditions such as molar ratio, catalyst concentration,, agitation speed were set on standard conditions. Boiling point of methanol is 60C so which can cause evaporation of methanol and then alcohol will not be available for transesterification so, it cannot be raised above this temperature (Pullen & Saeed, 2015). Effect of temperature as shown in the graph indicates that temperature has negative effect. Maximum yield was obtained at 50^0 C.

5.6.2 Effect of different catalyst concentration

Catalyst concentration is an important factor that affects the transesterification yield. A slight variation in concentration can either incomplete reaction or some unnecessary products maybe created resulting in low yield. In this experiment 1%, 1.5%, 2% concentrations were used. The highest yield was obtained at 1% catalyst concentration. If the concentration was increased it reduced the yield, excess catalyst reacts with TAG molecules and causes emulsification and it inhibits the proper layer formation.

5.6.3 Effect of different Reaction time

Two (2) hrs is the standard time for transesterification reaction. Process will be efficient if there is short lag phase, the phase where catalyst binds with methanol and later with sufficient agitation TAG molecules e enter this phase. In our study the maximum yield was obtained at 90 minutes. Further time increase decreased the yield as the prolonged time reverses the reaction and causes soap formation.

5.6.4 Effect of Different Molar Ratios

Oil : Methanol ratio is one of the important parameters for optimization of biodiesel yield. Different ratios such as 1:6, 1:9 and 1:12 were used. Maximum yield was obtained at 1;9. Higher ratio can efficiently increase the yield. Higher ratios can reduce the conversion time. Higher ratios has no significant effect on yield that is due to the reason that glycerol can get dissolved with FAME layers because of the polar -OH group in methanol that acts as an emulsifier. And this prevents glycerol to separate due to gravity and remains in biodiesel and decrease the yield as glycerol is removed.

5.6.5 Effect of Different Agitation Speed

Agitation is required for TAG molecules to enter the methanolic phase. The rate of FAME production depend upon the mass- transfer limitations. DAG and TAG molecules act as surfactants to enhance this transfer process. In our current study agitation speed optimized was 600 rpm further increase in speed does not allow the methanol, catalyst and TAG molecules to completely interact with each other hence reducing the yield and at low rpm mass transfer limitations were not enough to produce esters.

5.7 Biodiesel production using whole cell Catalyst

Already identified Q5 strain *Bacillus subtilis* C17 (KU681037) was used for biodiesel production. *C.sativa* seeds have antibacterial properties, so the toxicity of seed oil was assessed against the chosen bacterial strain in order to ascertain its antibacterial properties.

For whole cell approach parameters used were oil: methanol 1:9, temperature 37 degrees, solvent used is n hexane and time is 48 hrs. Biodiesel yield obtained from whole cell catalyst approach is 68%. This indicates that biological production technique is more efficient and sustainable than chemical transesterification. Although time is prolonged, but it does not involve down streaming costs and saves time. Further optimization can be done, and biodiesel yield can be optimized from biological catalysts.

The results of the current study demonstrate that the *Cannabis sativa* plant is an excellent feedstock for biorefineries. It can be used in pharmaceutical industry to prepare antifungal and antibacterial drugs The study's findings support the notion that *C. sativa* seed oil can be used to chemically trans esterify oil to produce biodiesel. It is possible to produce bioactive chemicals and biogas using the hemp de-oiled pressed cake as a feedstock.

Chapter 05

Conclusion and Future Prospects

Conclusion

Increased population and globalization has created number of problems that include energy shortage and health problems. Need of the hour is to develop sustainable development goals to combat the energy shortage and also health crisis which include AMR. Emerging global health concerns include antibiotic side effects, drug-resistant infections, and drug-resistant cancer cells. Nature has always provided us with solutions. Plants and their parts can be a useful resource for this purpose. The current study helped with the investigation of the antimicrobial properties of seed cake and seed oil extracts from *Cannabis sativa* against five fungal and three MDR bacterial strain along with the synergistic assay with their respective antimicrobial drug. The crude extracts were found to have antifungal activity against *Candida albicans, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Curvularia lunata*, mostly in hexane and methanolic extracts have high susceptibility. And for antibacterial activity seed cake extracts were tested against Gram-negative, Multi Drug Resistant, MDR, human pathogens(*Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella)* strains that showed slight activity but in synergistic assay these extracts when combined with antibacterial drug they gave antagonistic effect except *Klebsiella pneumonia strain*. Besides antimicrobial effect of seedcake the seed oil can be a resource for energy production i.e biodiesel. The study's findings support the notion that *C. sativa* seed oil can be used to chemically trans esterify oil to produce biodiesel. After optimization highest volumetric yield of biodiesel 84% was recorded with run with conditions: temperature 50 degrees, oil to methanol ratio 1:9, catalyst concentration 1%, RPM 600 and reaction time 90 minutes. Biological production by using whole cell catalyst lipase producing bacteria were also used and the yield obtained was almost 68% indicating that biological method is more cost effective and sustainable way for biodiesel production. Hence, the results of the current study demonstrate that the *Cannabis sativa* plant is an excellent feedstock for biorefineries.

Future Prospects

1. After further purification using a variety of methods, the crude phytochemicals isolated from seeds can be employed individually or in conjunction with other antibiotics to fight a variety of pathogenic strains.
2. After being purified from Cannbis extracts, bioactive chemicals can be employed for enzyme inhibition, cytotoxicity, and antioxidant tests both in vitro and in vivo.
3. Further optimization of biological transesterification of Hemp seed oil can also be performed either by using lipase enzyme or whole cell approach.
4. Biogas can also be produced by using the residues left after the extraction process and their effect can be checked on the stages of the anaerobic digestion.
5. It is possible to study how various pretreatments, such as ionic solvent, alkaline, and enzymatic pretreatments, affect seed cake residues (seed cake after extraction).

