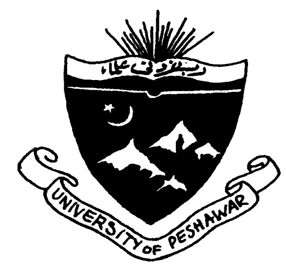
BIOACTIVE PRINCIPALS FROM *TEUCRIUM ROYLEANUM* WALL. EX BENTH. AND *PEROVSKIA ATRIPLICIFOLIA* BENTH.-ANTIMICROBIAL, ALLELOPATHY AND ANTIOXIDANT ASSAYS



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PAKISTAN

February 2009

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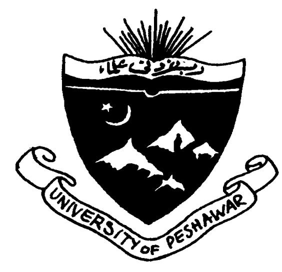

By

SHABIR AHMAD

Dissertation

Submitted to the University of Peshawar as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry.

INSTITUTE OF CHEMICAL SCIENCES

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It is recommended that this dissertation prepared by Mr. Shabir Ahmad entitled "Bioactive Principals from Teucrium royleanum Wall. Ex Benth. and Perovskia atriplicifolia Benth.-Antimicrobial, Allelopathy and Antioxidant Assays" be accepted as fulfilling this part of the requirements for the degree of

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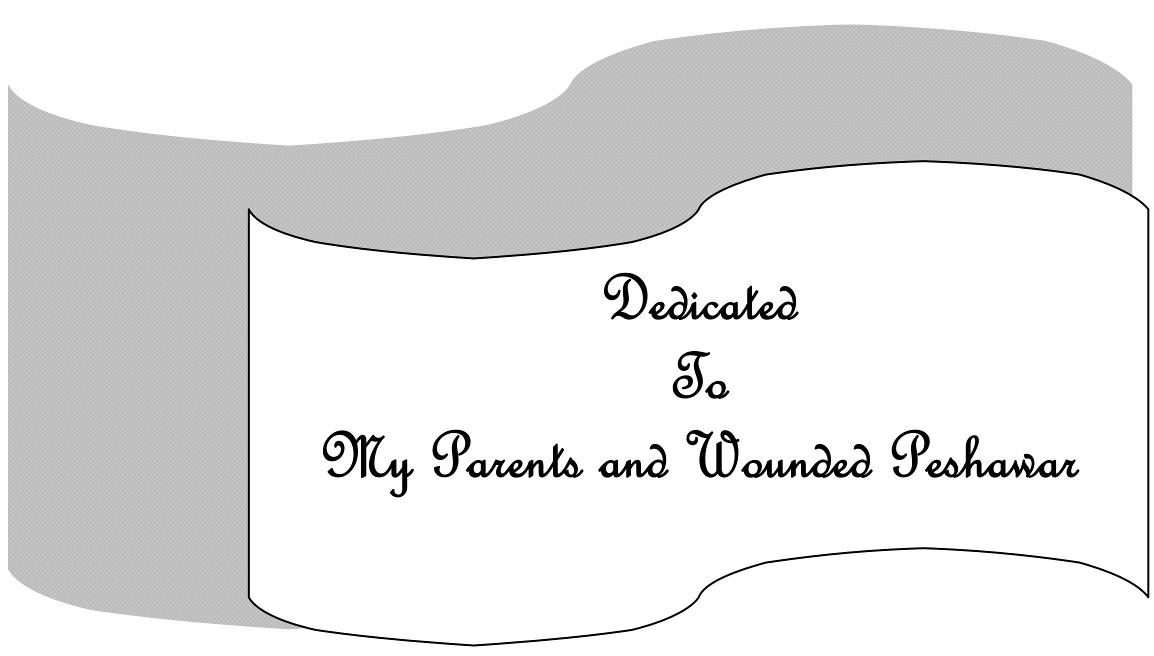
Dedicated
To
My Parents and Wounded Peshawar

TABLE OF CONTENTS

S #	TITLE	PAGE#
	ACKNOWLEDGEMENTS	**i**
	ABSTRACT	**ii**
	PART – A	
Chapter 1	**GENERAL INTRODUCTION**	2
1.1.	Scope of the Present Work	3
Chapter 2	**INTRODUCTION**	5
2.1.	Family Lamiacea	5
2.2.	Genus Teucrium	5
2.3.	Taxonomic Discription of *Teucrium Royleanum*	5
2.4.	Pharmacological Importance of Genus *Teucrium*	7
2.5	Terpenes	9
2.5.1.	Classification of Terpenes	9
2.5.1.1	Hemiterpenes	9
2.5.1.2	Monoterpenes	9
2.5.1.3	Sesquiterpenes	10
2.5.1.4	Diterpenes	10
2.5.1.5	Triterpenes	10
2.5.1.6	Tetraterpenes	10
2.6.	Terpenoid Biosynthesis	10
2.6.1	Mevalonic Acid	11
2.6.2	The Dihydroxyacetone Phosphate Pathway	14

2.6.3	Alkylation Step in Terpenoid Synthesis	15
2.7.	Biosynthesis of Diterpenoids	16
2.7.1.	Acyclic Diterpenoids	16
2.7.2.	Monocyclic Diterpenoids	17
2.7.3.	Bicyclic Diterpenoids	19
2.7.4	Tricyclic Diterpenoids	19
2.7.5	Tetra-and Pentacyclic Diterpenoid	24
2.8.	Biosynthesis of Triterpenoids	26
2.8.1.	Ambrein and Onocerin	27
2.8.2.	Tetracyclic Triterpenoids	28
2.8.3	Pentacyclic Triterpenoids	30
2.9.	Literature Survey on *Teucrium Royleanum*	36
2.10	A Brief Introduction on the Essential Oil	39
2.10.1	General Introduction	39
2.10.2	The Source of the Essential Oil	39
2.11	Methods for the Extraction of the Essential Oil	39
2.11.1.	Steam and Hydrodistillation	39
2.11.2.	Extraction with Solvent	40
2.11.3	Extraction of the Essential Oil Using Supercritical Fluid Extraction (SCFE)	40
2.11.4.	Chemical Composition of the Essential Oil	41
2.11.5.	Qualitative and Quantitative Analysis of the Essentials Oil	41

CHAPTER 3	RESULTS AND DISCUSSION	42
3.1	Ethnobotanical Survey	42
3.2.	Chemistry of the Essential Oil of *Teucrium Royleanum*	42
3.2.1.	Introduction	42
3.2.2.	Analysis of the Essential Oil of *Teucrium Royleanum*	43
3.2.3	Antioxidant Assays of the Essential Oil from *Teucrium Royleanum*	46
3.2.3.1.	The Action of 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) Stable Free Radical on the Tested Constituents	46
3.2.3.2.	Superoxide Anion Radical Scavenging Activity	47
3.3.	Phytochemical Investigation of *Teucrium Royleanum* Wall. Ex Benth.	48
3.3.1.	Introduction	48
3.3.2	Characterization of Royleanumin **1**	49
3.3.2.1	Structure Elucidation of Royleanumin	49
3.3.2.2.	Stereochemistry of Royleanumin	50
3.3.3	Characterization of Royleanumioside **2**	53
3.3.3.1	Structure Elucidation of Royleanumioside	53
3.3.4	Characterization of Royleanumoate **3**	59
3.3.4.1	Structure Elucidation of Royleanumoate	59
3.3.5.	Characterization 5-Hydroxy-4′, 7-Dimethoxyflavone **4**	63
3.3.5.1	Structure Elucidation of 5-Hydroxy-4′, 7-Dimethoxyflavone	63
3.3.6.	Characterization 5, 7 Dihdroxy 4′-3-Dimethoxy Flavone **5**	66

3.3.6.1.	Structure Elucidation Of 5, 7 Dihdroxy 4'-3-Dimethoxy Flavone	66
3.3.7.	Characterization 6, 4′ -Dihydroxy 5,7-Dimethoxy Flavone **6**	69
3.3.7.1.	Structure Elucidation Of 4′ 6-Dihydroxy 5,7-Dimethoxy Flavone	69
3.3.8	Characterization of 3, 4-Dihydroxymethyl Benzoate **7**	73
3.3.8.1	Structure Elucidation of 3, 4-Dihydroxymethyl Benzoate	73
3.3.9.	Characterization of Oleanolic Acid **8**	74
3.3.9.1.	Structure Elucidation of Oleanolic Acid	74
3.3.10	Characterization of β-Sitosterol **9**	78
3.3.10.1.	Structure Elucidation of β-Sitosterol	78
3.3.11.	Characterization of β-Sitosterol Glucoside **10**	80
3.3.11.1.	Structure Elucidation of β-Sitosterol Glucoside	80
3.4.	Allelopathy Assay of *Teucrium royleanum*	83
3.4.1.	Introduction	83
3.4.2.	Leaf Leachates Assay	84
3.4.2.1.	Sandwich Method	84
3.4.2.2.	Homogenated Sandwich Method	85
3.4.2.3	Comparison of Sandwich and Homogenated Sandwich Methods	85
3.4.3.	Essential Oil Phytotoxicity	86
3.4.4.	Methanol and Subsequent Subfractions Allelopathy	88
3.4.5.	Allelopathy of Royleanumin and Royleanumioside (New Compounds)	92
3.4.6	Allelopathy of Known Compounds from *Teucrium Royleanum*	94

CHAPTER 4	**EXPERIMENTAL**	98
4.1.	General Notes	98
4.1.1.	Material and Instrumentaiton	98
4.1.2.	Chromatography	99
4.1.3.	Spray Reagents	99
4.2.	*Teucrium royleanum* Essential oil Isolation	100
4.2.1.	Material	100
4.2.2.	Method	101
4.3.	Essential Oil Analysis	101
4.3.1	Material and Method	101
4.3.1.1	Gas Chromatogrpahy	101
4.3.1.2	Gas Chromatogrpahy-Mass Specrometry	101
4.3.2	Fractionation of the Essential oil	102
4.3.3	Idenificaiton by ^{13}C NMR Spectroscopy	102
4.4.	Antioxidant Activity	103
4.4.1	Material and Method	103
4.4.1.1	The Action of 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) Stable Free Radical with the Tested Constituents	102
4.4.1.2	Superoxide Anion Radical Scavenging Activity	103
4.5.	Phytochemical Investigations of *Teucrium royleanum*	104
4.5.1	Material and Method	104
4.5.2	Purification of Royleanumin **1**	107
4.5.2.1	Physical and Spectral Data of Royleanumin	107

4.5.3	Purification of Royleanumioside **2**	108
4.5.3.1	Physical and Spectral Data of Royleanumioside	107
4.5.3.2	Acid Hydrolysis of Royleanumioside	108
4.5.4	Purification of Royleanumoate **3**	108
4.5.4.1	Physical and Spectral Data of Royleanumoate	108
4.5.5	Purification of 5-Hydroxy-4′, 7-Dimethoxyflavone **4**	108
4.5.5.1	Physical and Spectral Data of 5-Hydroxy 4', 7-Dimethoxy Flavone	109
4.5.6.	Purification of 5, 7-Dihdroxy 4′-3-Dimethoxy Flavone **5**	110
4.5.6.1	Physical and Spectral Data of 5, 7-Dihydroxy-4′ ,3-Dimethoxy Flavone	109
4.5.7	Purification of 6, 4′-Dihydroxy, 5, 7-Dimethoxy Flavone **6**	110
4.5.7.1	Spectral Data of 4`, 6 Dihydroxy, 5, 7 Dimethoxy Flavone	110
4.5.8	Purification of 3, 4-Dihydroxymethyl Benzoate **7**	110
4.5.8.1	Physical and Spectral Data 3, 4 Dihydroxymethyl Benzoate	110
4.5.9	Purification of Oleanolic Acid **8**	111
4.5.9.1	Physical and Spectral Data of Oleanolic Acid	111
4.5.10	Purification of β-Sitosterol **9**	111
4.5.10.1	Physical and Spectral Data of β-Sitosterol	112
4.5.11	Purification of β-Sitosterol Glucoside **10**	112
4.5.11.1	Physical and Spectral Data of β-Sitosterol Glucoside	112
4.6.	Allelopathy Assay of *Teucrium royleanum*	113
4.6.1	Material and Method	113

4.6.1.1	Leaf Leachates Assay	113
4.6.1.2	Sandwich Method	113
4.6.1.3	Homogenated Sandwich Method	113
4.6.1.4	Essential Oil Isolation	114
4.6.1.5	Dish Pack Method	115
4.6.1.6	Total Activity	115
4.6.1.7	Statistical Analysis	116
4.6.1.8	Allelopathy of Purified Compounds	116
	References	117
PART – B		
CHAPTER 5	**THE CHEMISTRY OF THE ESSENTIAL OIL OF *PEROVSKIA ATRIPLICIFOLIA* BENTH.**	130
5.1	Introduction	130
5.1.1.	Genus Perovskia	130
CHAPTER 6	**RESULTS AND DISCUSSION**	132
6.1	Antimicrobial Activity	137
6.2.	Mutagenic and Antimutagenic Activity	142
CHAPTER 7	**EXPERIMENTAL**	146
7.1.	Materials and Methods	146
7.1.1.	Plant Material	146
7.1.2.	Isolation of Essential Oil	146
7.1.3.	Analysis of the Essential Oil	146
7.2.	Antimicrobial Activity Tests	147

7.2.1.	Material	147
7.2.2.	Method (Agar Diffusion)	148
7.2.3.	Minimum Inhibitory Concentration	149
7.3.	Mutagenicity and Antimutagenicity Tests	150
7.3.1.	Material	150
7.3.2.	Method	150
7.3.2.1.	S9 Fraction Preparation	150
7.4.	Cytotoxic Dose Measurements of Essential Oil	151
7.5.	Mutagenic Activities	151
7.6.	Antimutagenicity Test	152
7.7.	Statistical Analysis	152
	Refrences	154
	List of Publications	158

LIST OF TABLES

TABLE #	TITLE	PAGE #
Table 2.1:	Chemical Constituents of *Teucrium royleanum*.	36
Table 3.1:	Chemical Constituents of the Essential Oil of *Teucrium royleanum*	44
Table 3.2:	Classes of Compounds with Corresponding %Ages in v/wt	45
Table 3.3:	DPPH Radical Scavenging and Super Oxide Anion Radical Scavenging by *Teucrium royleanum*	48
Table 3.4:	HMQC of Royleanumin **1**	52
Table 3.5:	HMQC of Royleanumioside **2**	56
Table 3.6:	HMQC of Royleanumoate **3**	62
Table 3.7:	HMQC of 5-Hydroxy-4′, 7-Dimethoxyflavone **4**	65
Table 3.8:	HMQC of 5, 7 Dihdroxy 4′-3-Dimethoxy Flavone **5**	68
Table 3.9:	HMQC of 6, 4′-Dihydroxy 5,7-Dimethoxy Flavone **6**	72
Table 3.10:	HMQC of 3, 4-Dihydroxymethyl Benzoate **7**	74
Table3.11.	^{13}C-NMR Chemical Shifts and Multiplicity of Oleanolic Acid **(8).**	77
Table 3.12:	^{13}C -NMR Chemical Shifts and Multiplicity of ***β***-Sitosterol	79
Table 3.13:	HMQC of *β*-Sitosterol Glucoside **10**	81
Table 3.14:	Inhibitory Effect of Various Extracts of *Teucrium royleanum* on the Growth of Root and Hypocotyls	89
Table 6.1:	Chemical Constituents of *Perovskia atriplicifolia* Benth.	133
Table 6.2:	The Chemical Composition of the Essential Oil of *P. atriplicifolia* by Different Authors	134
Table 6.3:	Antibacterial Assays of the *P. atriplicifolia* Essential Oil	139
Table 6.4:	Antifungal Assays of the *P. atriplicifolia* Essential Oil	140
Table 6.5:	Minimum Inhibitory Concentration of the Essential Oil	141
Table 6.6:	The Antimutagenic and Mutagenicity Assays in Incorporated *Salmonella* Plate Test	144

LIST OF FIGURES

FIG. #	TITLE	PAGE #
Fig. 2.1:	Leaf of *Teucrium royleanum*	6
Fig. 2.2:	Aerial Parts of *Teucrium royleanum*	6
Fig.3.1:	2 D NOESY Correlations for Royleanumin **(1)**	51
Fig. 3.2:	HMBC Correlations for Royleanumin **(1)**	51
Fig. 3.3:	HMBC correlations of royleanumioside **(2)**	55
Fig. 3.4:	HMBC correlations of royleanumoate **(3)**	61
Fig. 3.5:	Effects of Leaf Leachates On the Inhibitions of Lettuce Seed by Sandwich Method	84
Fig.3.6:	Effects of Leaf Leachates on the Inhibitions of Lettuce Seeds by Homogenated Sandwich Method	85
Fig. 3.7:	Comparison of the Effects of Leaf Leachates on the Lettuce Seeds Inhibition by Both Methods.	86
Fig. 3.8:	Inhibition Rates of Lettuce Roots and Hypocotyl by Essential Oil of *Teucrium royleanum*	87
Fig. 3.9:	Effect of Various Concentration of Hexane On the Lettuce Roots and Hypocotyl Germination	90
Fig. 3.10:	Effect of Various Concentration of Chloroform on the Lettuce Roots and Hypocotyl Germination	91
Fig. 3.11:	Allelopathy of Compound **1**	93
Fig. 3.12:	Allelopathy of Compound **2**	93
Fig. 3.13:	Allelopathy of Compound **4**	95
Fig. 3.14:	Allelopathy of Compound **5**	95
Fig. 3.15:	Allelopathy of Compound **6**	96
Fig. 3.16:	Clevenger-type Apparatus	100
Fig. 6.1:	Mutagenicity and Anti Mutagenicity of *P. atriplicifolia*	142

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ABSTRACT

This dissertation describes the essential oil analysis, antioxidant, antimicrobial and allelopathy of two plants belonging to the genus *Teucrium* and *Perovskia* namely, *Teucrium royleanum* Wall. ex Benth., and *Perovskia atriplicifolia* Benth. belonging to the family Lamiaceae. The *Teucrium royleanum* was also subjected to the isolation and characterization of the secondary metabolites.

Investigation of *T. royleanum* resulted in isolation of three (3) new compounds, the skeletons of two belonging to terpenes, named as royleanumin **1**, and royleanumioside **2**, while the third was named as royleanumoate **3**.

The compounds **1** and **2** were subjected to allelopathy assays against lettuce seedlings (weed). Compound **1** exhibited remarkable phytotoxic activities and could prove as a lead compound for the development of environmentally friendly weedicides.

The known compounds isolated from this plant included, 5-hydroxy-4,7-dimethoxy flavone **4,** 5,7-dihydroxy-3,4−dimethoxyflavone **5**,4′, 6-dihydroxy 5,7-dimethoxyflavone **6**,3,4-dihydroxymethylbenzoate **7**, oleanolic acid **8**, β-sitosterol **9** and β-sitosterol glucoside **10**.

The literature survey reveals that flavonoids are good allelochemicals and thus the characterized 5-hydroxy-4,7-dimethoxyflavone **4,** 5,7-dihydroxy-3,4−dimethoxyflavone **5**, 4′,6-dihydroxy 5,7-dimethoxyflavone **6** were subjected to the allelopathy assays. All compounds exhibited strong allelopathy activities to the test lettuce seedlings. Consequently, these compounds can also be used as lead structures for studies on the development of new synthetic herbicides.

Analysis of the essential oil obtained from *Teucrium royleanum* Wall. ex Benth. by means of GC-MS resulted in qualitative and quantitative identification of forty six known compounds comprising of sesquiterpene hydrocarbons (42.2 %), oxygenated sesquiterpenes (21 %), monoterpene hydrocarbons (17.3 %), oxygenated monoterpenes (7.7 %). The antioxidant capacity of the essential oil was evaluated by means of 1, 1-Diphenyl-2-Picryl-Hydrazyl (DPPH) and superoxide anion methods.

The same essential oil was also subjected to allelopathy assays and showed promising results. *Teucrium royleanum* was tested by four variant assays in a separate set of experiments using lettuce seeds, showing excellent inhibition of the growth of lettuce seedlings. The plants leaf leachates were assayed by Sandwich and Homogenated Sandwich methods while Dish Pack Method was used for the evaluation of essential oils. The above stated methods confirmed the presence of allelochemicals in *T. royleanum* and were further evaluated in bioassay guided way by total activity approach. The crude methanolic extract fractionated with n-hexane, chloroform, ethylacetate, butanol and water fractions were assayed by the Total Activity Method. The essential oils, n-hexane and chloroform fractions played a significant role in our findings. The results from this study suggested that the secondary metabolites from *Teucrium royleanum* can be potential candidates for the phytotoxicity (Allelopathy).

Analysis of the essential oil obtained from *Perovskia atriplicifolia* Benth. by means of GC-MS resulted in the qualitative and quantitative identification of eighteen compounds. The major constituents were camphor (28.91%), limonene (16.72%), ά-globulol (10.21%), trans-caryophyllene (9.30%), and ά-humulene (9.25%). Antimicrobial activity of the oil was evaluated using Agar Diffusion Method and Agar Dilution Method. The antimicrobial test results showed that the oil possesses significant antimicrobial activity

against ten bacteria. The bacterial strains tested were *Bacillus cereus*, *Bacillus cerus* subsp. *mycoides*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecium*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (clinical isolate), and *Yersinia enterocolitica* respectively and five fungal strains namely *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus parasiticus*, and *Geotricum candidum* (wild type). The over all results of our studies on the essential oil of *P. atriplacifolia* suggests it to be a good source of antimutagenic and antimicrobial agents and thus can prove as a good candidate for utilization in the cosmetics, food and pharmaceutical industries.

PART A

PART A

TECRIUM ROYLEANUM WALL EX BENTH.

CHAPTER 1

GENERAL INTRODUCTION

Throughout the known history, mankind has depended and remained interested in natural medicines. Simple aqueous extractions of flowers, plants, and even insects and are being used as remedies for various diseases. Their taste, color, and odor could be used for various purposes. Healing creams and liniments were produced from plant extracts in particularly evidenced in all ancient cultures. Keeping in mind the centuries old practice of the use of plants as sources of medicinal agents for maintenance of human health, it is not surprising that natural product chemistry gained importance as part of organic chemistry around the world[1]. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousand of years in countries such as China, India, Egypt, and Greece. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization (WHO) that approximately 80% of the world's inhabitants, largely in the developing countries, rely mainly on traditional medicines for their primary health care. If one considers the therapeutic importance of morphine, quinine, digitalis, atropine, reserpine, vincristine, vinblastine, taxol etc., it is evident how great is the contribution of plant-derived drugs to medicine even today[1, 2].

The phytochemical studies on medicinal plants have served the dual purpose of bringing up new therapeutic agents, and providing useful leads for chemotherapeutic studies directed towards the synthesis of drugs modeled on the chemical structure of the natural products. Moreover, they have prompted studies on the correlation of chemical structure

and physiological activity through substituents and functional group variations in a lead structure[2].

1.1. SCOPE OF THE PRESENT WORK

The medicinal plants *Teucrium royleanum* and *Perovskia atriplicifolia* species have been used as a folk medicine for the treatment of various diseases; *Teucrium*, an important genus of the family Lamiaceae, has many pharmacological activities. Some of them have been used as medicinal plants since time immemorial and are still being used in folk medicine as antispasmodics, tonics, antipyretics and antiseptics[3, 4]. However, several publications have indicated the hepatotoxic effect when the decoction of the aerial parts of some *Teucrium* spp. are used in herbal medicine, the observed hepatotoxicity is due to some diterpenoid compounds present in these plants[5-8] some types of terpenoids have in general exhibited insect antifeedant activit[2,9-11]. The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease, diabetes and cancer is also raising interest among food manufacturers as the aware consumers move towards functional foods possessing specific health effects[12].

The plant *Perovskia atriplicifolia* has long been used in herbal medicine for treatment of fever[13]. Some Isetexane diterpene analogs and abietane diterpenoids were isolated from *Perovskia* plants that possess potent cytotoxic activities[14]. Perovskoate, an isorinic acid derivative has been isolated from the ethyl acetate soluble fraction of the whole plant of *Perovskia atriplicifolia* that showed significant inhibitory activity against lipoxygenase[15]. These observations support the uses of these plants as traditional medicine in the north-west areas of Pakistan, and they have the potential to provide new lead structures for drug discovery.

The present thesis entitled, “Bioactive principal from *Teucrium royleanum* Wall. ex Benth.

Perovskia atriplicifolia. Benth. antimicrobial, allelopathy and antioxidant assays", was completed in the Institute of Chemical Sciences University of Peshawar. In view of the importance of the *Teucrium and Perovskia* plants as remedies for diseases and as a source of medicinal and allelochemical lead compounds, there is a need for proper and systematic phytochemical investigations of these plants which was under taken in the present study.

CHAPTER 2

INTRODUCTION

2.1 FAMILY LAMIACEAE

The family Lamiaceae is a large family of order Lamials[16]. It contains about 170 genera and 300 species of worldwide distribution, growing under great variety of soils and climates but more abundant in Mediterranean and mountainous region[17]. Several genera of the family Lamiaceae contain biologically active compounds[18]. *Teucrium* is one of the important genus of this family[19].

2.2. GENUS TEUCRIUM

The genus *Teucrium* is comprised mainly of herbaceous plants. It contains about 7,000 species in temperate region, only four species of which are reported in Pakistan namely *Teucrium stocksianum, Teucrium scordium, Teucrium royleanum and Teucrium quadrifarium*[19].

2.3. TAXONOMIC DISCRIPTION OF *TEUCRIUM ROYLEANUM*

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Sub-class: Asteridae
Order: Lamiales
Family: Lamiaceae (Labiatae)
Tribe: Ajugeae
Genus: *Teucrium*
Species: *royleanum* Wall. ex Benth.
Altitude: 1300 – 2800 m

Fig. 2.1: Leaf of *Teucrium royleanum* Wall. Ex Benth.

Fig. 2.2: Aerial Parts of *Teucrium royleanum* Wall. Ex Benth.

2.4 PHARMACOLOGICAL IMPORTANCE OF GENUS *TEUCRIUM*

The genus *Teucrium* has been attributed many pharmacological activities. Some of the species have been used as medicinal plants since time immemorial and are still being used in folk medicine as antispasmodics, tonics, antipyretics and antiseptics[3,4]. However, as stated before, several publications have indicated the hepatotoxic effect when the decoction of the aerial parts of some *Teucrium* spp. are used in herbal medicine, probably due to the hepatotoxic diterpenoids involved present in these plants[5-8]. The terpenoids in these plants have also been reported to show insect antifeedant activity[3, 9-11]. The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among food manufacturers as consumers move towards functional foods with specific health effects[12]. *Teucrium* species are bitter, astringent, anti rheumatic herbs that reduce inflammation, stimulate the digestion and have been used as herbal medicines for coughs and asthma since ancient times. Several studies about bacteriostatic, spasmolytic, antioxidant and anti-inflammatory effects of *Teucrium* species have also been reported in the literature[20, 21].

The *Teucrium* genus is famous for the presence of essential oils which exhibit various activities like antibacterial, antifungal, and antioxidant.

Teucrium royleanum

Antibacterial activities of the crude methanolic extract and of sub-sequent fractions of *Teucrium royleanum* have been reported. Among all the fractions, ethyl-acetate and chloroform extracts exhibited significant inhibitory effects against selected bacterial cultures. Crude extract with mild activity, and sub-sequent fractions of *Teucrium*

royleanum had also a potential to show antifungal activity[22]. The crude methanolic extracts and its subsequent fractions of *Teucrium royleanum* showed 52-83% inhibitory effects against acetyl cholinesterase, while 19-93% inhibitory effects against butyrylcholinesterase.[23]

T. ramosiaaimum

The essential oil of this species harvested in the mountainous region of Tunisia, showed weak to moderate activity for its antifungal and antibacterial assays[24].

T. polium

Crude ethanolic extract of this plant of Iran origin showed good cytotoxic activity when compared to a known herbal anticancer agent[25]. The ethylacetate extract exhibits hepatoprotective activity[26]

T. sauvagei

This specie is endemic to Tunisia, the methanolic extract and the essential oil of this plant was tested for in vitro antifungal and antioxidant assays. Both gave promising results and due to this reason, it is being used as natural preservative ingredients in food and pharmaceutical industries[27]

T. stocksianum subsp. stocksianum

The plant collected from Oman at flowering stage, the essential oil when subjected to antimicrobial activity showed good results against some strains[28].

T. orieantale* L. var. *orientale

Aerial parts were collected at budding, flowering, and vegetative stages and extracted with petroleum ether, chloroform, acetone and methanol. It was found that the acetone and methanol extracts of all and the chloroform extracts of the budding and flowering stages showed highest antioxidant activity. Further, one new iridoid was isolated from the

acetone extract through column chromatography.[29]

T. mascatenses

The essential oil of this plant, indigenous to Oman, exhibited good antimicrobial activity[30]

2.5. TERPENES

Terpenes are the generic name of a group of natural products, structurally composed of isoprene units. The oxygenated derivatives of terpenes are known as terpenoids. The theory that provided the first conceptual framework for a common structural relationship among the terpenes was first formulated by Wallach in 1887, after carrying out structural investigations of several terpenes[31]. His theory stated that terpenes can be viewed as made up of isoprene (2-methyl-1, 3-diene) units joined together in a head to tail manner. Wallach's idea was further refined in the 1950 by Ruzicka's formulations of the biogenetic isoprene rule[32], emphasizing mechanistic considerations of terpenes synthesis in terms of electrophilic elongations, cyclizations and rearrangements.

2.5.1. CLASSIFICATION OF TERPENES

The normal classification of terpenes is based upon the number of isoprene units. These terpenes are further subdivided into several subclasses on the basis of a particular type of skeleton they posses.

2.5.1.1 Hemiterpenes

These are made up of one isoprene unit. The best example of hemiterpenes is isoprene itself [33].

2.5.1.2 Monoterpenes

These are characterized by two isoprene units in their basic skeleton and are widely distributed in nature as the major constituents of essential oils. They are also important in

marine organisms. The biosynthetic pathways of the main classes of monoterpenes have been well studied[34, 35].

2.5.1.3 Sesquiterpenes

The terpenes belonging to this class are the result of combination of three isoprene units. They are found in many living system but particularly in higher plants[36, 37]. There is a vast number of sesquiterpenoid carbon skeletons, arising from the common precursor, farnesyl pyrophosphate by various modes of cyclizations, followed in many cases by skeletal rearrangements[4, 38, 39].

