Synthesis and biological evaluation of metallic complexes of crude extracts of *Bergenia ciliata* and *Euphorbia wallichii*

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2013

Synthesis and biological evaluation of metallic complexes of crude extracts of *Bergenia ciliata* and *Euphorbia wallichii*

Submitted by

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Thesis Submitted to

Department of Biochemistry

Quaid-i-Azam University, Islamabad

In the partial fulfillment of the requirements for the degree of

Master of Philosophy

in

Biochemistry

Department of Biochemistry

Quaid-i-Azam University

Islamabad, Pakistan

2013

APPROVAL CERTIFICATE

This is to certify that the dissertation entitled **"Synthesis and biological evaluation of metallic complexes of crude extracts of *Bergenia ciliata* and *Euphorbia wallichii*"** submitted by **Abdul Rehman** is accepted in its present form by the Department of Biochemistry, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of M.Phil in **Biochemistry.**

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بسم الله الرحمن الرحيم

Dedicated

to my

loving parents

and

kind teachers

Dated: _____

Content

ACKNOWLEDGEMENT

Countless thanks to Almighty  **,** Creator, Omnipotent, Worthy of all praises, who helps in difficulties and director in darkness. All respect for Holy Prophet Muhammad (PBUH) who is forever source of knowledge and guidance of entire humanity.

I am expressing my sincere and unforgettable gratitude to Dr. Ihsan-ul-Haq, Department of Pharmacy, Quaid-i-Azam University Islamabad, Pakistan for continuous encouragement and sincere criticism throughout the study. Whenever there was any hurdle in the way, he was there to support and facilitate me. I am forever indebted to him.

I am thankful to Dr. Bushra Mirza, Professor and Chairperson, Department of Biochemistry, Quaid-i-Azam University Islamabad for research facilities to accomplish this work.

I am grateful to Dr. Muhammad Zia for facilitating to carry out research and remain kind and helping during research. I am also grateful to Mr. Muneer Ahmed and Dr. Nighat for their valuable suggestions and moral support during research.

I would like to extend my sincere thankfulness to those people, who helped me in one way or another during research. I worked with and wish to convey my gratitude to all of them specially Attarad Ali, Madiha Ahmed, Humaira Fatima and Tanveer Ahmed. Some of my academic colleagues and friends Waqas Ahmed Kiani, Mudassir Sardar, Atta Rasool, Altaf Hussain Shah, Akhtar Ali, Zahir, Hanif, Abdul Rehman, Muneer, Zeeshan, Majid, Sadam, Waqar, Muzfar, Shahab deserve special thanks for their inseparable support and wishes.

I would like to pay special tribute to my family as their love and devoted support provided me a great source of satisfaction. I owe non payable debt to my loving father Dr. Saeed Ahmed, mother Shahar Bano, uncle M. Panah and brother Abdul Manan. Their wishes motivate me to strive for higher studies. Without their understanding and continuous support, it would have been impossible for me to carry out this study. My special gratitude is for my grandmother, my sisters and brothers.

Finally I am thankful to all those, involved directly or indirectly in the accomplishment of this work.

Abdul Rehman

ml	Millilitre
mg	Milligram
^{o}C	Degree centigrade
EW	*Euphorbia wallichii*
BC	*Bergenia ciliate*
Al	Aluminium
Cd	Cadmium
Lan	Lanthium
Sn	Tin
Bi	Bismuth
Co	Cobolt
Pb	Plumbic (lead)
Mo	Molbdate
Cs	Cesium
Li	Lithium
Hg	Mercuric
Fs	Ferrus
Fc	Ferric
Ag	Silver
CME	Crude Methanolic Extract
DMSO	Dimethyl sulfoxide
DPPH	2’2-diphenyl-1-picrylhydrazyl
FTIR	Fourier Tranform infrared
LD_{50}	Concentration at which 50% shrimp die
$^{\bullet}OH$	Hydroxyl free radical
M. luteus	*Micrococcus luteus*
B. bronchiseptica	*Bordetella bronchiseptica*
E. coli	*Escherichia coli*
S. typhimurium	*Salmonella typhimurium*
A. aerogens	*Anterobacter aerogens*
A. niger	*Aspergillus niger*
A. fumigatus	*Aspergilus fumigatus*
A. flavus	*Aspergilus flavus*
F. solani	*Fusarium solani*

Abstract

Secondary metabolite obtained from plants play important role in treatment of various diseases, both in natural or coordinated forms. Metals combined with such compounds play key role in treatment strategies of different ailments. Current study deals with biological evaluation of fourteen metallic complexes, synthesized using extracts of *Bergenia ciliata* (BC) and *Euphorbia wallichii* (EW). Complexes showed increased reducing potential whereas decreased activity was observed in DPPH scavenging assay and total antioxidant assay. Cytotoxic potential of complexes was evaluated by performing brine shrimp assay. EWHg (LD_{50}= 96 µg/ml) and BCFs (LD_{50}=58 µg/ml) complexes showed higher cytotoxic potential comparative to their extracts. Two gram positive bacterial strains i.e. *M. luteus* ATCC 10240, *S. aureus* ATCC 6538 and four gram negative strains i.e. *A. aerogens* ATCC 13048, *E. coli* ATCC 15224, *S. typhimurium* ATCC 14028 and *B. bronchiseptica* ATCC 4617 were used for evaluation of antibacterial activity of complexes. EWHg complex was active against five bacterial strains and exhibited maximum inhibition zone (16±1.5 mm) among EW complexes. BCHg showed highest activity among BC complexes and produced inhibition zone of 17±0.58 mm against *M.luteus*. Five fungal strains, *A. fumigatus* (FCBP 66), *A. niger* (FCBP 0198), *F. solani* (FCBP 0291), *A. flavus* (FCBP 0064), and *Mucor* species (FCBP 0300) were used in present study to evaluate the antifungal potential of complexes. EWFs produced maximum zone (10±0.2 mm) against *A. flavus*. Five BC complexes were found to be active against *A. niger* and *A. fumigatus*. BCAl produced maximum zone of inhibition (16±2.0 mm) against *A. niger*. All complexes were evaluated for protein kinase inhibition potential in which, EWCd, EWCs, BCFs and BCFc complexes produced zones of inhibition ranging from 9 mm to 16 mm. FTIR of extracts and complexes was also performed which suggested the presences of various phytochemicals such as hydrocarbons, terpenoids, abietane steroids, aromaticeasters, phenolics, flavone glycosides and several bioactive constituents.

INTRODUCTION

1.1. Plants in drug discovery and as potential alternative therapies

Plants being natural sources have been used to cure most of the diseases and to maintain good health since ancient times. The World Health Organization (WHO) has estimated about US $14 billion demand for medicinal plants per year. Approximately 15 to 20% annual growth has also been assessed in the demand of medicinal plants based raw materials, which will increase up to US $5 trillion till 2050 (Kala *et al.,* 2006; Verma *et al.,* 2011). Various drugs and their derivatives present in current pharmacopoeias have been originated from plants. Twenty eight percent of chemical entities launched during last 20 years were comprised of natural products or their derivatives (Goyal *et al*., 2008).

Drug discovery from natural sources undoubtedly started in prehistoric times, that led to discovery of many compounds as digoxin from *Digitals spp.*, atropine from *Atropa belladonna*, morphine and codeine from *Papavor somniferum*, quinine and quinidine from *Cinchona* and vincristine and vinblastine from *Catharanthus roseus* (Rates, 2000). Many of the present medicines have been developed on the basis of traditionally used therapies (Rates, 2000). Natural products are the significant sources of new drugs particularly in the field of oncological and anti-hypertensive therapeutics. Due to better pharmacological responses drug obtained from plants constitute 25% of worldwide prescribed drugs (Rates, 2000; Verpoorte *et al*., 2006).

Botanical medicines are sharing a large market in the form of food, drug and dietary supplements. Plants have been used to cure numerous diseases for thousands of years and are grouped as complementary/alternative medicines (CAM) in western countries (Verpoorte *et al.,* 2006). Recent trend of taking complementary and alternative medicines is increasing, in order to relieve the symptoms because of perceived side effects of chemical drugs (Verpoorte *et al.,* 2006). Revolution in the field of organic chemistry has led towards the production of synthetic compounds for pharmacological treatment. Structural modification in pure compounds produces potent, safe and more active drugs which also increases the economy of pharmaceutical companies (Petrovick *et al*., 1997; Sharapin, 1997; Rates, 2000).

1.2. Coordination compounds

Metal complexes have essential role in biological systems. Biological compounds like carboxy-peptidase, hemoglobin and chlorophyll are metallic complexes containing zinc, iron and magnesium metals (Jain *et al.,* 2009). Alizarin dye is a bright red

coordination compound that was initially used by Egyptians and Persians. Modern coordination chemistry began to develop in 1798 by B. M. Tassaert. He observed brownish color of cobalt chloride in ammoniacal solution (Martell and Calvin, 1987). Number of theories regarding complexes properties and synthesis were originated during 19^{th} century like Chain theory by Christian Wilhelm Blomstrand (1869) and Alfred Werner's coordination theory (1893) (McCleverty and Meyer, 2003; Martell and Calvin, 1987).

1.2.1. Properties of coordination complex

i. Coordination number

Extensive studies on coordination compound reveal the presence of certain useful properties and unusual chemical nature. Nature of complex depends upon central metal atom, oxidation state, kind, number and ligand arrangement (Basolo and Johnson, 2003). Werner gave the concept of coordination number and describes ligand arrangement around metal. Number of bonds donated from ligand to metal atom is coordination number. Usually it ranges from 2 to 12. Tetra and hexa-coordinate are common (Basolo and Johnson, 2003).

ii. Ligands and chelates

In 1916 Alfred Stock (German chemist) proposed the term ligand and these can be mono, di or polydenate involving 1, 2 or many atoms respectively. Polydenate ligand containing complex are chelates. These are stable, useful and their synthesis is termed as chelation (McCleverty and Meyer, 2003). Coordination complexes having metals with valence electrons up to 18, are most stable. In 1920s, Gilbert N. Lewis (physical chemist, USA) described the coordinate bond. It is denoted by arrow from donor to acceptor (Basolo and Johnson, 2003).

1.2.2. Organic metal complex

Metals are the important parts of medicines, being used by humans for many years. Significance of essential metals has recently been realized, because varying quantities of these are needed in our diet (Jain *et al.,* 2009). On the other hand, certain metals are toxic even in trace amount and can leads to metal toxicity (Tripathi, 2009). Metal complexes are of different types. On the basis of charge these are negative, positive and neutral metallic complexes. In these complexes metal is coordinated by ligands (Anacona *et al*., 2010). Organometallic complexes are the result of combination of metal atoms and organic groups as ligand. Various organometallic complexes are present naturally as myoglobin, hemoglobin and methyl-cobalamin (Vitamin B type).

Properties of these complexes are in between ionic and covalent nature, because of metal-carbon bonding.

They can be divided into two general classes:

- Metal-unsaturated hydrocarbon π-bond containing complexes (C-C multiple bonds)
- Metal-carbon σ bonds containing complexes

In these organometallic complexes, the pi orbitals of the organic matter connect metal as as dibenzene-chromium or ferrocene (Basolo and Johnson, 2003).

1.2.3. Synthesis of metallic complexes (Coordination complexes)

Metallo-complexes can be synthesized through various methods. Direct combination of specific ligand and metal ion led to production of hexa-ammimine-nickel (2^{+}) ion through the series of reactions in aqueous solution of nickel salt and in the presence of ammonia. Finally precipitated sulphate salt ($[Ni(NH_3)_6]SO_4$) is obtained. Similarly in the presence of oxygen aqueous solution of cobalt salt and ammonia produce hexaamminecobalt(3+), $[Co(NH_3)_6]^{3+}$ as cobalt-ammine complex (Basolo and Johnson, 2003). Halide coordination compounds can be synthesized in solution of two halide salts in appropriate solvent or in molten state. For instance potassium tetrachloropalladate can be prepared by the reaction of potassium chloride and palladium chloride.

$PdCl_2 + 2\ KCl \rightarrow K_2[PdCl_4]$.

Treatment of particular precursor with specific reducing agents such as hydrazine or molecular hydrogen results in the synthesis of hydrido complexes of transition metal which are given as;

$2[Co(CN)_5]^{3-} + H_2 \rightarrow 2[CoH(CN)_5]^{3-}$

(Martell and Calvin, 1987; Basolo and Johnson, 2003).

1.3. Role of metallic complex in drug discovery

Medicinal utility of metals can be traced up to 5000 years back (Orvig *et al*., 1999)**.** The splendid array of metal complexes applications has been investigated in pharmaceutics (Sadler *et al*, 1999; Ali & van Lier, 1999; Louie & Meade, 1999; Volkert & Hoffman, 1999; Sakurai *et al*, 2002). Humans' well-being is affected in various ways by metal ions. Applications of metals in pharmaceutical substances are increasing rapidly due to their enhanced compatibility (Smith-Warner *et al*., 2000; Cragg *et al*., 2000). Administered metal complexes undergo redox reactions and ligand substitutions, which produce active biotransformation product. Metal

compounds can be used effectively as drugs, upon identification of their active species (Sadler and Guo, 1998).

Metal complexes are utilized in pharmaceutical preparation due to their potential applicability, several medicinal preparations have been investigated. Various reviews had focused on advances in the field of coordination compounds (Sakurai *et al*, 2002). Metals combines with ligands to form complexes and if ligand forms more rings, then resultant product will be chelate (Wayne, 1999). These metallo complexes had been found to possess importance in different fields, like biomedical, analytical, therapeutic, synthetic applications for example gold complexes (Auranofin etc.). These were important anti-arthritic drugs and capable of tumor cells inhibition during *in vitro* testing (Mckeage, 2002). Gromer *et al*. (1998) has found auranofin as potent inhibitor of enzyme thioredoxin reductase, which is involved in mitochondrial membrane permeability by redox control.

Metal complexes could be used for the treatment of cancer for example antibiotic bleomycin cut the DNA strand by synthesizing intermediate metal complex, having iron or copper as cofactor (Perry, 2002; Dorr & Von Hoff, 1994). Tetracyclines that are well known antibiotics are chelators of Ca^{2+} and Hg^{2+} ions (Venkateswarlu and Sailaja, 2011). Second generation platinum drugs are used for the cancer treatment like carboplatin, cisplatin (Kostova, 2006) and cisplatin tri peptide conjugate is used for platinum drug resistant tumor. Ruthenium complexes having ligand dimethylsulfoxides, amine and N-heterocyclesare have been to found to possess *in vivo* antitumor activity (Farkas & Sovago, 2002).

Antiviral agents are also complexes in their nature. Currently these complexes are found to possess enzyme inhibition activity for example, Ruthenium poly-aminocarboxylate complex are inhibitors of cysteine proteases. Enzyme inhibition activity of these complexes could be a suitable route for finding new antiviral substances and their utilization to inhibit diseases from progression (Chaterjee *et al*, 2006). Ruthenium complexes are also nitric oxide scavengers (Farkas & Sovago, 2002; Sangeeta *et al.*, 2009).

1.4. Plants used in current study

Extracts of two plant species were used in the current study. Their brief introduction is given as under;

1.4.1. *Bergenia Cilliata*

i. Taxonomy

Taxonomical classification of Bergenia ciliata is given bellow

Kingdom	Plantae
Subkingdom	Viridiaeplantae
Division	Tracheophyta
Class	Mangoliopsida
Order	Saxifragalea
Family	Saxifragaceae
Genus	*Bergenia*
Species	*Bergenia ciliata*
Common name	Urdu. Zakhm-e-hayat Arbic. Bergenia-Mehdiyata (Ghani *et al.,* 1996)

Fig1.1: *Bergenia ciliata* plant

Bergenia ciliata is the member of *Saxifragaceae* family. This family is comprised of 30 genera and 580 species. Three genera named as *Saxifrage*, *Bergenia* and *Astilbe* are commonly found in Pakistan. *Bergenia* genus comprises of six species which are widely distributed in East Asia, Central Asia and Himalayas. *Bergenia stracheye* and *B. ciliata* represent the *Bergenia* genus in Pakistan (Uddin *et al.*, 2012). *Bergenia ciliata* is ever green perennial herb (Bhakuni *et al*., 1974). This plant is widely used in Pakistan, India, Bhutan, Nepal and other countries, in ayurvedic and traditional medicines to treat several diseases (Rajkumar *et al.,* 2011).

ii. Phytochemistry

Bergenia ciliata contains different phytochemical. Studies on this plant show the presence of different constituents like paashaano lactone (Chandrareddy *et al*., 1998), catechin, berginin and gallic acid (Dhalwal *et al*., 2008).

iii. Ethenomedicinal importance

B. ciliata plant is used in Indian system of medicine for the treatment of pulmonary infections, fever, diarrhea (Nadkarni, 2000). Asokar *et al*. (1992) had reported that that *bergenia ciliata* rhizome be supportive in dissolution of kidney stone. Extract of *Bergenia ciliata* found to possess anti-tussive activity (Sinha *et al*., 2001) and showed significance in reducing inflammation and boost immunity against bacterial infections. It also demonstrated antiviral activity (Li *et al*., 2006). This plant exhibits significant anti-inflammatory, analgesic, diuretic property (Gehlot *et al.,* 2000). It is

evaluated for antioxidant activity and DNA protection abilities (Rajkumar *et al*., 2010). Due to the presence of anti-cancer metabolites, this plant could be considered as potential source of anti-cancer drug development (Rajkumar *et al.,* 2011).

1.4.2. *Euphorbia wallichii*

i. Taxonomy

Taxonomy of *Euphorbia wallichii* is given below:

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Euphorbiales
Family	Euphorbiaceae
Genus	*Euphorbia*
Specie	*wallichii*
Common name	Dodal

Fig1.2: *Euphorbia wallichii* plant

Euphorbiaceae families have 300 genera and 7500 species and it is counted as one of the largest families of higher plants. This family is widely spread in central and western Himalayas, middle-east, China and South Africa (Ali *et al*., 2009). *Euphorbia* genus is among the six largest genera of flowering plants, comprising 2160 species. *Euphorbia* plant is perennial herb, contains bright yellowish green flowers, dark green fruits and height of this plant is 75 cm (Ali and Ahmad, 2008). *Euphorbia* genus grows in the form of herbs few as shrubs and trees (Satyanarayana *et al*., 1991).

ii. Phytochemistry

Phytochemical studies on *E. wallichii*, mentions the presence of rearranged diterpenoids (Pan *et al*., 2006) and abietane (Wang *et al*., 2004). *Euphorbia* genus has been found rich in possessing steroids (Ahmed *et al*., 2005), aeromaticeasters (Ahmad *et al*., 2002), phenolics (Ahmad *et al*., 2002), essential oils (Feizbakhsh *et al*., 2004), tetracyclic triterpenoids (Jassbi *et al*., 2004), diterpenoids (Ahmad *et al*., 2002; Vasas *et al*., 2004; Parvez *et al*., 2004; Corea *et al*., 2005; Ahmad *et al*., 2005), penta cyclic triterpenoids (Ahmad *et al*., 2002) and several other bioactive constituents (Ravikanth *et al*., 2002; Hohmann *et al*., 2003).

iii. Ethno-medicinal importance

Some species of *Euphorbia* genus have been used in traditional medicines for hundreds of years and are used to treat tumors, cancers, migraine, gonorrhea, skin diseases, warts and intestinal parasites (Smith-Kielland *et al*., 1966; Singla *et al*., 1990). Plants of this genus are used to cure rheumatism and bronchitis (Satyanarayana *et al*., 1991). In Tibetan folk medicines, *E. wallichii* roots have been used as traditional medicine for the treatment of edema and skin diseases such as cutaneous anthrax and furuncle (Lal *et al*., 1990). It also showed significant cytotoxicity and phytotoxicity (Ali *et al*., 2009). In Indian folk medicines *E. antisyphilitica* was used to treat liver ailments (Saraf *et al*., 1994). *E. prostrate* is used as blood purifier and as an anti-inflammatory medicine (Singla *et al*., 1991). *E. lateriflora* latex is found to be effective against ring worm, also act as purgative (Fakunle *et al*., 1992) and remedy for spleeno-hepatomegaly (Satyanarayana *et al*., 1992). Ointments for a chronic skin disease in which red scaly pustule and patches appears on skin (psoriasis) are made from extract of *E. fischeriana* (Wu, L. *et al*., 1993).

1.5. Biological assays

Bioassay is the potential determination of activity of any chemical, physical or biological agent by using biological indicators. These indicators can be the living beings, organ, tissues, cells, components of cell.

Essential points of bioassays are;

- Stimulus (Test sample, drugs, bio active substance etc)
- Subject (Organism, Biochemical, living sample, etc.)
- Response (Response of the subject to stimulus at various level)

Concepts regarding bioassay characterization

- Potency of the sample compounds is not absolute but relative. Relative potential of bioactive compounds to reference drug or standard and both of these show similar biological response.
- Amount of biological active substance can be estimated when absolute amount of standard is known.
- Only estimated potential of bioactive substance is obtained by bioassays while precision of that amount is decided on the data of bioassay.
- Active potential is the property belongs to substance to be used in assay. It is not concerned to property of response. Usually association between dose of

active substance and change in indicator and dose effect curve is carried out as a part of assay (Ghosh 2008, Bohlin and Bruhn, 1999).

1.5.1. Broad categories of biological assays

i. Virtual or *in silico* screening

In medicinal research virtual research is thought to have an important role, by speeding the rate and reducing extra cost for lab work and clinical trials. During 2010, researchers have found the potential enzyme inhibitors associated with cancer, by using protein docking algorithum EA dock. These molecule were found to be 50% active enzyme inhibitors *in vitro* testing (Röhrig *et al.*, 2010; Ludwig Institute report, 2010).

ii. Primary bioassays

Bioassays of this category can be utilized rapidly to find if any desired activity is present in large number of samples. These can be sub classified into non-physiological assays, biochemical or mechanism-based, microorganism-based, cell-based, tissue-based and many other *in vitro* bioassays/assays. Examples of primary bioassays are given as antioxidant assays, enzyme inhibition assays, cytotoxicity bioassays, anti-bacterial bioassays, antifungal bioassays, insecticidal bioassays, *in vitro* antiparasitic bioassays, brine shrimp lethality bioassays, phytotoxicity bioassays and anti-cancer bioassays (Cancer Cell Lines) (Rahman *et al.,* 2005, Bohlin and Bruhn 1999).

iii. Secondary bioassays

Secondary biological assays require detailed sample testing on model living system so that sample compounds can be selected for clinical trials (Rahman *et al.,* 2005). These assays are usually slow, low capacity and expensive. Examples are given as;

a. Animal-based assays (i*n vivo*)

- Animal model with induced disease
- Animal model with induced injury

b. Toxicological assessments in whole animals

- Acute toxicity,
- Chronic toxicity

c. Preclinical Studies

These analysis of *in-vitro* (test tube) and *in-vivo* (animal) experiments using different doses of desired drug to obtain toxicity, preliminary efficacy and pharmacokinetics information.

Pharmaceutical companies assess whether studied drug has scientific value to develop that as investigational new drug.

1.5.2. Importance of bioassays

Bioassays play important role at the initial stages of drug discovery. Task of discovering new therapeutic agents for different diseases is becoming vast at the present time. The combination of bioassays provides the necessary screens in a timely manner to obtain data of active substance and its use in potential therapy against specific disease. Bioassays are very important tool in determining the quality of bioactive compounds (Bohlin and Bruhn 1999). In current study primary bioassays are mainly used because these are potently predictive and general in nature, unbiased, fast, cost-effective, reproducible, tolerant of impurities and compatible with DMSO (Rahman *et al.,* 2005, Ghosh, 2008).

1.6. Assays carried out in this study

1.6.1. Antioxidant assays

Antioxidants are class of highly reactive molecules. Reactive oxygen species (ROS) as hydrogen peroxide (H_2O_2), singled oxygen ($1O_2$), hydroxyl radical ($HO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are synthesized in living organisms (Halliwell *et al.,* 1999; Yldrm *et al.,* 2000; Gulcin *et al.,* 2002). Generally, synthesis of ROS is monitored by antioxidant system in living system. In many cellular functions, it may be necessary for killing phagocytes and redox regulation of signal transduction. In some conditions ROS are over produced in organisms that can lead to their attraction towards cellular components including protein, DNA and cell membrane and consequently induce destruction, degradation and toxicity of various molecules that play vital role in metabolism (Heo *et al*., 2005). Reactive oxygen species (ROS) can induce numerous diseases, like muscular dystrophy, cataracts, atherosclerosis, rheumatoid arthritis, some types of cancer, neurological disorders and aging (Kovatcheva *et al*., 2001; Ruberto *et al*., 2001). Currently different type of synthetic antioxidants like TBHQ (tertiary butyl hydroquinone), BHT (butylated-hydroxytoluene), BHA (butylated hydroxyl anisol) and propyl gallate are widely used in drugs, medicines and foods as materials or additives for oxygen suppression (Shon *et al*., 2003). However, usage of these synthetic antioxidants has been strictly banned due to toxicity that can lead to serious human health issues. Due to this fact, researchers are trying to find effective natural antioxidants having minimum side effects. Numerous antioxidants like carotenoids,

chlorophylls, polyphenolic compounds, isoprenoids and tocopherol have been isolated from plants and being used for several years (Ramarathnam *et al*., 1995; Wettasinghe *et al*., 1999; Shon *et al*., 2003).

i. DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is based on method of Blois (1958) in which radical scavenging activity of substrates, foods and beverages can be observed. Evaluation of scavenging capacities of various antioxidants is being carried out by using this method (Marxen *et al*., 2007; Perez-Jimenez and Saura-Calixto 2008; Perez-Jimenez *et al*., 2008). This method is simple, rapid, cheaper and accurate for free radical scavenging potential of samples (Prakesh, 2001). Methanol (Shikanga *et al*., 2010) and ethanol (Pavlov *et al*., 2002) can be used as solvent for DPPH. DPPH assay can be carried out by using various wavelengths like Kamkar *et al*. (2010) have used 517 nm for measuring absorbance of reaction mixture, Shikanga *et al*. (2010) have checked at 492 nm, Liebenberg (2004) at 540 nm, Lachman *et al*. (2008) used 515 nm and Gülcin *et al*. (2010) have used 516 nm. Various standards have been used for comparing radical scavenging activity among which ascorbic acid (vitamin C) is most widely used as standard drug (Kwon *et al*., 2003). DPPH is reduced upon reacting with sample. The reduced form (DPPH-H) results in decrease in absorbance. It can be explained by following reaction;

More the scavenging potential of antioxidant sample (hydrogen donating power), more discoloration will occur (Oktay *et al*., 2003). Given equation as described by Kamkar *et al.* (2010) was followed for calculation of scavenging activity of DPPH.

$$\text{Percantage scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

ii. Total antioxidant capacity

Various human pathological conditions are caused by reactive oxygen species. Imbalance in these species during synthesis and scavenging produces cellular damages that lead to disorder in proper functioning. Utilization of natural products and medicinal plants having significant antioxidant activity for prevention of disorders produced due to reactive oxidative species is a useful strategy (Shahwar *et al*., 2010). Phosphomolybdenum method was used for total antioxidant assay. In this method of total antioxidant capacity, sample reduces the Mo (VI) to green phosphate

Mo (V) complex at acidic pH. Phosphomolybdenum method for total antioxidant capacity is quantitative since the total capacity is expressed as equivalents number of ascorbic acid (Prieto *et al.,* 1999).

iii. Reducing power assay

Redox properties of sample determine its antioxidant activity and these properties play vital role in neutralizing and absorbing free radicals, decomposing peroxides or quenching singlet and triplet oxygen (Osawa, 1994). Antioxidant sample have potential of reducing potassium ferricyanide (Fe^{3+}) to potassium ferro-cyanide (Fe^{2+}), which result in formation of ferric ferrous complex. This assay is based on checking absorption of ferric ferrous complex, synthesized in reaction mixture, at 700 nm. Reaction can be explained as given below;

Potassium ferricyanide + Ferric chloride → Potassium ferro-cyanide + Ferrous chloride

1.6.2. Cytotoxicity assay

Brine shrimp lethality assay was performed for cytotoxic evaluation of complexes.

i. Brine shrimp lethality assay

Artemia salina (leach) is invertebrate organisms, commonly known as shrimps. These are found in lakes. Nauplii are the larvae of this organism. Human cytotoxicity and brine shrimp nauplii lethality may comparatively show positive correlation. Toxicological studies are carried out on these organisms for more than 50 years. (Krishnaraju *et al.,* 2006). At high doses, almost all biological active compounds are toxic for living systems. Simple zoologic organisms like shrimp larvae can be used conveniently for determination of lethal activity of natural bioactive compounds as well as derived or synthetic compounds. *Artemia salina* eggs remain viable for years in dry condition and are available in pet shops at low cost. Eggs are being placed in sea water and hatching occurs within 48 hours to provide nauplii for experimental purpose. The brine shrimp lethality assay is comparatively rapid, takes 24 hours for completion. It is also simple as it does not need any aseptic technique, is inexpensive and large numbers of organisms are utilized for statistical validation. It does not require any special equipment and usually small amount of sample from 2 to 20 mg or less is required for finding activity of compounds (Meyer *et al*., 1982; McLaughlin *et al*., 1991).

1.6.3. Antimicrobial assay

Search for medicine undoubtedly started in prehistoric times. Being core of health care, these relieve the symptoms (as analgesics) and cure diseases (as antibiotics). In 1928, Alexander Fleming discovered the first antibiotic penicillin, which was contemplating the main step in drug discovery. The exploration of specific potent antibacterial and antifungal drug, which can be used as therapeutic remedy using investigation tools, is biological research. Various antibiotics manufactured and discovered from natural sources. Antibacterial and antifungal activity of any compound, natural or synthetic, can be evaluated by comparative measurement of inhibited zone of standard drug and sample against particular microbes.