REFERENCES

Abbaszaadeh, A., Ghobadian, B., Omidkhah, M. R., & Najafi, G. (2012). Current biodiesel production technologies: A comparative review. *Energy Conversion and Management*, *63*, 138–148.

Afif, M. K., & Biradar, C. H. (2019). Production of biodiesel from Cannabis sativa (Hemp) seed oil and its performance and emission characteristics on DI engine fueled with biodiesel blends. *Int. Res. J. Eng. Technol*, *6*(8), 246–253.

Aftab, T. (2019). A review of medicinal and aromatic plants and their secondary metabolites status under abiotic stress. *Journal of Medicinal Plants*, *7*(3), 99–106.

Ajala, O. E., Aberuagba, F., Odetoye, T. E., & Ajala, A. M. (2015). Biodiesel: Sustainable Energy Replacement to Petroleum-Based Diesel Fuel--A Review. *ChemBioEng Reviews*, *2*(3), 145–156.

Amoah, J., Kahar, P., Ogino, C., & Kondo, A. (2019). Bioenergy and Biorefinery: Feedstock, Biotechnological Conversion, and Products. *Biotechnology Journal*, *14*(6). https://doi.org/10.1002/BIOT.201800494

Anand, U., Jacobo-Herrera, N., Altemimi, A., & Lakhssassi, N. (2019). A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites*, *9*(11), 258.

Andrei, S., Droc, G., & Stefan, G. (2019). FDA approved antibacterial drugs: 2018-2019. *Discoveries*, *7*(4).

Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., Smith, E., & Rahman, M. M. (2008). Antibacterial cannabinoids from Cannabis sativa: A structure- activity study. *Journal of Natural Products*, *71*(8), 1427–1430.

Asif, M., Salman, M. U., Anwar, S., Gul, M., & Aslam, R. (2022). Renewable and non-renewable energy resources of Pakistan and their applicability under the current scenario in Pakistan. OPEC Energy Review, 46(3), 310–339. https://doi.org/10.1111/opec.12230

Berardo, M. E. V., Mendieta, J. R., Villamonte, M. D., Colman, S. L., & Nercessian, D. (2024). Antifungal and antibacterial activities of Cannabis sativa L. resins. *Journal of Ethnopharmacology*, *318*, 116839.

Berchmans, H. J., & Hirata, S. (2008). Biodiesel production from crude Jatropha curcas L. seed oil with a high content of free fatty acids. *Bioresource Technology*, *99*(6), 1716–1721.

Bhatia, S. K., Bhatia, R. K., Jeon, J.-M., Pugazhendhi, A., Awasthi, M. K., Kumar, D., Kumar, G., Yoon, J.-J., & Yang, Y.-H. (2021). An overview on advancements in biobased transesterification methods for biodiesel production: Oil resources, extraction, biocatalysts, and process intensification technologies. *Fuel*, *285*, 119117.

Bourdy, R., & Befort, K. (2023). The Role of the Endocannabinoid System in Binge Eating Disorder. *International Journal of Molecular Sciences*, *24*(11), 9574.

Carriquiry, M. A., Du, X., & Timilsina, G. R. (2011). Second generation biofuels: Economics and policies. *Energy Policy*, *39*(7), 4222–4234.

Charlier, C. (n.d.). *Cannabis: Plant, Industry, and Ideology*.

Chavez, P. I., Sánchez, I. A., Gonzalez, F. A., Rodr\'\iguez, J. L., & Axelrod, F. (1997). Cytotoxicity correlations of Puerto Rican plants using a simplified brine shrimp lethality screening procedure. *International Journal of Pharmacognosy*, *35*(4), 222–226.

Chda, A., Mahou, Y., Znata, Y., El Fatemi, H., Boukir, A., Ananou, S., El Abida, K., & Bencheikh, R. (2023). Investigation on the gastrointestinal properties of ethanolic extract of Cannabis sativa through in vivo and in vitro approaches. *Journal of Herbmed Pharmacology*, *12*(3), 344–355.

Cheah, W. Y., Sankaran, R., Show, P. L., Ibrahim, T., Baizura, T. N., Chew, K. W., Culaba, A., & Chang, J.-S. (2020). Pretreatment methods for lignocellulosic biofuels production: current advances, challenges and future prospects. *Biofuel Research Journal*, *7*(1), 1115–1127.

Cherney, J. H., & Small, E. (2016). Industrial hemp in North America: Production, politics and potential. *Agronomy*, *6*(4), 58.

Chowdhury, H., Loganathan, B., Mustary, I., Alam, F., & Mobin, S. M. A. (2019). Algae for biofuels: the third generation of feedstock. In *Second and third generation of feedstocks* (pp. 323–344). Elsevier.

Chozhavendhan, S., Singh, M. V. P., Fransila, B., Kumar, R. P., & Devi, G. K. (2020). A review on influencing parameters of biodiesel production and purification processes. *Current Research in Green and Sustainable Chemistry*, *1*, 1–6.

Chuah, L. F., Klemeš, J. J., Bokhari, A., & Asif, S. (2021). A review of biodiesel production from renewable resources: chemical reactions. *Chemical Engineering Transactions*, *88*, 943–948.

Ciolkosz, D., & Wallace, R. (2011). A review of torrefaction for bioenergy feedstock production. Biofuels, Bioproducts and Biorefining, 5(3), 317–329. https://doi.org/10.1002/BBB.275

Crini, G., Lichtfouse, E., Chanet, G., & Morin-Crini, N. (2020). Traditional and new applications of hemp. *Sustainable Agriculture Reviews 42: Hemp Production and Applications*, 37–87.

