Phytochemical Investigation of *Salvia sclarea*

**Abstract**

*Salvia sclarea* (Lamiaceae) commonly known as clarysage, is a medicinally important herb showing broad range of biological activities. Phytochemical investigation of the aerial parts of this plant yielded nine compounds i.e. β-sitosterol, α-amyrin, ursolic acid, oleanolic acid, betulinic acid, sclareol, salvigenin, acacetin and norartocarpetin. The structures of these compounds have been established by spectroscopic methods (UV, IR, \(^1\)H NMR, \(^{13}\)C NMR and MS) in the light of literature. Ursolic acid, oleanolic acid, betulinic acid, salvigenin, acacetin and norartocarpetin are reported for the first time from this plant species.

**Keywords**: *Salvia sclarea*, triterpenes, flavones, sclareol, salvigenin, NMR.
3a.1. Introduction

Lamiaceae (formerly Labiatae) is commonly known as mint family and most of its species are aromatic and often used as spices, folk medicines and a source of fragrance.\(^1\) *Salvia* (commonly known as sage), the largest genus of the family Lamiaceae, represents an enormous and cosmopolitan assemblage of nearly 1000 species displaying a remarkable range of variation. The genus comprises 500 species in Central and South America, 250 species in Central Asia/Mediterranean and 90 species in Eastern Asia.\(^2\) Because they readily cross-pollinate, innumerable hybrids, both natural and manmade, are also found. India is a major diversity centre for most members of Lamiaceae in Asia.\(^3\) The name of the genus, *Salvia*, is derived from the Latin *salvere*, meaning ‘to save’ which sums up the folkloric belief of its ‘magical’ therapeutic properties for many kinds of ailments and its popularity in traditional medicine.\(^4\) Sage has been an important medicinal plant since earliest times. This herb has the reputation as one which wards off evil. It was thought to be efficacious against the biting of serpents and the dispelling of evil spirits.\(^5\) It has been employed in ancient Egypt to increase the fertility of women.\(^6\) It appears that sage was brought from ancient Egypt to our shores by the Romans.\(^7\) *Salvia* is a fascinating plant genus, it features prominently in the pharmacopoeias of many countries throughout the world. *Salvia* species have been used in many ways, e.g. essential oils used in perfumery, the flowers used as rouge, the leaves used for varicose veins, the seed oil as an emollient, the roots as a tranquiliser. The range of traditional applications of the herb in domestic medicine seems to be endless: it has been used as a medication against perspiration and fever; as a carminative; a spasmolytic; an antiseptic/bactericidal; an astringent; as a gargle or mouthwash against the inflammation of the mouth, tongue and throat; a wound-healing agent; in skin and hair care; and against rheumatism and sexual debility in treating mental and nervous conditions as well as an

insecticidal.\textsuperscript{8} Some species of \textit{Salvia} have been cultivated worldwide for use in folk medicine, in flavour and fragrance industry and for culinary purposes.\textsuperscript{9}

\section*{3a.2. Review of literature}

\subsection*{3a.2.1. Ethnopharmacology}

Fascinating plant folklore and ethnopharmacology leads to medicinal potential. \textit{Salvia} species are important group of useful plants which have not lost their medicinal importance since ancient times. An Anglo-Saxon manuscript reads “Why should a Man die, if sage grows in his garden?”.\textsuperscript{8} This reflects the medicinal importance of \textit{Salvia} species since the existence of ethnopharmacology. \textit{Salvia}, commonly known as sage, has multiple uses such as condiment, food additive, spice and herbal tea.\textsuperscript{10} The seeds of \textit{Salvia} species often produce mucilage on wetting which is used to produce pleasant drinks and in the treatment of eye diseases.\textsuperscript{11} In addition to this, \textit{Salvia} species have been used for memory-enhancing purposes in European folk medicine.\textsuperscript{12}