2.5.1.4 Diterpenes

Diterpenoids are made up of four isoprene units with a total of twenty carbon atoms in the nucleus of the molecule. They occur in several plant families and have more than 20 major structural types[40- 42].

2.5.1.5 Triterpenes

The combination of six isoprene units having thirty carbon atoms results in the basic skeleton of triterpenes. They are derived from squalene which in turn is formed upon head to head coupling of two sesquiterpenoid units[43-45].

2.5.1.6 Tetraterpenes

These are eight isoprene units containing class of terpenes. They are generally formed by head to head coupling of two geranyl-geranyl pyrophosphate molecules[46].

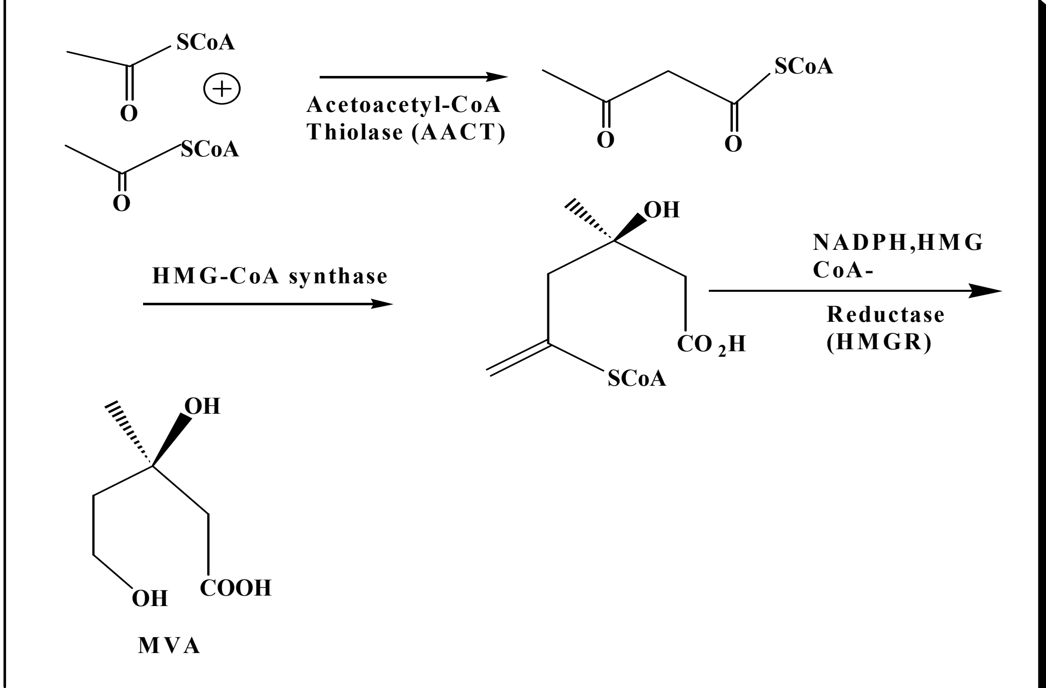
2.6. TERPENOID BIOSYNTHESIS

The pyrophosphate ester of an unsaturated five-carbon alcohol, 3-methyl-3-buten-1-ol is the structural building block of the naturally occurring terpenes. Pyrophosphoric acid is an anhydride of phosphoric acid and seems to be nature's tool for creating good leaving groups. 3-Methyl-3-buten-1yl pyrophosphate, known in the biochemical literature as

isopentenyl pyrophosphate, is isomerized enzymatically to 3-methyl-2-buten-1-yl (dimethylallyl) pyrophosphate in a reaction that may be regarded as a protonation at one sp^2-hybridized carbon atom and deprotonation of the incipient carbocation at another site to give the more highly substituted alkenes. The participation of an enzyme, a highly specific biological catalyst, ensures that no high-energy intermediate is formed at any point of the reaction[47].

2.6.1 MEVALONIC ACID (MVA)

The biosynthesis of almost all isoprenoids begins from mevalonic acids (MVA) which is synthesized by the condensation of two molecules of acetyl-CoA to produce an acetoaceyl-CoA. Further aldol type condensation of another molecule of acetyl-CoA with acetoacetyl-CoA yields 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is catalyzed by HMG-CoA synthase enzyme. HMG-CoA is then reduced to Mevalonic acid (MVA) by HMG-CoA reductase (HMGR) which uses NADPH[48].

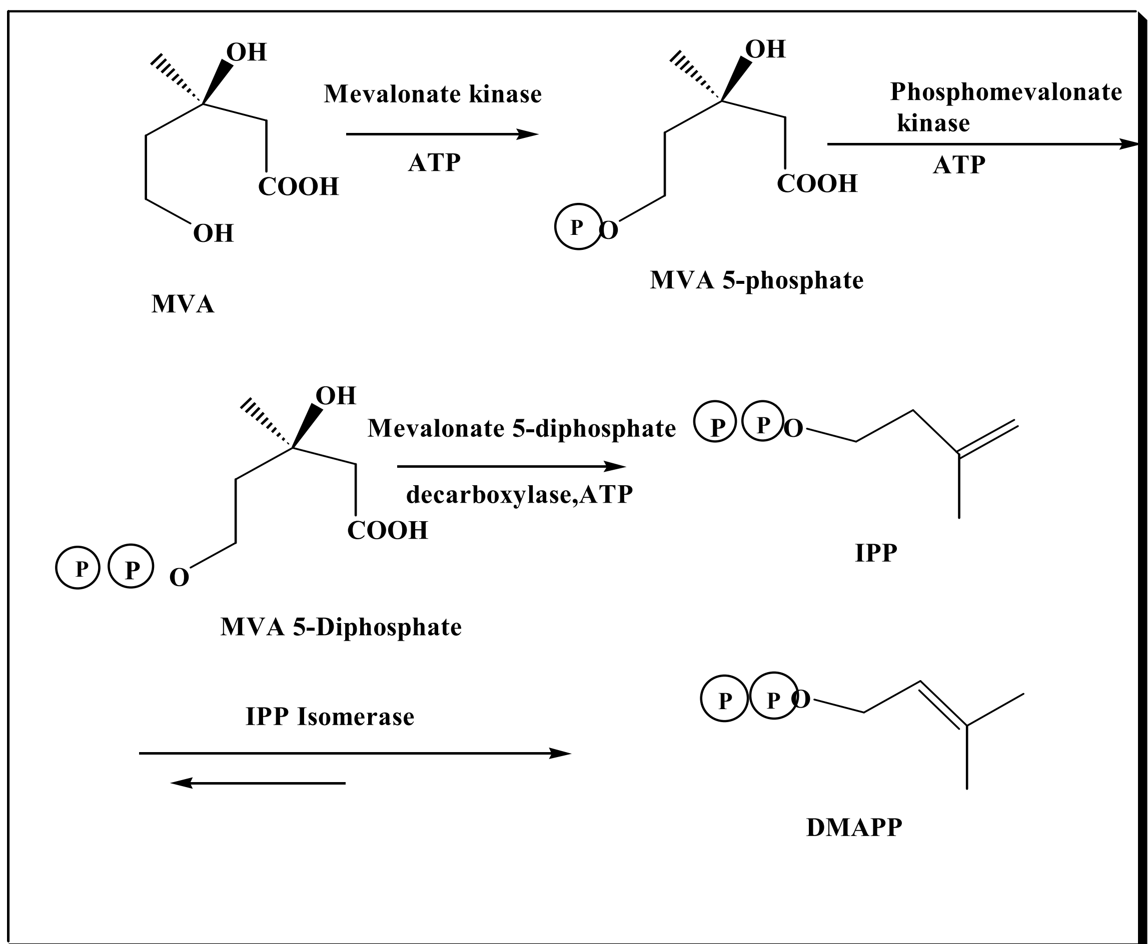


Scheme-2.1: Biosynthesis of Mevalonic acid

The suggested mechanism for the reduction of HMG-CoA to MVA is given in Scheme-2.2

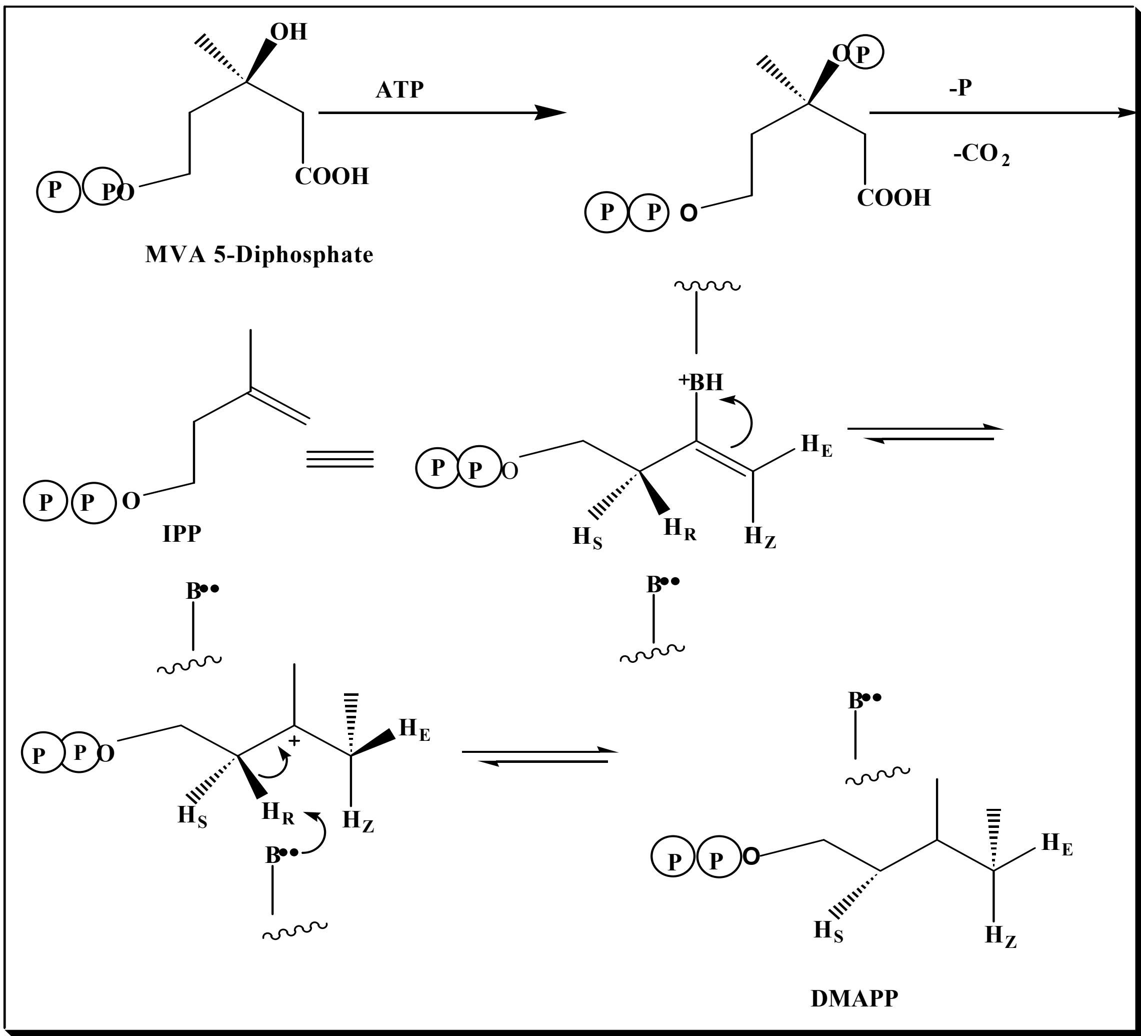
Scheme- 2.2: The mechanism of the enzyme catalysed reduction of HMG-CoA to MVA

ATP- dependent phosphorylation of mevalonate to mevalonate-5- diphosphate followed by enzymatic decarboxylation yields isopentenyl diphosphate (IPP) which can be converted to dimethylallyl diphosphate (DMAP) **(Scheme-2.3)**.



Scheme-2.3: Conversion of MVA to DMAPP and IPP.

The mechanism of decarboxylation of MVA-5-diphosphate involves two steps, the phosphorylation of the 3-hydroxy group followed by decarboxylation Scheme-2.4. The conversion of IPP to DMAPP is catalysed by IPP isomerase through a 1, 3-allylic rearrangement through a postulated two base cationic mechanism **(Scheme-2.4).**



Scheme-2.4: Decarboxylation of MVA 5-diphosphate and isomerization of IPP to DMAPP

2.6.2 THE DIHYDROXYACETONE PHOSPHATE PATHWAY

A number of studies showed that some bacteria employ a biosynthetic pathway to terpenoids which is different from the mevalonate pathway. In this pathway the C_5 framework of terpenoids is considered to be constructed by the condensation of a C_2 unit derived from the decarboxylation of pyruvate onto the C-2 carboxyl of a triose phosphate derivative. The dihydroxyacetone phosphate pathway to produce IPP and DMAP[48] is

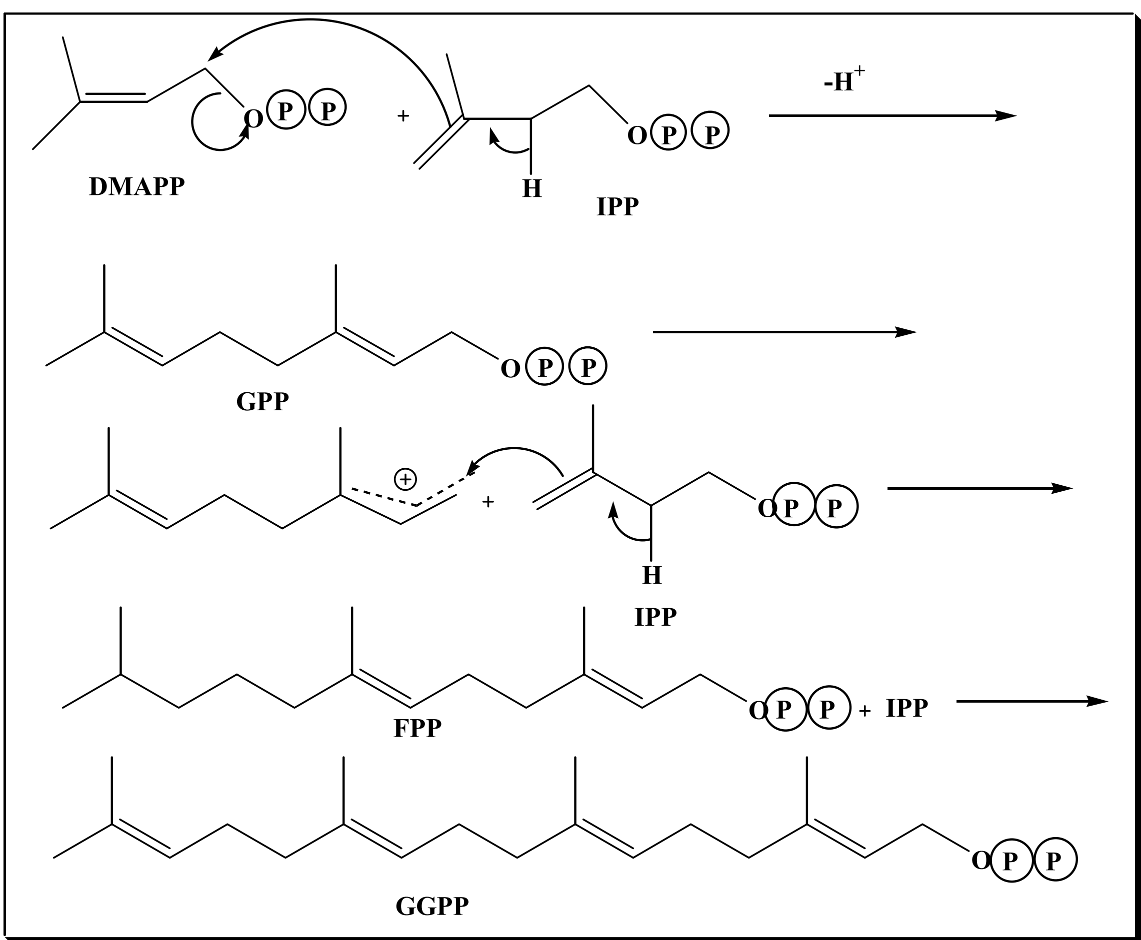
illustrated in Scheme-2.5.

Scheme-2.5: The dihydroxyacetone pathway for the biosynthesis of IPP and DMAPP

2.6.3 ALKYLATION STEP IN TERPENOID SYNTHESIS

The enzymes known as prenyltransferases are responsible for the alkylation steps involving DMAPP and one or more units of IPP. This reaction provides the precursors for terpenoids synthesis. The enzymes geranyl diphosphate synthase, farnesyl diphosphate

synthase and geranylgeranyl diphosphate synthase are the enzymes which produce geranyl-geranyl pyrophosphate (GGPP), the precursors of mono-, sesqui- and diterpenoids, respectively[32, 48] (**Scheme-2.6).**



Scheme-2.6: Biosynthesis of GPP, FPP and GGPP from DMAPP and IPP

2.7. BIOSYNTHESIS OF DITERPENOIDS

As mentioned earlier, almost all diterpenoids derive via cyclizations of geranyl- geranyl or geranyl-linalool either from a free radical or a cationic pathway. This biogenetic isoprene rule was first suggested by Ruzicka L. in 1953[32].

2.7.1. ACYCLIC DITERPENOIDS

The acyclic diterpenoids with the phytane framework are the most important linear

diterpenes, but, despite the fact that it is derived from geranyl-geranyl PP having the precursors of all diterpenes, however, the occurrence of these compounds is very rare. Some acyclic diterpenes are derived from precursors other than GGPP such as digeranyl and isodigeranyl and peucelinendiol which are named as non-geranyl-geranyl diterpenoids[49, 50].

2.7.2. MONOCYCLIC DITERPENOIDS

The biogenesis of these compounds involves the cyclizations of GGPP. The biogenesis of the retinane type skeleton and Trixagol can begin with the electrophilic attack of H^+ to the C-14/C-15 double bond of GGPP followed by dehydrogenation **(Scheme-2.7)**. The most important compound of this group, retinol, is produced in the body by cleavage of certain Carotenoids. Trixagol and related compounds are apparently synthesized by the cyclizations of GGPP [49, 51].

GGPP

Retinane type skeleton

Trixagol

Scheme-2.7: Cyclization of GGPP to retinane.

The intramolecular electrophilic attack of the C-14/C-15 double bond to the ionized GGPP at C-1 produces the macrocyclic diterpene, cembrene, which is widely distributed in resins, tobacco and marine species[52, 53]. Several diterpenoids with the cembrane skeleton have been isolated, flexibilene is a metabolite of the soft coral. From *Simdaria flexibilis* a diterpenoid with a 15 membered ring resulting from the C-1/C-15 cyclization of GGPP[54] **(Scheme-2.8)**.

Scheme 2.8: Biogenesis of macrocyclic diterpenoids from GGPP.

2.7.3 BICYCLIC DITERPENOIDS

Among the bicyclic diterpenoids is labdane are the most abundant group. The biogenesis of labdane is the continuation of the cyclizations of the second ring of GGPP (**Scheme-2.9)**. On the other hand, clerodanes, which exist in both antipodal types, are predominant in the forms which are derived from ent-labdane[55] **Scheme 2.9.**

Scheme 2.9: Biogenesis of bicyclic diterpenoids from GGPP.

2.7.4. TRICYCLIC DITERPENOIDS

Copalyl pyrophosphate, obtained from labdane, is a precursor for the tricyclic diterpenoids, pimarane via the internal attack of the double bond at C-13 which produces both of the epimers of pimarane[49, 48]

Copalyl pyrophosphate

epimer of Pimarane

epimer of Pimarane

Scheme- 2.10: Biogenesis of tricyclic diterpenoids.

Rosane, Ablietane and Cassane skeleton can be made from a series of hydride and methyl shift in the pimarane skeleton. They are considered as rearranged pimarane-type diterpenes[49] (**Scheme-2.11).**

H

Rosane

H^+

Abletane

Cossane

Scheme-2.11: Rearranged pimarane type diterpenoids.

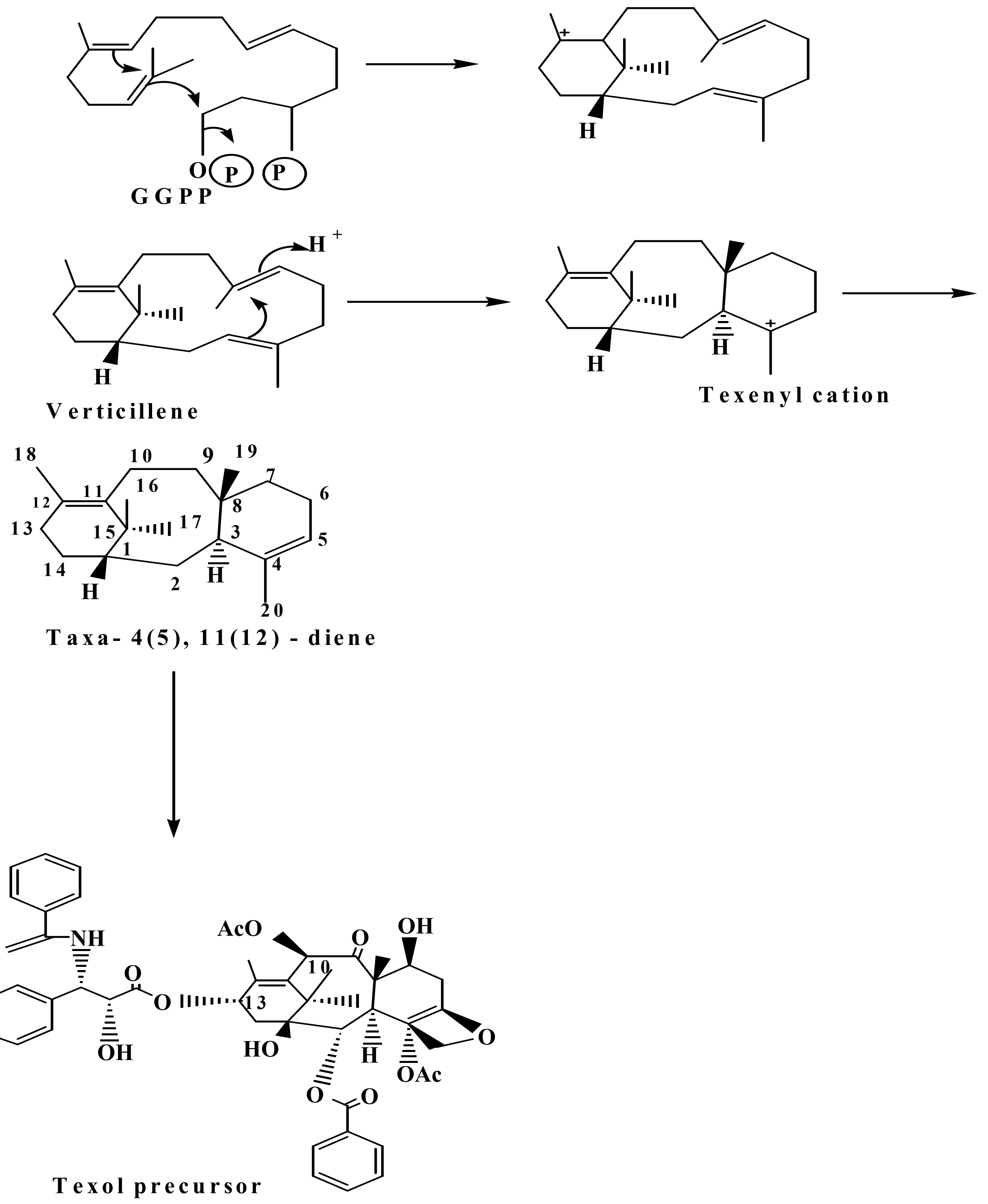
Taxenyl cation, the precursor of taxol, the most important anti-cancer diterpenoid, can be biosynthesized through a cembrane pathway[49] **(Scheme-2.12)** or directly from GGPP[48] (**Scheme-2.13)**.

3
7
1
14
11
Cembrene cation

H+
3

Taxenyl cation

Scheme-2.12: Biogenesis of taxol from cembrene.



Scheme-2.13: Biogenesis of taxol from GGPP.

2.7.5 TETRA-AND PENTACYCLIC DITERPENOID

Further cyclizations of the tricyclic pimarenyl cation generate the beyerane cation, the precursor of several tetra and pentacyclic diterpenes. A Wagner-Meerwein re-arrangement in the beyerane cation produces the kaurane skeleton which can be cyclized to trachielobane. Another important diterpene skeleton, atisane, is produced from the beyerane skeleton via a 1,3-H-Shift and Wagner-Meerwein rearrangement[49, 56] (**Scheme-2.14).**

Scheme-2.14: Biogenesis of tetra- and pentacyclic diterpenoids.

Andromedane type diterpenes are kaurane derivatives which are produced via expansion of the B ring of the kaurane skeleton[49, 48] **(Scheme-2.15)**

Kaurane

Andromedane cation

Scheme-2.15: Rearrangement of the kaurane to the Andromedane skeleton.

The biosynthesis of the gibberellins, the most important class of diterpenes which are found in almost all green plants, involves the contraction of ring B of an ent kaurane intermediate[49, 48] **(Scheme-2.16).**

COOH H H OH

Ent-kaurane

COOH CHO

Gibberellins

Scheme-2.16: Formation of the gibberellane from the kaurane skeleton

2.8. BIOSYNTHESIS OF TRITERPENOIDS

Triterpenoids can be classified in two broad classes: tetracyclic series structurally related to the steroids and a very large pentacyclic class, including a number of skeletal types. The cyclizations of squalene can be promoted either by oxidative or non oxidative agents. Two routes can be proposed for oxidative cyclizations a prior conversion of squalene into 2, 3-epoxy-squalene followed by its transformation into cyclic derivative via protonation and Markwnikoff's opening of the epoxide ring or HO+ (protonated oxene) attack on the 2, 3 double bond. The non-oxidative cyclizations involves the attack of a proton on 2, 3 double bond[57] (**Scheme 2.17).**

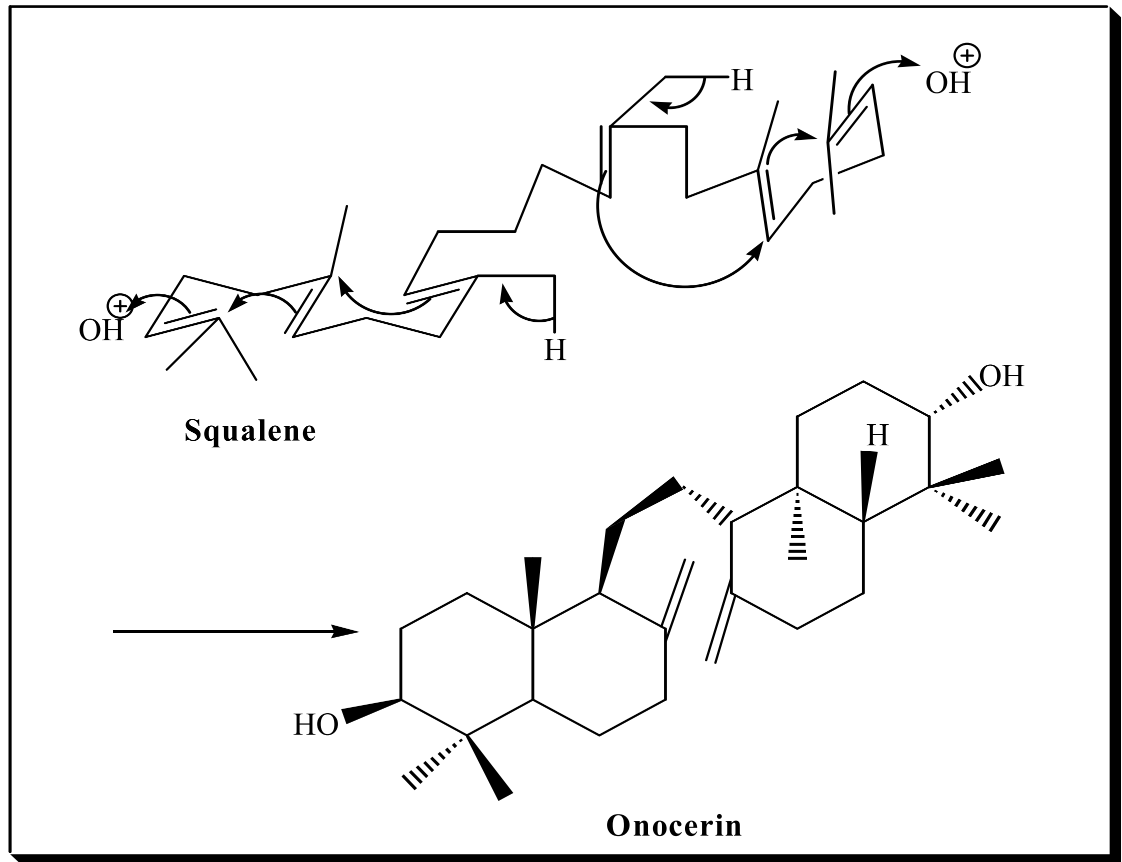
Scheme 2.17: Formation of 3-Hydroxy triterpene

2.8.1 AMBREIN AND ONOCERIN

Ambrein does not belong to any of the major class of triterpenoids and results from non-oxidative cyclizations of squalene in c-c-u-u-c conformation and is initiated by the attack of a proton. It starts from both ends of the squalene chain leaving an uncyclized portion in the middle[58, 59] (**Scheme 2.18**).

