"Screening of phytocomponents and antioxidant potential of *Ajuga bracteosa* and *Berberis aristata*"

Dissertation Report

Submitted in partial fulfillment of the
Requirement for the award of the
Degree of

Master of Technology in Biotechnology

By
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(2013-2014)

CERTIFICATE

This is to certify that the Report entitled "**Screening of phytocomponents and antioxidant potential of *Ajuga bracteosa* and *Berberis aristata***", that is being submitted by "**Simrandeep Kaur (Regd.No. 11300715)**" in partial fulfillment of the requirements for the award of degree **"MASTER OF TECHNOLOGY"** in **"BIOTECHNOLOGY"**, is a record of bonafide and authentic work done under my guidance. The contents of this Report, in full or in parts, have neither been taken from any other source nor have been submitted to any other Institute or University for award of any degree or diploma and the same is certified.

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DECLARATION

I hereby declare that the project work entitled "**Screening of phytocomponents and antioxidant potential of *Ajuga bracteosa* and *Berberis aristata***" is an authentic record of my own work carried out at "**Lovely Professional University, Phagwara"** as requirements of pre-dissertation project for the award of degree of **"M. Tech. in Biotechnology"** at **Lovely Professional University, Phagwara** under the guidance of "**Dr. Amit sehgal, Assistant Professor, L.P.U., Phagwara"** during August, 2014 to April, 2015. Taking all the responsibility regarding anti-plagiarism drive by our institute, I hereby declare that no previously published document has resemblance with my work to be identified as under act of plagiarism. All the writing and work in this Report is mine. Wherever I have borrowed material from other sources, I have diligently acknowledged the source of the borrowed material.

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Date: 4rth May, 2015 **Regd. No: 11300715**

AKNOWLEGEMENT

As I begin to write these lines after completion of my thesis, my heart is filled with deepest sense of gratitude. I shall ever remain thankfully indebted to all those who have encouraged me to achieve my goal and enlightened me with the touch of their encouragement.

First and foremost I want to thanks my beloved ***Mother, Father*** *and* ***Brother*** *for their affection, blessings, sacrifice, emotional support and love which has actually inspired me though out my dissertation work. Without them I would have never come to this proliferative stage and engaged myself in carrier building. Having such a wonderful family who supports me whole heartedly, no matter what I do, is something I feel unique to my life. I would like to thanks specially my mother who has listened to my problems patiently, suggested me the possible solutions and with whom I have shared each and every moment of excitement after getting positive results and disappointment after not getting success in some experiments during my dissertation work, I have no words to pay regards to this moral support.*

I consider it as a blessing to pursue my Dissertation under the guidance of ***Dr. Amit sehgal Asst Proff., LPU, Jalandhar****. I am grateful to my mentor for being a great support and a true guide throughout my dissertation work. No words are enough to express my gratitude for his whole hearted encouragement, supervision and support. He would take a great concern in troubleshooting problems and was always available to discuss the problems even in his busy schedules. I heartily thank him for being a patient listener to my problems which I have came across during my dissertation and for providing me his useful guidance.*

I am equally thankful to the administrative faculty of LPU ***Dr. Himanshu (HOD)*** *and Mr****. Ravi kant Pathak (HOL)*** *for their cooperation and support.*

I extend my special thanks to my dearest friend Navjeet for her motivation, encouragement, unconditional support. She was always beside me during the happy and hard moments to push me and motivate me.

The chain of my gratitude would be definitely incomplete if I forgot to thank ***Mr. Yogesh Dogra (lab technician)*** *who has always provided me with all those materials, lab equipments and useful suggestion which I needed for my the lab work. You provided such a positive environment in the lab that even prolonged lab work never seemed hectic to us.*

I would like to thank the whole ***Department of Biotechnology and Biosciences, Lovely Professional University, Jalandhar*** *for accepting and allowing me to conduct the experiments regarding my dissertation.*

At last, I would like to thank almighty ***GOD*** *for supporting me spiritually throughout my life and providing me everything that I needed.*

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List of Abbreviations

Abbrevations	Discription
DW	Distilled water
WHO	World Health Organization
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
HCL	Hydrochloric Acid
GAE	Gallic Acid Equivalents
IC_{50}	Half Maximal Inhibitory Concentration
QE	Quercetin Equivalents
ROS	Reactive Oxygen Species
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
HPLC	High pressure liquid chromatography

Units	Discription
µl	Microliter
µg	Microgram
g	Gram
mg	Miligram
ml	Mililiter
hr	Hour

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CHAPTER 1

INTRODUCTION

Nature has all the resources to heal every ailment of human body. From ancient time plants and plant products have played very important role as medicines. Herbal medicines are rich source of therapeutic agents in all over the world and are having higher safety margins with lesser costs. Recently, there is shift in the universal trend in use herbal medicine rather than synthetic medicines, which we can say 'Return to Nature' (Kavuthodi and Sebastian, 2013). "Rig-Veda"- the oldest known repository of Hindu culture, mentioned the use of medicinal plants to cure wide range of diseases (Joshi *et al.,* 2008). A vast variety of plants and their use as medicines has been mentioned in many ancient literatures such as Charak Samhita. Ayurveda wich is considered as foundation of medicinal science, also deals with specific properties of drugs, most of them are derived from plants (Sherpa *et al.,* 2014).

India has often been referred to as the Medicinal Garden of the world. In India, during the traditional systems of Unani, Ayurveda, Homeopathy and Siddha almost 95% of the treatments were based on medicinal plant. Indian Medicinal plants which are commonly used in home remedies against multiple ailments are considered as major source of several pharmacologically active compounds (Savithramma *et al.,* 2011).

The plant species having medicinal properties have made an outstanding contribution in the origin of many herbal therapies. Taking the advantage of modern medicines, herbal medicines have experienced ignorance, but during last two or three decades, the medicinal plants have attained a wide recognition due to an lesser side effects and lower costs (Sanhita *et al.,* 2012).

All parts of the plant produce different pigments, which are the major classes of secondary metabolites and are usually referred as bio-pigments. Pigments reflect only certain wavelengths of visible light that is between 380 to 730 nm. All biological pigments selectively absorb certain wavelength of light while reflect other (Mlodzinska *et al.,* 2009). For example anthocyanins absorb yellow-green light at wavelength of 520-530nm and generate mauve colors formed by the reflection of a mix of orange, red and blue wavelengths. There are various groups of plant pigments responsible for coloration: Flavonoids anathocyanins, betalains, carotenoids and chlorophyll's, they show different absorbance peaks. Flavonoids are secondary metabolities that belong to the class of phenyl propanoids,

having a wide range of color from pale-yellow to blue. Betalains are nitrogen containing compounds ranging from yellow to red color. Carotenoids are isoprenoids found in plants as well as in micro organisms. They are essential compounds of photosystem (Vargas *et al.,* 2000).

The medicinal activity of plants mainly depends on phytochemicals, which are being synthesized by the plant, which are also termed as secondary metabolites (Kaur *et al*., 2011). They are called secondary metabolites because they are not absolutely essential for the survival of plants though they play very important role as their defense mechanism to survive in various stress conditions (Lewis *et al.,* 2006). Most of these secondary metabolites have been proved to provide protection against various pathogenic microorganisms having potential to cause diseased condition in the plant. Therefore, these secondary metabolites, depending upon their phytochemical properties can be used as antimicrobial agents to provide protection and to design drugs against human pathogenic microorganisms in order to cure infectious diseases. These secondary metabolites are a source of biologically active chemicals with various functions, including antibacterial, antioxidant, antifungal, antiviral, and anticancer activities. Infectious diseases are one of the leading causes of deaths in developing countries (Gayathri *et al*., 2011).

Antioxidants are important part of food preservatives used to preserve food quality mainly because they prevent oxidative deterioration of lipids. The exploration of naturally occurring antimicrobials for food preservation is getting preference over synthetic preservatives due to consumer awareness of natural food products and growing concern of microbial resistance towards conventional preservatives. Such factors have inspired the screening of new plant species for possible medicinal and antioxidant properties (Alghazeer *et al.,* 2012). Antioxidants have the potential to prevent the oxidation of molecules to protect cells from damage that is caused by free radicals (unstable molecules) (Khaled, 2014).

In the previous years, the investigation of the natural products from different sources for the discovery of new antimicrobial and antioxidant agents has been increasing (Ayari *et al.,* 2013). Plants with medicinal properties have the potential of being novel source for antioxidants. Reactive oxygen species (ROS) cause oxidative stress to the human tissues which usually arise from disease pathogenesis and aging. Although, the effects of free radicals is being counteract by inherent antioxidative property of human body, there is often need of antioxidant supplements specially at old age and diseased condition of the body. Oxidative stress that is being caused when there is an imbalance between ROS and detoxification capacity of the body and leads to damage of DNA (Amoo *et al.,* 2012).

Plants are major sources of various natural antioxidants, which naturally produce various chemicals to arrest the activity of reactive oxygen species (ROS) in order to survive. Superoxide anion (O_2), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO) and reactive hydroxyl radicals (OH) are the common reactive oxygen species (Panchawat *et al.*, 2010). ROS can cause lipid peroxidation, which leads to the deterioration of the food and the oxidative deterioration of the lipid-containing food is responsible for the rancid odors during processing and storage. Moreover the addition of antioxidant increases the shelf life of the food (Gulcin, 2013 and Ramirez *et al*., 2014). Labiatae and berberidaceae are plant families that have very important role in the medicinal and ayurvedic world. Many plants of these families have medicinal properties.In this work,we will focus on "Neelkanthi" and "Daruhaldi" plants of these families, with botanical names *Ajuga bracteosa* and *Berberis aristata*.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Plant Extracts as a source of novel drug development

Nature has provided a complete set of remedies and sources to cure all kind of diseases.Many higher plants produce economically important organic compounds which possess pharmaceutical properties also (Adwan*et al.*, 2008). From the ancient time, plants and plant products played very significant role in maintaining good health of human whether it is by providing the nutrition or by preventing humans from diseases. The ancient Indian medicinal literature like CharakSamhita justifies the effective applications of plant and plant products to cure wide range of diseases (Jackson, *et al.*, 2013). In India 85% population is using plants products as source of effective anti-microbial agents for the treatment of wide range of diseases. The easy availability, low cost and negligible side effects are some of the major features of herbal compounds which make them superior over the synthetic ones.

Many part of the plant viz. seeds, roots, leaves, bark or flowers can be used for medicinal purpose and therefore called "Herbal medicine" (Barrett *et al.*, 1999). According to statistical analysis of World Health Organization (WHO), almost 84% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used as modern medicine(Newman*et al.*, 2003).

Based on this traditional and other uses of medicinal plants many studies have been conducted to assure their potentially pharmacologically active components having, antibacterial and antioxidant activities of the therapeutically important plants. Many readily available plants in India are used in traditional medicine for treating the cholera, diarrhea and dysentery (Brantner.*et al*,1994). The leaves of sweet basil (*Ocimumbasilicum*) are applied on cutaneous lesions and ring worm in the form of paste. While essential oils of the leaves are used to cure ear infections and infusion of the seeds to treat bronchial asthma (Rangasamy*et al.*, 2012).

Based on Ethnobotanical knowledge, for example, the antibacterial activity of medicinal plant such as tulsi, dalchini, neem, datura against potential pathogens such as *Bacillus cereus, Bacillus thuringiensis, Staphylococcus aureus,Salmonella typhi, Escherichia coli, Shigelladysentriae,Klebsiella pneumonia*andantioxidant properties of medicinal plants viz.Quercetin,*Sphaeranthusindicus*Linn.(Gorakmundi),*Glycyrrhizaglabra*(yastimadhu),Terminal iabelerica(Triphala) have already been determined (Panchawat*et al.*,2010 and Joshi, *et al.*, 2009). Antioxidant capacity of plant materials relies on the content and composition of phenolic compounds as well as other groups of natural substances. Compounds with antioxidant activity prevent the oxidative damage induced by reactive oxygen species (ROS) and other free radicalsuch as superoxide anion, hydroxyl radical, and hydrogen peroxide (Hajimehdipoor*et al.*,2012).The antioxidant property of phenolic compounds is confined to their redox potential, and ability of neutralizing the free radicals(Aryaand Yadav, 2010).

The plants synthesize various chemicals naturally termed as phytochemicals, which play many important roles in plant physiology and are responsible for survival potential of the plant under biotic or abiotic stress conditions. Many of these chemicals are essential for plant growth and metabolic processes termed as primary metabolites, where as another class of metabolites is there which do not have direct role in plant growth but provide protection against infectious microbes and responsible for plant's survival in stress conditions (Bernhoft*et al.*,2004).

Plants are rich source of secondary metabolites which are confined to antioxidant and antimicrobial properties (Lewis *et al,* 2006). Many ancient plants are found to be elective for treating number of infectious diseases with fewer side effects and lesser toxicity (Lee *et al.,* 2007). By finding new chemicals entities in the plants, the potential of plants of treating the disease can be increased. These new chemicals can serve as authentic drugs to fight against fatal disease and treating the diseased persons with lesser rate of toxicity and thus the human health can be improved (Raskin*et al.,* 2002).

2.2 Bioactive compounds or bio-pigments in medicinally important plants

Plants are a significant source of diverse organic compounds having no direct role in plant direct growth but have the potential of treating various diseases, termed as the secondary

metabolites (Reddy *et al.,* 2003). The specific function of many of such metabolites is already being investigated while the activity of many of such organic compounds is still unknown. The primary metabolites on the other hand, play a direct role in the growth and development of plant (Bajaj &Srinivasan, 1999).

Secondary metabolites are the bioactive chemicals. Many secondary metabolites are being identified, characterized and classified in various classes viz. tannins, phenolic compounds, steroids, flavonoids and fatty acids which have the potential of producing physiological action on the body. The distribution of the secondary metabolites varies with the species of plant (Rodney *et al.,* 2000).

Main groups of bioactive compounds in plants are categorized as:

- Phenolics
- Nitrogen containing compounds (alkaloid and terpenoids)

2.2.1 Phenolic compounds

Phytochemicals which are phenolic by nature are the largest category of phytochemicals. Phenolic compounds play an important role as defense compounds. Phenolic compounds are also confined to the antioxidant properties and plays important role as protecting agents against free radicals mediated disease. Flavonoids, tannins and phenolic acids are three most important groups of dietary phenolics(Reddy *et al.,* 2003).

2.2.1.1 Flavonoids

Flavonoids are low molecular weight polyphenolic secondary metabolites known forvariety of biological activities.More than 4,000 flavonoids have been recognized so far, many ofwhichoccurs in vegetables, fruits and fruit drinks. Theyprovide color and aroma to flowers and fruits (Abbas *et al.,* 2009).Isoflavons are nownasphytoestrogens. Flavonoids and proanthocyanidins are allpigments occurring in a long range of plant families. Isoflavones are produced by speciesof*Fabaeceae*(bean family) (Ayoola*et al.,* 2008).

Activity of Flavonoids

All compounds contain phenol-groups involved in an effect as general antioxidant.Flavonoids contain multiple biological properties including cytotoxicity, antimicrobial, anti-inflammatory and antitumor activities but the best property of flavonoids is to act asantioxidant which has the potential to protect human body from reactive oxygen (Saxena*et al.*,2013). Injuries Flavonoids can prevent injury caused by free radicals, by the direct scavenging of free radicals.Flavonoidsstabilize the reactive oxygen species by reacting with the reactive compound of the radical.Due to high reactivity of hydroxyl group of flavonoids, free radicals are made inactive. Flavonoids,radicals are made inactive.Flavonoids also possess anti-allergic, enzyme inhibition and antitumor activity(Nijveldt*et al.,* 2001).

2.2.1.2 Tannins

Tannins are commonly known as tannic acid and are water soluble phenoliccompounds having molecular weights between 500-3000Da. Tannins play a role in protection frompredation, as pesticides, and in plant growth regulation. On the basis of their structuralcharacteristics tannins are divided into four major groups i.e. ellagitannins,complextannins,Gallotanninsand condensed tannins (Srinivas*et al.*,2003).