\textit{Salvia officinalis} is reported in the Pharmacopoeias of Austria, Czechoslovakia, Germany, Hungary, Jugoslavia, Netherlands, Poland, Portugal, Roumania, Russia and Switzerland for the treatment of a host of diseases.\textsuperscript{8} Herbalists in Jordan, Syria and Lebanon consider \textit{Salvia} as a ‘panacea’ i.e. a universal drug.\textsuperscript{13} Traditionally, this plant has been used as anti-inflammatory in small doses and in the treatment of fevers by depressing fever control centre in the brain, as well as by relieving spasm in the smooth skeletal muscles.\textsuperscript{14} \textit{Salvia officinalis} has value as a carminative, spasmylytic, antiseptic, astringent, and is used in a variety of complaints, the most relevant being inflammation of the mouth, tongue and throat.\textsuperscript{15} It has been cited for use in bad breath and sore throat and is even said to be able to regulate the flow of saliva. Its leaves have been used to treat laryngitis in China.\textsuperscript{16} It has some value in relieving indigestion with gas or spasmodic pain.\textsuperscript{17} It has also been used against sexual debility, as skin care in the form of face packs and creams, as a wound healer in the form of lotion or compress, in the treatment of

\begin{thebibliography}
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painful rheumatic joints, as a hair tonic and is also used in nervous conditions, trembling, depression, and vertigo.\textsuperscript{8}

In South America, \textit{S. coccinea} is used to bathe varicosities, blood clots and congested blood.\textsuperscript{18} \textit{S. columbariae}, an annual herb, was an important food plant for the Native American tribes of south-western USA and northern Mexico, who believed that as little as a tablespoon of the seed would sustain a tribesman for 24 hours. It was also used in folk medicine for the treatment of diarrhoea. It was also used to treat eye inflammation. The seed absorbs water to form a mucilaginous coating around itself and when the seed is placed under the eyelid this gelatinous coating removes any foreign body that may be causing irritation without causing damage itself.\textsuperscript{19} In northern and central Yunnan, The root of \textit{S. digitaloides} is used to stimulate the circulation, treat women’s diseases, clear extra-vasated blood, and regulate the senses, also to allay the pains of swellings and clear out pus. It has also been found eminently successful for use in cardiovascular and coronary heart disorders.\textsuperscript{16} The Mazatec Indians of Oaxaca used leaves of \textit{S. divinorum} as the basis of an infusion known as "poyomatli" or "pipilzintzintli".\textsuperscript{20} It has been used by them as a vision-inducing plant in ritual curing.\textsuperscript{21} The seeds of \textit{S. hispanica} are ground and served in nutritious drinks in Mexico and South America. This plant was extensively used by Native Americans as a food source.\textsuperscript{22} In medicine, it was used to stimulate saliva, to relieve pain of the knees, for injured feet, as a lotion for stricken patients and for eruptions of the skin. Combined with a white willow, it was a cure for intestinal disorders and fevers.\textsuperscript{8} In Southern Europe, \textit{S. horminum} or the red-topped sage has been used as a good gargle for sore gums, and powdered makes a good snuff.\textsuperscript{8}

In China and Japan, the root of \textit{S. miltiorrhiza} has been used to encourage tissue growth, to invigorate and nourish the blood, and reduce swellings. It is antibacterial, anti-fungal, vasodilator, good for burns, ringworm, acne, hair loss, itching and urticaria.\textsuperscript{8} It has been employed as a female tonic in amenorrhoea, metrorrhagia, gastralgia, mastitis.\textsuperscript{23} In the Indo-Chinese region \textit{S. plebeian} has been traditionally used against stomach-ache, and

