CHEMICAL INVESTIGATION ON

PLECTRANTHUS COESTA

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SHOBHA WALDIA

M.Sc.

DEPARTMENT OF CHEMISTRY

L.S.M.GOVT. P.G. COLLEGE

PITHORAGARH (U.K.) - 262501

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Date (SHOBHA WALDIA)

CONTENTS

PREFACE

The system of herbal medicine known as Ayurveda has been followed in India from time immemorial. The germ plasm for this science which is now receiving world-wide attention can be traced to the most ancient of Sanskrit texts known as the Vedas. Near about 700 species of important medicinal plants from the real back bone of Ayurveda. Since disease, decay and death have always co-existed with life. The study of diseases and their treatment must have been contemporaneous with the dawn of the human intelligence. The primitive man must have used those things as therapeutic agents and remedial procedures which he able to produce easily. The importance is still retained in spite of spectacular advances in synthetic drugs in recent years. The whole Himalayan belt of Kumaun is a rich source of several medicinal plants in India. There are more than 8,000 plant species in South Asia with know medicinal uses.

Approximately one third of all pharmaceuticals are of plant origin. If bacteria and fungi are included, over 60% of the pharmaceutical preparations are plant based. So preparations based on natural products (mostly medicinal plants) have been employed since time immemorial for the treatment of human aliments.

The Lamiaceae (Labiateae) is an Angiospermic family represents a valuable pool of plant species which contains biological active molecules, found in most of the habitats and altitudes worldwide. It contains several genera, such as mint (Mentha), sage

(Salvia) and basil (Ocimum). Another important genus is *Plectranthus*. The family Lamiaceae consists of 3, 800 species grouped in 200 genera. In India about 64 genera with 380 species are found, mainly in hilly areas. The chemistry of *Plectranthus* remains relatively unknown, but several plants have been studied chemically, diterpenoids are the more common secondary metabolites in them. The majority of them are highly modified abietanoides in addition to ent-kauranes and phyllocladanes. Essential oils, triterpenoids, flavonoids and long-chain alkylphenols are also isolated. They are used all around the world as antiseptics, vermicides, purgatives, infections, toothache, stomachache and commonly used for the treatment of allergy.

The author has undertaken the study of *Plectranthus coesta* (Lamiaceae), widely used for medicinal purposes.

The subject matter of the thesis has been divided into six chapters, which are further sub divided into different parts.

Chapter 1 of the thesis is review on "Genus *Plectranthus* in India and its chemistry".

Chapter 2 is Chemical examination of *P. coesta* which comprises plant's previous and present work.

Chapter 3 of the thesis is Results and Discussion, in which the results of the plant constituents identified by different chemical and spectral methods have been discussed.

Chapter 4 is Experimental which comprises of plant collection and its identification by means of chemical methods as well as spectroscopic methods.

Chapter 5 includes Biochemical and Elemental analysis.

(5a) Material and methods

(5b) Results and Discussion

Chapter 6 includes Biological activity.

The references of literature concerned are given at the end of each chapter.

Date **Shobha Waldia**

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INTRODUCTION

Man has always utilized natural products for different purposes, of which medical application is the most common. Indeed, the first documents of traditional medicine trace back to the ancient ages[1]. Besides healing agents, natural products have been used as poisons, narcotics, hallucinogens, stimulants, perfumes, spices, dyes and so on[2].

More precisely, the term 'natural products' stands for secondary metabolites; compounds produced often species specific by microorganisms, fungi, plants or animals that are not essential for the survival of the organism. The biosynthesis and degradation of secondary and primary metabolites (sugars, polysaccharides, amino acids, proteins, common fatty acids, lipids, nucleotides, RNA, and DNA) are interrelated[2].

The relevance of natural product research in drug discovery is a subject of discussions in the pharmaceutical industry today. Nevertheless, according to a study in the 1990's more than 50% of the most often prescribed drugs in the US contained a natural product or a structurally related derivative[1]. Hence there is hardly any doubt about the importance of natural products. In fact, natural product research is much more than searching for compounds that are potentially beneficial to men (as medicines, fragrances, insecticides). Who knows what kind of role antibiotics from microorganisms play in nature other than defense or which additional physiological functions pheromones may have[3]? Investigating natural products requires joint work of organic

chemists, biochemists, biologists and medical scientists. It offers at the same time challenging problems to solve: ultra-trace analysis in complex matrices, synthesis of complicated structures, revealing biosynthetic mechanisms, also on the level of genetics (combinatorial biosynthesis), and exploration of substrate-receptor interactions[3].

Early milestones of the history of natural products are records about plants and extracts used for medical purposes in the ancient world. The use of different plant oils (from Cedrus, Cupressus species, and Papaver somniferum) were documented in Mesopotamia as early as in about 2600 BC. Several hundred drugs, some also of animal origin were recorded in Egyptian 1000 years later, although observations began long before. From about 1100-1000 BC stem the first documents from Asia (China, India). It were the Greeks that added new facts to the field of herbal drugs in the last hundred years of the ancient western culture. This medical knowledge was used in the early centuries of the mediaeval western world. However, Arabic and Persian scholars completed their own experience with the Greco-Roman and Asian heritage and became real experts at the same time.1 Personal documents about the use of natural products for healing purposes in the mediaeval ages are recipes of plant extracts from medical researchers in the 16th and 17th century. Nevertheless, the next stage of development, the isolation of active substances, such as morphine, strychnine, quinine, caffeine, nicotine, codeine, camphor, and cocaine was reported first

in the 19th century[2]. It was a great challenge for chemists to work out total synthetic routes and structure elucidation techniques. These efforts lead in the 20th century to the development of research methods to explore biosynthetic pathways. Due to the results of natural product research in the last decades we know more about the ecological function of theses substances, how they contribute to the competitiveness of the parent organisms[2].

Modern pharmaceutical research based on screening of natural products began with the exploration of antibiotics. The trigger of the research work directed to antibiotics was the isolation of penicillin in 1929, followed by intense screening of bacteria and fungi after the World War II. Interest in natural product research rose again after the thalidomide scandal in the 1960's. As a consequence, secondary metabolites were applied in other pharmaceutical research fields, for instance in the development of anti-cancer drugs. One of the success stories was the discovery of the diterpene Paclitaxel, isolated from the bark of the yew tree, Taxus brevifolia, used under the trade name Taxol for the treatment of ovarian and breast cancer4, and being tested against other cancer types. In a study of the pharmaceuticals on the market from 1981-2002, only 43% of the drugs were purely synthetic, while the remaining 57% were derived from a natural source[5].

In spite of the good results of natural product research combinatorial chemistry has been a major competitor in the pharmaceutical industry. Isolation and structure elucidation of the

compound responsible for the desired biological activity from the natural extract found by screening are highly time- and cost-intensive steps. In contrast, it is cheaper and faster to achieve the first clinical phase once a compound generated by combinatorial methods is proved to be active, since its structure is already known[4].

Nevertheless, combinatorial chemistry produces structures without known function in the environment. A further problem in natural product research is the difficulty of supply of source organism or the uncertainty of future supplies, for example of marine organisms. This is especially crucial in case of complicated natural products, where synthetic production is not possible. For this reason, microorganisms that easily grow in cell culture are still preferred. Some firms (Phytopharmaceuticals, Phytera) work with plant cell cultures to avoid the problem[4].

As a new trend, natural product research is integrated, even though to a small percent (5-20%), in the drug discovery program of large pharmaceutical companies (Merck, Novartis). It is not the principal goal any more to find ready-to-use natural drugs, but to perform a large scale biological screening and find as many interesting substances as possible with high structural diversity. These compounds are applied as lead molecules for further investigation: combinatorial development, or chemical modification to simplify the structure or improve pharmacological properties[1,4]. In addition, smaller companies have been established to devote their efforts fully to natural product research. They usually specialize in a

particular task. As a unique example, Shaman Pharmaceuticals Inc. in San Francisco organizes sample collections based solely on ethno-biological information (traditional medicine)[4]. Though investments are fluctuating, the role of natural products in drug discovery remains important. It is still nature that provides the highest structural diversity.

Exploration of the high complexity of natural products requires constant technical development, which again, results in discovery of novel structures. This automatism is the key of the future success of natural product research.

The aim of this Ph.D. work was chemical investigation of *Plectranthus coesta* for getting biologically active compounds.

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Chapter -1

REVIEW

GENUS PLECTRANTHUS IN INDIA AND ITS CHEMISTRY

ABSTRACT

In India genera *Plectranthus* is found in all the habitats and altitudes most likely in Himalaya, Southern Ghat and Nilgiri range, have been used in traditional medicines and ornaments with economical interest. The phytochemical study of the genera reveals that the most secondary metabolites are abietane diterpenoides with rich essential oils and triterpenoids. A group of long – chain alkylphenols, of possible taxonomic significance in *Plectranthus sp*., were also isolated.

KEY WORDS

Plectranthus, Lamiaceae, abietane diterpenoides, triterpenoids.

CONTENTS

INTRODUCTION

The Lamiaceae is an Angiospermic family represents a valuable pool of plant species which contains biological active molecules[1], found in most of the habitats and altitudes worldwide. It contains several genera, such as mint (Mentha), sage (Salvia) and basil (Ocimum). Another important genus is *Plectranthus*. The first discription about *Plectranthus L' He'r* was given by Charles Louis de L'Héritier breeding Elle was, published in 1788[2]. *Plectranthus* (harp shrubs) is a plant genus in the family Lamiaceae belongs to sub family Nepetoideae of tribe Ocimeae[3]. The genus *Plectranthus* name derives from the Greek for *plektron* Sporn and *anthos* for Blume, also translated means "Valerian", because the flowers at their base a tube (Sporn). Because of the scent of the plant are also many types moth King. *Plectranthus* genus is related to Salvia, Rabdosia and Coleus[4]. Some of the species were formally placed under *Plectranthus* which are being known as Isodon[5]. About 62 species of *Plectranthus* are used for ornaments, medicines with economic interest, along with a rich diversity of ethnobotanical uses. They are used all around the world as antiseptics, vermicides, purgatives, infections, toothache, stomachache and commonly used for the treatment of allergy[6]. The chemistry of *Plectranthus* remains relatively unknown, but several plants have been studied chemically, diterpenoids are the more common secondary metabolites in them. The majority of them are highly modified abietanoides in addition to ent-kauranes and phyllocladanes.

Essential oils, triterpenoids, flavonoids and long-chain alkylphenols are also isolated[7].

The genus *Plectranthus* consists of some 300 species, distributed from Africa through to Asia and Australia[8]. About 40 *Plectranthus* species grow in India[9], of which *P. macranthus, P. mollis, P. stocksii, P. coesta and P. incanus* are the more common species[10].

(I)Essential Oils

Essantial oils are isolated from different parts of the plant and are complex, volatile mixture of secondary metabolites and responsible for the fragrance, aromatic as well as the medicinal properties. Due to those virtue most of the plants are used in perfumery, food, bevrages industry and also used in therapeutic applications[11]. Essential oils are generally extracted by distillation. Other process include solvent extraction. The genera *Plectranthus* of sub family Nepetoideae, is rich–in essential oils[12]. The main constituents of essential oils of *Plectranthus* are mono and sesquiterpenes. On the GC column (fused silica capillary), essential oil of *P. amboinicus* was separated into α-pinene, camphene, 1-octen-3-ol, β-pinene, myrcene, α-phellandrene, Δ^3-carene, α-terpinene, *p*-cymene, limonene, (Z)-β-ocimene, (E)-β-ocimene, α-phelandrène, γ-terpinene, α-terpinolene, linalool, camphor, 1-terpinen-4-ol, α-terpineol, thymol, carvacrol, α-cubebene, β-cubebene, β-elemene, β-caryophyllene, α-bergamotene, (Z)-β-

farnesene, α-humulene, β-guaiene, (-)-α-selinene, β-bisabolene, δ-cadinene, caryophyllene oxide, δ-cadinol, α-cadinol, farnesol, calamenol and (-)-4β-7β-aromadendrandiol[13]. The major component of essential oil of *P. amboinicus* was carvacrol[14]. The major constituents of *Plectranthus barbatus Andr*. are α–pinene (22.2%) in the leaves, β–phellandrene (26.1%) in the stems and (Z)-β–ocimene (37.6%) in the root while analyzed by GC/MS[15]. Essential oil of *P. fruticosus* contains linalool, α-thuyene, aromadendrene, β-bourbonene, terpinen-4-ol, γ-cadinene, sabinene, α-elemene, sabinyl acetate, γ-terpinene, α-humulene, α-cubene, β-bisabolene, *trans*-copaene and *trans*-farnesol[16]. The main component of the oils in *P. madagascariensis* is diterpene, 6,7-dehydroroyleanone, isolated as orange-to-reddish crystals, which represented 28%, 87% and 41% of the oils from the flowers and the leaves collected during the flowering and vegetative phases, analyzed by hydrodistillation and distillation extraction[21]. *P. melissoides* contain mostly oxygenated monoterpenes and monoterpene hydrocarbons. The main components of the oil were carvacrol (41.3%), p-cymene (17.4%), γ–terpinene (10.1%), methyl thymol (3.0%), thymol (7.9%) and carvacrol acetate (4.6%) by GC and GC/MS[22]. While examining *P. rugosus* by GLC, NMR and mass spectrometry the main constituents are germacrene D, caryophyllene, α–pinene, α–phellandrene, caryophyllene oxide, δ-cadinene, p-cymene, α–cadinol, limonene, β–phellandrene and myrcene[9]. In *P. vestitus* some of the following essential oils were

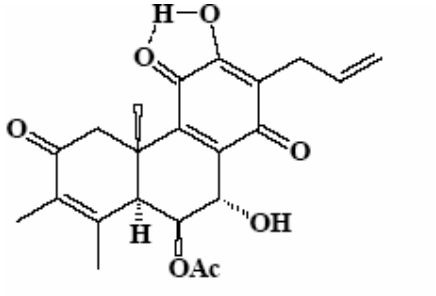
found with their respective concentration– β-caryophyllene (26.5%), cadinene (21.7%), geranyl acetate (9.3%), humulene (6.5%), farnesene (6.2%), 1,8-cineole (2.7%), neral (5.1%), isobornyl acetate (4.6%), and germacrene D (2.2%)[23].