De Faria, L., Mezey, L., & Winkler, A. (2021). Cannabis legalization and college mental health. *Current Psychiatry Reports*, *23*, 1–9.

de Medeiros Dantas, J. M., Chastel, C. F., Wolfaardt, F. J., Ghislain, T., & Lavoie, J.-M. (2023). Cannabis-based biofuels in a biorefinery approach. *Industrial Crops and Products*, *204*, 117225.

De Prato, L., Ansari, O., Hardy, G. E. S. J., Howieson, J., O'Hara, G., & Ruthrof, K. X. (2022). The cannabinoid profile and growth of hemp (Cannabis sativa L.) is influenced by tropical daylengths and temperatures, genotype and nitrogen nutrition. *Industrial Crops and Products*, *178*, 114605.

De Vita, S., Finamore, C., Chini, M. G., Saviano, G., De Felice, V., De Marino, S., Lauro, G., Casapullo, A., Fantasma, F., Trombetta, F., Bifulco, G., & Iorizzi, M. (2022). Phytochemical Analysis of the Methanolic Extract and Essential Oil from Leaves of Industrial Hemp Futura 75 Cultivar: Isolation of a New Cannabinoid Derivative and Biological Profile Using Computational Approaches. *Plants*, *11*(13). https://doi.org/10.3390/plants11131671

Echeverry, C., Reyes-Parada, M., & Scorza, C. (2021). Constituents of Cannabis sativa. *Cannabinoids and Sleep: Molecular, Functional and Clinical Aspects*, 1–9.

Freeman, T. P., Craft, S., Wilson, J., Stylianou, S., ElSohly, M., Di Forti, M., & Lynskey, M. T. (2021). Changes in delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) concentrations in cannabis over time: systematic review and meta-analysis. *Addiction*, *116*(5), 1000–1010.

Ghaly, A. E., Dave, D., Brooks, M. S., Budge, S., & others. (2010). Production of biodiesel by enzymatic transesterification. *Am J Biochem Biotechnol*, *6*(2), 54–76.

Ghelani, A. (2023). Perspectives on cannabis risks and harm reduction among youth in Early Psychosis Intervention programs: a qualitative study. *Mental Health and Social Inclusion*.

Ghosh, N., Rhithuparna, D., Rokhum, S. L., & Halder, G. (2023). Ethical issues pertaining to sustainable biodiesel synthesis over trans/esterification process. *Sustainable Chemistry and Pharmacy*, *33*, 101123.

Haq, A., Mushtaq, S., Khan, A., Islam, A., Khan, H., Malik, Z. A., Younas, F., Khan, S., Shah, A. A., & Badshah, M. (2021). Evaluation of phytochemical, bioactive, and antifungal potential of Jatropha curcas seed oil and de-oiled seed cake extracts against phytopathogenic fungi. *Journal of Plant Pathology*, *103*(3), 863–873.

Hernandes, C., Coppede, J. D. S., Bertoni, B. W., França, S. D. C., & Pereira, A. M. S. (2013). Flash microbiocide: A Rapid and Economic Method for Determination of MBC and MFC. *Am. J. Plant Sci*, *4*, 850–852.

Hoque, M. E., Singh, A., & Chuan, Y. L. (2011). Biodiesel from low cost feedstocks: The effects of process parameters on the biodiesel yield. *Biomass and Bioenergy*, *35*(4), 1582–1587.

Hossain, A. B. M. S., & Mazen, M. A. (2010). Effects of catalyst types and concentrations on biodiesel production from waste soybean oil biomass as renewable energy and environmental recycling process. *Australian Journal of Crop Science*, *4*(7), 550–555.

Isahq, M. S., Afridi, M. S., Ali, J., Hussain, M. M., Ahmad, S., & Kanwal, F. (2015). Proximate composition, phytochemical screening, GC-MS studies of biologically active cannabinoids and antimicrobial activities of Cannabis indica. *Asian Pacific Journal of Tropical Disease*, *5*(11), 897–902. https://doi.org/10.1016/S2222-1808(15)60953-7

Karas, J. A., Wong, L. J. M., Paulin, O. K. A., Mazeh, A. C., Hussein, M. H., Li, J., & Velkov, T. (2020). The antimicrobial activity of cannabinoids. *Antibiotics*, *9*(7), 406.

Kasim, F. H., & Harvey, A. P. (2011). Influence of various parameters on reactive extraction of Jatropha curcas L. for biodiesel production. *Chemical Engineering Journal*, *171*(3), 1373–1378.

Khurshid, H., Qureshi, I. A., Jahan, N., Went, T. R., Sultan, W., Sapkota, A., & Alfonso, M. (2021). A systematic review of fibromyalgia and recent advancements in treatment: is medicinal cannabis a new hope? *Cureus*, *13*(8).

Kraszkiewicz, A., Kachel, M., Parafiniuk, S., Zaj\kac, G., Niedziółka, I., & Sprawka, M. (2019). Assessment of the possibility of using hemp biomass (Cannabis sativa L.) for energy purposes: a case study. *Applied Sciences*, *9*(20), 4437.

Li, S. Y., Stuart, J. D., Li, Y., & Parnas, R. S. (2010). The feasibility of converting Cannabis sativa L. oil into biodiesel. *Bioresource Technology*, *101*(21), 8457–8460. https://doi.org/10.1016/j.biortech.2010.05.064

Lone, T. A., & Lone, R. A. (2012). “Extraction of cannabinoids from cannabis sativa L

plant and its potential antimicrobial activity." *Universal Journal of Medicine and Dentistry*, *1*(4), 51–055. https://doi.org/10.13140/RG.2.2.21906.94401

Lu, H.-C., & Mackie, K. (2021). Review of the endocannabinoid system. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, *6*(6), 607–615.