Animal and plant diseases are caused by pathogenic microorganisms, like bacteria and fungi. These microbes produce bacterial diseases such as hemolytic uremic syndrome, toxic shock syndrome and fungal diseases, such as keratitis, otitis, lung infections and chronic pulmonary infections in immune compromised hosts. Due to over resistance of antimicrobial drugs, demand of new and potent drug has been increasing. Biological activities of metallic complexes are checked against different gram positive and gram negative bacterial species. Quarter of deaths occur due to bacterial infections in spite of using broad spectrum antibiotics. This has led to new dimension of research for drug discovery (Wyke, 1987).

i. Antibacterial assay

Resistant bacteria are the main problem, emerging due to inappropriate use of antibiotics. In previous days these antibiotics were considered as vital for saving life but after 1950s resistant bacteria have become multi drug resistant (Hawkey, 2008). This problem can be solved by introducing new drugs or derivatives of existent drug. In this study antibacterial activity of complexes were observed using disc diffusion methods against various pathogenic bacteria as *Staphylococcus aureus, Salmonella typhimurium, Bordetella bronchiseptica, Escherichia coli* and *Micrococcus luteus,*

a. *Escherichia coli*

Escherichia coli (*E. coli*) are gram-negative, facultative anaerobic and rod shaped bacteria. These are harmless and opportunist bacteria found in intestine and can cause various problems like neonatal meningitis (Bentley and Meganathan, 1938), gastroenteritis (Chekeri *et al.,* 2008) and food poisoning (Hayhurst, 2004) in humans. This bacterium is used in research laboratories as research model due to its genetics,

simple growth, easy duplication and manipulation through metagenics. Virulence factors in the pathogenic strains cause pathogenesis (Elena *et al*., 2005).

b. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*is) are gram positive and facultative anaerobes, included in normal flora but causes atopic dermatitis in humans (Horn *et al.*, 2005). This group of bacteria is present in nose, on skin and it can contaminate the food and cause food poisoning in humans (Howardand Kloos, 1990).

c. *Enterobacter aerogenes*

Enterobacter aerogenes (*E. aerogenes*) are opportunist, gram negative bacterium. It is harmless and found in gastrointestinal tract, soil, water and hygienic chemicals. This bacterium is valuable, due to their importance in fermentation technology for the production of hydrogen (Chavarria *et al.*, 2000). It causes various infections such as urinary tract, respiratory tract, skin, eyes and can cause, endocarditis, osteomyelits (Jarvis and Martone, 1992) and nosocomial infections (Goshiab *et al.*, 2002).

d. *Bordetella bronchiseptica*

Bordetella bronchiseptica (*B. bronchi*) are gram negative bacteria responsible for snuffles in rabbits, kennel cough in dogs and bacterimia in humans. It is rarely associated with infections and found in respiratory system of humans. This specie causes infections in severely immune compromised hosts (Woolfrey and Moody, 1991). It is the causative agent of bronchitis (Rayn and Ray, 2004). Infection in Small animals such as rabbits, cats and dogs was reported (Finger *et al*., 1996).

e. *Salmonella typhimurium*

Salmonella typhimurium (*S. typhi*) is gram negative pathogen of humans that causes typhoid fever, systemic infection (Mastroeni *et al.*, 2009; Humphrey, 2004). It is resistant against innate immune system (Falkow *et al.*, 2004) and also involved in splenomegaly, hepatomegaly and white nodules in finches (Une *et al.*, 2008). This bacterium causes typhoid fever-like disease. Raw or poorly cooked food, contaminated water, sanitation are the important factors contributing for spreading of this bacteria. So this bacterium is used in labs for evaluating the antibacterial potential of various natural and synthetic products.

f. *Micrococcus luteus*

Micrococcus luteus (*M. luteus*) is gram-positive and was isolated by Fleming in 1922 from nasal secretion of patient of acute coryza. Kocur in 1992 detected *M. luteus* in water, soil and also in the mucosal membranes. This organism is recognized as

opportunist pathogen involved in recurrent bacteremia (Peces, 1997), endocarditis, septic arthritis, meningitis, cavitating pneumonia and intracranial suppuration in immune suppressed patients.

ii. Antifungal assay

Fungal strains can become resistant due to inappropriate and misuse of antibiotics. Resistant fungi have become critical issue towards human health and antifungal drugs have been observed useless against them. Studies on environmental issues suggest that population rising is main cause for anti-microbial resistance (Barret, 2002). There is need of exploring novel antifungal drugs against the resistant strains. Current study is emphasizing to synthesize organometallic complexes and to explore their antifungal potential. For this reason different fungal strains were used like *Fusarium solani, Mucor* species, *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus.*

a. *Mucor species*

Mucor species are olive gray to dark gray in color, fast growing mostly found in debris, soil, dairy products, leather, jute, meat, dead and decaying plants. These species are responsible for thrombosis in hepatic artery (Marco *et al.*, 2000), mucrosis (Crissy *et al.*, 1995), prosthetic mitral valve mucormycosis (Rama *et al.*, 2004) and majorly affect lungs, nasal cavity, sinuses of individual.

b. *Aspergillus niger*

Aspergillus niger (*A. niger*) is filamentous fungus, member of Ascomycota group and naturally found in environment. Initially, colonies of this fungus appeared white and finally black due to the production of conidia. Genome of this fungus is up to 38.5 megabite (MB) that could be useful as microbial research model for morphology, pathogencity and fermentation process (Baker, 2006). This fungus is used in fermentation industries for the synthesis of citric acid. Nakagawa *et al.* (1999) reported that *A. niger* is more dangerous against immune-compromised patients. it cause crown rot of ground nut in plant (Bobbarala, 2009).

c. *Aspergillus flavus*

Aspergillus flavus (*A. flavus*) fungi are dangerous to human health due to the production of toxins. Various types of fungal strains grow on improperly stored grains. It is also involved in harming cash crops. Alfa toxins produced by these strains are more dangerous for spreading different detoriating conditions. This strain also causes chronic pulmonary aspergillosis, invasive and non-invasive aspergillosis,

dermal infection, fungal sinusitis (Pasqualotto, 2009). Flavous alpha toxins can cause hepatitis, liver cancer and immune-suppression (Hedayati *et al.,* 2007).

d. *Aspergillus fumigatus*

Aspergilus fumigatus (*A. fumigatus*) is pathogenic fungus mostly found on soil, fruits, grains, vegetables and grow above 40ºC temperature. This fungus causes invasive, non-invasive infections in immune-compromised patients and also involved in systemic diseases, ormycotoxicosis and allergic reactions in individuals. Aspergillosis in immune-compromised individuals is caused by *Aspergilus fumigatus* (Latge, 2001). Working with this strain following poor managed protocols may lead to hypersensitivity pneumonitis (Suberosis) (Villar *et al.,* 2009).

e. *Fusarium solani*

Among various species of *Fusarium,* genus *Solani* specie is commonly found in humans and animals. Cottony or woolly colonies, having white to cream mycelium, microconidia and monophialides are the characteristics of this specie. *Fusarium solani* (*F. solani*) is also involved in pathogenesis, lignin biodegradation and phytohormones synthesis in plants (Nazareth and Bruschi, 1994) and causes mycetoma, oncomycosis, endocarditits, septic arthritis, pulmonary diseases, sinusitis and invasive epidermal infections in humans (Summerbell and Schroers, 2002).

1.6.4. Kinases inhibition assay

Protein kinases are vital enzymes for cell growth, cell cycle progression, and signal transduction across nuclear membrane. Human genome codes for 500 different protein kinases and classified on the basis of sequence of amino acid in catalytic domain (Manning *et al.,* 2002). Modulation in kinases activities could be potential source of cancer and other signalopathies. Its inhibitors can be used in various disease treatments (Li *et al.,* 2004). Kinases inhibitors are important drugs for the treatment of cancer. Selectivity of inhibitors as drugs is challenge for researchers (Lynette *et al.,* 2009) particularly, targeting oncogenic kinases involved in cancer (Arslan *et al.,* 2006).

1.7. Chemical and spectroscopic analysis

Chemical analysis of all complexes of EW and BC were carried out by measuring phenolics and flavonoid contents. FT-IR was used for the spectroscopic analysis of complexes.

i. Total phenolic content

Phenolics are group of secondary metabolites, which are synthesized by animals, plants and microorganisms. Phenolics of plants give them taste, odor, appearance and oxidative stability. These compounds are present in pericarp of cereal grains (Naczk *et al.,* 2004). Ferulic and p-coumaric acids are major phenolics (Zhou *et al.,* 2004; Mattila *et al.,* 2005; Holtekjolen *et al.,* 2006). It is observed that methanolic extracts of plants have more significant content of phenolics (Iqbal *et al*., 2006). Andersen *et al.* (2007) and Hurst *et al.* (2002) have classified the phenolic compound in different groups as under

- Benzenediols class contains hydrooxy phenol based simple structures.
- Phenolic acid class contains derivatives of cinnamic acid (C6-C3), benzoic acid (C6-C1) and associated as long polymers form tannins and lignans.
- Coumarins class contains compounds having 2H-1-benzopiran-2-one as basic structure.
- Flavonoids class contains compound having diarylpropane (C6-C3-C6) as basic structure.

ii. Total flavonoid contents

Flavonoids are plants' secondary metabolites group, having two benzene ring attached through pyrane ring (Bagchi *et al*., 1999). Basic structure comprises of 2-phenyl-benzo [α] pyrane and found in strawberries, green, black teas and vegetables e.g. kaempferol from brussel, quercetin from beans. This group has again three sub-classes as flavones, iso-flavonoids and neo-flavonoids. Flavonoids are well known for *in vitro* antioxidant activity that may be stronger than vitamins C and E (Bagchi *et al*., 1999). These secondary metabolites have anti-diarrheal (Schuier *et al*., 2005), anti-inflammatory, anti-microbial and anti-allergic activities (Cushnie and Lamb, 2005). These were observed to enhance DNA changes in cultured blood stem cells (Barjesteh *et al*., 2007). They inhibit germination of plant pathogen spores, so could be used against human pathogens (Harborne and Williamsn 1992). Currently inhibitory effect of flavonoids has been observed against human immunodeficiency virus (HIV). These compounds were checked against some pandemic HIV-1 strain and studies shows that HIV-1 replication is inhibited by baicalein (5,6,7-trihydroxyflavone) (Li *et al.,* 2000). Flavonoids class contains diarylpropane (C6-C3-C6) as basic structure and

subclassified as catechins, flavones, flavonols, flavanones, isoflavonoids and anthocyanes (Andersen *et al.,* 2007).

iii. FT-IR spectroscopy

Structural analysis of complex organic compounds was being carried out by using IR spectroscopy during 1950 to 1970. Characteristic functional group's determination of various compounds is performed by using this technique. FTIR spectroscopy gives the impulse about IR spectroscopy. This new technique is used for analytical study of biologically important compounds. It is rapid, sensitive, inexpensive and traditionally have an advantage of providing practically complete analysis, while information about characteristic functional groups of compounds is being cleared from IR spectra. This technique performs fast analysis, computerized data processing, requires very small amount of sample, database, and spectral data banks are the main beneficial features of this technique. Infrared spectroscopy was not widely applied for the structure of carotenoids (Bernhard *et al.,* 1995, Britton, 1989; Lammers *et al*., 2009). Structural elucidation of carotenoids was carried out by NMR, UV, and MS spectroscopy. Recently FT-IR spectroscopic studies give the confirmation of these compounds (Tamas Lorand *et al.,* 2001). This is often used for complex carbohydrates and plant sugars (Kristin Lammers *et al*., 2009). Recently this technique has been considered as nondestructive and successful technique for rapid structural features, characterization and minimum requirement of sample (Sarah, 2007).

1.8. Aims and objectives

Aim and objectives of present study were

- Preparation of crude methanolic extract of *Bergenia ciliata* and *Euphorbia wallichii*.
- Synthesis of the metallic complexes of crude extracts of both plants.
- Antioxidant evaluation of synthesized complexes.
- Determination of cytotoxic capacities of complexes.
- Determination of antibacterial and antifungal potential of complexes.
- Evaluation of protein kinase inhibition potential of complexes for characterization of its anticancer potential.
- Chemical and spectroscopic analysis by measuring phenolics and flavonoid contents and FTIR respectively.

2.1. Plant collection and extraction

Bergenia ciliata and *Euphorbia wallichii* plants were collected from Nathia galli, KPK, Pakistan during June, 2012. Dr. Rizwana Alim Qureshi (Taxonomist, Quaid-i-Azam University Islamabad) had identified the plants. Roots (*E. wallichii*) and rhizome (*B. ciliata*) were dried under shade and crushed in a grinder. The dried plants were crushed in a grinder. Extraction was carried by using analytical grade methanol. Each plant material was extracted with methanol (2 liter/0.5 kg) for three days. Process was repeated three times. Methanol collected after each process was combined and filtered using filter paper (whatman No. 1). Than filtrates were concentrated using rotary evaporator (R-200 Buchi, Switzerland). Extracts were further dried in vacuum oven (Vacucell, Einrichtungen GmbH). Finally crude methanolic extracts of *Bergenia ciliata* and *Euphorbia wallichii* plants were used for the synthesis of metallic complexes. While remaining extracts were stored at 4ºC.

2.2. Synthesis of metallic complexes

In present study two plants, *Bergenia ciliata* and *Euphorbia wallichii* were used for the synthesis of complexes with thirteen metals. Metallic salts i.e. aluminum chloride ($AlCl_3$), cadmium chloride ($CdCl_2$), lanthium chloride ($LaCl_3$), tin chloride ($SnCl_2$), bismuth nitrate ($BiNO_3$), cobalt sulphate ($CoSO_4$), plumbic acetate ($Pb(CH_3COO)$), sodium molybdate ($NaMoO_4$), cesium chloride ($CsCl_2$), lithium acetate (CH_3COOLi), mercuric chloride ($HgCl_2$), ferrous chloride ($FeCl_2$), ferric sulphate ($FeSO_4$) and silver nitrate ($AgNO_3$) were used as source of metals. Complexes were synthesized by following the method as described by Saha *et al.* (2009) and Thakum *et al.* (2012).

i. Requirements

Metal salts, crude extracts of *Bergenia ciliata* and *Euphorbia wallichii*, distilled water, methanol, stirrer (Gallen hamp, England), vortex (Scientific Industries Inc. USA, model No. SI-T256), vacuum chamber (VWR-1575R-2), sonicator (E30H-elma sonic sonicator), hot plate (Nuova 11, model No. SP 18420-26 hot plate), centrifuge (Eppendorf centrifuge, 5810R).

ii. Procedure

Metallic complexes of crude extracts of *E. wallichii* and *B. ciliata* were synthesized through series of steps as given;

- Salts solutions were prepared, by dissolving 4 g of respective salt in 40 ml distilled water (10%). These solutions were subjected to vortex for complete dissolution.
- Crude extract solution was prepared by dissolving (2 g/50 ml methanol) in separate eppendroffs. For complete dissolution of extract and to make homogeneous solution, this solution was stirred for half an hour at room temperature.
- After that equal amount of salt and extract solutions (1:1) were mixed. This mixture of aqueous solution of metal salt and methanolic solution of extracts of both plants were mixed vigorously.
- This was followed by 10 minutes sonication and incubation of one hour at room temperature. Later on turbidity was observed in mixtures of both solutions. That was indication for complex synthesis.
- Metal complexes were separated by centrifuging the solutions at 4000 rpm for 15 minutes.
- Supernatant was removed and solid complexes settled in bottom, were washed with distilled water and centrifuged again for 5 minutes. Supernatant was poured off and complexes were kept for further processing.
- Finally all complexes were dried in vacuum chamber for 24 hours at 35ºC and led for biological evaluation.

2.3. Bioassays

2.3.1. Antioxidant assays

Antioxidant capacities of metal complexes were determined using different assays which are described as under;

a. DPPH free radical scavenging assay

Antioxidant potential of samples against DPPH was determined using method described by Clarke *et al.* (2013) with slight modification.

i. Requirement

Solid DPPH (Sigma U.S.A), methanol, DMSO, complex samples, ascorbic acid, microplate reader

ii. Samples preparation

Four mg of complex samples were dissolved in 1 ml of DMSO for the preparation

stock solution of each sample. Further, 0.4 µg/µl final concentration of complexes was used in this assay.

iii. DPPH solution preparation

DPPH solution was prepared by dissolving 10 mg in 100 ml of methanol. Freshly prepared DPPH solution was sonicated for 10 minutes before use.

iv. Assay procedure

DPPH assay was performed in 96 wells plate. Firstly, 20 µl of test sample from stock solution was poured in respective well of plate and then 180 µl of DPPH solution was added in each well to make 200 µl final volume. Ascorbic acid and DMSO were used as reference standard and negative control respectively. Mixture was incubated at room temperature for half an hour. Change in color from violet to yellow was observed. Oxidation potential of complexes determined the color changing. Absorbance of reaction mixture was measured at 517 nm on microplate reader. Given formula was used for calculation of scavenging activity of sample.

$$\text{Scavenging percentage} = \frac{\text{OD of control} - \text{OD of test sample}}{\text{Absorbance of control}} \times 100$$

OD = optical density (absorbance)

b. Total antioxidant capacity

Phosphomolybdenum method as described by Dillard *et al.* (2000) was used for measuring total antioxidant capacity.

i. Requirement

Complex solution stock (4 mg/ml), DMSO, sodium phosphate buffer (28 mM), ammonium molybdate (4 mM), sulphuric acid (0.6 M), microplate reader, sodium phosphate (28 mM), ammonium molybdate (4 mM), sulphuric acid (0.6 M).

ii. Sample preparation

Sample complex solution was prepared by dissolving 4 mg in one ml of DMSO as stock and further 0.4 mg/ml final concentration was used in this assay.

iii. Solution of positive control

Ascorbic acid was used as standard (positive control). Four mg of each complex was dissolved in 1 ml of DMSO for stock preparation. Final concentration (400 µg/ml) was used in this assay.

iv. Assay procedure

One ml reaction mixture was produced by addition of reagent solution and 100 µl of sample. Reagent solution comprised of ammonium molybdate (4 mM), sodium

phosphate (28 mM) and sulfuric acid (0.6 M). This reaction mixture was incubated for 90 minutes at 95^{o}C. After incubation, mixture was cooled and absorbance was recorded at 695 nm. DMSO was used instead of complex sample, as blank. Antioxidant potential of complexes was expressed as ascorbic acid equivalent.

c. Reducing power assay

Reducing power of metal complexes of EW and BC complexes was determined using the method as described by Ullah *et al.* (2013).

i. Requirement

Phosphate buffer (0.2 M, pH 6.6), 1% potassium ferricyanide $K_3[Fe(CN)_6]$, ascorbic acid, 10% trichloroacetic acid, 0.1% ferric chloride ($FeCl_3$), complex samples, distilled water, microplate reader, DMSO.

ii. Preparation of complex solution

Stock solutions of complexes were prepared by dissolving 4 mg in 1 ml of DMSO. Further final concentration 100 µg/ml was used for assay.

iii. Standard solution preparation

Ascorbic acid was used as standard and final concentration 392 µg/ml.

iv. Assay procedure

Reducing potential of sample was determined by taking 100 µl of sample, 250 µl phosphate buffer and 250 µl potassium ferricyanide to make 600 ml reaction mixture in eppendrof. Reaction mixture was subjected to incubation at 50^{o}C for 30 minutes. Incubation was followed by addition of 250 µl of trichloroacetic acid (10%). Resultant solution was centrifuged at 3000 rpm for ten minutes. Then, 250 µl of supernatant of each complex was taken carefully and poured in their respective well. Finally, 20 µl of 0.1% ferric cyanide solution and 30 µl of distilled water were added in all wells. Absorbance of solution was recorded at 700 nm. DMSO was used as blank. Ascorbic acid equivalents were used for expression of reducing power of complexes.

2.3.2. Brine shrimp cytotoxic lethality assay

Cytotoxic effect of metal complex was determined using brine shrimp assay. Complexes were evaluated for cytotoxic nature by following the method as described by McLaughlin and Rogers (1998).

i. Requirements

Brine shrimps eggs (Ocean Star Inc., USA), DMSO, terbinafine, portioned dish, sea salt, complex samples, vials, pasture pipette, magnifying glass, lamp, distilled water.

ii. Preparation solution of complex samples

Fifty mg of complex sample was dissolved in 1 ml of DMSO to prepared stock solution of each sample. Further three dilutions i.e. 1000 µg/ml, 100 µg/ml and 10 µg/ml were prepared from the stock solution. Table 2.1, shows the detailed dilutions procedure used in this assay.

Table 2.1: Detail of dilutions used in cytotoxicity assay

S. no.	Stock in (µg/ml)	Test compound (µl)	Sea water (µl)	Final volume (ml)	Final test conc. (µg/ml)
1	50,000	100	4900	5	1000
2	10,000	100	4900	5	100
3	1,000	100	4900	5	10

iii. Standard solution preparation

Terbinafine was used as standard drug. One mg of standard was dissolved in 1 ml of DMSO to prepare stock solution. Three concentrations i.e. 10 µg/ml, 1 µg/ml and 0.1 µg/ml were used.

iv. Sea water

Sea water produced by dissolving 34 g of sea salt in one 1000 ml. Solution was stirred constantly using magnetic stirrer. Sea water was aerated in open mouth beaker for sufficient time.

v. Hatching of shrimp

Hatching of shrimp eggs was carried in compartmentalized rectangular dish (22 × 23 cm), which was filled with sea water. One large and other small compartment with separating wall containing holes were present in dish. Eggs ware poured in large compartment. Lamp light was showered on smaller compartment, after covering larger compartment with aluminum foil. Newly hatched nauplii moved towards small compartment due to light. These were picked with pasture pipette and dispensed in small beaker having sea water.

vi. Assay procedure

Initially all vials were labeled, followed by addition of 2 ml sea water in all vials. After that 0.1 ml sample solution from their respective stock solutions were added in their respective vials. Ten nauplii were counted using pasture pipette and 3 X magnify glass and transferred to vials. Simultaneously volume was raised up to 5 ml with sea

water. So it does not affect the shrimps and desired concentration of sample attained. These vials were incubated for 24 hours at room temperature. After that shrimps were taken out from vials using pasture pipette and counted under magnifying glass. Finally lethal dose (LD_{50}) of metal complexes was determined.

vii. Determination of LD_{50}

For the determination of lethal dose (LD_{50}) of metal complexes samples against brine shrimps finny (1971) software was used.

2.3.3. Antimicrobial assays

a. Antibacterial assay

Antimicrobial activity was determined by using disc diffusion method as described by Islam *et al.* (2013).

i. Requirement

Metal complexes, DMSO, roxythromycin, cefixime-USP, whatman No. 1 filter paper discs, vernier caliper, nutrient broth (Merck, Gemany), nutrient agar (Merck, Gemany), petriplates, Six bacterial strains were used for antibacterial potential of complexes. Two gram positive strains (*M. luteus* ATCC 10240, *S. aureus* ATCC 6538) and four gram negative (*B. bronchiseptica* ATCC 4617, *E. coli* ATCC 15224, *S. typhimurium* ATCC 14028, *A. aerogens* ATCC 13048). Nutrient broth was used for the maintenance of bacterial cultures for 24 hours at 4^{o}C. Next day cold cultures of these strains in nutrient broth (Merck, Germany) were used for assay.

ii. Samples preparation of EW and BC metal complexes

Four mg of each complex were dissolved in 1 ml of DMSO, for the production of stock solution of each complex. Final concentration of 30 µg/disc of each complex was used in assay.

Table 2.2: Description of EW and BC metal complexes concentration in antibacterial assay.

S. no.	Stock (µg/ml)	Test compound (µl)	Final test concentration (µg/disc)
1	4,000	7.5	30

iii. Standard solution

Cefixime-USP and roxythromycin were used as standard drugs. Stock of each standard drug was prepared by dissolving 4 mg 1 ml of DMSO. DMSO was used as negative control.

iv. Assay procedure

Bacterial inoculum was prepared in nutrient broth. It was prepared by dissolved 2 grams of nutrient broth in 100 ml of water and pH was maintained to 7. Nutrient agar medium was used for the growth of bacterial strains that was produced by dissolving 2 grams in 100ml of water. Filter paper discs of 6 mm in size ware prepared from whatman no. 1 filter paper. Media, filter paper discs along with other apparatus required in this assay were autoclaved for sterilization. After autoclaving, whole experiment was carried out in microbiological safety cabinet. Solidified plates of nutrient agar were labeled and respective bacterial strain was streaked. Then 7.5 µl of each complex solution was absorbed on disc. These discs were placed on respective places in petri plate. These peteri plates were incubated for 24 hours at 28^{o}C. Zones of inhibitions were measured after 24 hours.

b. Antifungal assay

All complexes of EW and BC were evaluated for antifungal capacities. This assay was performed by following disc diffusion method as described by Islam *et al.* (2013).

i. Requirement

Metal complexes samples, DMSO, terbenafine, whatman No. 1 filter paper discs, vernier caliper, sabouraud dextrose agar (SDA) (Merck, Germany), petriplates, antifungal activity of complexes were determined against five fungal strains. Fungal cultures (*A. fumigatus* FCBP 66, *F. solani* FCBP 0291, *Mucor* species FCBP 0300, *A. niger* FCBP 0198, *A. flavus* FCBP 0064) were maintained on SDA at 4^{o}C.

ii. Solution of complex samples

Four mg of each complex was dissolved in 1 ml of DMSO to prepare stock solution of each sample.

Table 2.3: Description of EW and BC metal complexes concentration in antifungal assay.

S.no.	Stock (µg/ml)	Test compound (µl)	Final test concentration (µg/disc)
1	4,000	7.5	30

iii. Standard drug solution

Terbinafine was used as standard drug (positive control) and DMSO was used as negative control. Stock solution of standard was produced by dissolving 4 mg of

standard drug in 1 ml of DMSO and 10 µg/disc concentration of standard drug was used.

iv. Assay procedure

Fungal cultures were grown on SDA medium at the 6.5 pH. Sabouraud dextrose agar (SDA) media was prepared by dissolving 6.5 grams in 100 ml of distilled water and adjusted pH of 6.5. Whatman no.1 filter paper was used for the preparation of circular discs of 6 mm size. All apparatus required in assay were autoclaved for sterilization. Experiment was carried out in microbiological safety cabinet. SDA media was poured in petri plates and left for solidification. Filter paper discs were used for the absorption of sample solution and 7.5 µl of complex solution absorbed on disc. After absorption of complex solution on disc, were place in their respective places on solidified SDA plates.

Contamination was prevented by wrapping petri plates with parafilm. After that plates were incubated for 24 hours at 28^{o}C. On next day inhibition zones were measured by using venire caliper.

2.3.4. Protein kinases inhibition assay

i. Requirement

Growth media, streptomyces (Actinobacteria) culture, filter paper discs (6mm), pipette, spatula, cotton swab, petriplates.

ii. Procedure

Disc diffusion method was used for the kinases inhibition assay. Media along with spatula, cotton swab, discs were autoclaved prior to use for actinobacterial growth. Whole experiment was carried out in sterilized environment. Streptomyces actinobacteria was used in assay. Media was poured in petri plates and test microorganisms were grown. Test sample was absorbed on discs and placed above media at their respective positions. These plates were incubated at 28ºC for 48-72 hours. After that balled zones were measured for determination of protein kinase inhibition activity.

2.4. Chemical and spectroscopic analysis

Chemical analysis of complexes was carried by measuring the phenolic and flavonoid content. Spectroscopic study of complexes was also performed using FT-IR.

a. Determination of phenolic content

Phenolic content was determined by using Folin-ciocalteu reagent as described by Clarke *et al.* (2013) with slight modification.

i. Requirement

Six percent sodium carbonate ($NaCO_3$) solution, gallic acid, DMSO, metal complex samples, spectrophotometer.

ii. Preparation of sample solution

Stock solution of 4 mg/ ml of each complex was produced by dissolving 4 mg of complex in 1 ml of DMSO.

iii. Preparation of positive control

Gallic acid was used as positive control at final concentration of 400 µg/ml.

iv. Assay procedure

Initially 0.1 ml of each metal complex solution was taken in vial. Then, 0.95 ml of Folin-Ciocalteu reagent (ten times diluted) was added in all vials. These mixtures were left for 5 minutes. Further 0.95 ml of sodium carbonate (6%) solution was added in mixture to prepare reaction mixture. This solution was incubated at room temperature for 60 minutes. Incubation was followed by recording absorbance of reaction mixture at 715 nm. Gallic acid was used as standard. Phenolic content was expressed as gallic acid equivalent.

b. Determination of total flavonoids

Determination of flavonoids content was carried out by following aluminum colorimetric method Ullah *et al.* (2012).

i. Requirements

Aluminum chloride (10%), 1.0 M potassium acetate, Samples (4mg /ml), Quercitin, DMSO.

ii. Preparation of sample

Stock solutions of complexes were prepared by dissolving 4 mg of each metallic complex in 1 ml of DMSO.

iii. Preparation of positive control

Quercetin was used as positive control. Final concentration of 100 µg/ml was used for determination of total phenolics.

iv. Procedure for flavonoids

Reaction mixture was made by mixing 100 µl metal complex solutions, 0.4 ml of methanol, 66 µl of potassium acetate (1M), 66 µl of aluminum chloride solution (10%). Finally 2 ml volume was made with the addition of 1.360 ml of distilled water. Mixture was incubated at room temperature for half an hour and absorbance was

recorded at 415 nm. Quercetin was used as standard. Flavonoids content was expressed as quercetin equivalent.

c. Fourier transforms infrared spectroscopy (FTIR)

i. Requirement

FT-IR spectroscope, complex samples, methanol, spatula, tissue

ii. Procedure

Synthesized complexes were further studied by FTIR spectroscopy. All instruments have their own characteristics for measurement that change with temperature, ambient environment and time. Experiment was carried out at room temperature. Firstly FTIR was scanned without sample and this measurement was stored as background. After that sample was placed in sample compartment for FTIR measurement. Instrument automatically subtracted the background measurement and show the net measurement of sample. Sample was removed and methanol was used for cleaning after subsequent measurement. All the spectra were printed and interpreted.