\begin{thebibliography}{99}
\bibitem{18} Rosita A. 1993. \textit{Hundred healing herbs of Belize}. Lotus Press/Twin lakes WI 53181, USA.
\bibitem{22} Noll, Robert. 1951. Southwest Desert Botanicals. \textit{Cosmetics and Toiletries} Vol. 109, June, p. 35.
\end{thebibliography}
the whole plants and the flowers are prescribed to treat cholic, cholera, and dysentery.\textsuperscript{24} The dried leaves \textit{S. pomifera}, the applebearing sage, have been used medicinally as an infusion which produces profuse perspiration, laziness, and even faintness if used to excess. \textit{S. repens}, \textit{S. rugosa}, \textit{S. runcinata}, and \textit{S. sisymbrifolia} have all been used at various times by different Southeastern African tribes for treating bed sores, herpes lesions, and swellings due to insect or mosquito bites and wasp stings.\textsuperscript{8} \textit{S. serotina} has been used in the form of either a tea or lotion for treating scratches, eczema, rash, itching, cuts, and burns on the skin by native blacks throughout the West Indies.\textsuperscript{8} In Yunnan \textit{S. yunnanensis} (Syn. \textit{S. bodinieri}; \textit{S. esquirolii}) has been used in medicine in stimulating the circulation, stopping pain, and lowering the effects of stress.\textsuperscript{16} \textit{S. canariensis}, a plant endemic to Canary Islands,\textsuperscript{25} has been used in folk medicine as an antispasmodic, febrifuge and hypoglycemicant.\textsuperscript{8} The aerial parts of \textit{S. plebeian} have been traditionally used in folk medicine for treatment of hepatitis in Taiwan.\textsuperscript{26}

\subsection*{3a.2.2. Phytochemistry and Pharmacology}

Genus \textit{Salvia} has attracted great interest primarily because of its pharmacological potential and has been the subject of numerous chemical studies. The main secondary metabolite constituents of \textit{Salvia} species are terpenoids and flavonoids. The aerial parts of these plants contain flavonoids, triterpenoids, and monoterpenes, particularly in the flowers and leaves, while diterpenoids are found mostly in the roots. However, literature reveals that some American \textit{Salvia} species also contain diterpenoids in the aerial parts, and in certain \textit{Salvia} species, triterpenoids and flavones are present in the roots.\textsuperscript{27} Various species of the genus \textit{Salvia} are well known throughout the world for their ethnopharmacological properties which have made it an attractive choice for many researchers. Some \textit{Salvia} species have been scientifically studied in many parts of the world and are reported to have various biological activities including antibacterial,\textsuperscript{28} antioxidant,\textsuperscript{29} anti-inflammatory,\textsuperscript{30} anticancer\textsuperscript{31} and anticholinesterase.\textsuperscript{30}

\begin{thebibliography}{99}
\item 27 Topcu G. 2006. \textit{J. Nat. Prod.}, 69, 482.
\end{thebibliography}
Salvia species are considered as a rich source of polyphenolics and flavonoids. More than 160 polyphenols have been identified, some of which are unique to the genus. A large number of these polyphenolic compounds are apparently constructed from the caffeic acid building block via a variety of condensation reactions. The polar phenolic acids supposedly constitute the major part of the Salvia decoction. Caffeic acid plays a central role in the biochemistry of Lamiaceae and occurs predominantly in the dimer form as rosmarinic acid. In Salvia species, caffeic acid is the building block of a variety of the plant metabolites from the simple monomers to multiple condensation products that give rise to a variety of oligomers. Phenolic acids such as rosmarinic, caffeic and salvimanolic acids have been isolated. The phenolic glycosides are not very common in Salvia. The rosmarinic acid 3'-glucoside and its methyl ester, as well as the cis- and trans-p-coumaric acid 4-O-(2-O-apyiosyl) glucosides (figure 3a.1), are the only examples of the glycosylated phenolic acids. In contrast, flavonoids are widely distributed in Salvia species and they are mostly present as flavones, flavonols and their glycosides. The majority of O-flavonoids are the flavones, apigenin and luteolin. The 6-hydroxy flavones are the flavonoids that characterise the species of Salvia and they include a variety of 6-hydroxylated apigenin and luteolin derivatives with cirsimaritin. Flavonols are mostly those of kaempferol and quercetin. Flavonone O-glycosides are apparently common in Salvia and most of them are flavone 7-glucosides represented by cosmosiin and cinaroside (figure 3a.1).

The review of literature indicated that the genus Salvia has been a popular topic in phytochemical and ethnobotanical research. The solvent extracts, essential oils and compounds isolated from various Salvia species unveiled their broad range of pharmacological properties, both in vitro and in vivo. Table 3a.1 lists some examples of biological properties of Salvia species.