Scheme 2.18: Formation of Ambrein from Squalene

The tetracyclic onocerin like ambrein is not included in any of the major classes of triterpenoids and arises via cyclizations of squalene in c-c-u-c-c- conformation and again starts from both ends of the molecule, though in this case it is oxidative[60] **(Scheme 2.19).**



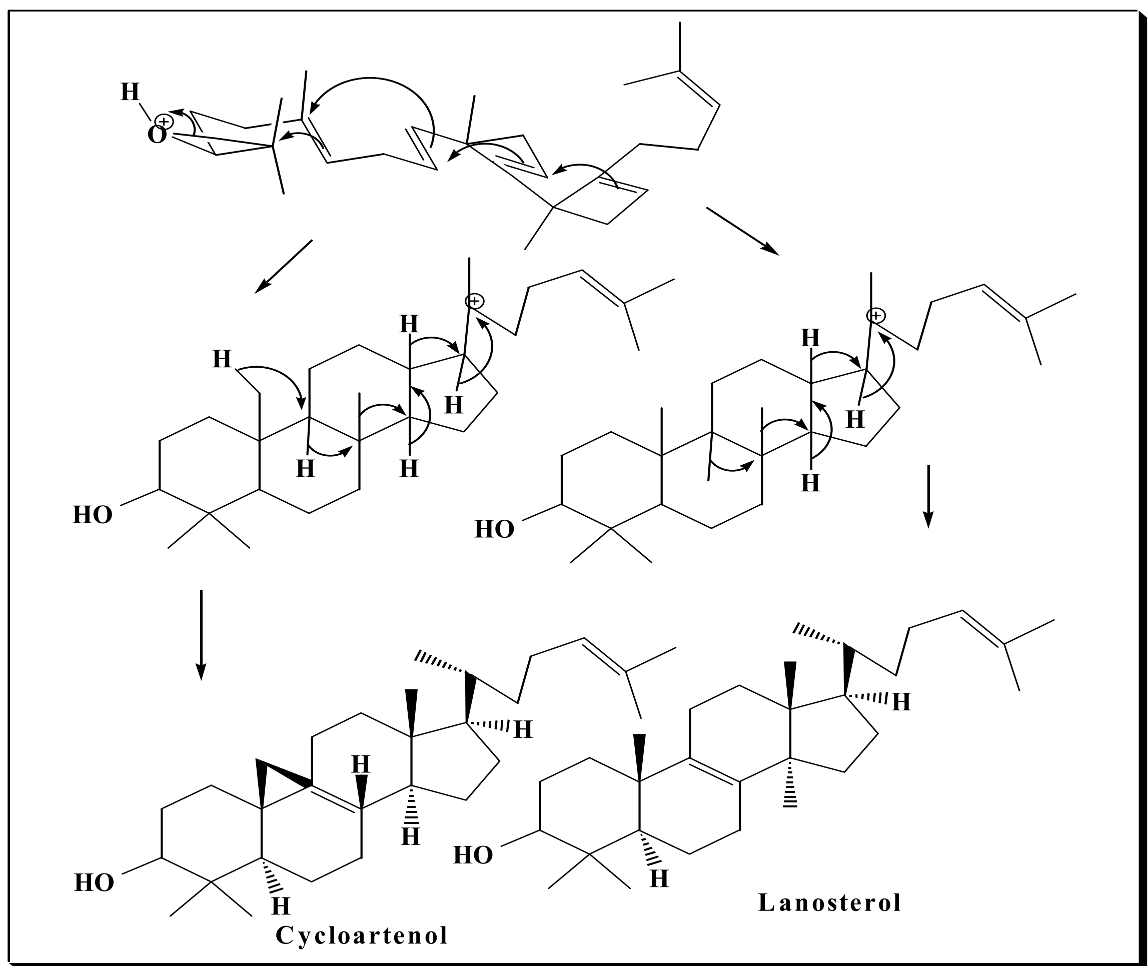
Scheme 2.19: Formation of Onocerin from Squalene

2.8.2. TETRACYCLIC TRITERPENOIDS

Most of the members of this group belong to either of two series i.e. lanosterol series on the one hand and euphol series on the other differing in the stereochemistry of C/D ring junction and the side chain.

Lanosterol and cycloartenol

The genesis of this skeleton requires oxidative cyclizations of squalene in c-b-c-b-u conformation. The intermediate ionic species undergo a series of 1, 2-migrations and eventual protonation leading to lanosterol or cycloartenol[61, 62] (**Scheme 2.20).**



Scheme 2.20: Formation of cycloartenol and lanosterol

Euphol and Tirucallol

This skeleton may be generated by oxidative cyclizations of squalene in c-c-c-b-u conformation to the intermediate (a), which on discharge by hydroxyl ion gives dammarendiol[63, 64]. Alternatively the intermediate (a) may rearrange via the cation (b) to euphol or via the cation (c) and (d) to tirucallol[65, 66]**(Scheme 2.21).**

Scheme 2.21: Formation of Euphol and Tirucallol

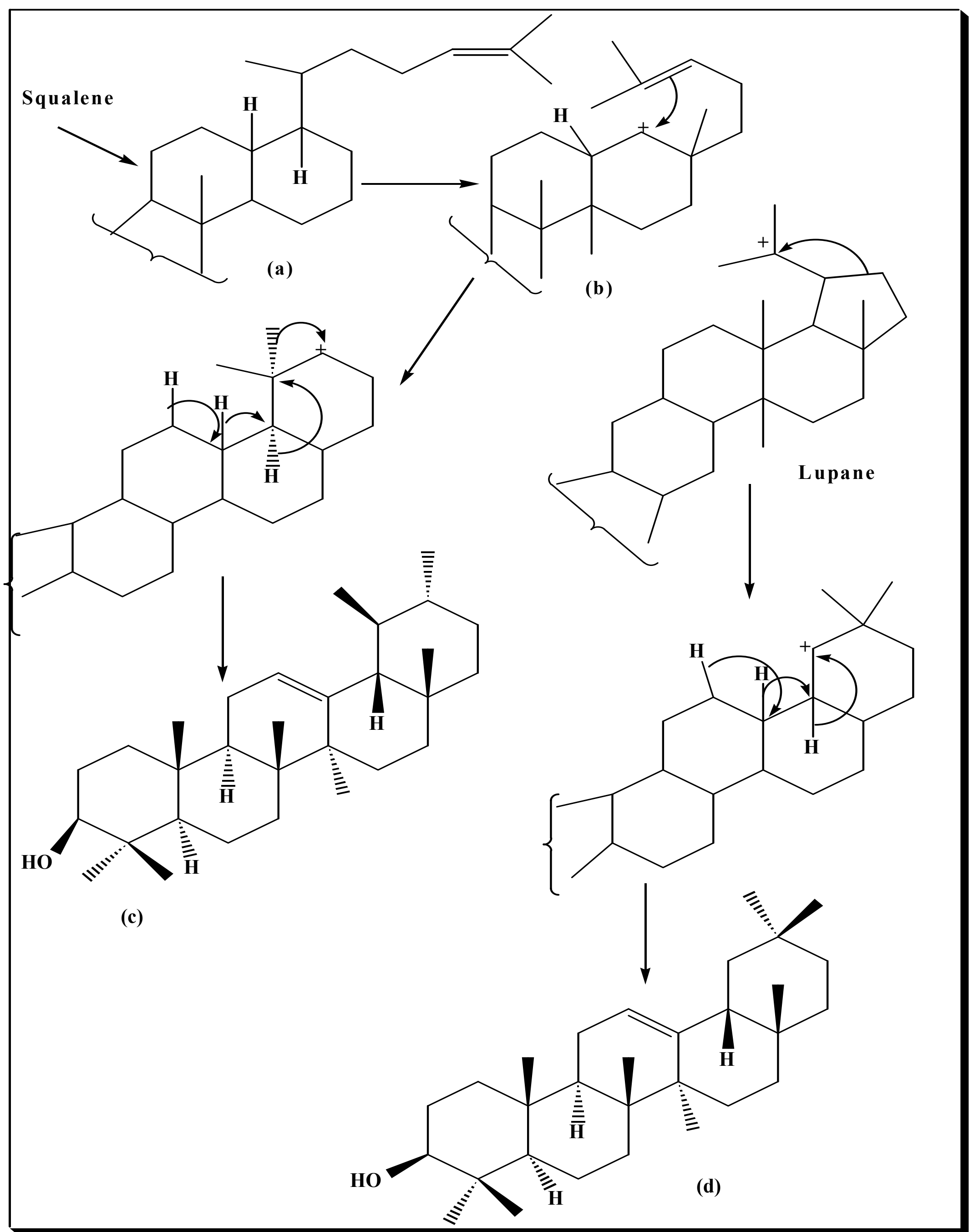
2.8.3 PENTACYCLIC TRITERPENOIDS

There are also two series of Pentacyclic triterpenoids having all six membered rings or with a five membered ring "E" bearing an isopropyl group.

Oleanane (β-Amyrin) And Ursane (ά -Amyrin) Series

The triterpenoids of these two series produced like that of the euphol series form the

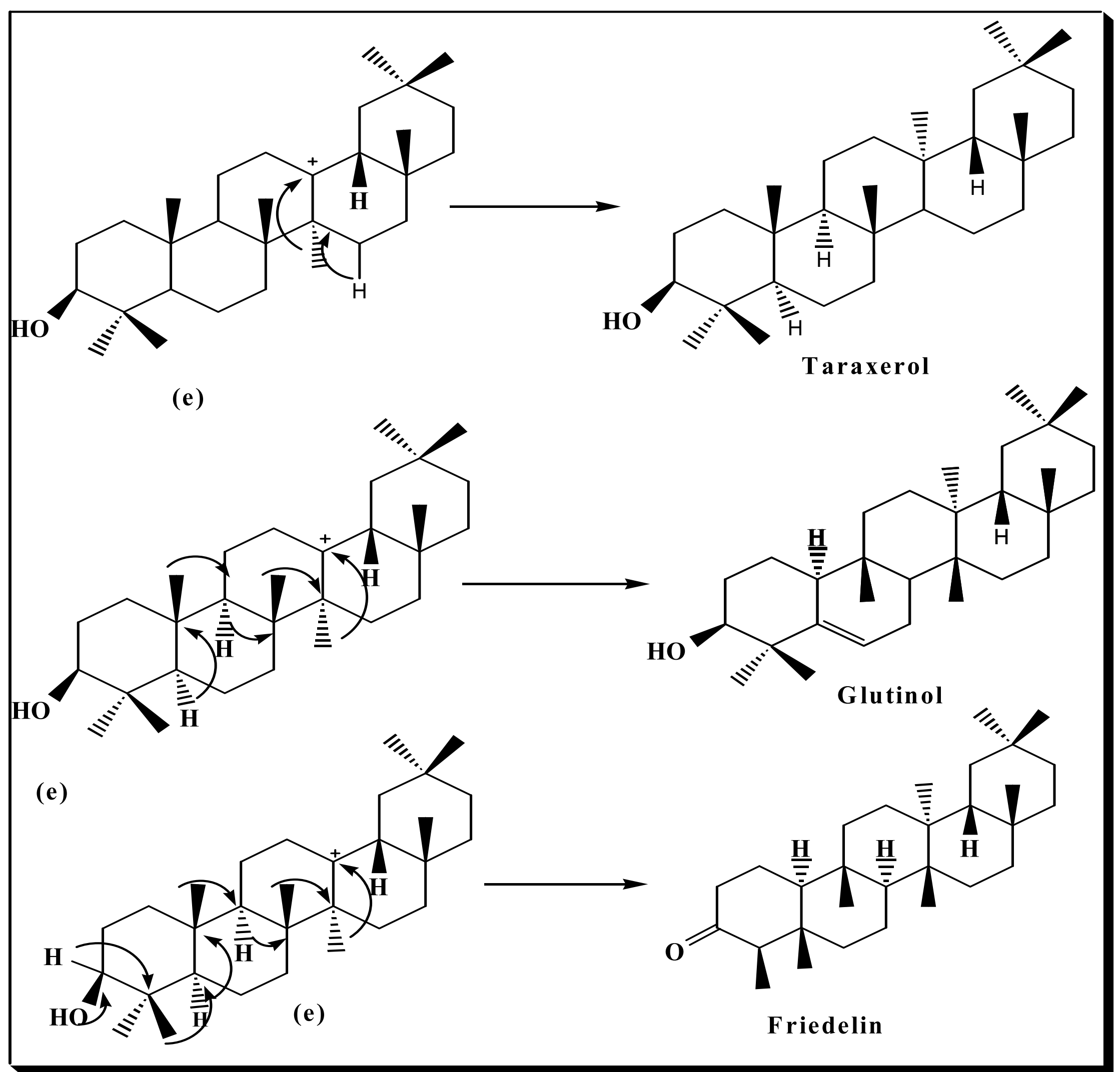
c-c-c-b-u conformation of squalene. Thus, oxidative cyclizations of squalene to cation (a), followed by ring enlargement through two different ways yielding either ά-amyrin (c) or β-amyrin (d) [67, 68] (**Scheme 2.22).**



Scheme-2.22: Formation of ά-Amyrin and β −Amyrin from squalene

Skeleton Modification in β-Amyrin

D-Friedoolean-14-ene (taraxerol), D-β-Friedoolean-5-ene (glutinol), and -ά-Friedooleanane (friedelin) skeletons arise directly from β-amyrin cation (e), involving a number of stereo specific 1,2-migrations along the back bone of the molecule[69, 70] **Scheme 2.23**



Scheme 2.23: Skeleton modification in **β**−Amyrin

Lupane and Hopane Series

These two small but biogenetically important groups are considered here together for convenience since both have five membered rings E. The lupane skeleton originates by the same biosynthetic processes as the ursane and oleanane skeletons. It is derivable by proton elimination from the intermediate (a) in the biogenesis of β-amyrin[71, 72] (**Scheme 2.24).**

Scheme 2.24: Formation of Lupane

The hopane skeleton can result through direct cyclizations of a folded squalene molecule without rearrangement. It arises by the attack of water and $^{+}OH_3$ on the all chair conformation of squalene[71, 72] (**Scheme 2.25).**

Scheme-2. 25: Formation of hopane

The other less commonly occurring pentacyclic triterpenes of the series include arborinol that results by the oxidative cyclizations of squalene in c-b-c-c-b conformation. While moretenol and fernenol arise from c-c-c-c-b conformation of squalene[73] (**Scheme 2.26).**

Scheme-2.26: Formation of Femenol and Moretenol from Squalene

2.9. LITERATURE SURVEY ON TEUCRIUM ROYLEANUM

A thorough literature search showed that a total of sixteen chemical constituents have been isolated from the aerial parts of *Teucrium royleanum* previously[74] (**Table 2.1).**

Table 2.1: Chemical constituents of *Teucrium royleanum* Wall. ex Benth.

No	Chemical Structure	Name
1.	$HOOC-(CH_2)_{16}-CH_3$	Octadecanoic acid
2.	$HOOC-(CH_2)_{24}-CH_3$	Hexacosanoic acid
3.		Lupeol
4.		β -Amyrin
5.	$HOOC-(CH_2)_{29}-CH_3$	Hentriacontane
6.	$H_3C-(CH_2)_{31}-CH_3$	Tritriacontane

7.	$H_3C-(CH_2)_{33}-CH_3$	Pentatriacontane
8.	$H_3C-(CH_2)_{28}-CH_3$	Triacontane
9.	$H_3COOC-(CH_2)_{22}-CH_3$	Tetracosanoic acid, methyl ester
10.	$HOOC-(CH_2)_{16}-CH_3$	Octadecadienoic acid
11.		5,22,25- Stigmastatrien-3-ol
12.		5,25- Stigmastadien-3-ol
13.	$H_3C-(CH_2)_{23}-CH_3$	Pentatriacontane

14.	$H_3C-(CH_2)_5-CH{=}CH-C(=O)-HC{=}HC-(CH_2)_5-CH_3$	7,12-Nonadecadien-9-one
15.	HO, HO, HO, OH, O, H, O, CH_3, CH_3, H_3C, C_2H_5, Pr-i, H, R, S	5,23-stigmastadien-3-O-glactoside
16.	$H_3C-(CH_2)_{23}-OH$	Hentriacontadien-1-ol

2.10 BRIEF INTRODUCTION OF THE ESSENTIAL OIL

2.10.1 GENERAL INTRODUCTION

Essential oil, ethereal oil, or volatile oil are the volatile constituents of a plant, which are extracted by means of steam distillation. They are oily material, insoluble in water, soluble in alcohol and ether, and are distinguished from fixed or non-volatile oils by their volatility. A spot of an essential oil on a piece of paper will evaporate after some time, unlike a fixed oil, which remains for a long period of time[75, 76].

About 200 essential oils are commercially known, among which five are produced on a large scale, for instance citrus, peppermint, spearmint, orange, lemon and some spice oils, such as clove and nutmeg. The essential oils are widely used in different industries, e. g. as ingredients in many pharmaceutical products, from antiseptics and flavouring agents to analgesic and antimicrobial components in mouth washes or gargles. In fragrances, perfumes and different foodstuffs the essential oils are important ingredients[77, 78].

2.10.2 THE SOURCES OF THE ESSENTIAL OIL

Essential oil can be isolated from almost every part of a plant, for instance, some of them are biosynthesized in flowers (rosemary and jasmine), in fruits (fennel, orange, lemon, star anise). In flower buds (clove oil), in seeds (mustard), in leaves (geranium, mint, sage), in woods and inner bark of the shoots (camphor, cinnamon), and they are either extracted from gum (turpentine oil)[77, 79].

2.11. METHODS FOR THE EXTRACTION OF THE ESSENTIAL OIL

2.11.1. STEAM AND HYDRODISTILLATION

The essential oils can be extracted by different methods. Among them steam and hydrodistillation of the plant material are the most common methods. In steam distillation,

a steam produced in a separate boiler is passed through plant material and the volatile substances are carried along with the steam. After condensation in a condenser, the volatile oil being immiscible is separated or it is extracted by suitable organic solvent from the aqueous solution. In the hydrodistillation methods a mixture of the plant material and water are heated. In the case of substances which are sensitive to water it is not a good method, but for dry plant material, it has great application.

2.11.2. EXTRACTION WITH SOLVENT

Extraction with a solvent is used for very sensitive plant materials such as flowers. The plant material is extracted by soaking in a non-polar solvent like hexane at room temperature, or they can be extracted by Soxhlet extractor. After removing the solvent at reduced pressure the viscous residue is named concrete. The concrete contains both volatile and non-volatile/non-polar compounds upon dissolving in ethanol, the non-polar, non-volatile materials are precipitated which can be separated by filtration. The filtrate is concentrated under reduced pressure, is named absolute and it contains the essential oil. This method has less application in comparison to steam distillation[75].

2.11.3. EXTRACTION OF THE ESSENTIAL OIL USING SUPERCRITICAL FLUID EXTRACTION (SCFE)

In 1879 Hanny and Hogarth demonstrated the solubilizing properties of supercritical fluids. Recently, this method of extraction has been applied for different classes of natural products, including essential oils.

The advantage of carbon dioxide (CO_2) supercritical fluid extraction for the isolation of the essential oils over steam-and hydrodistillation and solvent extraction are the mild conditions and the low cost as well as the lack of toxicity and the absence of problems of disposal of the waste organic[80]. In the SCFE apparatus the plant material is extracted by

fluid CO_2 at different densities, controlled by pressure and temperature of the CO_2[81].

2.11.4. CHEMICAL COMPOSITION OF ESSENTIAL OIL

The distinction between essential oil and flavour and fragrant compounds is not at all clear, but the essential oil are typically volatile terpenes and esters, while the flavour and fragrance compounds mostly consist of other volatiles like phenols, alkanes, aldehydes, etc. The sulfur and nitrogen-containing compounds which are available to be extracted by steam distillation also can be considered as components of essential oils[78, 80].

2.11.5. QUALITATIVE AND QUANTITATIVE ANALYSIS OF ESSENTIAL OIL

The most important method for identification of the essential oils is gas chromatography (GC) and its combination with mass spectrometry (GC/MS), in which even a trace amount of a compound can be detected and identified. This method is applicable for the identification of those compounds which have been identified previously, and for which GC and mass spectral data are available for comparison[82].

For the determination of the structure of a new compound, it must be first purified and then, utilizing a variety of different spectroscopic techniques such as NMR and MS, the structure is elucidated. For this purpose, several methods including vacuum distillation, flash chromatography, and preparative gas chromatography must be employed in order to obtain a pure compound[83]

The other methods include the coupling of GC and infrared spectroscopy (GC/IR) and co-injection with authentic samples in gas chromatography are also applied[84].

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ETHNOBOTANICAL SURVEY

Ethnobotanical studies on *T. royleanum* are not reported from any part of its growing habitat. However, according to our survey, *T. royleanum* is an endemic species of Asia. It grows in northern province, NWFP, of Pakistan, in the districts of Swat and Kohat. Besides plant collection for phytochemical studies, ethnobotanical survey regarding *T. royleanum* was also carried out. The preference for interview was given to the elders of the community. The plant is used by the local people for stomach disorders in the district of Kohat and locally it is known as *Mastiyara*. But in Kurram Agency in North Western part of the province, the name *Mastiyara* is used for *Artemisia absinthium* and is used for the same purpose. The local people of the district of Swat, Pakistan take the leaves of *T. royleanum* and touch it on the surface of milk, due to which the milk converts into yogurt. In June and July, summer months of the year, the local people of district Swat, crush the leaves of the plant and boil them in water. The decoction of the plant is used as cooling agent. The local communities also collect the plant and use it as a fuel.

3.2. THE CHEMISTRY OF THE ESSENTIAL OIL OF *TEUCRIUM ROYLEANUM*

3.2.1. INTRODUCTION

The genus *Teucrium* (Lamiaceae) consist of more than 300 species 4 of which are growing wild in Pakistan[20]. Several among these species have been used as medicinal plants since an immemorial time and still being utilized in folk medicines as tonic, antiseptic and antipyretic[3,4]. When the decoction of the aerial parts of some of Teucrium

species have used it showed hepatotoxic effects, the credit of this activity may be due to the diterpenoids presence in the species [5-8]; this class of terpenoids (neo-clerodane type) has in general shown insect antifeedant activity[3, 9-11]**.** Several *Teucrium* species have been subjected so for to the analysis of essential oil. The essential oil content varies from 0.5-1.5% in the different species growing in different parts of the world and the percentage of the major chemical constituents (mainly terpenoids) invariably differ from species to species[3, 85-90].

3.2.2. ANALYSIS OF THE ESSENTIAL OIL OF *TEUCRIUM ROYLEANUM*

The extracted essential oil was yellow in colour and with a characteristic odour, and had an optical rotation of $[\alpha]_D^{25} = -11.43$ (pentane, *c*, 0.07). The identified volatile components are listed according to their retention indices in **Table 3.1**, including their retention indices and their percentage contributions. In total, 46 chemical constituents were recognized, contributing to 90.1% of the oil. The essential oil comprising of a complex mixture of different substances, with sesquiterpene hydrocarbons as the dominating constituents (42.2%). Among them, β- santalene (20.7%) and *cis*-α-bisabolene (11.8%) were the predominant compounds. When the chemical profile of the essential oil studied was compared to previously investigated essential oils from *Teucrium* spp., it appeared some what different in the sense that α-Pinene a common constituent of other *Teucrium* essential oils[3, 85, 88] was totally absent in our specimen.

Table 3.1: Chemical constituents of the Essential oil of *Teucrium royleanum* Wall. ex Benth.

No.	Compounds	RI[1]	RI[2]	Percentage
1	α-Thujene	929	1028	1.9
2	2, 4(10)-Thujadiene	955		1.8
3	Sabinene	972	1123	1.2
4	1-Octen-3-ol	975	1441	1.3
5	6-Methyl-5-hepten-2-one	985	1335	0.6
6	Myrcene	990	1160	0.1
7	p-Cymene	1022	1269	1.4
8	o-Cymene	1026	1016	8.7
9	β-Phellandrene	1030	1211	0.1
10	*Trans*-β-Ocimene	1050	1248	0.0
11	γ-Terpinene	1060	1244	0.7
12	*Cis*-Linalool Oxide	1087	1464	0.1
13	*p*-Cymenene	1091	1431	1.2
14	Linalool	1097	1537	2.9
15	α-Thujone	1102	1437	0.1
16	Oct-1-3-yl acetate	1113	1372	0.1
17	Sabinol	1143	1683	0.1
18	Umbellulone	1171		0.1
19	4-Terpineol	1177	1592	3.9
20	Myrtenal	1196	1196	tr
21	α-Terpineol	1189	1620	0.1
22	Cuminal	1242	1783	0.1
23	Piperitone	1250	1739	tr
24	*p*-Cuminol	1290	1395	tr
25	Thymol	1290	2213	0.1
26	Carvacrol	1299	2159	0.1
27	*Trans*-β-Damascenone	1381	1663	0.1
28	*Cis*-α-Bergamotene	1412	1577	0.1
29	β-Caryophyllene	1415	1584	3.2
30	α-Humulene	1448		0.6
31	*Trans*-β-Farnesene	1454	1659	1.4
32	β-Santalene	1458	1642	20.7

33	Germacrene D	1480	1707	4.3
34	α-Amorphene	1482	1685	0.1
35	β-Selinene	1488	1717	0.1
36	*Cis*-α-Bisabolene	1508	1729	11.8
37	*Cis*-Nerolidol	1531	2017	0.1
38	Caryophyllene Oxide	1578	1987	1.2
41	Nerolidol Oxide	1640		1.3
42	β-Eudesmol	1649	2234	3.0
43	T-Cadinol B	1640	2191	2.0
44	*Cis*-α-Santalol	1671	1721	2.9
45	α-Bisabolol	1682	2222	6.0
46	Acorenone B	1700	2196	0.9
	Total			90.3

The yield (% v/dry weight in g) obtained was 1.8. The **Table 3.2** categorizes the classes of compounds with their corresponding percentages in v/g dry weight.

Table 3.2: Classes of Compounds with corresponding %ages in v/wt

Class	% v/g dry weight
Aliphatic	2.0
Alcohols	1.3
Ketones	0.6
Fatty acids and aliphatic esters	0.1
Terpenoids	88.3
Monoterpenes hydrocarbons	17.3
Oxygenated monoterpenes	7.7
Sesquiterpene hydrocarbons	42.2
Oxygenated sesquiterpenes	21
Compounds with C-13	0.1

Components listed in order of elution from a HP 5MS column. RI, Kovats indices calculated against n-alkanes (C_9-C_{24}) on HP 5MS column (1) and HP Innowax (2) capillary columns, respectively. In the table tr is used for the concentrations in traces (between 0.01 and 0.05).

3.2.3 ANTIOXIDANT ASSAYS OF THE ESSENTIAL OILS FROM *TEUCRIUM ROYLEANUM*

The antioxidant activity of the essential oils of *T. royleanum* has been evaluated by two *in vitro* assays **(Table 3.3).** In view of the differences among the assays systems available, the consequences of a single assay can elaborate only an idea on the defensive abilities of phytochemicals.

3.2.3.1. The Action of 1, 1-Diphenyl-2-Picryl-Hydrazyl (DPPH) Stable Free on the Tested Constituents

Several assays have been put forward for the determination of the antioxidant assays but DPPH assay is handy and the method is simpler, lesser time consuming and applicable to both lipophilic and hydrophilic samples. Literature reports showed that many studies relating the antioxidant assays of essential oils treated with DPPH have been reported[91-94]**.** The tested sample was found to react with the DPPH and in a time-dependent manner. DPPH can gain an electron from the antioxidant and hence its typical purple colour vanished and the reaction can be monitored spectrophotometrically at 517 nm. This interaction shows its radical scavenging capability in an iron-free system, while dealing with plant extracts or other crude samples this method will give data on the basis of reduction potential of the test sample and hence this method can be used for comparing the reduction potential of unknown samples. The resultant data obtained were compared to the assays of synthetic antioxidants and to commercial origanum essential oil, to provide a reference with a natural phytocomplex which shows antioxidant properties.

3.2.3.2. Superoxide Anion Radical Scavenging Activity

Xanthine–xanthine oxidase system generates the superoxide anion radical and was determined by monitoring its capability to bring about reduction of nitroblue tetrazolium (NBT) to formazan. The activity was adopted to help the ability of antioxidants to react with O_2^{-}.The tested sample (100 µl) at a final volume of 700 µl, showed a mild ability to scavenge superoxide anion radical (19.7%) however, it was to difficult to measure the scavenging activity of the samples at higher concentrations, due to solubility problem. In contrast, lower concentrations did not show any scavenging effect. The oxidation of hypoxanthine to xanthine was carried out by the catalytic activity of a key enzyme namely xanthine oxidase. In the presence of O_2 as electron acceptor, xanthine produces uric acid, superoxide anions and hydrogen peroxide[95]. The inhibition of this enzyme is handy in the treatment of several diseases[96]. Several natural products are inhibitors of XOD and possess antioxidant properties[96].

Table 3.3: DPPH radical scavenging and super oxide anion radical scavenging by *Teucrium royleanum* and *Origanum vulgare* essential oils used as reference oil.

Test oil	% Interaction[(a)] DPPH 20 min	% Interaction DPPH 60 min	% Scavenging [(b)] O_2^{-}.
Oil (0.018g/1 mL EtOH)	3.5 ± 0.8	7.2 ± 0.7	19.7 ± 0.8
Commercial *Origanum valgare* essential oil	15.2 ± 0.1	11.6± 0.4	-

a. Acetylsalicylic acid (Standard), 80.6% (± 5), BHT (standard), 36.1 % (± 3) at 0.1 mm.

b. Caffeic acid (standard), 70% (±3.2) at 1 mm.

SD < 10 %

3.3. PHYTOCHEMICAL INVESTIGATION OF *TEUCRIUM ROYLEANUM* WALL. EX BENTH

3.3.1. INTRODUCTION

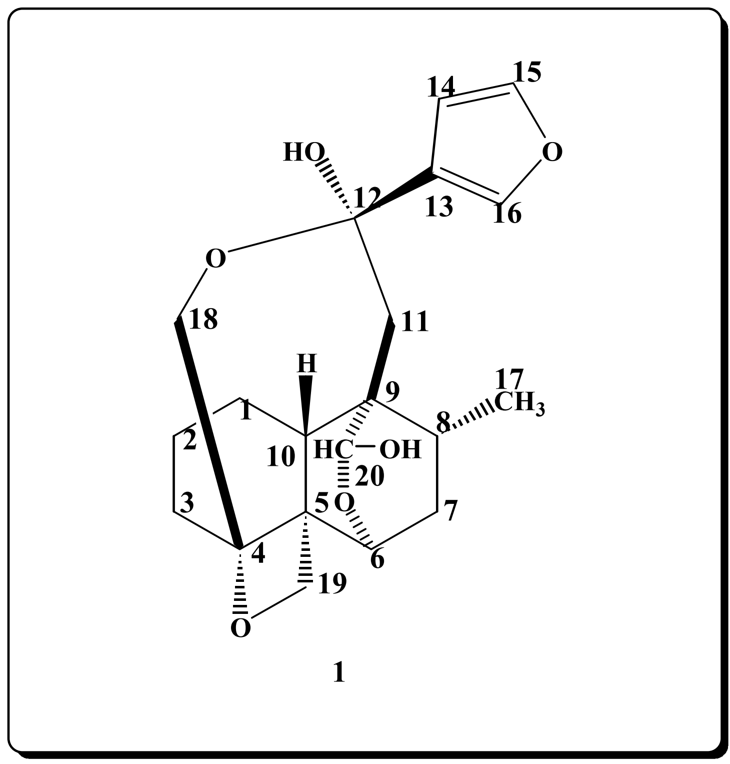
Teucrium royleanum Wall. Ex Benth. belonging to the family Lamiaceae, grows wild in different parts of Pakistan at high altitude and is indigenous to Asia[20]. Literature survey reveals that only 16 secondary metabolites have been investigated so far from this plant[74]**.** The medicinal properties attributed to the genus *Teucrium* have prompted us to investigate *T. royleanum* for its chemical constituents. Investigation of the chloroform extract of the whole plant resulted in the isolation of three new secondary metabolites two of them related to terpenes (neo-clarodane diterpene and a lupane type triterpene) and one was a phenolic derivative. These new compounds were named as royleanumin **1**, royleanumioside **2** and royleanumoate **3**.