(II) Diterpenoides

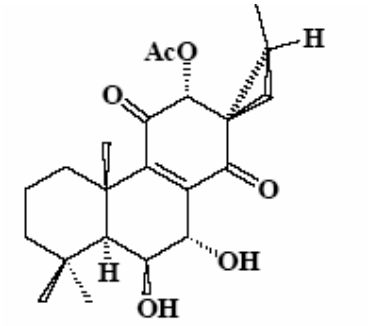
Diterpens are hydrocarbons or their derivatives containing four isoprene units hence containing twenty carbon atoms and four branched methyl groups. eg.–Vitamin A, retinene, aconitina, derived from geranylgeraniol pyrophosphate.

Diterpenoids, a class of naturally occurring secondary metabolides are found mainly in Angiospermae plants with a large variety of skeleton[24]. These are found mostly in Lamiaceae[25] and Asteraceae[26], among Angiospermae. The majority of the diterpenoids are isolated from the leaf–glands of *Plectranthus* species. Most of them are highly abietanoids, in addition to some ent-kaurenes and phyllocladanes. In table-1 the name of diterpenoids are listed. About 41 diterpenoids (1-41) and other chemical constituents in *Plectranthus* species are shown in table-2.

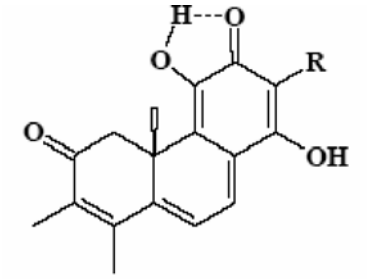
Structure of diterpenoids



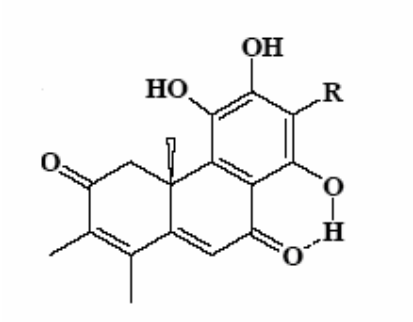
[1]



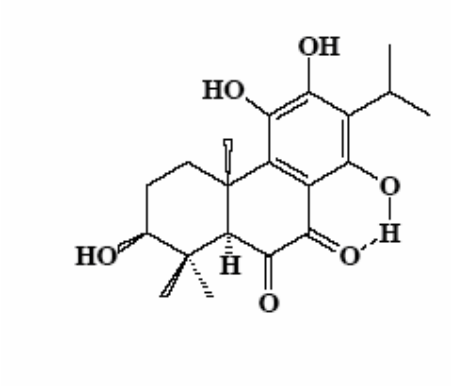
[2]



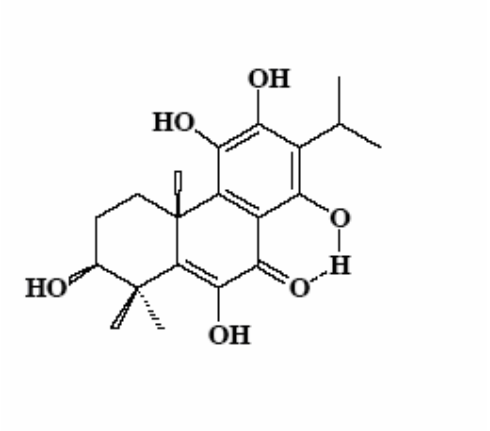
[3] R= $CH_2CH=CH_2$
[4] R=(S)-$CH_2CH(OH)CH_3$



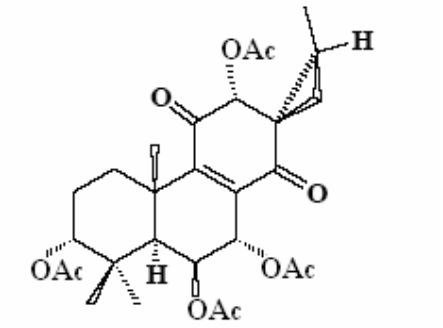
[5] R=$CH_2CH=CH_2$
[6] R=(R)-$CH_2CH(OH)CH_3$
[7] R=CH $(OH)CH_3$



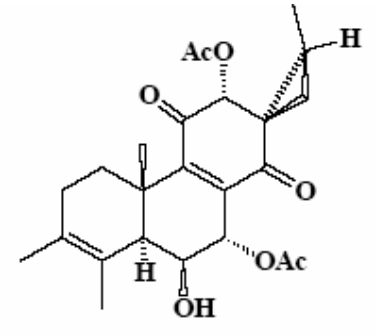
[8]



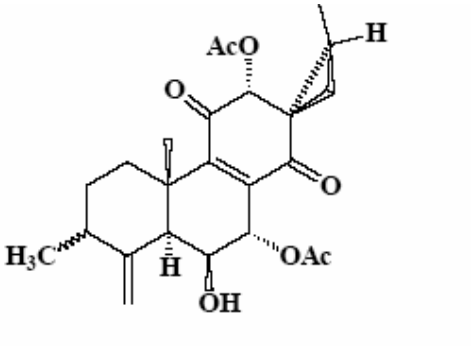
[9]



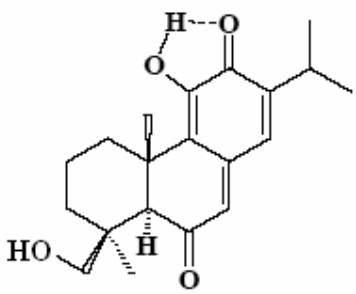
[10]



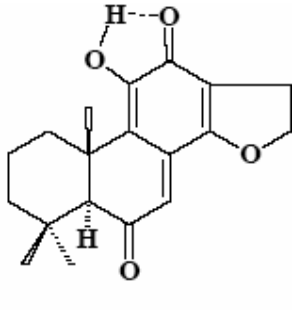
[11]



[12] α- CH_3
[13] β- CH_3



[14]



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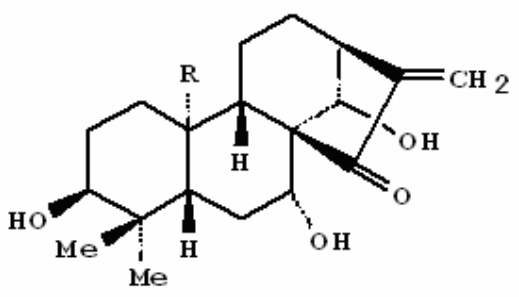
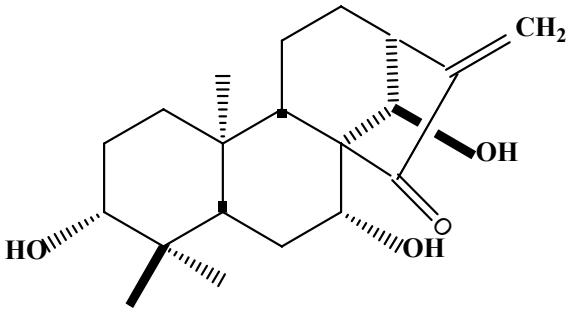


[16] R=CH_2OH



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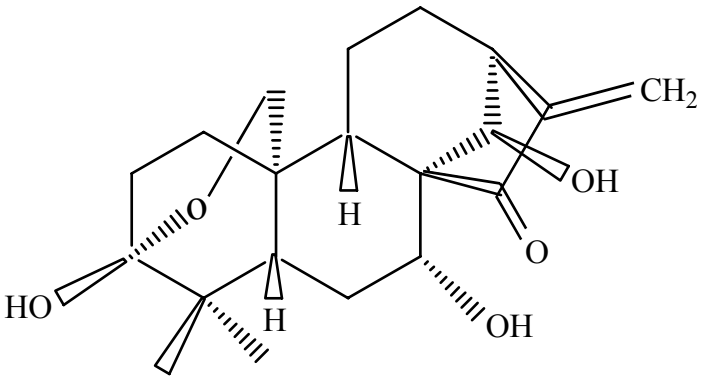

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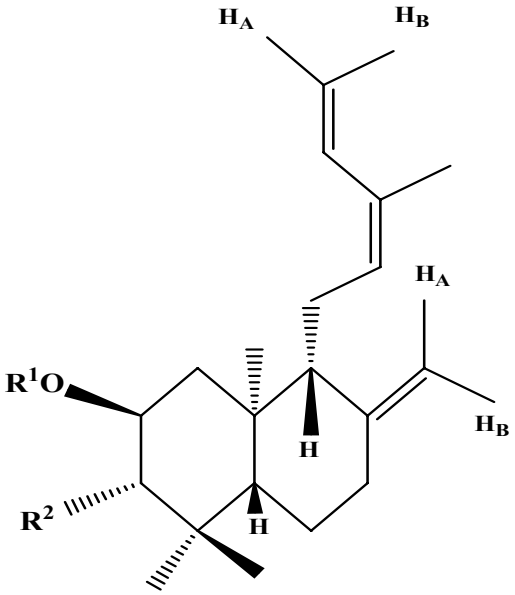
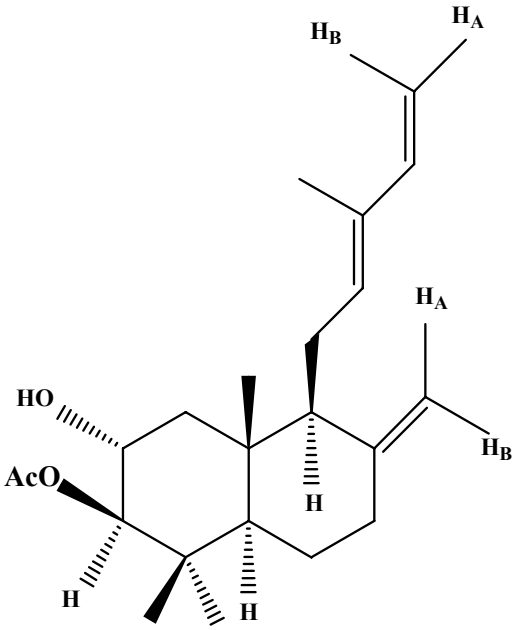