Activity of Tannins

The tannin-containing plant extracts are used as astringents, against stomach tumorsagainstdiarrhea, as diuretics (Ahmad *et al.*,2001) and as anti-inflammatory, antiseptic,antioxidant(Reddy *et al.,* 2003). In food industrytannins are used to clarify wine, beer, and fruit juices. Tannins are considered as "health –promoting" components in plant derived foods. Tannins do not function solely as primary antioxidants; they donate hydrogen atom or electrons so they function as secondary antioxidants (Amarowicz*et al.*, 2007).

2.2.3 Nitrogen containing compounds

Nitrogen containing secondary metabolites isclassified in two categories as follows:

2.2.3.1 Alkaloids

Alkaloids are nitrogen containing natural chemical compounds.These chemical compounds are known for various potent pharmacological activities such as analgesics, anti-malarial and anti-

tumor activities(Saxena*et al.*,2013). The alkaloids have bitter taste and are heterocyclic nitrogen containing compounds. Depending upon their chemical properties alkaloids has diverse clinical properties. The compounds have anticholinergic activityand are used medically to reduce smoothmuscle spasms, hypersecretion and pain(Ramsewak*et al.* 1999).

2.2.3.2 Terpenoids

Terpenoids are the components of essential oil of the plants.Terpenoids present in this essential oil possess a broad spectrum of antimicrobial activities which is due to the high content of phenolic derivatives. Some essential oils are used to cure fungal infections and further exploration reveals a broad spectrum effect against other pathogenic manifestations (Ramsewak*et al.* 1999). Terpenes are the compounds contain additional elements, usually oxygen which are derived from five carbon isoprene units. They are having various medicinal properties such as anti- carcinogenic, antimicrobial and anti-malarial activities (Ramar*et al.*, 2010).

2.3 The classification, distribution, description and uses of plants used in this research:

2.3.1 Berberisaristata

Scientific classification (Sharma *et al.*, 2011).

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Ranunculales

Family : Berberidaceae

Genus : Berberis

Species : Aristata

Berberisaristata belongs to the family Berberidaceae and the genus Berberis. In India it is also known as Indian berberry or DaruHaidra. The species of this genus are found in the temperate and sub-tropical region of Asia Europe and America. In India *Berberisaristata are native* to the Himalayas(Sharma*et al.,* 2011).For the treatment of oxidative stress induced ailments, fruits of *Berberisaristata*are consumed by local people of north western Himalayan states of India from ancient time. Moreover plants of Genus Berberis are either singly used or in combination with other medicinal plants for treating a variety of ailments like jaundice, enlargement of spleen, leprosy and rheumatism(Srivastava*et al.,* 2013). The plants of Genus Aristata are being examined for their medicinal properties and pharmacological uses by various scientists.From long time *B.aristata* is used in ayurvedic medicines. The plant is used for the treatmentofinflammation, wound healing and skindisease(Mitra*et al.*,2013).It also has antiamoebic, antifungal, antihelminthic properties and some central nervous system activity as well. Bacteria related diarrhea, parasitic intestinal infections and ocular infections are the most prominent clinical uses of berberine. It has been reported that berberine exhibits local anesthetic, enzyme inhibitory, antipyretic and antiamnesic activities (Sharma *et al.,* 2011). Stem barks of *B.aristata* have astringent, febrifuge and stomachic effect and are used to treat piles, spleen and diarrhea, malaria, menorrhagia, diabetes (Ahamad*et al.,* 2012).

Gahlaut*et al.,* 2013 have observed the antibacterial potential of extracts of berberisaristata against six bacterial strains i.e. *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa*. The extracts were prepared in five different solvents according to increasing polarity. Soxhlet extraction method was used to prepare extracts. The percentage yield with the alcoholic solvent was observed to bemaximum.

Bhandari*et al.,* 2000 have evaluated the Antimicrobial activity of crude extracts from Berberisasiatica stem bark using their aqueous and methanolic extracts. The disc diffusion and agar dilution methods were used to check the antimicrobial activities. The methanolic extracts showed the better result than aqueous extracts. This proves the potential of Berberis as antimicrobial agent. The result proves its antibacterial activity.

Porwal*et al.,* 2010 have evaluated aqueous and alcoholic extracts of *Berberisaristata* for its antitumouractivity in Swiss albino mice. The antitumor activity was more potent with the

alcoholic extracts as compared to the aqueous extracts. Decrease in cancer cell number and increase in life span was observed in the mice after treatment with the *B. aristata* extracts thus confirming its anti-tumor activity.

Shamkuwar*et al.,* 2013, have investigated antispasmodic effect of aqueous extract of *Berberisaristata* stem (100, 200, 400 mg/kg) using charcoal meal test and castor oil induced intestinal secretions method for testing anti-motility and anti-secretory activity in mice. The results indicate that *B.aristata* produces antispasmodic effect through inhibiting the intestinal motility.

Dar *et al.,* 2013, have investigated the antioxidant property of the methanolic extract which were estimated by DPPH assay and nitric oxide scavenging method.The methanolic extract was able to reduce stable purple coloring 50% reduction with IC_{50} values as .90mg/ml and gallic acid 0.61mg/ml.The concentration of DPPH free radical with an efficacy near to that standard gallic acid.

2.3.1.1Chemical Constituents

The main constituent of *B.aristata* stem and root is Berberine which is a quanternaryisoquinolinealkaloid(Pasrija*et al.,* 2011).The most important property of berberine is itsantibiotic properties (Patel *et al.,* 2013). The berberine is being found to be responsible for hepatoprotective activity. The Alcoholic extracts of bark of *B.aristata*yielded berberine chloride, palmatine chloride and berberine(Sharma*et al.,* 2011).

*B.Aristata*have shown antimicrobial activity against many bacteria including *Salmonella typhii, Pseudomonas aeruginosa, Escherichia coli, and fungal strain Aspergillusniger*(Pasrija*et al.,* 2011). Study has been conducted to examine phytochemical, antioxidant, antimicrobial and anticancer activity of methanol extract of *B.aristita*. Plant extractshowed the presence of phytochemicals like alkaloid, saponin, terpenoids, coumarin, flavonoids, tannin,glycoside and steroid.Antioxidant activity was evaluated using DPPH assay IC50 shown by extract was 33.31µg/ml with L Ascorbic acid as standard showing IC50 9.6µg/ml.Antimicrobial screening

showed good positive result with *Candida albicans*, *Salmonella typhii*, *Pseudomonas aeruginosa* and *Escherichia coli*(Lamichhane*et al.,* 2014).

2.3.2 Ajugabracteosa(http://www.gbif.org/species/3887998)

Kingdom : Plantae

Phylum : Magnoliophyta

Class : Magnoliopsida

Order : Lamiales

Family : lamiaceae(Labiatae)

Genus : *Ajuga*

Species:*Ajugabracteosa*

Ajugabracteosa is also known as Neelkanthi,leelkounthe,ratpacho,lilkounthe, belongs to the family lamiaceae(Labiatae) and the genus *Ajuga*(Chauhan*et al.*,1999). Plants of this genus are greatly used in ayurvedic world. Many plants of this family have medicinal properties. In India *Ajugabracteosa*it is given in the treatment of fevers and neuro diseases (Vohra and Kaur, 2011).

It is anerect or ascending hairy herb, spathulate,obtuse leaves, with slight purple tinge from lower surface.Flowers are purplish-violet and grows up to 5-50cm tall (Chauhan*et al.*,1999). The distribution of *A.bracteosa* is in subtropical and tempered regions such as Bhutan, Pakistan,China, Afganistan and Malasia. (Jan *et al.*, 2014). It abounds in western Himalaya at an altitude of 1300m (Chandel and Bagai, 2010).

Leaves of *A.bracteosa*are being used in treatment of various disease lie gout, rheumantism and amenorrhea. For antimalarial properties, the ethanolic leaves extracts at various concentrations demonstrated chemosuppression during infections with significant repository activity (Chandel

and Bagai.,2010). The compound was found effective against a broad category of pathogenic microbes.

Mothana*et al.*,2012 investigated the antimicrobial and antioxidant activity and gas chromatography mass spectrometry (GC/MS) analysis of the essential oilsof *A.bracteosa*. The main constituent of the oil of *A.bracteosa* was aliphatic acids (30.3%), Borneol (20.8%) and hexadecanoic acid (16.0%).Along with antimicrobial activity it showed a great radical scavenging activity (78%) at 1 mg/ml comparing with the high antioxidant activity of ascorbic acid (96%).

2.3.2.1 Chemical constituents

Previous Studies have shown that the essential oil, as well as their active principles possess several pharmacological properties like anti-microbial, anti-tumor, anti-inflammatory, anti-plasmodial, anti-helmenthic, antipyretic, hepatoprotective, cytotoxic and insect-antifeedant ones (Chauhan*et al.,* 1991). A large variety of compounds have so far been isolated from *A.bracteosa*including essential oils, phenolic compounds, flavonoids and tannins. Major components present in essential oil of leaves are limonene, α-humulene, β-myrcene, elemol, camphene, β-caryophellene, α-phellendrene (Vohra and Kaur, 2011).*A.bracteosa*has important biological activities and acts against different types of diseases. The present literature study further reveals that the biological properties of the *A.bracteosa*are not only due to their essential oils and their main compounds such as limonene, α-humulene, β-myrcene, elemol, but also to the other polar constituents acting synergistically or possessing different biological activites (Jan *et al.,* 2014).

*Ajugabracteosa*have shown antimicrobial activity against many bacterial strains including *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*(Vohra and Kaur, 2011).Studies have been conducted to examine phytochemical, antioxidant, antimicrobial and anticancer activity of methanol extract of *A.bracteosa*. Leaf in water extract of *A.bracteosa*presented the highest activity in DPPH radical scavenging activity (Chao*et al.*,2012).The essential oil of *A.bracteosa*exhibit interesting potent antimicrobial activity and the results show that oil of*A.Bracteosa*present antioxidant activity and that might be useful for

therapeutic purposes to prevent ROS disorders and explain their use in the treatment of various inflammatory disorders (Mothana*et al.*,2012).

CHAPTER-3

SCOPE OF THE STUDY

Every day, our body got exposed to xenobiotics which are metabolized by our body and may lead to production of reactive oxygen species or free radicals. Free radicals induces damage due to peroxidation to biomembrane and also to DNA, which lead to tissue damage, thus become the cause of occurrence of diseases like cancer, heart disease and osteoporosis. Antioxidants neutral lise the effect of free radicals by, reducing their unwanted and uncontrolled production in our body and may prevent the body from various diseases. In addition, antioxidants have many industrial uses, such as preservatives in food and cosmetics industry.

Antioxidants may be synthetic or natural, synthetic antioxidants such as butylated hydroxytoluene(BHT) and butylated hydroxyanisole(BHA) have recently been reported to be having side effects for human health. The antioxidants isolated from the natural plant extracts are very useful for human health in term of avoiding the side effect of using synthetic antioxidants.

After understanding the present scenario of free radical which leads to cause many diseases, we need to discover new effective, non-toxic natural antioxidant products. That is the reason of current research, to look for the antioxidant properties of plant, that widens the futuristic scope for the development of possible alternatives of synthetic antioxidants, to cure various diseases.

The curative properties of medicinal plants are mainly due to the presence of various secondary metabolities such as alkaloids, Flavonoids, anthocyanin, phenols, saponins *etc*. The preliminary screening tests may be useful in the detection of the bioactive compounds and this may lead to the drug discovery and development.

CHAPTER-4

AIM AND OBJECTIVES

Aim

The aim of our study wasto evaluate the antioxidant activities of plant extracts derived from *Berberis aristata* and *Ajuga bracteosa*. We also aim to perform the phytochemical analysis to investigate presence of different components in extracted plant samples.

The main objectives of this study are:

1. Phytochemical analysis of plant extracts.
2. In-vitro investigation of the antioxidant properties of plant extracts.
3. Quantitative analysis of total phenolic andFlavonoid content.
4. HPLC for evaluation of berberine content in *Berberisaristata*.

CHAPTER-5

MATERIALS & METHODOLOGY

5.1 Materials

5.1.1 Instruments and Equipments

Table 1- Instruments and Equipments Used in Laboratory

Sr. No.	Materials	Company
1.	Water bath	Labtronics
2.	Microwave	IFB
3.	Micropipette	P' Fact A
4.	Micro tips	TARSON
5.	Refrigerator	LG
6.	Spectrophotometer	Systronic spectrophotometer
7.	Weighing balance	Adventurer
8.	Plastic wares	Poly lab
9.	Face mask	Smart care
10.	Glass wares	Borosil glass
11.	pH meter	Hanna
12.	Soxhlet	Borosilicate

Table 2: Media, reagents and Chemical Used

Sr. No.	Chemical Name	Company
1.	Methanol	LOBA chemie
2.	Ethanol	Changhsuyangyuan chemical
3.	Hexane	LOBA chemie
4.	Ethyl acetate	Central drug home
5.	Butanol	Lobachemie
6.	Ammonia	Lobachemie
7.	Ferric Chloride	Molychem
8.	Fehling Solution A	Lobachemie
9.	Fehling Solution B	Lobachemie
10.	Sodium Hydroxide	Lobachemie
11.	HCL	Lobachemie
12.	DMSO	HIMEDIA
13.	Lead acetate	CDH
14.	Glacial acetic acid	MOLY chem.
15.	Ferric chloride	TITAN
16.	Ascorbic acid	CDH
17.	Quercitine	HIMEDIA

18.	DPPH	HIMEDIA
19.	ABTS	HIMEDIA
20.	Sulpheuric acid	RANKEM

5.2 Collection of Plants Material:

The following plants were taken for evaluation

Plant Name	**Parts Collected**
Berberisaristata	Root
Ajugabracteosa	leaves

Location: The plant sample of *BerberisAristata* was collected from HHRC laboratory at Amritsar, while the leaves of *AjugaBracteosa* were collected from the botanical garden of Lovely Professional University.

5.3 Preparation of plants extracts

5.3.1 Drying and grinding of samples

The plant sample i.e. root of plant *Berberisaristata*washed thoroughly with tap water followed by washing with distilled water to remove the dust particles and were dried in shadow. After air drying, samples were dried in the oven at 30˚temperature for 1 hour till the water content got evaporated. The samples were then grinded by mechanical grinder to make fine powder (Gul*et al.*, 2012).

Figure 1: Root of *B.aristata* and its dried powder.

Figure 2: Leaves of *Ajugabracteosa* and its dried powder.

5.3.2 Soxhlet extraction (Abbas, *et al*., 2009)

The samples of dried root (250gm) were placed in glass thimble with whattman filter paper around it. The Soxhlet extraction was processed using three solvent i.e. Ethanol, Methanol (Polar), Ethyl acetate (intermediate polar), Hexane, Butanol. 250 ml of solvent was poured into the round bottom extraction flask and placed on the heating mantle on boiling temperature of solvent. After this, the thimble containing the sample was placed into the extraction chamber. Lastly, the condenser was placed on top of the extraction flask and all these parts were fixed vertically. The operation was run for 6 -8 hrs with continuous supply of water to go through the apparatus. The extract was collected from the round bottom flask and stored into an air tight sterile glass jar at 4°C temperature for further use.

Figure 3: Soxhlet apparatus

5.4 Preliminary Phytochemical Qualitative Analysis

5.4.1 Test for Alkaloids

Concentration of 2 ml extract was taken and few drops of Wagner's reagent(dissolve Iodine(1.27gm) and potassium iodide(2gm) in 5ml DW and then make up the volume up to 100ml) were added to the extract. Formation of reddish brown precipitates considered as positive result for Alkaloids (Joshi *et al.,* 2011).