The known compounds purified for the first time from the plant and from the genus were

5-hydroxy-4′, 7-dimethoxy flavone **4**, 5,7-dihydroxy-4′, 3-dimethoxy flavone **5**, 6, 4′ −dihydroxy 5, 7-dimethoxy flavone **6**, 3, 4-dihydroxymethyl benzoate **7**, oleanolic acid **8**, β-sitosterol **9**, β-sitosterol glucoside **10**.

3.3.2 CHARACTERIZATION OF ROYLEANUMIN (1)

Royleanumin **1** was isolated as an amorphous powder from methanol fraction using chloroform-methanol (9.5: 0.5).



3.3.2.1 Structure Elucidation of Royleanumin (1)

The IR spectrum indicates the presence of hydroxyl group (3400 cm^{-1}) and furan moiety (3130, 1660, 1505, 874 cm^{-1})[97]. The molecular formula $C_{20}H_{26}O_6$ was determined by HR-EI-MS showing molecular ion peak [M^+] at *m/z* 362.1720 (calc. for $C_{20}H_{26}O_6$, 362.1729) exhibiting eight degrees of unsaturation. The ^{1}H-NMR spectrum showed the presence of a furan ring δ 7.48 (1H, s), 7.45 (1H, d, *J* = 1.5 Hz) and 6.42 (1H, d, *J* = 1.5 Hz), an acetal proton at δ 5.09 (1H, s), an oxymethine δ 5.04 (1H, t, *J* = 7.1 Hz), four

overlapped signals for two cyclic oxymethylenes at δ 4.14, 4.11, 3.88, 3.83 and a methyl group δ 0.96 (1H, d, J = 6.5 Hz). The ^{13}C-NMR spectrum of **1** disclosed 20 carbon signals for one -CH_3, seven -CH_2, seven -CH and five quaternary carbon atoms. The signals at δ 144.8, 140.7, 128.1 and 109.5 indicated the presence of a furan moiety[97]; where as the signal of two double oxygenated carbons at δ 109.5 and 102.6 give indication about the presence of two acetal moieties in the molecule. The downfield signal of oxymethylene at δ 76.5 displayed the presence of oxetane ring between carbons 4 and 5 in the molecule[98]. The comprehensive ID- and 2D-NMR spectroscopic data and comparison with the already reported studies of the molecules isolated from *Teucrium*[97-101], let us deduce that **1** may exhibit a *neo*-clerodane type nucleus. After the cautious examination of HMQC and ^{1}H–^{1}H COSY experiments, two sequences, –CH-CH_2-CH_2-CH_2- linking C-10→C-4 and –CH (O)-CH_2-CH-CH_3 C-6→C-17 linking C-6→C-17 moieties were established. The linkages of these fragments to the furan ring were established by the attachment of two acetals and an oxetane ring, with the help of examination of ^{1}H–^{13}C long rang correlation spectroscopy (HMBC). Besides connecting the two fragments it also resolute two acetals between C-4/C-12, C-6/C-20 and an oxetane ring between C-4/C-5.

3.3.2.2. Stereochemistry of Royleanumin (1)

The 2D-NOESY data was used for the assignment of the relative stereochemistry at various chiral centers of compound **1**, in which H-6 (δ 5.04) showed NOESY correlations with H-10 (δ 2.17), and H-10 (δ 2.17) with CH_2-11 (δ 1.91) and CH_2-18 (δ 4.14, 3.88) indicating configurations "*S*" at C-6, "*R*" at C-10. However, H-20 (δ 5.09) showed NOESY correlations with CH_3-17 (δ 0.96) and CH_2-19 (δ 4.11, 3.83) deduced geometries "*S*" at C-4, "*S*" at C-5, "*R*" at C-8 and "*S*" at C-20, which is also

confirmed by the construction of Drieding model. On the basis of these evidences the structure **1** could be assign to royleanumin.

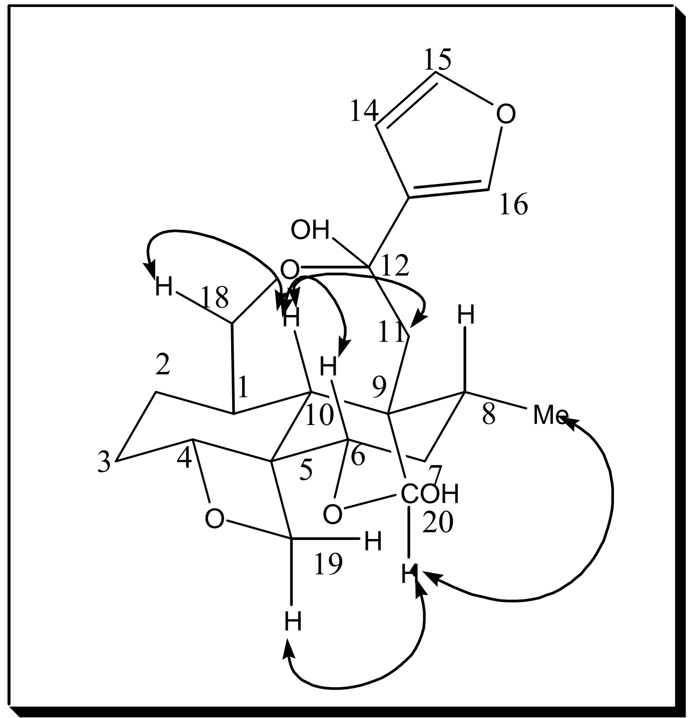


Fig.3.1: 2 D NOESY Correlations for Royleanumin **(1)**

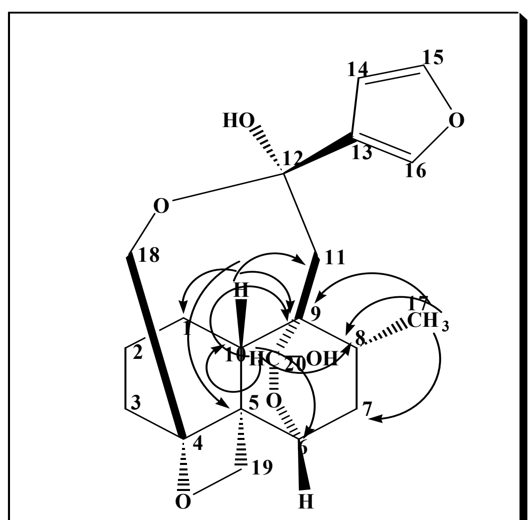


Fig. 3.2: Important HMBC Correlations for Royleanumin **(1)**

Table-3.4: ^{13}C-NMR (C_5D_5N, 100 MHz), chemical shifts, multiplicities and one-bond ^{1}H-, ^{13}C -NMR connectivities (HMQC) of (**1**).

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 1	CH_2	24.6		
C-2	CH_2	25.3		
C-3	CH_2	31.1		
C-4	C	82.4		
C-5	C	50.1		
C-6	C	73.3	5.04	t, J = 7.1
C-7	CH_2	39.0		
C-8	CH	36.3	1.83	m
C-9	C	49.0	-	-
C-10	C	45.6	2.17	dd,14.2, 4.8
C-11	CH_2	41.2		
C-12	C	109.0	-	-
C-13	C	128.1	-	-
C-14	CH	109.5	6.42	d, J = 1.5
C-15	CH	144.2	7.45	d, J = 1.5
C-16	CH	140.7	7.48	s
C-17	Me	16.6		s
C-18	CH_2	60.1	4.11,3.8	1H each signal overlapped

C-19	CH_2	76.5	4.14, 3.83	1H each signal overlapped
C-20	CH	102.6	5.09	s

3.3.3 CHARACTERIZATION OF ROYLEANUMIOSIDE (2)

The second compound **(2)** purified was as an amorphous powder from the methanol fraction (chloroform-methanol (9.4: 0.6).

3.3.3.1 Structure Elucidation of Royleanumioside (2)

The HRFABMS (positive ion mode) provided molecular ion peak $[M+H]^+$ at *m/z* 853.6929, corresponding to the molecular formula $C_{54}H_{92}O_7$ which indicated nine degrees of unsaturation.

Scheme-3.1: The mass spectral fragmentation pattern for royleanumioside

The IR spectrum showed strong absorptions at 3360 (O-H), 2900 (C-H), 1740 (C=O), 1630 (C=C) and 1250 (C-O) cm^{-1}. The ^{1}H-NMR spectrum exhibited seven tertiary methyl singlet at δ 0.67, 0.77, 0.85, 0.89, 0.97, 0.99 and 1.54 (all, 3H, s), a trisubstituted double bond at δ 5.33 (1H, t, J = 3.5 Hz) together with two broad doublets at δ 4.70 and 4.62 (1H each, J = 2.0 Hz). Keeping in view the above data, it clearly indicated a lupine type triterpene with additional bond[102] The signals for sugar moiety appeared at δ 4.35 (1H, d, J = 7.6 Hz, H-1′), 3.33 (1H, m, H-2′), 3.54 (1H, m, H-3′), 3.45 (1H, m, H-4′), 3.42 (1H, m, H-5′) and oxymethylene at δ 4.43 (1H, dd, J = 12.0, 4.6 Hz) and 4.25 (1H, dd, J = 12.0, 1.9 Hz). It also showed signals for the presence of acyl group at δ 0.66 (3H, t, J = 7.4 Hz), 1.23 (26H, br s) and 2.32 (2H, t, J = 7.4 Hz). The ^{13}C-NMR

spectra (BB and DEPT) of **2** revealed the presence of eight methyl, twenty seven methylene, eleven methine and eight quaternary carbon atoms. It includes signals for four olefinic carbons (δ 140.2, 121.4 and 147.4, 111.1), a hydroxyl bearing carbon (δ 79.4), sugar moiety (δ 101.1, 73.3, 76.2, 70.2, 73.6 and 63.5) and an acyl moiety (δ 174.2, 34.1, 24.8, 29.5 and 13.8). A triplet at δ 5.33 (1H, J = 3.5 Hz) in ^{1}H-NMR spectrum and characteristics EIMS fragments at *m/z* 216 and 201 resulted from *retro*-Diels-Alder (RDA) cleavage in the ring C indicating the position of second double bond at C-12[103] The fragments at *m/z* 208 and 189 indicated hydroxyl group at ring A or B. The hydroxyl group was assigned to C-3 on biogenetic grounds and confirmed through the characteristic shifts of C-2 and C-4 in ^{13}C-NMR spectrum. The β-orientation of this group was decided by measuring the J value of the double doublet at δ 3.57 (1H, dd, J = 9.5, 4.8 Hz). The acid hydrolysis of **2** provided various products amongst which the glycone was separated and identified as D-glucose through its optical rotation sign, retention time in GC and by co-TLC with authentic sample.

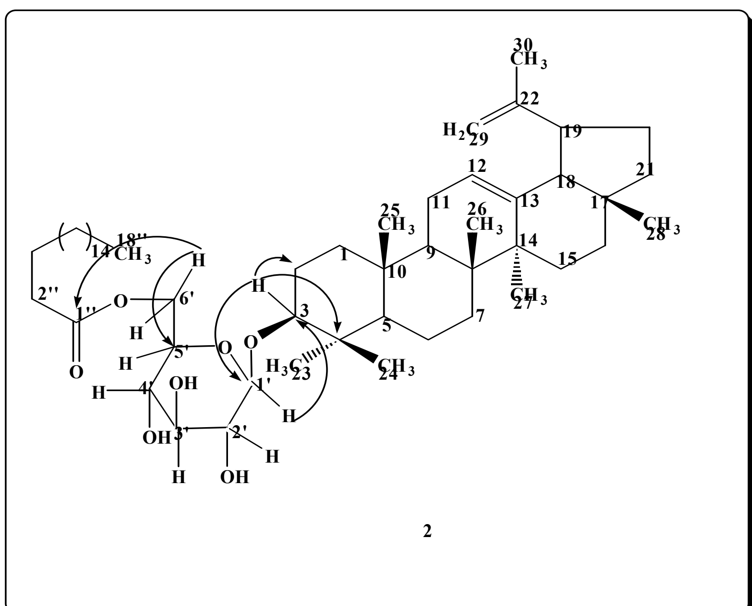


Fig. 3.3: Important HMBC correlations of royleanumioside **(2)**

The position of sugar moiety and aliphatic ester was finally confirmed by long range HMBC experiments in which the C-3 proton (δ 3.57) showed ^{3}J correlation with anomeric carbon (δ 101.1) and the anomeric proton (δ 4.35) showed ^{3}J correlation with C-3 (δ 79.4). The position of aliphatic ester was deduced at C-6′ of sugar moiety which is due to the downfield shifts of H-6′ (δ 4.43 and 4.25), C-6′ (δ 63.5) and up field shift of C-5′ (δ 73.6) and finally confirmed by HMBC interactions in H-6′ (δ 4.43 and 4.25) showed ^{3}J correlation with C-1″ (δ 174.0). On the basis of the evidences, the structure of **2** was elucidated as lupa-12, 20(29)-dien-6′-stearoyl 3-O-β-D-glucopyranoside.

Table-3.5: ^{13}C-NMR (C_5D_5N, 125 MHz), chemical shifts, multiplicities and one-bond ^{1}H-, ^{13}C -NMR connectivities (HMQC) of (**2**).

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 1	CH_2	38.6	-	-
C-2	CH_2	24.1		
C-3	CH	79.4	3.57	dd, *J*=9.5, 4.8
C-4	C	42.1		
C-5	CH	55.9		
C-6	CH_2	22.5		
C-7	CH_2	31.7		
C-8	C	37.3		
C-9	CH	50.0		
C-10	C	36.5		
C-11	CH_2	31.8		

C-12	CH	121.4	5.33	t, *J* = 3.4
C-13	CH	140.2		
C-14	C	42.0		
C-15	CH_2	27.9		
C-16	CH	37.2		
C-17	C	36.4		
C-18	CH	48.7		
C-19	CH	49.3	2.76	m
C-20	C	150.8		
C-21	CH_2	39.6		
C-22	CH_2	147.4		
C-23	CH_3	17.5	0.98	s
C-24	CH_3	18.4	0.97	s
C-25	CH_3	11.8	0.77	s
C-26	CH_3	11.6	0.67	s
C-27	CH_3	19.1	0.99	s
C-28	CH_3	13.9	0.85	s
C-29	CH_2	111.9	4.70 Ha 4.62 Hb	d, *J*= 2.0 d, *J*= 2.0
C-30	CH_3	18.5	1.54	s
C-1$^{/}$	CH	101.1	4.35	d, *J*= 7.6
C-2$^{/}$	CH	73.3	3.33	m

C-3$^{/}$	CH	76.2	3.54	m
C-4$^{/}$	CH	70.2	3.45	m
C-5$^{/}$	CH	73.6	3.42	m
C-6$^{/}$	CH_2	63.5	4.43	dd, *J*=12.0, 4.6 dd, *J*= 12.0,1.9
C-1$^{//}$	C	174.2		
C-2$^{//}$	CH_2	34.1	2.32	t, *J*= 7.4
C-3$^{//}$	CH_2	24.8	1.60	m
C-4$^{//}$-16$^{//}$	CH_2	29.0	1.23	br s
C-17$^{//}$	CH_2	20.9		
C-18$^{//}$	CH_3	13.8	0.65	t, *J*= 7.4

3.3.4 CHARACTERIZATION OF ROYLEANUMOATE (3)

Compound **3** was isolated from the VLC fraction of the chloroform soluble part obtained from the methanol extract of *T. royleanum* Wall. Ex Benth. (See experimental section Part A) as white amorphous powder.

3.3.4.1 Structure Elucidation of Royleanumoate (3)

To compound **3** was assigned on the basis of the M^+ at *m/z* 333 $[M+1]^+$ in FAB +ve, and NMR spectral data, with a total of 5 degrees of unsaturation. The fragmentation pattern of compound **3** is shown in Scheme-3.2. The prominent peaks observed were at *m/z* 57, 71, 107 and a base peak at 120.

The IR spectrum of compound **3** exhibited an absorption bands at 1735 (ester C=O), 3430 for (OH), and 1617 for (aryl).

Scheme-3.2: The mass spectral fragmentation pattern for royleanumoate **(3)**

In the ^{1}H-NMR spectrum, one methyl, thirteen methylene and aromatic groups were observed at the high-field region. In the downfield region of the spectrum two doublets at δ 7.05 (2H, d, *J*=10 .0 Hz) and δ 6.75 (2H, d, *J*=10.0 Hz) were assigned to C-2′, C-6′ and C-3′, C-5′ aromatic protons. A triplet at δ 0.88 (3H, t, J=6.36) was assigned to the methyl group at the terminal position. Similarly, at δ 4.21 and 2.81 two triplets each of 2 H integration with a *J* value of 7.3 Hz, were assigned to the presence of methylene protons to C-1′′ and C-2′′ respectively.

The ^{13}C-NMR spectrum (BB, DEPT) (**Table-3.6**) showed twenty one signals, including one methyl, thirteen methylene, four methines, and three quaternary carbons. In the down field region signals appear at δ 138.1, 130.04 (2C), 115.38 (2C) and 153.84 which were assigned to the C-1′, C-2`, C-6`, C-3`, C-5`, and C-4` aromatic carbons. While a signal at δ 173.7 indicated the presence of a carbonyl carbon in the molecule. Similarly, two

signals at δ 34.4 and 64.9 were assigned to the methylene carbons present in between ether oxygen and aromatic ring. While in the up field region a signal at δ 14.1 was assigned to the methyl carbon attached at terminal position. The ^{1}H-^{13}C correlations were determined by HMQC spectrum, while the long-range ^{1}H-^{13}C connectivities were established through HMBC technique.

Fig. 3.4: Important HMBC correlations of royleanumoate **(3)**

In the HMBC spectrum (**Fig. 3.4)** the C-2″ methylene protons (δ 2.81, t) showed ^{2}J correlations with C-1′ (δ138.1), C-1″ (δ 64.87), and ^{3}J correlations with C-2′ (δ130.1) and C-6′ (δ130.1) thus supporting the attachment of -CH2-CH2- to the phenol ring at *para* position.

On the basis of all the above spectral data, and comparison with the analogous structures in the literature[104] the compound **3** was named as 2-(4-hydroxyphenyl) ethyltridecanoate commonly named as royleanumoate.

Table-3.6: ^{13}C-NMR (C_5D_5N, 100 MHz), chemical shifts, multiplicities and one-bond ^{1}H-, ^{13}C -NMR connectivities (HMQC) of (**3**).

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 1	C	173.8	-	-
C-2	CH_2	32.1	2.23	t, *J*= 2.3
C-3-11	CH_2	29.25	1.23	brs
C-12	CH_3	14.11	0.88	t, *J* = 6.36
C-1′	C	138.1	-	-
C-2′-6C′	CH	130.1	7.05	d, *J* =10.0
C-3′-C-5′	CH	115.3	6.75	d, *J* = 10.0
C-4′	C	153.84	-	-
C-1′′	CH_2	64.87	4.21	t, *J* = 7.09
C-2′′	CH_2	34.38	2.83	t, *J* = 7.09

3.3.5. 5-HYDROXY-4′, 7-DIMETHOXYFLAVONE (4)

5-Hydroxy-4′, 7-dimethoxyflavone **4** was isolated as yellow coloured needle like crystals from the chloroform soluble part of methanolic extract of *Teucrium royleanum* Because of the fluorescence under UV light and yellow appearance on silica gel plate after spraying with ceric sulfate reagent, **4** was considered as flavonoid.

3.3.5.1 Structure Elucidation of 5-Hydroxy-4′, 7-Dimethoxyflavone (4)

To compound **4** was assigned the molecular formula $C_{17}H_{14}O_5$ on the basis of EI-MS and NMR spectral data. The molecular ion peak appear at *m/z* 298, the mass fragmentation pattern of compound **4** is shown in Scheme-3.3.

The UV spectrum of **4** showed maxima at 330, 265 and 255 nm. The absorptions at 3250, 1670 and 1610 cm^{-1} in the IR spectrum of **4** revealed the presence of hydroxy, carbonyl and olefinic functions, respectively in the molecule.

The 15 signals appearing in the broad-band spectrum of **4** were resolved with the help of DEPT experiment into two methyls, five methines and eight quaternary

carbons. The ^{1}H-NMR spectrum (C_5D_5N, 500 MHz) of **4** showed two singlets at δ 3.87 and 3.88 each of three protons integration indicating the presence of two methoxyl groups in the molecule.

Scheme-3.3 The mass spectral fragmentation pattern for 5-Hydroxy-4′, 7-dimethoxyflavone **(4)**

The two doublets, each of one proton integration, at δ 6.36 (2.3 Hz) and 6.47 (2.3 Hz) were assigned to H-6 and H-8, respectively on the basis of comparison with reported chemical shifts for similar type of compounds[105]. The ^{1}H-NMR of **4** displayed a doublet of two protons at δ 7.01 (H-3′, H-5′) having a coupling constant of 9.0 Hz. Another doublet of two protons resonated at δ 7.84 (H-2′, H-6′) with the same coupling constant of 9.0 Hz. The same magnitude of coupling constant (9.0 Hz) showed that the carbon corresponding to both doublets were

adjacent to each other. The HMQC experiment showed that the protons at δ 7.01 and 7.84 were linked with the carbons at δ 122.1 and 115.6, respectively. The DEPT and HMQC experiments revealed that two protons integration of each doublet was actually due to two aromatic methines having similar environment.

The assignments of protonated carbons in ^{13}C–NMR spectrum were carried out with the help of HMQC and the long range were verified by HMBC experiments. The structure of **4** was finally, confirmed by comparing its m.p and NMR (^{1}H, C ^{13}) data with reported values[105] and thus it was characterized as 5-hydroxy-4′, 7-dimethoxyflavone.

Table-3.7: ^{13}C-NMR (C_5D_5N, 100 MHz), chemical shifts, multiplicities and one-bond ^{1}H, ^{13}C -NMR connectivities (HMQC) of **(4)**.

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 2	C	164.5	-	-
C-3	CH	104.3	6.57	s
C-4	C	182.6	-	-
C-5	C	162.7	-	-
C-6	CH	98.1	6.36	d, J = 2.3
C-7	C	162.3	-	-
C-8	CH	92.7	6.47	d, J= 2.3
C-9	C	157.8	-	-
C-10	C	103.9	-	-
C-1′	C	123.7	-	-

C-2′	CH	115.6	7.84	d, J = 9.0
C-3′	CH	122.1	7.01	d, J = 9.0
C-4′	C	164.1	-	-
C-5′	CH	122.1	7.01	d, J = 9.0
C-6′	CH	115.6	7.84	d, J = 9.0
C-7′	OMe	55.6	3.87	s
C-4″	OMe	55.8	3.88	s

3.3.6. CHARACTERIZATION OF 5, 7-DIHDROXY 4′, 3-DIMETHOXY FLAVONE (5)

The compound was considered as a flavone due to its yellow appearance on silica gel TLC card after spraying with ceric sulfate reagent.

HO
O
OCH_3
OCH_3
OH
O
5

3.3.6.1 Structure Elucidation of 5, 7-Dihdroxy-4′, 3-Dimethoxy Flavone (5)

Its UV spectrum showed absorption maxima at 360, 315, 260 nm. The presence of intense absorptions at 1685, 1605, 1505 cm-1 in the IR spectrum of the compound indicated the presence of a cross conjugated carbonyl, olefinic, and aromatic functions in the molecule. The broad absorption at 3400 cm-1 appeared due to the hydroxyl function.

The mass spectrum of the compound displayed molecular ion peak at *m/z* 314. The molecular formula, $C_{17}H_{14}O_6$ corresponding to the exact mass 314.0794 (calculated 314.0794) was established through high resolution mass measurements.

Scheme-3.4 The mass spectral fragmentation pattern for 4', 5-Dihdroxy-3, 7-Dimethoxy Flavone **(5)**

A pair of doublets, integrating for two protons each appeared at 7.95 and 7.26 in the ^{1}H-NMR spectrum with an identical coupling constant of 8.5 Hz. The doublets corresponded to the methine at δ 128.9 and 116, respectively, in the ^{13}C-NMR, revealed the presence of AABB system of ring B.

Two OMe singlets resonated at δ 3.99 and δ 3.87 in the 1H-NMR spectrum, and corresponded to the carbon signals at δ 56.3 and 60.5, showed the attachment of the two methoxy groups in the molecule at C-7 and C-3, respectively.

Another pair of doublets resonated in the aromatic region of the spectrum at δ 6.79

and δ 6.95 having almost same coupling constant of 2.0 Hz indicating *meta* coupling and they were assigned to the aromatic protons H-8 and H-6 in ring A. The corresponding evidence and the comparative literature report[106] led to identification of **5** as 4′, 5-dihydroxy- 3, 7-dimethoxy flavone**.**

Table 3.8: ^{13}C-NMR Chemical Shifts, Multiplicities and one-band ^{1}H-^{13}C-NMR connectivities (HMQC) of **(5).**

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 2	C	164.8	-	-
C-3	C	135	-	-
C-4	C	183	-	-
C-5	C	157	-	-
C-6	CH	103.7	6.95	s
C-7	C	159	-	-
C-8	CH	91.5	6.79	s
C-9	C	153	-	-
C-10	C	106	-	-
C-1′	C	123	-	-
C-2′. C-6′	CH	128	7.95	d, J = 8.5
C-3′. C-5′	CH	116	7.26	d, J = 8.5
C-4′	C	162.9	-	-
C-4′	OMe	56.3	3.99	s
C-3	OMe	60.5	3.87	s

3.3.7. CHARACTERIZATION OF 6, 4′DIHYDROXY-5, 7-DIMETHOXY FLAVONE (6)

The compound was considered flavone due to its yellow appearance on silica gel plate after spraying with ceric sulphate reagent.

3.3.7.1 Structure Elucidation of 4′, 6-Dihydroxy 5,7 Dimethoxy Flavone (6)

UV spectrum showed absorption maxima at 323, 282, 267, 244 and 205 nm. The presence of intense absorptions at 1685, 1605, 1505 cm^{-1} in the IR spectrum of the compound indicated the presence of a cross conjugated carbonyl, olefinic, and aromatic functions in the molecule. The broad absorption at 3550 cm^{-1} appeared due to the OH function.

The mass spectrum of the compound displayed a very intense molecular ion peak that appeared as a base peak in the spectrum at m/z 314. The molecular formula was established as $C_{17}H_{14}O_6$ on the basis of high resolution mass measurement which provided exact mass at m/z 314.0800 and was consistent with eleven degrees of unsaturation. The other abundant fragments in the spectrum appeared at m/z 286, 196,

and 118. These fragments ions and their relative intensities were in accordance with the literature[107] (**Scheme 3.5).**

-CO

(A)$C_9H_8O_5$ *m/z* 196

C_9H_6O *m/z* 118

$C_{16}H_{14}O_5$ *m/z* 286

Scheme-3.5 The mass spectral fragmentation pattern for 4′6, dihydroxy 5,7 dimethoxy flavone **(6)**

The 15 signals appearing in the broad-band spectrum of **6** were resolved with the help of DEPT experiment into two methyls, four methines and nine quaternary carbons. The ^{1}H-NMR spectrum (C_5D_5N, 500 MHz) of **6** showed two singlets at δ 3.87 and 3.9 each of three protons integration indicating the presence of two methoxyl groups in the molecule.

A singlet at δ 6.79 was assigned to H-8. The ^{1}H-NMR of **6** displayed a doublet of

two protons at δ 7.26 (H-3′, H-5′) having a coupling constant of 9.0 Hz. Another doublet of two protons resonated at δ 7.95 (H-2′, H-6′) with the same coupling constant of 8.5 Hz. The same magnitude of coupling constant (8.5 Hz) showed that carbons corresponding to both doublets were adjacent to each other. The HMQC experiment showed that the protons at δ 7.26 and 7.95 were linked with the carbons at δ 116 and 128, respectively. The DEPT and HMQC experiments revealed that two protons integration of each doublet was actually due to two aromatic methines having similar environment.

The assignments of protonated carbons in ^{13}C–NMR spectrum were carried out with the help of HMQC and the long range were verified by HMBC experiments. The structure of **6** was finally, confirmed by comparing its NMR (^{1}H,^{13}C) data with reported values[107] and to the compound was assigned structure **6** i.e., 6, 4′ -dihydroxy 5, 7-dimethoxyflavone **6**.

Table 3.9: ^{13}C-NMR Chemical Shifts, Multiplicities and one-band ^{1}H-^{13}C-NMR connectivities (HMQC) of **6.**

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C-2	C	164.8	-	-
C-3	C	135	6.57	s
C-4	C	183	-	-
C-5	C	157	-	-
C-6	C	163	-	-
C-7	C	159	-	-
C-8	CH	91.5	6.79	s
C-9	C	153	-	-
C-10	C	106	-	-
C-1′	C	123	-	-
C-2′. C-6′	CH	128	7.95	d, J = 8.5
C-3′. C-5′	CH	116	7.26	d, J = 8.5
C-4′	C	162.9	-	-
C-5	OMe	60.3	3.8	s
C-7	OMe	56.3	3.99	s

3.3.8 CHARACTERIZATION OF 3, 4-DIHYDROXYMETHYL BENZOATE (7)

3, 4-Dihydroxymethylbenzoate **7** was isolated as brown coloured crystals from the chloroform soluble part of methanolic extract of *Teucrium royleanum.*

3.3.8.1 Structure Elucidation of 3, 4-Dihydroxymethyl Benzoate (7)

The HREIMS of **7** gave the molecular ion peak at m/z 168.0371 corresponding to the molecular formula $C_8H_8O_4$. The IR spectrum of this compound showed the absorption bands at 3370 (O-H), 1694 cm-1 for the C=O, and 1619 cm-1 (aromatic).

