Chapter 1
Phytochemical Analysis
Introduction

Phytochemicals are natural bioactive compounds found in plants, works as nutrients, dietary fiber and protects against diseases. Research results reveals that, various phytochemicals, together with nutrients found in fruits, vegetables and nuts may help to reduce the rate of aging process and decrease the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, cataracts, osteoporosis and urinary tract infections. Many phytochemicals used as drugs to protect human health. They can have complementary and overlapping mechanisms of action in the body, including antioxidant effects, modulation of detoxification enzymes, stimulation of the immune system, modulation of hormone metabolism, antibacterial and antiviral effects.

Phytochemicals are typically related to plant pigments. So, fruits and vegetables that are bright colors - yellow, orange, red, green, blue and purple - usually contains most of the phytochemicals and nutrients.

Ethnomedicine may be defined broadly as the use of plants as medicines by people (Farnsworth, 1990; 1994). But this use could be called more truly as ethnobotanic medicine. Traditional medicine is a broad term, used to define any non-western medical practice (Bannerman, 1983). Ethnopharmacology is a highly expanded approach used to refer drug discovery, involving observation, description and experimental investigation of original drugs and their biological activities. It is based on botany, chemistry, biochemistry, pharmacology and many other science disciplines that contribute to the
discovery of natural products with biologic activity. Historically, plants have played a significant role in medicine. Through the observation and experimentation, humans have learnt that, plants boost up the health and welfare of mankind. The use of these herbal remedies is not only cost effective but also safe and almost free from serious side effects.

According to WHO, over 80% of the world’s population depends on traditional forms of medicine, principally plant based products to meet primary health care needs. In India, the contribution of medicinal plants and plant products play a major part in the national economy. The plants are one of the most vital sources of medicines. The application of plants as medicines dated back to prehistoric period. In India, the references to the curative properties of some herbs are mentioned in the Rigveda which seems to be the earliest records regarding the use of plants in medicines. The medicinal plants are widely utilized throughout the world, in two distinct areas of health management, viz., traditional and contemporary system of medicine. The traditional system of medicine mainly purposes through local or folk or tribal system and also through the organized Indian system of medicines like Ayurveda, Siddha and Unani etc. Inspite of fabulous advance made in the contemporary medicine there are still a large number of ailments for which suitable drugs are yet to be examined.

The information of the biological activities and chemical constituents of plants are desirable, not only for the discovery of new therapeutic materials. Thus an extensive study on medicinal plants is essential to identify new plant based drugs to estimate its efficacy to combat the diseases. The recent research in the area of pharmacology has resulted in the production of many pharmaceutical substances for new therapeutics. Developments in the area of phytochemistry and medicinal plants for the production of
Bioactive compounds has made possible for the production of wide variety of pharmaceuticals such as alkaloids, flavonoids, tannins, steroids, saponins (Vanisree et al., 2004).

In India, Seigler and David (1994) have dedicated their great work on the family Euphorbiaceae. They have recorded large number of compounds belongs to different chemical classes. According to them, the phytochemistry of the Euphorbiaceae family is the most diverse and interesting and is comparable to be biological diversity of the family. With this background, through present investigation, the phytochemical analysis of one of the unexplored medicinally important plant Epiprinus mallotiformis belongs to Euphorbiaceae has been considered for further studies.

**Review of Literature**

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). These medicines originally took the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (Balick and Cox, 1997). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. In more recent, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001).

Drug discovery from medicinal plants led to the isolation of early drugs, such as cocaine, codeine, digitoxin and quinine, in addition to morphine, of which some are still in use (Newman et al., 2000; Butler, 2004; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants
continued. More recently, drug discovery techniques have been applied to the standardization of herbal medicines. The following information provides a brief review on the importance of medicinal plants in drug discovery, including, noteworthy compounds isolated from plant source.

Drug discovery from medicinal plants has evolved to comprise many fields of investigation and several methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist or plant ecologist, who gathers and identifies the plant(s) of interest. Collection may involve species, with known biological activity for which active compound(s) have not been isolated or may contain taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plant(s) of interest are collected (Baker et al., 1995). Phytochemists prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically applicable assays and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and application of suitable screening assays directed towards physiologically related molecular targets.

According to Kinghorn (2001) and Samuelsson (2004) Pharmacognosy summarizes all of these fields into a distinct interdisciplinary science. The definition and practice of pharmacognosy have been evolving since the term was first introduced about 200 years ago, as drug use from medicinal plants has progressed from the formulation of crude drugs to the isolation of active compounds in drug discovery. The American Society of Pharmacognosy mentions to pharmacognosy as “the study of the physical,
chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources”. As practiced today, pharmacognosy includes the broad study of natural products from numerous sources including plants, bacteria, fungi and marine organisms.