The acetone, ethanol, butanol and water extracts of S. sclareaoides have shown potent inhibitory activity against acetylcholinesterase (AChE) and butyrilcholinesterase (BChE) enzymes which play an important role in the Alzheimer disease. The acetone extract of the same plant has led to the isolation of (1β, 3β)-lup-20(29)-ene-1, 3, 30-triol along with

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Figure 3a.1. Chemical structures of commonly occurring Salvia phenolics and flavonoids.
Table 3a.1. Some examples of biological activities of *Salvia* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Plant parts used</th>
<th>Bioactive fraction/compound</th>
<th>Activity</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>S. aethiopis</em></td>
<td>Roots</td>
<td>Aethiopinone</td>
<td>Anti-inflammatory</td>
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<tr>
<td><em>S. sclareoides</em></td>
<td>Whole plant</td>
<td>Rosmarinic acid</td>
<td>Acetylcholinesterase (AChE inhibition)</td>
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<tr>
<td><em>S. africana-lutea</em></td>
<td>Leaves</td>
<td>Solvent extract</td>
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<td><em>S. sahendica</em></td>
<td>Roots</td>
<td>Abietane diterpenoids</td>
<td>Anti-oxidant</td>
<td>38</td>
</tr>
<tr>
<td><em>S. chorassanica</em></td>
<td>Roots</td>
<td>Diterpenoids</td>
<td>Cytotoxic</td>
<td>39</td>
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<tr>
<td><em>S. fruticosa</em></td>
<td>Whole plant</td>
<td>Rosmarinic acid</td>
<td>Anti-oxidant</td>
<td>40</td>
</tr>
<tr>
<td><em>S. hypargeia</em></td>
<td>Roots</td>
<td>Crude extracts, taxodione</td>
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<td><em>S. lavandulaefolia</em></td>
<td>Aerial parts</td>
<td>Essential oils</td>
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<td>42</td>
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<td>Roots</td>
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<tr>
<td></td>
<td>Aerial parts</td>
<td>Polysaccharide (SMP1)</td>
<td>Cardio-protective</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>Polysaccharides</td>
<td>Cerebroprotective</td>
<td>44</td>
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<td><em>S. officinalis</em></td>
<td>Leaves</td>
<td>Ursolic acid</td>
<td>Anti-inflammatory</td>
<td>45</td>
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<tr>
<td></td>
<td>Aerial parts</td>
<td>Essential oils</td>
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<td>Leaves</td>
<td>Cirsimartinin</td>
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<td><em>S. prionitis</em></td>
<td>Leaves</td>
<td>3-Keto-4-hydroxy saprothorquinone</td>
<td>Anticancer</td>
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<tr>
<td><em>S. repens</em></td>
<td>Whole plant</td>
<td>Hydroalcoholic extract</td>
<td>Antimalarial</td>
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<td><em>S. viridis</em></td>
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<td><em>S. chamelaegna</em></td>
<td>Aerial parts</td>
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<td>Whole plant</td>
<td>Diterpenes and Triterpenes</td>
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<td>Leaves</td>
<td>Polyphenolics</td>
<td>Hypoglycemic, anti-inflammatory and antioxidant</td>
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</tr>
</tbody>
</table>

References:
nepetidin, nepeticin, lupendiol, (1β, 11α)-dihydroxy-lup-20(29)-en-3-one, ursolic acid, sumaresinolic acid and hederagenin.\textsuperscript{55} The water and methanolic extracts of \textit{S. officinalis} have been found to protect HepG2 cells from tert-butyl hydroperoxide (t-BHP) induced oxidative damage.\textsuperscript{56} The essential oil of \textit{S. officinalis} along with its monoterpenes have shown good antimutagenic effects against UV-induced mutations in \textit{Escherichia coli} and \textit{Saccharomyces cerevisiae}.\textsuperscript{57} In the British Pharmacopeia, \textit{S. officinalis} (Herba Salviae) is an official drug. This drug contains an ethereal oil (1-2%), diterpenoids, triterpenoids, and tannins. “Herba Salviae” and the extracts prepared from it have been used as anti-inflammatory, stomachic and antihydrotic agent. The plant extract, essential oil, and its constituents have shown various biological activities, including antioxidative, anti-inflammatory, immunomodulatory, HIV-1 reverse transcriptase-inhibitory, cholinesterase inhibitory, anti-Alzheimer’s disease, and insulin-like activities, as well as inhibition of pancreatic lipase activity.\textsuperscript{27}