The H^1NMR spectrum of 7 displayed a three proton singlet at 3.80 (3H, s, OMe). In the aromatic region, two sets of doublets at 6.81(1 H, d, H-5) and 7.46 (1H, d, H-2) with the coupling constants of 8.2 Hz and 1.83 Hz respectively and a doublet of a doublet at 7.52(1H, dd, H-6) having coupling constants of 8.2 and 1.9 Hz.

C13-NMR (BB and DEPT) spectrum of **7** disclosed the presence of eight carbons signals of one methyl, three methine, and four quaternary carbon atoms. The downfield signals at 170.6, 146.7, and 140.0 were assigned to ester carbonyl and aromatic oxygenated quaternary carbon atoms respectively, whereas other signals at 116.3, 110.3, 107.3, 119.9

and 56.4 were assigned to aromatic methines, aromatic quaternary, and methoxy carbons atoms, respectively

On the basis of the above evidences as well as by comparison from the literature[108], it was deduced that compound **7** was a 3, 4 dihydroxymethyl benzoate**.**

Table 3.10: ^{13}C-NMR Chemical Shifts, Multiplicities and one-band ^{1}H-^{13}C-NMR connectivities (HMQC) of **(7).**

C. No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	^{1}H-NMR (δ)	$^{1}J_{HH}$ (Hz)
C-1	C	146.7	-	-
C-2	C	140.0	7.46	d, J = 1.83
C-3	C	119.9	-	-
C-4	CH	107.3	-	-
C-5	CH	110.3	6.81	d, J = 8.2
C-6	CH	116.3	7.52	dd, J = 8.2,1.9
C-7	OCH_3	56.4	3.80	s
C-8	C=O	170.6	-	-

3.3.9. CHARACTERIZATION OF OLEANOLIC ACID (8).

Oleanolic acid **8** was isolated from the chloroform soluble part of *T. royleanum*.

3.3.9.1. STRUCTURE ELUCIDATION OF OLEANOLIC ACID

The UV spectrum gave band at 218 nm which confirms the absence of conjugated unsaturation. The IR spectrum of the compound showed the presence of COOH and olefinic function in the molecule supported by bands at 2400-3500, 1705 and 1640 cm^{-1}

respectively.

The EI of the compound showed molecular ion peak at m/z 456 and the HR provided molecular ion peak at m/z 456.3430, corresponding to molecular formula $C_{30}H_{48}O_3$.

In the HR mass spectrum, the retro Diels-Alder gave a fragment at m/z 248 as a base peak, with other peaks at m/z 255.223 and 203 further supported the presence of COOH function at C-17 and showed the compound to be a pentacyclic diterpene of β-amyrin series with a Δ^{12} unsaturation. The mass fragmentation further indicated that presence of hydroxyl function in ring A, as the major fragment at m/z 248 would not then be obtained which was also favored by its presence on biogenetic grounds. The appearance of a major fragment at m/z 203 due to ready loss of carboxylic group from the most abundant fragment at m/z 248 was in complete agreement with its positioning at C-17 (**Scheme 3.6).**

Scheme-3.6 The mass spectral fragmentation pattern for oleanolic acid **(8).**

The ^{1}H-NMR spectrum of the compound exhibited an olefinic triplet for one proton at δ 5.27 (*J*=3.5 HZ) due to the hydrogen at position 12 (H-12) confirming the presence of unsaturation in the molecule. In the downfield region at δ 3.19 (*J*=6.8 HZ) a triplet signal appeared in the ^{1}H-NMR and was assigned to compound **8** by comparing it to the already reported data[109]. A one proton double doublet appeared at δ 2.70 (*J*=13.7 and 4.0 Hz) and was assigned to H-18 by keeping in view its

chemical shift value all along with the multiplicity pattern reported for H-18 with β-stereochemistry.

The 1H-NMR spectrum of the compound showed the presence of seven tertiary methyl signals appearing as sharp singlet at δ 1.13, 0.98, 0.92, 0.91, 0.90, 0.77 and 0.75 and attached to C-14, C-4, C-20, C-20, C-10 and C-8, respectively.

The ^{13}C-NMR spectrum (BB, DEPT) **(Table-3.11)** showed thirty carbon signals including seven methyls, ten methylenes, five methines, and eight quaternary carbons. Finally the structure was assigned by comparative spectral assignments reported in the literature[110-112] and the compound **8** was thus identified as 3β-Hydroxy-olean-12-en-28-oic acid, i.e. oleanolic acid.

Table3.11. C^{13}-NMR Chemical Shifts and Multiplicity of Oleanolic Acid **(8).**

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**C. No**	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**
1	CH_2	38.5	16	CH_2	23.4
2	CH_2	27.3	17	C	46.6
3	CH	79,1	18	CH	41.2
4	C	38.7	19	CH_2	45.9
5	CH	55.3	20	C	30.6
6	CH_2	18.4	21	CH_2	33.9
7	CH_2	32.7	22	CH_2	32.5
8	C	39.4	23	CH_3	28.2
9	CH	47.7	24	CH_3	15.6
10	C	36.9	25	CH_3	15.4

11	CH_2	23.4	26	CH_3	17.2
12	CH	125.1	27	CH_3	25.8
13	C	138.7	28	C	177.8
14	C	42.1	29	CH_3	33.1
15	CH_2	30.6	30	CH_3	23.6

3.3.10. CHARACTERIZATION OF β-SITOSTEROL (9)

24-ethylcholest-5en-3ol is known as β -sitosterol. It was purified from Tb-SA4 (see experimental section) from the initial column chromatography step using chloroform-methanol (1:1).

3.3.10.1. STRUCTURE ELUCIDATION OF β-SITOSTEROL

The mass spectrum of the compound exhibited molecular ion peak at *m/z* 414 appeared as a base peak, indicating the high stability of the compound. The high resolution mass spectrum established the molecular formula as $C_{29}H_{50}O$ corresponding to exact mass 414.3857 and was consistent with five degrees of unsaturation.

10

The broad band ^{13}C-NMR spectrum showed twenty nine carbon signals. By comparative study of physical properties and NMR spectroscopy[113, 114] the compound was characterized as *β*-Sitosterol **10**.

Table- 3.12: 13C-NMR Chemical Shifts and Multiplicity of *β*-Sitosterol

C. No.	Multiplicity (DEPT)	^{13}C-NMR (δ)	C. No.	Multiplicity (DEPT)	^{13}C-NMR (δ)
1	CH_2	37.2	19	CH_3	19.3
2	CH_2	28.7	20	CH	36.7
3	CH	71.6	21	CH_3	19.3
4	CH_2	40.4*	22	CH_2	34.5
5	C	141.0	23	CH_2	30.1
6	CH	122.4	24	CH	46.6
7	CH_2	32.4**	25	CH	29.8
8	CH	32.5**	26	CH_3	20.0
9	CH	50.9	27	CH_3	19.6
10	C	37.3	28	CH_2	23.6
11	CH_2	21.6	29	CH_3	12.2
12	CH_2	40.3*	-		
13	C	42.9			
14	CH	57.4			
15	CH_2	24.7			
16	CH_2	26.8			
17	CH	56.7			
18	CH_3	12.1			

*, **: Interchangeable assignments

3.3.11 CHARACTERIZATION OF *β*-SITOSTEROL GLUCOSIDE (10)

β-Sitosterol glucoside **10** was isolated from the chloroform soluble part of *T. royleanum* using chloroform-methanol (8.7:1.3).

3.3.11.1 Structure Elucidation of *β*-Sitosterol Glucoside

The negative FAB mass spectrum of **10** exhibited the molecular ion peak at *m/z* 575 $[M-H]^-$, which revealed the molecular peak at *m/z* 576. The ^{13}C-NMR spectrum indicated the presence of 35 signals, which were resolved through DEPT experiments into six methyls, 12 methylenes, fourteen methines and three quaternary carbons. By a comparative study of NMR spectroscopy with literature data[114, 115.] compound **10** was established as *β*-sitosterol glucoside.

β-Sitosterol glucoside **(10)**

Table-3.13: ^{13}C-NMR Chemical Shifts, Multiplicities and one-band ^{1}H-^{13}C-NMR connectivities (HMQC) of **(10).**

C. No.	**Multiplicity (DEPT)**	**^{13}C-NMR (δ)**	**^{1}H-NMR (δ)**	**$^{1}J_{HH}$ (Hz)**
C- 1	CH_2	39.2	-	-
C-2	CH_2	28.7		
C-3	CH	79.6		
C-4	CH_2	40.4*		
C-5	C	141.0		
C-6	CH	122.4	5.32	br. s
C-7	CH_2	32.4**		
C-8	CH	32.5**		
C-9	CH	50.9		
C-10	C	37.3		
C-11	CH_2	21.6		
C-12	CH_2	40.3*		
C-13	C	42.9		
C-14	CH	57.4		
C-15	CH_2	24.7		
C-16	CH_2	26.8		
C-17	CH	56.7		
C-18	CH_3	12.1	0.66	

C-19	CH_3	19.3	0.99	s
C-20	CH	36.7		
C-21	CH_3	19.3	0.89	d, *J*=6.5 Hz
C-22	CH_2	34.5		
C-23	CH_2	30.1		
C-24	CH	46.6		
C-25	CH	29.8		
C-26	CH_3	20.0	0.80	d, *J*=6.2 Hz
C-27	CH_3	19.6	0.77	d, *J*=6.2 Hz
C-28	CH_2	23.6		
C-29	CH_3	12.2	0.79	t, *J*= 7.1 Hz
C-1$^{/}$	CH	101.8	4.35	d, *J*= 7.7
C-2$^{/}$	CH	71.0		
C-3$^{/}$	CH	76.6		
C-4$^{/}$	CH	74.3		
C-5$^{/}$	CH	77.2		
C-6$^{/}$	CH_2	62.4		

*, **: Interchangeable assignments

3.4. ALLELOPATHY ASSAY OF *TEUCRIUM ROYLEANUM*

3.4.1. INTRODUCTION

Generally the allelopathy is accepted as an important ecological factor in determining the chemistry of plant communities.[116-118] The dependence of allelopathic effect is upon release of certain compounds into the environment known as allelochemicals[119]. These are biosynthesized in the plants as secondary metabolites such as tannins, phenolics acids, lignins, alkaloids, flavonoids, coumarins, and as terpenoids, and may be present in all tissues including leaves, stems, roots rhizomes, flowers, fruits, and seeds and even trichomes and pollens.[120]

The concept of allelopathy is still a matter of controversy[121] and is plagued with methodological problems, particularly those of the distinguishing effects of allelopathy from those of competition[122]. Only few investigations have separated the components of interference because of the complexity of the ecological phenomenon[123].

In our studies on the allelopathy of *Teucrium royleanum* we have utilized two methods, namely, Sandwich and Homogenated Sandwich methods for the initial screening to know the effect of secondary metabolites in the plant leaves on the lettuce seed.

The genus *Teucrium* is famous for the presence of essential oils, which have been recognized since ancient times to exhibit biological activities. The phytotoxicity of the essential oil was determined by the Dish Pack Method[124]. Terpenoids and flavonoids are also reported from the genus *Teucrium* [125- 135] which are more polar and less volatile so may not be a part of the essential oils. To know their combined effects in the methanolic extract and subsequent sub-fractions on the growth of lettuce seedlings, approach of total activity[136] was followed.

The objective of our research was to know the allelopathic potential of *Teucrium royleanum*, and to screen it for the presence of some environmentally friendly weedicides. Our final findings suggested that some interesting weedicides can be isolated from the bioactive subfractions.

3.4.2 LEAF LEACHATES ASSAY

Dried leaves of *Teucrium royleanum* were subjected for the preliminary screening of phytotoxicity by Sandwich and Homogenated Sandwich method.

3.4.2.1 Sandwich Method

The lengths of both the roots and hypocotyl were measured, more the inhibition rates showed higher phytotoxicity. The test plant has inhibited the lettuce roots and hypocotyl elongation of germination to 60 % and 22 % respectively at 50 mg concentration. While at 10 mg roots and hypocotyl were inhibited to 45% and 20% respectively **(Fig 3.5)**. *T. royleanum* leaf leachates exhibited more phytotoxicity at 50mg compared to 10mg concentrations to both the roots and hypocotyl elongations.

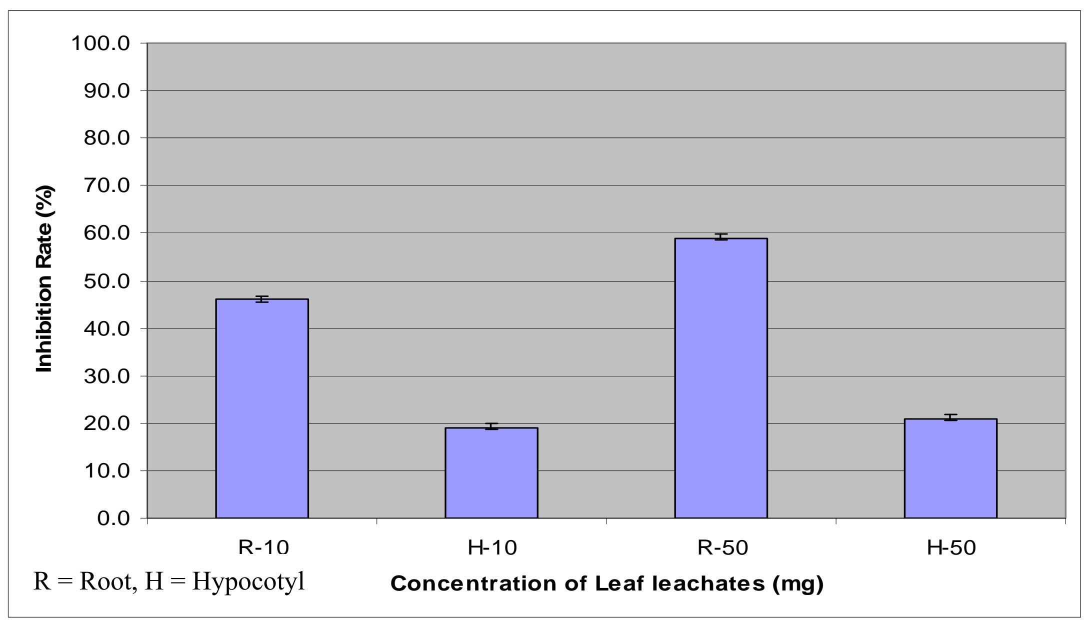


Fig. 3.5 Effects of leaf leachates on the inhibitions of Lettuce seed by Sandwich method

3.4.2.2 Homogenated Sandwich Method

The inhibitions of the roots and hypocotyl of lettuce seeds were 50% and 5% at 300 mg respectively while at 60 mg, the roots were inhibited to 42% and hypocotyl to 18% **(Fig. 3.6).**

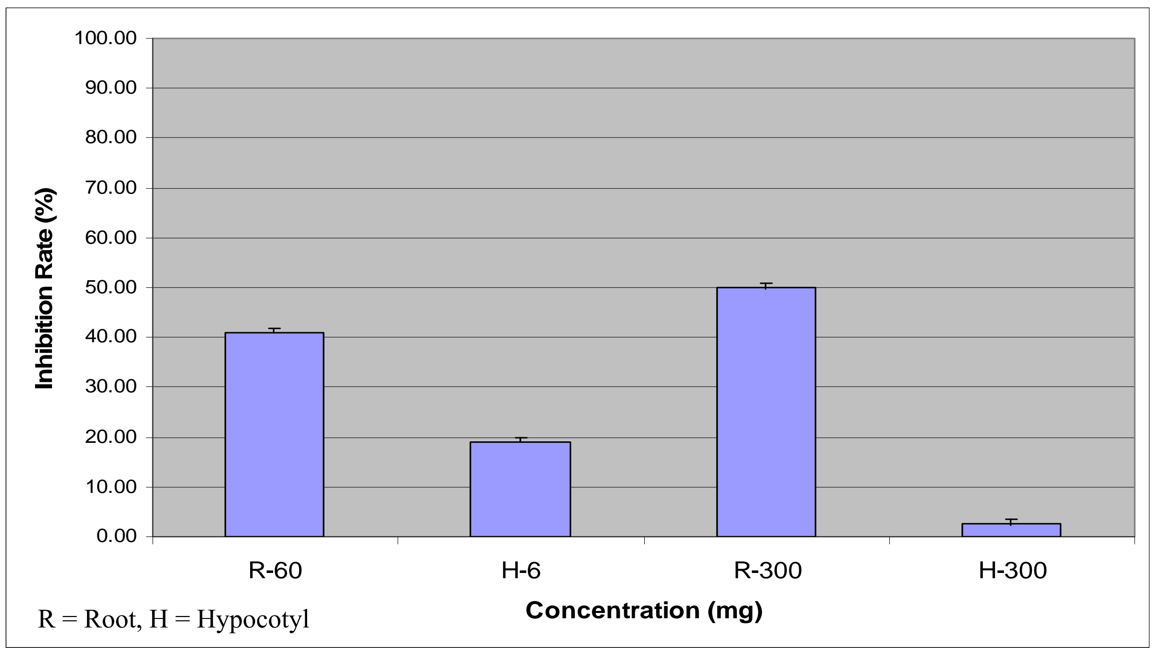


Fig.3.6. Effects of leaf leachates on the inhibitions of Lettuce seeds by homogenated Sandwich method

3.4.2.3 Comparison of Sandwich and Homogenated Sandwich Methods

Two different approaches were used in both the methods. In Sandwich method, the plant leaves as such were sandwiched between the agar layers so all the phytotoxic volatile and nonvolatile compounds leaches out from the leaves slowly thus were in direct contact with the lettuce seedlings. While in the Homogenated Sandwich Method the powdered plant material was taken in a falcon tubes containing water thus only the water soluble compounds were taken in consideration which were responsible for showing inhibitory activity. Comparing the results of both the assays (**Fig. 3.7)** the Sandwich method exerts

more inhibitory effects which may be due to the absence of the more volatile oils (monoterpenes or other volatile sesquiterpenes) from the aqueous extract (Homogenated Sandwich Method consideration) due to its insoluble nature in water.

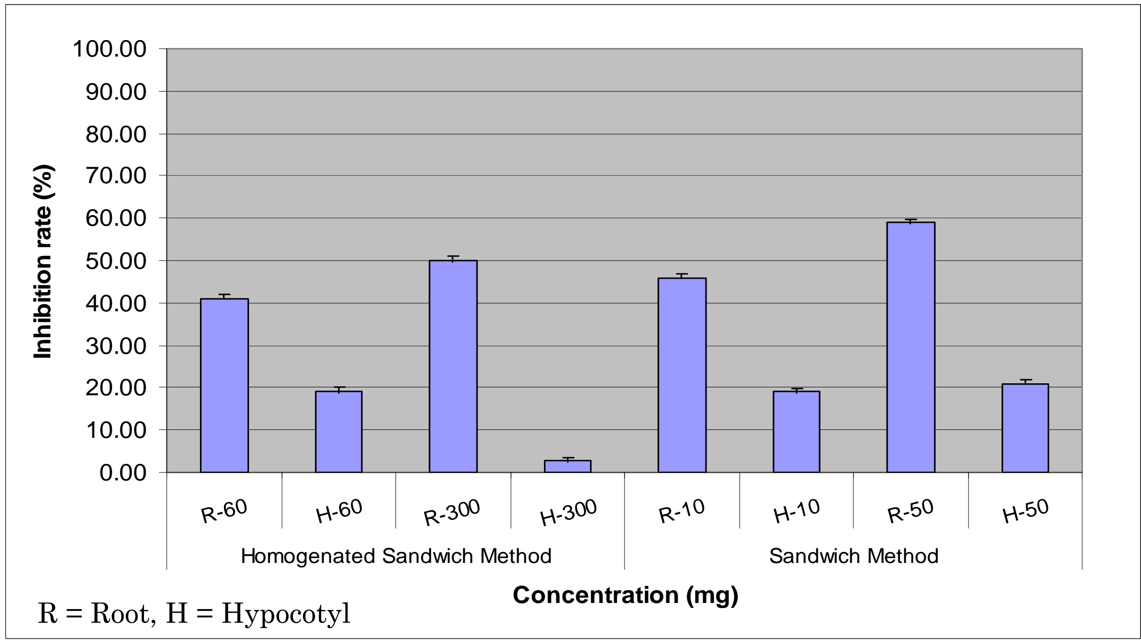


Fig. 3.7. Comparison of the effects of leaf leachates on the lettuce seeds inhibition by both methods.

These preliminary results showed the presence of some phytotoxic compounds in the leaves of the test plant. These results prompted us to check the presence of phytotoxic compounds in the volatile oils and in the subfractions of hexane, chloroform, ethylacetate, butanol and water.

3.4.3 ESSENTIAL OILS ALLELOPATHY

The essential oils were subjected to phytotoxic studies using Dish Pack Method. The nearest 2 wells to essential oils have the distance of 41 mm. The upper middle well had distance of 58 mm, furthest lower right 82 mm and furthest upper right had 92 mm distance. The data was recorded according to the distance of the wells from the essential

oils that how far it is from each well. Results showed that essential oils of *Teucrium royleanum* exhibited inhibitory rates of 65 to 100 % on shoots and 47 to 65 % on roots growth of lettuce seeds. The essential oil showed maximum inhibitory effects at 65 and 100% on both the roots and hypocotyl in 41 mm wells respectively **(Fig. 3.9**).

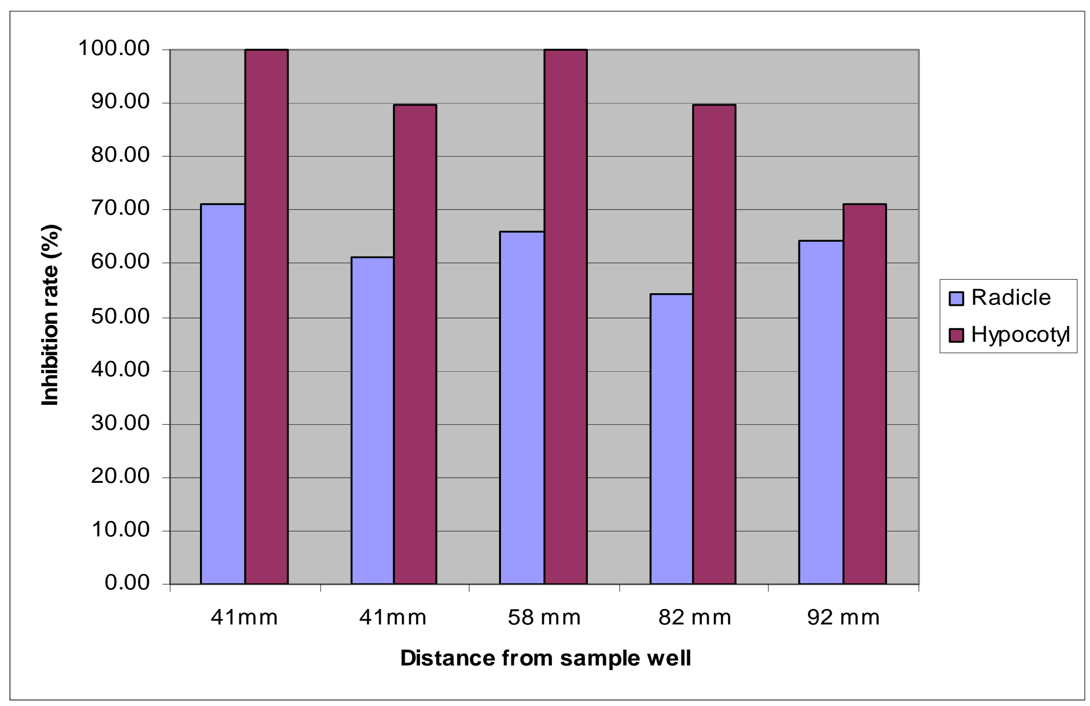


Fig. 3.8. Inhibition rates of lettuce roots and hypocotyl by essential oils of *Teucrium royleanum*

Nearest wells were affected to great extents as these compounds are released by processes such as volatilization. The volatiles oil and terpenoids has the tendency to strongly inhibit the germination[137-144]. The GC-MS analysis of *T. royleanum* in our present work showed that major chemical constituents are monoterpenes hydrocarbons, 17.1% oxygenated monoterpenes 7.6% sesquiterpene hydrocarbons, 42.3%, oxygenated sesquiterpenes 21.0 %[145]. Thus the inhibition caused by the essential oil can be attributed to the presence of these terpenoids.[145]

3.4.4 METHANOL AND SUBSEQUENT SUBFRACTIONS

From the preliminary results of above mentioned studies, it was confirmed that *Teucrium royleanum* contained allelochemicals. After confirming their presence in essential oils, the test plant was further subjected to extraction with 20% methanol and subsequently partitioned into hexane, chloroform, ethylacetate, butanol, and water sub-fractions. Hexane fraction showed slightly stimulatory effects on roots at 3, 10, and 30 ppm but at 100 and 300 ppm concentrations, it exhibited 43.3 and 100% inhibitory effects, respectively **(Table 3.14)**, while hypocotyl inhibition was observed in all the concentration except 10 ppm **(Table 3.14)**. The sub-fraction of chloroform showed stimulatory effects in all the concentration except higher concentration of 300 ppm for roots and inhibition was caused in shoots lengths except at 30 ppm.

Since the other sub-fractions of ethylacetate, butanol, and water have not shown 50 % of the inhibition to the growth of lettuce seed, therefore, they have no EC_{50} value and the compounds of our interest may be absent or of low concentration in these subsequent fractions **(Table 3.14)**

Table 3.14: Inhibitory effect of various extracts of *Teucrium royleanum* on the growth of root and hypocotyl

Conc.	Hexane		$CHCl_3$		EtOAc		BuOH		Water	
	R	**H**	**R**	**H**	**R**	**H**	**R**	**H**	**R**	**H**
0 ppm	0	0	0	0	0	0	0	0	0	0
3 ppm	-0.2	3.7	-11.2	9.2	4.2	-3.7	-5.9	-1.8	-15.6	-3.7
10 ppm	-7.3	-6.7	-7.3	1.2	-17.8	-4.3	-10.3	6.7	-29.7	0.6
30 ppm	-7.7	9.2	-4.6	-1.8	-17.4	7.4	15.2	19.6	-12.1	5.5
100 ppm	43.3 (c)	54.0 (b)	-13.0	6.7	-28.4	7.4	1.5	8.0	-13.4	3.7
300 ppm	100.0 (a)	100.0 (a)	35.4 (c)	58.9 (b)	-2.0	8.6	4.6	12.9	-10.3	6.1
ANOVA Test	**SS**	**dF**	**MS**		**F**		**P-Value**		**F crit**	
Between groups	8588.083	9	954.2314		1.94149		0.067083		2.073349	
Within groups	24574.73	50	491.494		-		-		-	

R= Root, H = Hypocotyl; ppm= parts per millions; a= highly significant, b = significant and c= moderate level of inhibition rate by the extract's concentration. SS = Sum of squares; dF = denominator of factors; MS = means of square; F = factor; P-value = alfa value; F crit = factorial critical value

ANOVA results showed that inhibitory effects were significantly concentrations dependent. Higher concentrations had higher inhibitory effects and vice versa **(Table 3.14)**. The probability value (P – value) was 0.1 for the ANOVA. The resultant P – value is 0.067083, which is less than the 0.1 probability level, thus presenting highly significant value. The higher concentration of leaf leachates furnish significant inhibitory values which can help in identification of allelopathic potential of the plant species[136].