The foundation for the use of therapeutic drugs in modern day medicine is the history of natural product use in prehistoric times and in traditional medicine in the globe. Primitive cultures used plants as a source not only of medicines but also of toxic substances for killing animals and for stimulants and hallucinogens used in religious rituals. Traditionally, natural plant products have been the source for the search for new drugs, by pharmaceutical companies. Plant sources of herbal medicines rich in polyphenols are being studied in detail to find active molecules with curative properties (Dillard and German, 2000). The plant products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although, herbs had been assessed for their medicinal flavouring and aromatic qualities.

Among prehistoric civilizations, India has been rich storehouse of medicinal plants. The different types of forests in India is the major responsibility of large number of medicinal and aromatic plants, which are largely collected as raw materials for manufacture of drugs and perfumery products. About 8,000 herbal medicines have been codified in Ayurveda. The Rigveda has recorded 67 medicinal plants, Yajurveda 81 species, Atharvaveda (290), Charaka Samhita (1100) and Sushruta Samhita (1270) had described its medicinal properties.

India is a known mega biodiversity center harboring a multitude of medicinal plants species each presumably studies with as yet mysterious genetic and chemical
variation of economic important. Out of an estimated 17,000 higher plants species occurring in India, more than 1000 species are used over several centuries in the traditional systems of medicines viz., Ayurveda, Sidda, Unani and Amchi. The villages and tribal folks spread across the length and breadth of the country make use of more than 7000 plant species through oral traditions (Pushpangadan, 1997). Nearly \( \frac{3}{4} \) of the herbal drugs and perfumery products used in the world are available in natural state in India. Therefore, the rich and varied plant diversity, especially genetic diversity of medicinal and aromatic plants, in one of India's important strength and is the bedrock for all for all future bio industrial development. The detailed report of literature survey pertaining to phytochemical analysis of medicinal plants is given below:

In India, Karnataka state is endowed with rich natural resources especially along the Western Ghats ranges, arises from abruptly in the west from the Arabian Sea coast and descends gradually towards the dry Deccan plains in the east. The total area of the Western Ghats is estimated to be about 20,000 sq. km. The good climatic conditions and altitude gradients have resulted on the development of a variety of forest forms evergreen to deciduous and scrub jungles. It is one of the richest biodiversity centers and is considered as one among the eighteenth hotspots of the world. This region comprises about 4,000 species of angiosperms of which 2,280 species are endemic to this region (Pascal et al., 1982).

Local knowledge of indigenous peoples includes information about the ecosystem in general, but also about specific plants used as medicine, food, building material and the like (Leonti et al., 2003). Establishing the historical depth of medicinal plant use is relevant from a variety of perspectives. Not only would it show definitely that indigenous
cultures have an in depth knowledge of certain botanical taxa, which has been transmitted over centuries prior to it becoming important in the context of developing novel pharmaceuticals, but as importantly, such research would demonstrate the historical development of an intricate relationship between a culture and its environment (Posey, 2002).

Parnitha et al. (2004) have conducted the field visit and evaluated the ethnobotanical wealth of Bhadra Wildlife Sanctuary in Karnataka and they have indicated that 60 plant species belonging to 50 genera 35 families were used to preparing herbal drugs. In 2005, Parnitha et al. an ethnomedicinal survey was conducted in three villages of Shimoga district of Karnataka. The survey revealed that utilization of 47 species of plant belonging to 46 genera in 28 families.

Rajakumar and Shivanna (2009) have documented the 85 plant species belonging to 41 families which are used to treat the Human and veterinary ailments in the eastern region of Shimoga district, Karnataka. In 2010, they have conducted an ethno-botanical field survey in Sagar taluk of Shimoga district of Karnataka. In this study a total of 48 plant species belonging to 44 genera and 31 families used by folk practitioners to treat various common to chronic human and veterinary ailments. In the same year, Shivanna and Rajakumar have documented 40 medicinal plant species belongs to 26 families in Bhadravathi taluk of Shimoga district Karnataka. In 2011, they have documented ethnomedico-botanical knowledge Hosanagara taluk in Shimoga district of Karnataka, India. In this study 86 plant species belonging 44 families to treat human and veterinary ailments. In 2012, Rajakumar and Shivanna have evaluated the traditional veterinary health care practices in Shimoga district of Karnataka, India. In this study a total of 52 plant species of 48 genera and 38 families were used to treat the veterinary ailments.
Achar et al. (2010) conducted a field work on Khare-vokkaliga Community in Uttara Kannada District of Karnataka, and documented 57 plant species of 56 genera and 38 families. Medicinal plants which are used to cure the human diseases and disorders.

Rajasab and Isaq (2004) have documented the folk knowledge on edible wild plants of North Karnataka, in this study 51 plant species 46 genera belongs to 37 families were recorded in the north Karnataka.

Bhat et al. (2012) have documented the 106 medicinal plant species of 55 families which are uses for treatment of wounds in ethnomedicinal practices in different communities of Uttara Kannada district of Karnataka.

Prasad and Kumar (2013) have conducted the work on the Ethno-botanical potential of medicinal legumes in the Western Ghats of Karnataka in this study 28 medicinal legume species are recorded in the different region of Karnataka.

Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. Scientific analysis of plant components follows a logical pathway. Some of the important isolated compounds are reviews are mentioned below:

Jisaka et al. (1993) isolated the Steroid glucosides from vernonia amygdalina, Ivanovaa et al., 2011 have studied on Smilax aspera and isolated two new cis-fused A/B rings furostanol saponins (25S)-26-O-β-D-glucopyranosyl-5β-furostan-1β, 3β, 22α, 26-tetraol-1-O-β-D-glucopyranoside and (25S)-26-O-β-D-glucopyranosyl-5β-furostan-1β, 2β, 3β, 5β, 22α, 26-hexaol and the known compounds(25S)-26-O-β-D-glucopyranosyl-5β-furostan-3β, 22α, 26-triol-3-O-α-L-rhamnopyranosyl-0-β-D-
glucopyranoside and (25S)-26-O-β-D-glucopyranosyl-5β-furostan-3β,22α,26-triol-3-O-β-D-glucopyranosyl-(1 → 2)-O-β-D-glucopyranoside.

Hussein et al. (1997) isolated a new di-C-glucosylflavone, 2",2"-di-O-β-glucopyranosyl-vicenin II and a new flavonol di-O-glycoside, herbacetin 3-0-α-rhamnopyranoside-8-0-β-glucopyranoside, from the aerial parts of Ephedra aphylla.

Staubmann et al. (1999) have been studied on Jatropha curcas and isolated from compound 5-hydroxypyrrolidin-2-one and pyrimidine-2,4-dione.

Seidela et al. (2000) have studied on The aerial parts of Goniothalamus gardneri (Annonaceae) and isolated the known flavonoids 2'-hydroxy-4,4',6'-trimethoxychalcone (flavokawain A), 2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone, 4,2',4'-tri hydroxy-6'-methoxydihydrochalcone, 5,7,4'-trimethoxyflavanone (naringenin trimethyl ether) and 7-hydroxy-5,4'-dimethoxyflavanone (tsugaflolin) together with three novel compounds, the dimer characterised as (ref)-1β, 2α-di-(2,4-dihydroxy-6-methoxybenzoyl)-3β, 4α-di-(4-methoxyphenyl)-cyclobutane, 2',4'-dihydroxy-4',6'-dimethoxychalcone and 2'-hydroxy-4,4',6'-trimethoxydihydrochalcone.

Kamiya et al. (2001) have studied on Helicteres isora and isolated five flavonoids, they are isoscutellarein 4'-methyl ether 8-O-β-D glucuronide 6"-n- butyl ester, isoscutellarein 4'-methyl ether 8-O-β-D glucuronide 2"4"-disulfate, isoscutellarein 8-O-β-D glucuronide 2"4"-disulfate, isoscutellarein 4'-methyl ether 8-O-β-D glucuronide, isoscutellarein 4'-methyl ether 8-O-β-D glucuronide 2"-sulfate.

In 2002, Patora and Klimek, have been isolated six flavonoids from the leaves of Lemon balm. Their structures were determined on the basis of spectral data (UV, 1R, 1H NMR, 13C NMR and FAB MS) as luteolin, luteolin 7-O-beta-D-glucopyranoside, apigenin 7-O-beta-D-glucopyranoside, luteolin 7-O-beta-D-glucuronopyranoside, luteolin
3'-O-beta-D-glucuronopyranoside and luteolin 7-O-beta-D-glucopyranoside-3'-Obeta-D-glucuronopyranoside.

Madureira et al. (2003) have studied on a phytochemical reinvestigation of the whole plant of *Euphorbia segetalis* and isolated five tetracyclic triterpenes: 3'-hydroxy-cycloart-25-en-24-one, cycloart-25-ene-3, 24-diol, cycloart-23-ene-3,25-diol, lanosta-7,9(11),24-trien-3-ol and lanosta-7, 9(11), 24(31)-trien-3-ol. 3-acetoxy-cycloart-25-en-24-one (1a) and glutinol, lupenone, dammaranodienol, cycloartenol acetate, 24-methylenecycloartanolacetate and sitosterol.

Saxena and Goutam (2008) have studied and isolation and structural elucidation of flavone-0-glycoside, characterized as; luteolin-8-O-β-D-glucopyranoside isolated from the seeds of the *Capparis decidua*.

In 2009, Grzywacz and Krzaczek have isolated nine flavonoid compounds and two coumarin glycosides from the inflorescences and the herb of *Hieracium pilosella* glycosides were isolated by preparative thin layer chromatography. Subsequent UV, NMR and MS analyses have led to identification of the following flavonoid derivatives: known for the species - apigenin, luteolin, luteolin 7-O-β-glucopyranoside, luteolin 4'-O-β-glucopyranoside, isoetin 7-O-β- -glucopyranoside, isoetin 4'-O-β-glucuronide and new for the species-kaempferol 3-methyl ether and apigenin 7-O-β-glucopyranoside. Hesperidin was isolated from *Citrus sinensis* (Aghel et al., 2008).