[19] $R^1 = R^2 = H$
[20] $R^1 = Ac$, $R^2 = OH$



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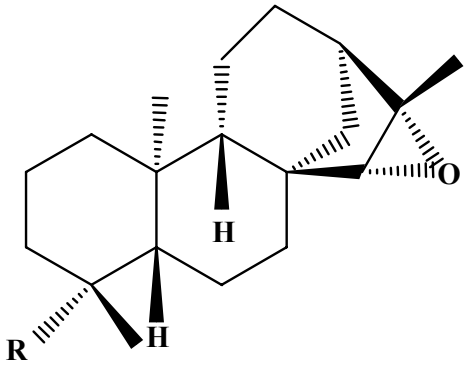
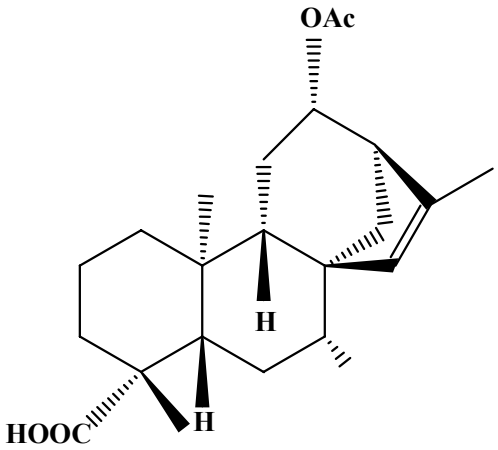
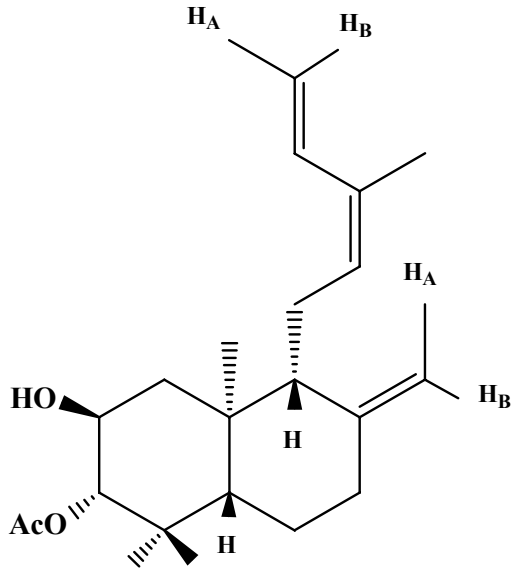


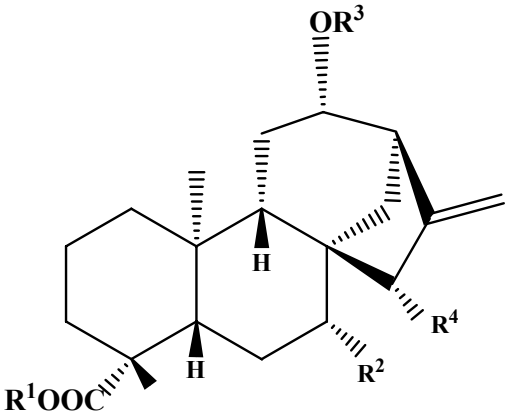
[22] R= COOH
[23] R= CH_2OH



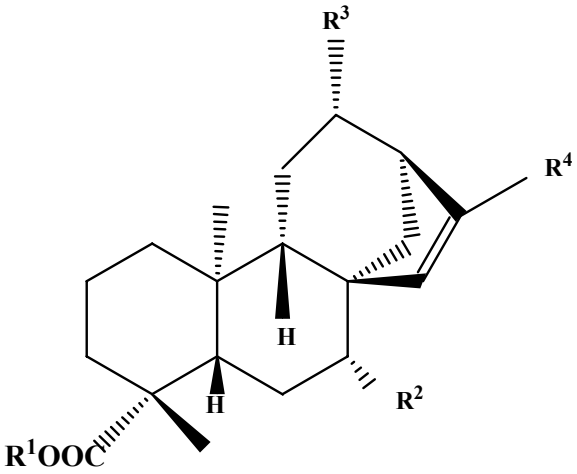
[24]



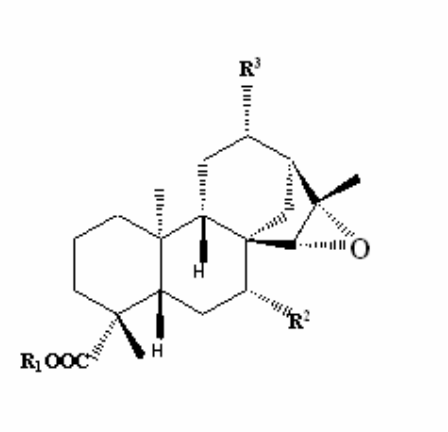
[25]



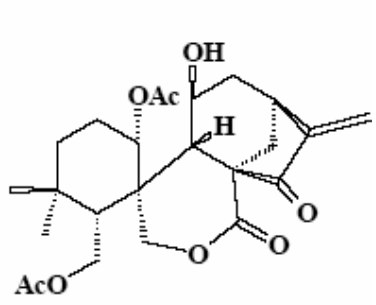
[26] $R^1 = R^2 = H$, $R^3 = Ac$, $R^4 = OH$
[27] $R^1 = R^4 = H$, $R^2 = OH$, $R^3 = Ac$



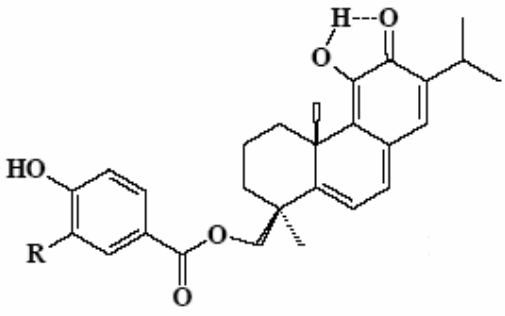
[28] $R^1 = R^3 = H$, $R^2 = OH$, $R^4 = Me$
[29] $R^1 = H$, $R^2 = OH$, R3 = OAc, $R^4 = Me$
[30] $R^1 = R^2 = H$, $R^3 = OAc$, $R^4 = CHO$



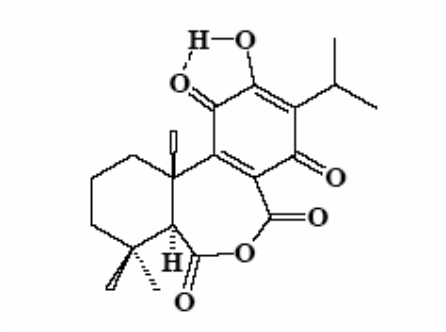
[31] $R^1 = R^2 = H$, $R^3 = OAc$
[32] $R^1 = R^3 = H$, $R^2 = OH$



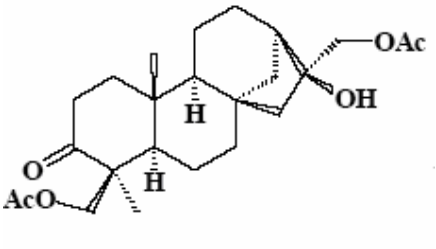
[33]



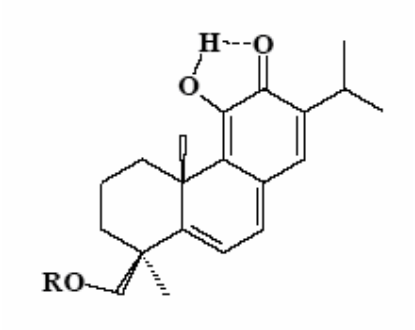
[34] R = H
[35] R = OH
[39] R = OCH_3



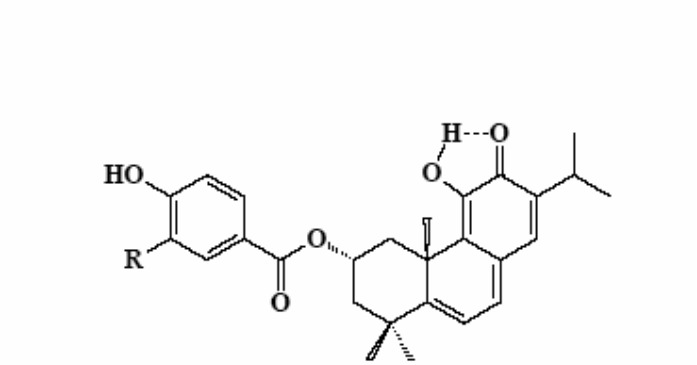
[36]



[37]



[38] R= COCH=C $(CH_3)_2$



[40]R=H
[41]R=OH

Table-1

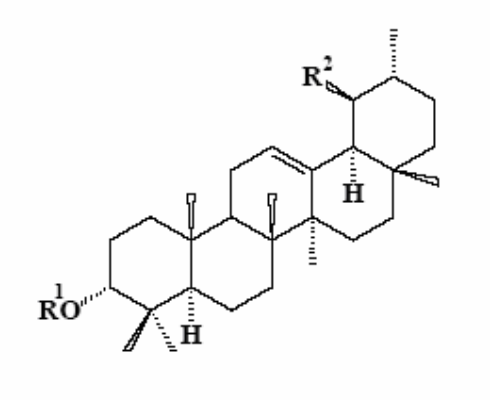
Names of Diterpenoids in Genus Plectranthus in India

Diterpenoid	Name
1	Plectranthone J
2	Plectrin
3	Coleon F
4	(16S)- Coleon E
5	Plectrinone B
6	(16R)-Plectrinone A
7	Plectrinone A
8	Coleon T
9	Coleon S
10	Coleon R
11	Coleon M
12	7, 12-Diacetylcoleon J
13	Coleon N
14	Coleon Q
15	Coleon P
16	Coestinol
17	ent-3α,7β, 14α–trihydroxykaur–16–en-15- one
18	Plecostonol
19	ent-Labda-8(17), 12Z,14-trien-2 α-ol

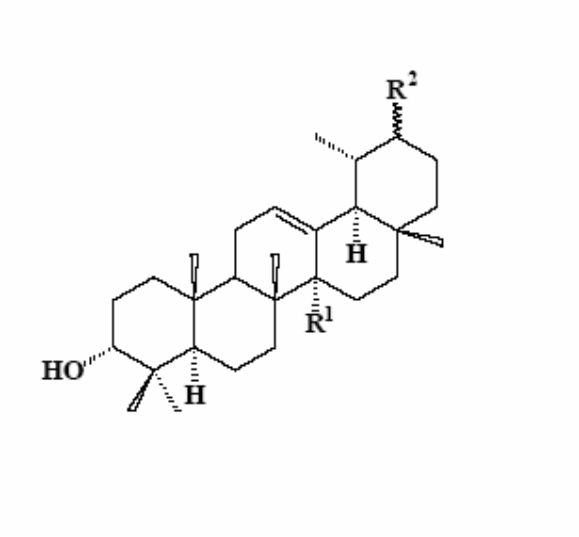
20	ent- 2α–Acetoxylabda-8(17), 12Z,14-trien-3β –ol
21	3β–Acetoxylabda-8(17), 12E,14-trien-2α –ol
22	ent-15β, 16β–Epoxykauran-19-oic acid
23	ent-15β, 16β–Epoxykauran-19-ol
24	Kauran-19-oic-acid
25	ent-3β-Acetoxylabda-8(17),12Z,14-trien-2α-ol
26	ent-12β–Acetoxy-15β-hydroxykaur-16-en-19-oic acid
27	ent-12β–Acetoxy-7β–hydroxykaur-16-en-19-oic acid
28	ent-7β–Hydroxykaur-15-en-19-oic acid
29	Methyl ent-12β-acetoxy-7β hydroxykaur-15-en-19-0ate
30	ent-12β-Acetoxy-17-oxokaur-15-en-19-oic acid
31	Methyl ent-12 β-acetoxy-15β**,** 16β-epoxykauran-19-oate
32	ent-7β-Hydroxy-15β, 16β-epoxykauran-19-oic acid
33	Rabdosin B
34	Parviflorone C
35	Parviflorone E
36	Sanguinon A
37	(16R)-17, 19-Diacetoxy-16-hydroxy-13β-kauran-3-one
38	Parviflorone A [=11- Hydroxy-19-senecioyl- oxy-5, 7, 9(11), 13-abietatetraen-12-one]
39	Parviflorone B
40	Parviflorone D
41	Parviflorone F

(III) Triterpenoids

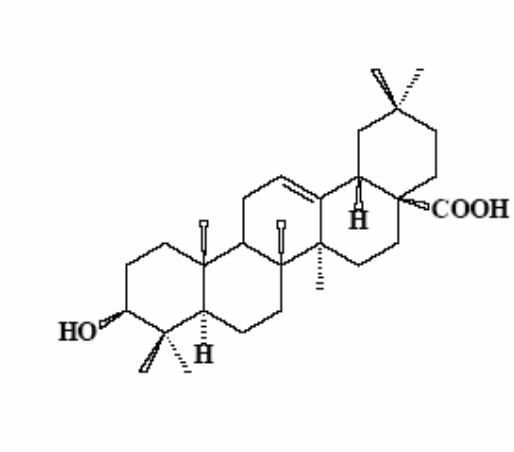
Triterpenoids contain thirty carbon atoms and have been isolated from plants as well as from animals. The important triterpenoids are squalene (acyclic), ambrein (tricyclic), ianosterol (tetratcyclic) and amyrins (pentacyclic). Five triterpenoids plectranthoic acid (**42**), acetylplectranthoic acid (**43**), plectranthadiol (**44**), plectranthoic acid A (**45**) and plectranthoic acid B (**46**) in addition to β-sitosterol were isolated from *P. rugosus*[38-39]**.** Oleanolic acid (**47**), ursolic acid (**48**) and betulin (**49**) in addition to β-sitosterol and hexacosanol were isolated from same species by Misra et al[40]**.** From the roots of *P. straitus* ursolic acid (**48**), Oleanolic acid (**47**) in addition to β-sitosterol were isolated[41]. From *P. fruticosus* aromadendrane-type sesquiterpenoid namely 10(14) – Aromadendrene-4β, 15 –diol (**50**) isolated[34].