3.1. Extraction and complex synthesis

Extraction of both *Euphorbia wallichii* (EW) and *Bergenia ciliata* (BC) was carried using methanol. From EW, BC plants 6.3 and 5.2% extract was obtained respectively. Complexes of fourteen metals with extract of both plants were formed. Percent yield of all complexes are given in table 3.1. Varying content of each complex was obtained, that was due to the nature of metal and ligand.

Table 3.1: Percentage yields of metallic complexes of *Euphorbia wallichii* and *Bergenia ciliata.*

EW complexes			BC complexes	
S. No.	Sample code	% yield	Sample code	% yield
1	EWAl	4.9	BCAl	5.7
2	EWCd	4.7	BCCd	5.2
3	EWLan	5.3	BCLan	4.9
4	EWSn	4.5	BCSn	3.2
5	EWBi	3.3	BCBi	4.3
6	EWCo	3.7	BCCo	2.9
7	EWPb	4.9	BCPb	5.2
8	EWMo	7.6	BCMo	6.9
9	EWCs	4.3	BCCs	5.6
10	EWLi	3.1	BCLi	3.9
11	EWHg	4.2	BCHg	3.7
12	EWFs	7.2	BCFs	6.4
131	EWFc	5.9	BCFc	6.1
14	EWAg	2.7	BCAg	2.1

3.2. Antioxidant assays

Antioxidant capacities of all EW complexes were checked. All complexes showed decrease in DPPH % scavenging capacities and total antioxidant capacities as compared to their crude extracts. Increased reducing capacities comparative to their extract were observed. EWMo complex was found to have 86% of scavenging potential comparative to extract. EWCd complex had significant highest total antioxidant potential among EW complexes and 13% lesser then EW extract. EW complexes showed increased reducing power to that of their extracts. EWFs was found have 51% increased reducing power among EW complexes to that of extract. EWLan and EWPb had increased reducing power 3.6% and 4.3% comparative to

extract. Detailed results are given in table 3.2. Experiment was performed in triplicate. Values were mean of three independent replicate along with standard deviation. Ascorbic acid was used as standard for antioxidant capacities of complexes. Antioxidant potential was expressed in A.A µg equivalent per 100 µg complex.

Table 3.2: Antioxidant capacities of *Euphorbia wallichii* metal complexes

S. No.	Sample	DPPH % Scavenging	Total antioxidant AA µg/100µg sample	Reducing power AA µg/100µg sample
1	EWAl	41.380	39.326±0.532	41.291±1.008
2	EWCd	33.016	55.326±0.645	39.717±0.582
3	EWLan	27.685	28.008±0.505	36.266±1.118
4	EWSn	28.181	50.925±0.577	46.801±1.210
5	EWBi	30.743	35.041±0.565	50.856±0.367
6	EWCo	28.140	47.408±0.527	51.401±1.175
7	EWPb	30.041	31.161±0.974	36.498±0.985
8	EWMo	67.231	46.217±0.841	47.922±1.386
9	EWCs	37.396	44.741±0.509	39.063±0.672
10	EWLi	29.545	32.645±2.053	49.815±1.536
11	EWHg	28.429	53.411±0.513	47.678±1.266
12	EWFs	22.024	51.262±0.518	53.030±1.455
13	EWFc	43.884	49.411±0.502	43.197±0.955
14	EWAg	38.842	37.466±0.749	45.801±1.604
15	EW	77.201	62.985±0.577	34.985±0.610

[± = Standard deviation (S.D)]

Antioxidant capacities of BC complexes were checked. All complexes showed decrease in DPPH % scavenging capacities and total antioxidant capacities. While increased reducing capacities comparative to their extract were observed. BCMo complex was found to have 88% of scavenging potential to extract. BCMo and BCCs showed 4% decreased total antioxidant potential among BC complexes comparative to BC extract. BCFc was found to 51% increased reducing power among BC complexes respect with to extract. BCFc had increased 3% reducing power comparative to BC extract. Detailed results antioxidant capacities of BC complexes are given in table 3.3.

Table 3.3: Antioxidant capacities of *Bergenia ciliata* metal complexes

S. No.	Sample code	DPPH % Scavenging	Total antioxidant AAeq: µg/100µg sample	Reducing power AAeq: µg/100µg sample
1	BCAl	47.355	53.075±0.519	44.078±0.572
2	BCCd	35.826	40.917±1.104	52.338±1.224
3	BCLan	34.256	39.312±0.541	47.590±1.674
4	BCSn	41.570	48.985±0.509	50.617±0.850
5	BCBi	34.669	44.792±0.50	51.010±0.528
6	BCCo	34.793	34.281±0.518	46.258±0.549
7	BCPb	31.570	38.132±0.547	43.170±0.333
8	BCMo	70.371	57.023±1.011	41.899±0.825
9	BCCs	41.404	56.987±0.547	37.914±0.50
10	BCLi	37.892	45.162±0.536	54.364±2.280
11	BCHg	42.975	35.283±0.577	43.473±0.505
12	BCFs	37.685	41.162±1.154	47.043±1.793
13	BCFc	42.603	36.191±1.103	54.169±1.285
14	BCAg	37.314	54.052±0.743	49.528±0.609
15	BC	79.297	58.943±0.577	35.802±0.310

[± = Standard deviation (S.D)]

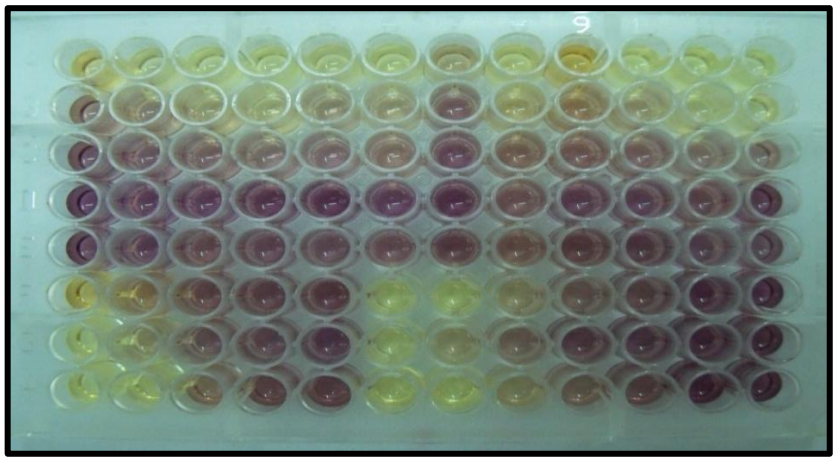

Fig 3.1: Pictorial view of DPPH free radical scavenging assay in 96-well format

3.3. Brine shrimp cytotoxicity assay

In the case of brine shrimp cytotoxicity of EW complexes, significant results were observed using EWHg complex (LD_{50} = 96 µg/ml), followed by EWFs complex (LD_{50} = 96.69 µg/ml). The metal complexes EWLi complex, EW Bi complex and EWLan complex also showed comparatively better results. The maximum LD_{50} was

found using EWCs complex (LD_{50} = 382 µg/ml), which indicate the least cytotoxic nature of EWCs complex among EW complexes. Detailed results are given in table 3.4. The assay was performed in triplicate. The values are the mean of three independent replicates along with standard deviation.

Table 3.4: Brine shrimps cytotoxicity assay of metal complexes of *Euphorbia wallichii* crude extracts

S. No.	Sample code	Number of brine shrimps killed after 24hours			LD_{50} µg/ml
		1000 µg/ml	100 µg/ml	10 µg/ml	
1	EWAl	9±1.53	2±1.5	1±1.0	191.6
2	EWCd	8±0.58	2±0.6	1±0.6	263
3	EWLan	10±0.58	3±1.2	1±1.0	117
4	EWSn	8±0.57	3±0.6	2±1.0	171
5	EWBi	9±1.15	3±1.0	2±0.6	123
6	EWCo	9±1.15	3±1.2	1±0.6	153
7	EWPb	8±1.0	3±0.6	2±0.6	209
8	EWMo	9±1.53	2±0.6	1±0.0	191
9	EWCs	7±1.15	2±1.0	1±0.6	382
10	EWLi	9±1.15	3±1.0	2±0.6	123
11	EWHg	9±1.53	4±1.0	1±0.6	96
12	EWFs	9±1.15	4±0.6	2±0.6	96.96
13	EWFc	8±1.53	3±1.0	1±0.6	315
14	EWAg	8±.58	3±0.6	1±1.0	209
15	EW	5±1.53	3±0.6	1±0.6	889

[± = Standard deviation (S.D)]

For the determination of cytotoxic effect of BC complexes this assay was performed. Comparatively BCFs complex was found more cytotoxic among BC complexes because significant lowest LD_{50} (LD_{50} = 58 µg/ml) was observed, Followed by BCFc (LD_{50} = 72 µg/ml) and BCHg (LD_{50} = 74 µg/ml). BCAg has shown comparatively maximum LD_{50} (LD_{50} = 392 µg/ml) which was different than the rest of metal complexes. Results of BCE metal complexes brine shrimps cytotoxicity assay are given in the table 3.5. The assay was performed in triplicate. The values are the mean of three independent replicate along with standard deviation.

Table 3.5: Brine shrimps cytotoxicity assay *Bergenia ciliata* crude extract metal complexes

S. No.	Sample Name	Number of brine shrimps killed after 24 hours			LD_{50} µg/ml
		1000 µg/ml	100 µg/ml	10 µg/ml	
1	BCAl	8±1.52	3±1.0	2±1.0	171
2	BCCd	9±0.57	3±1.0	2±0.57	123
3	BCLan	9±1.15	4±1.15	1±1.0	123
4	BCSn	7±0.57	3±1.0	2±1.15	261
5	BCBi	9±0.57	2±1.0	1±1.0	191
6	BCCo	10±1.52	3±0.57	1±0.57	117
7	BCPb	8±2.0	2±0.52	1±0.6	263
8	BCMo	8±0.57	3±1.0	2±1.0	171
9	BCCs	8±1.15	3±1.0	2±1.0	171
10	BCLi	9±1.0	2±0.57	1±0.6	191
11	BCHg	10±1.52	4±1.15	2±1.0	74
12	BCFs	8±0.57	7±2.0	2±1.0	58
13	BCFc	9±1.0	4±1.15	3±.57	72
14	BCAg	7±1.52	2±1.15	1±0.6	392
15	BC	5±1.15	2±0.57	1±0.6	1193

[± = Standard deviation]

3.4. Antibacterial capacities of metallic complexes

Antibacterial activity of EW complexes was checked against six bacterial strains, *E. coli, M. lutus, B. bronchiseptia, S. aureus, S. typhimurium* and *E. aerogenes*. Five EW complexes were active against *M. lutus* strain, four complexes were active against *E. coli*. EWHg produced maximum zone of inhibition (16±1.527 mm) againt *S.aureus*. The complex EWSn, EWPb, EWMo, EWFs, EWFc and EWAg were found to have no activity against any bacterial strains. While EWHg complex were found active against five bacterial strains, followed by EWAl, EWLi complexes which were active against three strains. Detailed results are given in table. 3.6. The assay was performed in triplicate. The values are the mean of three independent replicate along with standard deviation. DMSO was used as negative control and cefixime (10 µg/disc) was used as positive control.

Table 3.6: Antibacterial activity of complexes of *Euphorbia wallichii* crude extract

S. No.	Sample code	*E. coli*	*M. luteus*	*B. bronch iseptica*	*S. Aureus*	*S. typhi murium*	*E. aerogens*
1	EWAl	11±0.57	--	--	--	10±0.57	11±1.15
2	EWCad	8±0.581	--	--	--	--	--
3	EWLan	--	8±0.5773	--	--	--	--
4	EWSn	--	--	--	--	--	--
5	EWBi	--	--	10±1.15	--	--	--
6	EWCo	--	13±0.587	--	--	--	--
7	EWPb	--	--	--	--	--	--
8	EWMo	--	--	--	--	--	--
9	EWECs	--	14±0.577	--	--	--	--
10	EWLi	12±0.57	13±1.154	--	--	10±0.57	--
11	EWHg	15±1.22	6.5±0.577	--	16±1.52	15±1.15	16±1.15
12	EWFs	--	--	--	--	--	--
13	EWFc	--	--	--	--	--	--
14	EWAg	--	--	--	--	--	--
15	EW	8.5±0.56	11±0.577	8±0.577	--	9±0.577	15±1.15
16	Cefixime	23±1.52	15±1.527	22±1.52	18±1.15	24±1.15	19±1.52
17	DMSO	--	--	--	--	--	--

[-- = no activity, ± = standard deviation]

Antibacterial activity of BC complexes was checked against six bacterial strains, *E. coli, M. lutus, B. bronchiseptia, S. aureus, S. typhimurium* and *E. aerogenes*. Five BC complexes were active against *B. bronchiseptia* strain, four complexes were active against *S. typhimurium* and *E. aerogenes*. BCHg produced maximum zone of inhibition (17±0.58 mm) againt *M. lutus*. The complex BCCs was found to have no activity against any fungal strain. While BCCd and BCHg complexes were found active against four bacterial strains. BCAl and BCFc complexes were active against three strains. Detailed results are given in table. 3.7. The assay was performed in triplicate. The values are the mean of three independent replicate along with standard deviation. DMSO was used as negative control and cefixime 10 µg/disc was used as positive control. Zones of inhibition using complex sample was determined at final concentration of 30 µg/disc.

Table 3.7: Antibacterial activity of complexes of *Bergenia ciliata* crude extract

S. No.	Sample Code	*E. coli*	*M. luteus*	*B. bronchiseptia*	*S. aureus*	*S. typhi murium*	*E. Aerogenes*
1	BCAl	9±1.527	--	--	--	7.5±0.577	8±0.571
2	BCCd	--	--	9±0.577	9.5±1.15	9±0.577	20±1.52
3	BCLan	--	--	--	--	10±1.1547	--
4	BCSn	--	--	--	--	--	--
5	BCBi	--	--	--	--	--	--
6	BCCo	--	--	--	8.5±0.58	--	--
7	BCPb	--	--	--	--	--	--
8	BCMo	--	--	--	--	--	--
9	BCCs	--	--	--	--	--	--
10	BCLi	--	--	--	--	--	--
11	BCHg	--	15.5±0.58	17±0.58	15±0.523	13±0.577	--
12	BCFs	--	--	7±0.577	--	--	--
13	BCFc	13±0.52	--	8±0.58	--	--	9.5±0.76
14	BCAg	--	--	8±0.521	--	--	11±1.52
15	BC	--	9±0.58	7.5±0.57	--	8±0.57	9±1.53
16	Cefixime	23±1.53	15±1.527	22±0.866	18±0.58	24±1.52	20±0.57
17	DMSO	--	--	--	--	--	--

[-- = no activity, ± = standard deviation]

3.5. Antifungal capacities of metallic complexes

Antifungal activity of EW complexes was checked against five fungal strain, *A. niger*, *A. fumigatus*, *Mucor* species, *A. flavus* and *F. solani*. Six EW complexes were active against *fumigatus,* five complexes were active against *niger*. EWFs produced maximum zone of inhibition (10±0.288 mm) against *flavus*. The complex BCCs was found to have no activity against any fungal strain. EWAg complex was active against four fungal strains. Followed by EWAl and EWFs complexes these were active against three strains. Detailed results are given in the table. 3.8. The assay was performed in triplicate. The values are the mean of three independent replicate along with standard deviation. DMSO was used negative control and terbinafine at the final

concentration of 10 µg/disc was used as positive control. Inhibition was determined at final concentration of 30 µg/disc.

Table 3.8: Antifungal activity of complexes of *Euphorbia wallichii* crude extract

S. No.	Sample name	*Aspergillus niger*	*Aspergilus fumigatus*	*Aspergilus flavus*	*Fusarium solani*	*Mucor species*
1	EWAl	--	--	9.5±0.288	8.5±1.040	8±0.866
2	EWCd	8±1.04	8±0.50	--	--	--
3	EWLan	--	--	--	--	7±0.28
4	EWSn	9±0.00	--	--	--	--
5	EWBi	--	8.5±1.892	--	--	--
6	EWCo	--	--	--	8±0.86	7.5±0.10
7	EWPb	--	--	--	9±0.50	--
8	EWMo	--	7±0.322	--	--	--
9	EWCs	--	--	--	--	--
10	EWLi	7.5±0.25	--	9±0.00	--	--
11	EWHg	7.5±0.577	--	--	9.5±.866	--
12	EWFs	7±0.155	7.5±0.577	10±0.288	--	--
13	EWFc	--	8±0.862	--	--	--
14	EWAg	--	9.5±1.322	9±0.763	8.5±0.915	7±0.280
15	EW	7±0.250	7±1.0	8±0.7637	8±1.0	7±0.151
16	Terbinafine	33±1.527	30±1.0	35±1.527	38±2.081	30±1.52
17	DMSO	--	--	--	--	--

[-- = no activity, ± = standard deviation]

Antifungal activity of BC complexes was determined against five fungal strain, *Aspergillus niger*, *Aspergilus fumigatus*, *Mucor species*, *Aspergilus flavus* and *Fusarium solani*. Most of complexes were active against fungal species used in this study. The significant maximum zone of inhibition (16±2.081mm) was observed againt *Aspergillus niger* using BCAl complex. The complexes BCMo, BCCs and BCFc were found to have no activity against any fungal strain. While BCAg complex was found active against four fungal strains. The assay was performed in triplicate. The values are the mean of three independent replicate along with standard deviation. DMSO was used negative control and terbinafine 10 µg/disc was used as positive control. Zones of inhibition were measured (mm) at final concentration of 30 µg/disc.

Table 3.9: Antifungal activity of complexes of *Bergenia ciliata* crude extract

S. No.	Sample name	*A. niger*	*A. fumigatus*	*A. flavus*	*F. solani*	*Mucor species*
1	BCAl	16±2.081	14±1.527	--	9±1.527	--
2	BCCd	--	--	8±0.577	--	--
3	BCLan	--	9±1.52753	--	--	8±1.52753
4	BCSn	--	--	--	--	--
5	BCBi	--	--	--	--	7.5±0.86
6	BCCo	--	--	--	8±0.577	--
7	BCPb	8± 1.527	--	--	--	--
8	BCMo	--	--	--	--	--
9	BCCs	--	--	--	--	--
10	BCLi	--	8±1.0	8±1.32	--	--
11	BCHg	9±1.0	--	9.5±.92	--	--
12	BCFs	7±0.58	8.5±0.288	--	--	--
13	BCFc	--	--	--	--	--
14	BCAg	8±0.57	9±0.58	--	7.5±0.28	10±1.15
15	BC	7±0.58	8±0.40	8±0.288	7.5±	7.5±
16	Terbinafine	35±2.516	27±2.516	40±1.54	40±2.0	28±2.0
17	DMSO	--	--	--	--	--

[-- = no activity, ± = standard deviation]

3.6. Protein kinase inhibition assay.

All the EW and BC complexes were evaluated for protein kinase inhibition capacities. BC complexes were found to have more inhibitory capacity than EW complexes. Capacities were measured in mm inhibitory zone which may be balled or clear zone. Clear zone indicated the bactericidal effect, while balled zone represented the kinase inhibition potential. Activity was checked at final concentration of 30 µg/disc and DMSO was used as negative control that shows no activity in this assay. EWCd (9 mm), EWCs (15 mm) complexes from EW complexes and BCFs (10 mm), BCFc (16 mm) from BC complexes were observed to have kinase inhibitory potential. EW and BC extracts did show kinase inhibition capacity.

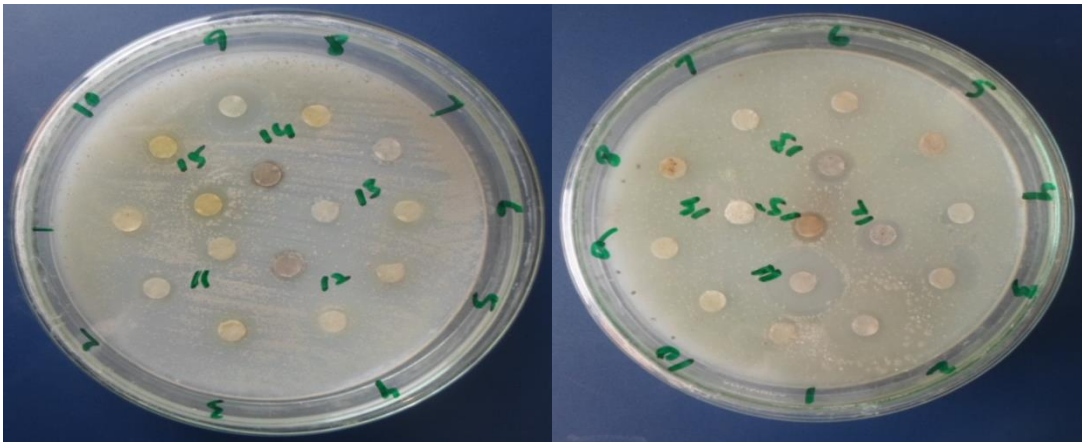

Fig 3.2: Protein kinase inhibition zones produced by EW and BC complexes

3.7. Phenolic and flavonoid contents of metallic complexes

Comparatively highest phenolic contents in EW complexes was observed in EWMo complex (1.8551±0.115) followed by EWCs (1.6815±0.059). While lowest phenolic content was observed in EWBi (0.3719±0.006). Significant highest flavonoids content was observed in EWMo complex (0.719±0.010) and lowest was observed in EWLan complex (0.094±0.005). In BC complexes comparatively highest phenolic content was observed in EWMo complex (2.0265±0.045). Significant lowest phenolic content was found in BCLan (0.3835 ±0.014). BCCs complex contained highest flavonoids content (0.236 ±0.009) and lowest content was observed in BCAl (0.057 ±0.005). detailed results are given in table 3.10. Experiment was performed in triplicate. Values are mean of three independent replicate along with standard deviations. Gallic acids and Quercetin were used as standard for phenolics and flavonoids respectively. Content was expressed as µg equivalent per 100 µg complex.

Table 3.10: Phenolic and flavonoid content of EW and BC complexes.

S. No.	EW complex			BC complex		
	Sample code	Phenolic content	Flavonoids content	Sample code	Phenolic content	Flavonoids content
1	EWAl	0.9030±0.012	0.214±0.025	BCAl	1.5408 ±0.030	0.057 ±0.005
2	EWCd	0.9758±0.006	0.225±0.019	BCCd	0.6968 ±0.014	0.123 ±0.018
3	EWLan	0.5950±0.021	0.094±0.005	BCLan	0.3835 ±0.014	0.134 ±0.026
4	EWSn	0.4852±0.005	0.166±0.011	BCSn	1.2411 ±0.046	0.128 ±0.024
5	EWBi	0.3719±0.006	0.143±0.005	BCBi	0.6826 ±0.007	0.184 ±0.016
6	EWCo	1.2206±0.015	0.279±0.011	BCCo	0.7521 ±0.005	0.145 ±0.010

S. No.	EW complex			BC complex		
	Sample code	Phenolic content	Flavonoids content	Sample code	Phenolic content	Flavonoids content
7	EWPb	1.4322±0.005	0.164±0.001	BCPb	0.5345 ±0.022	0.152 ±0.008
8	EWMo	1.8551±0.115	0.719±0.010	BCMo	2.0265 ±0.045	0.171 ±0.013
9	EWECs	1.6815±0.059	0.189±0.003	BCCs	1.4293 ±0.025	0.236 ±0.009
10	EWLi	1.1213±0.012	0.204±0.005	BCLi	0.7803 ±0.017	0.167 ±0.005
11	EWHg	1.0336±0.017	0.165±0.014	BCHg	1.3383 ±0.028	0.158 ±0.004
12	EWFs	1.2750±0.015	0.265±0.013	BCFs	1.2724 ±0.005	0.182 ±0.010
13	EWFc	1.0514±0.017	0.278±0.016	BCFc	1.0270 ±0.037	0.172 ±0.010
14	EWAg	1.1884±0.017	0.247±0.010	BCAg	1.4830 ±0.021	0.159 ±0.011
15	EW	15.686±0.014	4.720±0.199	BC	16.056 ±0.115	3.930 ±0.253

Fig 3.3: Phenolic content of EW complexes

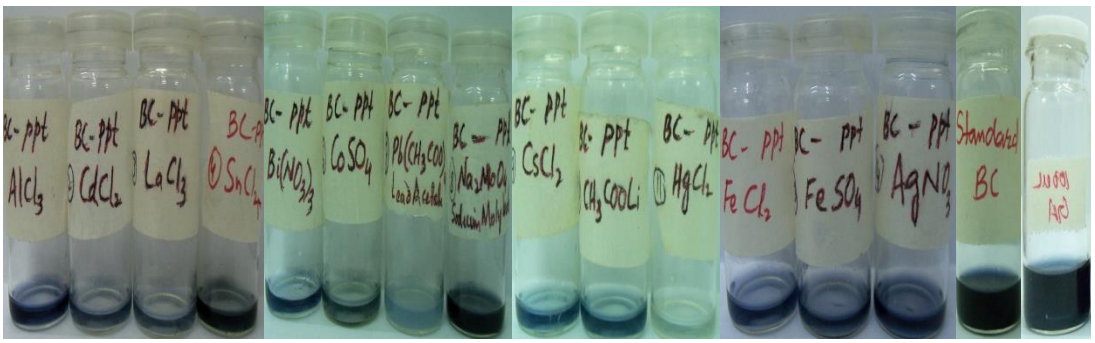

Fig 3.4: Phenolic content of BC complexes

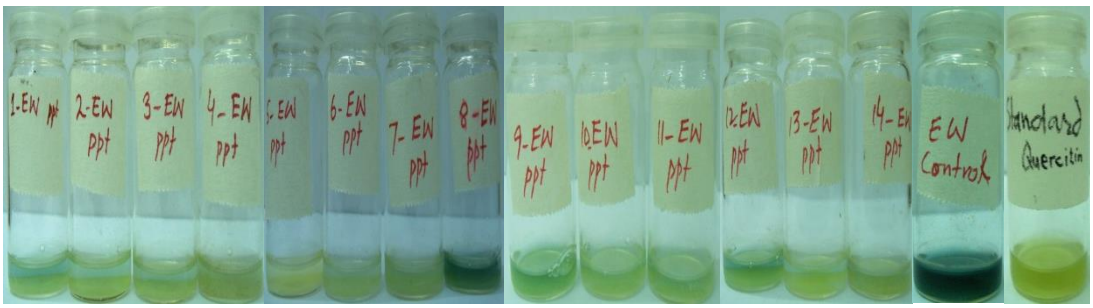

Fig 3.5: Flavonoid content of EW complexes

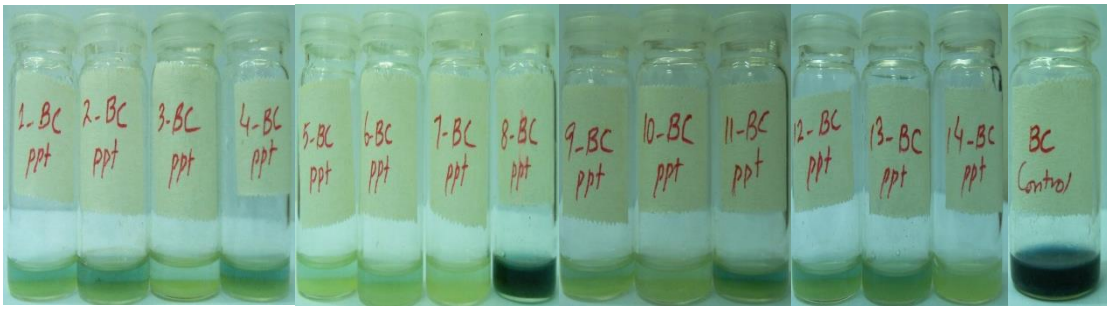

Fig 3.6: Flavonoid content of BC complexes

3.8 FTIR results

All the spectra were recorded in the range of 4000 to 600cm^{-1}, these are given bellow

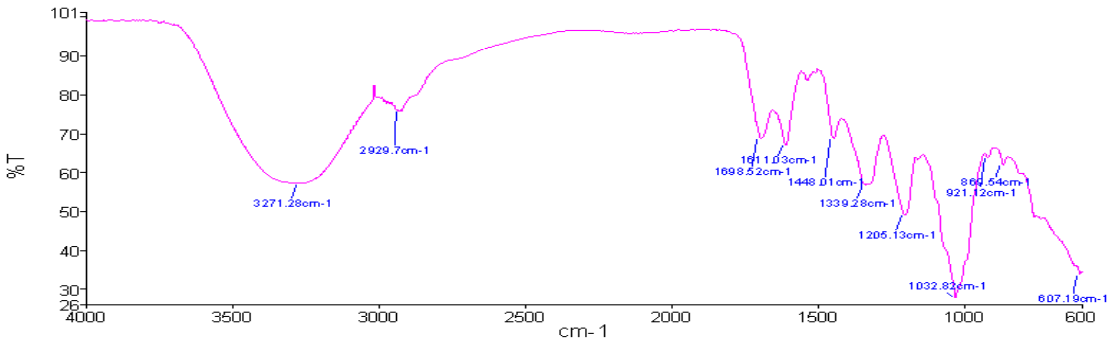

Fig 3.7: FTIR spectra of EW crude extract

Table 3.11: Wave numbers and suggested functional groups in FTIR spectra of EW crude extract

S. No.	Wave number (cm^{-1})	Suggested functional groups
1	921.66	Carboxylic acid, =C-H (Alkene)
2	1032.82	(C-O) Ester, (C-O) Alcohol
3	1205.13	(C-O) Ester, (C-N) amine, phenolic (-OH)
4	1339.28	(C-N) amine
5	1448.01	(C=C) aromatic (-C-H) alkane, acetonitrile
5	1611.03	(N-H)) Amides,
7	1698.52	(C=O) Ketone
8	2929.28	(-C-H) alkane
9	3271	(-OH)

Spectra of extract deduce the presence of various functional groups as carboxylic acid, (=C-H) Alkene, (C-O) Ester, (C-N) amine, (C-O) Ester, (C-N) amine, (C=C) aromatic (-C-H) alkane, acetonitrile, (N-H)) Amides, Ketone, (-C-H) alkane, (-OH) which are presented in above table with their peak values.