Tanshinone IIA, an abietane-type nor-diterpenoid quinone natural product from \textit{S. miltiorrhiza}, is a coronary vasodilator and antiischemic agent. It has also shown sedative and tranquilising effects and has been employed in the treatment of neurasthenic insomnia.\textsuperscript{8,58} Dihydratanshinone I, hydroxytanshinone II-A, kryptotanshinone, methyl tanshinate, and tanshinone II-B from the same plant have been reported to be bacteriostatic against \textit{Staphylococcus aureus}.\textsuperscript{59} In pharmacological activity, the constituent tanshinone have been found to be anti-inflammatory in rats with infective arthritis.\textsuperscript{59} Bioactive flavones, homoplantagenin, hispidulin, nepetin, nepetin, nepetrin, and eupafolin along with protocatechuic acid, 4-hydroxypropionic acid, saponins, sterols and terpenes have been reported from \textit{S. plebeia}.\textsuperscript{60} This herb has been used as Anthelmintic agent in India\textsuperscript{61} and Korea.\textsuperscript{62} The organic extracts from \textit{S. plebeia} have been reported as antitussive, anti-asthmatic, anti-inflammatory and as a cure for bronchitis.\textsuperscript{60} Diterpenes brussonol, iguestol, 7-oxodehydroabietane, 11-hydroxy-12-methoxyabietatriene, taxodione, inuroyleanol, ferruginol, deoxocarnosol 12-methyl ether,
cryptojaponol, pisiferal, sugiol, isomanool, 14-deoxycoleon U, 6-α-hydroxydemethylcryptojaponol, demethylsalvicanol, and demethylcryptojaponol from the root of *S. broussonetii* have been found to be insecticidal against *Spodoptera littoralis* and *Leptinotarsa decemlineata*.63 Various constituents from different *Salvia* species have shown promising biological activities which may form the basis for some pharmacological studies. Salvanolic acid A, the stilbenoid caffeic acid trimer has been shown to possess a strong oxygen free radical scavenging activity. Rosmarinic acid, the most abundant caffeic acid dimer in *Salvia* species, has been reported to be the major phenolic compound responsible for the high antioxidant activity of *Salvia* samples. Danshensu, a caffeic acid monomer, isolated from *S. miltiorrhiza* has been found to be a coronary vasodilator and to scavenge the free oxygen radicals generated during ischemia reperfusion injury in the myocardium as effectively as superoxide dismutase (SOD).9 The dried root and aerial part of sage has been shown to contain an acceptable quantity of polar polyphenols including flavonoids and phenolic acids which could potentially be used as herbal materials.

*Salvia sclarea* commonly known as clary sage, has vast traditional uses. The herb is antispasmodic and balsamic in nature and has been used for digestive difficulties as a stomachic. It has also been employed in treatment of kidney disease with good results. The mucilage of the seeds has been used in ophthalmic disorders and a decoction of the herb was considered by herbalists to be efficacious in any complaint of the eyes.64 Mucilage of the seeds is used in tumours.14 Cold extract of clary sage have been used to draw out thorns and splinters and reduce inflammation. The powdered roots have been used as snuff to clear the head and ease a headache.65 This herb has been used by the native Jamaicans, for cooling and cleansing of ulcers, and also for the treatment of inflammation of the eyes. A decoction of the leaves boiled in coconut oil has been considered beneficial for the stings of scorpions.66 The essential oil from *S. sclarea* has come under increasing attention for its use in aromatherapy. It is said to act on the brain’s thalamus which helps to relieve anxiety.
states, including those involving fear, paranoia and delusions.\textsuperscript{67} It is considered as a good relaxing oil with euphoric effect on sensitive people and may help in the treatment of insomnia.\textsuperscript{68} The oil has also been used for its antidepressant, antiseptic, antispasmodic, astringent, carminative and deodorant properties. These properties make it useful in the treatment of boils and infections as well as a useful skin care product. In cosmetics and toiletries, clary sage baths have been used for its warming and relaxing effects. The oil has been used externally as anti-inflammatory agent, and has been extensively used in skin care because of its scent.\textsuperscript{69} Because of the non-toxic nature, the essential oil has been used as a fragrance component and a flavouring in the food industry.\textsuperscript{8} The essential oil has shown \textit{in vivo} antidepressant-like effects by modulation of the DAnergic pathway.\textsuperscript{70}