5.4.2 Test for Anthocyanin

Concentration of 2 ml plant extract was added to 2 ml HCl and ammonia. The appearance of pin red turns blue violet indicated the presence of Anthocyanins (Savithramma*et al.,* 2011).

5.4.3 Test for Cardiac Glycosides (Killer Killanis Test)

To 2 ml of extract was added 2 ml glacial acetic acid containing one drop of 5 % w/v ferric chloride solution. Further, 1 ml of was added by the walls of test tube. Formation of a brown colored ring at the interface confirmed the presence of cardiac glycosides (Ayoola*et al.,* 2008).

5.4.4 Test for Coumarins

To 3ml of 10% w/v NaOH was added to 2 ml plant's extracts. Formation of yellow color indicated the presence of Coumarins (Savithramma*et al.,* 2011).

5.4.5 Test for Flavonoids

Take 2ml extract was added to 10% w/v NaOH till the color turned to bright yellow. Hydrochloric acid was added drop wise and observed for the color change. Change of color from yellow to colorless confirmed the presence of Flavonoids (Lamichhane *et al.,* 2014).

5.5.6 Test for Glycosides (Fehling' Test)

In 2 ml extract was added NaOH and then HCl to neutralize till the yellow color turned transparent. Then drop wise added the Fehling solution 1 followed by Fehling solution 2. Test tubes were left still for 2-3 min. appearance of reddish brown precipitates was considered as positive result for Glycosides (Lamichhane *et al*., 2012).

5.5.7 Saponin(Frothing test)

Take 2ml extract was mixed with 2 ml distilled water and was shaken vigorously on Vortex Mixer. Formation of foam indicated the presence of Saponin (Savithramma*et al.,* 2011).

5.5.8 Test for Steroids

Take 1ml of extract was taken in a test tube and dissolved in 10ml by sides of the test tube. The formation of distinct layers with upper layer containing red color and sulpheuric acid layer of yellow color with green fluorescence indicated the presence of steroids in the samples (Savithramma*et al.,* 2011).

5.5.9 Test for Tannin and Phenols (Lead Acetate Test)

Few drops of 1% w/v Lead Acetate was added to 2ml plant extract. Formation of Cream yellowish colored precipitates was considered as positive result for Tannins (Savithramma*et al.,* 2011)

5.5.10 Test for Terpenoids

Concentration of 2 ml extract was added to 2 ml of glacial acetic anhydride and conc. H_2SO_4. Formation of blue green rings indicated the presence of terpenoids. (Savithramma*et al.,* 2011)

5.6 Anti-oxidant activity

For evaluation of antioxidant activity of all plant extracts different methods followed were as:

5.6.1 DPPH assay(Soni*etal.*,2009)

The ability of the plant extracts to scavenge the stable fre radical DPPH was assayed by the method of Mensor*et al*., 2001

Reagents

1. DPPH (0.1mM in Methanol)
2. Methanol

The antioxidant activity of all plant extracts was measured with 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) spectrophotometrically at 518 nm. The stable DPPH was used for determination of free-radical-scavenging activity of the extracts. The 0.1mM solution of DPPH in methanol (11.1mg in 500ml) was freshly prepared. Different concentrations of extracts(10,20,30,40,50 µg/ml) were prepared by serially diluting stock solution(5mg extract in 100ml methanol) methanolic solution of DPPH is added to make the final volume 2ml.After 30min at room temperature, the absorbance was recorded.IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. Radical scavenging activity was calculated by following formula.

%Radical scavenging Activity=$A_{control} – A_{sample} / A_{control}$*100
Where, $A_{control}$=Absorbance of control
A_{sample}= Absorbance of sample

5.6.2ABTS RADICAL SCAVENGING ASSAY (Re *et al*., 1999)

This assay was based on the ability of different substances to scavenge 2,2'- azino-bis(ethylbenzthiazoline-6-sulfonic acid) ABTS in comparison to a standard (ascorbic acid, at 10mg/ml). By oxidation of ABTS with potassium persulfate, monocation of ABTS is generated and level of monocation is reduced in the presence of antioxidants. The radical cation was prepared by mixing a 7 mM ABTS stock solution with 3 mM potassium persulfate (1/1, v/v) and placed the mixture for 16 h in dark at room temperature until the reaction was completed and the absorbance was stable. The ABTS solution was diluted with 50% methanol to an absorbance of 0.900 ± 0.02 at 734 nm for measurements. The photometric

assay was conducted on 2ml of ABTS solution and various concentration of test samples (100µg to 300 µg/ml) were mixed, absorbance of respective samples were taken at 734 nm after 1 min.

5.7 TOTAL PHENOLIC AND FLAVONOID ASSAY

5.7.1 Determination of Total phenolic Content (Kaur *et al.*,2014)

Folin-Ciocalteu method was used to estimate the total phenolic content with catechol as standard. Plant extract (10mg/ml) was diluted to get the dilutions i.e. 10,20,30,40µlwith distilled water and 0.5ml of Folin-Ciocalteu reagent was added.2ml of 20% sodium carbonate was added after 3min to the same solution and the contents were mixed thoroughly and incubated at room temperature for 30min.Absorbance was measured at 650nm in spectrophotometer after the color developed. The total phenolic content was expressed as gallic acid equivalents(GAE)mg/g.The standard curve was obtained by taking gallic acid ranging from 10 to 50 µg/ml as standard.

5.7.2 Total flavonoid assay

The aluminum chloride method was used for the determination of the total flavonoid content of the plant extracts.0.5ml (10mg/ml) of extract solutions were taken and then 0.1ml of $AlCl_3$ (10%),0.1ml potassium acetate and 2.8ml of distilled water were added sequentially.The test solution was vigorously shaken and incubated for 30 minutes. Absorbance at 415 nm waso bserved. A standard calibration plot was generated at 415nm using known concentrations of quercetin. The concentration of flavonoid in the test samples were calculated from the calibration plot.

CHAPTER-6

RESULTS AND DISCUSSION

The present work was undertaken to explore medicinal value of two different plants i.e. *B.aristata* and *A.bracteosa* belonging to different families Berberidaceae and lamiaceae.

6.1 Yeild of plant extracts

The weight of dried plant extracts was measured in grams. Different amount of extracts was obtained in different solvents. For B*erberis aristata* Highest percentage yield was obtained in methanolic extract is 16.52%, the minimum percentage yield was obtained in hexane extract is 2.1%. Earlier research work done on *B.aristata* by Upwar *et al*.,2011 has given the percentage yield of methanolic extract 4.2% w/w. Chhillar *et al*.,2013 has also mentioned the percentage yield of methanolic and water extracts of plant isolated by Soxhlet's method was 4.77% and 5.76%.

While For *Ajuga bracteosa* highest percentage of yield was calculated in Methanolic extracts that is 11.79% the minimum percentage yield was obtained in hexane extract 2.24%. Mothana *et al*., 2008 has mentioned yield of methanolic and hot aqueous extracts of *A.bracteosa* as 10.94% and 8.05%. Difference in yield obtained in previous and current study may be due to variation in the methodology of extraction.

Table 4: Percentage yield of plant extracts in different solvent.

Plant	Solvent	Dry weight taken(g)	Solvent volume(ml)	Final extract weight(g)	Percentage yield (%)
B. aristata (Root)	Methanol	150gm	300ml	24.78	16.52
	Ethanol	150gm	300ml	16.44	10.96
	Hexane	150gm	300ml	3.15	2.1
	Ethyl acetate	150gm	300ml	13.46	8.97
	Butanol	150gm	300ml	18.24	12.16

	Water	200gm	300ml	21.63	10.82
A.bracteosa (Leaves)	Methanol	150gm	300ml	17.68	11.79
	Ethanol	150gm	300ml	14.28	9.52
	Hexane	150gm	300ml	3.37	2.24
	Ethyl acetate	150gm	300ml	10.75	7.16

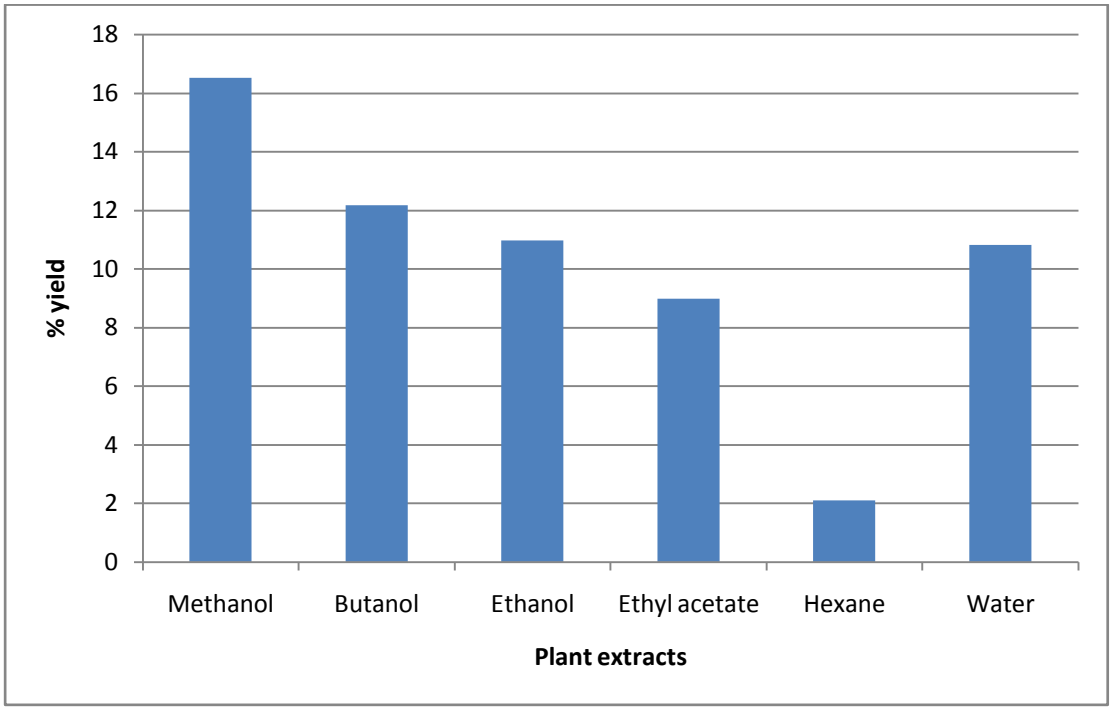

Figure 1: Yield of different extracts of *B.aristata.*

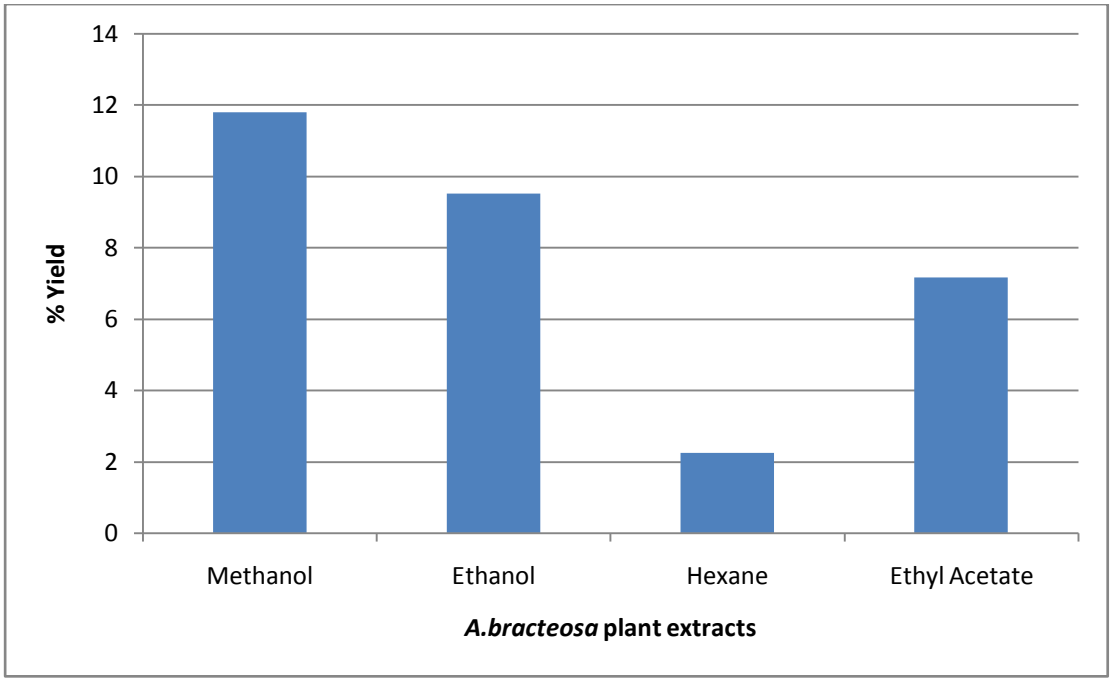

Figure 2: Yield of different extracts of *A.bracteosa.*

6.2 Phytochemical Analysis

Plant extracts 5mg/ml were taken for phytochemical analysis

6.2.1 Test for Alkaloids

Formation of red colored precipitates was considered as positive result. The positive results were observed in all the extracts (Methanol, Ethanol, Hexane, Aqueous, Butanol) of *B.aristata* except in aqueous extract.

6.2.2 Test for Tannins

Formation of white precipitates was considered as positive result. The positive results were observed in *B.aristata*(Aqueous, Methanol, Ethanol, Ethyl acetate), whereas the test was negative for Butanol and Hexane extracts.

6.2.3 Test for Terpenoids

The Salkowski Test was performed for estimation of Terpenoids in plant extracts. Formation of brown colored ring at the interface was considered as positive result. The positive results were observed in all the plant extracts.

6.2.4 Test for Steroids

Formation of transparent layer with dark brown ring confirmed the presence of steroids. The positive results were observed in all the extracts of B. aristata.

6.2.5 Test for Coumarins

The appearance of yellow color indicates the presence of Coumarins. The positive results were observed in Aqueous, Methanol, Ethanol, Ethyl acetate extracts of plant *B.aristata*, while the Negative results was observed in Hexane and Butanol extracts.

6.2.6 Test for Flavonoids

Change of color from yellow to colorless indicated the presence of Flavonoids. The positive results were observed in Methanol,Hexane, Ethyl acetate,Butanol, and Negative result was observed in Aqueous and Ethanolic extract of plant.

6.2.8 Test for Saponin

Formation of froth kind of bubbles after vigorous shaking was considered as positive result. The positive results were observed in only Aqueous and ethanolic extract whereas for other solvents the result was negative.

6.2.8 Test for reducing sugar

Formation of brick red precipitates after heating was considered as positive result. The positive results were observed in Aqueous, Hexane and ethanolic extract whereas for other solvents the result was negative.