EC_{50} values of all the subfractions of hexane, chloroform, ethylacetate, butanol and water was calculated and the results of the inhibitory activity on the lettuce seeds germination showed that the major activity of the original plant was accumulated into the hexane and chloroform fractions (**Figure 3.10,** and **3.11).**

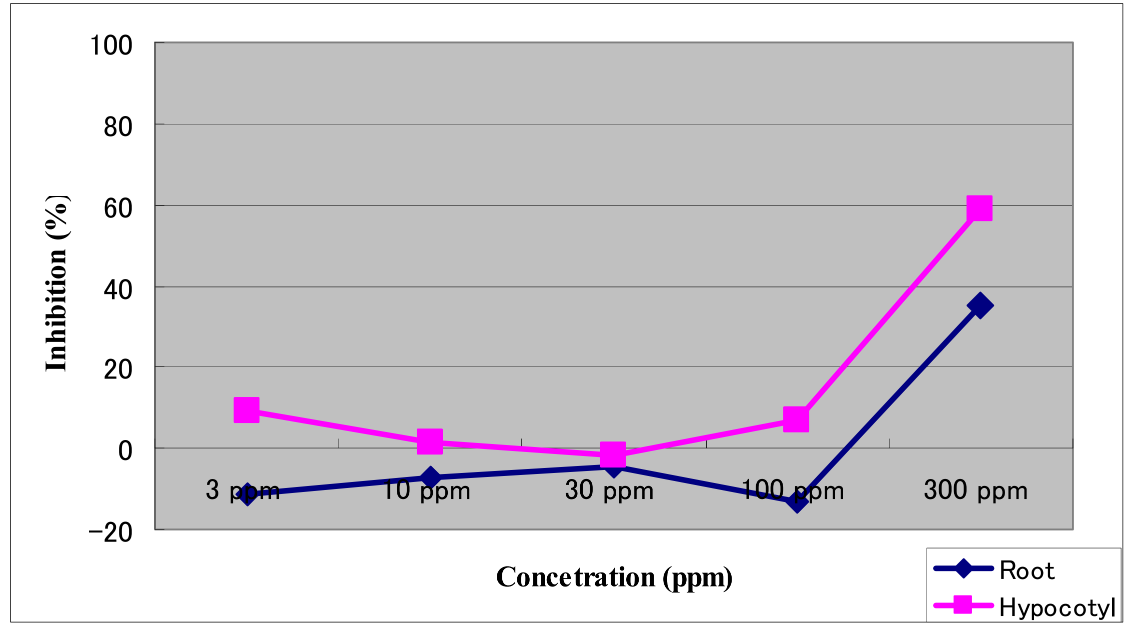


Fig. 3.9. Effect of various concentration of hexane on the lettuce roots and hypocotyl germination

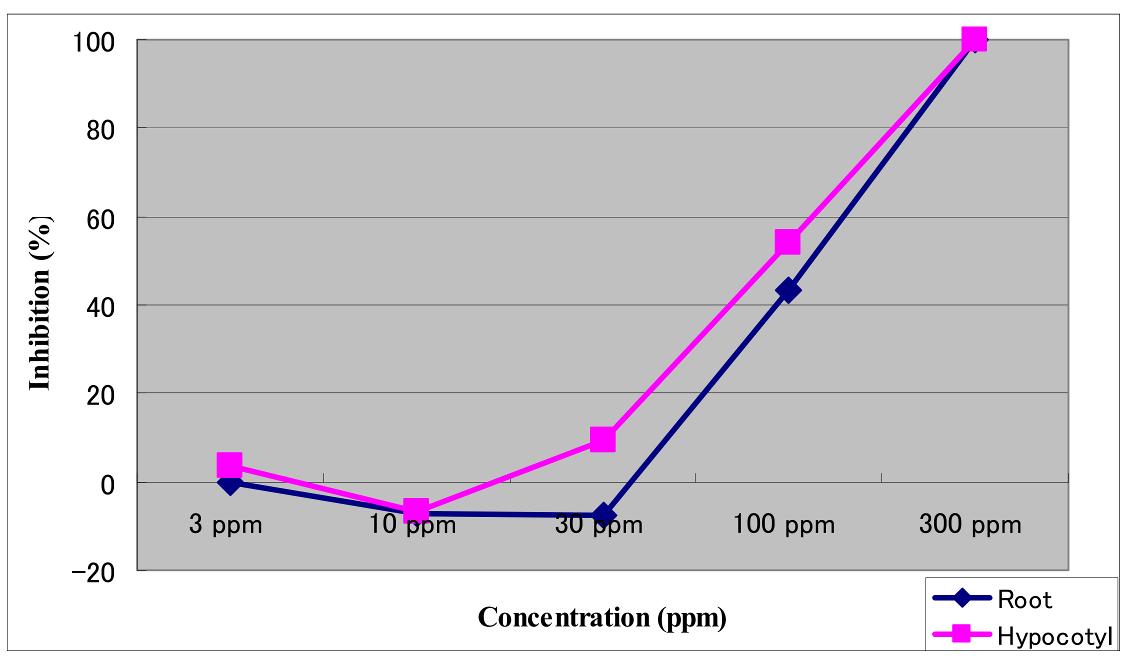


Fig. 3.10. Effect of various concentration of chloroform on the lettuce roots and hypocotyl germination

Although the focus of our study was on the inhibition but negative values for some of the concentrations of hexane and chloroform and almost all concentrations of ethylacetate, butanol and water subfractions showed stimulatory effects rather than inhibition. Our results supports the findings reported by Rice and Batish[146, 147], that allelochemicals are inhibitory to plants at one concentration but are stimulatory to the same plant or different plant at another concentration.

The EC_{50} value of hexane extract was 53.9% while the concentration that caused 50 % inhibition of the lettuce seed was 100 ppm. The 300 ppm concentration of hexane extract showed 100 % inhibition. The total activity of the hexane extract was 1.85, calculated by the following formula:

Total activity = $1/EC_{50}$ x Concentration

The EC_{50} value of chloroform extract was 60.4% at 300 ppm. The total activity of the extract was 5.09. The plants of genus *Teucrium* generally possess *neo*-clerodane diterpenoids[125- 132]. Some flavonoids have also been reported from this genus[133, 135]. The allelopathic properties of some flavonoids for some species of other families have been extensively studied[148-151]. Similarly some sesquiterpenes lactones exhibit strong inhibition to the growth of roots and hypocotyl of lettuce seedlings[152, 153]. Thus it may be concluded that diterpenoids and flavonoids are responsible for the allelopathy which are found in the chloroform fraction.

Our results of essential oils, hexane and chloroform showed the possible presence of phytotoxic terpenes and flavonoids in the *Teucrium royleanum*. The global effort today is to identify and isolate environmentally friendly weedicides and herbicides. Further analysis for the targeted isolation and characterization of potential allelochemicals in *T. royleanum* is worth while to pursue. However, here we contribute to the global goal of introducing environmentally friendly herbicides and weedicides.

3.4.5 ALLELOPATHY OF NEW COMPOUNDS FROM *TEUCRIUM ROYLEANUM*

The results of the phytotoxic bioassay against lettuce seedlings (*Lactuca sativa* cv. Great Lakes 366) are reported in Figure 3.12 and 3.13. Compound **1** (Royleanumin), which exhibits a neo-clarodane diterpenes skeleton. The neo-clarodane diterpenes are extensively studied for their insect antifeedant assays[11, 10, 154] and no literature is available on the allelopathy of this class. Our study on the bioassay of Royleanumin **1**, which exhibits a neo-clerodane skeleton, has shown higher phytotoxicity as compared to royleanumioside **2**.

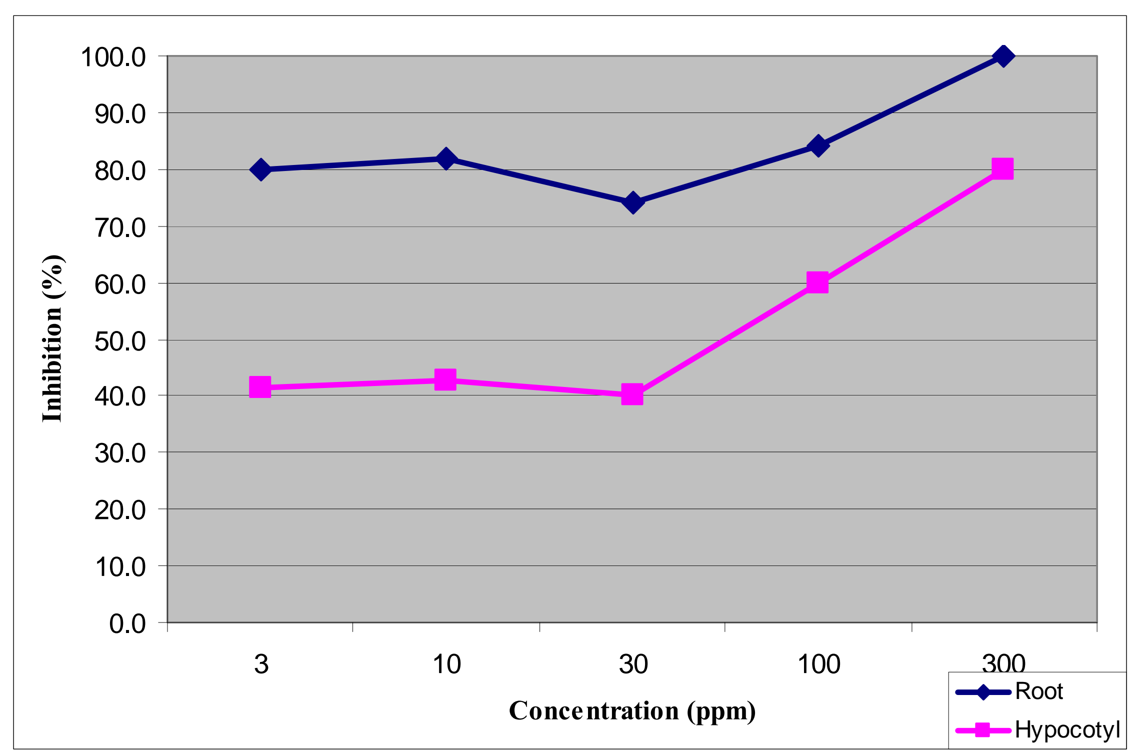


Fig. 3.11. Allelopathy of Compound **1**

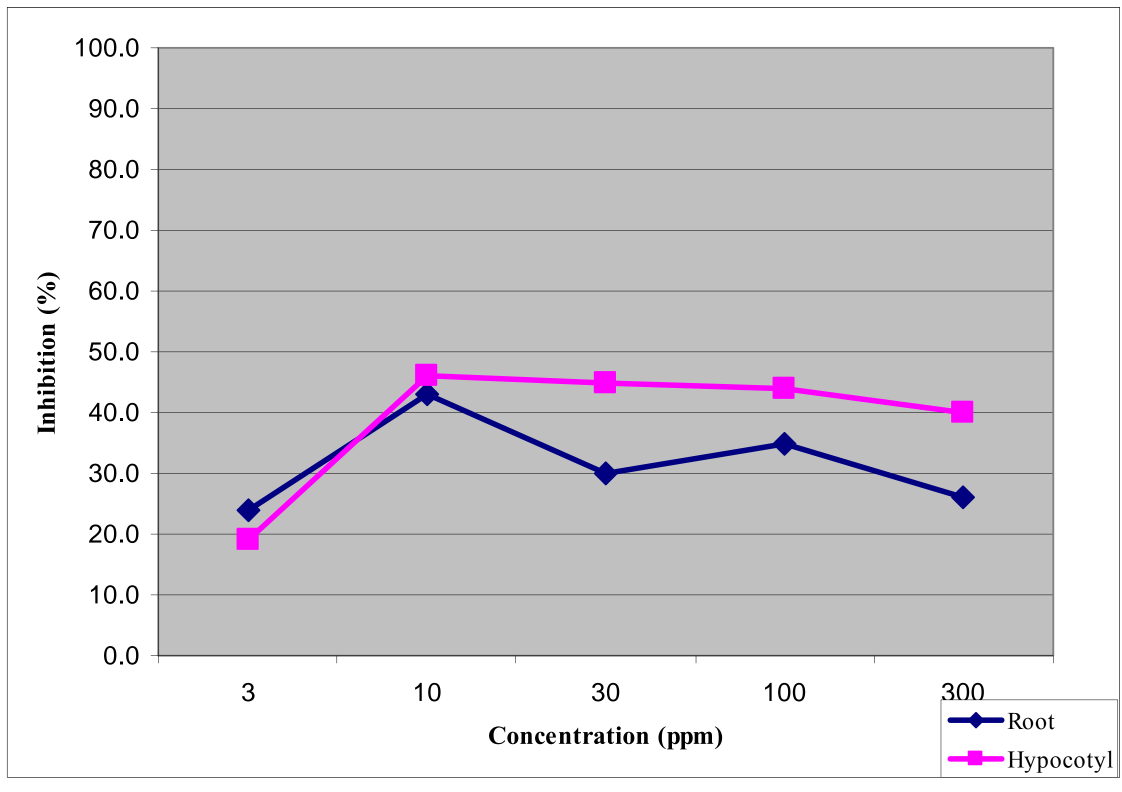


Fig. 3.12. Allelopathy of Compound **2**

The phytotoxicity of the compound **1** is clear from the **(Figure 3.12).** Remarkable inhibition of the roots has been shown at all concentrations (74.3-100 %). A dose dependant inhibition was observed, i.e. the inhibition increases with increase in concentration except at 30 ppm. This inhibition can be attributed to the basic skeleton of diterpenes[155-157]

The overall effect on *Lactuca sativa* (lettuce seeds) inhibition was 22 to 28.6% for compound **2**, interestingly lower inhibition was shown at higher concentrations. Non dose dependent results were obtained. The effects produced by our tested compound **2** on the roots inhibition are presented in **(Figure 3.13)**. The compound **2** exhibited lower inhibitory activities as none of the concentration has EC50 value. The sugar linkages followed by the ester linkage might be responsible for showing the slight inhibition as the lupanic skeleton is responsible for stimulation. The available data on the lupanic triterpenes showed stimulation of the germination of *Lactuca sativa*[158].

3.4.6 ALLELOPATHY OF KNOWN COMPOUNDS FROM *TEUCRIUM ROYLEANUM*

The results of the allelopathy of three flavonoids from *T. royleanum* against lettuce seedlings (*Lactuca sativa* cv. Great Lakes 366) are shown in **(Figure 3.14, 3.15,** and **3.16).** The compound **4** exhibited strong phytotoxicity to the root and hypocotyl germination at 30, 100, and 300 ppm where as 80% at 3 and 10 ppm concentrations (**Figure 3.14)**. Similarly compound **5** and **6** showed almost same results. Remarkable inhibition of 90 % at lower concentration of 3 ppm was observed for both the compounds, while the lettuce seedlings fail to germinate at the remaining all test concentrations (**Figure 3.15** and **3.16)**. Compound **5** and **6** are equally potent inhibitors showing 100% inhibition at 10 ppm concentration while compound **4** exerted 80% inhibition at 10 ppm

concentration and 100% inhibition were caused at 30 ppm concentration.

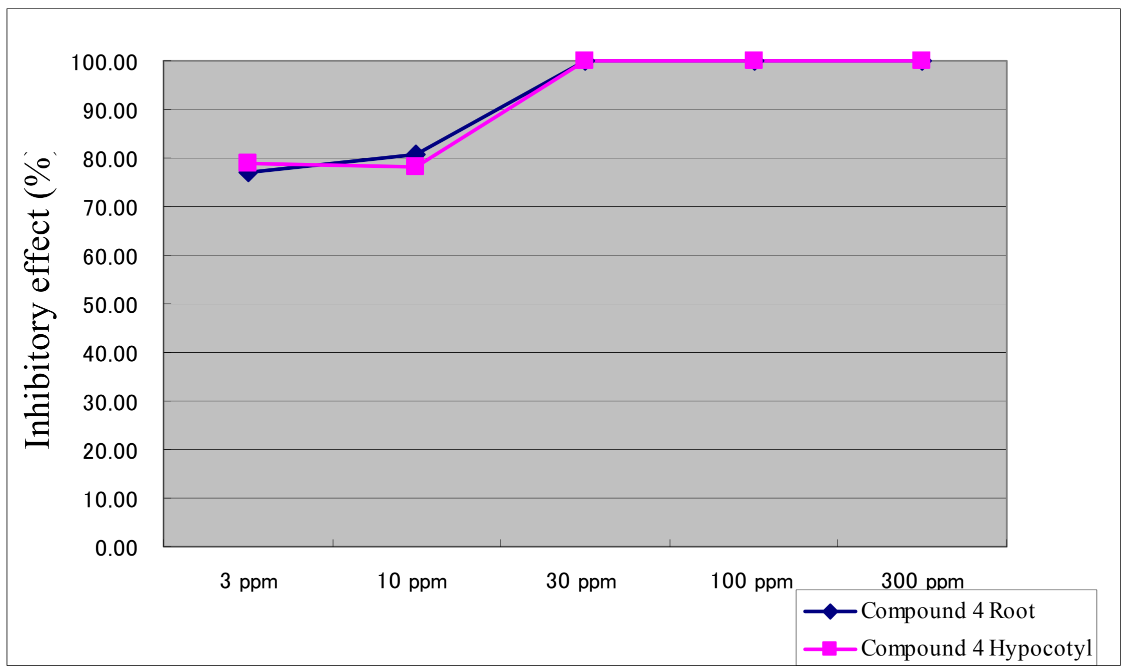


Fig. 3.13. Allelopathy of Compound 4

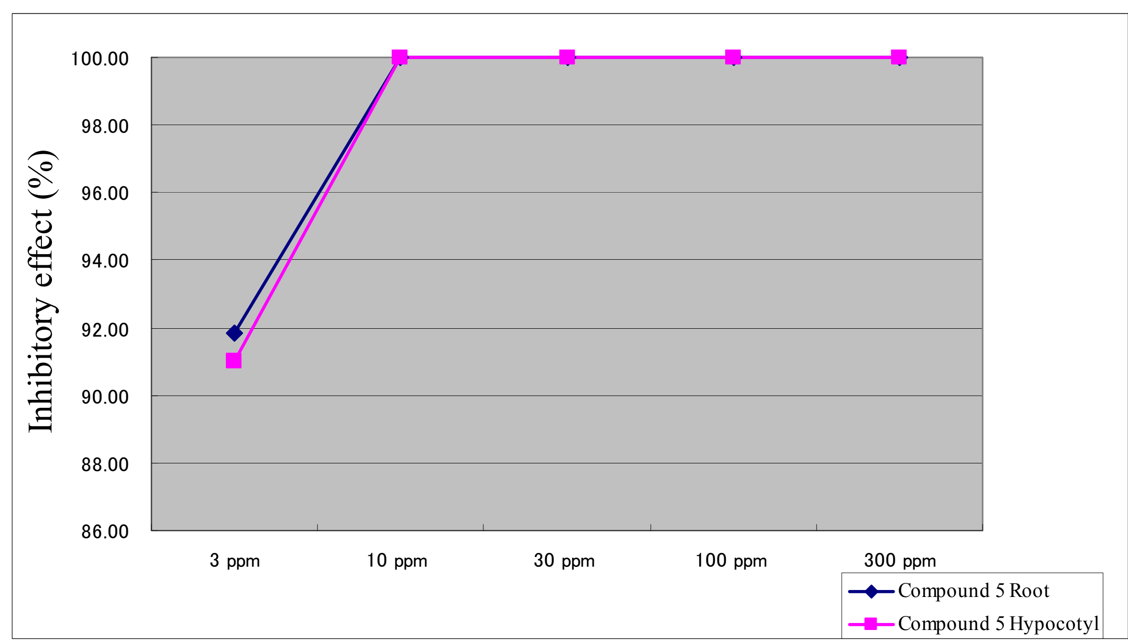


Fig. 3.14. Allelopathy of compound 5

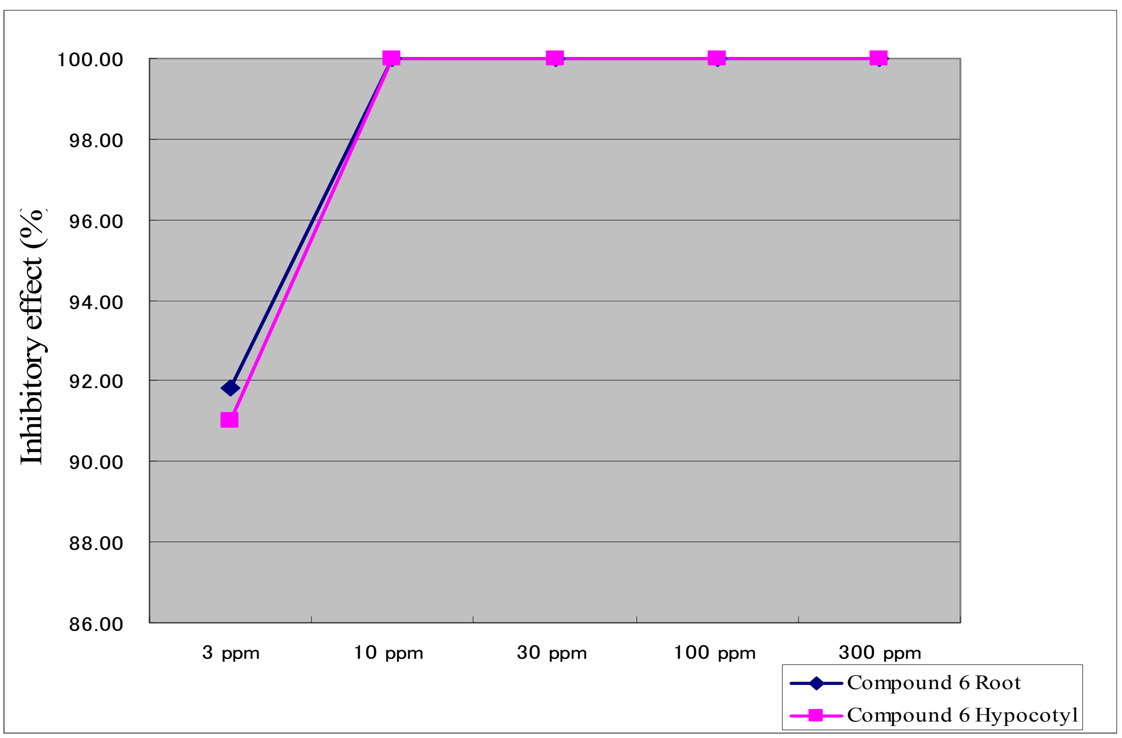


Fig. 3.15. Allelopathy of compound **6**

Plenty of information is available on the phenomenon of allelopathy but least is known about the allelopathy activities of purified compounds. Higher plants have rich distribution of flavonoids in different tissues like flowers, roots, leaves and stems. Flavonoids exhibited strong allelopathic effects on the lettuce seeds germination[161]. Some of the flavonoids like quercetin and kaempferol have already been isolated as allelopathic substances[159, 160]; however, no one knows the details of their mechanisms and functions as allelopathic agents.

The results obtained are in agreement with those reported in the literature. The existence of a methyl group in the flavonoid nucleus has an important role in inhibiting lettuce seedlings growth[162] but still it is difficult to answer as to why the activity of compound **4** is different than the compound **5** and **6**.

From the present study, it is clear that royleanumin **1**, 5-hydroxy-4′, 7-dimethoxy flavone **4**, 5, 7-dihydroxy-3, 4′ −dimethoxy flavone **5**, 4′, 6-dihydroxy-5, 7-dimethoxy flavone **6** exhibit strong allelopathy activities to the test lettuce seedlings. Consequently, these can be proposed as lead compounds for studies on the development of new herbicides and weedicides.

CHAPTER 4

EXPERIMENTAL

4.1 GENERAL NOTES

All the studies were carried out at the Institute of Chemical Sciences University of Peshawar, International Center for Chemical and biological sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Pakistan, and Gene Research Center, Graduate School of Life and Environmental Sciences 1-1-1 Tennoudai, University of Tsukuba, Tsukuba, Japan. The commercial grade solvents were distilled and used for thin layer and column chromatographic techniques.

4.1.1. INSTRUMENTATION

A Büchi m.p. apparatus was used for the measurements of melting points. A JASCO DIP-360 (Japan Spectroscopic Co. Ltd., Tokyo, Japan) digital Polarimeter was used for the measurement of optical rotations while Shimadzu UV-240 (Shimadzu Corporation, Tokyo, Japan) spectrophotometer was used for recording of UV spectra.

Shimadzu IR-460 (Shimadzu Corporation, Tokyo, Japan) instrument was used for the measurements of IR spectra. The ^{1}H-NMR spectra were recorded on a Bruker AM 300 FT NMR, AM 400 FT NMR and AM 500 FT NMR spectrophotometers using TMS as an internal reference standard. The ^{13}CNMR spectra were scanned on 75, 100 and 125 MHz on a Bruker A.M 300 FTNMR, AM 400MHz FTNMR and AM 500 FTNMR spectrophotometers, respectively. The chemical shift values are described in ppm (δ) units and the coupling constants (*J*) are reported in Hz.

The mass spectra were recorded on Varian-MAT 112S and Finnigan MAT-112 and 312A

double focusing mass spectrometers attached to DEC PDP 11/34 and IBM-AT compatible PC based system, respectively. Electron impact, peak matching, field desorption (FD) and fast atomic bombardment (FAB) studies were either taken on a Varian/Finnigan-MAT-312A or on a Jeol-JMS HX-110 mass spectrometers. FABMS were taken in a glycerol-water (1:1) matrix in the presence of KI. High Resolution electron impact mass spectra (HREIMS) were scanned on a Jeol-JMS H X-110 mass spectrometer.

4.1.2. CHROMATOGRAPHY

For thin layer chromatography (TLC) pre-coated aluminium sheets, silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E-Merck) and for column chromatography silica gel having mesh size of 60, 70-230, 230-400, (E. Merck) were utilized. Gas chromatography mass spectrometry was determined on Hewlett-Packard 5973–6890 system.

4.1.3. SPRAY REAGENTS

Ceric sulphate reagent was used for the detection of compounds and aniline phthalate reagent for detection of carbohydrates.

- **Ceric Sulphate Reagent**

Ceric sulphate (0.2 g) and trichloroacetic acid (2 gm) were solubalised in 8 mL distilled water. The solution was boiled and conc. H_2SO_4 was added drop-wise until the disappearance of turbidity.

- **Aniline Phathalate Reagent**

Aniline (0.93 g) and *o*-phthalic acid (1.66 g) were dissolved in 100 mL *n*-butanol saturated with water.

4.2. *TEUCRIUM ROYLEANUM* ESSENTIAL OIL ISOLATION

4.2.1. MATERIAL

T. royleanum aerial parts were collected at the flowering season during the month of June 2003 from a locality in Shamozai, Swat, Pakistan. A voucher specimen [No. Shabir 2651979 (PUP)] and deposited after being identified by Professor Dr. Abdul Rashid in the Botany Department Herbarium, University of Peshawar, Pakistan.

4.2.2. METHOD

The air dried aerial parts of the plants were subjected to extraction bye the process of hydrodistillation for three hours using a Clevenger-type apparatus[163] (**Fig. 3.14)**. The essential oil obtained was taken up in diethyl ether; moisture contents were removed by anhydrous magnesium sulphate, and filtered. The solvent, diethyl ether, was evaporated using a gentle stream of anhydrous nitrogen gas and the oil obtained was stored at 4 °C in a sample vial in the dark. The yield of the essential oil was 1.8% (v/w).

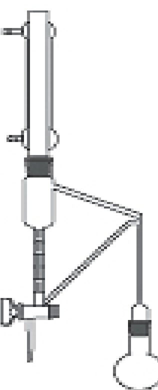


Fig. 3.16: Clevenger-type apparatus

4.3. ESSENTIAL OIL ANALYSIS

4.3.1. MATERIAL AND METHOD

The composition of the volatiles was determined utilizing flame ionization detection–gas chromatography (FID–GC) and GC–MS analyses.

4.3.1.1. Gas Chromatography

The Perkin-Elmer 8500 gas chromatograph with FID, fitted with a Supelcowax 10 fused silica capillary column (30 m x 0.32 mm i.d.; film thickness 0.25 µm) was used for the analysis. The column temperature programmed was 75 °C to 260 °C at a rate of 2.5 °C/min. The injector and detector temperatures were programmed at 230 °C and 300 °C, respectively.

4.3.1.2. Gas Chromatography–Mass Spectrometry

The Hewlett-Packard 5973–6890 system working in EI mode (70 eV) equipped with a split/ splitless injector (220 °C); split ratio, 1:10, utilizing two different columns; fused silica HP-5 MS capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm) and a HP-Innowax capillary column (30 m x 0.25 mm i.d., film thickness 0.50 µm) were used for GC-MS analyses. The temperature maintained for the HP-5 MS column was 60 °C (5 min) to 280 °C at a rate of 4 °C/min, and for the HP-Innowax column 60 °C to 260 °C at a rate of 3 °C/min; helium was used as carrier gas, flow maintained at a rate of 1.0 ml/min; injection volume, 2 µl. Van den Dool approach[164] was used for the determination of the retention indices for all compounds, making use of the *n*-alkanes as standards. The chemical constituents were then identified by comparing their mass spectra with those of Wiley and NBS Libraries[165] and those described by Adams, [166] in addition to some were also confirmed by comparing their retention indices with literature records.[166, 167] Some times the essential oil were also subjected to co-chromatography with reliable authentic

compounds (Fluka, Sigma). The optical rotation values were determined at 25 °C at 589 nm in *n*-pentane using Perkin Elmer 341 polarimeter.

4.3.2. FRACTIONATION OF THE OIL

After preliminary identification of the component by GC and MS for confirming the results, the oil was fractionated and subjected to ^{13}C-NMR spectroscopy experiments. The oil (1 g) was resolved into non polar and polar fractions by performing column chromatography over 40 g of silica gel (70-230 mesh), the column was eluted by hexane to recover hydrocarbons and then ethyl acetate to obtain oxygenated compounds. Each fraction after concentration in vacuum was subjected to ^{13}C-NMR spectroscopy experiments. The non polar fraction contained monoterpenes hydrocarbons and methyl carvacrol and the polar fraction consisted of almost pure carvacrol.

4.3.3. IDENTIFICATION BY ^{13}C-NMR SPECTROSCOPY

Identification by GC/MS was further confirmed by comparison of the ^{13}C-NMR spectra (recorded in deuterated benzene) of the mixture with those measured for the pure authentic compounds in the literature[168, 169] The chemical shift value obtained for the compounds in the oil were in good agreement with the reported ones.

4.4. ANTIOXIDANT ACTIVITY

4.4.1. MATERIAL AND METHOD

The isolated oil obtained was assayed by two methods.

4.4.1.1. 1, 1-Diphenyl-2-Picryl-Hydrazyl (DPPH) Stable Free Radical Interaction with the Tested Compounds

Stock solution (20 µl) of the test sample was diluted in absolute ethyl alcohol to a total volume of 1 ml and followed by its addition to 1 ml DPPH (0.1 mg in absolute ethanol).

This reaction mixture was then strongly stirred for 10 s and kept at room temperature (25 ^{0}C) for 20 and 60 min. As control, a solution with the same chemicals, except for the samples, was used.[170] Ethanol was used as blank. The optical density (OD) of the solution was measured and the percentage reduction was estimated, using the following equation at 517 nm. The optical densities of samples without DPPH were recorded and subtracted from the corresponding OD with DPPH:

% reduction = control OD (mean) – sample OD (mean) / control OD mean X 100

Acetylsalicylic acid was employed as the suitable standard gave 80.6% (± 5) in 0.1 mm **(Table 3.2)**, whereas butylated hydroxytoluene (BHT) presents 36.1% (± 3) interactions at 0.1 mm.