She et al. (2009) have isolated of two new flavonoid glycosides, apigenin 4'-Oα-D-glucopyranoside and 5,7,3',4'-tetrahydroxy-5'C-prenylflavone-7-O-β-glucopyranoside from *Elsholtzia rugulosa*.

Ying et al. (2009) investigation on the herbal of *Euphorbia humifusa* based on the spectral analysis, five apigenin glycosides were identified as apigenin-7-O-(6'-O-
galloyl)-beta-D-glucopyranoside (1), apigenin-7-O-beta-D-apiofuranosyl (1-->2)-beta-D-glucopyranoside (2), apigenin-7-O-beta-D-lutinoside (3), apigenin-7-O-beta-D-glucopyranoside (4) and apigenin (5)

Bhujbal et al. (2010) have investigated and isolated the Apigenin-7-glucosideo 7-(P-D-glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), from the roots of Clerodendrum serratum.

In 2010, Iwashina and Kokubugata have isolated the ten flavonoids, one anthocyanin and two organic acids from the leaves and flowers of Myoporum bontoides. Each one anthocyanin and organic acid and two flavonoids from the flowers were cyanidin3, 5-di-O-glucoside, acteoside and apigenin 7-O-glucuronide and luteolin 7-O-glucuronide. On the other hand, fourteen foliar flavonoids were characterized as apigenin 7-O-glucuronide, luteolin 7-O-glucoside, luteolin 7-O-glucuronide, chrysoeriol 7-O-glucoside, chrysoeriol 7-O-glucuronide, selagin 7-O-glucoside, selagin 7-O-glucuronide, tricin 7-O-glucuronide, quercetin 3-methyl ether, chrysoeriol, isokaempferide, apigenin, luteolin, quercetin 3,4-dimethyl ether. Two organic acids were identified as chlorogenic acid and acteoside.

Okwu and Ohenhren (2010) have done chemical investigation of the bioactive constituents from the leaves of Stachyctapheta jamaicensis resulted in the isolation of two new steroidal glucosides 16β-(β-D glucopyranosyl, 3,8,22-trihydroxy) Cholestan-1β-yl-6-O-(3,4,5-trimethoxybenzoyl)β-D, glucopyranoside1 and 16-β(β-D-glucopyranosyl 3,8,22-trihydroxy-cholest-5,14,16, 23 tetraiene 1β-yl,6-O-(3,4,5-trimethoxybenzoyl)β-D glucopyranoside2). The structures were elucidated using NMR spectroscopy in combination with IR and MS spectral data.
In 2011, Gohari et al. studied on *Salvia macrosiphon* and isolated four flavonoids plus a steroid compound from the ethyl acetate and methanol extracts of the aerial parts of *S. macrosiphon*, using different chromatographic methods on the silica gel and sephadex LH20. The structures of the isolated compounds were determined to be apigenin-7,4'-dimethyl ether (1), β-sitosterol (2), salvigenin (3) apigenin-7-O-glucoside (4) and luteolin-7-O-glucoside (5) using the $^1$H, $^{13}$C-NMR and MS spectra.

Obmann et al. (2011) have isolated 18 flavonoids from aqueous extract of the aerial parts of *Dianthus versicolor*. b-sitosterol 3-O-b-d-glucopyranoside and jaceidin, were isolated from aerial parts of *Centaurea bracteata* (Flamini *et al.*, 2001).

Kassem and Hashim (2013) have separated the compound from the leaves extract of *Antidesma Bunius* Corilagin (1-O-galloyl 3,6-O-hexahydroxydiphenoyl-β glucopyranoside), Gallic acid (3,4,5-trihydroxybenzoic), Ferrulic acid, Ellagic acid, Vicenin II (Apigenin-6, 8-di-C-β-D-glucopyranoside) Amentoflavone (3',8''-Biapigenin).

Kissa *et al.* (2013) have isolated the compounds from *Aconitum napellus*. Diterpene alkaloids neoline (1), napelline (2), isotalatizidine (3), karakoline (4), senbusine A (5), senbusine C (6), aconitine (7) and taurenine (8) were identified. Four (2-4, 6) of which are reported for the first time from this plant.

Literature survey revealed that, most of the investigators studied other genera of Euphorbiaceae on phytochemical and pharmacological activity. So far, no work has been attempted to study the phytochemical analysis and pharmacological activity of *E. mallotiformis* except its diversity. The traditional practitioners, living in the vicinity of the forest of the Western Ghats are using the bark and leaves of this species, for treating infectious diseases and disorders. With the light of existing literature the phytochemical investigation of *E. mallotiformis* has carried out.
Materials and Methods

Study area

Agumbe lies in the West region of Karnataka of South India. It is 55 km away from the Arabian Sea and the coast of Udupi. As a part of the Western Ghats mountain range, it is rich in biodiversity of flora and fauna. Agumbe lies in a rainforest region with a warm and humidity with a tropical monsoon climate. A dense silvery fog forms over the Western Ghats contributing to the natural beauty of Agumbe. In the month of July with an average rainfall of about 2,647 mm, the mean annual rainfall is 7,620 mm and the average temperature is about 23.6°C.