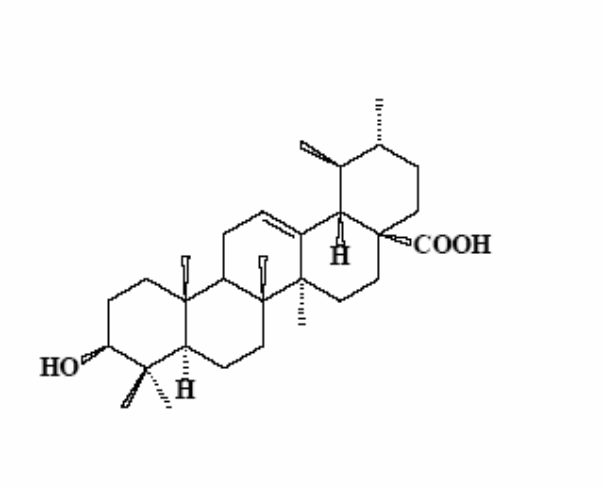
[42] R^1=H, R^2=COOH
[43] R^1=Ac, R^2=COOH
[44] R^1=H, R^2=CH_2OH



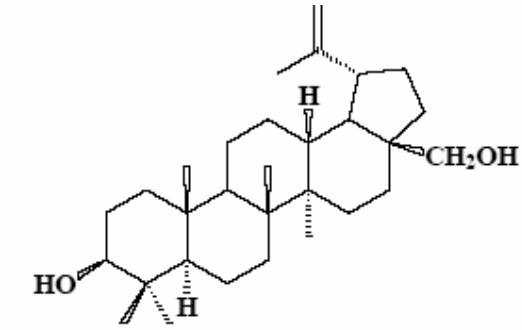
[45] $R^1 = CH_3$, $R^2 = \beta$-COOH
[46] R^1=COOH, $R^2 = \alpha$ -CH_3



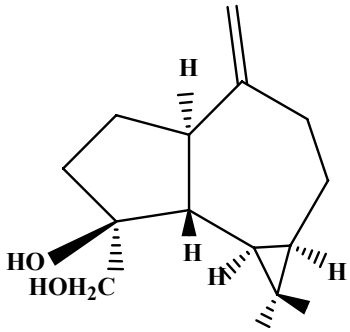
[47] Oleanolic acid



[48] Ursolic acid



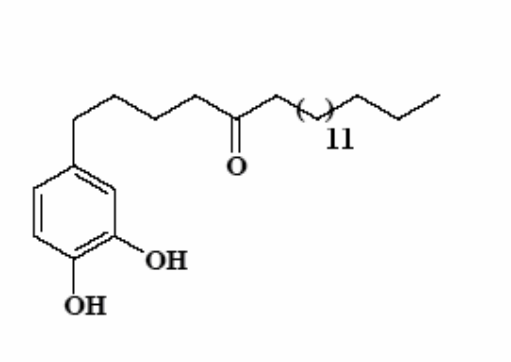
[49] Betulin



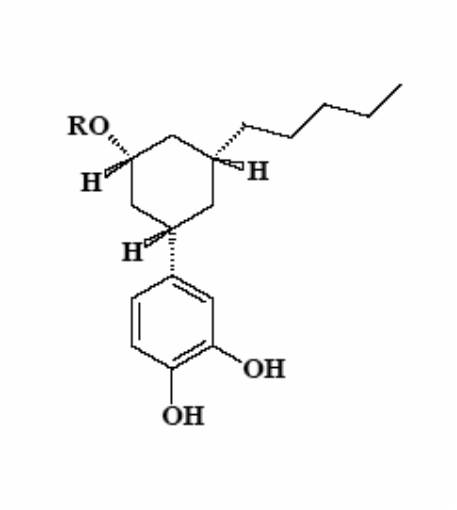
[50]

(iv) Long-Chain alkylphenols

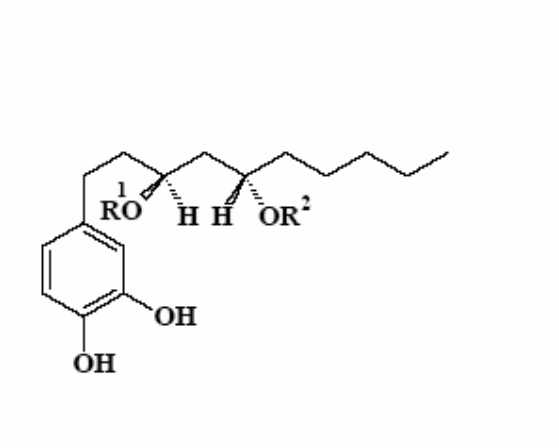
A group of long-chain alkylphenols has been isolated from fractionation of extracts of P. sylvestris[42], showed antioxidant activity. Long-Chain alkylphenols **(51-57)** yielded by HPLC separation.



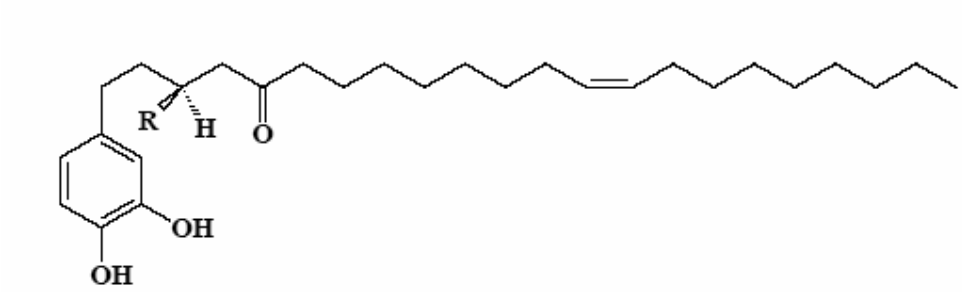
[51]



[52] R= Ac
[53] R=H



[54] R^1=H, R^2=Ac
[55] R^1=Ac, R^2=H



[56] R=OH
[57] R=H

(V) Miscellaneous

From *P.mollis(= P. incanus)* vernolic and cyclopropenoid fatty acids were isolated, reported by Mahmood et al.[19]. From *P. scutellarioides* sugar and protein contents isolated[43].

Table-2

List of Plectranthus species with Isolated Compounds

Plectranthus Species	Isolated chemical constituents	References
P. ambonicus (Lour)	Essential Oil	13-14
P. barbatus (Andrews)	Essential Oil **1 -7**	15 27-28
P. caninus (Roth)	**8-15**	29-30
P. coesta (Buch- Ham)	**16 -18** Triterpenoids **47- 48** and β -sitosterol	31-32 32
P. fruticosus (wight)	Essential Oil **19 – 32** and **50**	16 33-34
P. incanus (Link) (= *P. mollis)*	Essential Oil Fatty acids	17-19
P. japonicus (Burm. F.)	**33** Essential Oil	35 20
P. madagascariensis (Lam.)	Essential Oil	21
P. melissoides (Benth)	Essential Oil	22
P. nilgherricus (Benth)	**34 -37**	36
P. parviflorus(Poir)	**34 - 35 , 38- 41**	37
P. rugosus (wall.)	Essential Oil Triterpenoids **42- 49** and β -sitosterol and hexacosanol	9 38-40
P. scutellarioides (L.)	Sugar and Protein	43
P. straitus (Benth)	Triterpenoids **47- 48** and β –sitosterol	41
P. sylvestris	**51 -57**	42
P. vestitus (Benth)	Essential Oil	23

3.BIOLOGICAL ACTIVITY

Some of the *Plectranthus* species for which biological activity has been reported in the literature are listed in the table-3.

Table-3

Plectranthus species	Activity	Reference
P. amboinicus	Antimicrobial Antiviral Anti-HIV inhivition Anti-tumour Anti-cancer	44 - 45 46 47 48 49
P. barbatus	Antibacterial , Antiviral Antifungal Antiprotozoa Anti-tumour Anti-inflammatory Anti-feedant Anti-candida	50 - 52 53 - 55 56 57 55 58 59
P. caninus	Anti-tumour	60
P. fruticosus	Antimicrobial	33
P. incanus	Antimicrobial	61
P. japonicus	Antifungal activity	20
P. mollis	Anti-tumour	62
P.madagascariensis	Antibacterial Anti – oxidant	9
P. sylvestris	Anti- inflammatory , Anti – oxidant	42

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Chapter -2

CHAMICAL EXAMINATION

Of

Plectranthus coesta

INTRODUCTION

In India the plant *Plectranthus coesta* (Buch- Ham) is commonly known as merudh or molchara. It is a deciduous or subdeciduous undershrub with erect stems, belonging to the family Lamiaceae (Labiatae)[1]. Family Lamiaceae consists of 3,800 species grouped in 200 genera. In india about 64 genera with 380 species are found, chiefly in mountainous regions.

OCCURANCE

This plant occurs throughout the hills between 3,000 and 7,500 feet very common in forest and open waste lands[1,2].

CLASSIFICATION

Kingdom - Plantae
Division - Magnoliophyta
Class - Magnoliopsida
Order - Lamiales
Family - Lamiaceae
Genus - *Plectranthus*
Species - *coesta*

TAXONOMICAL DISCRIPTION

A decidious or subdecidious undershrub with erect stem 2-6 feet high herbacious in the upper portions. Branches quadrangular, each face more or less grooved down the centre, with large pith clothed with pale relaxed hair. Leaves opposite, very variable in size, lower ones up to 6 by 3.5 inches, ovate, dentate, base truncated cuneate narrrowed into a short petiolar wing, hairy on both

surfaces, dull green above; prominent beneath, lateral nerves 4-6 pairs. Patiole up to 1.5 inches long, flattened above. Flowers 0.3 to 0.35 inch long, pale lavender-blue with 2-4 purple spots or stripes on the upper lip, in few flower cymes arranged in axillary and terminal pubescent panicled racemes 3-6 inches long. Calyx .05-0.1 inch long, ribbed decurved in fruit. Corolla-tube bent at right angles near the base; upper lip obscurely lobed. Nutlets broadly ellipsoid and smooth[1].

Flower - Sep.- Oct.

Fruit - December.

The paste of crushed leaves of *Plactranthus coesta* is commonly used in the indegenous system of medicine for healing wounds and itches.

PREVIOUS CHEMICAL INVESTIGATION

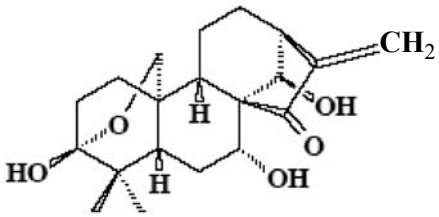
Three ent-kaurene diterpenoids named plecostonol[3], coestinol, ent-3α, 7β, 14α- trihydroxykaur-16-en-15-one[4] and triterpenoid acids viz oleanolic, ursolic acids along with β-sitosterol[3] were isolated from *P. Coesta*.

(I) Plecostonol

It is a new ent-kaurene, possessing a rare 3, 20-epoxy-ent-kaurene type skeleton, along with tripernoid acids, viz.ursolic and oleanolic acids and β-sitosterol. It was assigned the structure ent-3β,

20β-epoxy-3α, 7β, 14α-trihydroxy kaur-16-ene-15-one on the basis of its spectral properties and x-ray crystallography analysis.

An acetone extract of the whole shade-dried plant of *P.coesta* afforded, on repated chromatography, a pure crystalline compound, plecostonol-$C_{20}H_{28}O_5$ (M^+-348), m.p. 246-248^0, $[\alpha]_D$ -178.72^0.