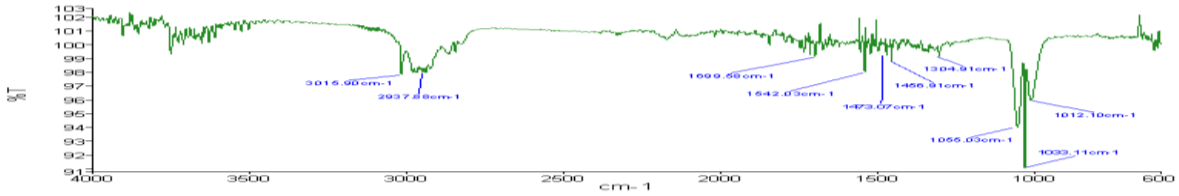

Fig 3.8: FTIR spectra of EWAl complex

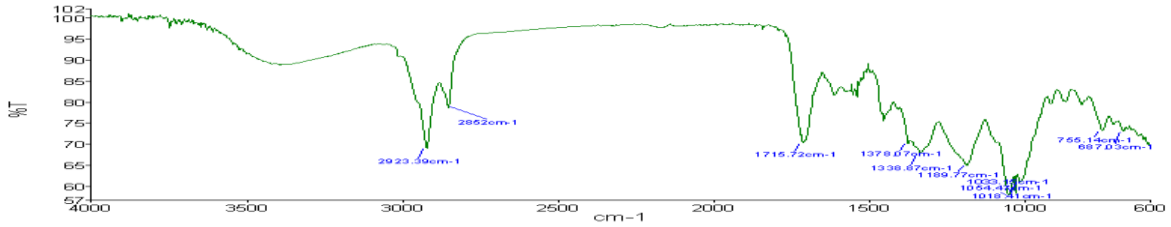

Fig 3.9: FTIR spectra of EWCd complex

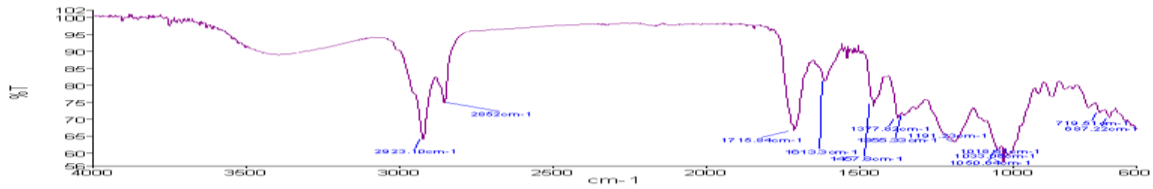

Fig 3.10: FTIR spectra of EWLan complex

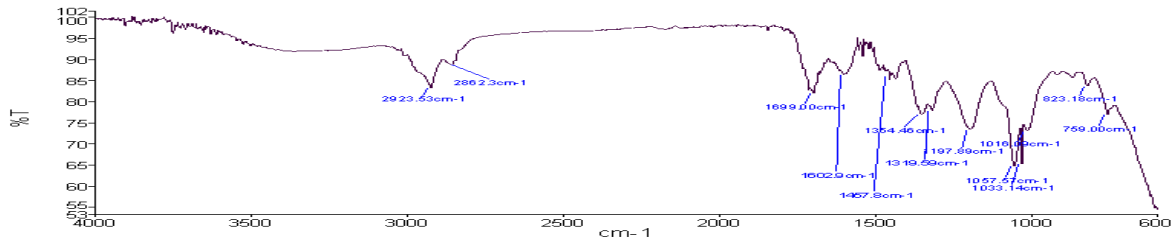

Fig 3.11: FTIR spectra of EWSn complex

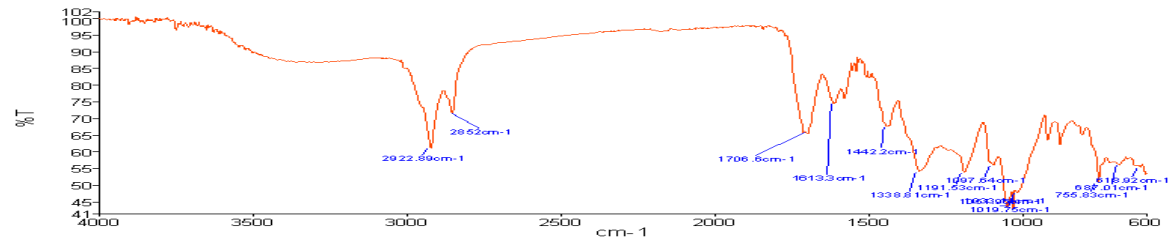

Fig 3.12: FTIR spectra of EWBi complex

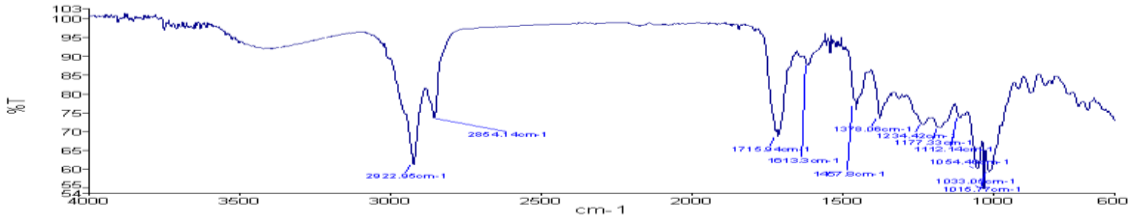

Fig 3.13: FTIR spectra of EWCo complex

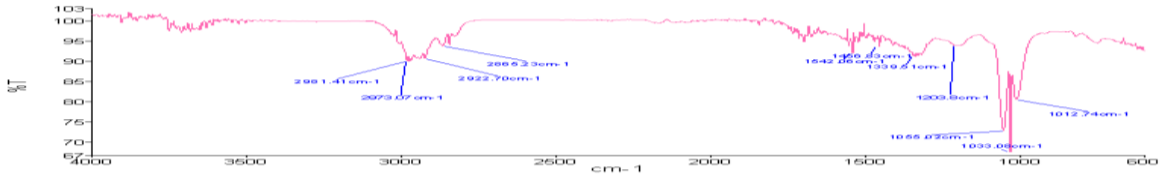

Fig 3.14: FTIR spectra of EWPb complex

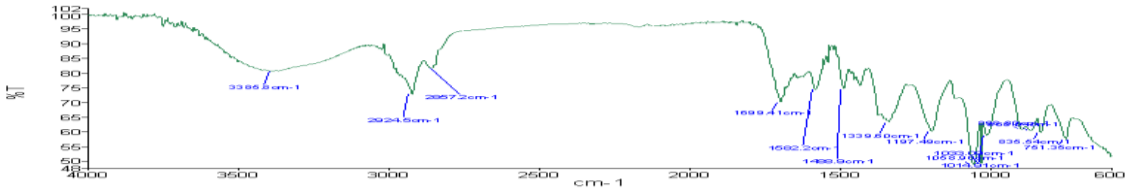

Fig 3.15: FTIR spectra of EWMo complex

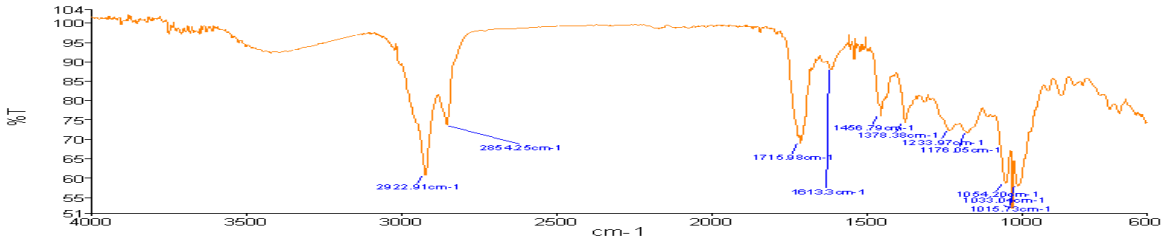

Fig 3.16: FTIR spectra of EWCs complex

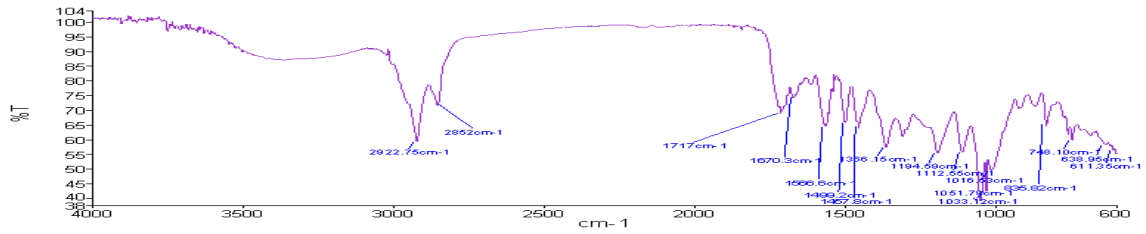

Fig 3.17: FTIR spectra of EWLi complex

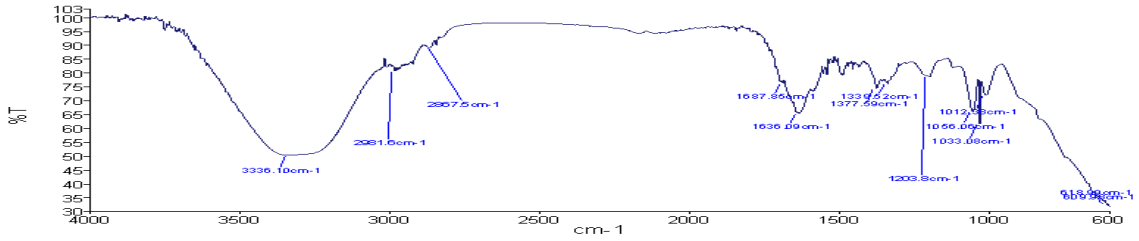

Fig 3.18: FTIR spectra of EWHg complex

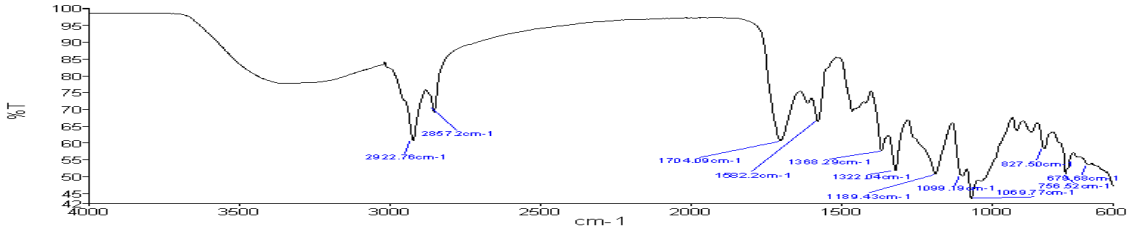

Fig 3.19: FTIR spectra of EWFs complex

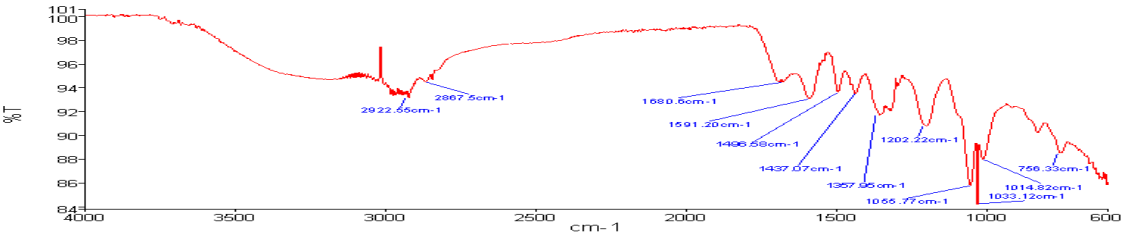

Fig 3.20: FTIR spectra of EWFc complex

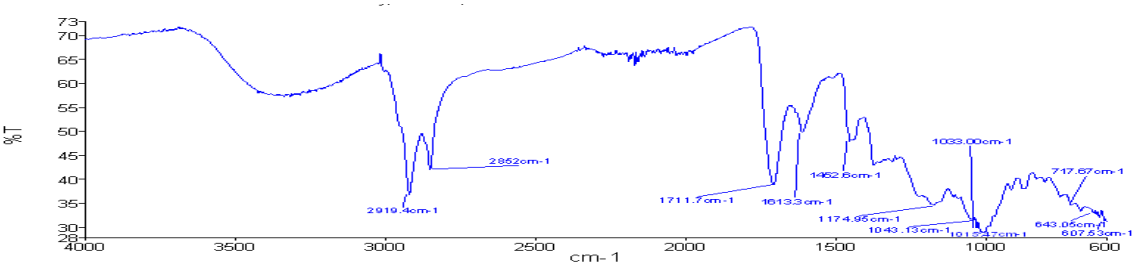

Fig 3.21: FTIR spectra of EWAg complex

Characteristic peaks appeared in the range of 1000-600cm^{-1} along with peak at 1205cm^{-1} in the spectra of EW extract, were disappeared in the spectra of EWAl complex. Variations in other peaks were also observed in this complex. Changed spectral orientations in EWCd complex were observed comparative to EW extract.

Peaks at 3271and 1611cm^{-1} in EW spectra were found missing in spectra of EWCd complex. EWLan, EWSn and EWBi complexes showed the similar pattern of spectral pattern and major change was found at 900 to 600 cm^{-1}. Broad peak that was originated in EW spectra at 3271.28 cm^{-1} was extinct and shifting in peak values were noticed in EWCo complex. EWPb complex spectra displayed a peak at 2865 cm^{-1} splitting into doublet, which is characteristic peak of carbonyl functional group. EWPb exhibited different spectra relative to EW extract and sharp peak was established at 1033.08 cm^{-1} in it. Characteristic peak observed in spectra of EW extract above 3000cm^{-1} was reappeared in the EWMo and EWHg complexes. Shifting in peak values were perceived in the spectra of these complexes. EWFs showed fused and transformed peaks. Spectra of EWCs, EWLi, EWFc, EWAg complexes showed the fused peaks in spectral positioning and peak values transformed indicating the different nature of complex formation.

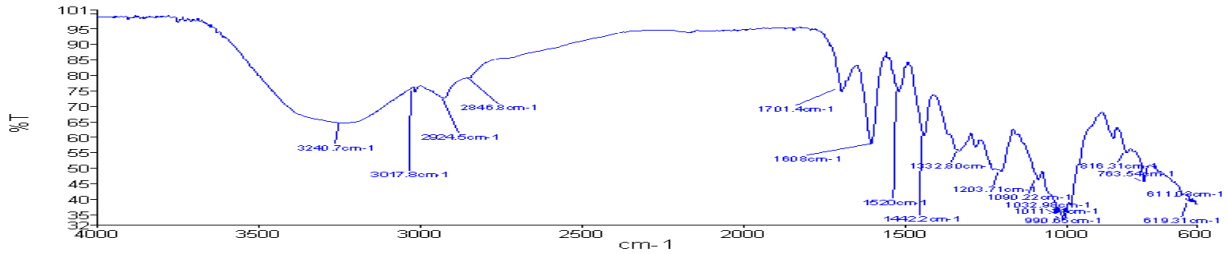

Fig 3.22: FTIR spectra of BC extract

Table 3.12: Wave numbers and suggested functional groups in FTIR spectra of BC crude extract

S. No.	Wave number (cm^{-1})	Suggested functional groups
1	921	Alkene
2	1032.98	(C-O) alkyl aryl ethers
3	1090.22	(C-N) amine (a)
4	1203.71	(C-O) Ester, (C-N) amine
5	1332.80	(C-N) amines(t)
6	1442.2	Alkanes
7	1520.0	(N-H) Nitro compounds (amide)
8	1701.4	(C=O) alkyl aryl ketone
9	2846.8	(CH)
10	2924.05	(-C-H) alkane, (C=O) acid
11	3017.8 3240.7	(N-H) amide

a= aliphatic, t=tertiary

Peaks along with their values appeared in IR spectra of BC extract are mentioned in table. 3.12. Which indicating the presence of various functional groups as (C-O) alkyl

aryl ethers, (C-N) amine, (C-O) Ester, alkane, (N-H) Nitro compounds (amide), (C=O) alkyl aryl ketone, (-C-H) alkane, (C=O) acid, (-OH) Alcohol and phenol in extract of BC.

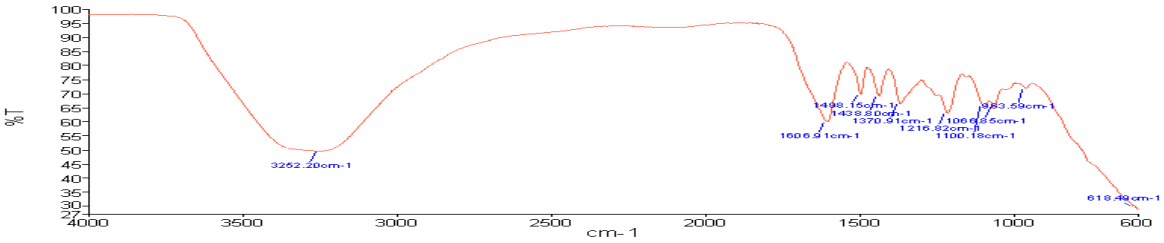

Fig 3.23: FTIR spectra of BCAl complex

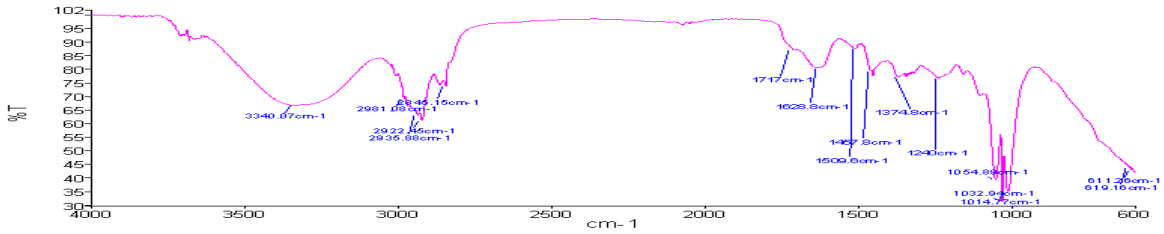

Fig 3.24: FTIR spectra of BCCd complex

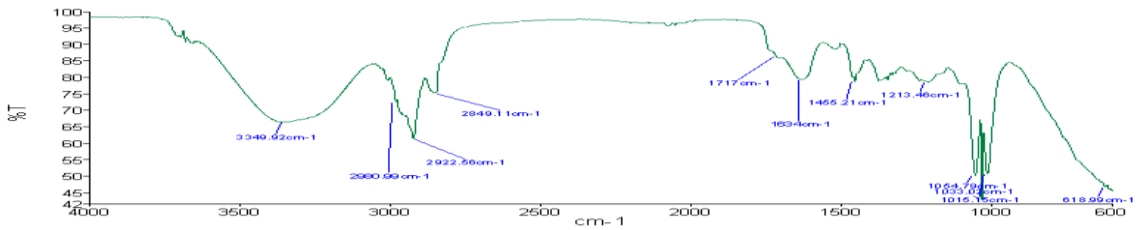

Fig 3.25: FTIR spectra of BCLan complex

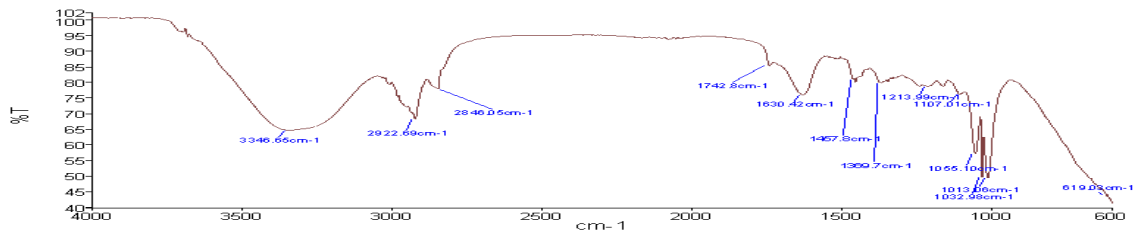

Fig 3.26: FTIR spectra of BCSn complex

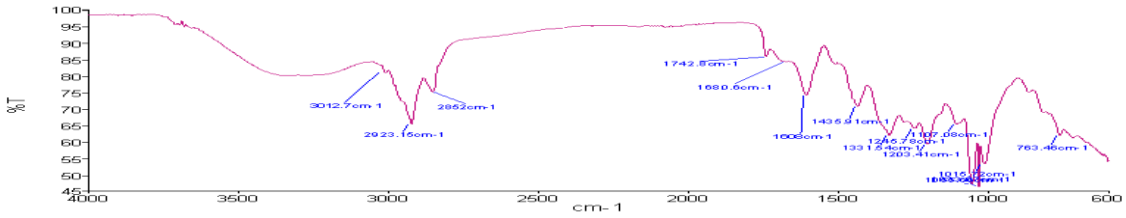

Fig 3.27: FTIR spectra of BCBi complex

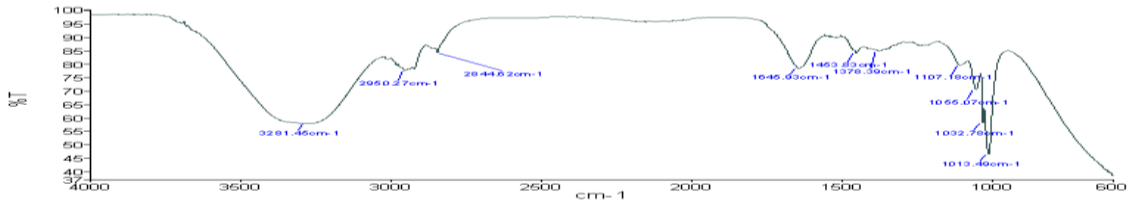

Fig 3.28: FTIR spectra of BCCo complex

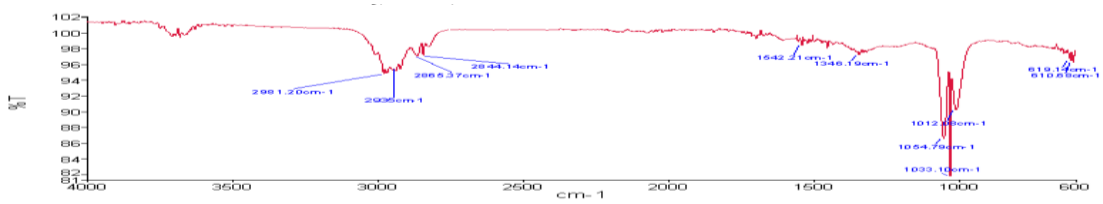

Fig 3.29: FTIR spectra of BCPb complex

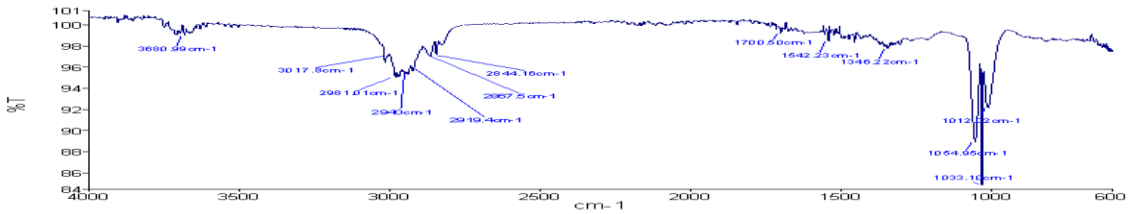

Fig 3.30: FTIR spectra of BCMo complex

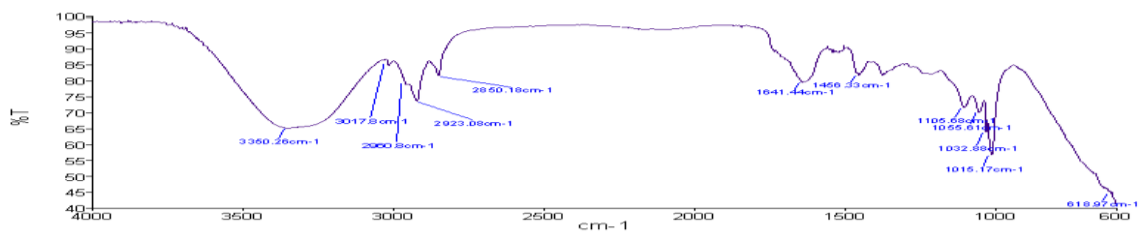

Fig 3.31: FTIR spectra of BCCs complex

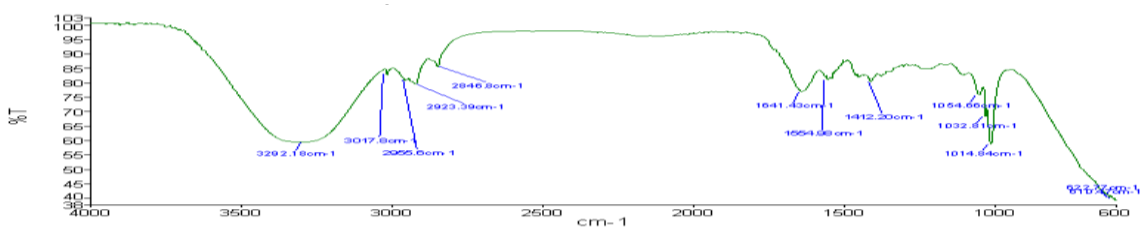

Fig 3.32: FTIR spectra of BCLi complex

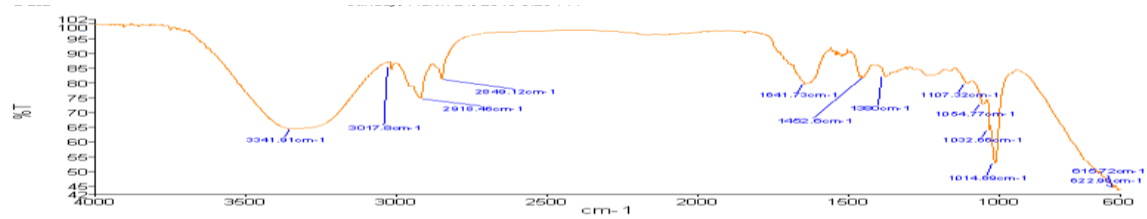

Fig 3.33: FTIR spectra of BCHg complex

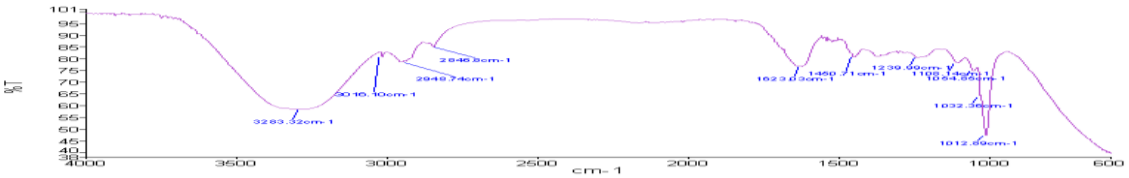

Fig 3.34: FTIR spectra of BCFs complex

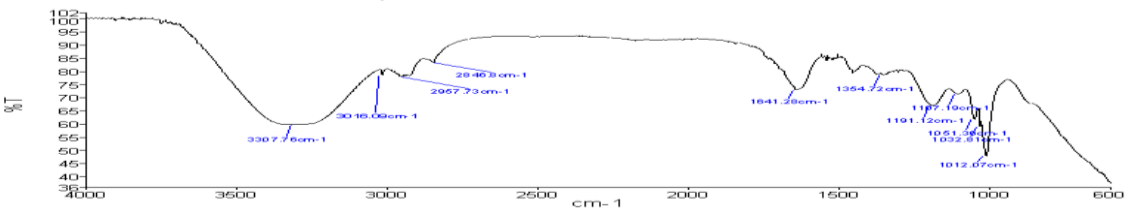

Fig 3.35: FTIR spectra of BCFc complex

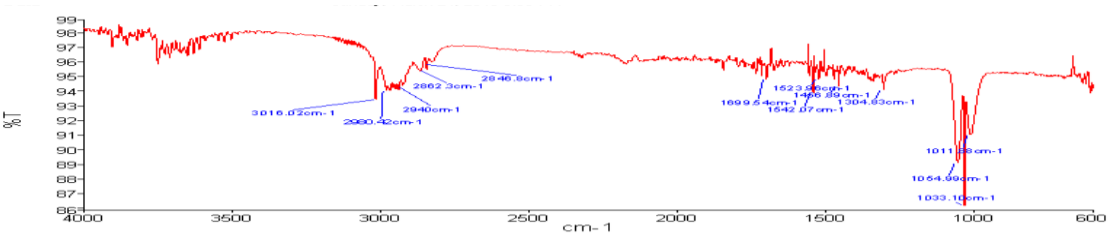

Fig 3.36: FTIR spectra of BCAg complex

All complexes showed changed spectral orientation comparative to spectra of BC extract. Characteristic peaks appeared at 3017.8, 2924.05, 2846.8, 1701.4 cm^{-1} were missing in in the spectra of BCAl complex. BCCd, BCLan, BCCo, BCSn, BCCs, BCLi, BCHg, BCFs, BCFc showed the similar spectral orientation for complexation with shifted in their values of peaks. Spectra of these complexes were differing in the range of 1000-600cm^{-1} along with other shifting in values of peaks. Peaks at 3017.8 and 1520cm^{-1} observed in BC spectra were extinct in spectra of BCSn complex. Characteristic peak of BC spectra at 1701.4, 1332.8 and 1203.71cm^{-1} were not exhibited in spectra of BCCo complex. BCPb, BCAg and BCMo complexes have showed different spectral orientation. Sharp peaks were observed near to 1000cm^{-1} in spectra of these complexes. Two peaks at 1442.2, 1203.71cm^{-1} were disappeared and specific peak at 3680.99 cm^{-1} was appeared in the spectra of BCMo complex. Nature of ligand can be predicted by observing the change in orientation and peaks of spectra of complexes, comparative to spectra of BC extract.