Madaras-Kelly, K. J., Ostergaard, B. E., Hovde, L. B., & Rotschafer, J. C. (1996). Twenty-four-hour area under the concentration-time curve/MIC ratio as a generic predictor of fluoroquinolone antimicrobial effect by using three strains of Pseudomonas aeruginosa and an in vitro pharmacodynamic model. *Antimicrobial Agents and Chemotherapy*, *40*(3), 627–632.

Magaldi, S., Mata-Essayag, S., De Capriles, C. H., Pérez, C., Colella, M. T., Olaizola, C., & Ontiveros, Y. (2004). Well diffusion for antifungal susceptibility testing. *International Journal of Infectious Diseases*, *8*(1), 39–45.

Malabadi, R. B., Kolkar, K. P., & Chalannavar, K. (2023). MEDICAL CANNABIS SATIVA (MARIJUANA OR DRUG TYPE): THE STORY OF DISCOVERY OF $Δ$9-TETRAHYDROCANNABINOL (THC). *International Journal of Innovation Scientific Research and Review*, *5*, 3.

Mancianti, F., & Ebani, V. V. (2020). Biological activity of essential oils. In *Molecules* (Vol. 25, Issue 3, p. 678). MDPI.

Mat Aron, N. S., Khoo, K. S., Chew, K. W., Show, P. L., Chen, W.-H., & Nguyen, T. H. P. (2020). Sustainability of the four generations of biofuels--a review. *International Journal of Energy Research*, *44*(12), 9266–9282.

Mathew, G. M., Raina, D., Narisetty, V., Kumar, V., Saran, S., Pugazhendi, A., Sindhu, R., Pandey, A., & Binod, P. (2021). Recent advances in biodiesel production: Challenges and solutions. *Science of the Total Environment*, *794*, 148751.

Mechoulam, R. (2019). The pharmacohistory of Cannabis sativa. *Cannabinoids as Therapeutic Agents*, 1–20.

Mishra, V. K., & Goswami, R. (2018). A review of production, properties and advantages of biodiesel. *Biofuels*, *9*(2), 273–289.

Morano, A., Fanella, M., Albini, M., Cifelli, P., Palma, E., Giallonardo, A. T., & Di Bonaventura, C. (2020). Cannabinoids in the treatment of epilepsy: current status and future prospects. *Neuropsychiatric Disease and Treatment*, 381–396.

Morrison, L., & Zembower, T. R. (2020). Antimicrobial resistance. *Gastrointestinal Endoscopy Clinics*, *30*(4), 619–635.

Musa, I. A. (2016). The effects of alcohol to oil molar ratios and the type of alcohol on biodiesel production using transesterification process. *Egyptian Journal of Petroleum*, *25*(1), 21–31.

Nafis, A., Kasrati, A., Jamali, C. A., Mezrioui, N., Setzer, W., Abbad, A., & Hassani, L. (2019). Antioxidant activity and evidence for synergism of Cannabis sativa (L.) essential oil with antimicrobial standards. *Industrial Crops and Products*, *137*, 396–400.

Naik, S. N., Goud, V. V, Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: a comprehensive review. *Renewable and Sustainable Energy Reviews*, *14*(2), 578–597.

Nunes, L. J. R., Causer, T. P., & Ciolkosz, D. (2020). Biomass for energy: A review on supply chain management models. In Renewable and Sustainable Energy Reviews (Vol. 120). Elsevier Ltd. https://doi.org/10.1016/j.rser.2019.109658

Pagano, C., Navarra, G., Coppola, L., Avilia, G., Bifulco, M., & Laezza, C. (2022). Cannabinoids: Therapeutic use in clinical practice. *International Journal of Molecular Sciences*, *23*(6), 3344.

Patil, P. D., & Deng, S. (2009). Optimization of biodiesel production from edible and non-edible vegetable oils. *Fuel*, *88*(7), 1302–1306.

Pattnaik, F., Nanda, S., Mohanty, S., Dalai, A. K., Kumar, V., Ponnusamy, S. K., & Naik,

S. (2022). Cannabis: Chemistry, extraction and therapeutic applications. *Chemosphere*, *289*, 133012.

Peters, L., Olson, L., Khu, D. T. K., Linnros, S., Le, N. K., Hanberger, H., Hoang, N. T. B., Tran, D. M., & Larsson, M. (2019). Multiple antibiotic resistance as a risk factor for mortality and prolonged hospital stay: a cohort study among neonatal intensive care patients with hospital-acquired infections caused by gram-negative bacteria in Vietnam. *PloS One*, *14*(5), e0215666.

Pierrehumbert, R. (2019). There is no Plan B for dealing with the climate crisis. *Bulletin of the Atomic Scientists*, *75*(5), 215–221.

Pullen, J., & Saeed, K. (2015). Investigation of the factors affecting the progress of base-catalyzed transesterification of rapeseed oil to biodiesel FAME. *Fuel Processing Technology*, *130*, 127–135.

Radwan, M. M., Chandra, S., Gul, S., & ElSohly, M. A. (2021). Cannabinoids, phenolics, terpenes and alkaloids of cannabis. *Molecules*, *26*(9), 2774.

Ramasar, V., Busch, H., Brandstedt, E., & Rudus, K. (2022). When energy justice is contested: A systematic review of a decade of research on Sweden's conflicted energy landscape. *Energy Research \& Social Science*, *94*, 102862.