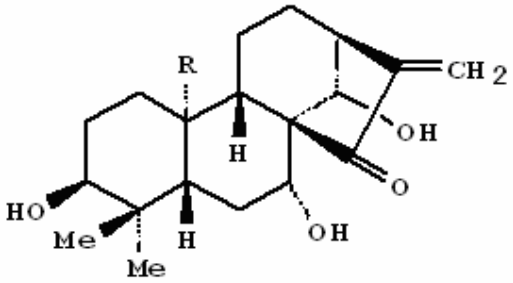


Plecostonol

(II) Coestinol

Coestinol, $C_{20}H_{30}O_5$, (M^+-350) was obtained as a crystalline solid, m.p.246-248^0, $[\alpha]_D$ -40.84^0, from the acetone extract of whole shade dried plant and characterized from their spectral data and chemical transformations.

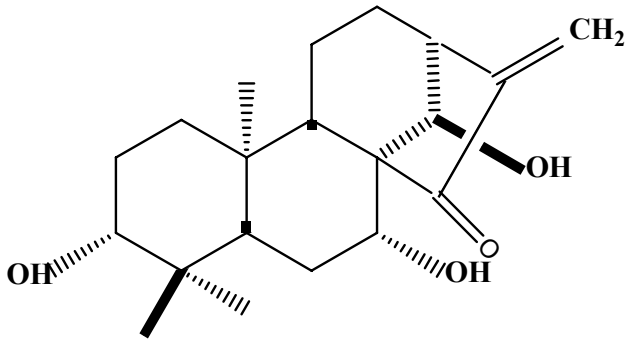
It is a new ent-kaurene diterpenoid, possessed 15-oxo-ent-kaur-16-en as its basic skeleton. It has been identified as ent-3α, 7β, 14α, 20-tetrahydroxykaur-16-en-15-one.



Coestinol; R=CH_2OH

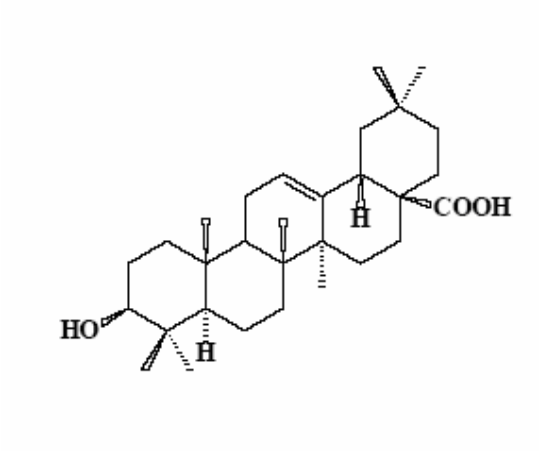
(III) ent-3α,7β,14α-trihydroxykaur-16- en- 15- one

$[\alpha]_D$ -80^0 It is a ent-kaurene diterpenoid, which is isolated from the acetone extract of whole shade dried plant and characterized by its spectral data. It is identified as its triacetate. The triacetate is identified from its spectral data.



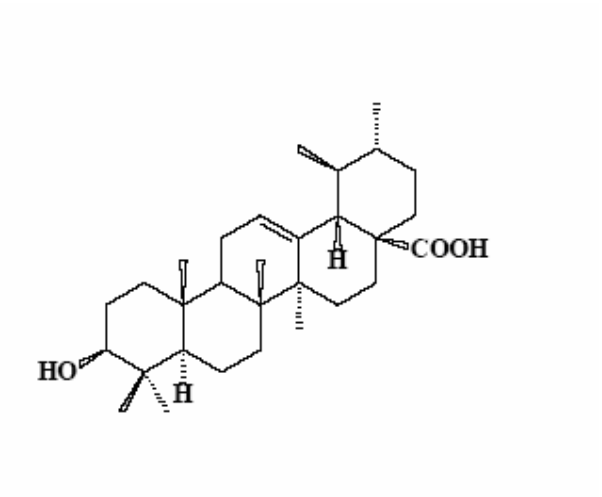
(IV) Oleanolic acid

m.p. 302^0



(V) Ursolic acid

m.p.280^0, $[\alpha]_D$ 64.2^0 (C 0.8, 1N. alc. KOH)



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Chapter -3

RESULTS AND DISCUSSION

Previous work on the chemical examination of *Plectranthus coesta* has been described in the introduction section. A number of diterpenoids, fatty acids, essential oils have been reported from this plant. We were interested in the isolation of diterpenoids as they are reported to exhibit various biological activity. Repeated chromatography process on the methanol extract of the shade dried leaves of *Plectranthus coesta* allowed the isolation of the compounds. Chemical examination of *Plectranthus coesta* in our hands led to the isolation of three kauran diterpenoids which are designated as 1, 2 and 3 for the purpose of discussion that follows.

Compound 1

Compound **1** was isolated from methanol extract and it was purified by column chromatography and preparative TLC. The compound was a solid, m.p. 267^0C. Combustion analysis and low resolution mass spectrometry indicated a molecular formula $C_{20}H_{30}O_3$ for compound **1**. IR spectrum showed strong absorption band at 3430 cm^{-1} and at 1690 cm^{-1}. The peak at 3430 cm^{-1} indicate the presence of hydroxyl group and peak at 1690 cm^{-1} indicate the presence of carbonyl group.

The ^{1}H and ^{13}C NMR spectra of this compound were showed the characteristics signals of kaur-15-ene diterpenoid derivative. Table-1 and table-2 showed the ^{1}H and ^{13}C NMR spectral data for compound **1** respectively.

^{13}C NMR spectral data for compound 1

Table–1

Carbon	Chemical shift (δ) (In $CDCl_3$ + MeOD solution)
1	39.1 (CH_2)
2	18.1 (CH_2)
3	37.2 (CH_2)
4	42.0 (C)
5	52.8 (CH)
6	30.4 (CH_2)
7	78.5 (CH)
8	55.2 (C)
9	48.2 (CH)
10	39.4 (C)
11	20.1 (CH_2)
12	26.8 (CH_2)
13	47.5 (CH)
14	34.2 (CH_2)
15	125.4 (CH)
16	138.1 (C)
17	15.3 (CH_3)
18	27.7 (CH_3)
19	180.5 (C)
20	16.7 (CH_3)

^{1}H NMR spectral data for compound 1

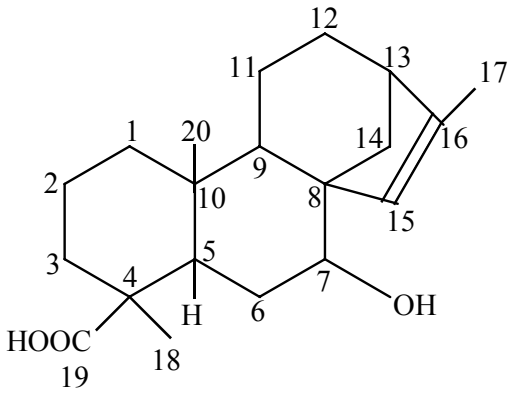
Table– 2

Proton no.	Chemical shift (δ) (In $CDCl_3$ + MeOD solution)
1	0.78, m , 1.89, m
2	1.31, m
3	2.15, m
5	1.15, d
6	1.15, d
7	3.45, m
9	3.55, d
11	1.58, m
12	1.49, m
13	2.29, m
14	2.05, m
15	5.11, q
17	1.63, s
18	1.18, s
20	0.82, s

In the ^{1}H NMR spectrum H-1 appeared as multiplet at δ 0.78 and δ 1.89 and H-2 appeared as multiplet at δ 1.31. H-3 also appeared as multiplet at δ 2.15. H-5 appeared as a doublet at δ 1.15. H-7 appeared as a doublet at δ 3.55. The down field shift of H-7 showed the proton under oxygen. H-20 appeared as a singlet at δ 0.82.

The ^{1}H and ^{13}C NMR spectra showed signals for a C-19 carboxyl group and 7-hydroxy substituent. This conclusion was also supported by the IR spectrum.

H-17 appeared as a singlet at δ 1.63 indicates the attachment with double bond. ^{13}C NMR spectra showed the signal for carboxyl group (δ 180.5) and a secondry hydroxyl group (δ 78.5).



On the basis of the above spectral data the compound **1** was identified as kaurane diterpenoid[1] and named as ent-7-Hydroxykaur-15-en-19-oic acid. Compound **1**, a kaurane diterponid has been isolated for the first time from this plant.

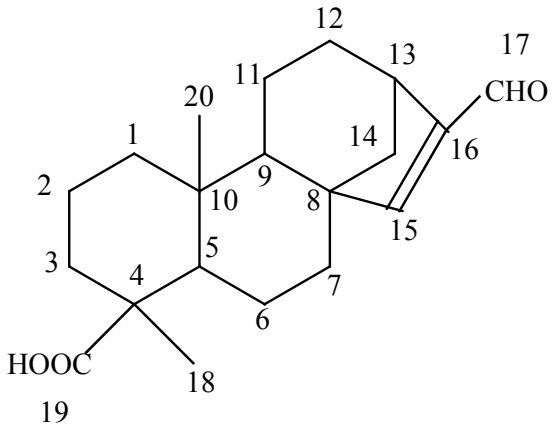
Compound 2

Compound **2** was isolated from methanol fraction and it was purified by column chromatography and HPLC.

The compound was a solid, m.p. $103^{0}C$. The EIMS indicated a molecular formula $C_{20}H_{28}O_3$ for compound **2**. The IR and UV

spectra of compound **2** showed absorptions typical for an α,β–unsaturated aldehyde function and its kaur-15-en-17-ol partial structure was supported by ^{1}H and ^{13}C NMR data.

In the ^{1}H NMR spectrum H-1 appeared as doublet and multiplet at δ 0.76 and δ 1.70 respectively. H-2 appeared as multiplet at δ 1.80. H-5 appeared as a doublet at δ 1.07. H-3 appeared as multiplet at δ 0.98. H-6 appeared as a multiplet at δ 1.79. H-7 appeared as a multiplet at δ 1.68. H-9 appeared as broad doublet at δ 1.22. H-11 appeared as a multiplet at δ 1.82. H-12 appeared as a multiplet at δ 1.81. H-14 appeared as a multiplet at δ 1.35. H-15 appeared as a doublet at δ 6.62. H-17 appeared as a singlet at δ 9.68. At δ 1.16 and δ 0.88 singlets for H-18 and H-20 respectively.



On the basis of the above spectral data the compound **2** was identified as kaurane diterpenoid[1] and named as 17-oxokaur-15-ene-19-oic acid. Compound **2,** a kaurane diterponoid has been isolated for the first time from this plant.

Compound 3

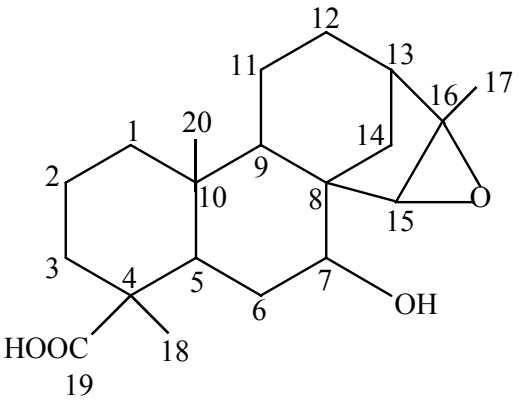
Compound **3** was isolated from methanol extract and it was purified by repeated column chromatography. The compound was white solid, m.p. 202^0C. The EIMS indicated a molecular formula $C_{20}H_{30}O_3$ for compound **3**. The IR spectrum showed strong band between 3420-2650 cm^{-1}, 2930 cm^{-1}.

In the ^{1}H NMR spectrum H-1 appeared as a multiplet at δ 1.85. H-2 appeared as a multiplet at δ 1.39. H-3 appeared as a multiplet at δ 2.15. H-5 appeared as a multiplet at δ 1.03. H-6 appeared as a multiplet at δ 1.78 and δ 1.65. H-7 appeared as a multiplet at δ 1.39. H-9 appeared as a multiplet at δ 1.12. H-11 appeared as a multiplet at δ 1.51. H-12 appeared as a multiplet at δ 1.56. H-14 appeared as a multiplet at δ 1.40. and δ 1.02. H-15 appeared as a singlet at δ 2.60. H-17 appeared as a singlet at δ 1.40. H-18 appeared as a singlet at δ 1.22. Carboxylic acid proton appeared at δ 11.43. H- 20 appeared as a singlet at δ 0.93.

The compound **3** possessed a 15, 16–epoxyde [δ_H 2.60 (1H, s, H-15), 1.40 (2H, s, Me-17), δ_C 67.9 (CH, C-15), 60.8 (C, C-16) and 14.1 (CH_3, C-17)].