Discussion

Drug discovery from natural sources started in prehistoric times that led for discovery of many compounds. Most of present medicines have been developed on the basis of traditionally used therapies (Rates, 2000). Drugs obtained from plants possess better pharmacological responses, due to which 25% of worldwide prescribed drugs have been obtained from plants (Rates, 2000). Recent trend of taking complementary and alternative medicines is increasing, in order to relieve the pathological symptoms, This is because of perceived side effects of chemical drugs (Verpoorte *et al.*, 2006). Revolution in the field of organic chemistry has led to the production of potent, safe and more active synthetic drugs by structural modification in pure compounds (Petrovick *et al*., 1997; Sharapin, 1997; Rates, 2000). Coordination compounds are playing vital role in homeostasis of biological systems. Various metal complexes like carboxy-peptidase, hemoglobin and chlorophyll containing zinc, iron and magnesium metals respectively are important biological compounds in living system (Orvig *et al*., 1999)**.** Medicinal utility of coordination compounds formed by the combination of metals can be traced up to 5000 years back (Orvig *et al*., 1999). The splendid array of metal complexes applications has been investigated in pharmaceutical substances (Sadler *et al.*, 1999; Ali & van Lier, 1999; Louie & Meade, 1999; Volkert & Hoffman, 1999 Sakurai *et al.*, 2002), which has been increasing rapidly due to enhanced compatibility (Smith-Warner *et al*., 2000; Cragg *et al*., 2000). Various researchers have focused on advances in the field of coordination compounds (Sakurai *et al.*, 2002). Metal combines with ligands to form complexes and if ligand forms more rings, then resultant product will be chelate (Wayne, 1999). These metallo complexes have been found to possess importance in different fields, like biomedical, analytical, therapeutic, synthetic applications as gold complexes (Auranofin etc) (Perry, 2002; Dorr & Von Hoff, 1994). New methodologies have continuously been applied for the development of new drugs as combinatorial drug chemistry. But it is necessary to determine the toxicity of chelation based therapies in clinical trials. In current study, coordination compounds of *B. ciliata* extract (BC) and *E. wallichii extract* (EW) were synthesized. These were evaluated for antioxidant potential, coordinated phenolic and flavonoids contents, cytotoxic effect, antimicrobial and kinase enzyme inhibition activity. Complexes were also subjected to FTIR spectroscopy in order to evaluate the nature of ligand.

Antioxidants work in diverse ways, so the mechanism of action of all antioxidants in a complex system cannot be reflected in any single assay (Prior *et al.,* 2005). There are two methods that describe mechanism of antioxidants i.e. free radical scavenging by hydrogen donation to form stable compound (Prior *et al.,* 2005) and reduction of free radical by donating electron.

DPPH, an organic nitrogen radical, is commercially available with deep purple color. Measurement of antioxidant potential of sample using DPPH was first time reported by Williams *et al.* (1995). All complexes showed the decrease in DPPH activity. This assay provides the basic information regarding free radical scavenging potential of different compounds. Reduction of DPPH by the compound produces diphenylpicryl hydrazine, a pale yellow compound. This property was utilized to determine the scavenging potential of EW and BC complexes. All EW and BC complexes showed decrease in DPPH % scavenging capacities. EWMo complex was found to have 14% and EWFs showed 70% less scavenging potential comparative to extract. BCMo complex was found to have 12% less scavenging potential as compared to extract. This shows the higher antioxidant potential of extract as also indicated by its significant reducing capacity (Rajkumar *et al.,* 2010). Phenolics are important antioxidant phytochemical (Javanmardi *et al.,* 2003) and these are nucleophilic in nature, which is responsible for the chelating potential of these compounds (Morgan *et al.,* 1997, JUN *et al.,* 2003). Total antioxidant capacities of complexes were checked using phosphomolybdenum method and capacities were expressed in ascorbic acid equivalent. All complexes have showed the lower total antioxidant capacities. EWCd complex showed 13% lesser total antioxidant potential then EW extract. Among BC complexes BCMo and BCCs were found to have significant 4% less total antioxidant potential with respect to BC extract. In this assay complexes reduce the Mo (VI) to Mo (V) in acidic condition that led to the formation of green colored phosphate Mo (V) complex (Prieto *et al.,* 1999). In both the DPPH and total antioxidant tests, metallo complexes showed reduced antioxidants activities as compared to crude extracts. It showed that incorporation of metals in the complexes of extracts changed the properties of ligands present in extracts, by reducing the binding sites of the free radicals and producing an antagonistic effect, which resulted in reduced antioxidant capacities (Thakum *et al.,* 2012).

EW and BC complexes were also assessed for the reducing capacities that were expressed in ascorbic acid equivalent. Complexes showed higher reducing power

comparative to extract. Higher ascorbic acid equivalent number indicates the more reducing power. EWFs complex had increased reducing power (51%) comparative to EW extract. Slight elevation in reducing power was produced by EWLan and EWPb, that was 3.6%, 4.3% with respect to EW extract. BCCd was also found to have 46% increases in reducing power among BC complexes. In our study, compound with increased reducing power were not active in DPPH assay. It may be due to the change in the chemical properties of the extracts after combination with the metal. Metal may had increased the reducing potential of electron donating substances in complexes, however failed to produce any significant activity in quenching capabilities of free radicals, resulting in the reduction of scavenging potential in DPPH assay. This idea was supported by Thakam *et al*. (2012), who also reported increase in reducing potential of complexes comparative to their extracts.

EW and BC complexes were evaluated for coordinated phenolic and flavonoid contents. Among EW complexes, highest phenolic contents were observed in EWMo complex (1.855 ± 0.115 µg/100 µg of sample) and lowest were observed in EWBi (0.372 ± 0.006 µg/100 µg of sample). Whereas, EWMo complex (0.719 ± 0.010 µg/100 µg of sample) had highest flavonoids, while lowest were observed in EWLan complex (0.094 ±0.005 µg/100 µg of sample). Among BC complexes, highest phenolic contents were observed in EWMo complex (2.0265±0.045 µg/100 µg of sample) and lowest in BCLan (0.3835 ±0.014 µg/100 µg of sample). BCCs complex contained highest flavonoids content (0.236 ±0.009 µg/100 µg of sample) and lowest value was observed in BCAl (0.057±0.005 µg/100 µg of sample). Haq *et al.,* (2012) had reported 20% phenolic and 0.39% flavonoids in EW extract. Rajkumar *et al.* (2010) had also found 38% phenolic contents in BC extract. In current study 16% phenolic was observed in extract of BC and 15% from EW extract. These differences may depend on variations in collection time and site. Due to the presence of aromatic rings phenolic and flavonoids are nucleophilic in nature, which is responsible for the chelating potential of these compounds (Morgan *et al.,* 1997, Jun *et al.,* 2003). Coordination of these compounds with metal is further confirmed by measuring their content in complexes. It is also evident from the above results that other compounds which are not phenolics and flavonoids are also involved actively in complex formation with metals as indicated by the lower content of phenolic and flavonoids measured in content determination.

Cytotoxic effects of complexes were also investigated by using the brine shrimp assay. It is cost effective, rapid and feasible to carry out for cytotoxic studies (Kivack *et al.,* 2001; Carballo *et al.,* 2002). Larval tissues of shrimps respond similarly as mammalians carcinoma cells. Cytotoxic effect of complexes towards shrimp's larvae can be correlated with anticancer potential and complexes could be alternative source of anticancer drugs (Ullah *et al.,* 2012). These days much attention is being given on metallo complexes and their anticancer activity. In order to discover new potent anticancer drug various studies has been done for example synthesis, modification, chemical and structural behavior of metal complexes (Saha *et al.,* 2009). In current study, significant cytotoxic activity was observed from EWHg (LD_{50} = 96 µg/ml), followed by EWFs (LD_{50} = 96.69 µg/ml). EWCs showed comparatively maximum LD_{50} value indicating its least activity among EW complexes. Among BC complexes, BCFs was more cytotoxic having LD_{50} (LD_{50} = 58 µg/ml), while BCAg has maximum LD_{50} value (LD_{50} = 392 µg/ml) showing lowest cytotoxic nature. Haq *et al.* (2012) reported the promising cytotoxic potential of EW extract, which was also observed in current study. Concerning the complexes of transition metal, these had been proved to be biologically active and were studied to have anticancer properties (Ali *et al.,* 2008; Ali *et al.,* 2009). Various complexes having organic ligand had also been found to have antitumor activity as amines ligand in ruthenium complexes (Jain *et al*., 2009). In current study, metal complexes were also found to have significant cytotoxic capacities as also reported by Islam *et al.* (2007).

Bacterial resistance against existing drugs has led to severe health concerns throughout the world. It imposes for more research to find new classes of potent antibiotics. Various studies had been carried out for the investigation of potent and effective antibacterial agents. In present study, all complexes were evaluated for antibacterial capacities using disc diffusion method against six types of bacterial strains. Activity was calculated by measuring inhibition zone around disc. Complexes were found to have enhanced antibacterial activity comparative to their extracts. Among EW complexes five were found active against *M. lutus* strain, four against *E. coli*. While EWHg exhibited maximum inhibition zone (16 ± 1.527 mm) against *S. aureus* and observed active against five bacterial strains. Varying antibacterial activities were observed in BC complexes. BCHg was observed significant active among BC complexes and produce inhibition zone of 17 ± 0.58 mm against *M.lutus*. Five complexes were found active against *B. bronchiseptia* strain, four were against *S.*

typhimurium and *E. aerogenes* among BC complexes. The complex EWSn, EWPb, EWMo, EWFs, EWFc and EWAg, BCCs were found to have no activity against any bacterial strain. Different studies being carried on toxicological, antimicrobial activities of metal complexes having mixed ligands. Ivanova1 *et al.* (2012) have also report the antimicrobial potential of metal complexes. Similarly Islam *et al.* (2013) have also reported the antimicrobial activity of metal complexes. It was observed that nature of ligands, metal and synergetic effect determines the antibacterial potential of metal complex (Chohan *et al.,* 2006). Selective antibacterial effect of the complexes depends on metals, ligands having different functional groups and donor atoms that produce selective toxic effect which resulted in varying antimicrobial activities of all complexes as also reported by Shaikh *et al.* (2004).

Pathogenic fungal strains increase the human mortality and morbidity rate. Multidrug resistance in microbe is fast growing and major global health issue (Chohan *et al.,* 2006). Saha *et al.* (2009) have reported various fungal strains showing drug resistance e.g *F. solani, Candida albicans, A. flavus* and *Candida glaberata.* This generates the interest for discovering new antifungal agent or to modify the existing drugs. Various studies have been carried for characterization of chemical and structural nature of metal complexes, which can be used as alternative antifungal drug (Saha *et al.,* 2009). All complexes of EW and BC were tested for antifungal potential against five fungal strains as *A. niger*, *A. fumigatus*, *Mucor species*, *A. flavus* and *F. solani.* Among EW complexes, six were detected active against *A. fumigatus.* EWFs has produce significant maximum zone of inhibition (10 ± 0.288 mm) against *A. flavus*. Five of BC complexes were found active against *A. niger* and *A. fumigatus.* BCAl have produced maximum zone of inhibition (16 ± 2.081 mm) against *A. niger.* EWCs, BCMo, BCCs and BCFc were observed inactive against any fungal strain. Antifungal potential of complexes was also reported by Chohan *et al.* (2006) and Saha *et al.* (2009). Metal, ligand and nature of complex decide the antimicrobial potential of coordinated compound (Chohan *et al.,* 2006). Metal complexes have more antifungal activity than free ligand as biological activity of complexes can be explained using overtone's concept and chelation theory (Chohan *et al.,* 2006). Liposolubility is the main factor that determines the antifungal activity of sample (Sadeek *et al.,* 2009). Lipid membrane around the cell controls the permeability and only allows lipid soluble material in to cell. Chelation decreases the metal ion polarity, due to overlap ligand orbital and partial sharing of the positive charge of the metal ion with donor

groups. Delocalization of p-electrons increase over whole chelate that favor the lipophilicity of the complex (Sadeek *et al*., 2009). Due to increase the lipophilicity complexes are penetrated into lipid membrane easily and consequently block metal binding sites on the enzymes of the microorganism (Raman *et al.,* 2003; Sadeek *et al.,* 2009). Various studies were carried on cytotoxic and anticancer property of metal complexes, for example, Islam *et al.* (2007).

Protein kinase inhibitors are important drugs for cancer treatment. Most of inhibitors are investigated for cancer and but deregulation of kinase function is also involved in neurological, immunological, infectious and metabolic diseases. Researchers are diverted for development of kinase inhibitors, so cancer along with these disorders can be treated (Cohen *et al.,* 2002). There are different methods for the measurement of inhibition of kinases. Currently election of inhibitor against specific protein kinase that can be used as drug remains challenge for researchers. Cohen was the first to screen kinase inhibitors for various enzymes and substrates using microplate reader in 96 well formats (Davies *et al.,* 2000; Bain *et al.,* 2003; Bain *et al.,* 2007). In present study metal complex of both plants were observed to have capacity of inhibiting protein kinases. EWCd (9mm), EWCs (15mm), BCFs (10mm), BCFc (16mm) complexes showed significant kinase inhibition potential among EW and BC complexes.

FTIR is prerequisite technique in pharmacopoeia of various countries for identification of medicines (Liu *et al.,* 2006). It is widely used for recognition (Griffiths and De Haseth, 1986) as well as accurate and rapid identification of biological compounds (Sukmaran *et al.,* 2011; Thenmozhi *et al.,* 2011). Infrared region ranges from 0.8 to 10^3 µm. Limited portion of this range from 4000 to 625 cm^{-1} (2.5-16 µm) is useful for organic compounds (Younas, 2004). Haq *et al*. (2012) reported the presence of various phytochemicals like tannins, alkaloids, terpenoids, glycosides and flavonoids in EW extract. FTIR spectra in the range of 4000-600 cm^{-1} of crude extract of *E. wallichii*, suggested the presence of hydrocarbons, terpenoids, abietane steroids, aromatic esters, phenolics, flavone glycosides and several bioactive constituents. These compounds having different functional groups are represented by their characteristics peaks in the range of. Phenolic, flavonoid content in both extracts and that attached with metals was confirmed by measuring the amount of these phytochemicals. Characteristic peaks appeared in the range of 1000-600cm^{-1} along with peak at 1205cm^{-1} in the spectra of EW extract, were disappeared in the spectra of

EWAl complex. Variations in other peaks were also observed in this complex. Changed spectral orientations in EWCd complex were observed comparative to EW extract. Peaks at 3271and 1611cm^{-1} in EW spectra were found missing in spectra of EWCd complex. EWLan, EWSn and EWBi complexes showed the similar pattern of spectral pattern and major change was found at 900 to 600 cm^{-1}. Broad peak that was originated in EW spectra at 3271.28 cm^{-1} was extinct and shifting in peak values were noticed in EWCo complex. EWPb complex spectra displayed a peak at 2865 cm^{-1} splitting into doublet, which is characteristic peak of carbonyl functional group. EWPb exhibited absence of peak of OH and downward shifting of amine group peak to 1033.08 cm^{-1}. EWMo exhibited downward shifting of peak of OH. Characteristic less intense peaks were observed in spectra of EWHg complex above 3000cm^{-1} as compared to crude extract. Shifting in peak values were perceived in the spectra of these complexes. EWFs showed fused and transformed peaks in whole spectra. Spectra of EWCs, EWLi, EWFc, EWAg complexes showed the fused peaks in spectral positioning and peak values transformed indicating the different nature of complex formation. FTIR spectra of extract of *B. ciliata* suggested the presence of different compounds like paashaanolactone, catechin, anthraquinone, steroids, phenolic, flavonoids, saponins, tannins and terpenoids. All complexes of BC showed changed spectral orientation comparative to spectra of BC extract. Characteristic peaks appeared at 3017.8, 2924.05, 2846.8, 1701.4 cm^{-1} were missing in in the spectra of BCAl complex. BCCd, BCLan, BCCo, BCSn, BCCs, BCLi, BCHg, BCFs, BCFc showed the similar spectral orientation for complexation with shifted in their values of peaks. Peaks at 3017.8 and 1520cm^{-1} observed in BC spectra were extinct in spectra of BCSn complex. Characteristic peak of BC spectra at 1701.4, 1332.8 and 1203.71cm^{-1} were not exhibited in spectra of BCCo complex. BCPb, BCAg and BCMo complexes showed different spectral orientation. Sharp peaks were observed near to 1000cm^{-1} in spectra of these complexes unlike crude BC extract. Two peaks at 1442.2, 1203.71cm^{-1} were disappeared and specific peak at 3680.99 cm^{-1} was appeared in the spectra of BCMo complex. The shifting of peaks of various functional groups to lower absorption ranges, in spectra of both BC and EW complexes as compared to the spectra of the crude extracts of plants indicated the higher potential of coordination of those particular functional groups such as OH, alkenes, amines and carboxylic acid etc. with metals. In FTIR spectroscopy, coordination complexes frequently shows the absence as well as shifting of peaks lower absorption range as

compared to ligands. Singh (2012) indicated the shifting of bands of coordination compounds of schiff ligand, from 1492 cm^{-1} to 1490 cm^{-1} and 1456 cm^{-1} to 1453 cm^{-1} respectively as compared to the individual schiff ligand, indicating its potential to coordinate with the metals. Similarly Thakam *et al*. (2012) also observed the decrease in intensity of carbonyl group band in the complex of metal and curcumin showing its involvement in the coordination.

Conclusion and future strategies

Successful complexation of metals with *Bergenia ciliata* and *Euphorbia wallichii* extracts was carried out in current study. Complexes showed enhanced biological capacities and lower antioxidant activities. Due to lower antioxidant capacities complexes cannot be considered as potential antioxidant agents because metal changed the chemical properties of complex. Significant enhanced biological capacities of metallic complexes showed their potential to be used as an alternative source of cytotoxic and antimicrobial drugs. Few sample showed the potential of inhibiting protein kinases, so could be used as kinase inhibitors as well. However, there is need of further evaluation of chemical nature of ligands and *in vivo* and mechanistic based studies of these complexes. There is further need of investigation of actual mechanism of cytotoxicity and probable effect of complexes on cell lines and animal model. Studies may be performed for the size determination and structure elucidation of these compounds by means of various analytical procedures.

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Synthesis and biological evaluation of metallic complexes of crude extracts of Bergenia ciliata and Euphorbia wallichii By Abdul Rehman Abstract Secondary metabolite obtained from plants play important role in treatment of various diseases, both in natural form or as coordinated with other agents. Metal combined with such compounds play key role in treatment strategies of different ailments. Current study deals with synthesis of metallic complexes of Bergenia ciliata (BC) and Euphorbia wallichii (EW) extracts and their biological evaluation. Total 14 different salts were used for the synthesis of metallic complexes. These were subjected to different in vitro tests for determination of their activities, which were compared with the activities of crude extract. In DPPH free radical scavenging test complexes showed reduced activities compared to crude extract. EWFs and BCMo complexes showed 70% and 12% decrease in DPPH activities respectively, comparative to crude extracts. In total antioxidant capacity assay, EWCd exhibited 13%, while BCMo and BCCs had 4% lesser activity comparative to extracts. In reducing power assay, complexes had enhanced activities. EWFs and BCCd showed 51% and 46% greater reducing activity,

comparative to their extracts. Contents of phenolics and flavonoids were also measured. Highest phenolic contents were observed in EWMo (1.8551±0.115 µg/100 µg of sample) and BCMo (2.0265±0.045 µg/100 µg of sample) complexes. While maximum flavonoid contents was observed in EWMo (0.719±0.010 µg/100 µg of sample) and BCCs (0.236 ±0.009 µg/100 µg of sample) complexes. Cytotoxic potential of complexes were evaluated by performing brine shrimp assay. EWHg (LD50= 96 µg/ml) and BCFs (LD50=58 µg/ml) showed minimum LD50 value exhibiting their high cytotoxic potential. Two gram positive bacterial strains i.e. M. luteus ATCC 10240, S. aureus ATCC 6538 and four gram negative strains i.e. A. aerogens ATCC 13048, E. coli ATCC 15224, S. typhimurium ATCC 14028 and B. bronchiseptica ATCC 4617 were used for evaluation of antibacterial capacity of complexes. Complexes showed varying degree of antibacterial activities. Among EW complexes, five complexes were observed active against M. lutus, four against E. coli. While EWHg exhibited maximum inhibition zone (16±1.527mm) against S. aureus and observed active against five bacterial strains. BCHg showed highest activity among BC complexes and produced inhibition zone of 17±0.58 mm against M.lutus. Five complexes were found active against B. bronchiseptia, four against S. typhimurium and E. aerogenes among BC complexes. Five fungal strains, A. fumigatus (FCBP 66), A.

12**niger (FCBP 0198),** F. **solani (FCBP 0291),** A. **flavus (FCBP 0064), and Mucor species (FCBP 0300)**

were used for evaluation of antifungal capacities of complexes. Six EW complexes showed activity against A. fumigatus. Maximum inhibition zone (10±0.288mm) was produced by EWFs against A. flavus. Five BC complexes were found active against A. niger and A. fumigatus. BCAl produced maximum zone of inhibition (16±2.081mm) against A. niger. Complexes were evaluated for protein kinase inhibition potential and four complexes showed the inhibition activity i.e. EWCd, EWCs, BCFs and BCFc complexes. FTIR of complexes was also performed which showed the presences of various phytochemicals such as hydrocarbons, terpenoids, abietane steroids, aeromaticeasters, phenolics, flavone glycosides and several bioactive constituents, which had already been confirmed in crude extract of E. wallichii. FTIR spectra of extract and metallic complexes of B. ciliata suggested the presence of different compounds like paashaanolactone, catechin, anthraquinone, steroids, phenolic, flavonoids, saponins, tannins and terpenoids. FTIR spectra suggested the presence of important phytochemicals in crude extracts as well as metallic complexes of both plants. In conclusion, it can be suggested that metallic complexes of BC and EW possess varying degree of biological activities and can be further studied for development of pharmacologically important medicinal agents. Introduction INTRODUCTION 1.1. Plants in drug discovery and as potential alternative therapies Plants being natural sources have been used to cure most of the diseases and to maintain good health since ancient times. The World Health Organization (WHO) has estimated about US $14 billion demand for medicinal plants per year. Approximately 15 to 20% annual growth has also been assessed in the demand of medicinal plants based raw materials, which will increase up to US $5 trillion till 2050 (Kala et al., 2006; Verma et al., 2011). Various drugs and their derivatives present in current pharmacopoeias, have been originated from plants. Twenty eight percent of chemical entities launched during last 20 years were comprised of natural products or their derivatives (Goyal et al., 2008). Drug discovery from natural sources undoubtedly started in prehistoric times, that led to discovery of many compounds as digoxin from Digitals spp.,

18**atropine from Atropa belladonna, morphine and codeine from** Papavor **somniferum,**

18**quinine and quinidine from Cinchona** and **vincristine and vinblastine from Catharanthus roseus**

(Rates, 2000). Many of the present medicines have been developed on the basis of traditionally used therapies (Rates, 2000). Natural products are the significant sources of new drugs particularly in the field of oncological and anti-hypertensive therapeutics. Due to better pharmacological responses drug obtained from plants constitute 25% of worldwide prescribed drugs (Rates, 2000; Verpoorte et al., 2006). Botanical medicines are sharing a large market in the form of food, drug and dietary supplements. Plants have been used to cure numerous diseases for thousands of years and are grouped as complementary/alternative medicines (CAM) in western countries (Verpoorte et al., 2006). Recent trend of taking complementary and alternative medicines is increasing, in order to relieve the symptoms because of perceived side effects of chemical drugs (Verpoorte et al., 2006). Revolution in the field of organic chemistry has led towards the production of synthetic compounds for pharmacological treatment. Structural modification in pure compounds produces potent, safe and more active drugs which also increases the economy of pharmaceutical companies (Petrovick et al., 1997; Sharapin, 1997; Rates, 2000). 1.2. Coordination compounds Metal complexes have essential role in biological systems. Biological compounds like carboxy-peptidase, hemoglobin and chlorophyll are metallic complexes containing zinc, iron and magnesium metals (Jain et al., 2009). Alizarin dye is a bright red coordination compound that was initially used by Egyptians and Persians. Modern coordination chemistry began to develop in 1798 by B. M. Tassaert. He observed brownish color of cobalt chloride in ammoniacal solution (Martell and Calvin, 1987). Number of theories regarding complexes properties and synthesis were originated during 19th century like Chain theory by Christian Wilhelm Blomstrand (1869) and Alfred Werner's coordination theory (1893) (McCleverty and Meyer, 2003; Martell and Calvin, 1987). 1.2.1. Properties of coordination complex i. Coordination number Extensive studies on coordination compound reveal the presence of certain useful properties and unusual chemical nature. Nature of complex depends upon central metal atom, oxidation state, kind, number and ligand arrangement (Basolo and Johnson, 2003). Werner gave the concept of coordination number and describes ligand arrangement around metal. Number of bonds donated from ligand to metal atom is coordination number. Usually it ranges from 2 to 12. Tetra and hexa-coordinate are common (Basolo and Johnson, 2003). ii. Ligands and chelates In 1916 Alfred Stock (German chemist) proposed the term ligand and these can be mono, di or polydenate involving 1, 2 or many atoms respectively. Polydenate ligand containing complex are chelates. These are stable, useful and their synthesis is termed as chelation (McCleverty and Meyer, 2003). Coordination complexes having metals with valence electrons up to 18, are most stable. In 1920s, Gilbert N. Lewis (physical chemist, USA) described the coordinate bond. It is denoted by arrow from donor to acceptor (Basolo and Johnson, 2003). 1.2.2. Organic metal complex Metals are the important parts of medicines, being used by humans for many years. Significance of essential metals has recently been realized, because varying quantities of these are needed in our diet (Jian et al., 2009). On the other hand, certain metals are toxic even in trace amount and can leads to metal toxicity (Tripathi, 2009). Metal complexes are of different types. On the basis of charge these are negative, positive and neutral metallic complexes. In these complexes metal is coordinated by ligands (Anacona et al., 2010). Organometallic complexes are the result of combination of metal atoms and organic groups as ligand. Various organometallic complexes are present naturally as myoglobin, hemoglobin and methyl-cobalamin (Vitamin B type). Properties of these complexes are in between ionic and covalent nature, because of metal-carbon bonding. They can be divided into two general classes: • Metal-unsaturated hydrocarbon π-bond containing complexes (C-C multiple bonds) • Metal-carbon σ bonds containing complexes In these organometallic complexes, the pi orbitals of the organic matter connect metal as as dibenzene-chromium or ferrocene (Basolo and Johnson, 2003). 1.2.3. Synthesis of metallic complexes

(Coordination complexes) Metallo-complexes can be synthesized through various methods. Direct combination of specific ligand and metal ion led to production of hexa-ammimine-nickel (2+) ion through the series of reactions in aqueous solution of nickel salt and in the presence of ammonia. Finally precipitated sulphate salt ([Ni(NH3)6]SO4) is obtained. Similarly in the presence of oxygen aqueous solution of cobalt salt and ammonia produce hexaamminecobalt(3+), [Co(NH3)6]3+ as cobalt-ammine complex (Basolo and Johnson, 2003). Halide coordination compounds can be synthesized in solution of two halide salts in appropriate solvent or in molten state. For instance potassium tetrachloropalladate can be prepared by the reaction of potassium chloride and palladium chloride. PdCl2 + 2 KCl ? K2[PdCl4]. Treatment of particular precursor with specific reducing agents such as hydrazine or molecular hydrogen results in

66**the synthesis of** hydrido **complexes** of **transition metal** which **are** given **as;**

2[Co(CN)5]3-+ H2? 2[CoH(CN)5]3- (Martell and Calvin, 1987; Basolo and Johnson, 2003). 1.3. Role of metallic complex in drug discovery Medicinal utility of metals can be traced up to 5000 years back (Orvig et al., 1999). The splendid array of metal complexes applications has been investigated in pharmaceutics