Previous phytochemical studies on acetone extracts of the whole plant of \textit{S. sclarea} from Turkey have resulted in the isolation of sixteen diterpenes, sclareol, manool, salvipisone, ferruginol, microstegiol, candidissiol, 7-oxoroyleanone, 2,3-dehydrosalvipisone, 7-oxoferruginol-18-al, aethiopinone, 1-oxoaethiopinone, salvinolone, cryptojaponol, \(\delta^7\)-manool, sclareapinone and acetylsalvipisone; two sesquiterpenes, caryophyllene oxide and spathulenol (Figue 3.2); and the flavonoids; apigenin, luteolin, 4′-methylapigenin, 6-hydroxyluteolin-6,7,3′,4′-tetramethyl ether, 6-hydroxy apigenin-7,4′-dimethyl ether along with \(\alpha\)-amyrin, 3-oxo-oleanolic acid and \(\beta\)-sitosterol. The diterpenoids and the sesquiterpenoids were shown to have antimicrobial activity against standard bacterial strains and fungal strains.\textsuperscript{71,72} Recently two amphilectane diterpenes, salviatriene A and salviatriene B have been isolated from n-hexane extract of the full bloom stage calyx of \textit{S. sclarea}. These diterpene molecules are considered as the first representatives of this family to be described from the plant kingdom.\textsuperscript{73}

\textsuperscript{67} Holmes P. 1993. \textit{The Int. J. Aromather.}, 5, 1.
\textsuperscript{68} Hoffmann D. 1991. \textit{Thorsons Guide to Medical Herbalism-a comprehensive and practical introduction}.
\textsuperscript{70} Seol GH, Shim HS, Kim PJ, Moon HK, \textit{et al.}, 2010. \textit{J. Ethnopharmacol.}, 130, 187.
3a.3. Objectives of present work

- Procurement of *Salvia sclarea*.
- Extraction and isolation of chemical constituents from *Salvia sclarea* extracts using column chromatography.
- Identification and characterization of natural isolates using spectral data analysis in the light of literature.
- Synthetic modification of major isolate (sclareol) using terminal double bond as a template.
- Bioevaluation of all the semi-synthetic analogs of sclareol for cytotoxic/anticancer activity against target cell lines for the development of lead molecules.

![Previously reported compounds from *Salvia sclarea*.](image-url)
Thorough review of literature revealed no reports of phytochemistry on *S. sclarea* of Kashmir origin. This encouraged us to undertake the further phytochemical investigation of this plant species. Repeated column chromatography on silica gel and recrystallization techniques of hexane and acetone extract of the floral parts led to the isolation of nine natural compounds (Figure 3a.3).

### 3a.4. Results and discussion

Floral parts of *S. sclarea* were collected from IIIM Gene Bank, Kashmir, India in September 2012. The air dried powdered material was extracted with hexane and acetone. The concentrated hexane and acetone extracts were subjected to column chromatography over silica gel. Repeated column chromatography of hexane and acetone extracts using varied solvent polarity (hexane:ethyl acetate) and recrystallization techniques afforded nine compounds (Figure 3a.3).