Rashid, U., Bhatti, S. G., Ansari, T. M., Yunus, R., & Ibrahim, M. (2016). Biodiesel production from Cannabis sativa oil from Pakistan. *Energy Sources, Part A: Recovery, Utilization and Environmental Effects*, *38*(6), 865–875. https://doi.org/10.1080/15567036.2013.803179

Saleemi, M. A., Yahaya, N., Zain, N. N. M., Raoov, M., Yong, Y. K., Noor, N. S., & Lim, V. (2022). Antimicrobial and Cytotoxic Effects of Cannabinoids: An Updated Review with Future Perspectives and Current Challenges. *Pharmaceuticals*, *15*(10). https://doi.org/10.3390/ph15101228

Sales, M. B., Borges, P. T., Ribeiro Filho, M. N., da Silva, L. R., Castro, A. P., Sanders Lopes, A. A., de Lima, R. K., de Sousa Rios, M. A., & Santos, J. C. S. dos. (2022).

Sustainable feedstocks and challenges in biodiesel production: An advanced bibliometric analysis. *Bioengineering*, *9*(10), 539.

Setti, L., Samaei, S. P., Maggiore, I., Nissen, L., Gianotti, A., & Babini, E. (2020). Comparing the effectiveness of three different biorefinery processes at recovering bioactive products from hemp (Cannabis sativa L.) byproduct. *Food and Bioprocess Technology*, *13*, 2156–2171.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*, *113*(4), 1202–1205.

Singh, D., Sharma, D., Soni, S. L., Sharma, S., Sharma, P. K., & Jhalani, A. (2020). A review on feedstocks, production processes, and yield for different generations of biodiesel. *Fuel*, *262*, 116553.

Small, E. (2015). Evolution and classification of Cannabis sativa (marijuana, hemp) in relation to human utilization. *The Botanical Review*, *81*, 189–294.

Small, E., Pocock, T., & Cavers, P. B. (2003). The biology of Canadian weeds. 119. Cannabis sativa L. *Canadian Journal of Plant Science*, *83*(1), 217–237.

Sorrentino, G. (2021). Introduction to emerging industrial applications of cannabis (Cannabis sativa L.). *Rendiconti Lincei. Scienze Fisiche e Naturali*, *32*(2), 233–243.

Stella, N. (2023). THC and CBD: Similarities and differences between siblings. *Neuron*.

Vasudevan, P. T., & Briggs, M. (2008). Biodiesel production—current state of the art and challenges. *Journal of Industrial Microbiology and Biotechnology*, *35*(5), 421.

Viana, M. de B., Aquino, P. E. A. de, Estadella, D., Ribeiro, D. A., & Viana, G. S. de B. (2022). Cannabis sativa and Cannabidiol: A Therapeutic Strategy for the Treatment of Neurodegenerative Diseases? *Medical Cannabis and Cannabinoids*, *5*(1), 207–219.

Walker, M. J., Burns, D. T., Axford, I., & Moss, G. P. (2021). Cannabinoids—a tutorial review psychoactivity, regulation, common and IUPAC nomenclature, structures

and abbreviations in relation to cannabidiol (CBD) products. *J. Assoc. Publ. Analysts*, *49*, 1–28.

Withanarachchie, V., Rychert, M., & Wilkins, C. (2023). Barriers and facilitators to prescribing medicinal cannabis in New Zealand. *Journal of Primary Health Care*.

Xie, Z., Mi, Y., Kong, L., Gao, M., Chen, S., Chen, W., Meng, X., Sun, W., Chen, S., & Xu, Z. (2023). Cannabis sativa: origin and history, glandular trichome development, and cannabinoid biosynthesis. *Horticulture Research*, uhad150.

Abbaszaadeh, A., Ghobadian, B., Omidkhah, M. R., & Najafi, G. (2012). Current biodiesel production technologies: A comparative review. *Energy Conversion and Management*, *63*, 138–148.

Afif, M. K., & Biradar, C. H. (2019). Production of biodiesel from Cannabis sativa (Hemp) seed oil and its performance and emission characteristics on DI engine fueled with biodiesel blends. *Int. Res. J. Eng. Technol*, *6*(8), 246–253.

Aftab, T. (2019). A review of medicinal and aromatic plants and their secondary metabolites status under abiotic stress. *Journal of Medicinal Plants*, *7*(3), 99–106.

Ajala, O. E., Aberuagba, F., Odetoye, T. E., & Ajala, A. M. (2015). Biodiesel: Sustainable Energy Replacement to Petroleum-Based Diesel Fuel--A Review. *ChemBioEng Reviews*, *2*(3), 145–156.

Anand, U., Jacobo-Herrera, N., Altemimi, A., & Lakhssassi, N. (2019). A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites*, *9*(11), 258.

Andrei, S., Droc, G., & Stefan, G. (2019). FDA approved antibacterial drugs: 2018-2019. *Discoveries*, *7*(4).

Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., Smith, E., & Rahman, M. M. (2008). Antibacterial cannabinoids from Cannabis sativa: A structure- activity study. *Journal of Natural Products*, *71*(8), 1427–1430.

Berardo, M. E. V., Mendieta, J. R., Villamonte, M. D., Colman, S. L., & Nercessian, D. (2024). Antifungal and antibacterial activities of Cannabis sativa L. resins. *Journal of Ethnopharmacology*, *318*, 116839.

Berchmans, H. J., & Hirata, S. (2008). Biodiesel production from crude Jatropha curcas L. seed oil with a high content of free fatty acids. *Bioresource Technology*, *99*(6), 1716–1721.

Bhatia, S. K., Bhatia, R. K., Jeon, J.-M., Pugazhendhi, A., Awasthi, M. K., Kumar, D., Kumar, G., Yoon, J.-J., & Yang, Y.-H. (2021). An overview on advancements in biobased transesterification methods for biodiesel production: Oil resources, extraction, biocatalysts, and process intensification technologies. *Fuel*, *285*, 119117.

Bourdy, R., & Befort, K. (2023). The Role of the Endocannabinoid System in Binge Eating Disorder. *International Journal of Molecular Sciences*, *24*(11), 9574.