Collection of plant materials

The leaf and bark Sample of *Epiprinus mallotiformis* were collected from the Agumbe, Shimoga district of Karnataka. The samples were authenticated and herbarium was kept in the Department of Applied Botany, Kuvempu University, Shankaraghatta, Shimoga district, Karnataka. Samples were cleaned and air dried, then powdered for future work.

Extraction of plant materials

The leaf and bark of the plant *E. mallotiformis* were collected during the winter season. The leaves were shade dried for about 20 to 25 days and mechanically powdered. The powdered material was subjected to Soxhlet extraction by successively with petroleum ether, chloroform, methanol and aqueous one by one. After each extraction, the remaining residue was shade dried and then used for next extraction. Each extraction was carried for about 20 hours (approximately 40 cycles) .The pet ether, chloroform, methanol and aqueous extracts were concentrated to dryness in a rotary flash evaporator under reduced pressure and controlled temperature.
Plate -1.1

Map showing location of the sampling site
Phytochemical investigation of crude extracts

Each extracts was subjected to phytochemical investigation, to study the presence of the following constituents.

- Alkaloid
- Flavonoids
- Glycosides
- Saponin
- Steroids
- Tannins

Qualitative tests

1) Test for alkaloids

a) Mayer’s test (potassium mercuric iodide)

To a few drops of the Mayer’s reagent, 2 mg of extract was added, formation of white or pale yellow precipitation, indicated the presence of alkaloids.

b) Wagner’s test (solution of iodine in potassium iodide)

2 mg of extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner’s reagent were added. A yellow or brown precipitation indicates the presence of alkaloids.

2) Test for Flavonoids

a) Ferric chloride test

Test solution (small quantity of extract +2 ml of water) in a test tube and a few drops of ferric chloride solution was added intense green colour was appeared.
b) Alkaline reagent test

Test solution (small quantity of extract +2 ml of water) in a test tube sodium hydroxide solution was added, solution mixture shows increase in the intensity of yellow color which becomes colourless on addition of few drops of dilute acids.

3) Test for Glycosides

a) Keller-killiani test

To the test solution (small quantity of extract) few drops of ferric chloride solution was added and mixed well. Then concentrated sulphuric acid was added slowly two layers are formed. The upper layer was bluish green coloured and lower layer was reddish brown coloured.

b) Bromine water test

Test solution (small quantity of extract) was dissolved in bromine water gives yellow precipitate was obtained.

4) Test for saponins

a) Foam test

Small quantity of extract was treated with 5 ml of water and shaked well. It shows formation of froth which was found to be stable for about 15 min.

5) Test for steroids

a) Salkowski test

2 mg of extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of the test tube. Formation of red colour indicates the presence of steroids.
b) Lieberman buchard test

2 mg of extracts were dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid added along the sides of the test tube. A brown ring was formed at the junction of the two liquids and the upper layer colour turned to green.

6) Test for tannins

a) Ferric chloride test

0.2 gm of extract was boiled in 5 ml of water. The mixture was cooled and filtered. A few drop of 5% ferric chloride solution were added to the filtrate. Blue-Black precipitation was formed

b) Gelatin test

Test solution (small quantity of extract) taken in a test tube, was treated with gelatin solution, gives white precipitate.

Isolation of pure compound

Isolation work was carried out by adsorption of sample on silica gel and adsorbed sample kept for complete drying and later used for column elution. During the column elution process, fraction of methanol and chloroform shows, different banding pattern in 12:88, 17:83, 22:78 and 28:72 ratio (for 100ml). Thus, shows single, banding pattern and they were kept separately for drying under room temperature. Later, the residue was rinsed with acetone. The sample was air dried and subjected to spectral analysis. The other samples were not worked out, because of low yield and impurity. The pure compound was characterized by spectral analysis through IR, MASS, $^{13}$C NMR and $^1$H NMR.
Results

Table 1.1. Details of preliminary qualitative phytochemical analysis of crude leaf extracts

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phytochemical test for different compounds</th>
<th>Petroleum extract</th>
<th>Chloroform extract</th>
<th>Methanol extract</th>
<th>Water extract</th>
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<tr>
<td>1</td>
<td>Alkaloids</td>
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<td></td>
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</tr>
<tr>
<td>a</td>
<td>Mayer’s test</td>
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<tr>
<td>2</td>
<td>Flavonoids</td>
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<td>Gelatin test</td>
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Note: “+” Presence; “-” Absence
Table 1.2. Preliminary qualitative phytochemical analysis of crude bark extracts

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<th>Methanol extract</th>
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<td>Lieberman-burchard test</td>
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<td>a</td>
<td>Ferric chloride test</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>b</td>
<td>Gelatin test</td>
<td>-</td>
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Note: "+" Presence; "-" Absence