The ^{1}H and ^{13}C NMR spectra of compound **3** were in agreement with the presence of a C-19 carboxyl group.

The spectroscopic data of compound **3** confirmed a Kaurane type structure and which must be ent-15, 16-Epoxykauran-19-oic acid[2]. Compound 3 was isolated first time from this plant.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Chapter -4

EXPERIMENTAL

General Remarks

All melting points were determined in open capillaries on an electrically heated melting point apparatus and are uncorrected. IR spectra were recorded using either KBr pallets or in neat on Perkin Elmer 1710 FT-IR spectrophotometer. The NMR spectra were recorded on a Bruker WM – 400 (400 MHz) or a Bruker DRX – 300 (300 MHz) or Bruker Avance DPX – 200 (200 MHz) using TMS as internal standard. Mass spectra were recorded under electron impact at 70 eV on JEOL JMS D – 100 Spectrometer. FABMS were carried out with JEOL SX 102/DA – 6000 mass spectrometer using Argon/xenon (6kV, 10mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature. m – Nitrobenzyl alcohol (NBA) was used as the matrix unless specified otherwise.

The ESMS were recorded on Micromass Quattro II Triple Quadeupole Mass Spectrometer. The samples (dissolved in appropriate solvent) were introduced into the ESI source through a syringe pump at the rate of 5µl per min. The ESI capillary was set at 3.5 kV and the cone coltage was 40V unless stated otherwise. The spectra were collected in 6 scans and the print outs are averaged spectra of 6 – 8 scans. Optical rotations were recorded on JASCO DIP 180 digital polarimeter using the sodium D-line (c in g/100 ml).

TLC and prep. TLC were performed on silica gel G (Qualigens) and plates were activated at 110^0 C for 1 hr. Chromatograms were visualized on TLC by spraying with 50%

sulphuric acid and heating. On prep. TLC chromatograms were visualized in I_2 atmosphere before scratching the band. For column chromatography silica gel (60-120 mesh, qualigens) was used. All solvents were distilled as well as dried before use. Work up reaction mixtures were dried over anhydrous Na_2SO_4.

The two-dimensional NMR experiments were carried out on a Bruker DRX – 300 spectrometer operating at 300 MHz in $CDCl_3$. Assignments of ^{13}C signals have been made on the basis of DEPT spectrum.

Plant material

The dried leaves of *Plectranthus coesta* was collected in September 2003 from Jajardawal forest, Pithoragarh, Uttarakhand. The identification of the plant was carried out by Prof. Y. P. S. Pangati, Botany Department, D. S. B. Campus, Kumaun University, Nainital, where a voucher specimen has been deposited.

Extraction

2 kg of the shade dried and powdered leaves was extracted with cold methanol (3x2L). The MeOH extract was concentrated in vacuo. It was filtered and most of the methanol was removed under

reduced pressure. The residue was fractioned with iso-octane, chloroform and methanol (5x200 ml each) and than dried and evaporated to obtain iso-octane fraction (5.03g), chloroform fraction (3.63g) and methanol fraction (12.8g).

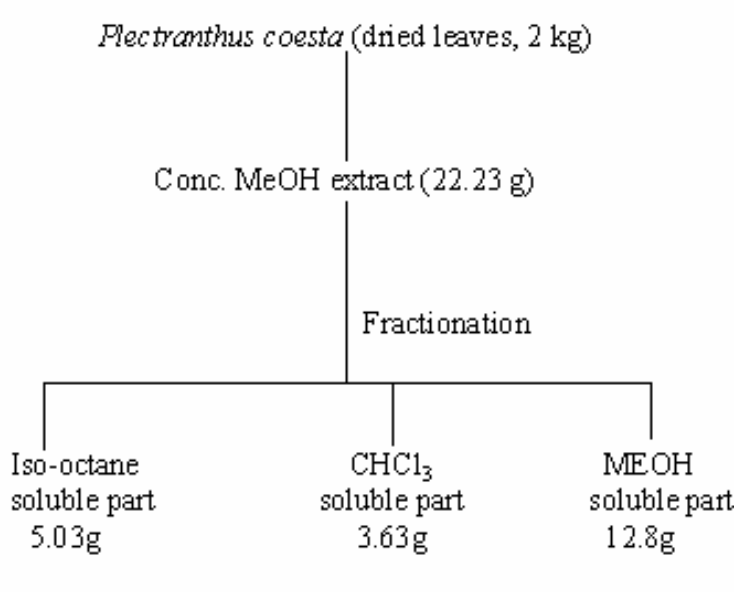

Chart -1

Column Chromatography of MeOH Fraction

Table – 1

S.No.	Fr.	Eluent	Quantity	Physical nature	Colour	Spots observed on TLC plate
1.	1-3	100% TMP	55mg	Oil	Colourless	3 major and 5 minor
2.	4-6	TMP+10%EtOAc	60 mg	Oil	Colourless	2 major and 4 minor
3.	7-9	TMP+10%EtOAc	150 mg	Oil	Dark yellow	3 major and 3 minor
4.	10-13	TMP+10%EtOAc	170 mg	Oil	Yellow-greenish	3 major and 3 minor
5.	14-25	TMP+50%EtOAc	260 mg	Oil	Light yellow	1 major and 1minor
6.	26-38	TMP+20%EtOAc	325 mg	Solid	Green	1 major and 3 minor
7.	39-59	TMP+25%EtOAc	610 mg	Solid	Brownish yellow	1 major and 2 minor
8.	60-62	TMP+35%EtOAc	312 mg	Solid	Green	1 major and 1 minor
9.	63-83	TMP+50%EtOAc	800 mg	Solid	Green	2 major and 2 minor
10.	84-93	TMP+70%EtOAc	915 mg	Solid	Light green	1 major and 1 minor
11.	94-106	100%EtOAc	186 mg	Solid	Light green	1 major and 1 minor
12.	107-112	EtOAc+10%MeOH	324 mg	Solid	Light green	2 major and 1 minor
13.	113-125	EtOAc+15%MeOH	860 mg	Solid	Light green	1 major and 4 minor
14.	126-132	EtOAc+18%MeOH	1.0g	Solid	Light green	1 major and 1 minor

Column Chromatography of $CHCl_3$ Fraction

Table – 2

S.No.	Fr.	Eluent	Quantity	Physical nature	Colour	Spots observed on TLC plate
1.	1-6	100% TMP	20 mg	Oil	Yellow	1 major and 1 minor
2.	7-8	TMP+3%EtOAc	35 mg	Oil	Colourless	2 major and 2 minor
3.	9-10	TMP+4%EtOAc	22 mg	Oil	Slightly green	1 major and 1 minor
4.	11-28	TMP+5%EtOAc	60 mg	Oil	Slightly green	2 major and 4 minor
5.	29-35	TMP+8%EtOAc	84 mg	Oil	Greenish	1 major and 3 minor
6.	36-42	TMP+10%EtOAc	85 mg	Oil	Light green	1 major and 3 minor
7.	43-50	TMP+14%EtOAc	69 mg	Solid	Light green	1 major and 1 minor
8.	51-60	TMP+18%EtOAc	53 mg	Solid	Light yellow	1 major and 1 minor
9.	61-70	TMP+20%EtOAc	101 mg	Solid	Light yellow	2 major and 2 minor
10.	71-78	TMP+30%EtOAc	200 mg	Solid	Colourless	1 major and 2 minor
11.	79-85	TMP+40%EtOAc	168 mg	Solid	Colourless	1 major and 1 minor
12.	86-88	TMP+50%EtOAc	125 mg	Solid	Dark brown	2 major
13.	89-92	TMP+60%EtOAc	54 mg	Solid	Light brown	2 major and 1 minor
14.	93-95	TMP+70%EtOAc	305 mg	Solid	Light brown	1 major and 2 minor
15.	96-98	TMP+80%EtOAc	120 mg	Solid	Light brown	2 major
16.	99-103	100%EtOAc	125 mg	Solid	Light brown	2 major and 1 minor

Isolation of compounds 1, 2 and 3

Fraction 39-59 (610 mg) was rechromatographed over silica gel (60 g) TMP and increasing amount of EtOAc. The eluent obtained using 10% EtOAc in TMP furnished compound **1** (25 mg), m.p., 267^0C and compound **2** (15 mg), m.p., 103^0C respectively and 50% EtOAc in TMP furnished compound **3** (22 mg), m.p. 202^0C.

Compound 1

Solid, m.p. 267^0C, $C_{20}H_{30}O_3$.

^{1}H NMR ($CDCl_3$, δ) : 0.78, 1.89 (2H, m, H-1), 1.31 (2H, m, H-2), 2.15 (2H, m, H-3), 1.15 (1H,d, H-5), 1.15 (2H, d, H-6), 3.45 (1H, m, H-7), 3.55 (1H,d, H-9), 1.58 (2H, m, H-11), 1.49 (1H, m, H-12), 2.29 (1H, m, H-13), 2.05 (2H, m, H-14), 5.11 (1H, q, H-15), 1.63 (3H, s, H-17), 1.18 (3H, s, H-18), 0.82 (3H, s, H-20).

^{13}C NMR ($CDCl_3$, δ) : 39.1(C-1), 18.1 (C-2), 37.2 (C-3), 42.0 (C-4), 52.8 (C-5), 30.4 (C-6), 78.5 (C-7), 55.2 (C-8), 48.2 (C-9), 39.4 (C-10), 20.1 (C-11), 26.8 (C-12), 47.5 (C-13), 34.2 (C-14), 125.4 (C-15), 138.1 (C-16), 15.3 (C-17), 27.7 (C-18), 180.5 (C-19), 16.7 (C-20).

^{KBr}IRν_{max} cm^{-1} : 3430, 2870, 1690, 1470, 1280, 1050, 890, 810

EIMS m/z : 341[M^+ +Na], 318[M^+]

Compound 2

Solid, m.p.103^{0}C, $C_{20}H_{28}O_3$.

IRν_{max}^{KBr}: 3425, 2930, 2850, 2730, 1735, 1690, 1675, 1605, 1450, 1365, 1205, 1025, 985, 970, 855, 755 cm^{-1}.

UV (MeOH) λ_{max}: 249 nm.

^{1}H NMR ($CDCl_3$, δ): 0.76 (1H, dd, J=13.2, 4.1, H-1α), 1.70(1H, m, H-1β), 1.80 (2H, m, H-2), 1.07 (1H, d, J=11.6 Hz, H-5), 0.98 (2H, m, H-3), 1.79 (2H, m, H-6), 1.68 (2H, m, H-7), 1.22 (1H, brd, J=9.5 Hz, H-9), 1.82 (2H, m, H-11), 1.81 (2H, m, H-12), 1.35 (1H, m ,H-14), 6.62 (1H, d, J=0.8 Hz, H-15), 9.68 (1H, s, H-17), 1.16 (3H, s, Me-18), 0.88 (3H, s, Me-20).

^{13}C NMR ($CDCl_3$, δ) : 40.2 (C-1), 19.2 (C-2), 37.2 (C-3), 42.9 (C-4), 57.1 (C-5), 21.2 (C-6), 36.1 (C-7), 44.1 (C-8), 50.3 (C-9), 40.5(C-10), 19.3 (C-11), 27.3 (C-12) , 40.4 (C-13), 33.1 (C-14), 139.3 (C-15) , 142.2 (C-16), 15.6 (C-17), 27.8 (C-18), 182.3 (C-19).

EIMS m/z: 316 [M^+], 268, 147, 133, 131, 119, 117, 109, 43.

Compound 3

Solid, m.p. 202^0C, $C_{20}H_{30}O_3$.

IRν_{max}^{KBr} cm^{-1}: 3420-2650 br., 2930, 2870, 1690, 1450, 1250, 982, 850, 790.

^{1}H NMR ($CDCl_3$, δ) : 1.85 (2H, m, H-1), 1.39 (2H, m, H-2), 2.15 (2H, m, H-3), 1.03 (1H, m, H-5), 1.78, 1.65 (2H, m, H-6), 1.39 (2H, m, H-7), 1.12 (1H, m, H-9), 1.51(2H, m, H-11), 1.56 (2H, m, H-12), 1.40, 1.02 (2H, m, H-14), 2.60 (1H, s, H-15), 1.40 (3H, s, H-17), 1.22 (3H, s, H-18), 11.43 (1H, s, -COOH), 0.93 (3H, s, H-20).