Carriquiry, M. A., Du, X., & Timilsina, G. R. (2011). Second generation biofuels: Economics and policies. *Energy Policy*, *39*(7), 4222–4234.

Charlier, C. (n.d.). *Cannabis: Plant, Industry, and Ideology*.

Chavez, P. I., Sánchez, I. A., Gonzalez, F. A., Rodr\'\iguez, J. L., & Axelrod, F. (1997). Cytotoxicity correlations of Puerto Rican plants using a simplified brine shrimp lethality screening procedure. *International Journal of Pharmacognosy*, *35*(4), 222–226.

Chda, A., Mahou, Y., Znata, Y., El Fatemi, H., Boukir, A., Ananou, S., El Abida, K., & Bencheikh, R. (2023). Investigation on the gastrointestinal properties of ethanolic extract of Cannabis sativa through in vivo and in vitro approaches. *Journal of Herbmed Pharmacology*, *12*(3), 344–355.

Cheah, W. Y., Sankaran, R., Show, P. L., Ibrahim, T., Baizura, T. N., Chew, K. W., Culaba, A., & Chang, J.-S. (2020). Pretreatment methods for lignocellulosic biofuels production: current advances, challenges and future prospects. *Biofuel Research Journal*, *7*(1), 1115–1127.

Cherney, J. H., & Small, E. (2016). Industrial hemp in North America: Production, politics and potential. *Agronomy*, *6*(4), 58.

Chowdhury, H., Loganathan, B., Mustary, I., Alam, F., & Mobin, S. M. A. (2019). Algae for biofuels: the third generation of feedstock. In *Second and third generation of feedstocks* (pp. 323–344). Elsevier.

Chozhavendhan, S., Singh, M. V. P., Fransila, B., Kumar, R. P., & Devi, G. K. (2020). A review on influencing parameters of biodiesel production and purification processes. *Current Research in Green and Sustainable Chemistry*, *1*, 1–6.

Chuah, L. F., Klemeš, J. J., Bokhari, A., & Asif, S. (2021). A review of biodiesel production from renewable resources: chemical reactions. *Chemical Engineering Transactions*, *88*, 943–948.

Crini, G., Lichtfouse, E., Chanet, G., & Morin-Crini, N. (2020). Traditional and new applications of hemp. *Sustainable Agriculture Reviews 42: Hemp Production and Applications*, 37–87.

De Faria, L., Mezey, L., & Winkler, A. (2021). Cannabis legalization and college mental health. *Current Psychiatry Reports*, *23*, 1–9.

de Medeiros Dantas, J. M., Chastel, C. F., Wolfaardt, F. J., Ghislain, T., & Lavoie, J.-M. (2023). Cannabis-based biofuels in a biorefinery approach. *Industrial Crops and Products*, *204*, 117225.

De Prato, L., Ansari, O., Hardy, G. E. S. J., Howieson, J., O'Hara, G., & Ruthrof, K. X. (2022). The cannabinoid profile and growth of hemp (Cannabis sativa L.) is influenced by tropical daylengths and temperatures, genotype and nitrogen nutrition. *Industrial Crops and Products*, *178*, 114605.

De Vita, S., Finamore, C., Chini, M. G., Saviano, G., De Felice, V., De Marino, S., Lauro, G., Casapullo, A., Fantasma, F., Trombetta, F., Bifulco, G., & Iorizzi, M. (2022). Phytochemical Analysis of the Methanolic Extract and Essential Oil from Leaves of Industrial Hemp Futura 75 Cultivar: Isolation of a New Cannabinoid

Derivative and Biological Profile Using Computational Approaches. *Plants*, *11*(13). https://doi.org/10.3390/plants11131671

Echeverry, C., Reyes-Parada, M., & Scorza, C. (2021). Constituents of Cannabis sativa. *Cannabinoids and Sleep: Molecular, Functional and Clinical Aspects*, 1–9.

Freeman, T. P., Craft, S., Wilson, J., Stylianou, S., ElSohly, M., Di Forti, M., & Lynskey, M. T. (2021). Changes in delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) concentrations in cannabis over time: systematic review and meta-analysis. *Addiction*, *116*(5), 1000–1010.

Ghaly, A. E., Dave, D., Brooks, M. S., Budge, S., & others. (2010). Production of biodiesel by enzymatic transesterification. *Am J Biochem Biotechnol*, *6*(2), 54–76.

Ghelani, A. (2023). Perspectives on cannabis risks and harm reduction among youth in Early Psychosis Intervention programs: a qualitative study. *Mental Health and Social Inclusion*.

Ghosh, N., Rhithuparna, D., Rokhum, S. L., & Halder, G. (2023). Ethical issues pertaining to sustainable biodiesel synthesis over trans/esterification process. *Sustainable Chemistry and Pharmacy*, *33*, 101123.

Haq, A., Mushtaq, S., Khan, A., Islam, A., Khan, H., Malik, Z. A., Younas, F., Khan, S., Shah, A. A., & Badshah, M. (2021). Evaluation of phytochemical, bioactive, and antifungal potential of Jatropha curcas seed oil and de-oiled seed cake extracts against phytopathogenic fungi. *Journal of Plant Pathology*, *103*(3), 863–873.

Hernandes, C., Coppede, J. D. S., Bertoni, B. W., França, S. D. C., & Pereira, A. M. S. (2013). Flash microbiocide: A Rapid and Economic Method for Determination of MBC and MFC. *Am. J. Plant Sci*, *4*, 850–852.

Hoque, M. E., Singh, A., & Chuan, Y. L. (2011). Biodiesel from low cost feedstocks: The effects of process parameters on the biodiesel yield. *Biomass and Bioenergy*, *35*(4), 1582–1587.