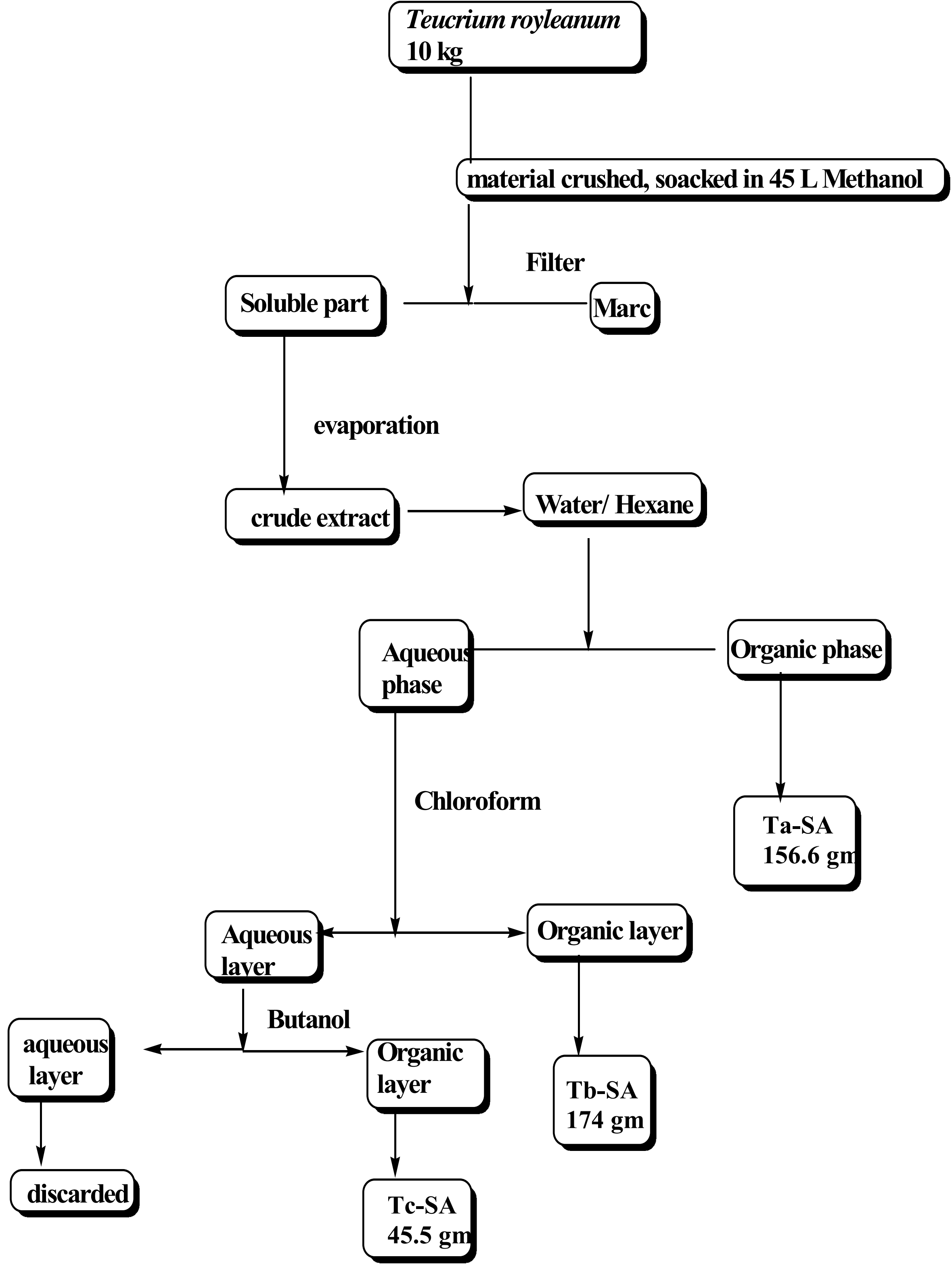
4.4.1.2. Superoxide Anion Radical Scavenging Activity

The xanthine–xanthine oxidase system was used for the generation of the superoxide anion and calculated by the nitroblue tetrazolium (NBT) method.[171] Reaction mixture, containing 350 µl xanthine, 150 µl NBT and 100 µl each sample in phosphate buffer, pH 7.4 (0.1 mol /l) was prepared; followed by the subsequent addition of 40 µl (50 U/2.6 ml) xanthine oxidase. Then it was incubated for 10 min at room temperature, the absorbance was then measured at 560 nm (**Table 3.2)**. Both of the above stated experiments were carried out in triplicate and the standard deviation of absorbance was <10% of the mean. Standard deviation of the mean (four to six values) is given in parentheses. Caffeic acid used as an appropriate standard gave 70% (± 3.2) at 1 mM concentration (**Table 3.2)**.

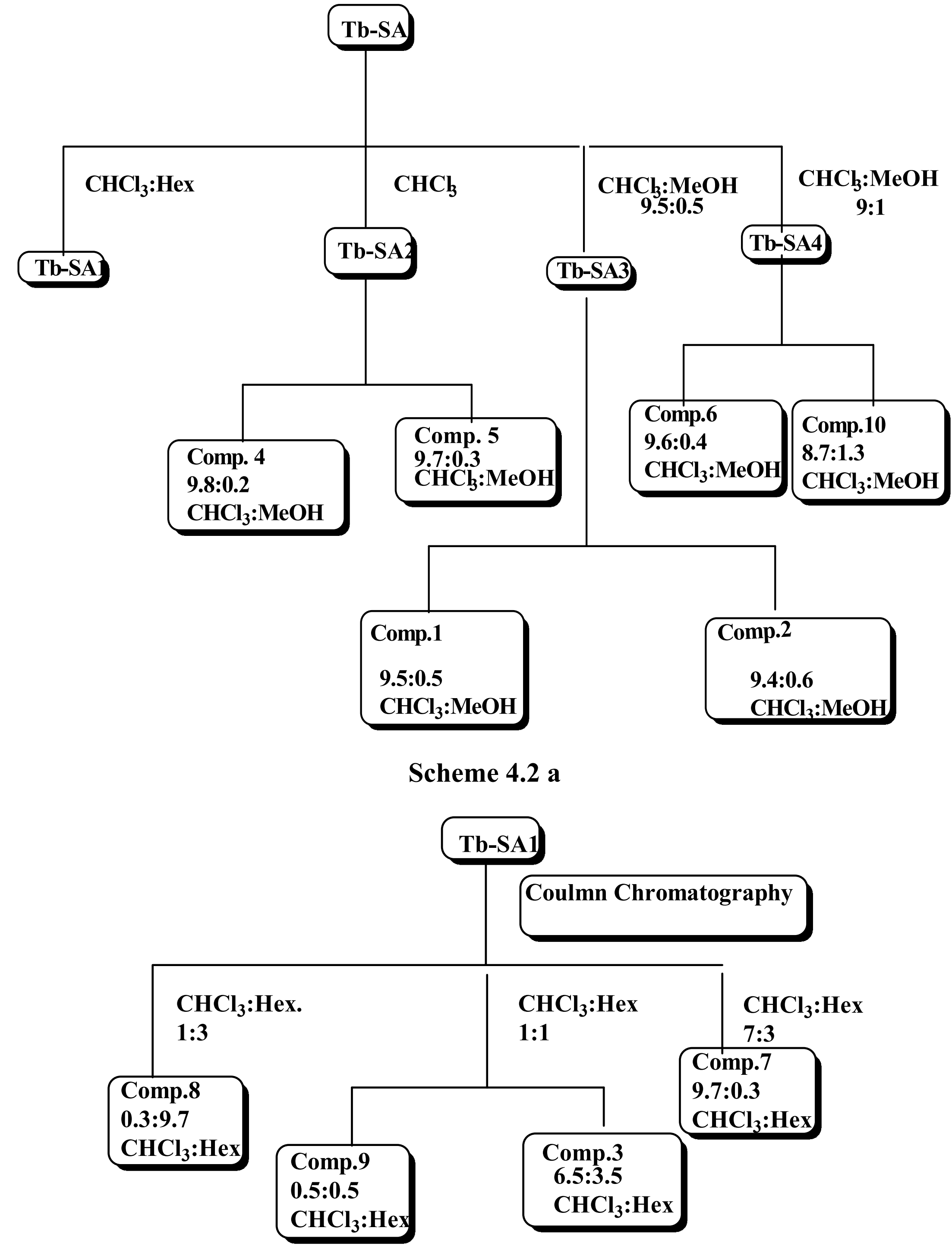
4.5. PHYTOCHEMICAL INVESTIGATIONS OF *TEUCRIUM ROYLEANUM*

4.5.1. MATERIAL AND METHOD

The powdered air dried aerial parts of *T. royleanum* (10 kg) was soaked in MeOH (3 × 45L) at room temperature for about 24 hours. The combined methanolic extract was then concentrated via rotavapour to get a thick gummy extract (850 g). The resultant concentrated extract was then dissolved in water and was subjected to solvent-solvent extraction process using *n*-hexane, chloroform and *n*-butanol **(Scheme 4.1).** The column chromatography over Silica gel was performed for chloroform soluble fraction Tb SA (174 g), using a gradient of organic solvents including *n*-hexane, *n*-hexane-chloroform, chloroform, and chloroform-methanol in the polarity increasing order of the solvent (**Scheme-4.1 a, b**).



Scheme 4.1. Extraction scheme of *Teucrium royleanum*



Scheme 4.2 a

Scheme- 4.2 b

Scheme-4.2 a, b Purified compounds from $CHCl_3$ soluble fraction of *Teucrium royleanum*

4.5.2. PURIFICATION OF ROYLEANUMIN (1)

Fraction Tb-SA3 obtained as a result of the column chromatography of chloroform soluble part (Tb-SA), was re-chromatographed over a silica gel column and the column was fractionated using gradient elution technique. The fraction eluted in chloroform-methanol (9.5:0.5) afforded compound **1** as colorless amorphous solid (14 mg) **(Scheme 4.2 a).**

4.5.2.1 Physical and Spectral Data of Royleanumin (1)

$[\alpha]_D^{25}$ (c, 1.2 $CHCl_3$) -36°

IR ν_{max} (KBr) cm^{-1}: 3400, 3130, 1660, 1505, 874.

ElMS *m/z*: 362 (18), 344 (97), 285 (28), 267 (14), 251 (18) 191 (38), 134 (14), 105 (23), 95 (25), 94 (100).

HREIMS *m/z*: 362.1720 (Calculated for $C_{20}H_{26}O_6$, 362.1729)

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz) : **Table-3.4.**

4.5.3 PURIFICATION OF ROYLEANUMIOSIDE (2)

The fraction Tb-SA3 gave two spots on TLC. The upper one resulted in compound **1** and the lower one resulted in compound **2** with the TLC solvent system of chloroform-methanol (9.4: 0.6) as a colorless amorphous solid (11mg) **(Scheme.4.2a).**

4.5.3.1. Physical and Spectral Data of Royleanumioside (2)

$[\alpha]_D^{25}$ (c, 1.2 $CHCl_3$) -34°

IRν_{max} (KBr) cm^{-1}**:** 3360, 2900, 1740, 1630, 1250.

ElMS *m/z***:** 423 (15), 407 (10), 216 (20), 208 (25), 201 (17), 189 (34), 69 (24), 175 (19).

HRFABMS *m/z*: 853.6929 (Calcd for $C_{54}H_{93}O_7$, 853.6929) $[M+H]^+$.

^{1}H-NMR, ^{13}C-NMR (C_5D_5N, 500 MHz and 125 MHz) : **Table-3.5.**

4.5.3.2. Acid Hydrolysis of Royleanumioside (2)

A solution of the 8 mg of test compound **2** in methanol (5 mL) having 1N HCl (4 mL) was refluxed for 4 hours. Then it was concentrated under reduced pressure followed by dilution with H_2O (8 mL). It was taken up with Ethyl acetate. The organic phase had provided the aglycones while the aqueous phase in each case was concentrated to obtain the glycone. The glycone was recognized as D-glucose by the optical rotation sign of $[\acute{\alpha}]_D^{20}$ +52°. Further confirmation was also carried out based on the retention time of its TMS ether (ά -anomer 4.1 min, β -anomer 7.8 min) with a standard.

4.5.4. PURIFICATION OF ROYLEANUMOATE (3)

The fraction Tb-SA1 was eluted in the chloroform-hexane (1:1), from a silica gel column loaded with initial chloroform soluble part (Tb-SA), on further chromatography provided compound **3** in chloroform- hexane (6.5:3.5). The compound was purified as amorphous solid (7mg) by repeated column chromatography **(Scheme-4.2b)**.

4.5.4.1. Physical and Spectral Data of Royleanumoate (3)

IRν_{max} (KBr) cm^{-1} 3440, 1735, 1617

ElMS *m/z*: 121 (100), 107 (6), 71 (11), 57 (40)

FAB+MS *m/z*: 333 (caled. for $C_{21}H_{34}O_3$)

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz): **Table-3.6.**

4.5.5. PURIFICATION OF 5-HYDROXY 4', 7-DIMETHOXY FLAVONE (4)

The fraction Tb-SA2 obtained as a result of column chromatography of chloroform soluble part Tb-SA in Chloroform was repeatedly chromatographed over a silica gel column. Chloroform-methanol (9.8:0.2) afforded compound **4** with some minor impurities. The impure solid obtained on drying and after washing with methanol

provided compound **4** as a yellowish solid in pure form (11mg) **(Scheme-4.1 a).**

4.5.5.1. Physical and Spectral Data of 5-Hydroxy 4', 7-Dimethoxy Flavone (4)

M.P: 170-171 ^{o}C.

IR ν_{max} (KBr) cm^{-1}: 3250 (OH), 1670 (C0), 1610 (C=C), 1505 and 840.

UV λ_{max} (MeOH) nm: 330, 265, 255.

EI-MS *m/z* (rel. int. %): 298 [M^{+}], (100%), 270 (10), 269 (3), 166 (12), 162 (3), 138 (10), 150 (17), 132 (17) and 95 (14).

HR-EI-MS *m/z* (formula, calculated value): 298.0838 ($C_{17}H_{14}O_5$, 298.0841).

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz): **Table-3.7.**

4.5.6. PURIFICATION OF 5, 7-DIHYDROXY-4′,3-DIMETHOXY FLAVONE (5)

The fraction Tb-SA2 obtained as a result of column chromatography of chloroform soluble part, Tb-SA repeated chromatographed over a silica gel column in chloroform-methanol (9.7:0.3) afforded compound **5** with some minor impurities. The impure solid obtained on drying and after washing with methanol provided compound **5** as a yellowish solid in pure form (13mg) **(Scheme.4.2a)**.

4.5.6.1 Physical and Spectral Data of 5, 7-Dihydroxy-4′ ,3-Dimethoxy Flavone (5)

M.P: 170-171 ^{o}C

IR ν_{max} (KBr) cm^{-1}: 3450 (OH), 1685(C0), 1610 (C=C), 1505 and 840.

UV λ_{max} (MeOH) nm: 360, 315, 260.

EI-MS *m/z* (rel. int. %): 314 [M^{+}], (100%), 286 (10), 152 (3), 132 (17) and 95 (14)

HR-EI-MS *m/z* (formula, calcd. value): 314.0838 ($C_{17}H_{14}O_5$, 314.0841),

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz) : **Table-3.8.**

4.5.7. PURIFICATION OF 6, 4′-DIHYDROXY 5, 7-DIMETHOXY FLAVONE (6)

The fraction Tb-SA2 was obtained as a result of column chromatography of chloroform soluble part, Tb-SA. Repeated chromatographed over a silica gel column in chloroform-methanol (9.6:0.4) afforded compound **6** with some minor impurities. The impure solid obtained on drying and after washing with methanol provided compound **6** as a yellowish solid in pure form (13mg) **(Scheme. 4.2a).**

4.5.7.1 Spectral Data of 4`, 6 Dihydroxy, 5, 7 Dimethoxy Flavone (6)

IR ν_{max} (KBr) cm^{-1}: 3250 (OH), 1670 (C0), 1610 (C=C), 1505 and 840.

UV λ_{max} (MeOH) nm: 330, 265, 255.

EI-MS *m/z* (rel. int. %):314 [M^+], (100%), 286 (10), 196 (3), 118 (12), and 95 (14).

HR-EI-MS *m/z* (formula, calcd. value): 314.0838 ($C_{17}H_{14}O_5$, 314.0841).

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz): **Table-3.9.**

4.5.8. PURIFICATION OF 3, 4-DIHYDROXYMETHYL BENZOATE (7)

The column chromatography of chloroform soluble part, Tb-SA, yielded a fraction Tb-SA1. The continuous CC over silica using chloroform-hexane (9.7:0.3) resulted in the purification of compound **7** as a white crystalline solid (14 mg) **(Scheme. 4.2 b)**.

4.5.8.1. Physical and Spectral Data 3, 4 Dihydroxymethyl Benzoate (7)

M.P. 157-159 ^{0}C

UV (CD_3OD) λ_{max} log E: 276 (3.82), 283 (4.01) nm.

IR (KBr) ν_{max} Cm^{-1}: 3370 (O-H), 1694 (C=O), 1619 (Ar. C=C).

HREIMS m/z: 168.0371 (calcd. for $C_8H_8O_4$, 168.0360).

^{1}H-NMR ^{13}C-NMR (C_5D_5N, 400 MHz and 100 MHz) : **Table-3.10.**

4.5.9. PURIFICATION OF OLEANOLIC ACID (8)

The column chromatography of chloroform soluble part Tb-SA, gave a fraction TbSA1. The CC over silica using hexane-chloroform (9.7:0.3) as solvent system gave compound **8** in an impure form. This was later dried and washed with methanol, as result pure oleanolic acid was obtained as white crystalline solid (7mg) **(Scheme-4.2 b)**.

4.5.9.1. Physical and Spectral Data of Oleanolic Acid

M.P.: 306- 308 ^{0}C

$[ά]^{25}_{D}$: (c, 1.2 CHCl3) + 79.5

UV (Methanol): UV $\lambda_{max: 218}$

IR ν_{max} (KBr) cm^{-1}: 3500- 2400 (O-H, acidic O-H), 1705 (C=O), 1640 (C=C), 1210, 1120 (C-O).

EIMS m/z: (rel. Int. %): 456 (M+, 18) 248 (100), 235 (87), 203 (55), 189(35), 119 (28).

HREIMS m/z (formula, calcd.Value): 456.3600 ($C_{30}H_{48}O_3$)**.**

^{1}H-NMR ($CDCl_3$), 400 MHz) : 5.27 (1 H, t, j= 3.5 Hz, H-12), 3.19 (1H, t, J= 6.8 Hz, H-3), 2.79 (1H, dd, J= 13.7, 4.0 Hz, H-18), 1.13 (3 H, s, H-27), 0.98 (3 H, s, H-23), 0.92 (3H,s, H-24), 0.91 (3 H,s, H-30), 0.90 (3H, s, H-29) o.77 (3H, s, H-25), 0.75(3H, s, H-26).

^{13}C-NMR ($CDCl_3$, 100 MHz): Table-3.11

4.5.10. PURIFICATION OF β-SITOSTEROL (9)

The column chromatography of chloroform soluble part Tb-SA, gave a fraction TbSA1. The compound **9** was purified by column chromatography over silica gel eluted with hexane-chloroform (5:5) in an impure form which was then dried and washed with

methanol, as result pure β-Sitosterol was obtained as white crystalline solid (32mg) **(Scheme-4.2b).**

4.5.10.1. Physical and Spectral Data of β-Sitosterol

M.P.: 136-137 ^{0}C

$[\alpha]^{25}_{D}$: [C. 1.3 $CHCl_3$] - 35^0

EIMS e/z (rel. Int. %): 414 [m^+, 100], 399 (21), 371 (11), 329 (17), 273 (25), 222 (29), 141 (14), 138 (10), 99 (20), 43 (57).

HREIMS m/z (formula, calcd. Value): 414.3855 ($C_{29}H_{50}O$, 414. 3861),

^{I}**H-NMR ($CDCl_3$. 500 MHz)** δ: 0.66 {3H, s, H-18}, 0.78 {3H, d. J=6.0 Hz. 11-27}, 0.811 {H. d. J=6.2 Hz. H-26}, 0.82 {3H, d, J=7.5 Hz, H-29}, 0.90 {3H, d, J=6.5 Hz, H-21}, 0.98 {3H, s, 11-19}, 5.32 {IH, brs., H-6}.

^{13}C-NMR $CDCl_3$, 125 MHz: Table-3.12.

4.5.11. PURIFICATION OF β-SITOSTEROL GLUCOSIDE (10)

The fraction Tb-SA4 obtained as a result of column chromatography of chloroform soluble part, Tb-SA. Repeated chromatography over silica gel column in chloroform-methanol (8.7:1.3) offered compound **10** as amorphous solid (32 mg) **(Scheme-4.2a)**..

4.5.11.1. Physical and Spectral Data of β-Sitosterol Glucoside

M.P. : 287-289 ^{o}C.

IR ν_{max} (KBr) cm^{-1}: 3600-3400 (O-H), 1570 (C=C).

FAB-MS (-ve) *m/z*: 573, 413.

Peak Matching *m/z* (formula): 575.4315 ($C_{35}H_{59}O_6$).

^{1}H-NMR, ^{13}C-NMR ($CDCl_3$+CD_3OD, 500 MHz and $CDCl_3$+CD_3OD, 125 MHz) : **Table-3.13.**

4.6. ALLELOPATHY ASSAY OF *TEUCRIUM ROYLEANUM*

4.6.1. MATERIAL AND METHOD

4.6.1.1 Leaf Leachates Assay

Two assays namely Sandwich[172] and a newly established method of Watanabe lab as homogenated sandwich method were used for the preliminary screening of the *Teucrium royleanum.* Details of these two methods are as follows.

4.6.1.2. Sandwich Method[172, 136]

Agar Powder (Nacalai Tesque Kyoto, Japan) having gelling temperature of 30~ 31 ºC was used as for this methods and 0.75% (w/v) solution was prepared in distilled water. Two different weights (10mg and 50 mg) of plant leaf leachates were taken and placed in each well of the 6 – wells micro-plat [172, 136]. Then to each well 5 mL of 0.75% of agar solution was added. It was kept for 30-60 min at room temperature (ca. 25ºC) for solidification followed by another 5mL agar addition to each well. The plant material becomes embedded between two agar layers. The experiment was performed in triplicate. To ensure the re-use of microplate these were washed with 10% contamin for 20 minutes followed by keeping in sonicator for 20 minutes.

4.6.1.3. Homogenated Sandwich Method

Agar solution of 0.5% and 1% of was prepared and for 15 minutes it was autoclaved at 115 ºC. After that, the agar solution was shaked well and then cooled down to *ca*. 45ºC using a water bath. Like sandwich method two different weights 60 mg (10 mg/well) and 300 mg (60 mg/well) of the plant material was taken and fine grinded using liquid nitrogen. The grinded material was placed in 50 mL Falcon tubes and 20 mL of distilled water (DW) was added subsequently. This was centrifuged (Kobota 5220) at a speed of 3000 rpm for 20 minutes, resultant supernatant was transferred into a new Falcon Tube

while the residue was discarded. Further the supernatant was filtered using Syringe driven filter unit made of Millipore Corporation, Bedford USA having 0.45 µm (Millex-HV PVDF membrane) with the help of 50 mL disposable syringe and transferred in to a new falcon tube. Then to the filtrate 1% of equal volume of 20 mL agar solution was added, followed by the addition of 5 mL of this solution to each well of a 6-well multidish plastic plate (3.5 cm x 3.5 cm D x W x H) which made the same concentration of leaf leachates as sandwich method i.e. 10mg /well and 50mg /well. It was left for 30 minutes to make ensure the solidification of the solution. 0.5% agar solution was prepared in distilled water separately, 5 mL of which was then added to each well of multidish plastic plate. The solution was then left undisturbed to be solidifying.

Five Lettuce seeds (*Lactuca sativa* L. Great Lakes 366, Takii Seed Co. Ltd, Japan) were added to each well of the multidish plastic plate. Then were sealed with plastic tape, labeled and kept in an incubator (BIOTEC 300-L) (Shimadzu Rika Institute Co. Ltd, Kyoto, Japan) so that to grow the lettuce seeds for 72 h at 25 ºC under complete darkened conditions. The results are based on the inhibition and promotion of hypocotyl and roots of lettuce seedlings. The negative or lesser values represent promotion of the root or hypocotyl in comparison with controls. The roots and hypocotyls were measured and percentage of seed germination was also recorded. The experiment was repeated in triplicate.

4.6.1.4. Essential Oil Isolation

The essential oil isolation was carried out by means of hydrodistillation process for 3 h using a Clevenger-type apparatus (1.8% v/w).The essential oils extracted were obtained with diethyl ether, the later then evaporated to give essential oil which was dried with anhydrous magnesium sulphate.

4.6.1.5. Dish Pack Method[124]

Essential oil of *Teucrium royleanum* was taken in one well of 6-well multi dish plate and lettuce seeds on filter paper in rest of the five wells. 50 µl of essential oil was weighted and was added into 0.25 ml sample cup (11.0 x 13.5 x 16.3 mm). The cup was placed in the lower left well of the 6 well multi dish plate (0 mm distance well). In the rest of the five wells filter paper was placed. To each well 0.7 ml distilled water was added followed by placement of 7 seeds for germination. All the plates were later on sealed with plastic tapes and then incubation was carried out for 72 h in dark at 24 ºC. After the incubation (72 Hours) the lengths of hypocotyl and roots were recorded.

4.6.1.6. Total Activity[136]

The aerial crushed parts of the plant (1.5 Kg) were subjected to extract with 20 % Methanol for 15 days. Then it was suspended in water and was subjected to solvent-solvent partition to get fractions inorder of increasing polarity, hexane, chloroform, ethylacetate, butanol, and water sub-fractions. Total activity was measured with the help of following formula:

Total activity = $\frac{1}{EC_{50}} \times Concentration$

4.6.1.6.1. EC_{50} Value Calculation

1000 ppm (1mg/ml) of stock solution was prepared in 1% DMSO for each of the above sub-fractions. Working standards: 3, 10, 30, 100, and 300 ppm solutions of each sub-fraction were prepared. A glass petri dish (27 mm ø) was taken and a filter paper (27 mm ø, Type Roshi Kaisha, Ltd, Tokyo) was placed inside. The different working standards solutions were added to the filter paper in the petridish, followed the placement of seven lettuce seedlings (*Lactuca sativa* cv. Great Lakes 366) in on the filter paper, and

was incubated for 72 h at 20 ^{0}C in the dark. The inhibitory effects at each concentration of all sub-fractions were calculated by measuring the length of both roots and shoots and comparing it with the control.

4.6.1.7. Statistical Analysis

For the resultant data from the aforementioned experiments, means, standard deviation and standard error were calculated to determine the inhibition pattern of different concentrations. The extension rate is the actual rate of root and hypocotyl while inhibition rate shows the suppression of growth against control. The negative values present stimulation while higher inhibition rate shows restraint of lettuce seeds. One way ANOVA was applied to know the significance of concentration on the rate of inhibition of lettuce seeds **(Table-3.14).**

4.6.1.8. Allelopathy of Purified Compounds

A filter paper (27 mm Ø, Type 1, Toyo Roshi Kaisha, Ltd, Tokyo) was placed in a glass petri dish (27 mm Ø). Then the test solution (different concentrations of purified compounds) was added to the filter paper in the petri dish and dried completely in vacuo at 40 ºC. Followed by the addition of distilled water (0.7 ml), and six pre-germinated (16 h at 20 ºC in the dark) lettuce seeds (*Lactuca sativa* cv. Great Lakes 366) were also placed on the filter paper, and incubation was carried out for 48 h at 20 ºC in the dark. The inhibitory was measured for the root and hypocotyl elongation in germination and by comparison of the data obtained with that of untreated controls.

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PART B

PEROVSKIA ATRIPLICIFOLIA BENTH.

CHAPTER 5

THE CHEMISTRY OF THE ESSENTIAL OIL OF *PEROVSKIA ATRIPLACIFOLIA* BENTH

5.1. INTRODUCTION

5.1.1. GENUS *PEROVSKIA*

Perovskia is a genus belonging to the family lamiaceae, comprising of seven species. *Perovskia atriplicifolia* Benth. grows wild in the rocky places in Central Asia, Pakistan, Iran, and Afghanistan[1]. Due to the sweet flavour of the flower it can be used as salad and as garnish. The plant is also use in farming decorative hedge of a moderate height[2]. Due to its sweet flavor the flowers are favorite of honey bees, the honey obtained after the fed is colorless and has a typical aroma and flavor. This specie can be used as cooloing medicine and also exhibit antibacterial assay.[3] The crude methanolic extract of the plant showed positive result for cytotoxicity via brine shrimp lethality test[4]. A new secondary metabolite Perovskoate, an isorinic acid derivative exhibited significant enzyme inhibition activity against lipoxygenase enzyme and against cholinesterase it shows weak to moderate activity[5].

The cultivated *P. atriplicifolia* results in the identification of 1, 8-cineole + limonene, ά-pinene, β-pinene, camphene, and camphor with 40.13%, 9.13%, 6.59%, 6.17%, and 5.36% respectively[6]. The *P. atriplicifolia* cultivated in an experimental plot at the Botanical Garden (University of Turin) gave camphor as a major constituent (14.9%) [7, 8]. Another reported the chemical constituents of the oil at three stages of growth in Iran[9]. Nineteen chemical components have been identified from the plant *P .atriplicifolia* wild

growing in Pakistan by the GC-MS analysis[10]. 1, 8-cineol limonene, camphor, β-caryophyllene, γ-cadinene, α-pinene, and α-terpinyl acetate have been identified as the major chemical constituents of the supercritical fluid extraction (SFE) of the aerial parts of the *P. atriplicifolia*[2].

Previous reports of the supercritical fluid extraction (SFE) of the aerial parts of *P. atriplicifolia* showed major components α -pinene, camphor, 1,8-cineol limonene, β -caryophyllene, α -terpenyl acetate and γ -cadinene[2]. The same oil extracted by steam distillation have 1, 8-cineol camphene, and ά-humulene as the major chemical constituent[2]. Keeping in view the difference in the chemical composition of the *P. atriplicifolia* essential oil based on geographical location, growth stage and method of extraction, the present work was carried out with the aims to determine the percentage of the oil contents, constituents as well as their composition and to evaluate antimicrobial, mutagenic and antimutagenic activities of the essential oil obtained through hydrodistillation techniques[11].