^{13}C NMR ($CDCl_3$, δ) : 39.8 (C-1), 19.2 (C-2), 37.9 (C-3), 43.2 (C-4), 56.1 (C-5), 20.1 (C-6), 34.2 (C-7), 42.8 (C-8), 49.1 (C-9), 38.9 (C-10), 17.8 (C-11), 26.1 (C-12), 38.7 (C-13), 31.9 (C-14), 67.9 (C-15), 60.8 (C-16), 14.1 (C-17), 28.1 (C-18), 182.3 (C-19), 15.2 (C-20).

EIMS m/z : 318 [M^+], 300, 285, 273, 257, 239, 201, 159, 147, 107, 91, 79, 55, 43.

Chapter -5

BIOCHEMICAL AND ELEMENTAL ANALYSIS

Nutrition is a most important basic need, being a major detriment of health, labour productivity and mental development. With a high mineral content along with energy value, leaves have been recognized for their nutritional importance. Within the leaves, wild edible are getting special attention due to the fact that they are rich of carbohydrate, protein, mineral, vitamins etc and more importantly they grows in natural habitat without fertilizers. Generally, the wild leaves are highly variable in nutrients content. Through investigation of nutrient contents of wild edible plant it was revealed that they contain surprisingly high contents of some nutrients.

Plectranthus spp. are commonly used as fodder[1].

Material and methods

The fresh leaves of *Plectranthus coesta* was collected in September 2003 from Jajardawal forest, Pithoragarh, Uttarakhand. Shade dried leaves were powdered and kept in air tight container. The fine powder was used for biochemical analysis. Following parameters were analyzed.

Total soluble solids

The estimation of TSS was made from the juice of fresh leaves directly by using Erma hand refractrometer (Model A-Erma optical works Pvt. Ltd. Tokyo-Japan). The leaves were crushed in an electric

blender and juice was extracted. It was taken into refractrometer and TSS percentage was read out from the instrument on the principle of refractrometery. The observed readings was corrected at 20^0C by temperature correction chart supplied with the instrument.

Moisture

It is estimated by AOAC method[2]. Approximately 10 g fresh sample was taken in a dried weighed dish and it was kept in an air circulating oven first at 40^0C for two hours, then followed at 60^0C and 80^0C respectively. Finally, the sample was kept between 100^0C to 105^0C for another hour. Thereafter the samples were cooled and again weighed using electronic balance. The loss in weight of the sample was reported as moisture percentage and calculated by following formula:

% Moisture = w X 100/W

Where: w = loss in weight of fresh sample and W = weight of fresh sample taken.

Dry matter

It is calculated by the following formula:

Dry matter % = 100-moisture %

Organic Matter

It is calculated by the following formula:

Organic Matter = 100-(moisture % + mineral %)

Crude fat

The content of crude fat was estimated by AOAC method[3]. Dry powdered (1g) sample was taken in an extraction thimble and placed in a soxhlet extractor fitted with condenser and a flask containing petroleum ether (40^{o}C to 60^{o}C BP). Thereafter the flask was put on a heating mantle especially designed for this purpose and fixed at 40^{o}C. The extraction was carried out for 6 hours. The thimble was then removed and flask was again heated, so that the extractor was filled up about two third with petroleum ether and only a small quantity of it was left in the flask. The residue of petroleum ether containing crude fat was filtered. Flask and filter paper were washed carefully. Filtrate was evaporated and the beaker was again weighed after cooling. Weight of residue was calculated on the fresh weight basis and reported as crude fat.

Crude fat (%) = (w/W) x 100

Where: w = weight of residue, W = weight of sample

Crude protein

Crude protein was calculated as N x 6.25 (based on assumption that nitrogen constitutes 16.0% of a protein). Total nitrogen was estimated by Snell's method[4]. Triple acid (perchloric acid,

hydrochloric acid and hydrogen peroxide 2.5: 2.5:1.0) digested sample was used for the analysis of nitrogen. 1ml of prepared acid solution of plant material was pipetted into 50 ml volumetric flask. To this aliquot added 1ml of 10% sodium hydroxide and 1ml of 10% sodium silicate, then solution was diluted to 35 ml. To this mixture, 1ml of Nesselar's Reagent was added and volume make up to the mark. The colour intensity was measured by spectrophotometer HITACHI, model U-2001 after 15 min at 420 nm using a reagent blank as reference with the help of standard curve and the amount of nitrogen in sample was calculated. Ammonium chloride was used as a standard for nitrogen. The crude protein was calculated by the following formula.

Crude protein % = Nitrogen % x 6.25

Crude fiber

Crude fiber was estimated on dried powdered sample by Maynard's method[5].This method is based on the imitation of gastric and intestinal action. In a 250 ml beaker, 2 g of powdered sample were taken. The beaker was previously marked at 100 ml level. To the beaker, 200 ml of 1.25 % Sulphuric acid (25 ml of 10 % Sulphuric acid in 175 ml water) was added and digested on a hot plate for two hours and stirred regularly. The solution was filtered through a muslin cloth by giving suction for filtration. The residue

was washed several times by distilled water till it was acid free. Again the solution transferred to original beaker and 200 ml of 1.25 % NaOH were added (25 ml of 10 % NaOH +175 ml water). This was again digested on hot plate, filtered and washed to make it free from alkali. The residue was thereafter washed three times with rectified spirit to remove moisture. The residue was washed with three times with acetone to make it free from fatty compounds. Then it was dried, weighed, ignited to ash in a muffle furnace and weight of ash was determined. This weight of ash was substracted from the weight of residue and crude fiber was calculated as following:

Crude fiber (w) = weight of residue – weight of ash

Crude fiber (%) = (w / W) X 100

Where: w = weight of crude fiber, W = weight of sample taken

Carbohydrate

Total carbohydrate content in plant leaves was estimated by the Phenol sulphuric acid method[6]. The carbohydrate present in leaves powder was first hydrolyzed into simple sugar by acid hydrolysis (5 ml of 2.5 N HCl) for 3 hours and resultant monosaccharide. It was neutralize with solid sodium carbonate until the effervescence ceases and centrifuge then make up volume to 100 ml. 0.5 to 1 ml supernatant were pipetted for analysis. 1 ml of 5 % phenol solution

and 5 ml of 96 % sulphuric acid was added to 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard concentration of 100 mg/lit in test tube. The absorbance was measured at 490nm after 10min. Total carbohydrate percent in sample solution was calculated using the standard graph.

Absorbance corresponds to 0.1ml of test = x mg of glucose

Total carbohydrate (%) = x / 0.1 x 100 mg of glucose

Where; x = spectrophotometer reading

Ash and silica

Ash content and ash insoluble content were estimated by AOAC[7], Peach[8] and Mishra[9] methods. The samples were burned in a muffle furnace at 550 ^{0}C and then oxidized. Ash was dissolved in acidic water and insoluble part was reported as silica. Acid soluble part is reported as acid soluble ash.

Ash

The total ash was described by AOAC[7] and Peach[8] methods. Dried sample (2 g) was taken in a silica crucible and burnt in muffle furnace at 550 ^{0}C until it was free from carbon. Muffle furnace was allowed to cool. Crucible was taken out of furnace, cooled in a

dissecator, and weighted. Total ash was calculated by following formula:

%Total ash= w x 100/ W

Where: w - Weight of ash, W - Weight of sample

Silica

Silica was analysed by Peach[8] and Mishra[9] methods. Conc. HCl (10 ml) was added to the ash in the crucible and covered by a clock glass. The slolution was heated for 30 minutes and clock glass was removed by rinsing and heated again to dryness. Dilute HCl was added to the residue and heated for dissolving the soluble ash and contents were filtered through Whattman No. 44 paper in a 100 ml volumetric flask. The crucible and residue on filter paper were rinsed with water and volume was made up to 100ml. The residue left on paper was burnt, weighed and percentage of silica was calculated by following formula:

%Silica= w /W x 100

Where: w = Weight of silica, W = Weight of sample

Phenolics

Total phenolic content was estimated by Singleton method[10] with minor modification. Dry leaves powder (0.5 g) was extracted

with 10 time volume of 80 % ethanol. The Homogenate was centrifuged at 10,000 rpm for 20 minutes and collected the supernatant. The residue re-extracted three times with 80 % ethanol, centrifuged and supernatant was collected. The supernatant was evaporated to dryness. The residue was dissolved in 5 ml distilled water. 1 ml aliquots taken into test tube and added 0.5 ml Folin-Ciocalteau reagent. After three minutes 2 ml of 20 % sodium carbonate solution was added to each test tube and the absorbance was measured at 650 nm. Catechol was used as a standard. The concentration of phenols in test sample was calculated according to standard graph of phenolics and expressed as mg/100g.

Minerals

The total ash was analyzed by AOAC[7] and Peach[8]. Dried sample (2g) was taken in a silica crucible and front in muffle furnace at 550^0C until it was free from carbon. Muffle furnace was allowed to cool, crcucible was taken out of the furnace and cooled in a dissector and weighted. Total ash was calculated as follows.

%Total ash=Weight of ash x 100/Weight of sample

Chlorophyll

The chlorophyll content in dry leaves powder was estimated by Witham method[11]. Dry leaves powder (0.5 g) was extracted with 20

ml 80 % acetone (prechilled), centrifuged at 5000 rpm for 5 min and transferred the supernatant to a 100 ml volumetric flask. The residue was extracted with 20 ml of 80 % acetone, centrifuged and collected supernatant to same volumetric flask. This procedure was repeated until the residue was colorless. The volume was made up to 100 ml with 80% acetone and the absorbance was measured at 645 and 663 nm. The amount of chlorophyll a and b present in sample was calculated by following formula:

Chlorophyll a (mg /100g) = 12.7 (A_{663}) – 2.69 (A_{645}) x V/10 x W
Chlorophyll b (mg /100g) = 22.9 (A_{645}) – 2.69 (A_{663}) x V/10 x W

Where: A = Absorbance of specific wavelengths, V = Final volume of chlorophyll extract in 80% acetone, W = Weight of sample.

Solubility

AOAC methods[12] were used for the estimation of solubility percentage. 1g. each of dried powdered leaves were taken in extraction thimbles. These were then placed in a soxhlet extraction fitted with condenser and different flasks containing different solvents viz: petroleum ether, ethyl alcohol, chloroform, acetone, benzene and methanol. These were put on their heating mental specially designed for this purpose controlled at 60^0C. The extraction was carried out for 6 hours. The thimbles were then removed and

flasks were again heated so that the extractors were filled up about two third with above solvents and only a small quantity of these were left in the flasks. The boiling was stopped. The residue of above solvents was filtered. Flasks and filter papers were washed carefully. Filterates were evaporated and the beakers were again weighed after cooling. Weight of residue were calculated on the dry weight basis and reported as solubility.

Elemental analysis

Elements are important for metabolism. They are necessary part of many important enzymes and play a vital role as antioxidant and catalyst. Elements are required to activate hundred of enzymatic reactions within the body[13].

One gram dried sample was first digested with 15 ml of triple acid mixture (10 part HNO_3+ 4 parts of $HClO_4$ + 2 parts H_2SO_4) at 110^0C and reduced to about 1.0 ml. The digested residue was dissolved in triple distilled water, filtered and diluted to 100 ml. This solution was used for the estimation of elements. In similar condition blank samples were also prepared. Samples of plant material taken in three replications.

Reference standards were prepared from E. Merck, AAS Spectrosol (1000 mg L-1). Estimation of element like Na, K, Ca and Li were carried out by AIMIL, flame Photometer, New Delhi, while Fe, Co, Mn, Cu and Zn were determined by Atomic Absorption

Spectrophotometer, model 4129, Electronic Corporation of India Ltd, Hyderabad (A.P.). The instruments were calibrated by using standard solutions (0.20-10 mgL-1) of above mentioned elements.

The concentration of Na, K, Ca, Li, Fe, Co, Mn, Cu and Zn was calculated by using the following formula:

E mg/100g =A x V/ W x 10

Where: E= Elements viz- Na, K, Ca, Li, Fe, Co, Mn, Cu and Zn.