8**(Sadler et al, 1999; Ali & van Lier, 1999; Louie & Meade, 1999; Volkert & Hoffman, 1999;**

Sakurai et al, 2002). Humans' well-being is affected in various ways by metal ions. Applications of metals in pharmaceutical substances are increasing rapidly due to their enhanced compatibility (Smith-Warner et al., 2000; Cragg et al., 2000). Administered metal complexes undergo redox reactions and ligand substitutions, which produce active biotransformation product. Metal compounds can be used effectively as drugs, upon identification of their active species (Sadler and Guo, 1998). Metal complexes are utilized in pharmaceutical preparation due to their potential applicability, several medicinal preparations have been investigated. Various reviews had focused on advances in the field of coordination compounds (Sakurai et al, 2002). Metals combines with ligands to form complexes and if ligand forms more rings, then resultant product will be chelate (Wayne, 1999). These metallo complexes had been found to possess importance in different fields, like biomedical, analytical, therapeutic, synthetic applications for example gold complexes (Auranofin etc.). These were important anti- arthritic drugs and capable of tumor cells inhibition during in vitro testing (Mckeage, 2002). Gromer et al. (1998) has found auranofin as potent inhibitor of enzyme thioredoxin reductase, which is involved in mitochondrial membrane permeability by redox control. Metal complexes could be used for the treatment of cancer for example antibiotic bleomycin cut the DNA strand by synthesizing intermediate metal complex, having iron or copper as cofactor (Perry, 2002; Dorr & Von Hoff, 1994). Tetracyclines that are well known antibiotics are chelators of Ca2+ and Hg2+ ions (Venkateswarlu and Sailaja, 2011). Second generation platinum drugs are used for the cancer treatment like carboplatin, cisplatin (Kostova, 2006) and cisplatin tri peptide conjugate is used for platinum drug resistant tumor. Ruthenium complexes having ligand dimethylsulfoxides, amine and N- heterocyclesare have been to found to possess in vivo antitumor activity (Farkas & Sovago, 2002). Antiviral agents are also complexes in their nature. Currently these complexes are found to possess enzyme inhibition activity for example, Ruthenium poly-aminocarboxylate complex are inhibitors of cysteine proteases. Enzyme inhibition activity of these complexes could be a suitable route for finding new antiviral substances and their utilization to inhibit diseases from progression (Chaterjee et al, 2006). Ruthenium complexes are also nitric oxide scavengers (Farkas & Sovago, 2002; Sangeeta et al., 2009). 1.4. Plants used in current study Extracts of two plant species were used in the current study. Their brief introduction is given as under;

1.4.1. Bergenia Cilliata i. Taxonomy Bergenia ciliata is the member of Saxifragaceae family. This family is comprised of 30 genera and 580 species. Three genera named as Saxifrage, Bergenia and Astilbe are commonly found in Pakistan. Bergenia genus comprises of six species which are widely distributed in East Asia, Central Asia and Himalayas. Bergenia stracheye and B. ciliata represent the Bergenia genus in Pakistan (Uddin et al., 2012). Bergenia ciliata is ever green perennial herb (Bhakuni et al., 1974). This plant is widely used in Pakistan, India, Bhutan, Nepal and other countries, in ayurvedic and traditional medicines to treat several diseases (Rajkumar et al., 2011). ii. Phytochemistry Bergenia ciliata contains different phytochemical. Studies on this plant show the presence of different constituents like paashaano lactone (Chandrareddy et al., 1998), catechin, berginin and gallic acid (Dhalwal et al., 2008). iii. Ethenomedicinal importance B. ciliata plant is

47**used in Indian system of medicine for the treatment of**

pulmonary infections, fever, diarrhea (Nadkarni, 2000). Asokar et al. (1992) had reported that that bergenia ciliata rhizome be supportive in dissolution of kidney stone. Extract of Bergenia ciliata found to possess anti-tussive activity (Sinha et al., 2001) and showed significance in reducing inflammation and boost immunity against bacterial infections. It also demonstrated antiviral activity (Li et al., 2006). This plant exhibits significant anti- inflammatory, analgesic, diuretic property (Gehlot et al., 2000). It is evaluated for antioxidant activity and DNA protection abilities (Rajkumar et al., 2010). Due to the presence of

33**anti-cancer metabolites,** this plant **could be considered as potential source** of **anti-cancer drug development**

(Rajkumar et al., 2011). 1.4.2. Euphorbia wallichii i. Taxonomy Euphorbiaceae families have 300 genera and 7500 species and it is counted as one of the largest families of higher plants. This family is widely spread in central and western Himalayas, middle-east, China and South Africa (Ali et al., 2009). Euphorbia genus is among the six largest genera of flowering plants, comprising 2160 species. Euphorbia plant is perennial herb, contains bright yellowish green flowers, dark green fruits and height of this plant is 75 cm (Ali and Ahmad, 2008). Euphorbia genus grows in the form of herbs few as shrubs and trees (Satyanarayana et al., 1991). iii. Ethno medicinal importance Some species of Euphorbia genus have been used in traditional medicines for hundreds of years and are used to treat tumors, cancers, migraine, gonorrhea, skin diseases, warts and intestinal parasites (Smith-Kielland et al., 1966; Singla et al., 1990). Plants of this genus are used to cure rheumatism and bronchitis (Satyanarayana et al., 1991). In Tibetan folk medicines, E. wallichii roots

22**have been used** as **traditional medicine for the treatment of**

edema and skin diseases such as cutaneous anthrax and furuncle (Lal et al., 1990). It also showed significant cytotoxicity and phytotoxicity (Ali et al., 2009). In Indian folk medicines E. antisyphilitica was used to treat liver ailments (Saraf et al., 1994). E. prostrate is used as blood purifier and as an anti-inflammatory medicine (Singla et al., 1991). E. lateriflora latex is found to be effective against ring worm, also act as purgative (Fakunle et al., 1992) and remedy for spleeno-hepatomegaly (Satyanarayana et al., 1992). Ointments for a chronic skin disease in which red scaly pustule and patches appears on skin (psoriasis) are made from extract of E. fischeriana (Wu, L. et al., 1993). 1.5. Biological assays Bioassay is the

potential determination of activity of any chemical, physical or biological agent by using biological indicators. These indicators can be the living beings, organ, tissues, cells, components of cell. Essential points of bioassays are; • Stimulus (Test sample, drugs, bio active substance etc) • Subject (Organism, Biochemical, living sample, etc.) • Response (Response of the subject to stimulus at various level) Concepts regarding bioassay characterization ? Potency of the sample compounds is not absolute but relative. Relative potential of bioactive compounds to reference drug or standard and both of these show similar biological response. ? Amount of biological active substance can be estimated when absolute amount of standard is known. ? Only estimated potential of bioactive substance is obtained by bioassays while precision of that amount is decided on the data of bioassay. ? Active potential is the property belongs to substance to be used in assay. It is not concerned to property of response. Usually association between dose of active substance and change in indicator and dose effect curve is carried out as a part of assay (Ghosh 2008, Bohlin and Bruhn, 1999). 1.5.1. Broad categories of biological assays i. Virtual or in silico screening In medicinal research virtual research is thought to have an important role, by speeding the rate and reducing extra cost for lab work and clinical trials. During 2010, researchers have found the potential enzyme inhibitors associated with cancer, by using protein docking algorithum EA dock. These molecule were found to be 50% active enzyme inhibitors in vitro testing (Röhrig et al., 2010; Ludwig Institute report, 2010). ii. Primary bioassays Bioassays of this category can be utilized rapidly to find if any desired activity is present in large number of samples. These can be sub classified into non- physiological assays, biochemical or mechanism-based, microorganism-based, cell-based, tissue-based and many other in vitro bioassays/assays. Examples of primary bioassays are given as antioxidant assays, enzyme inhibition assays, cytotoxicity bioassays, anti-bacterial bioassays, antifungal bioassays, insecticidal bioassays, in vitro antiparasitic bioassays, brine shrimp lethality bioassays, phytotoxicity bioassays and anti-cancer bioassays (Cancer Cell Lines) (Rahman et al., 2005, Bohlin and Bruhn 1999). iii. Secondary bioassays Secondary biological assays require detailed sample testing on model living system so that sample compounds can be selected for clinical trials (Rahman et al., 2005). These assays are usually slow, low capacity and expensive. Examples are given as; a. Animal-based assays (in vivo) • Animal model with induced disease • Animal model with induced injury b. Toxicological assessments in whole animals • Acute toxicity, • Chronic toxicity c. Preclinical Studies ? These analysis of in-vitro (test tube) and in vivo (animal) experiments using different doses of desired drug to obtain toxicity, preliminary efficacy and pharmacokinetics information. ? Pharmaceutical companies assess whether studied drug has scientific value to develop that as investigational new drug. 1.5.2. Importance of bioassays Bioassays play important role at the initial stages of drug discovery. Task of discovering new therapeutic agents for different diseases is becoming vast at the present time. The combination of bioassays provides the necessary screens in a timely manner to obtain data of active substance and its use in potential therapy against specific disease. Bioassays are very important tool in determining the quality of bioactive compounds (Bohlin and Bruhn 1999). In current study primary bioassays are mainly used because these are potently predictive and general in nature, unbiased, fast, cost-effective, reproducible, tolerant of impurities and compatible with DMSO (Rahman et al., 2005, Ghosh, 2008). 1.6. Assays carried out in this study 1.6.1. Antioxidant assays Antioxidants are class of highly reactive molecules. Reactive oxygen species (ROS) as hydrogen peroxide (H2O2), singled oxygen (1O2),

24**hydroxyl radical (HO•), superoxide anion radical (O2•−) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)** are synthesized **in**

living organisms (Halliwell

4**et al., 1999;** Yldrm **et al., 2000;** Gulcin **et al.,**

2002). Generally, synthesis of ROS is monitored by antioxidant system in living system. In many cellular functions, it may be necessary for killing phagocytes and redox regulation of signal transduction. In some conditions ROS are over produced in organisms that can lead to their attraction towards cellular components including protein, DNA and cell membrane and consequently induce destruction, degradation and toxicity of various molecules that play vital role in metabolism (Heo et al., 2005). Reactive oxygen species (ROS) can induce numerous diseases, like muscular dystrophy, cataracts, atherosclerosis, rheumatoid arthritis, some types of cancer, neurological disorders and aging (Kovatcheva et al., 2001; Ruberto et al., 2001). Currently different type of synthetic antioxidants like TBHQ (tertiary butyl hydroquinone), BHT (butylated- hydroxytoluene), BHA (butylated hydroxyl anisol) and propyl gallate are widely used in drugs, medicines and foods as materials or additives for oxygen suppression (Shon et al., 2003). However, usage of these synthetic antioxidants has been strictly banned due to toxicity that can lead to serious human health issues. Due to this fact, researchers are trying to find effective natural antioxidants having minimum side effects. Numerous antioxidants like carotenoids, chlorophylls, polyphenolic compounds, isoprenoids and tocopherol have been isolated from plants and being used for several years

48**(Ramarathnam et al., 1995; Wettasinghe** et al., **1999; Shon et al., 2003).**

i. DPPH free

55**radical scavenging assay DPPH (2,2-diphenyl-1-picrylhydrazyl)**

is based on method of Blois (1958) in which radical scavenging activity of substrates, foods and beverages can be observed. Evaluation of scavenging capacities of various antioxidants is being carried out by using this method (Marxen et al., 2007;

1**Perez-Jimenez and Saura-Calixto 2008; Perez-Jimenez et al., 2008).** This method **is**

simple, rapid, cheaper and accurate for free radical scavenging potential of samples (Prakesh, 2001). Methanol (Shikanga et al., 2010) and ethanol (Pavlov et al., 2002) can be used as solvent for DPPH. DPPH assay can be carried out by using various wavelengths like Kamkar et al. (2010) have used 517 nm for measuring absorbance of reaction mixture, Shikanga et al. (2010) have checked at 492 nm, Liebenberg (2004) at 540 nm, Lachman et al. (2008) used 515 nm and Gülcin et al. (2010) have used 516 nm. Various standards have been used for comparing radical scavenging activity among which ascorbic acid (vitamin C) is most widely used as standard drug (Kwon et al., 2003). DPPH is reduced upon reacting with sample. The reduced form (DPPH-H) results in decrease in absorbance. It can be explained by following reaction; (DPPH) + (H-A) DPPH-H + (A) (Purple) (Yellow) More the scavenging potential of antioxidant sample (hydrogen donating power), more discoloration will occur (Oktay et al., 2003). Given equation as described by Kamkar et al. (2010) was followed for calculation of scavenging activity of DPPH. ii. Total antioxidant capacity Various human pathological conditions are caused by reactive oxygen

species. Imbalance in these species during synthesis and scavenging produces cellular damages that lead to disorder in proper functioning. Utilization of natural products and medicinal plants having significant antioxidant activity for prevention of disorders produced due to reactive oxidative species is a useful strategy (Shahwar et al., 2010). Phosphomolybdenum method was used for total antioxidant assay. In this method of total antioxidant capacity, sample reduces the Mo (VI) to

> 46**green phosphate Mo (V) complex at acidic pH. Phosphomolybdenum method**

for total antioxidant capacity

> 1**is quantitative since the total** capacity **is expressed as** equivalents **number of ascorbic acid (Prieto et al., 1999).**

iii. Reducing power assay Redox properties of sample determine its antioxidant activity and these properties play vital

> 11**role in neutralizing and** absorbing **free radicals, decomposing peroxides** or **quenching** singlet **and triplet oxygen**

(Osawa, 1994). Antioxidant sample have potential of reducing potassium ferricyanide (Fe3+) to potassium ferro-cyanide (Fe2+), which result in formation of ferric ferrous complex. This assay is based on checking absorption of ferric ferrous complex, synthesized in reaction mixture, at 700 nm. Reaction can be explained as given below; Potassium ferricyanide + Ferric chloride → Potassium ferro-cyanide + Ferrous chloride 1.6.2. Cytotoxicity assay Brine shrimp lethality assay was performed for cytotoxic evaluation of complexes. i. Brine shrimp lethality assay Artemia salina (leach) is invertebrate organisms, commonly known as shrimps. These are found in lakes. Nauplii are the larvae of this organism. Human cytotoxicity and brine shrimp nauplii lethality may comparatively show positive correlation. Toxicological studies are carried out on these organisms for more than 50 years. (Krishnaraju et al., 2006). At high doses, almost all biological active compounds are toxic for living systems. Simple zoologic organisms like shrimp larvae can be used conveniently for determination of lethal activity of natural bioactive compounds as well as derived or synthetic compounds. Artemia salina eggs

> 34**remain viable for years in dry**

condition and are

> 34**available in pet shops at low cost.**

Eggs are being placed in sea water and hatching occurs within 48 hours to provide nauplii for experimental purpose. The brine shrimp lethality assay is comparatively rapid, takes 24 hours for completion. It is also simple as it does not need any aseptic technique, is inexpensive and large numbers of organisms are

utilized for statistical validation. It does not require any special equipment and usually small amount of sample from 2 to 20 mg or less is required for finding activity of compounds

70**(Meyer et al., 1982; McLaughlin et al., 1991).**

1.6.3. Antimicrobial assay Search for medicine undoubtedly started in prehistoric times. Being core of health care, these relieve the symptoms (as analgesics) and cure diseases (as antibiotics). In 1928, Alexander Fleming discovered the first antibiotic penicillin, which was contemplating the main step in drug discovery. The exploration of specific potent antibacterial and antifungal drug, which can be used as therapeutic remedy using investigation tools, is biological research. Various antibiotics manufactured and discovered from natural sources. Antibacterial and antifungal activity of any compound, natural or synthetic, can be evaluated by comparative measurement of inhibited zone of standard drug and sample against particular microbes. Animal and plant diseases are caused by pathogenic microorganisms, like bacteria and fungi. These microbes produce bacterial diseases such as hemolytic uremic syndrome, toxic shock syndrome and fungal diseases, such as keratitis, otitis, lung infections and chronic pulmonary infections in immune compromised hosts. Due to over resistance of antimicrobial drugs, demand of new and potent drug has been increasing. Biological activities of metallic complexes are checked against different gram positive and gram negative bacterial species. Quarter of deaths occur due to bacterial infections in spite of using broad spectrum antibiotics. This has led to new dimension of research for durg discovery. (Wyke, 1987). i. Antibacterial assay Resistant bacteria are the main problem, emerging due to inappropriate use of antibiotics. In previous days these antibiotics were considered as vital for saving life but after 1950s resistant bacteria have become multi drug resistant (Hawkey, 2008). This problem can be solved by introducing new drugs or derivatives of existent drug. In this study antibacterial activity of complexes were observed using disc diffusion methods against various pathogenic bacteria as Staphylococcus aureus, Salmonella typhimurium, Enterobacter aerogenes, Bordetella bronchiseptica, Escherichia coli and Micrococcus luteus, a. Escherichia coli

38**Escherichia coli (E. coli) are gram-negative, facultative anaerobic** and **rod shaped bacteria.**

These are harmless and opportunist bacteria found in intestine and can cause various problems like neonatal meningitis (Bentley and Meganathan, 1938), gastroenteritis (Chekeri et al., 2008) and food poisoning (Hayhurst, 2004) in humans. This bacterium is used in research laboratories as research model due to its genetics, simple growth, easy duplication and manipulation through metagenics. Virulence factors in the pathogenic strains cause pathogenesis (Elena et al., 2005). b. Staphylococcus aureus Staphylococcus aureus (S. aureusis) are gram positive and facultative anaerobes, included in normal flora but causes atopic dermatitis in humans (Horn et al., 2005). This group of bacteria is present in nose, on skin and it can contaminate the food and cause food poisoning in humans (Howardand Kloos, 1990). c. Enterobacter aerogenes Enterobacter aerogenes (E. aerogenes) are opportunist, gram negative bacterium. It is harmless and found in gastrointestinal tract, soil, water and hygienic chemicals. This bacterium is valuable, due to their importance in fermentation technology for the production of hydrogen (Chavarria et al., 2000). It causes various infections such as urinary tract, respiratory tract, skin, eyes and can cause, endocarditis, osteomyelits (Jarvis and Martone, 1992) and nosocomial infections (Goshiab et al., 2002). d. Bordetella bronchiseptica Bordetella bronchiseptica (B. bronchi) are gram negative bacteria responsible for snuffles in rabbits, kennel cough in dogs and bacterimia in humans. It is rarely associated with infections and found in respiratory system of humans. This specie causes infections in severely

immune compromised hosts (Woolfrey and Moody, 1991). It is the causative agent of bronchitis (Rayn and Ray, 2004). Infection in Small animals such as rabbits, cats and dogs was reported (Finger et al., 1996). e. Salmonella typhimurium Salmonella typhimurium (S. typhi) is gram negative pathogen of humans that causes typhoid fever, systemic infection (Mastroeni et al., 2009; Humphrey, 2004). It is resistant against innate immune system (Falkow et al., 2004) and also involved in splenomegaly, hepatomegaly and white nodules in finches (Une et al., 2008). This bacterium causes typhoid fever-like disease. Raw or poorly cooked food, contaminated water, sanitation are the important factors contributing for spreading of this bacteria. So this bacterium is used in labs for evaluating the antibacterial potential of various natural and synthetic products. f.

54**Micrococcus luteus Micrococcus luteus (M. luteus) is gram-positive**

and was isolated by Fleming in 1922 from nasal secretion of patient of acute coryza. Kocur in 1992 detected M. luteus in water, soil and also in the mucosal membranes. This organism is recognized as opportunist pathogen involved in recurrent bacteremia (Peces, 1997), endocarditis, septic arthritis, meningitis, cavitating pneumonia and intracranial suppuration in immune suppressed patients. ii. Antifungal assay Fungal strains can become resistant due to inappropriate and misuse of antibiotics. Resistant fungi have become critical issue towards human health and antifungal drugs have been observed useless against them. Studies on environmental issues suggest that population rising is main cause for anti-microbial resistance (Barret, 2002). There is need of exploring novel antifungal drugs against the resistant strains. Current study is emphasizing to synthesize organometallic complexes and to explore their antifungal potential. For this reason different fungal strains were used like Fusarium solani,

12**Mucor species, Aspergillus niger, Aspergillus flavus** and **Aspergillus fumigatus.** a. **Mucor species Mucor species**

are olive gray to dark gray in color, fast growing mostly found in debris, soil, dairy products, leather, jute, meat, dead and decaying plants. These species are responsible for thrombosis in hepatic artery (Marco et al., 2000), mucrosis (Crissy et al., 1995), prosthetic mitral valve mucormycosis (Rama et al., 2004) and majorly affect lungs, nasal cavity, sinuses of individual. b. Aspergillus niger Aspergillus niger (A. niger) is filamentous fungus, member of Ascomycota group and naturally found in environment. Initially, colonies of this fungus appeared white and finally black due to the production of conidia. Genome of this fungus is up to 38.5 megabite (MB) that could be useful as microbial research model for morphology, pathogencity and fermentation process (Baker, 2006). This fungus is used in fermentation industries for the synthesis of citric acid. Nakagawa et al. (1999) reported that A. niger is more dangerous against immune-compromised patients. It cause crown rot of ground nut in plant (Bobbarala, 2009). c. Aspergillus flavus Aspergillus flavus (A. flavus) fungi are dangerous to human health due to the production of toxins. Various types of fungal strains grow on improperly stored grains. It is also involved in harming cash crops. Alfa toxins produced by these strains are more dangerous for spreading different detoriating conditions. This strain also causes chronic pulmonary aspergillosis, invasive and non-invasive aspergillosis, dermal infection, fungal sinusitis (Pasqualotto, 2009). Flavous alpha toxins can cause hepatitis, liver cancer and immune-suppression (Hedayati et al., 2007). d. Aspergillus fumigatus Aspergilus fumigatus (A. fumigatus) is pathogenic fungus mostly found on soil, fruits, grains, vegetables and grow above 40ºC temperature. This fungus causes invasive, non- invasive infections in immune-compromised patients and also involved in systemic diseases, ormycotoxicosis and allergic reactions in individuals. Aspergillosis in immune-compromised individuals is caused by Aspergilus fumigatus (Latge, 2001). Working with this strain

following poor managed protocols may lead to hypersensitivity pneumonitis (Suberosis) (Villar et al., 2009). e. Fusarium solani Among various species of Fusarium, genus Solani specie is commonly found in humans and animals. Cottony or woolly colonies, having white to cream mycelium, microconidia and monophialides are the characteristics of this specie. Fusarium solani (F. solani) is also involved in pathogenesis, lignin biodegradation and phytohormones synthesis in plants (Nazareth and Bruschi, 1994) and causes mycetoma, oncomycosis, endocarditits, septic arthritis, pulmonary diseases, sinusitis and invasive epidermal infections in humans (Summerbell and Schroers, 2002). 1.6.4. Kinases inhibition assay Protein kinases are vital enzymes for cell growth, cell cycle progression, and signal transduction across nuclear membrane. Human genome codes for 500 different protein kinases and classified on the basis of sequence of amino acid in catalytic domain (Manning et al., 2002). Modulation in kinases activities could be potential source of cancer and other signalopathies. Its inhibitors can be used in various disease treatments (Li et al., 2004). Kinases inhibitors are important drugs for the treatment of cancer. Selectivity of inhibitors as drugs is challenge for researchers (Lynette et al., 2009) particularly, targeting oncogenic kinases involved in cancer (Arslan et al., 2006). 1.7. Chemical and spectroscopic analysis Chemical analysis of all complexes of EW and BC were carried out by measuring phenolics and flavonoid contents. FT-IR was used for the spectroscopic analysis of complexes. i. Total phenolic content

7**Phenolics are group of secondary metabolites, which are synthesized by** animals, **plants and**

microorganisms. Phenolics of plants give them

1**taste, odor,** appearance **and oxidative stability. These compounds are** present **in pericarp**

of cereal grains (Naczk et al., 2004).

1**Ferulic and p-coumaric acids** are major phenolics **(Zhou et al., 2004; Mattila et al., 2005; Holtekjolen et al., 2006).**

It is observed that methanolic extracts of plants have more significant content of phenolics (Iqbal

4**et al., 2006).** Andersen **et al. (2007)** and Hurst **et al. (2002)**

have classified the phenolic compound in different groups as under ? Benzenediols class contains hydrooxy phenol based simple structures. ? Phenolic acid class contains derivatives of cinnamic acid (C6-C3), benzoic acid (C6-C1) and

23**associated as long polymers form tannins and lignans. ? Coumarins**

class contains compounds having

23**2H-1-benzopiran-2-one** as basic structure. ? **Flavonoids**

class contains compound having diarylpropane (C6-C3-C6) as basic structure. ii. Total flavonoid contents Flavonoids are plants' secondary metabolites group, having two benzene ring attached through pyrane ring (Bagchi et al., 1999). Basic structure comprises of 2-phenyl-benzo [α] pyrane and

1**found in strawberries, green, black teas** and vegetables **e.g. kaempferol from brussel, quercetin from beans.**

This group has again three sub- classes as flavones, iso-flavonoids and neo-flavonoids. Flavonoids are well known for in vitro antioxidant activity that may be stronger than vitamins C and E (Bagchi et al., 1999). These secondary metabolites have anti-diarrheal (Schuier et al., 2005), anti- inflammatory, anti-microbial and anti-allergic activities (Cushnie and Lamb, 2005). These were observed to enhance DNA changes in

1**cultured blood stem cells (Barjesteh et al., 2007).** They **inhibit germination of plant**

pathogen spores, so could be used against human pathogens (Harborne and Williamsn 1992). Currently inhibitory effect of flavonoids has been observed against human immunodeficiency virus (HIV). These compounds were checked against some pandemic HIV-1 strain and studies shows that HIV-1 replication is inhibited by baicalein (5,6,7-trihydroxyflavone) (Li et al., 2000). Flavonoids class contains diarylpropane (C6-C3-C6) as basic structure and subclassified as catechins, flavones, flavonols, flavanones, isoflavonoids and anthocyanes (Andersen et al., 2007). iii. FT-IR spectroscopy Structural analysis of complex organic compounds was being carried out by using IR spectroscopy during 1950 to 1970. Characteristic functional group's determination of various compounds is performed by using this technique. FT-IR spectroscopy gives the impulse about IR spectroscopy. This new technique is used for analytical study of biologically important compounds. It is rapid, sensitive, inexpensive and traditionally have an advantage of providing practically complete analysis, while information about characteristic functional groups of compounds is being cleared from IR spectra. This technique performs fast analysis, computerized data processing, requires very small amount of sample, database, and spectral data banks are the main beneficial features of this technique. Infrared spectroscopy was not widely applied for the structure of carotenoids (Bernhard et al., 1995, Britton, 1989; Lammers et al., 2009). Structural elucidation of carotenoids was carried out by NMR, UV, and MS spectroscopy. Recently FT-IR spectroscopic studies give the confirmation of these compounds (Tama´s Lo´ra´nd et al., 2001). This is often used for complex carbohydrates and plant sugars (Kristin Lammers et al., 2009). Recently this technique has been considered as nondestructive and successful technique for rapid structural features, characterization and minimum requirement of sample (Sarah, 2007). 1.8. Aims and objectives Aim and objectives of present study were ? Preparation of crude methanolic extract of Bergenia ciliata and Euphorbia wallichii. ? Synthesis of the metallic complexes of crude extracts of both plants. ? Antioxidant evaluation of synthesized complexes. ? Determination of cytotoxic capacities of complexes. ? Determination of antibacterial and antifungal potential of complexes. ? Evaluation of protein kinase inhibition potential of complexes for characterization of its anticancer potential. ? Chemical and spectroscopic analysis by measuring phenolics and flavonoid contents and FTIR respectively. Material and methods 2.1. Plant collection and extraction Bergenia ciliata and Euphorbia wallichii plants were collected from Nathia galli,

KPK, Pakistan during June, 2012. Dr. Rizwana Alim Qureshi (Taxonomist, Quaid-i-Azam University Islamabad) had identified the plants. Roots (E. wallichii) and rhizome (B. ciliata) were dried under shade and crushed in a grinder. Extraction was carried by using analytical grade methanol. Each plant material was extracted with methanol (2 liter/0.5 kg) for three days. Process was repeated three times. Methanol collected after each process was combined and filtered using filter paper (whatman No. 1). Than filtrates were concentrated using rotary evaporator (R-200 Buchi, Switzerland). Extracts were further dried in vacuum oven (Vacucell, Einrichtungen GmbH). Finally crude methanolic extracts of Bergenia ciliata and Euphorbia wallichii plants were used for the synthesis of metallic complexes. While remaining extracts were stored at 4ºC. 2.2. Synthesis of metallic complexes In present study two plants, Bergenia ciliata and Euphorbia wallichii were used for the synthesis of complexes with fourteen metals. Metallic salts i.e. aluminum chloride (AlCl3), cadmium chloride (CdCl2), lanthium chloride (LaCl3), tin chloride (SnCl2), bismuth nitrate (BiNO3), cobalt sulphate (CoSO4), plumbic acetate (Pb(CH3COO)), sodium molybdate (NaMoO4), cesium chloride (CsCl2), lithium acetate (CH3COOLi), mercuric chloride (HgCl2), ferrous chloride (FeCl2), ferric sulphate (FeSO4) and silver nitrate (AgNO3) were used as source of metals. Complexes were synthesized by following the

60**method as described by** Saha **et al.** (2009) **and** Thakum **et al.**

(2012). i. Requirements Metal salts, crude extracts of Bergenia ciliata and Euphorbia wallichii, distilled water, methanol, stirrer (Gallen hamp, England), vortex (Scientific Industries Inc. USA, model No. SI-T256), vacuum chamber (VWR-1575R-2), sonicator (E30H-elma sonic sonicator), hot plate (Nuova 11, model No. SP 18420-26 hot plate), centrifuge (Eppendorf Centrifuge, 5810R). ii. Procedure Metallic complexes of crude extracts of E. wallichii and B. ciliata were synthesized through series of steps as given; ? Salts

15**solutions were prepared, by dissolving 4 g of** respective salt **in** 40 **ml**

distilled water (10%). These solutions were subjected to vortex for complete dissolution. ? Crude extract solution was prepared by dissolving (2 g/50 ml methanol) in separate eppendroffs. For complete dissolution of extract and to make homogeneous solution, this solution was stirred for half an hour at room temperature. ? After that equal amount of salt and extract solutions (1:1) were mixed. This mixture of aqueous solution of metal salt and methanolic solution of extracts of both plants were mixed vigorously. ? This was followed by 10 minutes sonication and incubation of one hour at room temperature. Later on turbidity was observed in mixtures of both solutions. That was indication for complex synthesis. ? Metal complexes were separated by centrifuging the solutions at 4000 rpm for 15 minutes. ? Supernatant was removed and solid complexes settled in bottom, were washed with distilled water and centrifuged again for 5 minutes. Supernatant was poured off and complexes were kept for further processing. ? Finally all complexes were dried in vacuum chamber for 24 hours at 35ºC and led for biological evaluation. 2.3. Bioassays 2.3.1. Antioxidant assays Antioxidant capacities of metal complexes were determined using different assays which are described as under; a. DPPH free radical scavenging assay Antioxidant potential of samples against DPPH

62**was determined using method** described **by** Clarke **et al.** (2013) **with** slight **modification.**

i. Requirement Solid DPPH (Sigma U.S.A), methanol, DMSO, complex samples, ascorbic acid, microplate reader (biotek ELx800, USA). ii. Samples preparation Four mg of complex samples were dissolved in 1 ml of DMSO for the preparation stock solution of each sample. Further, 0.4 µg/µl final concentration of complexes was used in this assay. iii.