The result of leaf and bark extracts shows that, the highest yield was obtained in the methanol extract, followed by aqueous, chloroform and petroleum ether. In general, the total yield of extract obtained from leaves was found to be more when compared to bark.
The data of preliminary phytochemical screening of secondary metabolites of different solvent extracts of *E. mallotiformis* leaf and bark has given in the Table 1.1 and Table 1.2. Preliminary phytochemical analysis of leaf extract of *E. mallotiformis* revealed, the presence of flavonoids, glycosides, saponin, steroids and tannins whereas alkaloid was found to be absent. The petroleum ether leaf extract shows positive for saponin and shows negative for alkaloids, flavonoids, glycosides, steroids, and tannins. Similarly, the chloroform leaf extract shows the presence of saponin, glycosides and steroid but remaining secondary metabolites were absent in chloroform extract. The analysis of methanol leaf extracts reveals the presence of flavonoids, glycosides, saponin, steroids and tannins; whereas, alkaloids were found to be absent. The analysis of aqueous leaf extract reveals the presence of flavonoids, glycosides, saponin, steroids and tannins. Whereas alkaloids are found to be absent.

Preliminary phytochemical analysis of bark extracts of *E. mallotiformis* revealed the presence of flavonoids, glycosides, saponin, steroids, tannins, whereas, alkaloid was found to be absent. The petroleum ether bark extract showed the presence of saponin and absence of alkaloids, flavonoids, glycosides, steroids, and tannins. The chloroform bark extract shows the presence of saponin, glycosides and steroid and the absence of remaining secondary metabolites. During the analysis of methanol bark extract revealed the presence of flavonoids, saponin, steroids, tannins, whereas, alkaloids and glycosides is found to be absent. The aqueous bark extract revealed the presence of flavonoids, glycosides, steroids and tannins, and the absence alkaloids and saponin.
Isolation and characterization of phytochemical constituents from the leaf of
E. mallotiformis

From the methanolic leaf extract of E. mallotiformis, four different compounds
were isolated and characterized by IR, MS, \(^1\)HNMR and \(^{13}\)CNMR viz., Hesperidin
(Fig. 1.1), Apigenin-7-glycosides (Fig. 1.2), aliphatic tetrol (Fig. 1.7) and 13-labdene-
2,3,8,15-tetrol (Fig. 1.13) were isolated. All the above isolated compounds are found to
be first reported from the test plant E. mallotiformis.

Characterization of the Compounds from Methanol Extract

The details of scientific procedure followed in the isolation and characterization
of above compounds are as below

Spectral Characteristics of Compound F-28:

Physical State: Brownish Yellow powder

Melting point: 258°C-260°C

Soluble in: Methanol

Stability: Hygroscopic, light sensitive, Stable at room temperature

\( \lambda \text{ max: } 255\text{nm- }351\text{ nm (Fig. 1.3)} \)

\(^1\)HNMR (\( \delta, \text{ ppm} \)): 7.0-7.5 (m, Ar-H, Ar-OH)

3.2 (s -O- CH₃)

0.89- 0.91 (s - CH₃)

1.2-1.3 (t, Cyclic Aliphatic hydrogens, alcoholic –OH) (Fig. 1.4)

\(^{13}\)CNMR (\( \delta, \text{ ppm} \)): As the compound is likely to be isomer i.e., mixture of R&S
form lots of peaks are observed in \(^{13}\)CNMR Spectra. 109, 110, 111, 113, 114, 121, 124 are
the peaks at representatives of aromatic carbons. 30, 31, 32, 33, 34, 38, 42, 51, 53, 59,
60, 62, 65, 67 are the representatives of aliphatic carbons (Fig. 1.5).
Mass Spectra: From the mass spectra two molecular mass peaks i.e., at m/e 663.6 and 611.4. Appearance indicate the possibility of Hesperidin corresponds to molecular mass of 611.4 (610) and Apigenin-7-glycosides corresponds to molecular mass 663.6 (662) (Fig. 1.6). From the above observations NMR and mass spectral details the possible structure of fraction F-28 can be mentioned below.

![Fig. 1.1. Hesperidin](image1)

![Fig. 1.2. Apigenin-7-glycosides](image2)
Fig. 1.3. $A_{max}$ Spectra of Compound of F-28 (Hesperidin and Apigenin-7-glycosides)
Fig. 1.6. Mass spectra of compound F-28 (Hesperidin and Apigenin-7-glycosides)
Compound F-22

Physical state: Brownish yellow powder

Soluble in: Methanol

Stability: Hygroscopic, Stable at ordinary temperature

$A_{max}$: 270 nm (Fig. 1.8)

IR (KBr cm\(^{-1}\)):
- 3547.21 (O-H Stretch)
- 2920.32 (CH Aliphatic Stretch) (Fig. 1.9)

\(^1\)H NMR (δ, ppm):
- 3.3-3.8 (t- Aliphatic Hydrogens)
- 1.22-1.3 (s, CH\(_3\) Hydrogens)
- 0.8-0.9 (Alcoholic hydrogens) (Fig. 1.10)