![Molecular structures of the isolated compounds.](image)

**Figure 3a.3.** Molecular structures of the isolated compounds.

#### 3a.4.1. Compound 1 (SSf-1)

This compound was isolated as a colourless crystalline solid. The HR-EIMS showed a molecular ion peak at \( m/z \) 414.3837. From elemental analysis, HR-EIMS and other spectral data (\(^1\)H NMR, \(^{13}\)C NMR), **SSf-1** was assigned the molecular formula \( C_{29}H_{50}O \).
The IR spectrum showed a hydroxyl band at 3440 cm$^{-1}$ and bands at 3060, 1655 and 815 cm$^{-1}$ due to trisubstituted double bond.

**Figure 3a.4.** Structure of SSf-1 ($\beta$-Sitosterol).

SSf-1, in its $^1$H NMR, displayed resonance signals due to two quaternary methyl groups at $\delta$ 0.69 (3H, s, H-18) and 0.97 (3H, s, H-19) and three secondary methyl groups at $\delta$ 0.90 (3H, d, $J$ = 6.5 Hz, H-21) and 0.87 (6H, d, $J$ = 5.0, H-26, H-27), besides a resonance signal due to a primary methyl group at $\delta$ 0.80 (3H, t, H-29). The $^1$H NMR spectrum of this compound displayed one resonance signal at $\delta$ 5.34 (1H, d, $J$ = 3.1 Hz, H-6), due to olefinic proton. The $^1$H NMR spectrum of this compound also showed one resonance corresponding to the proton connected to the C-3 (-OH carbon) which appeared as a triplet of doublet of doublets at $\delta$ 3.42. $^{13}$C NMR-DEPT spectrum exhibited the presence of twenty nine carbon signal including six methyls, eleven methylenes, ten methine and three quaternary carbons. This compound showed positive Liebermann-Burchard and Salkowski test specific for $\Delta^5$ sterols.$^{74,75}$ The MS spectrum showed characteristic fragment ions at $m/z$ 399, 396, 381, 329 and 303. Fragment ions 329 and 303 are considered to be diagnostic for sterols having $\Delta^5$-unsaturation.$^{74,75}$ Comparison of physical characteristics and spectral data of SSf-1, with that reported in literature,$^{76}$ confirmed it to be $\beta$-Sitosterol.

Experimental studies have shown its effectiveness as an anticancer,$^{77,78}$ antipyretic,$^{79}$ Antihyperglycemic,$^{80}$ anti-HIV$^{81}$ and in the reduction blood levels of cholesterol.$^{82}$

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75 Williams BL, Goad CT, Goodwin TW. 1967. *Phytochemistry*, 6, 1137.
3a.4.2. Compound 2 (SSf-2)

This compound was obtained as white crystalline powder with melting point 179-183 °C. The HR-EIMS showed a molecular ion peak at $m/z$ 426.3848. From elemental analysis, HREIMS and other spectral data ($^1$H NMR, $^{13}$C NMR), SSf-2 was assigned the molecular formula C$_{30}$H$_{50}$O. The compound responded positively to Liebermann-Burchard test indicating the presence of triterpene skeleton. The IR spectrum showed a hydroxyl band at 3320 cm$^{-1}$ and the presence of gem dimethyl group (1380 and 1372 cm$^{-1}$).

![Figure 3a.5. Structure of SSf-2 (α-amyrin).](image)

In the $^1$H NMR spectrum, a multiplet centered at $\delta$ 5.53 integrating for one proton is assigned to an olefinic proton. Examination of the $^{13}$C NMR spectrum also reveals the presence of two olefinic carbons at $\delta$ 124.5 and 139.6 ppm, which suggested an urs-12-ene triterpene skeleton. The other downfield signal at $\delta$ 3.85 (1H, $J = 12$ Hz) was assigned to proton which is which is attached to a hydroxylated carbon. $^{13}$C NMR spectra revealed the presence of 30 carbon atoms including eight methyls, nine methylenes, seven methines and six quartenary carbons. The carbon bearing the hydroxyl group was located at $\delta$ 78.9 in $^{13}$C NMR spectrum. Since this compound was earlier reported from the root parts of this plant species, it was identified as α-amyrin from the spectral evidence and comparing the physical data with the literature values.