44**DPPH solution preparation DPPH solution was prepared by dissolving 3.** 32 **mg**

16**in 100 ml of methanol.** Freshly prepared **DPPH solution was sonicated for 10 minutes** before use. iv. **Assay procedure** DPPH **assay was performed** in **96** wells **plate** format. Firstly, 20 **µl of**

each complex sample and crude extracts of EW and BC were poured in their respective wells. After that DPPH solution (180 µl) was added in each well to produce 200 µl reaction mixture.

57**Ascorbic acid and** DMSO **were used as** reference **standard and** negative control **respectively.**

Mixture was incubated

69**at room temperature for half an hour.** Change **in**

color from violet to yellow was observed. Oxidation potential of complexes determined the color changing.

3**Absorbance of reaction mixture was measured at** 517 **nm**

on microplate reader. Given formula was used for calculation of scavenging activity of sample. OD = optical density (absorbance) b. Total antioxidant capacity Phosphomolybdenum method as described by Dillard et al. (2000) was used for measuring total antioxidant capacity. i. Requirement Complex solution stock (4 mg/ml), DMSO, sodium phosphate buffer

75**(28 mM), ammonium molybdate (4 mM),**

30**sulphuric acid (0.6 M),** microplate reader, **sodium phosphate (28 mM), ammonium molybdate (4 mM),** sulphuric acid **(0.**

6 M). ii. Sample preparation Sample complex solution

45**was prepared by dissolving** 4 **mg in** one **ml of DMSO** as stock **and**

further 0

22.**4 mg/ml** final **concentration was used in**

this assay. iii. Solution of positive control

20**Ascorbic acid was used as standard.** Four mg **of** each complex **was**

dissolved in 1 ml of DMSO for stock preparation. Final concentration (400 µg/ml) was used in this assay. iv. Assay procedure One ml reaction mixture was produced by addition of reagent solution and 100 µl of sample. Reagent solution comprised of

29**ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid (0.6 M).**

This

68**reaction mixture was incubated for** 90 minutes **at** 95oC. **After incubation,**

mixture was cooled and absorbance was recorded at 695 nm. DMSO was used instead of complex sample, as blank. Antioxidant potential of complexes

74**was expressed as ascorbic acid equivalent.**

c.

73**Reducing power assay Reducing power of**

metal complexes of EW and BC complexes

52**was determined using the method** as **described by** Ullah **et al.**

(2013). i. Requirement

32**Phosphate buffer (0.2 M, pH 6.6),** 1% **potassium ferricyanide** K3 **[Fe (CN)6],**

ascorbic acid, 10% trichloroacetic acid, 0.1% ferric chloride (FeCl3), complex samples, distilled water, microplate reader (biotek ELx800, USA), DMSO. ii. Preparation of complex solution Stock solutions of complexes were

> 1**prepared by dissolving** 4 **mg in 1 ml of DMSO. Further final** concentration 100 **µg/ml was**

used for assay. iii. Standard solution preparation Ascorbic acid was used as standard and final concentration 392 µg/ml was used in assay. iv. Assay procedure Reducing potential of sample was determined by taking 100 µl of sample, 400 µl phosphate buffer and 500 µl potassium ferricyanide to make 1 ml reaction mixture in eppendrof. Reaction mixture was subjected to incubation at 50oC for 30 minutes. Incubation was followed by addition of 500 µl of trichloroacetic acid (10%). Resultant solution

> 7**was centrifuged at 3000 rpm for** ten **minutes.** Then, 100 µl **of supernatant**

of each complex was taken carefully and poured in their respective well. Finally, 0.1% ferric cynide solution and 20 µl of distilled water were added in all wells.

> 51**Absorbance of solution was** recorded **at 700 nm.** DMSO **was** used **as**

blank. Ascorbic acid equivalents were used for expression of reducing power of metal complexes. 2.3.2. Cytotoxic lethality assay Cytotoxic effect of metal complex was determined using brine shrimp assay. Complexes were evaluated for cytotoxic nature by following the method as described by McLaughlin and Rogers (1998). i. Requirements Brine shrimps eggs (Ocean Star Inc., USA), DMSO, terbinafine, portioned dish, sea salt, complex samples, vials, pasture pipette, magnifying glass, lamp, distilled water. ii. Preparation solution of complex samples Fifty

> 5**mg of** complex **sample was dissolved in 1 ml of DMSO to prepared stock solution** of each sample. **Further** three **dilutions**

i.e.

> 26**1000 µg/ml, 100 µg/ml and 10 µg/ml were prepared from** the **stock solution.**

Table 2.1, shows the detailed dilutions procedure used in this assay. Table 2.1: Detail of dilutions used in cytotoxicity assay iii. Standard solution preparation Terbinafine was used as standard drug. One mg of standard

> 5**was dissolved in 1 ml of DMSO to** prepare **stock solution.**

Three concentrations i.e.

3710 µg/ml, 1 µg/ml and 0.1 µg/ml were

used. iv.

1Sea water Sea water produced by dissolving 34 g of sea salt () in one

1000 ml.

1Solution was stirred constantly using magnetic stirrer. Sea water was aerated

in open mouth beaker for sufficient time. v. Hatching of shrimp Hatching of shrimp eggs was carried in compartmentalized rectangular dish (22 × 23 cm), which was filled with sea water. One large and other small compartment with separating wall containing holes were present in dish. Eggs ware poured in large compartment. Lamp light was showered on smaller compartment, after covering larger compartment with aluminum foil. Newly hatched nauplii moved towards small compartment due to light. These were picked with pasture pipette and dispensed in small beaker having sea water. vi. Assay procedure Initially all vials were labeled, followed by addition of 2 ml sea water in all vials. After that 0.1 ml sample solution from their respective stock solutions were added in their respective vials. Ten nauplii were counted using pasture pipette and 3 X magnify glass and transferred to vials. Simultaneously

7volume was raised up to 5 ml with sea water.

So it does not affect the shrimps and desired concentration of sample attained. These vials were incubated for 24 hours at room temperature. After that shrimps were taken out from vials using pasture pipette and counted under magnifying glass. Finally lethal dose (LD50) of metal complexes was determined. vii. Determination of LD50 For the determination of lethal dose (LD50) of metal complexes samples against brine shrimps finny (1971) software was used. 2.3.3. Antimicrobial assays a. Antibacterial assay All complexes of EW and BC were evaluated for

50antibacterial activity by using disc diffusion method as described by Islam et al.

(2013). i. Requirement Metal complexes, DMSO, roxythromycin, cefixime-USP, whatman No. 1 filter paper discs, vernier caliper, nutrient broth (Merck, Gemany), nutrient agar (Merck, Gemany), petriplates, Six bacterial strains were used for antibacterial potential of complexes. Two gram positive strains (M. luteus ATCC 10240, S.

25aureus ATCC 6538) and four gram negative (B. bronchiseptica ATCC 4617, E. coli ATCC 15224, S. typhimurium ATCC 14028,

A. aerogens ATCC 13048). Nutrient broth was used for the maintenance of bacterial cultures for 24 hours at 4oC. Next day cold cultures of these strains in nutrient broth (Merck, Germany) were used for assay. ii. Samples preparation of EW and BC metal complexes Four mg of each complex were dissolved in 1 ml of DMSO, for the production of stock solution of each complex. Final

4**concentration of 30 µg/disc** of each complex **was used in**

assay. Table 2.2: Dilutions of EW and BC metal complexes concentration in antibacterial assay. iii. Standard solution Cefixime-USP and roxythromycin were used as standard drugs. Stock of each standard drug

1**was prepared by dissolving** 4 **mg** 1 **ml of** DMSO. **DMSO was used as negative control.** iv. **Assay procedure**

Bacterial inoculum was prepared in nutrient broth. It

1**was prepared by** dissolved **2** grams **of nutrient** broth **in 100 ml of water** and **pH was** maintained **to 7.** Nutrient agar medium **was**

1**used for the growth of** bacterial strains that **was** produced **by dissolving** 2 grams **in 100ml of water.**

36**Filter paper discs of 6 mm** in size ware **prepared from whatman no. 1 filter paper.**

Media, filter paper discs along with other apparatus required in this assay were autoclaved for sterilization. After autoclaving, whole experiment was carried out in microbiological safety cabinet. Solidified plates of nutrient agar were labeled and respective bacterial strain was streaked. Then 7.5 µl of each complex solution was absorbed on disc. These discs were placed on respective places in petri plate. These peteri plates were incubated for 24 hours at 28oC. Zones of inhibitions were measured after 24 hours. b. Antifungal assay All complexes of EW and BC were evaluated for antifungal capacities. This assay was performed by following disc diffusion method as described by Islam et al. (2013). i. Requirement Metal complexes samples, DMSO, terbenafine, whatman No. 1 filter paper discs, vernier caliper, sabouraud dextrose agar (SDA) (Merck, Germany), petriplates, antifungal activity of complexes were determined against five fungal strains. Fungal cultures (A.

15**fumigatus FCBP 66,** F. **solani FCBP 0291, Mucor species FCBP 0300,** A. **niger FCBP 0198,**

A. flavus FCBP 0064) were maintained on SDA at 4oC. ii. Solution of complex samples Four

> 5**mg of each** complex **was dissolved in 1 ml of DMSO to** prepare **stock solution**

of each sample. Table 2.3: Detail of sample for antifungal assay of EWE and BC metal complexes iii. Standard drug solution

> 10**Terbinafine was used as** standard drug **(positive control) and DMSO was used as negative control.**

> 27**Stock solution of** standard **was** produced **by dissolving 4 mg of** standard drug **in 1 ml of DMSO and**

10 µg/disc concentration of standard drug was used. iv. Assay procedure Fungal cultures were grown on SDA medium at the 6.5 pH.

> 28**Sabouraud dextrose agar (SDA)** media **was prepared by dissolving 6.5** grams in **100 ml** of **distilled water** and adjusted **pH**

of 6.5. Whatman no.1 filter paper was used for the preparation of circular discs of 6 mm size. All apparatus required in assay were autoclaved for sterilization. Experiment was carried out in microbiological safety cabinet.

> 31**SDA media** was **poured in petri plates and left for solidification. Filter paper discs were**

used for the absorption of sample solution and 7.5 µl of complex solution absorbed on disc. After absorption of complex solution on disc, were place in their respective places on solidified SDA plates. Contamination was prevented by wrapping petri plates with parafilm. After that plates were incubated for 24 hours at 28oC. On next day inhibition zones were measured by using venire caliper. 2.3.4. Protein kinases inhibition assay i. Requirement Growth media, streptomyces fungal culture, filter paper discs (6mm), pipette, spatula, cotton swab, petriplates. ii. Procedure Disc diffusion method was used for the kinases inhibition assay. Media along with spatula, cotton swab, discs were autoclaved prior to use for fungal growth. Whole experiment was carried out in sterilized environment. Streptomyces fungus was used in assay. Media was poured in petriplates and test microorganisms were grown. Test sample was absorbed on discs and placed above media at their respective positions. These

> 63**plates were incubated at** 28ºC **for 48-** 72 **hours.** After that balled **zones**

were measured for determination of protein kinase inhibition activity. 2.4. Chemical and spectroscopic

analysis Chemical analysis of complexes was carried by measuring the phenolic and flavonoid content. Spectroscopic study of complexes was also performed using FT-IR. a. Determination of phenolic content Phenolic content

> 35**was determined** by **using Folin-** ciocalteu **reagent as described by** Clarke **et al.** (2013) **with slight modification.**

i. Requirement Six percent sodium carbonate (NaCO3) solution, gallic acid, DMSO, metal complex samples, spectrophotometer, vials. ii. Preparation of sample solution Stock solution of 4 mg/

> 10**ml of each** complex **was** produced **by dissolving 4 mg of** complex **in 1 ml of DMSO.**

iii. Preparation of standard solution Gallic acid was used as standard and final concentration of 400 µg/ml was used. iv. Assay procedure Initially

> 41**0.1 ml of** each metal complex **solution was**

taken in vial. Then, 0.95

> 41**ml of Folin- Ciocalteu reagent** (ten times **diluted)**

was added in all vials. These mixtures were left for 5 minutes. Further 0.95

> 53**ml of sodium carbonate** (6%) **solution was added** in mixture **to** prepare reaction **mixture.**

This solution was incubated at room temperature for 60 minutes. Incubation was followed by recording absorbance of reaction mixture at 715 nm. Gallic acid was used

> 59**as standard. Phenolic content was expressed as** gallic acid **equivalent.** b. Determination **of**

total flavonoids Determination of flavonoids content was carried out by following aluminum colorimetric method Ullah et al. (2012). i. Requirements Aluminum chloride (10%), 1.0 M potassium acetate, Samples (4mg /ml), Quercitin, DMSO. ii. Preparation of sample Stock solutions of complexes were

> 1**prepared by dissolving** 4 **mg of each** metallic complex **in 1 ml of DMSO.** iii. Standard **solution** Quercetin **was**

used as standard. Final concentration of standard as 100 µg/ml was used for determination of total phenolics. iv. Procedure for flavonoids Reaction mixture was made by mixing 100 µl metal complex solutions, 0.4 ml of methanol, 66 µl of potassium acetate (1M), 66 µl of aluminum chloride solution (10%). Finally 2 ml volume was made

11**with the addition of** 1.360 **ml of distilled water. Mixture was incubated at room temperature for**

half an hour and

40**absorbance was recorded at 415 nm.**

Quercetin was used as standard. Flavonoids

40**content was expressed as quercetin equivalent.**

c. Fourier transforms infrared spectroscopy (FTIR) i. Requirement FT-IR spectroscope, complex samples, methanol, spatula, tissue ii. Procedure Synthesized complexes were further studied by FT-IR spectroscopy. All instruments have their own characteristics for measurement that change with temperature, ambient environment and time. Experiment was carried out at room temperature. Firstly FT-IR was scanned without sample and this measurement was stored as background. After that sample was placed in sample compartment for FT-IR measurement. Instrument automatically subtracted the background measurement and show the net measurement of sample. Sample was removed and methanol was used for cleaning after subsequent measurement. All the spectra were printed and interpreted. Results 3.1. Extraction and complex synthesis Extraction of both Euphorbia wallichii (EW) and Bergenia ciliata (BC) was carried using methanol. From EW, BC plants 6.3 and 5.2% extract was obtained respectively. Complexes of fourteen metals with extract of both plants were formed. Percent yield of all complexes are given in table 3.1. Varying content of each complex was obtained, that was due to the nature of metal and ligand. Table 3.1: Percentage yields of metallic complexes of Euphorbia wallichii and Bergenia ciliata. EW complexes BC complexes s.no: Sample code % yield Sample code % yield 1 EWAl 4.9 BCAl 5.7 2 EWCd 4.7 BCCd 5.2 3 EWLan 5.3 BCLan 4.9 4 EWSn 4.5 BCSn 3.2 5 EWBi 3.3 BCBi 4.3 6 EWCo 3.7 BCCo 2.9 7 EWPb 4.9 BCPb 5.2 8 EWMo 7.6 BCMo 6.9 9 EWCs 4.3 BCCs 5.6 10 EWLi 3.1 BCLi 3.9 11 EWHg 4.2 BCHg 3.7 12 EWFs 7.2 BCFs 6.4 131 EWFc 5.9 BCFc 6.1 14 EWAg 2.7 BCAg 2.1 3.2. Antioxidant assays Antioxidant capacities of all EW complexes were checked. All complexes showed decrease in DPPH % scavenging capacities and total antioxidant capacities as compared to their crude extracts. Increased reducing capacities comparative to their extract were observed. EWMo complex was found to have 86% of scavenging potential comparative to extract. EWCd complex had significant highest total antioxidant potential among EW complexes and 13% lesser then EW extract. EW complexes showed increased reducing power to that of their extracts. EWFs was found have 51% increased reducing power among EW complexes to that of extract. EWLan and EWPb had increased reducing power 3.6% and 4.3% comparative to extract. Detailed results are given in table 3.2. Experiment was performed in triplicate. Values were mean of three independent replicate along with standard deviation.

20**Ascorbic acid was used as standard** for antioxidant capacities **of** complexes. Antioxidant potential **was**

expressed in A.A µg equivalent per 100 µg complex. Table 3.2: Antioxidant capacities of Euphorbia wallichii crude extract metal complexes S. no: Sample DPPH % Scavenging Total antioxidant A.A µg/100 µgsample Reducing power A.A µg/100µg sample 1 EWAl 41.380 39.326±0.532 41.291±1.008 2 EWCd 33.016 55.326±0.645 39.717±0.582 3 EWLan 27.685 28.008±0.505 36.266±1.118 4 EWSn 28.181 50.925±0.577 46.801±1.210 5 EWBi 30.743 35.041±0.565 50.856±0.367 6 EWCo 28.140 47.408±0.527 51.401±1.175 7 EWPb 30.041 31.161±0.974 36.498±0.985 8 EWMo 67.231 46.217±0.841 47.922±1.386 9 EWCs 37.396 44.741±0.509 39.063±0.672 10 EWLi 29.545 32.645±2.053 49.815±1.536 11 EWHg 28.429 53.411±0.513 47.678±1.266 12 EWFs 22.024 51.262±0.518 53.030±1.455 13 EWFc 43.884 49.411±0.502 43.197±0.955 14 EWAg 38.842 37.466±0.749 45.801±1.604 15 EW 77.201 62.985±0.577 34.985±0.610 [± = Standard deviation (S.D)] Antioxidant capacities of BC complexes were checked. All complexes showed decrease in DPPH % scavenging capacities and total antioxidant capacities. While increased reducing capacities comparative to their extract were observed. BCMo complex was found to have 88% of scavenging potential to extract. BCMo and BCCs showed 4% decreased total antioxidant potential among BC complexes comparative to BC extract. BCFc was found to 51% increased reducing power among BC complexes respect with to extract. BCFc had increased 3% reducing power comparative to BC extract. Detailed results antioxidant capacities of BC complexes

61**are given in table 3.3. Table 3.3:**

Antioxidant capacities of Bergenia ciliata crude extract metal complexes [± = Standard deviation (S.D)] S. Samplec DPPH Total antioxidant Reducing power no: ode % Scavenging AAeq: µg/100 µg sample A.A eq: µg/100 µg sample 1 BCAl 47.355 53.075±0.519 44.078±0.572 2 BCCd 35.826 40.917±1.104 52.338±1.224 3 BCLan 34.256 39.312±0.541 47.590±1.674 4 BCSn 41.570 48.985±0.509 50.617±0.850 5 BCBi 34.669 44.792±0.50 51.010±0.528 6 BCCo 34.793 34.281±0.518 46.258±0.549 7 BCPb 31.570 38.132±0.547 43.170±0.333 8 BCMo 70.371 57.023±1.011 41.899±0.825 9 BCCs 41.404 56.987±0.547 37.914±0.50 10 BCLi 37.892 45.162±0.536 54.364±2.280 11 BCHg 42.975 35.283±0.577 43.473±0.505 12 BCFs 37.685 41.162±1.154 47.043±1.793 13 BCFc 42.603 36.191±1.103 54.169±1.285 14 BCAg 37.314 54.052±0.743 49.528±0.609 15 BC 79.297 58.943±0.577 35.802±0.310 [± = Standard deviation (S.D)] Fig 3.1: DPPH free radical scavenging assay 3.3. Brine shrimp cytotoxicity assay In the case of brine shrimp cytotoxicity of EW complexes, significant results were observed using EWHg complex (LD50 = 96 µg/ml), followed by EWFs complex (LD50 = 96.69 µg/ml). The metal complexes EWLi complex, EW Bi complex and EWLan complex also showed comparatively better results. The maximum LD50 was found using EWCs complex (LD50 = 382 µg/ml), which indicate the least cytotoxic nature of EWCs complex among EW complexes. Detailed results are given in table 3.4. The assay was performed in triplicate. The

58**values are** the **mean of three** independent **replicates** along **with** standard deviation. **Table 3.**

4: Brine shrimps cytotoxicity assay of metal complexes of Euphorbia wallichii crude extracts [± = Standard deviation (S.D)] For the determination of cytotoxic effect of BC complexes this assay was performed. Comparatively BCFs complex was found more cytotoxic among BC complexes because significant lowest

LD50 (LD50 = 58 µg/ml) was observed, Followed by BCFc (LD50 = 72 µg/ml) and BCHg (LD50 = 74 µg/ml). BCAg has shown comparatively maximum LD50 (LD50 = 392 µg/ml) which was

1**different than the rest of** metal complexes. **Results of** BCE metal complexes **brine shrimps cytotoxicity assay are given in the table 3.**

5. The assay was performed in triplicate. The

3**values are** the **mean of three** independent **replicate** along with **standard deviation.**

Table 3.5: Brine shrimps cytotoxicity assay Bergenia ciliata crude extract metal complexes [± = Standard deviation] 3.4. Antibacterial capacities of metallic complexes

14**Antibacterial activity of** EW complexes **was** checked **against** six **bacterial strains, E.**

coli, M. lutus, B. bronchiseptia, S. aureus, S. typhimurium and E. aerogenes. Five EW complexes were active against M. lutus strain, four complexes were active against E. coli. EWHg produced maximum zone of inhibition (16±1.527 mm) againt S.aureus. The complex EWSn, EWPb, EWMo, EWFs, EWFc and EWAg were found to have no activity against any bacterial strains. While BCHg complex were found active against five bacterial strains, followed by EWAl, EWLi complexes which were active against three strains. Detailed results are given in table. 3.6. The assay was performed in triplicate. The

3**values are** the **mean of three** independent **replicate** along with **standard deviation.**

DMSO was used as negative control and cefixime (10 µg/disc) was used as positive control. Table 3.6: Antibacterial activity of complexes of Euphorbia wallichii crude extract S. no: Sample code E. coli M. luteus B. bronch iseptica S. Aureus S. typhi E. aerogens 1 EWAl 11±0.57 -- -- -- 10±0.57 11±1.15 2 EWCad 8±0.581 -- -- -- -- -- 3 EWLan -- 8±0.5773 -- -- -- -- 4 EWSn -- -- -- -- -- -- 5 EWBi -- -- 10±1.15 -- -- -- 6 EWCo -- 13±0.587 -- -- -- -- 7 EWPb -- -- -- -- -- -- 8 EWMo -- -- -- -- -- -- 9 EWECs -- 14±0.577 -- -- -- -- 10 EWLi 12±0.57 13±1.154 -- -- 10±0.57 -- 11 EWHg 15±1.22 6.5±0.577 -- 16±1.52 15±1.15 16±1.15 12 EWFs -- -- -- -- -- -- 13 EWFc -- -- -- -- -- -- 14 EWAg -- -- -- -- -- -- 15 EW 8.5±0.56 11±0.577 8±0.577 -- 9±0.577 15±1.15 16 Cefixime 23±1.52 15±1.527 22±1.52 18±1.15 24±1.15 19±1.52 17 DMSO -- -- -- -- -- -- [-- = no activity, ± = standard deviation]

14**Antibacterial activity of** BC complexes **was** checked **against** six **bacterial strains, E.**

coli, M. lutus, B. bronchiseptia, S. aureus, S. typhimurium and E. aerogenes. Five BC complexes were

active against B. bronchiseptia strain, four complexes were active against S. typhimurium and E. aerogenes. BCHg produced maximum zone of inhibition (17±0.58mm) againt M. lutus. The complex BCCs was found to have no activity against any fungal strain. While BCCd and BCHg complexes were found active against four bacterial strains. EWAl and EWFc complexes were active against three strains. Detailed results are given in table. 3.7. The assay was performed in triplicate. The

> 3**values are** the **mean of three** independent **replicate** along with **standard deviation.**

DMSO was used as negative control and cefixime 10 µg/disc was used as positive control. Zones of inhibition using complex sample was determined at final concentration of 30 µg/disc. Table 3.7: Antibacterial activity of complexes of Bergenia ciliata crude extract S. No Sample Code E. coli M. luteus B. bronchiseptia S. aureus S. typhi murium E. aer ogenes 1 BCAl 9±1.527 -- -- -- 7.5±0.577 8±0.571 2 BCCd -- -- 9±0.577 9.5±1.15 9±0.577 20±1.52 3 BCLan -- -- -- -- 10±1.1547 -- 4 BCSn -- -- -- -- -- -- 5 BCBi -- -- -- -- -- -- 6 BCCo -- -- -- 8.5±0.58 -- -- 7 BCPb -- -- -- -- -- -- 8 BCMo -- -- -- -- -- -- 9 BCCs -- -- -- -- -- -- 10 BCLi -- -- -- -- -- -- 11 BCHg -- 15.5±0.58 17±0.58 15±0.523 13±0.577 -- 12 BCFs -- -- 7±0.577 -- -- -- 13 BCFc 13±0.52 1 -- 8±0.58 -- -- 9.5±0.76 14 BCAg -- -- 8±0.521 -- -- 11±1.52 15 BC -- 9±0.58 7.5±0.57 -- 8±0.57 9±1.53 16 Cefixim e 23±1.52 7 15±1.527 22±0.866 18±0.58 24±±1.52 20±±0.57 [-- = no activity, ± = standard deviation] 3.5. Antifungal capacities of metallic complexes Antifungal activity of EW complexes was checked against five fungal strain, A. niger, A. fumigatus, Mucor species, A. flavus and F. solani. Six EW complexes were active against fumigatus, five complexes were active against niger. EWFs produced maximum zone of inhibition (10±0.288 mm) against flavus. The complex BCCs was found to have no activity against any fungal strain. BCAg complex was active against four fungal strains. Followed by EWAl and EWFs complexes these were active against three strains. Detailed results are given in the table. 3.8. The assay was performed in triplicate. The

> 3**values are** the **mean of three** independent **replicate** along with **standard deviation.**

DMSO was used negative control and terbinafine at the final

> 4**concentration of** 10 **µg/disc was used as positive control.**

Inhibition was determined at final concentration of 30 µg/disc. Table 3.8: Antifungal activity of complexes of Euphorbia wallichii crude extract S. Sample Aspergillus Aspergilus Aspergilus Fusarium Mucor no: name niger fumigatus flavus solani species 1 EWAl -- -- 9.5±0.288 8.5±1.040 8±0.866 2 EWCd 8±1.04 8±0.50 -- -- -- 3 EWLan -- -- -- -- 7±0.28 4 EWSn 9±0.00 -- -- -- -- 5 EWBi -- 8.5±1.892 -- -- -- 6 EWCo -- -- -- 8±0.86 7.5±0.10 7 EWPb -- -- -- 9±0.50 -- 8 EWMo -- 7±0.322 -- -- -- 9 EWCs -- -- -- -- -- 10 EWLi 7.5±0.25 -- 9±0.00 -- -- 11 EWHg 7.5±0.577 -- -- 9.5±.866 -- 12 EWFs 7±0.155 7.5±0.577 10±0.288 -- -- 13 EWFc -- 8±0.862 -- -- -- 14 EWAg -- 9.5±1.322 9±0.763 8.5±0.915 7±0.280 15 EW 7±0.250 7±1.0 8±0.7637 8±1.0 7±0.151 16 Terbinafine 33±1.527 30±1.0 35±1.527 38±2.081 30±1.52 17 DMSO -- -- -- -- -- [-- = no activity, ± = standard deviation] Antifungal activity of BC complexes was determined against five fungal strain, Aspergillus niger, Aspergilus fumigatus, Mucor species, Aspergilus flavus and Fusarium solani. Most of complexes were active against fungal species used in this study. The significant maximum zone of

inhibition (16±2.081mm) was observed againt Aspergillus niger using BCAl complex. The complexes BCMo, BCCs and BCFc were found to have no activity against any fungal strain. While BCAg complex was found active against four fungal strains. The assay was performed in triplicate. The