Table 5: Phytochemical profile of *B.aristata*

Phytochemicals	Aqueous	Methanol	Hexane	Ethly acetate	Butanol	Ethanol
Alkaloids	-	++	+	++	+	++
Reducing sugar	+	_	++	_	_	+
Coumarins	++	++	-	+	_	+
Flavonoids	_	+	++	++	++	_
Saponins	++	_	_	_	_	_
Terpenoids	+	++	+	++	++	++
Tanins	++	+	_	++	_	+

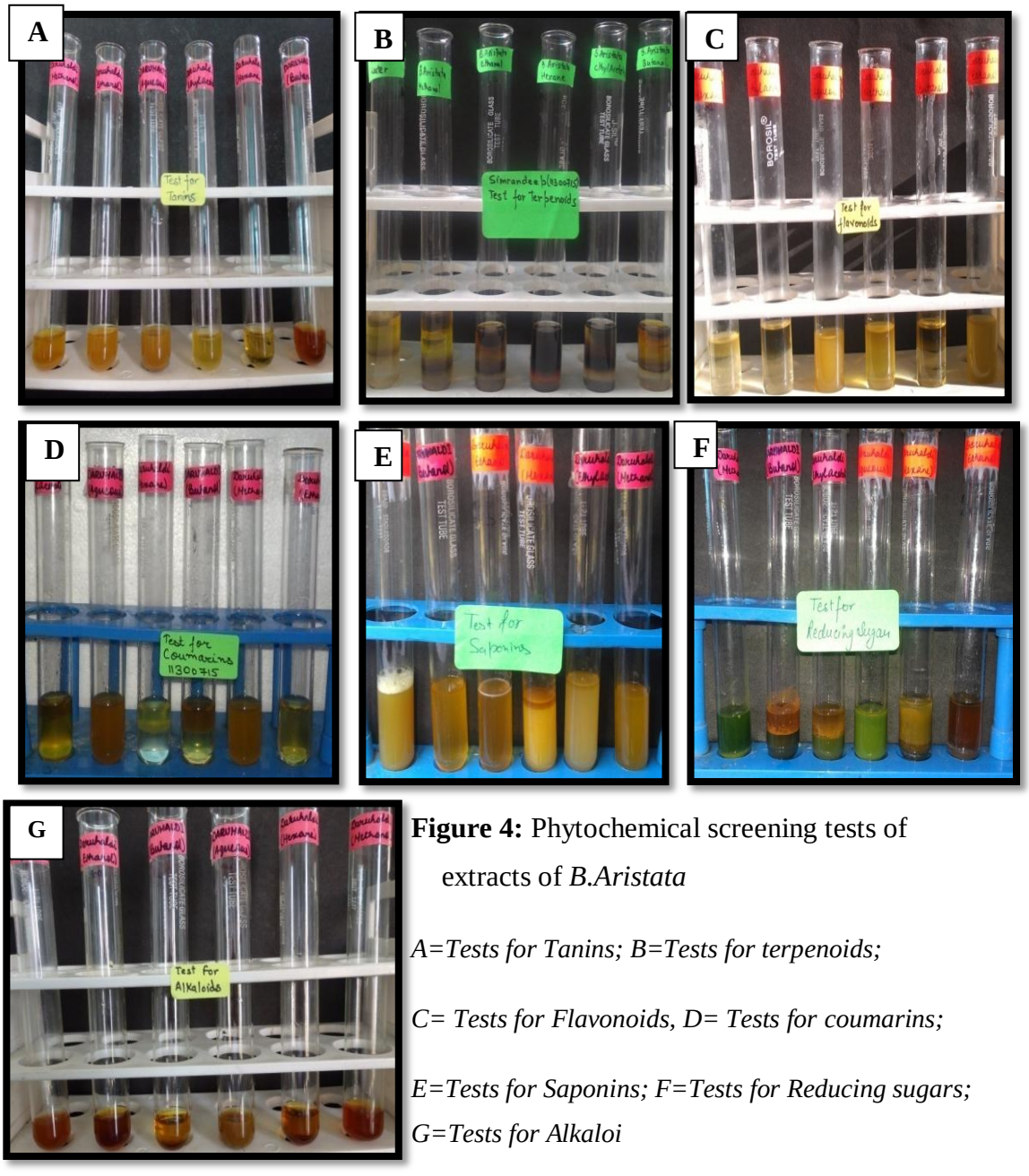

Figure 4: Phytochemical screening tests of extracts of *B.Aristata*

A=Tests for Tanins; B=Tests for terpenoids;

C= Tests for Flavonoids, D= Tests for coumarins;

E=Tests for Saponins; F=Tests for Reducing sugars; G=Tests for Alkaloi

Table 6: Phytochemical profile of *A.bracteosa*

Phytochemicals	Methanol	Ethanol	Hexane	Ethyl acetate
Alkaloids	+	+	++	+
Coumarins	++	+	+	+
Flavonoids	++	+	++	++
Saponins	++	++	-	-
Terpenoids	++	++	+	++
Tanins or phenols	+	++	+	++
Anthocyanin	++	++	++	++

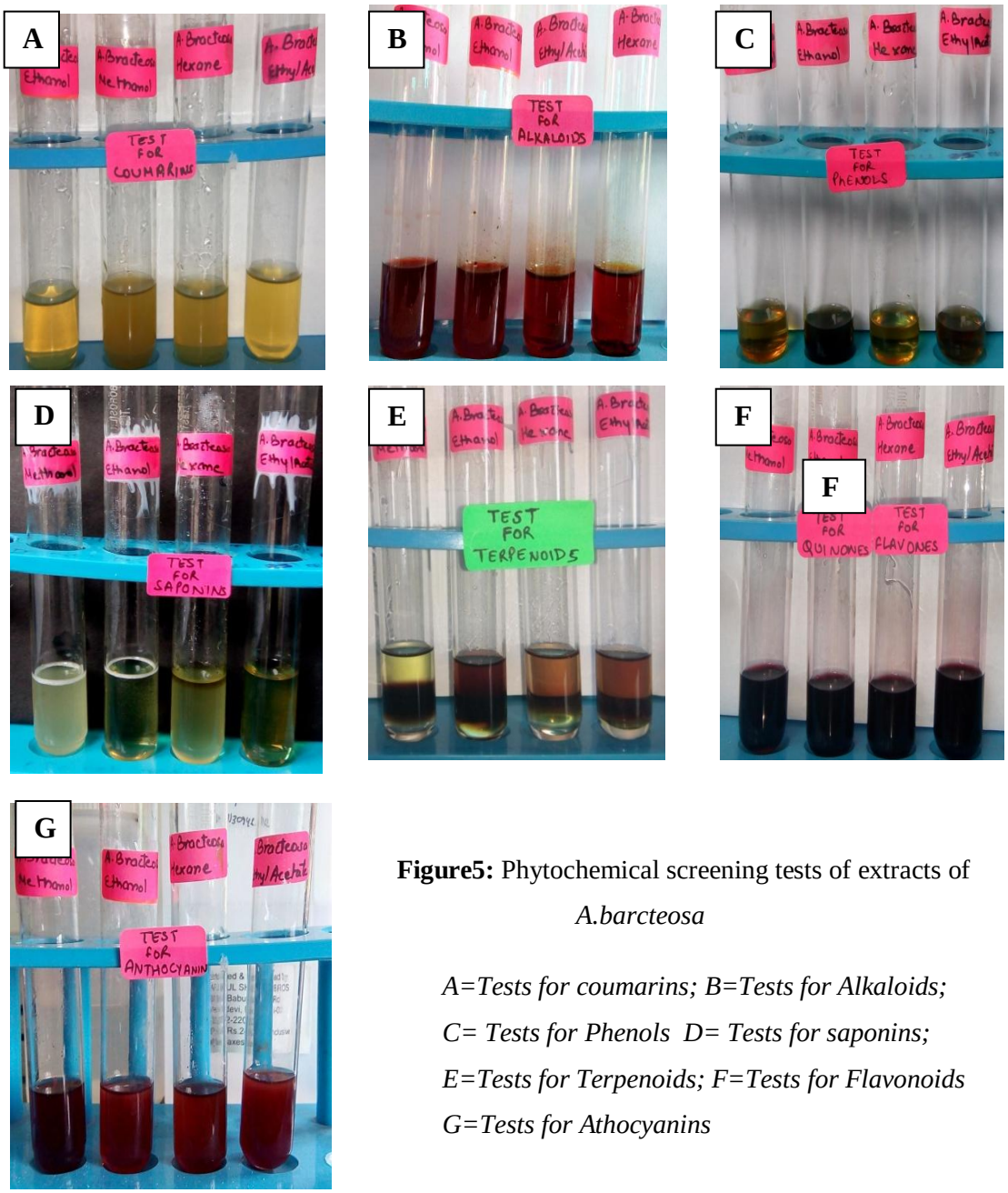

Figure5: Phytochemical screening tests of extracts of *A.barcteosa*

A=Tests for coumarins; B=Tests for Alkaloids;
C= Tests for Phenols D= Tests for saponins;
E=Tests for Terpenoids; F=Tests for Flavonoids
G=Tests for Athocyanins

The curative properties of medicinal plants are mainly due to the presence of various secondary metabolities such as alkaloids, Flavonoids, anthocyanin, phenols, saponins *etc*. The preliminary Phytochemical tests revealed the presences of alkaloids, Flavonoids, phenols, saponins, tannins, coumarins, anthocyanin in both the plants in different extracts.Thus, the preliminary screening tests may be useful in the detection of the bioactive compounds and this may lead to the drug discovery and development. Previous research work on these plant *B.aristata* has shown the presence of alkaloids and tannins in aqueous and alcoholic extracts by Rajan *et al*.,2012. Study by Lamichhane *et al*., 2014 on *B.aristata* showed the presence of alkaloids, saponin, terpenoids, coumarin, Flavonoids, tannin and steroids.

6.3 Antioxidant activity

To obtain information about the antioxidant potential of *A.bracteosa* and *B.aristata* effects of the extracts, two in-vitro tests, the DPPH radical scavenging and ABTS assay were used to assess the antioxidant properties of plant extracts of *A.bracteosa* and *B.aristata*. The results obtained from both the assays provide better assessment of antioxidant properties, which revealed that inhibitory activity was concentration dependent.

6.3.1 DPPH scavenging method:

The percentage of scavenging effect on DPPH free radical significantly increased with the increase in concentration of methanolic, ethanolic, ethyl acetate and hexane extractsof *B.aristata* from 10µg/ml to 50µg/ml (Figure 6,7,8 & 9) (Table 7, 8,9& 10).The inhibition was varying from 23% to 87% for methanolic extract, 31% to 85% for ethanolic extract, 38% to 70% for hexane extract, 21% to 61% for ethyl acetate extract.The IC_{50} value of methanolic,ethanolic, hexane and ethyl acetate extracts of *B.aristata* was calculated to be 20, 17, 36, 28 µg/ul respectively (Figure 10 and Table 11). Multiple comparison of methanolic, ethanolic, ethyl acetate and hexane were done and significant difference was observed among all extracts.

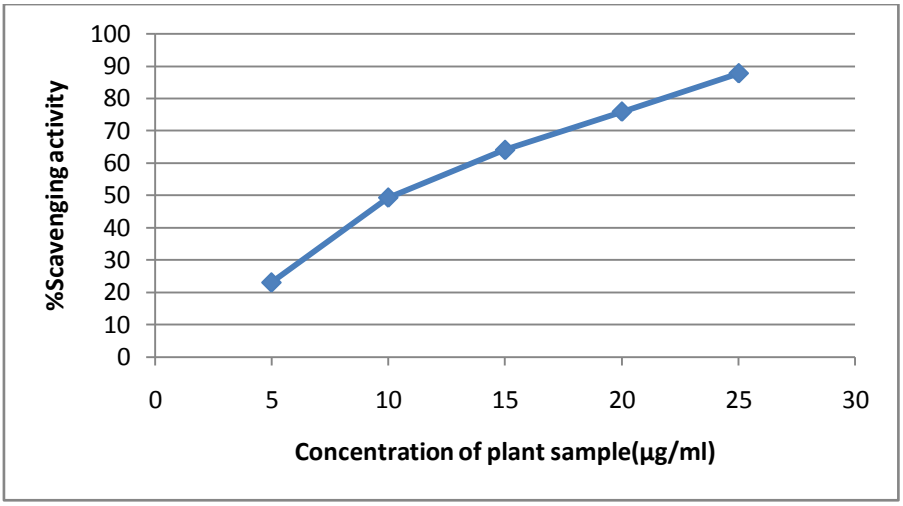

Figure 6: DPPH radical scavenging activity of methanolic extract of *B.aristata* (mean±sd, n=3).

Table 7: DPPH radical scavenging activity of methanolic extract of *B.aristata* (mean±sd, n=3).

Concentration of plant extract(ug/ml)	%Scavenging activity
10	23.06 ± 0.30^{a}
20	49.28 ± 0.29^{b}
30	64.01 ± 0.46^{c}
40	75.85 ± 0.35^{d}
45	87.69 ± 0.48^{e}

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at $P<0.05$ by Tukey's test.

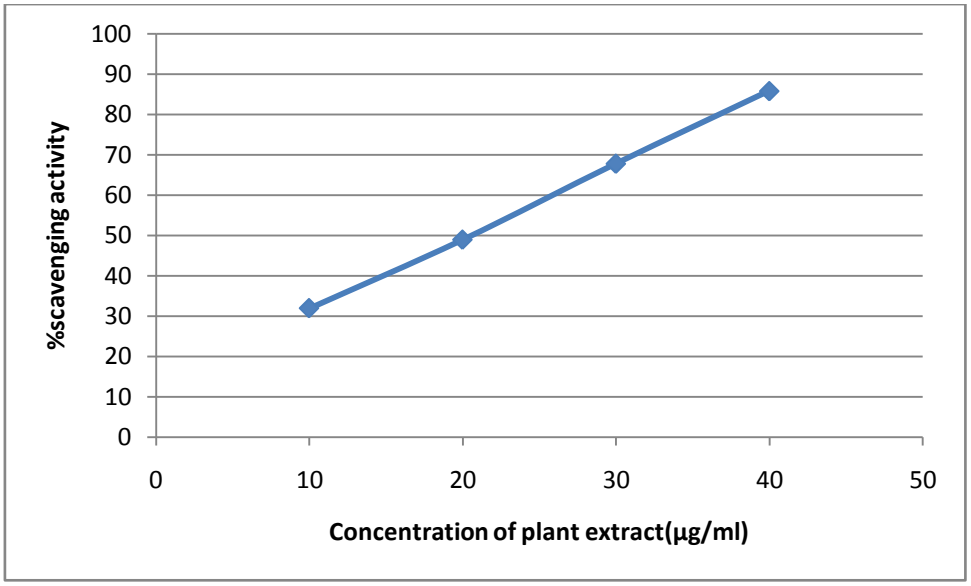

Figure 7: DPPH radical scavenging activity of Ethanolic extract of *B.aristata*(mean±sd, n=3).

Table 8: DPPH radical scavenging activity of Ethanolic extract of *B.aristata*(mean±sd, n=3).

Concentration of plant extract(µg/ml)	% Scavenging activity
10	31.88 ± 0.39^{a}
20	48.90 ± 0.47^{b}
30	67.77 ± 0.26^{c}
40	85.81 ± 0.45^{d}

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at $P<0.05$ by Tukey's test.

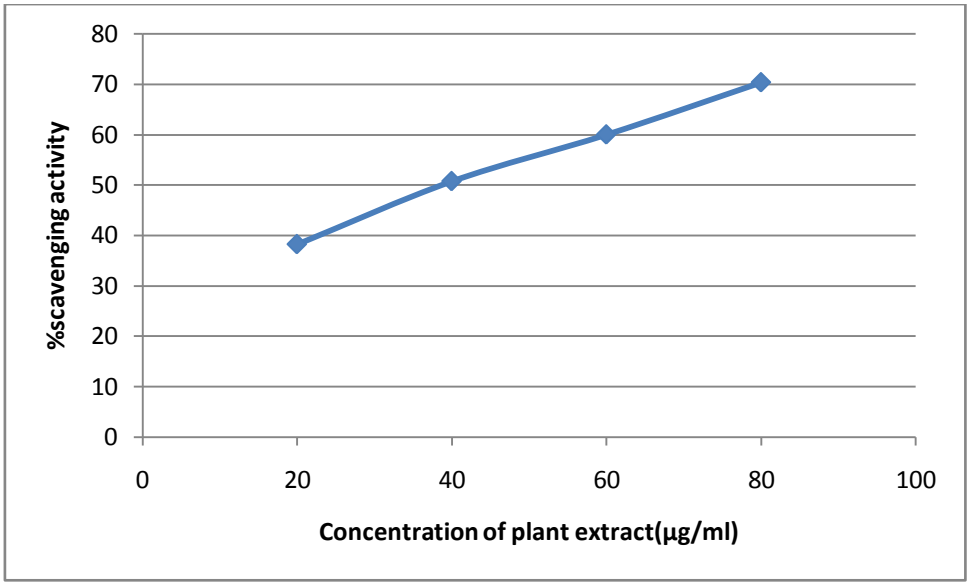

Figure 8: DPPH radical scavenging activity of Hexane extract of *B.aristata*(mean±sd, n=3).