Hossain, A. B. M. S., & Mazen, M. A. (2010). Effects of catalyst types and concentrations on biodiesel production from waste soybean oil biomass as renewable energy and environmental recycling process. *Australian Journal of Crop Science*, *4*(7), 550–555.

Isahq, M. S., Afridi, M. S., Ali, J., Hussain, M. M., Ahmad, S., & Kanwal, F. (2015). Proximate composition, phytochemical screening, GC-MS studies of biologically active cannabinoids and antimicrobial activities of Cannabis indica. *Asian Pacific Journal of Tropical Disease*, *5*(11), 897–902. https://doi.org/10.1016/S2222-1808(15)60953-7

Karas, J. A., Wong, L. J. M., Paulin, O. K. A., Mazeh, A. C., Hussein, M. H., Li, J., & Velkov, T. (2020). The antimicrobial activity of cannabinoids. *Antibiotics*, *9*(7), 406.

Kasim, F. H., & Harvey, A. P. (2011). Influence of various parameters on reactive extraction of Jatropha curcas L. for biodiesel production. *Chemical Engineering Journal*, *171*(3), 1373–1378.

Khurshid, H., Qureshi, I. A., Jahan, N., Went, T. R., Sultan, W., Sapkota, A., & Alfonso, M. (2021). A systematic review of fibromyalgia and recent advancements in treatment: is medicinal cannabis a new hope? *Cureus*, *13*(8).

Kraszkiewicz, A., Kachel, M., Parafiniuk, S., Zaj\kac, G., Niedziółka, I., & Sprawka, M. (2019). Assessment of the possibility of using hemp biomass (Cannabis sativa L.) for energy purposes: a case study. *Applied Sciences*, *9*(20), 4437.

Li, S. Y., Stuart, J. D., Li, Y., & Parnas, R. S. (2010). The feasibility of converting Cannabis sativa L. oil into biodiesel. *Bioresource Technology*, *101*(21), 8457–8460. https://doi.org/10.1016/j.biortech.2010.05.064

Lone, T. A., & Lone, R. A. (2012). "Extraction of cannabinoids from cannabis sativa L plant and its potential antimicrobial activity." *Universal Journal of Medicine and Dentistry*, *1*(4), 51–055. https://doi.org/10.13140/RG.2.2.21906.94401

Lu, H.-C., & Mackie, K. (2021). Review of the endocannabinoid system. *Biological*

Psychiatry: Cognitive Neuroscience and Neuroimaging, *6*(6), 607–615.

Madaras-Kelly, K. J., Ostergaard, B. E., Hovde, L. B., & Rotschafer, J. C. (1996). Twenty-four-hour area under the concentration-time curve/MIC ratio as a generic predictor of fluoroquinolone antimicrobial effect by using three strains of Pseudomonas aeruginosa and an in vitro pharmacodynamic model. *Antimicrobial Agents and Chemotherapy*, *40*(3), 627–632.

Magaldi, S., Mata-Essayag, S., De Capriles, C. H., Pérez, C., Colella, M. T., Olaizola, C., & Ontiveros, Y. (2004). Well diffusion for antifungal susceptibility testing. *International Journal of Infectious Diseases*, *8*(1), 39–45.

Malabadi, R. B., Kolkar, K. P., & Chalannavar, K. (2023). MEDICAL CANNABIS SATIVA (MARIJUANA OR DRUG TYPE): THE STORY OF DISCOVERY OF $Δ$9-TETRAHYDROCANNABINOL (THC). *International Journal of Innovation Scientific Research and Review*, *5*, 3.

Mancianti, F., & Ebani, V. V. (2020). Biological activity of essential oils. In *Molecules* (Vol. 25, Issue 3, p. 678). MDPI.

Mat Aron, N. S., Khoo, K. S., Chew, K. W., Show, P. L., Chen, W.-H., & Nguyen, T. H. P. (2020). Sustainability of the four generations of biofuels--a review. *International Journal of Energy Research*, *44*(12), 9266–9282.

Mathew, G. M., Raina, D., Narisetty, V., Kumar, V., Saran, S., Pugazhendi, A., Sindhu, R., Pandey, A., & Binod, P. (2021). Recent advances in biodiesel production: Challenges and solutions. *Science of the Total Environment*, *794*, 148751.

Mechoulam, R. (2019). The pharmacohistory of Cannabis sativa. *Cannabinoids as Therapeutic Agents*, 1–20.

Mishra, V. K., & Goswami, R. (2018). A review of production, properties and advantages of biodiesel. *Biofuels*, *9*(2), 273–289.

Morano, A., Fanella, M., Albini, M., Cifelli, P., Palma, E., Giallonardo, A. T., & Di

Bonaventura, C. (2020). Cannabinoids in the treatment of epilepsy: current status and future prospects. *Neuropsychiatric Disease and Treatment*, 381–396.

Morrison, L., & Zembower, T. R. (2020). Antimicrobial resistance. *Gastrointestinal Endoscopy Clinics*, *30*(4), 619–635.

Musa, I. A. (2016). The effects of alcohol to oil molar ratios and the type of alcohol on biodiesel production using transesterification process. *Egyptian Journal of Petroleum*, *25*(1), 21–31.

Nafis, A., Kasrati, A., Jamali, C. A., Mezrioui, N., Setzer, W., Abbad, A., & Hassani, L. (2019). Antioxidant activity and evidence for synergism of Cannabis sativa (L.) essential oil with antimicrobial standards. *Industrial Crops and Products*, *137*, 396–400.

Naik, S. N., Goud, V. V, Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: a comprehensive review. *Renewable and Sustainable Energy Reviews*, *14*(2), 578–597.

Pagano, C., Navarra, G., Coppola, L., Avilia, G., Bifulco, M., & Laezza, C. (2022). Cannabinoids: Therapeutic use in clinical practice. *International Journal of Molecular Sciences*, *23*(6), 3344.