A = Reading of instrument, W= Weight of sample

Estimation of phosphorus

Phosphorus was estimated by Allen method[14]. Samples were digested in triple acid and taken for the estimation of phosphorus. 1ml of 4% ascorbic acid and 4 ml of molybalate reagent were added into 1 ml of digested sample, mixed well and left for 1h for color developing. The standard curve was made by different concentration of standard 0-20 µg phosphorus per liter. The concentration of phosphorus was calculated by using the following formula:

Phosphorus (mg/100g) = (X / W) x 10

Where: X = ppm P in 1ml, W = weight of sample

Results and discussion

Biochemical constituents play a vital role for proper maintenance of body and are source of energy. Among those biochemicals moisture (75.18 ± 1.13), dry matter (24.34 ± 0.56), total carbohydrate (11.99 ± 0.35), Crude fiber (2.51 ± 0.40), crude fat (0.62 ± 0.02), crude protein (13.42 ± 0.37), and ash (4.57 ± 0.02) were found in the leaves of *P. coesta* (Table 1). They are vital part of many important enzymes and play a role of antioxidant and catalyst. As such carbohydrates comprise one of the most important classes of natural products called sugars or saccharides. Carbohydrates have a lot of biological importance are main source of energy. Carbohydrates are the structural backbone of the nucleic acids (DNA and RNA) Carbohydrates provide fuel to our body. Each gram of digestible carbohydrate contains four calories. About half of our energy needed per day is provided by carbohydrates. Hypoglycemia, hyperglycemia and glycosuria are the common diseases which are related with carbohydrates[15]. Lipids are important constituents of diet. They act as filling and protect vital organs. They act as fuel and yield energy.They are the building blocks for high molecular weight substances eg. Acetic acid is used for the synthesis of cholesterol and some hormones. They supply essential fatty acids, which are not synthesized with in the body. They also carry fat-soluble vitamins and also take a vital role as structural component of cell membrane. Due to low specific gravity, they float in water.

Excess of lipid in diet may lead to Ketosis, Atreosclerosis, Fatty liver and Gaucher's disease[15].

Likewise, the proteins are most important constituents of all living substances. They are basic structural constituents of body and provide structural skeleton for cells and tissues. Protein act as enzymes and hormones. They are essential for repair and maintenance of body tissues and synthesis of antibody, hormones, hemoglobin and enzymes. They help in cell mediated immunity and bactericidal action of leukocytes. Various diseases are caused by deficiency of protein. Two
important diseases due to deficiency of proteins in children are marasmus and kwashiorkor. Abnormal metabolism of protein may lead to diseases like Phenyl ketonuria, Alkaptonuria and Albinism[15].

Crude fiber is composed of mixture of substances like cellulose, hemicelluloses, lignin and pectin which are necessary constituents of cell wall of plants. They are in bulk in our vegetarian diet and hard to digest. They are helpful in digestion and absorption of food[16].

The presence of secondary metabolites i.e. (chlorophyll, phenols) indicates availability of active compounds in *P. coesta*. The range of chlorophyll-a content was 0.75 – 0.79 mg/100g. The content of chlorophyll- b was found 1.46±0.03 mg/100g on dry weight basis. The range of chlorophyll-b was found 1.45 – 1.49 mg/100g. The content of total phenolics in *P. coesta* fruits was found 0.49±0.02 mg/100g on dry weight basis. The range of phenolic content varied

0.47- 0.49 mg/100g. Presence of such biologically important groups is indicative of medicinal potential of the *P. coesta* leaves.

It is evident from the table–1 that the leaves of the *P. coesta* have great potential to meet the nutritional requirement for the animals.

Biochemical composition of *Plectranthus coesta* leaves.

Table 1

Biochemical Parameter	Composition	Range
1. Total Soluble Solids	10.0 ± 0.28	9.80-10.20
2. Moisture ($g.100g^{-1}$)	75.18 ± 1.13	74.36-75.96
3. Dry mater ($g.100g^{-1}$)	24.34 ± 0.56	23.96-24.32
4. Organic matter ($g.100g^{-1}$)	21.12 ± 0.14	21.03-21.23
5. Crude Protein ($g.100g^{-1}$)	13.42 ± 0.37	12.96-13.87
6. Crude Fat ($g.100g^{-1}$)	0.62 ± 0.02	0.61-0.63
7. Total Carbohydrate ($g.100g^{-1}$)	11.99 ± 0.35	11.58-12.44
8. Crude Fiber ($g.100g^{-1}$)	2.51 ± 0.40	2.19-2.73
9. Ash ($g.100g^{-1}$)	4.57 ± 0.02	4.55-4.57
10. Acid Soluble ash ($g.100g^{-1}$)	2.97 ± 0.02	2.96-2.98
11. Acid insoluble ash ($g.100g^{-1}$)	1.59 ± 0.02	1.58-1.60
12. Chlorophyll-a ($mg.100g^{-1}$)	0.77 ± 0.03	0.75 – 0.79
13. Chlorophyll-b (mg. 100 g^{-1})	1.46 ± 0.03	1.45 – 1.49
14. Phenolics (mg. 100 g^{-1})	0.49 ± 0.02	0.47 – 0.49

± denotes the standard error. All values are mean of triplicate determinations expressed on dry weight basis.

Dried plant material was extracted with solvents of different polarity viz, petroleum ether, benzene, chloroform, ethanol, methanol and acetone to know the solubility of phytochemicals present in the plant material in the solvents of different polarity. Because according to polarity of solvents. It dissolves phytochemicals of different groups in the solvents.

Solubility % in different solvents

Table 3

Solvent	Solubility %	Range
Acetone-	16.08 ± 0.03	16.06 – 6.10
Ethyl alcohol-	32.07 ± 0.04	32.05 –32.11
Benzene-	09.31 ± 0.05	09.28 – 9.35
Chloroform-	15.30 ± 0.02	15.29 – 5.31
Petroleum ether-	06.08 ± 0.02	06.07 – 6.10
Methanol-	30.09 ± 0.03	30.07 – 0.11

± denotes the standard error. All values are mean of triplicate determinations expressed on dry weight basis.

Elemental Composition of *Plectranthus coesta* leaves

Table 4

Element	Composition (mg/100g)	Range (mg.100g^{-1})
1. Potassium – K	275.40 ± 0.47	274.36 – 276.05
2. Sodium – Na	5.16 ± 0.02	5.15 – 5.18
3. Nitrogen – N	2151.85 ± 1.35	2150.49 – 2153.69
4. Lithium – Li	0.76 ± 0.02	0.75 – 0.78
5. Calcium - Ca	242.46 ± 0.56	241.68 – 242.96
6. Iron – Fe	2.15 ± 0.04	2.13 – 2.18
7. Cobalt – Co	1.86 ± 0.03	1.84 – 1.88
8. Zinc - Zn	0.95 ± 0.02	0.94 – 0.96
9. Manganese- Mn	1.35 ± 0.02	1.34 – 1.37
10. Copper- Cu	0.67 ± 0.03	0.65 – 0.69
11. Phosphorus – P	120.15 ± 0.92	120.14 – 120.17

± denotes the standard error. All values are mean of triplicate determinations expressed on dry weight basis.

The trace elements play important role in maintaining and regulating the metabolic activities. The content of K and Na were found 275.40±0.47 mg/100g and 5.16±0.02 mg/100g respectively on dry weight basis. Na and K take part in the ionic balance and maintain tissue functionality. Na plays an important role in transport of metabolites because of the solubility of the salts. K is diuretic and lowers the blood pressure[17]. The range of Cu was found 0.65-0.69 mg/100g on dry weight basis. Cu is a part of many enzyme systems such as cytochrome oxidase, celluloplasmin and lysyl oxidase, an iron oxidizing enzyme in blood and are essential in blood formation and concerned in normal carbohydrate and lipid metabolism[18]. The

content of Ca was found 242.46±0.56 mg/100g on dry weight basis. Ca constitutes a large proportion of bones, blood and extracellular fluid. It is essential for the normal functioning of cardiac muscles, milk clotting, blood coagulation and the regulation of cell permeability and plays an important role in nerve impulse, transmission and the mechanism of neuromuscular system. It also plays a crucial role in the transformation of light to electrical impulses in the retina. Ca lowers depression, allergies and irritability[19].The range of Co content was found 1.84-1.88 mg/100g on dry weight basis. Cobalt is one of the elements that is very important to life, including human life and health. Vitamin B-12 contains cobalt. In areas where there is little cobalt in the soil, farmers have to provide salt blocks containing cobalt for their animals to lick in order to provide enough cobalt in their diet.

The content of Zn was found 0.95±0.02 mg/100g on dry weight basis. Zn is present in every part of the body and has a wide range of functions. It helps with the healing of wounds and is a vital component of many enzyme reactions. As a component of many enzymes, Zinc is involved in the metabolism of proteins, carbohydrates, lipids and energy. It is also used in the growth and maintenance of muscles. Abnormally low Zn uptake has been found in Crohn's disease pancreatic insufficiency[20] and jejuno-ileostomy patients[21].

The content of Li was found 0.76±0.02 mg/100g on dry weight basis. Lithium salts are used in the treatment of manic-depressive illness. The range of Fe content was found 2.13-2.18 mg/100g on dry weight basis. Fe is necessary for many functions in the body including formation of hemoglobin, brain, development and function, regulation of body temperature, muscle activity and catecholamine metabolism. It is also essential for binding oxygen to the blood cells. The central function of the iron is oxygen transport and cell respiration. Iron deficiency affects the formation of hemoglobin as a result anemia occurs[22]. The content of Mn was found 1.35±0.02 mg/100g on dry weight basis. Mn is a trace element found in large quantities in plants and animals, which help the body, convert protein and fat to energy. It promotes normal bone growth, helps maintain healthy reproductive, nervous and immune systems and is involved in blood clotting and the formation of cartilage and lubricating fluid in the joints. It is a well know neurotoxic metal causing psychic and neurological disorders is exposed persons but little attension has been paid to the levels of Mn in blood and/or in air in relation to these disorders[23].

The range of P was found 120.14-120.17 mg/100g on dry weight basis. Phosphorus is found in every cell of the body. In the bones two elements, Ca and P are combined to give strength and firmness to the skeleton or bony structure of the body. Many chemical reactions of the body require both calcium and phosphorus.

Hence there is a constant interchange of these elements between the bones and the blood system. Deficiency of P can cause rickets. In chronic phosphorus deficiency animals may have stiff joints and muscular weakness. It also causes low milk yield in cows[24].

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Chapter -6

BIOLOGICAL ACTIVITY

Anti-mycobacterial Assay

The crude extract and pure compounds were tested for activity against *Mycobacterium marinum. M. marinum* was selected as the good target for several reasons. First, the nature of infection by *M. marinum* allows for safer anti-mycobacterial screening in a primarily UG setting. The microorganism grows optimally at 30ºC, so infection in humans occurs cutaneously and rarely spreads systemically[1]. Second, few effective treatments exists for M. *marinum* infection. Lastly, *M. marinum* has been targeted because of its close relation to *M. tuberculosis* both phenotypically and genetically. Consequently, many of the antibiotics used to treat tuberculosis are also used to treat infection by *M. marinum.* Therefore, it is thought that a novel antibiotic with inhibitory activity against *M. marinum* will most likely have inhibitory activity against *M. tuberculosis.* Biological activity against the *Mycobacterium marinum* uracil auxotroph was determined for extract and isolated fractions using the BD™ Oxygen Biosensor system 96 well format plate. Each well has an oxygen sensitive fluorophore compound, *tris*- 1,7-diphenyl-1,10-phenanthroline ruthenium(II) chloride, embedded in a silicone matrix on the bottom of the well. If the organism is growing (i.e. respiring), the oxygen in the well will be depleted, causing the fluorophore to fluoresce. With anti-mycobacterial compounds present, oxygen will not be depleted, and fluorescence will remain quenched. This assay is particularly appropriate for growth assays in bacteria like *M. marinum* which

form a biofilm at the air/liquid interface. For the assay, extract and compounds were dissolved in a 75:25 ethyl acetate: methanol mixture and then diluted to a concentration of 0.01 to 0.02 mg/ml using the same solvent mixture. An aliquot of 15 µl of the extract and compounds were then added to duplicate wells containing 185 µl of *M. marinum* at an OD of 0.8 – 0.9 l600 in Middlebrook 7H9UR media. A positive fluorescence control of 100 mM sodium sulfite and a negative fluorescence/growth control of sterile medium were included on each plate. The positive growth control for each plate consisted of the *M. marinum* and the solvent mixture. In addition, 2 sets of antibiotic controls were run for each plate: 185µl of *M. marinum* with 15µl of streptomycin or rifampicin added at concentrations of 0.5, 1.0 and 2.0 mg/ml. In addition, a single well was left empty for the entire incubation period as a background control. Plates were incubated at 31ºC and were read at 24 and 48 hr. Fluorescence readings were compared to the plate just before filling.

Antimycobacterial activities of iso-octane fraction, chloroform fraction and MeOH fraction of *Plectranthus coesta* were assayed against *Mycobacterium marinum* in a fluorescence based growth assay. Iso-octane fraction was inactive but other two fractions showed 90% inhibition at 0.2µg/mL concentration.

Reference:

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