Majority of the plants produce antibacterial and antifungal compounds either in response to the attack of a pathogen or stress. The antimicrobial compounds can also be produced by plants for the normal nourishment and growth. The essential oil can be utilized for the reduction of proliferation of the microorganism in a novel way. The essential oils can be used as natural additives in many food stuffs because of its antimicrobial, antimutagenic and antioxidant properties [12--18]. Depending upon the active constituents, testing methods, and concentrations the essential oils exhibit inhibitory effects against microbes having capability of causing food spoilage.[15]

CHAPTER 6

RESULTS AND DISCUSSION

The air dried aerial parts of *Perovskia atriplicifolia* were subjected to hydrodistillation using a clevenger type distillation apparatus. The extracted oil percentage yield was 3.2% (v/w). A total number of 18 chemical contents were identified with the help of Gas Chromatography Mass Spectrometry (GC-MS) analysis (**Table 6.1).** The 18-chemical components were group as 7-non-oxygented monoterpenoid (29.22%), 5-sesquiterpenes one of which was in oxygenated form (30.57%), 3-oxygenated monoterpenoid (3.84%), 2-esters (3.52%) and a ketonic monoterpene (28.91%). The camphor was the major chemical component (28.90%). Beside it the other prominent chemicals were limonene, α-globulol, trans-caryophyllene, and α-humulene with 16.72%, 10.21%, 9.30%, 9.25% respectively. The other minor constituents of the essential oil were linalool (0.30%), γ-terpinene (0.33%), β-pinene (0.57%), (-)-caryophyllene oxide (0.74%), T-cadinol (1.07%), terpenyl acetate (1.45%), bornyl acetate (2.07%), camphene (3.76%), ά-pinene (3.84%), and (E)-b-ocimene (4.00%) while trace amount of β-myrcene and 1,8-cineole were also identified. The comparison of the secondary metabolites identified from the essential oils of *Perovskia atriplicifolia* and *P. abrotanoides* exhibit variations.[19] The literature reports on the *Perovskia atriplicifolia* showed that even the chemical constituents can be varied with in the same plant collected from different localities and thus can be concluded as it may depend on the genetic features and the conditions under which the plant is grown[15]. The change of the geographic sources and the harvesting season also results in the variation of the chemical analytes of the essential oils for the

same specie.[12]

Table 6.1: Chemical constituents of *Perovskia atriplicifolia* Benth.

No.	Compounds	RT	Percentage
1	α -Pinene	6.11	3.84
2	Camphene	6.52	3.76
3	β -Pinene	7.21	0.57
4	β -Myrcene	7.56	T
5	(E)- β -Cimene	8.02	4.00
6	Limonene	8.57	16.72
7	1,8-Cineole	8.64	T
8	γ -Terpinene	9.30	0.33
9	Linalool	10.40	0.30
10	Camphor	11.59	28.91
11	Borneol	12.22	3.54
12	Bornyl acetate	14.88	2.07
13	Terpynil acetate	16.29	1.45
14	*Trans*-Caryophyllene	17.90	9.30
15	α -Humulene	18.67	9.25
16	(-)-Caryophyllene oxide	21.26	0.74
17	α -Globulol	21.51	10.21
18	T-Cadinol	22.43	1.07

Table-6.2: The chemical composition of the essential oil of *P. atriplicifolia* by different authors.

S. No.	Constituents	*Pourmortazavi et al., 2003	**Dabiri et al., 2001	Jassbi et al., 1999	Present studies
1	α-Pinene	7.3	7.77	5.9	3.84
2	Camphene	2.9	3.41	3.0	3.76
3	β-Pinene	2.7	2.93	2.9	0.57
4	Myrcene	0.7	0.68	1.1	0.3
5	δ-3-Carene	5.4	6.04	-	4.4
6	α-Terpinene	0.2	0.23	-	
7	1,8-Cineole	29	20.74	27.5	T
8	Limonene		8.58	-	16.72
9	(*E*)- β-Ocimene	0.2		-	
10	γ-Terpinene	0.3	0.29	0.6	
11	(*E*)-sabinene hydrate	0.3	0.31	-	
12	Terpinolene	0.6	0.51	1.0	
13	α-Thujone			-	
14	Camphor	14.8	14.52	2.6	28.91
15	Borneol	2.2	1.57	4.6	3.54
16	δ-Terpineol			-	
17	Linalyl acetate	0.4	0.46	-	
18	Bornyl acetate	3.0	2.2	1.0	2.07
19	α-Terpinenyl acetate	3.1	3.45	1.4	
20	α-Copaene	0.4	0.34	-	

21	α -Gurjanene	0.4		-	
22	β -Caryophyllene	8.7	7.91	0.8	
23	α -Humulene	6.7	6.28	5.7	9.25
24	Valencene			-	
25	γ -Cadinene	2.0	1.65	-	
26	Calamenene		0.20	-	
27	δ -Cadinene	1.6	1.21	-	
28	Caryophyllene oxide	0.8	1.32	0.9	
29	Globulol	0.3	0.69	-	
30	Cubenol	0.6	0.47	-	
31	T-Cadinol	3.5	2.14	-	1.07
32	γ -Eudesmol	0.4		-	
33	α -Eudesmol			-	
35	Tricylene		0.13	-	
36	α -Thujene		0.12	-	
37	Δ^3-Carene			22.3	
38	*p*-Cymene		0.41	0.6	
39	4-Terpeneol			0.8	
40	α -Terpeneol		0.27	1.5	
41	Guaiol			2.3	
42	β -Myrcene			-	T
43	(E)- β -Cimene			-	4.00
44	δ -Terpenene			-	0.33
45	Linalool			-	0.30

46	Terpynil acetate			-	1.45
47	*Trans*-caryophyllene			--	9.30
48	(-)-Caryophyllene oxide			-	0.74
49	α-Globulol			-	10.21
50	Tricylene		0.13		
51	(-Z)- β-Ocimene		0.26		
52	Sabinene				
53	α-Phellandrene				
54	Terpinen-4-ol		0.30		
55	Thymol				
56	α-Cubebene				
57	β-Bourbonene		Tr		
58	α-Gurjunene		0.78		
59	γ-Muurolene		0.26		
60	*allo*-Aromadendrene				
61	Germacrene D				
62	α-Muurolene				
63	α-Cadinene				
64	Hydroxygermacrene				
65	T-Muurolol		0.44		
66	Z, E- -Farnesol		0.25		
67	E, E-Farnesol		0.33		

* Only % of essential oil Obtained by Steam Distillation (SD) is taken in consideration for the comparison

** Only % of essential oil extracted from the *P. atriplicifolia* collected at complete flowering stage is taken in consideration for the comparison

6.1 ANTIBACTERIAL AND ANTIFUNGAL ASSAYS

The essential oil of *Perovskia atriplicifolia* was subjected to the *in vitro* antibacterial and antifungal assays with the employed micro-organism. The potential activities of the essential oil were measured both qualitatively and quantitatively, the criteria being kept as presence or the absence of the zone of inhibition, zone diameter and the minimum inhibition concentration values. In accordance to the prescribed results **(Tables 6.3, 6.4 and 6.5)**, the oils of the species under investigation had a significant activity against the tested ten strains of bacteria and five strains of fungi. The disk diffusion method was employed for the measurement of the antimicrobial assays, results showed variable activities against the tested strains for both antibacterial and antifungal assays. The results showed that the Gram-positive bacterial strains *Bacillus subtilis* (14mm), *Bacillus mycoides* (10 mm), and *Bacillus cereus* (10 mm) were more sensitive as it gave the strongest inhibition zones. *P. atriplicifolia* essential oil possessed high antibacterial assays against *Micrococcus luteus*. Moderate assays were obtained verses the *Staphylococcus aureus* (food-born pathogen) and the other gram-positive bacterial strains *Staphylococcus epidermidis* and *Streptococcus faecium* with eight to nine mm inhibitory zones. The comparison of the gram positive bacteria and gram negative shows that the later were less susceptible and hence showed variable degrees of susceptibilities verses the tested essential oil. Against *Yersinia enterocolitica* and *Enterobacter aerogenes* modest activity was observed. The test oil showed no activities against the Gram-negative bacterial strains namely *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The *P. aeruginosa* has a very resistive outer membrane cover thus possessed a high level of intrinsic resistance to virtually all so for known antibacterial, antifungal as

well antibiotics and even to the synthetic drugs.

The essential oil of *P. atriplicifolia* gave the highest activity against the fungal strain *Candida albicans* having fourteen mm inhibiting zone, while the ten mm for *Aspergillus flavus*, *Geotricum candidum*, and eight mm for *Aspergillus niger* and *Aspergillus fumigatus.*

For most of the organism the MIC values ranges from10 µL/mL to160 µL/mL as is shown in (**Table 6.5)**. The minimum inhibitory concentration values calculated for the strains *Streptococcus faecium*, *Bacillus cereus, Staphylococcus epidermidis* were 20 µL/mL and verses *Bacillus subtilis, Yersinia enterocolitica*, *Bacillus mycoides,* and *Micrococcus luteus* MIC were 10 µL/mL. No effects were observed by essential oil on the *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* up to 640 µL/mL concentration. The test oil had showed strong activity against *Aspergillus flavus* (80 µL/mL), *Candida albicans* (80 µL/mL) and *Aspergillus fumigatus* (40 µL/mL). The most significant feature of the essential oil is its hydrophobicity property, due to this property the oil got the ability to be partitioned between the cell membrane and bacterial mitochondria as a result structure is disturb to great extent. Certainly some quantitative leakage from the cells of bacteria may be tolerated with out losing viability, the exit of critical molecule or the extra ordinary loss of cell content and ions will results in death.[12] The constituents of the essential oil like α-pinene, 1, 8-cineole, β-pinene, (E) - β-ocimene can have contribution towards those activities. One can not neglect the contribution of the enantiomers of the constituents of the oil which exhibited activities to different extents. Enantiomers of limonene, α and β-pinene has exhibit strong antibacterial activity[20].

The α-pinene and β-pinene also have the ability to destroy cellular integrity and hence offer hurdles in respiration and ion transport mechanisms[21, 22, 12] These terpenoids are good source to enhance the yeast's cells and mitochondria membrane permeability.[23, 21] The effects exerted by various chemical constituents of the essential oil on the Gram-negative bacterial outer membrane permeability are cleared by from these studies[25].

The mode of action of the other components of the essential oil such as ά-humulene, bornyl acetate, camphene, trans-caryophyllene, and limonene are not been fully illustrated[26]. The susceptibility of the Gram-positive bacteria is more if compared with the Gram-negative bacterial strains[12]. According to the previous work *Escherichia coli* was found to be least susceptible. Prove of the presence of outer membrane of the Gram-negative bacterial strain were their weak antimicrobial assays. The walls contains hydrophilic polysaccharide chains which serve as a barrier to hydrophobic nature of the oil. In accordance, a high degree of susceptibility was not expected for the two strains namely, *K. pneumonia* and *P. aeruginosa*.

Table 6.3: Antibacterial assays of the *P. atriplicifolia* essential oil

Zone of inhibition (mm)				
Bacterial strains	**Essential oil (15 µL/disk)**	**Penicillin (10U /disk)**	**Tetracycline (30µg/disk)**	**Cephotaxime (30µg/disk)**
B. cereus NRRL B-3711	10	30	32	8
B. mycoides NRRL B-4379	10	11	33	10
B. subtilis NRRL B-209	14	31	36	26
M. luteus NRRL B-1018	12	46	40	46
S. aureus ATCC 25923	8	–	30	–

S. epidermidis NRRL B-4268	9	18	28	26
S. faecium NRRL B-3502	9	22	–	9
E. coli ATCC 25922	7	–	30	32
E. aerogenes NRRL B-3567	8	–	30	35
P. aeruginosa ATCC 10145	–	–	16	–
Y. enterocolitica	9	13	26	13
K. pneumoniae	–	–	25	32

Table 6.4: Antifungal assays of the *P. atriplicifolia* essential oil.

Fungal strains	**Essential oil (15 µL/disk)**	**Amphotericin B (10 µg/disk)**
C. albicans NRRL Y-12983	14	13
A. niger ATCC 10549	8	9
A. fumigatus NRRL 163	9	–
A. flavus NRRL 1957	10	8
A. parasiticus NRRL 465	–	7
G. candidum	10	8

–, No zone of inhibition.

Table 6.5. Minimum inhibitory concentration (μ L/mL) for the essential oil against bacterial and fungal strains.

Organisms	**MIC (μL/mL)**
Bacterial strains	
B. cereus NRRL B-3711	20
B. mycoides NRRL B-4379	10
B. subtilis NRRL B-209	10
M. luteus NRRL B-1018	10
S. aureus ATCC 25923	160
S. epidermidis NRRL B-4268	20
S. faecium NRRL B-3502	20
E. coli ATCC 25922	640
E. aerogenes NRRL B-3567	640
Y. enterocolitica	10
Fungal strains	
C. albicans NRRL Y-12983	80
S. cerevisiae	160
A. niger ATCC 10549	160
A. fumigatus NRRL 163	40
A. flavus NRRL 1957	80
A. parasiticus NRRL 465	640
G. candidum	320

6.2. MUTAGENIC AND ANTIMUTAGENIC ASSAYS

The essential oil of *P. atriplicifolia* was subjected to mutagenic and antimutagenic assays by make use of the *Salmonella* microsome test using *Salmonella typhimurium* TA98 and *S. typhimurium* TA100. **Table 6.6.** representing the results. The essential oil had no mutagenic effect on the strains ($p < 0.05$) on all concentrations. The antimutagenicity results are presented in the **Figure 6.1**.

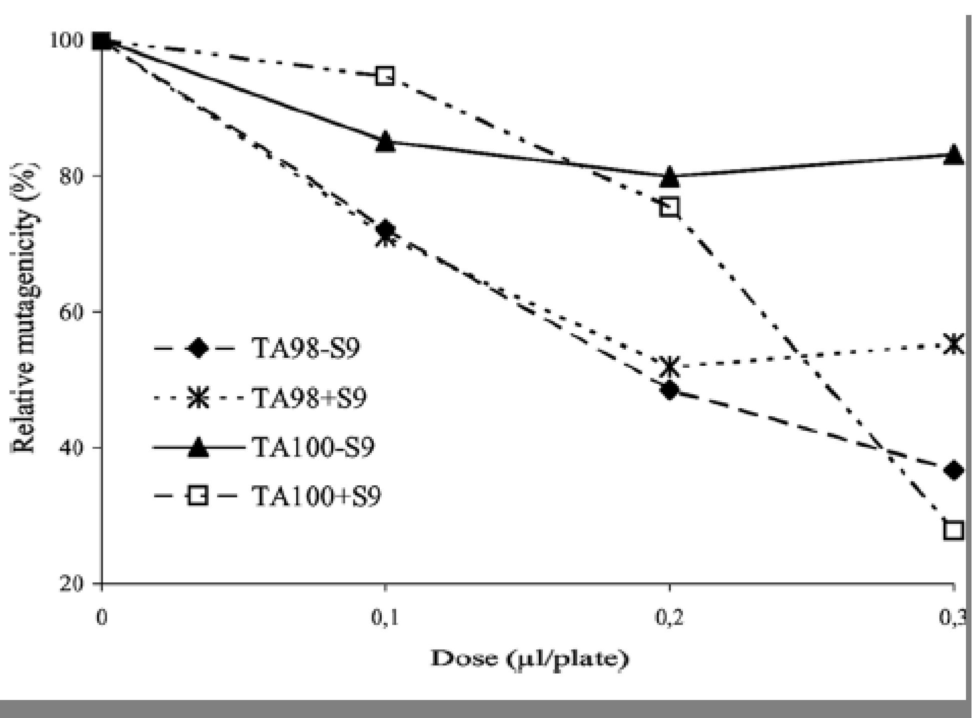

Fig. 6.1: Inhibitory effect of *Perovskia atriplicifolia* essential oil against the mutagenicity of daunomycin (6µg/ Plate), sodium azide (1.5µg/ plate), 2-aminofluorene (10 µg/plate) to S. typhimurium TA98 and TA100 with and without S9 fraction.

The Antimutagenic activity calculated for the essential oil of *P. atriplicifolia* was 72% in the TA100 strain when tested against 2- AF in the presence of S9 fraction. Mutagenic inhibitory effect was up to 63.3% verses daunomycin for TA98 with out S9 fraction and

for the strain TA98 having S9 fraction is about 44.7% when tested with 2-AF ($p < 0.05$). The camphor and limonene were the major chemical constituents of the *P .atriplicifolia* essential oil.[27] the same studies showed that camphor exhibit no mutagenic activity. Making use of the same test another group reported that there is mutagenicity activity exerted even when it was treated with the 4-methylbenzylidene camphor[28]. Similarly, analyzed essential oil showed that the limonene marked the 2nd highest concentration and exhibited no mutagenic activity[29, 30]**.** Moreover, some reported that the d-limonene exerts high antimutagenicity [31, 32]. The oil also contain the (-)-caryophyllene oxide and 1, 8-Cineol but there amount were very less. The same assay had showed no mutagenic activity for this chemical constituents.[27, 34] Moreover some studies[35] showed that the caryophyllene oxide reduce the mutagenicity of aflotoxin B-1 for *S. typhimurium* TA98 to 89% and for *S. typhimurium* TA100 to 71%. There is no literature available on the biological activities of the plant under our consideration namely *P. atriplicifolia*. Therefore, our studies on the essential oil analysis along with the antibacterial, antifungal, mutagenic and antimutagenic assays as the first report on the species of *P. atriplicifolia* Benth. The over all results of our studies on the essential oil of the specie under consideration suggests it to be a good source of antimutagenic and antimicrobial agents thus can be proved as a good candidate to be utilized in the cosmetics, food and pharmaceutical industries. Inorder to have in hand some more practical utilization of the essential oil of the *Perovskia atriplicifolia* more research will be required towards that aspect.

Table 6.6: The Antimutagenic and Mutagenicity assays in incorporated *Salmonella* plate test making use of TA98 and TA100 in the presence and absence of S9 fraction

Dose level (μL/plate)	**TA98**	**TA100**
Absence of S9 fraction		
Revertant	26.7 ± 5.1	170.3 ± 22.8
0.1	30.3 ± 5.8	141.3 ± 29.7
0.2	23.8 ± 3.7	108.2 ± 12.1
0.3	17.2 ± 2.8	106.5 ± 17.7
Daunomycin	233.0 ± 46.9	
NaN_3		606.2 ± 116.0
0.1+Dau	167.8 ± 33.2	–
0.2+Dau	112.8 ± 29.1*	–
0.3+Dau	85.5 ± 25.3*	–
Daunomycin	233.0 ± 46.9	–
0.1+NaN_3	–	515.5 ± 77.7
0.2+NaN_3	–	484.2 ± 119.9
0.3+NaN_3	–	504.2 ± 153.5**
NaN_3	–	606.2 ± 116.6**
Presence of S9 fraction		
Revertant	61.6 ± 11.0	177.4 ± 27.8
0.1	55.2 ± 8.8	126.1 ± 8.3
0.2	49.9 ± 5.7	120.1 ± 19.8
0.3	49.5 ± 9.6	104.8 ± 20.0

0.1+2-AF	1097.3 ± 101.0*	628.6 ± 148.3
0.2+2-AF	801.2 ± 98.2*	500.2 ± 100.4
0.3+2-AF	853.7 ± 104.2*	184.2 ± 51.5*
2-Aminofluorene	1544.8 ± 365.5	664.0 ± 122.9

Mean ± standard deviation of six plates without (-S9), and of ten plates with (+S9). Daunomycin (6µg / plate) for TA98-S9 and sodium azide (1.5 µg/plate) positive control for TA100-S9; 2-aminofluorene (10 µg/plate) positive control for both TA98 and TA100+S9.

*Statistically different groups from their positive control groups (p<0.05) for Games-Howell test.

**Statistically different groups from their positive control groups (p<0.05) for LSD test.

CHAPTER 7

EXPERIMENTAL

7.1. MATERIALS AND METHODS

7.1.1. PLANT MATERIAL

The air dried aerial parts of the plant were collected from the locality Tira of Khyber Agency, North-West Frontier Province (N.W.F.P), Pakistan at its flowering season in June. Dr. Habib Ahmad (Director of WWF, Peshawar, Pakistan) had identified the plant and a specimen with voucher number (no. LI-002-JZC) was deposited in the herbarium of the Department of Botany, Post Graduate College, Swat, The had identified the plant and voucher specimen was submitted in the Herbarium of Department of Botany, Jahan Zeb Postgraduate College, Swat, North West Frontier Province, Pakistan.

7.1.2. ESSENTIAL OIL EXTRACTION

The plant was dried in a shade at 25^0C and then subjected to grinding. The same grinded material was then subjected to hydrodistillation using Clevenger-type distillation assembly. Anhydrous sodium sulfate was used for the removal of some trace of water droplets followed by filtration through cotton. The moisture free oil was then stored at 4^0C till the analysis.

7.1.3. ANALYSIS OF THE ESSENTIAL OIL

The essential oil was subjected to the analysis process was done by Gas Chromatography Mass Spectrometry utilizing Gas Chromatograph (Perkin Elmer-Auto-system XL) and Mass Spectrometry (Perkin Elmer Turbo). PE-5ms (5% phenyl–95%methylpolysiloxane) column (20x 0.18mm Ø with 0.18-mm film thickness) was used with helium at 0.5mL/minute as the carrier gas; GC oven temperature was kept at 45^oC for 2 min and

programmed to 240 ^{o}C at a rate of 6 ^{o}C /min and kept constant at 240 ^{o}C for 5 min. The split ration was 1: 100, and the volume injected was 0.1 mL in amount. The EI-MS (Electron impact mass spectrometry) was obtained at 70eV ionization energy. 35 to 350 a.m.u. were the molecular mass ranges. Inorder to compare the analyzed constituents of the plants help was taken from the Wiley's GC-MS and NIST Library. The computerized integrator was used for the calculation of the relative percentage of separated compounds.

7.2. ANTIMICROBIAL ACTIVITY TESTS

7.2.1. MATERIAL

All the material for the present study was kindly provided by the Agriculture Research Service Culture Collection (NRRL), Peoria, Illinois, USA, and by the Department of Biology at Anadolu University, Eskisehir, Turkey. The tested bacterial strains were *Bacillus subtilis*, *Bacillus cereus*, *Bacillus cerus* subsp. *mycoides*, *Staphylococcus aureus*, *Streptococcus faecium*, *Micrococcus luteus*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Yersinia enterocolitica* and *Klebsiella pneumoniae* (isolated clinically) and five fungal strains namely *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus parasiticus*, and *Geotricum candidum (wild type)* were used in the study. Both the antibacterial and antifungal strains were kept on the agar nutrient make Merck, 1.05450, Schuchardt, OHG Germany and the agar malt extract make Merck, 1.05398 slants kept temperature around 4°C, respectively. The petri dishes were used for the sub culturing just before the use. The experiments were performed in triplicate.

7.2.2. METHOD (AGAR DIFFUSION)

The antimicrobial activities were performed by agar diffusion method. The bacterial strains (Gram-positive) such as *Bacillus subtilis, Micrococcus luteus, Bacillus cereus,*

Bacillus cerus subsp. *mycoides, Staphylococcus aureus, Staphylococcus epidermidis, and Streptococcus faecium.* Where as the Gram-negative bacterial strains were *Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella pneumoniae* and *Yersinia enterocolitica* were used for antibacterial activities make use of Mueller-Hinton agar with specification (MHA; Fluka, 70191, Steinheim, Germany).

The antifungal tested strains used in our experiments were *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus flavus*, and *Geotricum candidum* and the two yeast in the form of *C. albicans* and *S. cerevisiae* were also utilized made of Sabouraud glucose medium (4%).

The standards used in our study for antibacterial were tetracycline (Sigma, 20K1279, Steinheim, Germany), Penicillin G (Sigma, 111H0079), and Cephotaxime (Fluka, 22128). After autoclaving SGM and MHA, were added to the petri dishes obtain a uniform dept of approximately 4mm and were cooled to the room temp. The tubes containing four to five mL of Mueller-Hinton broth (MHB) were mixed with the test bacterial strains. Then it was incubated till visible turbidity keeping the temperature in the range of 35–37°C. The density of the tested bacterial cultured medium was accustomed with sterile saline to that of the 0.5 to the standard used (McFarland standards) keeping the λmax 625nm, while the absorbance rages from 0.08–0.1. the cultured medium of bacterial strains was adjusted to that standard have around 108 CFU/mL. Inorder to induce the spore formation these were growing on the potato dextrose agar slants at a fixed temperature at 27 ^{o}C for around five to seven days. The sterile 0.1% of Tween 80 was used for the adjustment of spore concentration at about 10^6 CFU/mL for each and every mold. The sterile saline was used for the adjustment of the yeast culture to that of the 0.5 McFarland which was then adjusted to 10^7 CFU/mL via dilution.

A streak of sterile swab was used for the inoculation of the inner surface of the MHA and SGM plates. An approximately a ø 6 mm (Schleicher & Schuell) of the paper disks impinged with15 μL of the P. atriplicifolia essential oil which were kept on surface of the agar plate. The pre-incubation of the plates were carried out at 4°C for 1 hour and incubated at 27 ^{o}C for fungi and at 4°C for bacterial cultures. Then these were incubated for 3 days for fungi and 18 hours for bacteria, all the plates were then examined and the diameters of the zones of complete inhibition were calculated, as well as with the diameter of the disk.[42]

7.2.3. MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) of the essential oil was determined by the agar-dilution method. The procedure was the addition of the essential into the agar medium in a way that each of the plate contains different concentrations from 10-640 μL/mL of the agent. Control for the experiment was prepared with out the addition of the essential oil. A concentrated bacterial culture (10^{8} CFU/mL) was subjected to dilution (1:10) to obtained an inoculums concentration (10^{7} CFU/mL). Later 2 mL was inoculated on an agar surface area of 5 to 8mm with unvarying loops. When the spots on the plates had dried up, these were inverted and subjected for incubation at 35°C temperature.[41, 43] Yeast and molds suspension of 10^{7}CFU/mL density were inoculated on agar surface in a similar way. MIC was measured as the minimum concentration of antibacterial and antifungal agents which totally cause growth inhibition.

7.3. MUTAGENICITY AND ANTIMUTAGENICITY TESTS

7.3.1. MATERIAL

The micro-organisms *Salmonella typhimurium* TA98 and TA100 were compassionately made available by Dr. Bruce Ames of the University of California, Berkeley, CA, USA.

7.3.2. METHOD

7.3.2.1. S9 Fraction Preparation

The liver S9 fraction was prepared from the Sprague-Dawley male rats. The test animals were cared properly keeping in view the principles and policies set by the Animal Welfare Act and the NIH Guide for Care and make use of Laboratory Animals (manuscript no. 86 to 23). For the introduction of rat liver enzymes phenobarbital and 3-methylcholanthrene were employed. The corn oil was used for the dilution of 3-methylcholanthrene (125 mg/kg body weight) and just 5 days before the sacrifice of the rat it was injected intraperitoneally to each one. Beside this phenobarbital was added to the drinking water (0.1%g/L) and monitored for five days before the sacrifice of the rat. The temperature maintained for the experiment was 21–23°C and during the whole experimental time the animals were kept in rooms illuminated from 7 to 19 h (12-hour dark cycle /12-hour light) cycles. Also the test animals were allowed to have full access to pellet food and water libitum.

The S9 fractions were prepared by the described method of Garner *et al*.[36] in this test 3-methylcholanthrene and Phenobarbital were utilized as inducers for S9 fraction. As a result, the cytochromes, cyt P-450 and cyt P-448 were activated with help of 3-methylcholanthrene and phenobarbital.[37] The protein proportion of S9 fraction was establish to be 12mg/mL. In pilot tests, the S9 fraction was treated on *S. typhimurium*

with 2-aminofluorene and in the presence of fraction S9 it is a positive mutagen. The number of revertant colonies give in with 2-aminofluorene was twenty five to thirty times more than that of the standard control grouping, and thus, the protein proportion of S9 was enough for the metabolic activation system. In view of that, the mutagenic activity experiment was performed using 12 mg/mL protein content with S9 fraction.

7.4. CYTOTOXIC DOSE MEASUREMENTS OF ESSENTIAL OIL

The utilized quantity of the oil in the mutation assays were chosen for the cytotoxicity assays. The underlying principle beyond this experiment was to know that whether the materials test doses would have any cytotoxicity. To the 2 mL of top agar, 0.1 mL of a suitable dilution of an overnight bacterial culture was added followed by the addition of different conc. of of the tested chemicals. The top agar was poured onto nutrient agar plates, and assessment of cytotoxicity was performed after overnight incubation at 37 ^{o}C [35].

7.5. MUTAGENIC ACTIVITIES

The method of Maron and Ames was used for the determination of mutagenic assays using *Salmonella*.[36] The non-cytotoxic doses of the test were utilized in our experiments. Dimethyl Sulfoxide was used as solvent in our experiment which is well-matched with the Ames test.[36] Overnight culture was performed with the oxoid nutrient broth no. 2. In the plate incorporation activities, 0.1mL of bacterial strain, 0.5mL of S9 fraction mix if suitable and to 2mL of the molten top agar the tested sample was added. All the proportions were then mix up and added on to the agar plates. Then it was incubated for 72 h, and after that the revertant colonies were measured by making use of the suggested method of Claxton *et al*. [38] With S9 fraction 6 plates were used for each dose and ten plates was used for each dose with out S9 fraction. Without S9 fraction, for TA98

daunomycin and for TA100 sodium azide were utilized as positive mutagens. With S9 fraction, 2-aminofluorene was used as positive mutagen for both the strains. All the strains were later examined regularly for resistance against ampicillin, sensitivity against UV-light, histidine requisite, crystal violet sensitivity and a spontaneous reversion rate. The storage of the material was done at a fixed temperature of 80 ^{o}C.

7.6. ANTIMUTAGENIC TEST

In the antimutagenic test 0.1 mL of the test sample was combined with the 0.1 mL of bacterial culture of the tester strain, and 0.1 mL of mutagen in agar and then was poured on to minimal agar plate. Then subjected to the incubation processes at 37°C for 48-72 hours, the inhibitory effects were then determined by counting the number of revertant colonies, and were expressed as the rate of inhibition. The % rate of inhibition was measured by the formula: rate of inhibition (%) = [(A -B) /A] x100. In the equation A represent the positive revertant colonies where as B represents the revertant colonies after the addition of test samples[42]. The antimutagenicity is clear by the positive rate of the tested substances. In the antimutagenic test for the TA100 and TA98 strains 2-aminofluorene was used as a positive mutagen for with S9 fraction and for with out S9 fraction, sodium azide was used as positive mutagen for TA 100 and for TA98 daunomycin were used as positive mutagen.

7.7. STATISTICAL ANALYSIS

The data was analyzed with the SOSS 11.00 software. Games-Howell test was utilized for to deposit the meaning full levels at $p < 0.05$ for the resolution of the groups gives clear differences consequence to antimutagenicity[40]. The same test was used with the 95% confidence level for to check the difference between the revertant colonies of the test group and the standard control groups. The mutagenic conditions caused by the dose

higher than the mean of the control group was defined ‘‘mutagenic’’ in contrast, an increase in dose reaching to, but not approaching a twice increase was elaborated as ‘‘weak mutagenic’’[38].

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