Table 9: DPPH radical scavenging activity of Hexane extract of *B.aristata*(mean±sd, n=3).

Concentration of plant extract(µg/ml)	% Scavenging activity
20	38.25 ± 0.43^{a}
40	50.74 ± 0.33^{b}
60	60 ± 0.57^{c}
80	70.34 ± 0.16^{d}

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at $P<0.05$ by Tukey's test.

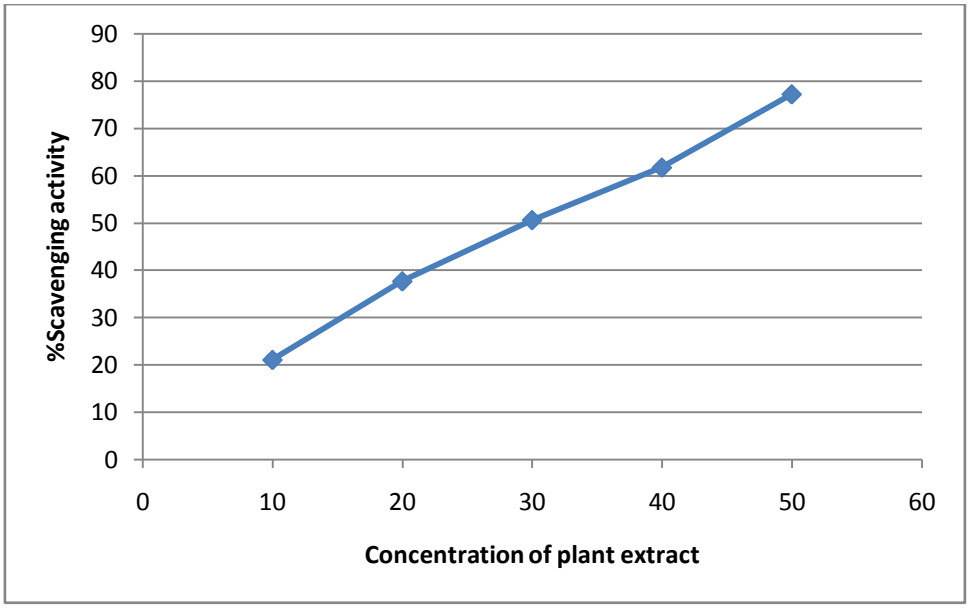

Figure 9: DPPH radical scavenging activity of ethyl acetate extract of *B.aristata*(mean±sd, n=3).

Table 10: DPPH radical scavenging activity of ethyl acetate extract of *B.aristata*(mean±sd, n=3).

Concentration of plant extract(µg/ml)	% Scavenging activity
10	21.07 ± 0.41^{a}
20	$37.80\ \pm0.72^{b}$
30	$50.64\pm.48^{c}$
40	61.34 ± 0.52^{d}

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

Table 11: IC_{50} value of various extracts of *B.aristata* (mean±sd, n=3).

Plant extract of *B.aristata*	IC_{50} (µg/ml)
Methanolic	$20\ \pm0.17^{a}$
Ethanolic	17.41 ± 0.06^{b}
Hexane	36.22 ± 0.60^{c}
ethyl acetate	$28.67\ \pm.18^{d}$

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

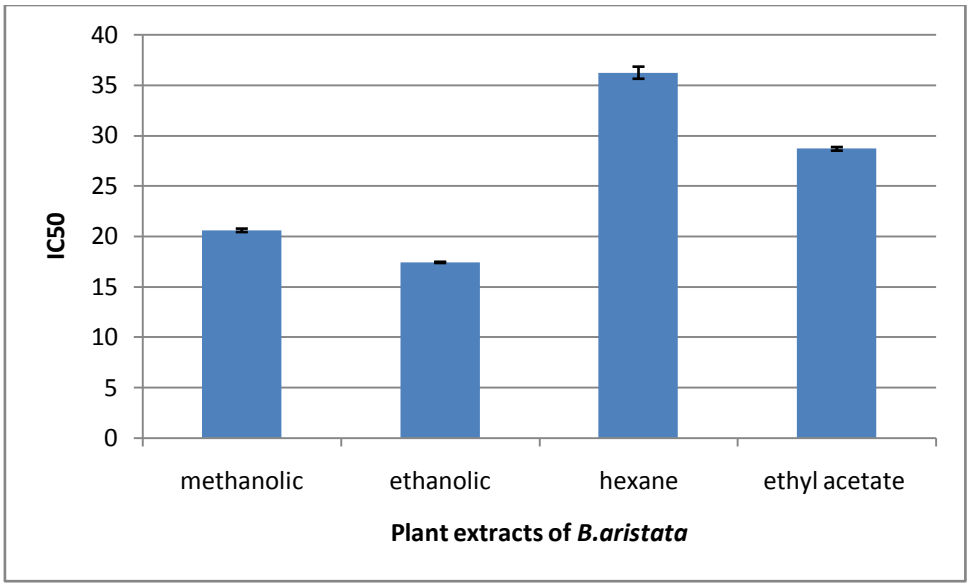

Figure 10: IC50 value of methanolic, ethanolic, hexane and ethyl acetate extracts of *B.aristata* (mean±sd, n=3).

6.3.2 DPPH assay for *A.bracteosa*

The percentage of scavenging effect on DPPH free radical increased with the increase in the concentration of methanolic, ethanolic, ethyl acetate and hexane extracts of *A.bracteosa* .1mg/ml to .5mg/ml (Figure 11,12,13 & 14) (Table 12, 13,14& 15). The inhibition was varying from 31% to 76% for methanolic extract, 39% to 77% for ethanolic extract, 24% to 86% for hexane extract, 30% to 76% for ethyl acetate extract. The IC_{50} value of methanolic, ethanolic, hexane and ethyl acetate extracts of *A.bracteosa* was calculated .19, .17, .24, .23 to be respectively(figure 15and table 16). Multiple comparison of methanolic, ethanolic, ethyl acetate and hexane were done and significant difference was observed among all extracts.

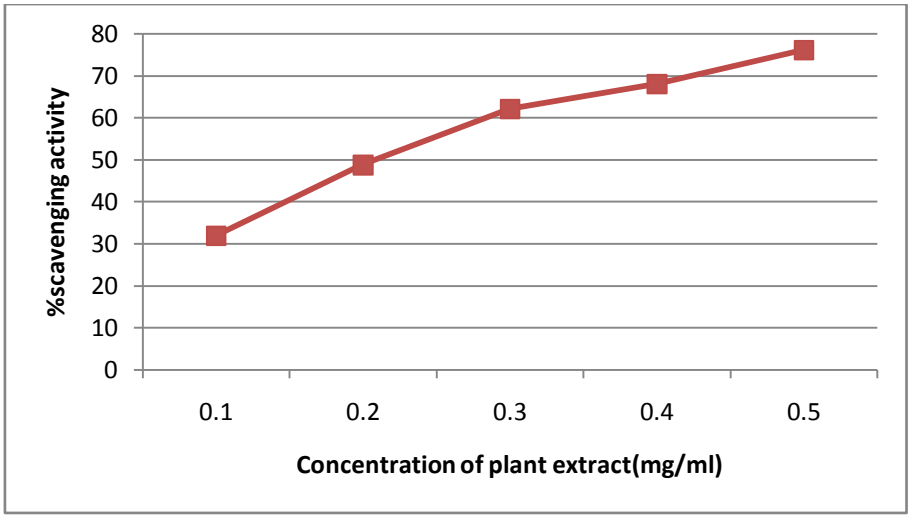

Figure 11: DPPH radical scavenging activity of methanolic extract of *A.bracteosa* (mean±sd, n=3).

Table 12: DPPH radical scavenging activity of methanolic extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract	%Scavenging activity
.1	$31.94 \pm .41^{a}$
.2	$48.76 \pm .54^{b}$
.3	$62.11 \pm .65^{c}$
.4	$67.97 \pm .46^{d}$
.45	$76.08 \pm .63^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

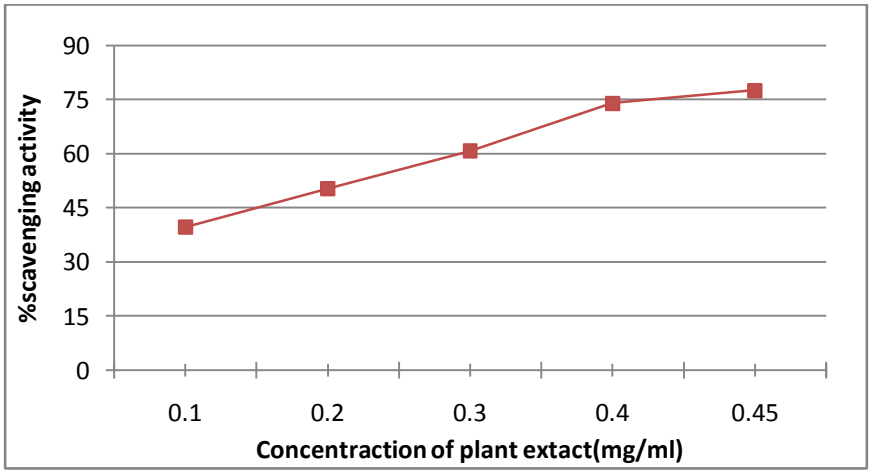

Figure 12: DPPH radical scavenging activity of Ethanolic extract of *A.bracteosa* (mean±sd, n=3).

Table 13: DPPH radical scavenging activity of Ethanolic extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract(mg/ml)	%Scavenging activity
0.1	$39.68 \pm .17^{a}$
0.2	$50.34 \pm .40^{b}$
0.3	$60.78 \pm .17^{c}$
0.4	$73.95 \pm .35^{d}$
0.45	$77.51 \pm .20^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

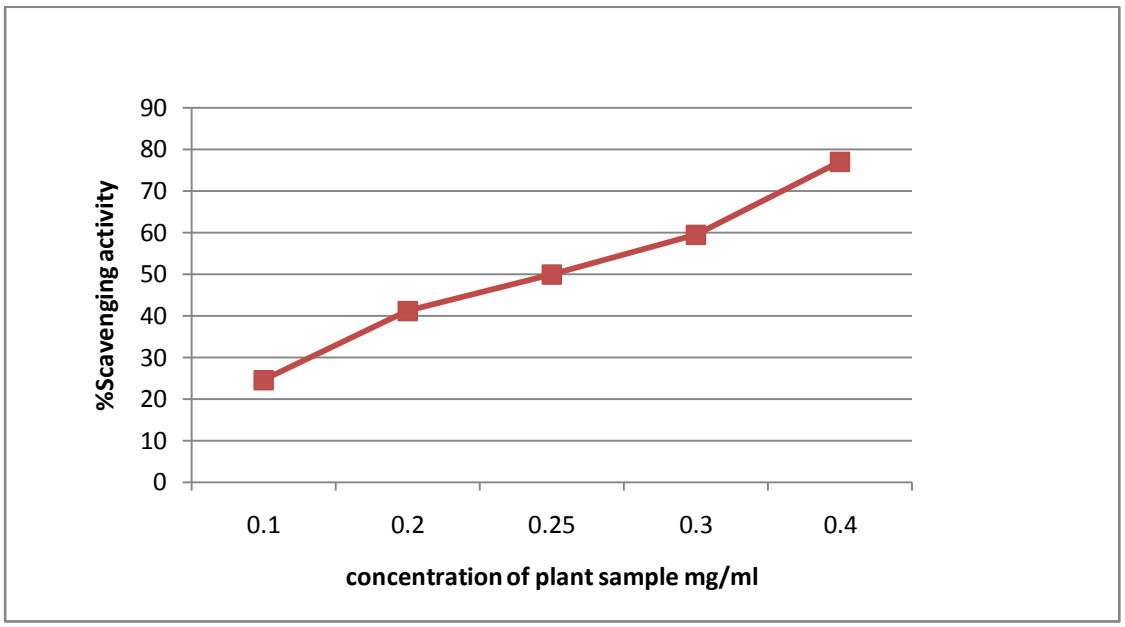

Figure 13: DPPH radical scavenging activity of Hexane extract of *A.bracteosa* (mean±sd, n=3).

Table 14: DPPH radical scavenging activity of Hexane extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant sample(mg/ml)	%Scavenging activity
0.1	$24.63 \pm .16^{a}$
0.2	$42.28 \pm .22^{b}$
0.25	$50.01 \pm .16^{c}$
0.3	$59.49 \pm .21^{d}$
0.4	$77.05 \pm .16^{e}$

0.45	$86.74 \pm .18^{f}$

Values within the column not sharing common superscript letters (a-f) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

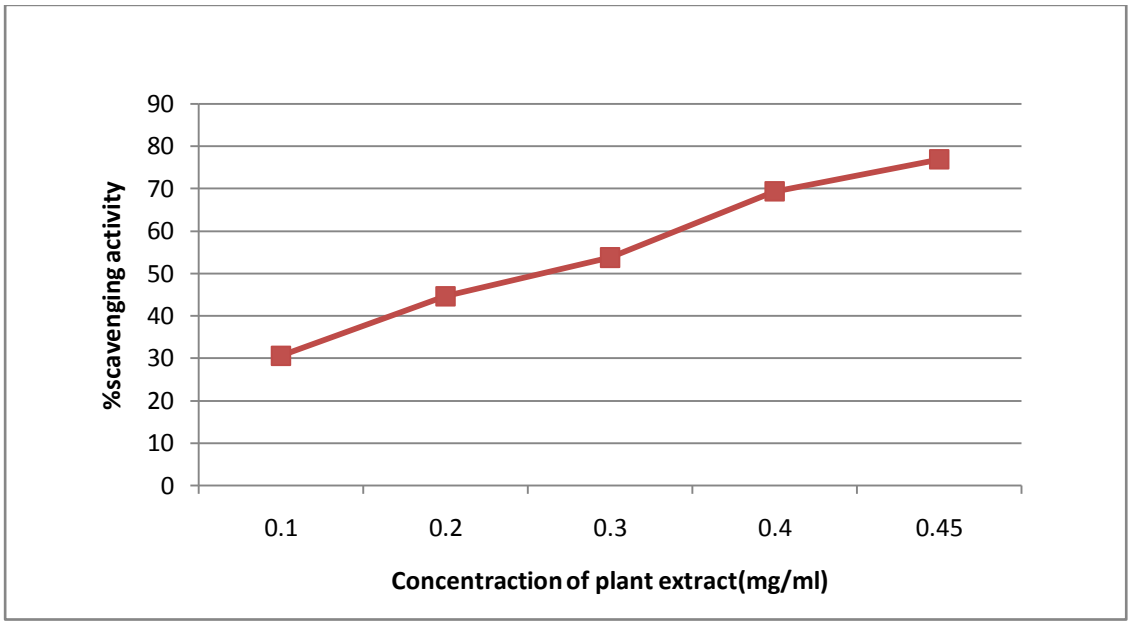

Figure 14: DPPH radical scavenging activity of ethyl acetate extract of *A.bracteosa* (mean±sd, n=3).