\(^13\)CNMR (δ, ppm):
- 14.4, 23.6, 26.9, 30.0, 30.1, 30.4, 30.5, 30.6, 31.7, 33.0, 34.8 (Aliphatic carbons and alcoholic carbons) (Fig. 1.11)

Mass spectra:
- Mass peak at m/e 322.2 almost near to mass peak of aliphatic tetrol (m/e 320) (Fig. 1.12)

The IR spectra of isolated fraction F-22 shows the peaks at 3547.21 and 2920.32 corresponding to OH stretch and CH aliphatic stretch. The \(^1\)H NMR spectra indicates the presence of aliphatic hydrogen at δ 3.2-3.8 methyl hydrogen at δ 1.22-1.3 and alcoholic hydrogen at δ 0.8-0.9. Similarly, \(^13\)CNMR shows the number of peaks corresponds to aliphatic hydrocarbons. These observation conforms that isolated fraction is aliphatic in nature and not aromatic (No multipletes in \(^1\)H NMR at δ 6.5-7.5 ppm are observed) in mass spectra mass peak at m/e 322.2 is observed and is almost near to the mass peak at aliphatic tetrol (m/e 320).
Fig. 1.7. Aliphatic tetrol

Fig. 1.8. $\lambda_{\text{max}}$ spectra of compound of F-22 (Aliphatic tetrol)
Fig. 1.10. $^1$HNMR ($\delta$, ppm) spectra of compound F-22 (Aliphatic tetrol)
Fig. 1.11. $^{13}$CNMR ($\delta$, ppm) spectra of compound F-22 (Aliphatic tetrol)
Compound F-12

Physical state: Greenish yellow

Soluble in: Methanol

Stability: Hygroscopic, Stable at ordinary temperature

$\lambda_{max}$: 325 nm (Fig. 1.14)

IR (KBr, Cm$^{-1}$): 3042.60 (O-H Stretch)

2849.20 (CH Aliphatic Stretch)

1649.26 (-C=C- Stretch Alkenes) (Fig. 1.15)

$^1$HNMR ($\delta$, ppm): 3.2-4.09 (t, Aliphatic hydrogens)

1.28-1.33 (d, Alkenes hydrogens)

0.85-1.0 (d, Alcoholic hydrogens) (Fig. 1.16)

$^{13}$CNMR ($\delta$, ppm): 132.3 and 129.8 (Alkenes carbons)

60.8 (Carbons linking between side chain and cyclic carbons)

72.0, 72.5, 72.9, 73.4, 73.7, 74.2, 84.9 (Cyclic carbons)

14.4, 19.4, 23.7, 26.0, 28.9, 30.2, 30.4, 30.7, 31.8, 33, 34.9

(Aliphatic carbons) (Fig. 1.17)

Mass Spectra: the mass spectra shows the appearance of mass peak at m/e 342.3 which is near to mass peak 340.26, which is 13-labdene-2,3,8,15-tetrol (Fig. 1.18).

The IR (KBr cm$^{-1}$) spectra of compound F-12 shows peaks at 3402.60 corresponds to O-H stretch of alcoholic group, 2849.20 corresponds to CH aliphatic stretch and 1649.26 corresponds to $-C=\equiv C-$ stretch of alkenes. Similarly, the peaks corresponds to aliphatic hydrogen, alkenes hydrogen and alcoholic hydrogen are observed at $\delta$ 3.2-4.09, 1.28-1.33 and 0.85-1.0 respectively in $^1$HNMR spectra. Also
$^{13}$CNMR spectra clearly indicate the presence of alkene carbons at δ 132.3, and 129.8, at δ 60.8, 72.0, 72.5, 72.9, 73.4, 73.7, 74.2, 84.9 the aliphatic carbons. The mass spectral studies reviles the appearance of mass peak at m/e 342.3 it is almost near to the mass peak of 13-labdene-2,3,8,15-tetrol (340.26). This is having all the structural features exhibited by the isolated compound. These observation matches with the chemical test performed on crud extracts, i.e., test for steroids. Here the isolated compound is having structural features matching with conventional steroids. Therefore, from the above observation of IR, NMR, Mass Spectra details the possible structure of the isolated fraction F-12 is as below.

![Fig. 1.13. 13-labdene-2,3,8,15-tetrol](image)
Fig. 1.14. $\lambda_{\text{max}}$ spectra of compound of F-12 (13-labdene-2,3,8,15-tetrol)
Fig. 1.15. IR spectra of compound F-12 (13-labdene-2,3,8,15-tetrol)
Fig. 1.17. $^{13}$CNMR (δ, ppm) spectra of compound F-22 (13-labdene-2,3,8,15-tetrol)
Fig. 1.18. Mass spectra of compound F-12 (13-labdone-2,3,8,15-tetrol)
Discussion

Plants synthesize and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations and industrial utilization. Medicinal plants are popularly known as "Chemical Goldmines" as they contain natural chemicals. All these chemicals cannot be synthesized in laboratories. Many secondary metabolites of plants are commercially important and find use in a number of pharmaceutical companies.