Patil, P. D., & Deng, S. (2009). Optimization of biodiesel production from edible and non-edible vegetable oils. *Fuel*, *88*(7), 1302–1306.

Pattnaik, F., Nanda, S., Mohanty, S., Dalai, A. K., Kumar, V., Ponnusamy, S. K., & Naik, S. (2022). Cannabis: Chemistry, extraction and therapeutic applications. *Chemosphere*, *289*, 133012.

Peters, L., Olson, L., Khu, D. T. K., Linnros, S., Le, N. K., Hanberger, H., Hoang, N. T. B., Tran, D. M., & Larsson, M. (2019). Multiple antibiotic resistance as a risk factor for mortality and prolonged hospital stay: a cohort study among neonatal intensive care patients with hospital-acquired infections caused by gram-negative bacteria in Vietnam. *PloS One*, *14*(5), e0215666.

Pierrehumbert, R. (2019). There is no Plan B for dealing with the climate crisis. *Bulletin of the Atomic Scientists*, *75*(5), 215–221.

Pullen, J., & Saeed, K. (2015). Investigation of the factors affecting the progress of base-catalyzed transesterification of rapeseed oil to biodiesel FAME. *Fuel Processing Technology*, *130*, 127–135.

Radwan, M. M., Chandra, S., Gul, S., & ElSohly, M. A. (2021). Cannabinoids, phenolics, terpenes and alkaloids of cannabis. *Molecules*, *26*(9), 2774.

Ramasar, V., Busch, H., Brandstedt, E., & Rudus, K. (2022). When energy justice is contested: A systematic review of a decade of research on Sweden's conflicted energy landscape. *Energy Research \& Social Science*, *94*, 102862.

Rashid, U., Bhatti, S. G., Ansari, T. M., Yunus, R., & Ibrahim, M. (2016). Biodiesel production from Cannabis sativa oil from Pakistan. *Energy Sources, Part A: Recovery, Utilization and Environmental Effects*, *38*(6), 865–875. https://doi.org/10.1080/15567036.2013.803179

Saleemi, M. A., Yahaya, N., Zain, N. N. M., Raoov, M., Yong, Y. K., Noor, N. S., & Lim, V. (2022). Antimicrobial and Cytotoxic Effects of Cannabinoids: An Updated Review with Future Perspectives and Current Challenges. *Pharmaceuticals*, *15*(10). https://doi.org/10.3390/ph15101228

Sales, M. B., Borges, P. T., Ribeiro Filho, M. N., da Silva, L. R., Castro, A. P., Sanders Lopes, A. A., de Lima, R. K., de Sousa Rios, M. A., & Santos, J. C. S. dos. (2022). Sustainable feedstocks and challenges in biodiesel production: An advanced bibliometric analysis. *Bioengineering*, *9*(10), 539.

Setti, L., Samaei, S. P., Maggiore, I., Nissen, L., Gianotti, A., & Babini, E. (2020). Comparing the effectiveness of three different biorefinery processes at recovering bioactive products from hemp (Cannabis sativa L.) byproduct. *Food and Bioprocess Technology*, *13*, 2156–2171.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*,

113(4), 1202–1205.

Singh, D., Sharma, D., Soni, S. L., Sharma, S., Sharma, P. K., & Jhalani, A. (2020). A review on feedstocks, production processes, and yield for different generations of biodiesel. *Fuel*, *262*, 116553.

Small, E. (2015). Evolution and classification of Cannabis sativa (marijuana, hemp) in relation to human utilization. *The Botanical Review*, *81*, 189–294.

Small, E., Pocock, T., & Cavers, P. B. (2003). The biology of Canadian weeds. 119. Cannabis sativa L. *Canadian Journal of Plant Science*, *83*(1), 217–237.

Sorrentino, G. (2021). Introduction to emerging industrial applications of cannabis (Cannabis sativa L.). *Rendiconti Lincei. Scienze Fisiche e Naturali*, *32*(2), 233–243.

Stella, N. (2023). THC and CBD: Similarities and differences between siblings. *Neuron*.

Vasudevan, P. T., & Briggs, M. (2008). Biodiesel production—current state of the art and challenges. *Journal of Industrial Microbiology and Biotechnology*, *35*(5), 421.

Viana, M. de B., Aquino, P. E. A. de, Estadella, D., Ribeiro, D. A., & Viana, G. S. de B. (2022). Cannabis sativa and Cannabidiol: A Therapeutic Strategy for the Treatment of Neurodegenerative Diseases? *Medical Cannabis and Cannabinoids*, *5*(1), 207–219.

Walker, M. J., Burns, D. T., Axford, I., & Moss, G. P. (2021). Cannabinoids—a tutorial review psychoactivity, regulation, common and IUPAC nomenclature, structures and abbreviations in relation to cannabidiol (CBD) products. *J. Assoc. Publ. Analysts*, *49*, 1–28.

Wang, H., Lei, Z., Zhang, X., Zhou, B., & Peng, J. (2019). A review of deep learning for renewable energy forecasting. In Energy Conversion and Management (Vol. 198). Elsevier Ltd. https://doi.org/10.1016/j.enconman.2019.111799

Withanarachchie, V., Rychert, M., & Wilkins, C. (2023). Barriers and facilitators to prescribing medicinal cannabis in New Zealand. *Journal of Primary Health Care*.

Xie, Z., Mi, Y., Kong, L., Gao, M., Chen, S., Chen, W., Meng, X., Sun, W., Chen, S., & Xu, Z. (2023). Cannabis sativa: origin and history, glandular trichome development, and cannabinoid biosynthesis. *Horticulture Research*, uhad150.

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