Table 15: DPPH radical scavenging activity of ethyl acetate extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract(mg/ml)	%Scavenging activity
0.1	$30.67 \pm .35^{a}$
0.2	$44.67 \pm .25^{b}$
0.3	$53.80 \pm .57^{c}$
0.4	$69.43 \pm .45^{d}$
0.45	$76.94 \pm .19^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

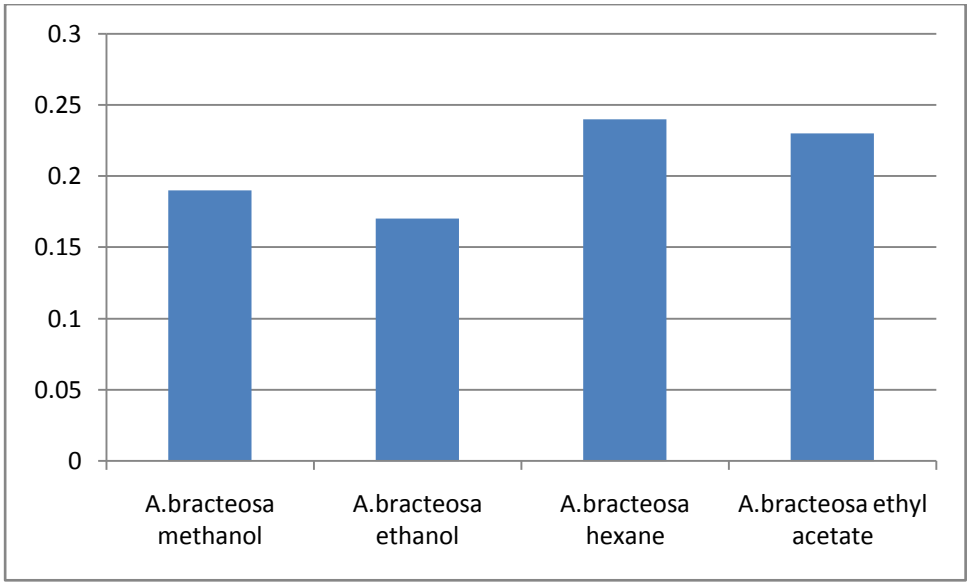

Figure 15: IC_{50} value of methanolic, ethanolic, hexane and ethyl acetate extracts of *A.bracteosa* (mean±sd, n=3).

Table 16: IC_{50}value of various extracts of *A.bracteosa* (mean±sd, n=3).

***A.bracteosa* extracts**	**IC_{50} (mg/ml)**
Methanol	0.19 ± 0.003^{a}
Ethanol	0.17 ± 0.0007^{b}
Hexane	0.24 ± 0.0004^{c}
ethyl acetate	0.23 ± 0.001^{d}

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

6.3.4 ABTS Scavenging activity for *B.aristata*

The percentage of scavenging effect on ABTS radical was increased with the increase in the concentration of methanolic, ethanolic , ethyl acetate and hexane extracts of *A.bracteosa* was taken between (10 to 50 µg/µl)(Figure 5,6 & 7) (Table 8, 10 & 12).The % inhibition was varying from 28.41% at 10µg/µl to 88.53% at 50µg/µl for methanolic extract, 33.46% at 10µg/µl to 88.20% at 50µg/µl for ethanolic extract, 22.65% at 10µg/µl to 63.43% 40µg/µl for ethyl acetate extract, 18.24% at 10µg/µl to 73.94% at 50µg/µl for hexane extract of *B.aristata*.The ic50 value of methanolic, ethanolic, ethyl acetate and hexane extracts of *B.aristata* was calculated to be 18.8,

16.64, 19.25,30.09 µg/µl respectively(figure & table).Significant difference was observed in the IC_{50} values of all the four extracts of *B.aristata*(Table)

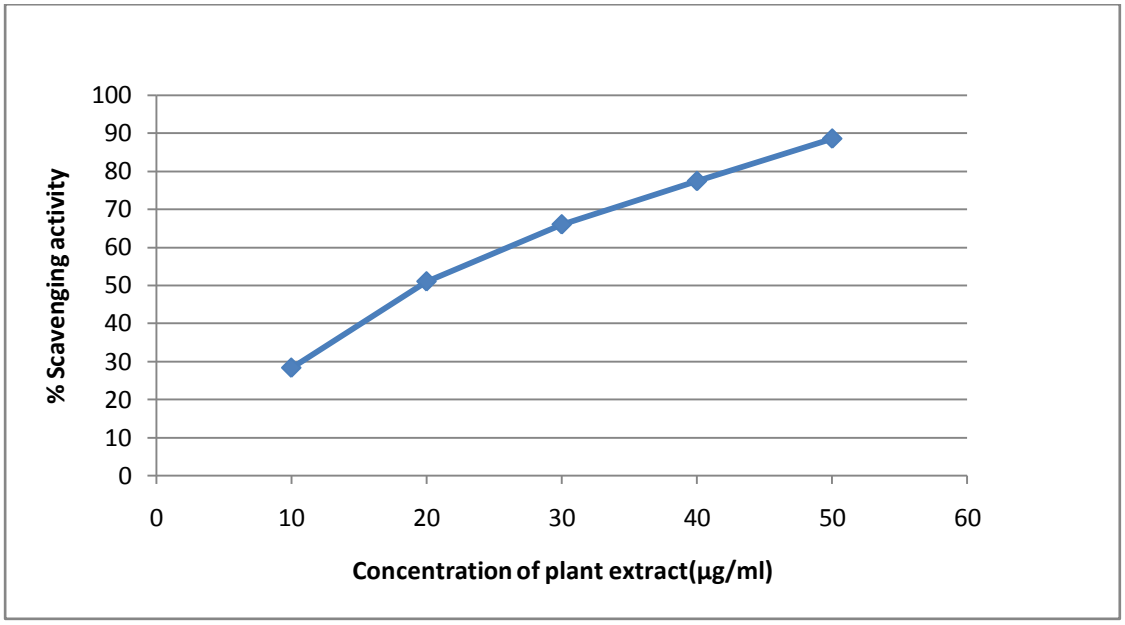

Figure 17: ABTS radical scavenging activity of methanolic extract of *B.aristata*(mean±sd, n=3).

Table 18: ABTS radical scavenging activity of methanolic extract of *B.aristata*(mean±sd, n=3).

Concentration of B.aristata	%Scavenging activity
10	28.41±.68[a]
20	50.97±.73[b]
30	65.97±.45[c]
40	77.35±.51[d]
50	88.53±.50[e]

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

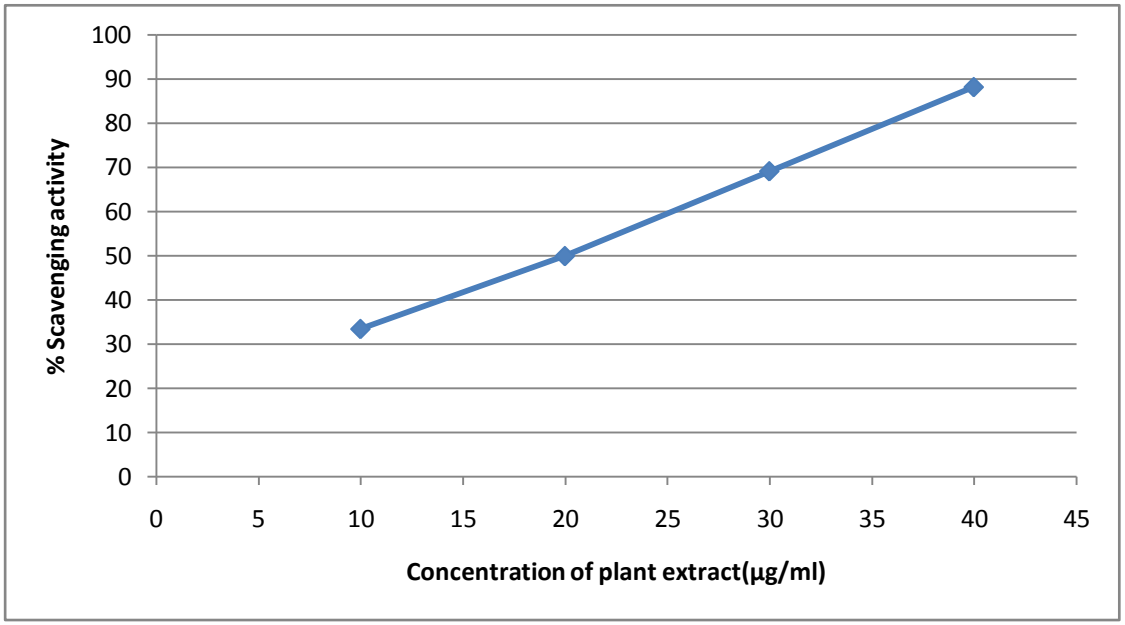

Figure 18: ABTS radical scavenging activity of Ethanolic extract of *B.aristata*(mean±sd, n=3).

Table 19: ABTS radical scavenging activity of Ethanolic extract of *B.aristata*(mean±sd, n=3).

Concentration of *B.aristata*	**%Scavenging activity**
10	33.46±.35[a]
20	49.96±.58[b]
30	69.16±.82[c]
40	88.20±.46[d]

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

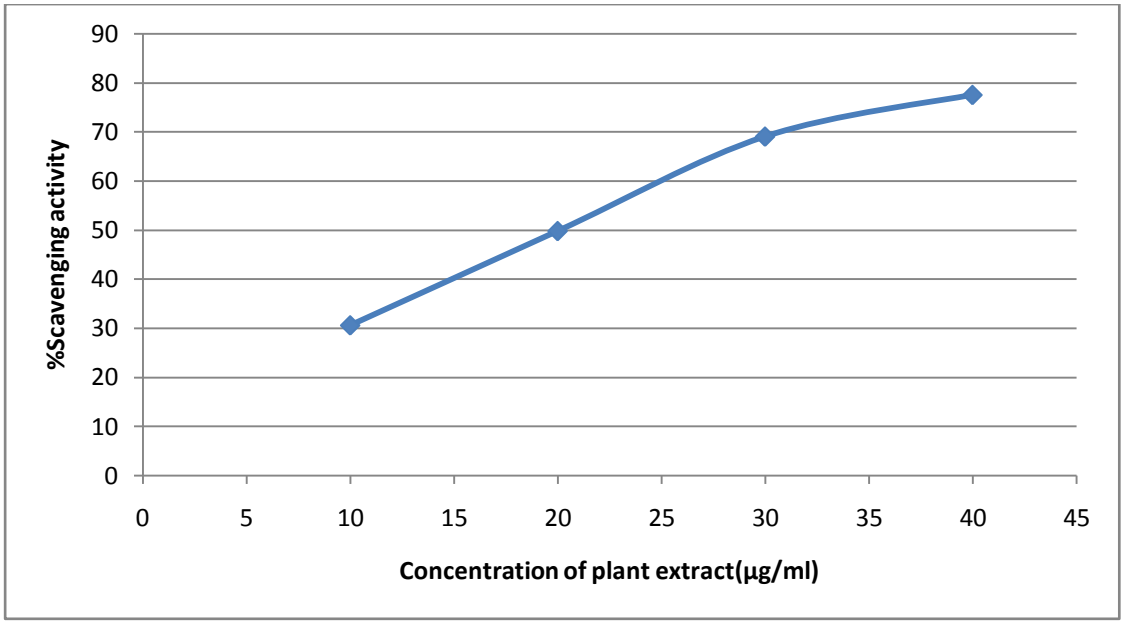

Figure 19: ABTS radical scavenging activity of ethyl acetate extract of *B.aristata* (mean±sd, n=3).

Table 20: ABTS radical scavenging activity of ethyl acetate extract of *B.aristata*(mean±sd, n=3).

Concentration of plant extract	**%Scavenging activity**
10	22.65±.82[a]
20	38.84±.71[b]
30	52.07±.35[c]
40	63.44±.25[d]

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

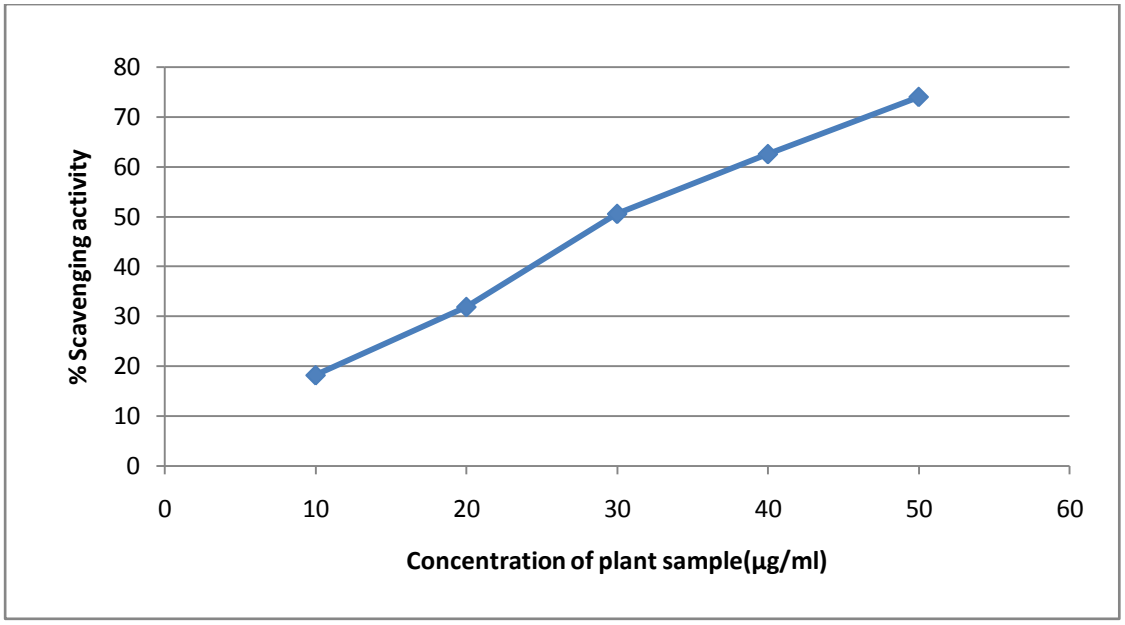

Figure 20: ABTS radical scavenging activity of Hexane extract of *B.aristata* (mean±sd, n=3).

Table 21: ABTS radical scavenging activity of Hexane extract of *B.aristata* (mean±sd, n=3).

Concentration of plant extract	%Scavenging activity
10	$18.24 \pm .92^{a}$
20	$31.96 \pm .90^{b}$
30	$50.53 \pm .60^{c}$
40	$62.50 \pm .70^{d}$
50	$73.94 \pm .70^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

Table 22: IC_{50} value of various extracts of *B.aristata* (mean±sd, n=3).

Plant extract of B.aristata	IC_{50}
Methanolic	$18.80 \pm .28^{a}$
Ethanolic	$16.64 \pm .12^{b}$
ethyl acetate	$19.25 \pm .24^{c}$
Hexane	$30.10 \pm .26^{d}$

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

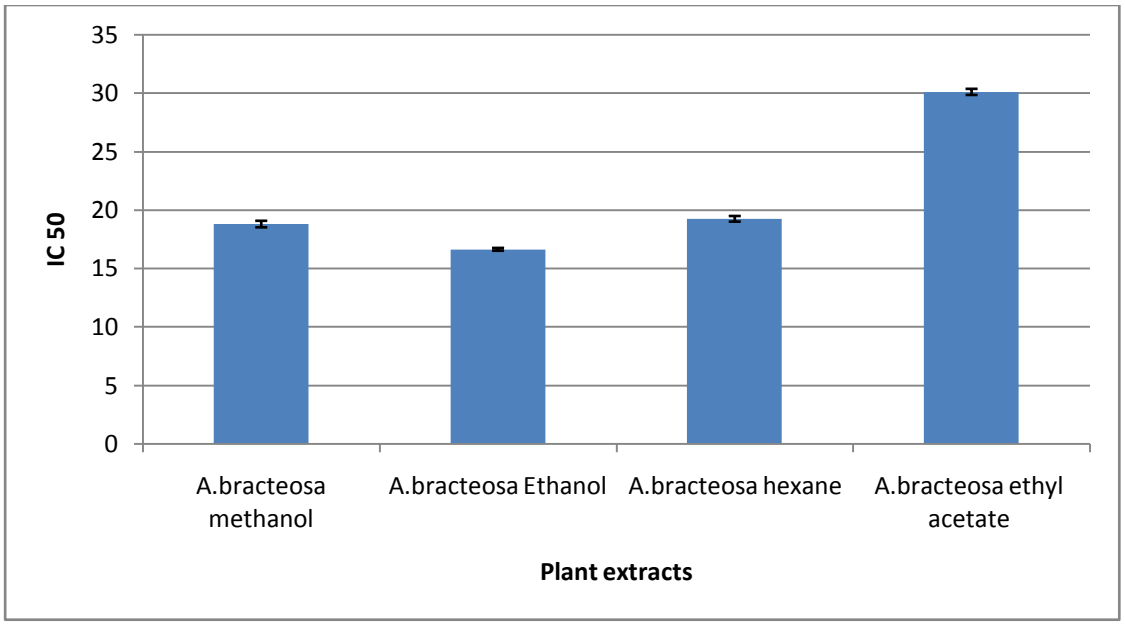

Figure 21: IC_{50} value of various extracts of *B.aristata* (mean±sd, n=3).