The aim of using plants as sources of therapeutic agents is to isolate bioactive compounds for direct use as drugs or yield bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and lower toxicity.

In the present study *E. mallotiformis*, a medicinal plant, grown in evergreen forest of the Western Ghats region of Karnataka, was evaluated for its phytochemical analysis and pharmacological activities. Preliminary phytochemical analysis of different solvent leaf extracts shows the presence of flavonoids, glycosides, saponins, steroids and tannins, while, alkaloid found to be absent. The different solvent bark extracts of *E. mallotiformis* showed the presence of flavonoids, glycosides, saponins, steroids and tannins, while, alkaloid found to be absent in all kinds solvent extracts. The above results are conformity with the findings of Savithramma *et al.* (2011a,b), Alphonso *et al.* (2012) and Edeoga *et al.* (2005).

Flavonoids are biologic antioxidant sources. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (Burlon and Ingold, 1984). Tannins are polyphenolic compounds and have a wide range of effects varying
from decreasing availability of proteins and other nutrients including amino acids and minerals to protecting ruminants from bloat, enhancing rumen bypass protein, improving meat quality and decreasing helminth infestation and a good astringency i.e., fasten the healing of wounds and inflamed mucous membrane. Tannins are also known to have antimicrobial, antimitagenic, antiinflammatory and antioxidant properties (Makkar et al., 2009).

Saponins have also been known to have several health beneficial effects, for example, enhancement of immunity, reduction in blood glucose and other antidiabetic effects and reduction in blood cholesterol (Franci et al., 2002). According to Argentieri et al. (2007) and Francis et al. (2002), saponins also have anti-protozoal effects and could potentially be used for controlling protozoal diseases and also has strong antifungal, antinematode, molluscicidal, and insecticidal properties.

Drug and pharmaceutical industries have great value of steroids and their related active metabolites. Steroids have diversified physiological functions and pharmacological effects. Most of these compounds contain alcoholic group. Steroid includes a variety of compounds, among which sapogenins hold a very important position. Sapogenins when linked with sugar constitutes the saponins (Singh and Kaushal, 2007).

From biological point of view, glycosides play a significant role in the life of plants involving its regulatory, transperatory and protective functions. It has wound healing properties and antimicrobial properties (Stevenson et al., 2002).

In order to promote Indian herbal drugs, there is an urgent need to evaluate the therapeutic potentials of the drugs as per WHO guidelines (Anonymous 1, 2000). Patwardhan et al. (2004) mentioned that, 30% of the global sales of drugs are based on
plant products. Traditional indigenous medicine is limited to small tribal and geographical areas called “little traditions” are an outstanding repository of knowledge about medicinal properties of botanical sources. Kamboj (2000) stated that the bioactive extract should be standardized on the basis of phytochemical compounds.

During the isolation of document of phytochemical constituents from methanolic leaf extract produce four distinct banding patterns, all these were eluted through TLC and they were isolated separately through the column chromatography. The eluted and isolated fractions from TLC and column chromatographic techniques were further subjected to spectral analysis viz., IR, NMR and MS. Based on the spectral analysis four different phytochemical compounds were recorded from leaf methanolic extract, They are Hesperidin, Apigenin-7-glycosides, aliphatic tetro, 13-labdone-2,3,8,15-tetrol the above finding are confirmatory with the findings of earlier researchers. The apigenin-7-glycosides were isolated from Euphorbia humifusa (Ying et al., 2009). Apigenin-7-glycosides from Salvia macrosiphon (Gohari et al., 2011), Hesperidin, was isolated from Citrus reticulata (Ma et al., 2008).

Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutically and industrially important compounds. It is imperative to initiate an urgent step for screening of plants for secondary metabolites. In present study assessment has done for phytochemical properties in leaf and bark extracts and isolated pure compounds Hespsridin, Apigenin-7-glycosides, aliphatic tetro and 13-labdone-2,3,8,15-tetrol of Epiprinus mallotiformis to improve the health status of people and also to use in pharmaceutical and nutraceutical products of commercial importance.
Plate -1.2

A - Habit of *Epiprinus mallotiformis*
B - Twig of *Epiprinus mallotiformis*
C - Inflorescence of the *Epiprinus mallotiformis*
D - Collection of leaf sample
E - Collection of bark sample
Plate -1.3

A - Soxhlet extraction unit
B - Column chromatography
C - Collecting different fractions of pure compound
References


Flamini G, Antognoli E, Morelli I. 2001. Two flavonoids and other compounds from the aerial parts of *Centaurea bracteata* from Italy. *Phytochem.*, 57: 559-564.


Seidela V, Bailleulb F and Watermanab PG. 2000. (Rel)-1β,2α-di-(2,4-dihydroxy-6-methoxybenzoyl)-3β, 4a-di-(4-methoxyphenyl)-cyclobutane and other flavonoids from the aerial parts of Goniothalamus gardneri and Goniothalamus thwaitesii. Phytochem., 55(5): 439-446.