3**values are** the **mean of three** independent **replicate** along with **standard deviation.**

DMSO was used negative control and terbinafine 10 µg/disc was used as positive control. Zones of inhibition were measured (mm) at final concentration of 30 µg/disc. Table 3.9: Antifungal activity of complexes of Bergenia ciliata crude extract S. no: Sample name A. niger A. fumigatus A. flavus F. solani Mucor species 1 BCAl 16±2.081 14±1.527 -- 9±1.527 -- 2 BCCd -- -- 8±0.577 -- -- 3 BCLan -- 9±1.52753 -- -- 8±1.52753 4 BCSn -- -- -- -- -- 5 BCBi -- -- -- -- 7.5±0.86 6 BCCo -- -- -- 8±0.577 -- 7 BCPb 8± 1.527 -- -- -- -- 8 BCMo -- -- -- -- -- 9 BCCs -- -- -- -- -- 10 BCLi -- 8±1.0 8±1.32 -- -- 11 BCHg 9±1.0 -- 9.5±.92 -- -- 12 BCFs 7±0.58 8.5±0.288 -- -- -- 13 BCFc -- -- -- -- -- 14 BCAg 8±0.57 9±0.58 -- 7.5±0.28 10±1.15 15 BC 7±0.58 8±0.40 8±0.288 7.5± 7.5± 16 Terbinafine 35±2.516 27±2.516 40±1.54 40±2.0 28±2.0 17 DMSO -- -- -- -- -- [-- = no activity, ± = standard deviation] 3.6. Protein kinase inhibition assay. All the EW and BC complexes were evaluated for protein kinase inhibition capacities. BC complexes were found to have more inhibitory capacity than EW complexes. Capacities were measured in mm inhibitory zone which may be balled or clear zone. Clear zone indicated the fungicidal effect, while balled zone represented the kinase inhibition potential. Activity was checked at final

4**concentration of 30 µg/disc** and DMSO **was used as** negative **control**

that shows no activity in this assay. EWCd (9 mm), EWCs (15 mm) complexes from EW complexes and BCFs (10 mm), BCFc (16 mm) from BC complexes were observed to have kinase inhibitory potential. EW and BC extracts did show kinase inhibition capacity. Fig 3.2: Protein kinase inhibition zones produced by EW and BC complexes 3.7. Phenolic and flavonoid contents of metallic complexes Comparatively highest phenolic contents in EW complexes was observed in EWMo complex (1.8551±0.115) followed by EWCs (1.6815±0.059). While lowest phenolic content was observed in EWBi (0.3719±0.006). Significant highest flavonoids content was observed in EWMo complex (0.719±0.010) and lowest was observed in EWLan complex (0.094±0.005). In BC complexes comparatively highest phenolic content was observed in EWMo complex (2.0265±0.045). Significant lowest phenolic content was found in BCLan (0.3835 ±0.014). BCCs complex contained highest flavonoids content (0.236 ±0.009) and lowest content was observed in BCAl (0.057 ±0.005). detailed results are given in table 3.10. Experiment was performed in triplicate. Values are mean of three independent replicate along with standard deviations. Gallic acids and Quercetin were used as standard for phenolics and flavonoids respectively. Content was expressed as µg equivalent per 100 µg complex. Table 3.10: Phenolic and flavonoid content of EW and BC complexes. Sr. no: EW complex BC complex Sample code Phenolic content Flavonoids content Sample code Phenolic content Flavonoids content 1 EWAl 0.9030±0.012 0.214±0.025 BCAl 1.5408 ±0.030 0.057 ±0.005 2 EWCd 0.9758±0.006 0.225±0.019 BCCd 0.6968 ±0.014 0.123 ±0.018 3 EWLan 0.5950±0.021 0.094±0.005 BCLan 0.3835 ±0.014 0.134 ±0.026 4 EWSn 0.4852±0.005 0.166±0.011 BCSn 1.2411 ±0.046 0.128 ±0.024 5 EWBi 0.3719±0.006 0.143±0.005 BCBi 0.6826 ±0.007 0.184 ±0.016 6 EWCo 1.2206±0.015 0.279±0.011 BCCo 0.7521 ±0.005 0.145 ±0.010 7 EWPb 1.4322±0.005 0.164±0.001 BCPb 0.5345 ±0.022 0.152 ±0.008 8 EWMo 1.8551±0.115 0.719±0.010 BCMo 2.0265 ±0.045 0.171 ±0.013 9 EWECs 1.6815±0.059 0.189±0.003 BCCs 1.4293 ±0.025 0.236 ±0.009 10 EWLi 1.1213±0.012 0.204±0.005 BCLi 0.7803 ±0.017 0.167 ±0.005 11 EWHg 1.0336±0.017 0.165±0.014 BCHg 1.3383 ±0.028 0.158 ±0.004 12 EWFs

1.2750±0.015 0.265±0.013 BCFs 1.2724 ±0.005 0.182 ±0.010 13 EWFc 1.0514±0.017 0.278±0.016 BCFc 1.0270 ±0.037 0.172 ±0.010 14 EWAg 1.1884±0.017 0.247±0.010 BCAg 1.4830 ±0.021 0.159 ±0.011 15 EW 15.686±0.014 4.720±0.199 BC 16.056 ±0.115 3.930 ±0.253 Fig 3.3: Phenolic content of EW complexes Fig 3.4: Phenolic content of BC complexes Fig 3.5: Flavonoid content of EW complexes Fig 3.6: Flavonoid content of BC complexes 3.8 FTIR results All the spectra were recorded in the range of 4000 to 600cm-1, these are given bellow EW extract Fig 3.7: FTIR spectra of EW crude extract Table 3.11: Wave numbers and suggested functional groups in FTIR spectra of EW crude extract S.No Wave number (cm) Suggested functional groups -1 1 921.66 Carboxylic acid, =C-H (Alkene) 2 1032.82 (C-O) Ester, (C-O) Alcohol 3 1205.13 (C-O) Ester, (C-N) amine, phenolic (-OH) 4 1339.28 (C-N) amine 5 1448.01 (C=C) aromatic (-C-H) alkane, acetonitrile 5 1611.03 (N-H)) Amides, 7 1698.52 (C=O) Ketone 8 2929.28 (-C-H) alkane 9 3271 (-OH) Spectra of extract deduce the presence of various functional groups as carboxylic acid,

> 49**(=C-H)** Alkene, **(C-O) Ester, (C-N)** amine, **(C-** O) Ester, **(C-** N) amine, **(C=**

C) aromatic (- C-H) alkane, acetonitrile, (N-H)) Amides, Ketone, (-C-H) alkane, (-OH) which are presented in above table with their peak values.

> 2**Fig 3.8: FTIR** spectra **of** EWAl complex **Fig 3.9: FTIR** spectra **of** EWCd complex **Fig 3.10: FTIR** spectra **of**

EWLan complex

> 2**Fig 3.11: FTIR** spectra **of** EWSn complex **Fig 3.12: FTIR** spectra **of** EWBi complex **Fig 3.13: FTIR** spectra **of** EWCo complex **Fig 3.**

14: FTIR spectra of EWPb complex

> 19**Fig 3.14: FTIR spectra of**

EWMo complex

> 13**Fig 3.15: FTIR** spectra **of** EWCs complex **Fig 3.16: FTIR** spectra **of** EWLi complex **Fig 3.17: FTIR** spectra **of** EWHg complex **Fig 3.18: FTIR** spectra **of** EWFs complex **Fig 3.19: FTIR** spectra **of** EWFc complex Fig **3.**

20: FTIR spectra of EWAg complex Characteristic peaks appeared in the range of 1000-600cm-1 along with peak at 1205cm-1 in the spectra of EW extract, were disappeared in the spectra of EWAl complex. Variation in other peaks were also observed in this complex. Changed spectral orientations in EWCd complex were observed comparative to EW extract. Peaks at 3271and 1611cm-1 in EW spectra were found missing in spectra of EWCd complex. EWLan, EWSn and EWBi complexes showed the similar

pattern of spectral pattern and major change was found at 900 to 600 cm-1. Broad peak that was originated in EW spectra at 3271.28 cm-1 was extinct and shifting in peak values were noticed in EWCo complex. EWPb complex spectra displayed a peak at 2865 cm-1 splitting into doublet, which is characteristic peak of carbonyl functional group. EWPb exhibited different spectra relative to EW extract and sharp peak was established at 1033.08 cm-1 in it. Characteristic peak observed in spectra of EW extract above 3000cm-1 was reappeared in the EWMo and EWHg complexes. Shifting in peak values were perceived in the spectra of these complexes. EWFs showed fused and transformed peaks. Spectra of EWCs, EWLi, EWFc, EWAg complexes showed the fused peaks in spectral positioning and peak values transformed indicating the different nature of complex formation. Fig 3.21: FTIR spectra of BC extract Table 3.12: Wave numbers and suggested functional groups in FTIR spectra of BC crude extract S. No Wave number (cm) Suggested functional groups -1 1 921 Alkene 2 1032.98 (C-O) alkyl aryl ethers 3 1090.22 (C-N) amine (a) 4 1203.71 (C-O) Ester, (C-N) amine 5 1332.80 (C-N) amines(t) 6 1442.2 Alkanes 7 1520.0 (N-H) Nitro compounds (amide) 8 1701.4 (C=O) alkyl aryl ketone 9 2846.8 (CH) 10 2924.05 (-C-H) alkane, (C=O) acid 11 3017.8 3240.7 (N-H) amide a= aliphatic, t=tertiary Peaks along with their values appeared in IR spectra of BC extract are mentioned in table. 3.12. Which indicating the presence of various functional groups as (C-O) alkyl aryl ethers, (C-N) amine, (C-O) Ester, alkane, (N-H) Nitro compounds (amide), (C=O) alkyl aryl ketone, (-C-H) alkane, (C=O) acid, (-OH) Alcohol and phenol in extract of BC.

19**Fig 3.** 22: **FTIR spectra of** BCAl complex Fig **3.**

23: FTIR spectra of BCCd complex

2**Fig 3.23: FTIR** spectra **of** BCLan complex **Fig 3.** 24: **FTIR** spectra **of**

BCSn complex Fig 3.25: FTIR spectra of BCBi complex

17**Fig 3.26:** FTIR **spectra of** BCCo complex **Fig 3.27:** FTIR **spectra of** BCPb complex **Fig 3.28:** FTIR **spectra of** BCMo complex **Fig 3.29:** FTIR **spectra of**

BCCs complex

2**Fig 3.30: FTIR** spectra **of** BCLi complex **Fig 3.31: FTIR** spectra **of** BCHg complex Fig **3.**

32: FTIR spectra of BCFs complex Fig 3.33: FTIR spectra of BCFc complex

19**Fig 3.34: FTIR spectra of**

BCAg complex All complexes showed changed spectral orientation comparative to spectra of BC extract. Characteristic peaks appeared at 3017.8, 2924.05, 2846.8, 1701.4 cm-1 were missing in in the spectra of BCAl complex. BCCd, BCLan, BCCo, BCSn, BCCs, BCLi, BCHg, BCFs, BCFc showed the similar spectral

orientation for complexation with shifted in their values of peaks. Spectra of these complexes were differing in the range of 1000-600cm-1 along with other shifting in values of peaks. Peaks at 3017.8 and 1520cm-1 observed in BC spectra were extinct in spectra of BCSn complex. Characteristic peak of BC spectra at 1701.4, 1332.8 and 1203.71cm-1 were not exhibited in spectra of BCCo complex. BCPb, BCAg and BCMo complexes have showed different spectral orientation. Sharp peaks were observed near to 1000cm-1 in spectra of these complexes. Two peaks at 1442.2, 1203.71cm-1 were disappeared and specific peak at 3680.99 cm-1 was appeared in the spectra of BCMo complex. Nature of ligand can be predicted by observing the change in orientation and peaks of spectra of complexes, comparative to spectra of BC extract. Discussion Discussion Drug discovery from natural sources started in prehistoric times that led for discovery of many compounds. Most of present medicines have been developed on the basis of traditionally used therapies (Rates, 2000). Drugs obtained from plants possess better pharmacological responses, due to which 25% of worldwide prescribed drugs have been obtained from plants (Rates, 2000). Recent trend of taking complementary and alternative medicines is increasing, in order to relieve the pathological symptoms, This is because of perceived side effects of chemical drugs (Verpoorte et al., 2006). Revolution in the field of organic chemistry has led to the production of potent, safe and more active synthetic drugs by structural modification in pure compounds (Petrovick et al., 1997; Sharapin, 1997; Rates, 2000). Coordination compounds are playing vital role in homeostasis of biological systems. Various metal complexes like carboxy-peptidase, hemoglobin and chlorophyll containing zinc, iron and magnesium metals respectively are important biological compounds in living system (Orvig et al., 1999). Medicinal utility of coordination compounds formed by the combination of metals can be traced up to 5000 years back (Orvig et al., 1999). The splendid array of metal complexes applications has been investigated in pharmaceutical substances

8**(Sadler et al., 1999; Ali & van Lier, 1999; Louie & Meade, 1999; Volkert & Hoffman, 1999**

Sakurai et al., 2002), which has been increasing rapidly due to enhanced compatibility (Smith-Warner et al., 2000; Cragg et al., 2000). Various researchers have focused on advances in the field of coordination compounds (Sakurai et al., 2002). Metal combines with ligands to form complexes and if ligand forms more rings, then resultant product will be chelate (Wayne, 1999). These metallo complexes have been found to possess importance in different fields, like biomedical, analytical, therapeutic, synthetic applications as gold complexes (Auranofin etc) (Perry, 2002; Dorr & Von Hoff, 1994). New methodologies have continuously been applied for the development of new drugs as combinatorial drug chemistry. But it is necessary to determine the toxicity of chelation based therapies in clinical trials. In current study, coordination compounds of B. cilitia extract (BC) and E. wallichii extract (EW) were synthesized. These were evaluated for antioxidant potential, coordinated phenolic and flavonoids contents, cytotoxic effect, antimicrobial and kinase enzyme inhibition activity. Complexes were also subjected to FTIR spectroscopy in order to evaluate the nature of ligand. Antioxidants work in diverse ways, so the mechanism of action of

6**all antioxidants in a complex system**

cannot be reflected in any single assay

6**(Prior et al., 2005).** There are two **methods that**

describe mechanism of antioxidants i.e. free radical scavenging

> 6**by hydrogen donation to form stable** compound **(Prior et al., 2005)**

and reduction of free radical by donating electron. DPPH, an organic nitrogen radical, is commercially available with deep purple color. Measurement of antioxidant potential of sample using DPPH

> 6**was first** time **reported by Williams et al. (1995).** All complexes showed **the** decrease in **DPPH**

activity. This assay provides the basic information regarding free radical scavenging potential of different compounds. Reduction of DPPH by the compound produces diphenylpicryl hydrazine, a pale yellow compound. This property was utilized to determine the scavenging potential of EW and BC complexes. All EW and BC complexes showed decrease in DPPH % scavenging capacities. EWMo complex was found to have 14% and EWFs showed 70% less scavenging potential comparative to extract. BCMo complex was found to have 12% less scavenging potential as compared to extract. This shows the higher antioxidant potential of extract as also indicated by its significant reducing capacity (Rajkumar et al., 2010). Phenolics are important antioxidant phytochemical (Javanmardi et al., 2003) and these are nucleophilic in nature, which is responsible for the chelating potential of these compounds (Morgan et al., 1997, JUN et al., 2003). Total antioxidant capacities of complexes were checked using phosphomolybdenum method and capacities were expressed in ascorbic acid equivalent. All complexes have showed the lower total antioxidant capacities. EWCd complex showed 13% lesser total antioxidant potential then EW extract. Among BC complexes BCMo and BCCs were found to have significant 4% less total antioxidant potential with respect to BC extract. In this assay complexes reduce the

> 72**Mo (VI) to Mo (V)** in acidic condition **that** led to **the**

> 56**formation of green** colored **phosphate Mo (V) complex (Prieto** et al., **1999).**

In both the DPPH and total antioxidant tests, metallo complexes showed reduced antioxidants activities as compared to crude extracts. It showed that incorporation of metals in the complexes of extracts changed the properties of ligands present in extracts, by reducing the binding sites of the free radicals and producing an antagonistic effect, which resulted in reduced antioxidant capacities (Thakum et al., 2012). EW and BC complexes were also assessed for the reducing capacities that were expressed in ascorbic acid equivalent. Complexes showed higher reducing power comparative to extract. Higher ascorbic acid equivalent number indicates the more reducing power. EWFs complex had increased reducing power (51%) comparative to EW extract. Slight elevation in reducing power was produced by EWLan and EWPb, that was 3.6%, 4.3% with respect to EW extract. BCCd was also found to have 46% increases in reducing power among BC complexes. In our study, compound with increased reducing power were not active in DPPH assay. It may be due to the change in the chemical properties of the extracts after combination with the metal. Metal may had increased the reducing potential of electron donating substances in complexes, however failed to produce any significant activity in quenching capabilities of free radicals, resulting in the reduction of scavenging potential in DPPH assay. This idea was supported by Thakam et al. (2012), who

also reported increase in reducing potential of complexes comparative to their extracts. EW and BC complexes were evaluated for coordinated phenolic and flavonoid contents. Among EW complexes, highest phenolic contents were observed in EWMo complex (1.855 ± 0.115 µg/100 µg of sample) and lowest were observed in EWBi (0.372 ± 0.006 µg/100 µg of sample). Whereas, EWMo complex (0.719 ± 0.010 µg/100 µg of sample) had highest flavonoids, while lowest were observed in EWLan complex (0.094 ±0.005 µg/100 µg of sample). Among BC complexes, highest phenolic contents were observed in EWMo complex (2.0265±0.045 µg/100 µg of sample) and lowest in BCLan (0.3835 ±0.014 µg/100 µg of sample). BCCs complex contained highest flavonoids content (0.236 ±0.009 µg/100 µg of sample) and lowest value was observed in BCAl (0.057±0.005 µg/100 µg of sample). Haq et al., (2012) had reported 20% phenolic and 0.39% flavonoids in EW extract. Rajkumar et al. (2010) had also found 38% phenolic contents in BC extract. In current study 16% phenolic was observed in extract of BC and 15% from EW extract. These differences may depend on variations in collection time and site.

71 **Due to the presence of** aromatic rings **phenolic and flavonoids**

are nucleophilic in nature, which is responsible for the chelating potential of these compounds (Morgan et al., 1997, Jun et al., 2003). Coordination of these compounds with metal is further confirmed by measuring their content in complexes. It is also evident from the above results that other compounds which are not phenolics and flavonoids are also involved actively in complex formation with metals as indicated by the lower content of phenolic and flavonoids measured in content determination. Cytotoxic effects of complexes were also investigated by using the brine shrimp assay. It is cost effective, rapid and feasible to carry out for cytotoxic studies (Kivack et al., 2001; Carballo et al., 2002). Larval tissues of shrimps respond similarly as mammalians carcinoma cells. Cytotoxic effect of complexes towards shrimp's larvae can be correlated with anticancer potential and complexes could be alternative source of anticancer drugs (Ullah et al., 2012). These days much attention is being given on metallo complexes and their anticancer activity. In order to discover new potent anticancer drug various studies has been done for example synthesis, modification, chemical and structural behavior of metal complexes (Saha et al., 2009). In current study, significant cytotoxic activity was observed from EWHg (LD50 = 96 µg/ml), followed by EWFs (LD50 = 96.69 µg/ml). EWCs showed comparatively maximum LD50 value indicating its least activity among EW complexes. Among BC complexes, BCFs was more cytotoxic having LD50 (LD50 = 58 µg/ml), while BCAg has maximum LD50 value (LD50 = 392 µg/ml) showing lowest cytotoxic nature. Haq et al. (2012) reported the promising cytotoxic potential of EW extract, which was also observed in current study. Concerning the complexes of transition metal, these had been proved to be biologically active and were studied to have anticancer properties (Ali et al., 2008; Ali et al., 2009). Various complexes having organic ligand had also been found to have antitumor activity as amines ligand in ruthenium complexes (Jain et al., 2009). In current study, metal complexes were also found to have significant cytotoxic capacities as also reported by Islam et al. (2007). Bacterial resistance against existing drugs has led to severe health concerns throughout the world. It imposes for more research to find new classes of potent antibiotics. Various studies had been carried out for the investigation of potent and effective antibacterial agents. In present study, all complexes were evaluated for antibacterial capacities using disc diffusion method against six types of bacterial strains. Activity was calculated by measuring inhibition zone around disc. Complexes were found to have enhanced antibacterial activity comparative to their extracts. Among EW complexes five were found active against M. lutus strain, four against E. coli. While EWHg exhibited maximum inhibition zone (16 ± 1.527 mm) against S. aureus and observed active against five bacterial strains. Varying antibacterial activities were observed in BC complexes. BCHg was observed significant active among BC complexes and produce inhibition zone of 17 ± 0.58 mm against M.lutus. Five complexes were found active against B. bronchiseptia strain, four were against S. typhimurium and E. aerogenes among BC complexes.

The complex EWSn, EWPb, EWMo, EWFs, EWFc and EWAg, BCCs were found to have no activity against any bacterial strain. Different studies being carried on toxicological, antimicrobial activities of metal complexes having mixed ligands. Ivanova1 et al. (2012) have also report the antimicrobial potential of metal complexes. Similarly Islam et al. (2013) have also reported the antimicrobial activity of metal complexes. It was observed that nature of ligands, metal and synergetic effect determines the antibacterial potential of metal complex (Chohan et al., 2006). Selective antibacterial effect of the complexes depends on metals, ligands having different functional groups and donor atoms that produce selective toxic effect which resulted in varying antimicrobial activities of all complexes as also reported by Shaikh et al. (2004). Pathogenic fungal strains increase the human mortality and morbidity rate. Multidrug resistance in microbe is fast growing and major global health issue (Chohan et al., 2006). Saha et al. (2009) have reported various fungal strains showing drug resistance e.g F. solani, Candida albicans, A. flavus and Candida glaberata. This generates the interest for discovering new antifungal agent or to modify the existing drugs. Various studies have been carried for characterization of chemical and structural nature of metal complexes, which can be used as alternative antifungal drug (Saha et al., 2009). All complexes of EW and BC were tested

> 43**for antifungal** potential **against five fungal strains** as **A. niger, A. fumigatus,** Mucor **species,**

A. flavus and F. solani. Among EW complexes, six were detected active against A. fumigatus. EWFs has produce significant maximum zone of inhibition (10 ± 0.288 mm) against A. flavus. Five of BC complexes were found active against A. niger and A. fumigatus. BCAl have produced maximum zone of inhibition (16 ± 2.081 mm) against A. niger. EWCs, BCMo, BCCs and BCFc were observed inactive against any fungal strain. Antifungal potential of complexes was also

> 67**reported by** Chohan **et al.** (2006) **and** Saha **et al. (2009).**

Metal, ligand and nature of complex decide the antimicrobial potential of coordinated compound (Chohan et al., 2006). Metal complexes have more antifungal

> 21**activity than free ligand** as biological **activity of complexes can be explained** using **overtone's concept and chelation theory**

(Chohan et al., 2006). Liposolubility is the main factor that determines the antifungal activity of sample (Sadeek et al., 2009).

> 64**Lipid membrane** around **the cell** controls **the** permeability and **only** allows **lipid soluble**

material in to cell. Chelation decreases the metal ion polarity,

> 9**due to overlap ligand orbital and partial sharing of the positive charge of the**

metal ion with donor groups. Delocalization of p **-electrons** increase over **whole chelate** that favor **the** lipophilicity **of the**

complex (Sadeek et al., 2009). Due to increase the lipophilicity complexes are penetrated

42**into lipid membrane** easily **and** consequently block **metal binding sites on** the **enzymes of the**

microorganism (Raman et al., 2003; Sadeek et al., 2009). Various studies are carried on cytotoxic and anticancer property of metal complexes and (Islam et al., 2007). Protein kinase inhibitors are important drugs for cancer treatment. Most of inhibitors are investigated for cancer and but deregulation of kinase function is also involved in neurological, immunological, infectious and metabolic diseases. Researchers are diverted for development of kinase inhibitors, so cancer along with these disorders can be treated (Cohen et al., 2002). There are different methods for the measurement of inhibition of kinases. Currently election of inhibitor against specific protein kinase that can be used as drug remains challenge for researchers. Cohen was the first to screen kinase inhibitors for various enzymes and substrates using microplate reader in 96 well formats

39**(Davies et al., 2000; Bain et al., 2003; Bain et al., 2007).**

In present study metal complex of both plants were observed to have capacity of inhibiting protein kinases. EWCd (9mm), EWCs (15mm), BCFs (10mm), BCFc (16mm) complexes showed significant kinase inhibition potential among EW and BC complexes. FTIR is prerequisite technique in pharmacopoeia of various countries for identification of medicines (Liu et al., 2006). It is widely used for recognition (Griffiths and De Haseth, 1986) as well as accurate and rapid identification of biological compounds (Sukmaran et al., 2011; Thenmozhi et al., 2011). Infrared region ranges from 0.8 to 103 µm. Limited portion of this range from 4000 to 625 cm-1 (2.5-16 µm) is useful for organic compounds (Younas, 2004). Haq et al. (2012) reported the presence of various phytochemicals like tannins, alkaloids, terpenoids, glycosides and flavonoids in EW extract. FTIR spectra in the range of 4000-600 cm-1 of crude extract of E. wallichii, suggested the presence of hydrocarbons, terpenoids, abietane steroids, aromatic esters, phenolics, flavone glycosides and several bioactive constituents. These compounds having different functional groups are represented by their characteristics peaks in the range of. Phenolic, flavonoid content in both extracts and that attached with metals was confirmed by measuring the amount of these phytochemicals. Characteristic peaks appeared in the range of 1000-600cm-1 along with peak at 1205cm-1 in the spectra of EW extract, were disappeared in the spectra of EWAl complex. Variations in other peaks were also observed in this complex. Changed spectral orientations in EWCd complex were observed comparative to EW extract. Peaks at 3271and 1611cm-1 in EW spectra were found missing in spectra of EWCd complex. EWLan, EWSn and EWBi complexes showed the similar pattern of spectral pattern and major change was found at 900 to 600 cm-1. Broad peak that was originated in EW spectra at 3271.28 cm-1 was extinct and shifting in peak values were noticed in EWCo complex. EWPb complex spectra displayed a peak at 2865 cm-1 splitting into doublet, which is characteristic peak of carbonyl functional group. EWPb exhibited absence of peak of OH and downward shifting of amine group peak to 1033.08 cm-1. EWMo exhibited downward shifting of peak of OH. Characteristic less intense peaks were observed in spectra of EWHg complex above 3000cm-1 as compared to crude extract. Shifting in peak values were perceived in the spectra of these complexes. EWFs showed fused and transformed peaks in whole spectra. Spectra of EWCs, EWLi, EWFc,

EWAg complexes showed the fused peaks in spectral positioning and peak values transformed indicating the different nature of complex formation. FTIR spectra of extract of B. ciliata suggested the presence of different compounds like paashaanolactone, catechin, anthraquinone, steroids, phenolic, flavonoids, saponins, tannins and terpenoids. All complexes of BC showed changed spectral orientation comparative to spectra of BC extract. Characteristic peaks appeared at 3017.8, 2924.05, 2846.8, 1701.4 cm-1 were missing in in the spectra of BCAl complex. BCCd, BCLan, BCCo, BCSn, BCCs, BCLi, BCHg, BCFs, BCFc showed the similar spectral orientation for complexation with shifted in their values of peaks. Peaks at 3017.8 and 1520cm-1 observed in BC spectra were extinct in spectra of BCSn complex. Characteristic peak of BC spectra at 1701.4, 1332.8 and 1203.71cm-1 were not exhibited in spectra of BCCo complex. BCPb, BCAg and BCMo complexes showed different spectral orientation. Sharp peaks were observed near to 1000cm-1 in spectra of these complexes unlike crude BC extract. Two peaks at 1442.2, 1203.71cm-1 were disappeared and specific peak at 3680.99 cm-1 was appeared in the spectra of BCMo complex. The shifting of peaks of various functional groups to lower absorption ranges, in spectra of both BC and EW complexes as compared to the spectra of the crude extracts of plants indicated the higher potential of coordination of those particular functional groups such as OH, alkenes, amines and carboxylic acid etc. with metals. In FTIR spectroscopy, coordination complexes frequently shows the absence as well as shifting of peaks lower absorption range as compared to ligands. Singh (2012) indicated the shifting of bands of coordination compounds of schiff ligand, from 1492

65**cm-1 to** 1490 **cm-1 and** 1456 **cm-1**

to 1453 cm-1 respectively as compared to the individual schiff ligand, indicating its potential to coordinate with the metals. Similarly Thakam et al. (2012) also observed the decrease in intensity of carbonyl group band in the complex of metal and curcumin showing its involvement in the coordination. Conclusion and future strategies Successful complexation of metals with Bergenia ciliata and Euphorbia wallichii extracts was carried out in current study. Complexes showed enhanced biological capacities and lower antioxidant activities. Due to lower antioxidant capacities complexes cannot be considered as potential antioxidant agents because metal changed the chemical properties of complex. Significant enhanced biological capacities of metallic complexes showed their potential to

14**be used as an** alternative **source of** cytotoxic **and antimicrobial drugs.**

Few sample showed the potential of inhibiting protein kinases, so could be used as kinase inhibitors as well. However, there is need of further evaluation of chemical nature of ligands and in vivo and mechanistic based studies of these complexes. There is further need of investigation of actual mechanism of cytotoxicity and probable effect of complexes on cell lines and animal model. Studies may be performed for the size determination and structure elucidation of these compounds by means of various analytical procedures.