6.3.5 ABTS assay for *A.bracteosa*

The percentage of scavenging effect on ABTS radical was increased with the increase in the concentration of methanolic, ethanolic , ethyl acetate and hexane extracts of *A.bracteosa* was taken between (10 to 50 µg/µl) (Figure 5,6 & 7) (Table 8, 10 & 12). The % inhibition was varying from 31.94% at .1mg/ml to 76.08% at .45mg/ml for methanolic extract, 40.53% at .1mg/ml to 78.69% at 0.45mg/ml for ethanolic extract, 30.62% at .1mg/ml to 81.94% .45mg/ml for ethyl acetate extract, 27.22% at 0.1mg/ml to 88.36% at .45mg/ml for hexane extract of *A.bracteosa*. The IC_{50} value of methanolic, ethanolic, ethyl acetate and hexane extracts of *A.bracteosa* was calculated to be 0.18, 0.16, 0.25, 0.20 mg/ml respectively (figure & table).Significant difference was observed in the IC_{50} values of all the four extracts of *A.bracteosa*(Table)

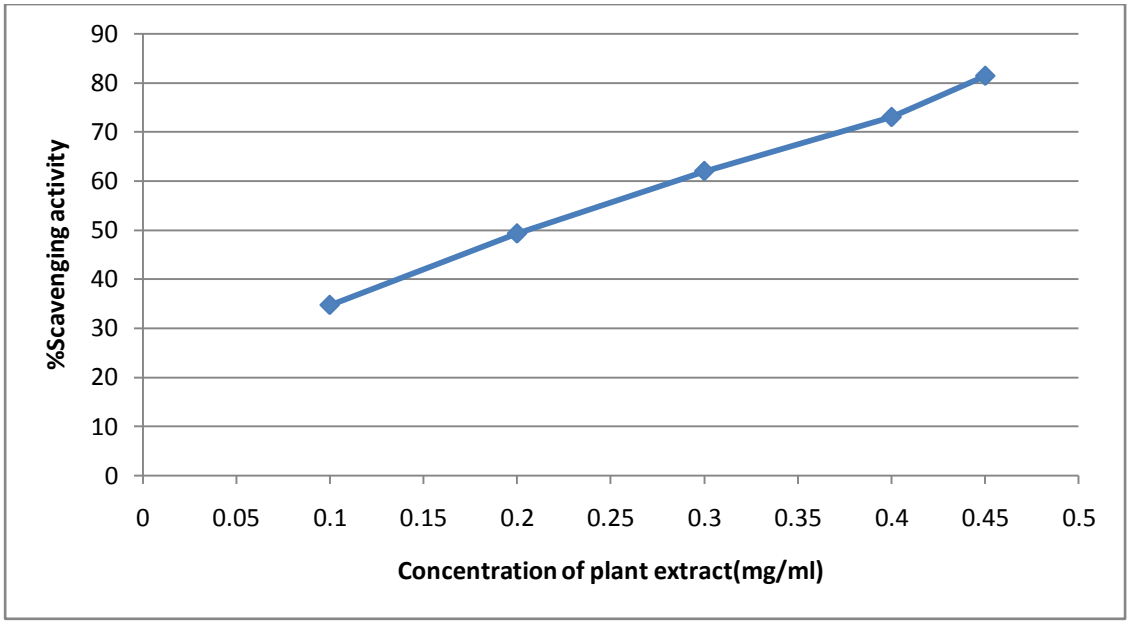

Figure 22: ABTS radical scavenging activity of Methanolic extract of *A.bracteosa* (mean±sd, n=3).

Table 23: ABTS radical scavenging activity of Methanolic extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract	%Scavenging activity
0.1	$34.73 \pm .44^{a}$
0.2	$49.27 \pm .44^{b}$
0.3	$61.98 \pm .39^{c}$
0.4	$72.97 \pm .21^{d}$
0.45	$81.44 \pm .81^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

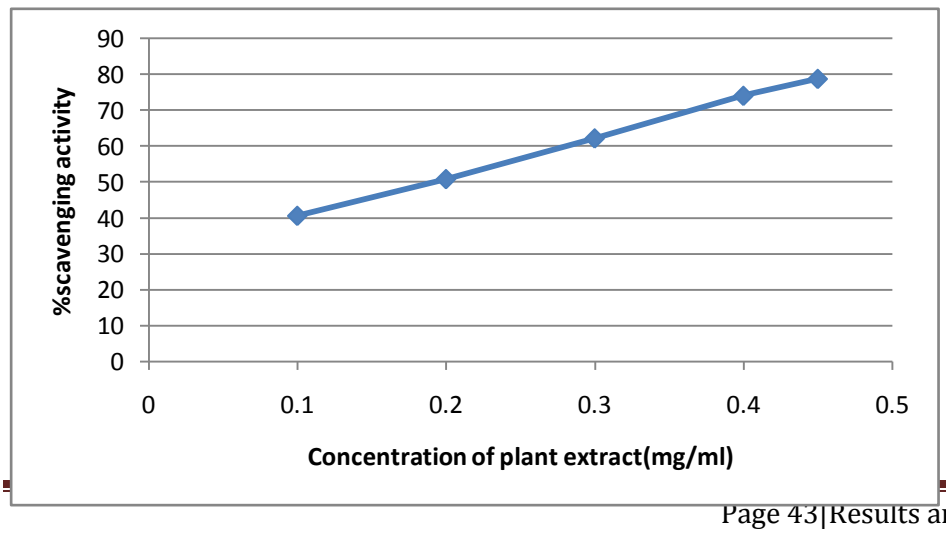

Figure 23: ABTS radical scavenging activity of Ethanolic extract of *A.bracteosa* (mean±sd, n=3).

Table 24: ABTS radical scavenging activity of Ethanolic extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract	%Scavenging activity
0.1	$40.54 \pm .57^{a}$
0.2	$50.77 \pm .36^{b}$
0.3	$62.11 \pm .54^{c}$
0.4	$73.97 \pm .91^{d}$
0.45	$78.69 \pm .40^{e}$

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

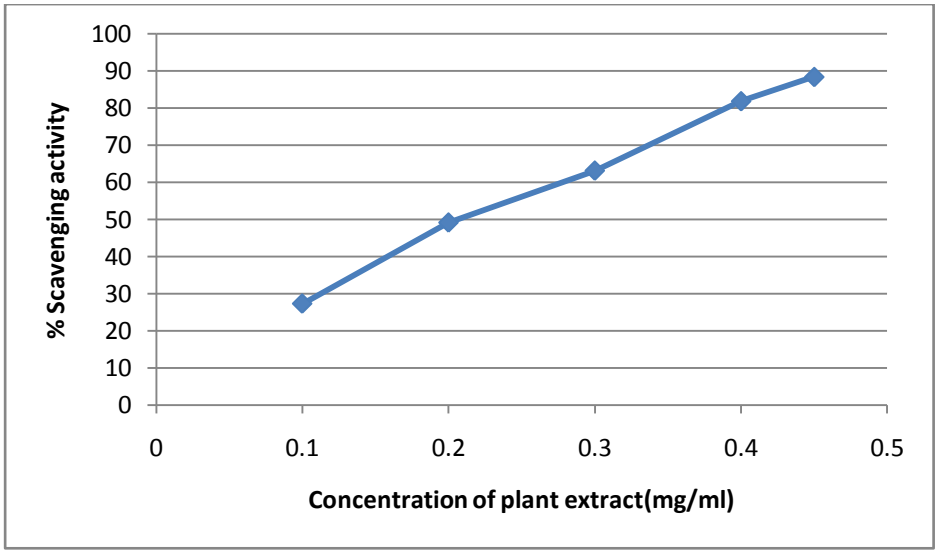

Figure 25: ABTS radical scavenging activity of Hexane extract of A.bracteosa (mean±sd, n=3).

Table 26: ABTS radical scavenging activity of Hexane extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract	%Scavenging activity
0.1	$27.22 \pm .68^{a}$
0.2	$49.09 \pm .82^{b}$
0.3	$64.09 \pm .39^{c}$
0.4	$81.81 \pm .49^{d}$
0.45	$88.35 \pm .66^{e}$

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

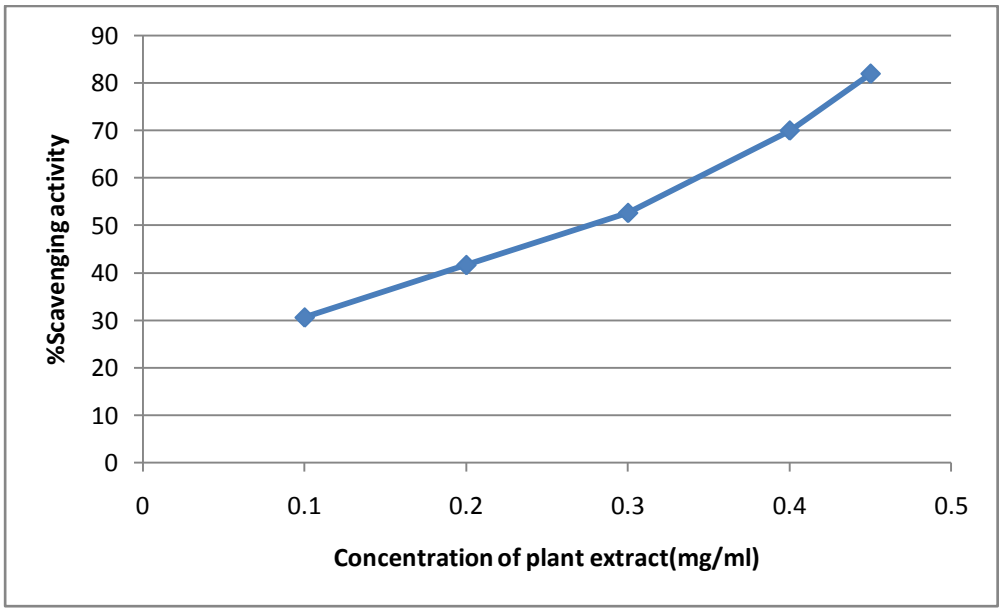

Figure 24: ABTS radical scavenging activity of ethyl acetate extract of *A.bracteosa* (mean±sd, n=3).

Table 25: ABTS radical scavenging activity of ethyl acetate extract of *A.bracteosa* (mean±sd, n=3).

Concentration of plant extract	%Scavenging Activity
0.1	$30.63 \pm .61^{a}$
0.2	$41.67 \pm .67^{b}$
0.3	$52.64 \pm .48^{c}$
0.4	$69.98 \pm .53^{d}$
0.45	$81.99 \pm .87^{e}$

Values within the column not sharing common superscript letters (a-e) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

Table 27: IC_{50} value of various extracts of *A.bracteosa* (mean±sd, n=3).

A.bracteosa extracts	IC_{50}
Methanolic	0.18 ± 02^{a}
Ethanolic	$0.16 \pm .05^{b}$
Butanolic	$0.25 \pm .01^{c}$
Hexane	$0.19 \pm .001^{d}$

Values within the column not sharing common superscript letters (a-d) indicate statistically significant difference among varying concentration at P<0.05 by Tukey's test.

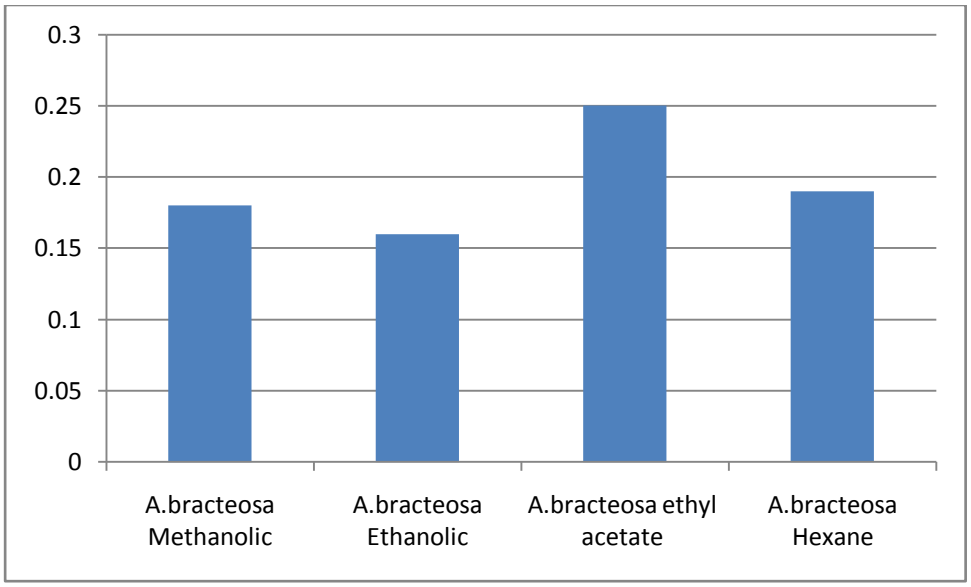

Figure 25: IC_{50} value of various extracts of *A.bracteosa* (mean±sd, n=3).

Antioxidant activity of both the plants (*B.aristata* and *A.bracteosa*) are carried out by DPPH and ABTS assay by using ascorbic acid as standard. The total phenolic and flavonoid content was also determined by using gallic acid and quercetin as standard.

Free radical are chemical entities that have one or more unpaired electrons. Presence of free radical in body can bring about thousands of reactions and have capability to cause extensive damage to tissues. Lipids, proteins and DNA are all susceptible to attack by free radicals (kalia *et al*.,2010). Antioxidants may offer resistance against oxidative stress by scavenging the free radicals and by their reducing ability. DPPH is purple colored stable nitrogen centered free radical, which show maximum

absorbance at 517nm (poonia *et al*.,2011). The change in absorbance of DPPH was due to reaction between the antioxidant molecule of extract which have hydrogen donating ability and the radical, it is visually noticeable by discoloration from purple to yellow. Extend of DPPH radical scavenged was determined by the decrease in intensity of violet colour in the form of IC_{50} values. In present study methanolic extract of *B.aristata* shows maximum scavenging activity that is 23.06% to 87.69% at concentration 5µg/ml to 25µg/ml. While *A.bracteosa* maximum scavenging activity was shown by ethanolic extract that is 39.68% to 77.51% at concentration varying from 0.1mg/ml to 0.45mg/ml. IC_{50} of methanolic and ethanolic extracts of B.aristata and *A.barcteosa* were found to be 10.30 µg/ml and 0.17 mg/ml. IC_{50} was compared respectively with the standard Ascorbic acid having IC_{50} value of 7.06 µg/ml. The present study revealed that *B.aristata* methnolic extracts has antioxidant activity near to standard ascorbic acid.

Previous antixodant studies on *B.aristata* stem methanolic extract of shown IC_{50} as 0.90mg/ml (Dar et al.,2014). Shakoor and Zaib.,2014, support the antioxidant activity of *A.bracteosa* by using DPPH assay and %RSA obtained was 33.22%. The dpph – radical scavenging assay of oil content for *A.barcteosa* which exhibit a significant antioxidant activity 78% at 1.0 mg/ml (Mothana et al.,2012).

The ABTS assay is based on the radical cation decolorization method based on the reduction of ABTS radicals by antioxidants of the plant extracts tested.The reaction's mechanism involves the electron-donating ability and result in the decolorization of the reaction mixture. In ABTS method, the highest antioxidant activities was given by ethanolic extract of *B.aristata* 33.46% to 88.20% at concentration varying from 10µg/ml to 40µg/ml. In case of *A.bracteosa*, maximum scavenging activity was shown by ethanolic extract that is 40.54 to 78.69 at 0.1 to 0.45mg/ml concentration. The IC_{50} of ethanolic extracts of *B.aristata* and *A.bracteosa* were found to be 16.64 µg/ml 0.16mg/ml. The ABTS scavenging capacities among *B.aristata* and *A.bracteosa* methanolic, ethanolic, ethyl acetate and hexane extracts were significantly different from each other ($p<0.05$). There were no study regarding antioxidant activity of *B.aristata* and *A.bracteosa* using ABTS assay.

6.4 TOTAL PHENOLIC CONTENT (TPC):

The total phenolic content (TPC) was determined using the standard curve of Gallic acid (Figure 26). The total phenolic content in methanolic, ethanolic and hexane extracts of *B.aristata was* measured to be 12.11, 13.18, 11.44 and 10.2 mg GAE/g.In case of *A.barcteosa* TPC measured was 8.16, 8.98, 7.02 and 7.49 mg GAE/g respectively (Table28).

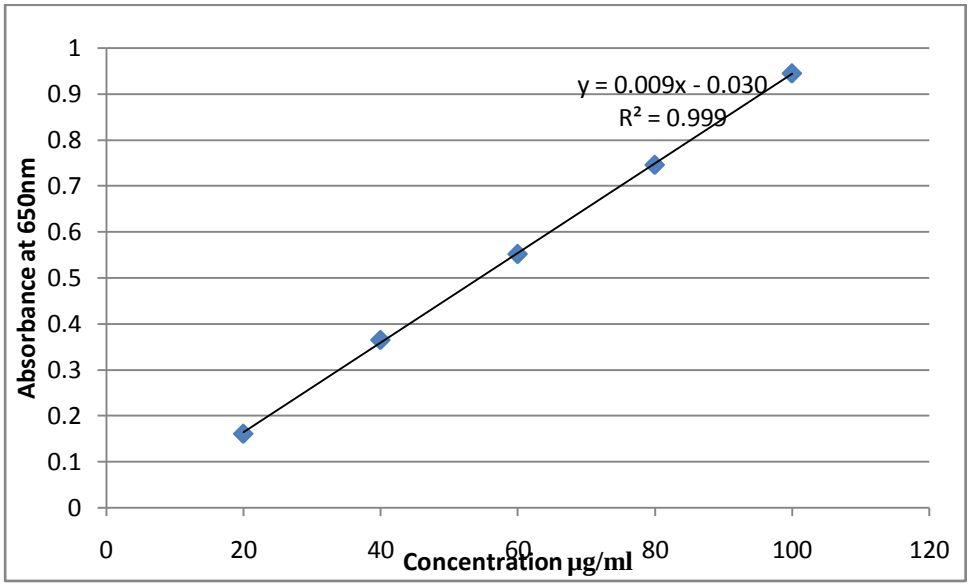

Figure 26: Standard curve of Gallic acid.

Table 28: Total phenolic content (TPC) in methanolic, ethanolic, ethyl acetate and hexane extracts of *B.aristata* and *A.bracteosa*.

Plant	Extracts	TPC(µg GAE/mg)
B.aristata	Ethanolic	13.18
	Methanolic	12.11
	Hexane	10.2
	Ethyle acetate	11.44
A.bracteosa	Ethanolic	8.16
	Methanolic	8.98
	Hexane	7.49
	Ethyle acetate	7.02

6.5 TOTAL FLAVONOID CONTENT:

The total flavonoid content (TFC) was calculated using the standard curve of Quercetin (Figure 27).The total flavonoid content in methanolic, ethanolic, Butanolic and hexane extracts of *B.aristata* and *A.barcteosa* was measured to be 121.8, 75.2, 160.2, 111.46, 75.26, 91.26, 56.73 and 51.2 mg QE/g respectively (Table 29).

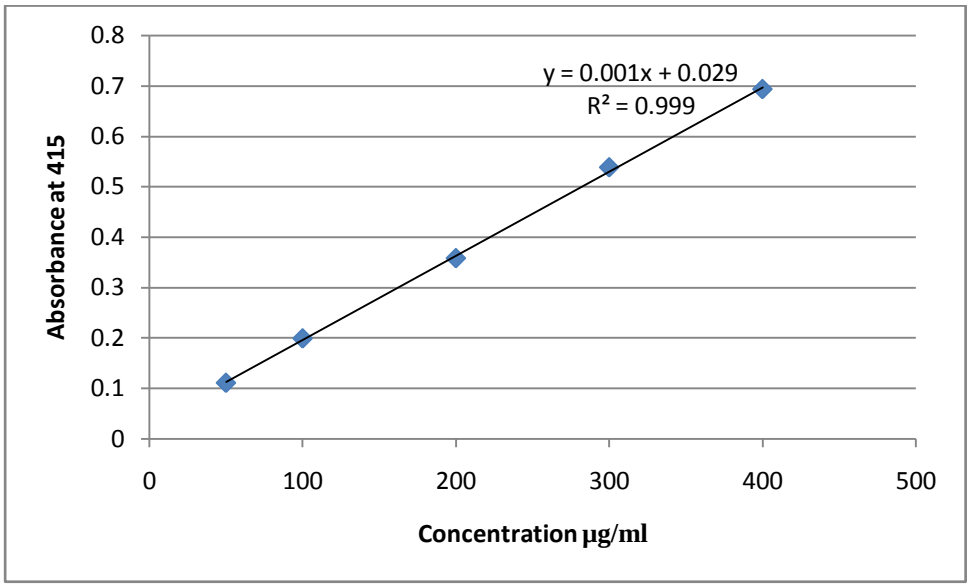

Figure 27: Standard curve of Quercetin.

Table 29: Total flavonoid content in methanolic, ethanolic, hexane and ethyle acetate extracts of *B.aristata* and *A.bracteosa*.

Plant	Extracts	Total flavonoid content µg QE/mg
B.aristata	Methanolic	121.8 ± 0.053
	Ethanolic	75.2 ± 0.015
	Hexane	160.2 ± 0.011
	Ethyl acetate	111.46 ± 0.015
A.bracteosa	Methanolic	75.26 ± 0.003
	Ethanolic	91.26 ± 0.003
	Hexane	56.73 ± 0.004
	Ethyl acetate	51.2 ± 0.005

The total phenolic content was calculated in refrence to gallic acid. The present study showed that methanolic, ethanolic, ethyl acetate and hexane extracts of *B.aristata* and *A.bracteosa* was calculated as 0.389, 0.384, 0.376, 0.382, 0.367, 0.371, 0.364, 0.362 µg GAE/mg. The total flavonoid content was calculated using quercetin as a standard. The present study show that the total flavonoid content of methanolic, ethanolic, ethyl acetate and hexane extracts of *B.aristata* and *A.bracteosa* were calculated as 29.89, 29.85, 29.77, 29.53, 29.35,29.43, 29.25, 29.25 µg QE/mg. The total flavonoid content in previous study for *A.bracteosa* was evaluated for water, ethanol and methanol extracts 7.25, 1.67, 3.22 µg QE/mg (Chao *et al.*, 2012).

6.6 High pressure liquid chromatography(HPLC) analysis of the plant extract

Previous studies on *B.aristata* has reported berberine as a major alkaloid present in this plant. It is a well-known isoquinoline alkaloid isolated from berberis, that are commonly used as herbal medicine for the treatment of patients with gastrointestinal disorders and as a antibiotic.

Area % Report

Data File:	C:\EZChrom Elite\Enterprise\Projects\April 2015\Data\Berberin Standard 030.dat
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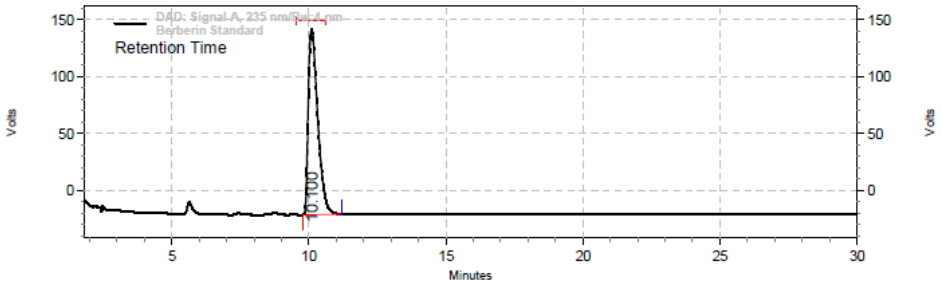
Analysis Time	4/9/2015 12:42:31 PM
Vial Number	Vial 1
Analyst	Aditya



DAD: Signal A, 235 nm/Bw:4 nm
Results

Retention Time	Area	Name
10.100	8242008	**Berberine**
Totals	8242008	

Figure 28 : Spectrum for HPLC of berberine standard

Area % Report

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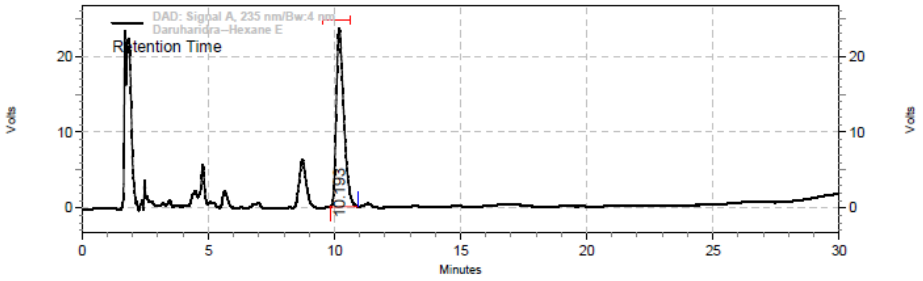
Analyst Aditya



DAD: Signal A, 235 nm/Bw:4 nm
Results

Retention Time	Area	Name
10.193	1018643	**Berberine**
Totals	1018643	

Figure 29: Spectrum of HPLC of ethanolic extract of *Berberis aristata*

Area % Report

Data File: C:\EZChrom Elite\Enterprise\Projects\April 2015\Data\Daruharidra--Methanol E 033.dat
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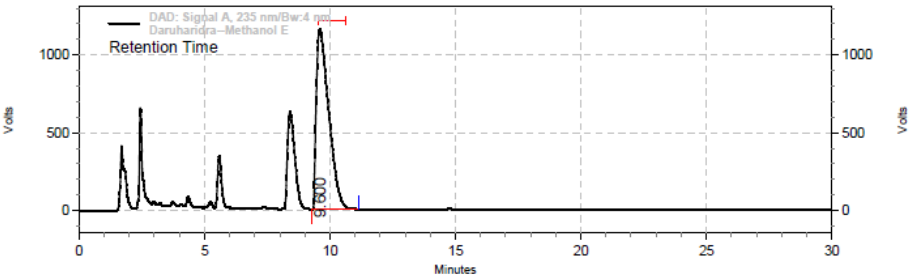
Analysis Time 4/9/2015 2:16:06 PM
Vial Number Vial 4
Analyst Aditya



DAD: Signal A, 235 nm/Bw:4 nm
Results

Retention Time	Area	Name
9.600	83799585	**Berberine**
Totals	83799585	

Figure 30: Spectrum of HPLC of methanolic extract of *Berberis aristata*.

Area % Report

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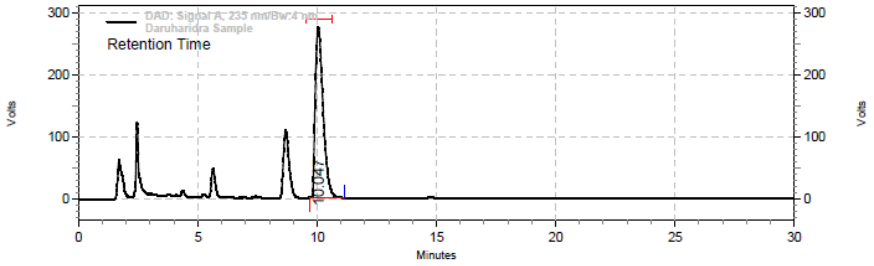
Analyst	Aditya



DAD: Signal A, 235 nm/Bw:4 nm
Results

Retention Time	Area	Name
10.047	13142402	**Berberine**

Totals	
	13142402

Figure 31: Spectrum of HPLC of hexane extract of *Berberis aristata*.

Area % Report

Data File:	C:\EZChrom Elite\Enterprise\Projects\April 2015\Data\Daruharidra Sample 032.dat
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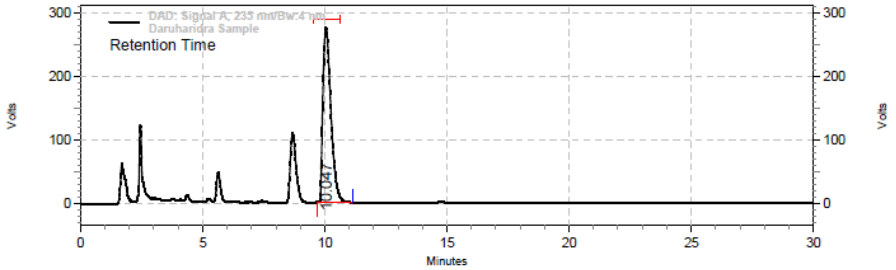
Analyst	Aditya



DAD: Signal A, 235 nm/Bw:4 nm
Results

Retention Time	Area	Name
10.047	13142402	**Berberine**

Totals	
	13142402

Figure 32: Spectrum of HPLC of ethyl acetate extract of *Berberis aristata*.

HPLC result revealed the presence of berberine in all the plant extract of *berberis aristata* in varying concentrations. The amount of berberine content was found

highest in ethanolic extract 78.65% and lowest in hexane extract .49% compared to standard shown in figure 28,29, 30, 31, 32. Decreasing order of berberine content is as followes in ethanolic, methanolic, ethyl acetate and hexane 78.65%, 41.04%, 3.14%, 049%.

CHAPTER-7

CONCLUSION AND FUTURE SCOPE

The variation in antioxidant activity of different extracts may be affected mainly by the solvent used for extraction. All the plant extracts of *B.aristata*and*A.bracteosa* were observed to possess significant antioxidant potential. Methanol and ethanol solvent extracts of both plants have shown best results, yielding the strong antioxidant activity. The Phytochemical analysis of the extracts indicates the presence of alkaloids, Flavonoids, phenols, terpenoids, anthocyanin andcoumarines.The results of antioxidant activity indicate higher free radical scavenging activity in ethanolicextract of *B.aristata* in comparison to *A.bracteosa*. This may be due to the amount of alkaloids is high, because the plant have higherberberine content, a major plant alkaloid in ethanolic extracts reported by HPLC analysis. The correlation to previous research conducted, our results elucidate that *B.aristata* root have more potential antioxidant property then stem and other parts. Another reason that contribute to the antioxidant potential of plant extract is presence of high phenols and Flavonoids content.

In future studies the results of this research should be extended to other methods for determination of antioxidant activity. The future research work may be aimed to identification of main Compounds responsible for high antioxidant activity. The identification of these compounds may lead to find out the percentage contribution of those compounds to the TAC.

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