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α-Amyrin is a pharmacologically important bioactive molecule. A recent study has shown its sedative, anxiolytic and antidepressant effects.\(^ {84}\) It could be potentially a good candidate for the development of a topical agent for the management of inflammatory diseases as it exerts significant anti-inflammatory effects when assessed \textit{in vivo}.\(^ {85,86,87}\) Furthermore, some \textit{in vitro} studies have shown it to be a relatively specific inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase.\(^ {88}\) In addition, it is effective in inhibiting collagenase.\(^ {89}\)

\subsection{3a.4.3. Compound 3 (SSf-3)}

It was obtained as colourless crystals with melting point 282-286 °C. The HR-EIMS showed a molecular ion peak at \textit{m/z} 456.3594. From elemental analysis, HR-EIMS and other spectral data (\(^1\)H NMR, \(^{13}\)C NMR), SS-3 was assigned the molecular formula C\(_{30}\)H\(_{48}\)O\(_3\). The compound was positive to Liebermann-Burchard test giving brownish violet colour indicating the presence of triterpene skeleton. The IR spectrum showed a hydroxyl band at 3440 cm\(^{-1}\), an acid carbonyl band at 1700 cm\(^{-1}\) and bands at 1380 and 1372 cm\(^{-1}\) due to a gem dimethyl group.

![Figure 3a.6. Structure of SSf-3 (Ursolic acid) and its significant HMBC correlations.](image)

In the \(^1\)H NMR spectrum, a multiplet centered at \(\delta\) 5.50 integrating for one proton is assigned to an olefinic proton. Examination of the \(^{13}\)C NMR spectrum also reveals the

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presence of two olefinic carbons at 125.7 and 139.3 ppm, which suggested an urs-12-ene triterpene skeleton. This was also supported by the presence of a 19β equatorial methyl group which is in close proximity to the double bond (γ and δ to C-13 and C-12 respectively) in urs-12-ens. The other downfield signal at δ 3.46 (dd, J = 6.4, 9.6 Hz) was assigned to a proton connected to hydroxylated carbon (δC 79.0). The above interpretations were substantiated by 2D NMR experiments. The two methyl singlets at δH 1.23 and 1.01 (Me-23 and Me-24) showed strong correlations with the hydroxylated carbon resonating at δC 79.0 in its HMBC spectrum. Thus the signal at δC 79.0 was therefore assigned to C-3 (Figure 3a.6). Further 13C NMR-DEPT spectra revealed the presence of 30 carbon atoms including seven methyls, one acid carbonyl, nine methylenes, seven methines and six quaternary carbons. In HMBC, the methyl protons resonating at δH 1.21 (Me-27) showed correlation with the olefinic carbon at δC 139.3 (C-13), the methyl protons at δH 1.04 and 1.21 (Me-26 and Me-27) showed correlation with a quaternary carbon resonating at δC 42.5 (C-14) and the methyl protons at δH 1.01 and 0.87 (Me-24 and Me-25) showed strong correlation with a carbon resonating at δC 52.8 (C-5). The presence of carboxylic group at C-17 was confirmed through HMBC interactions in which H-18 (δ 2.62) showed 2J correlations with C-17 (δ 48.1) and 3J correlation with C-28 (δ 179.8) of the carboxylic carbon atom. From the spectral evidence and comparing the physical data with the literature values, SSf-3 was identified as Ursolic acid (Figure 3a.6). This compound is reported for the first time from this plant species.

Ursolic acid is relatively non-toxic and could be used as chemopreventive/chemoprotective agent in clinical practices because of its anti-inflammatory, hepatoprotective, gastroprotective, anti-ulcer, anti-HIV, cardiovascular, hypolipidemic, antiatherosclerotic and immunoregulatory effect. Ursolic acid is of interest to scientists in the area of oncology because of its cytotoxicity, induction of differentiation, anti-mutagenic, antiviral and anti-invasive activities. It is capable of inducing apoptosis in tumour cells on one side and to prevent malignant transformation of normal cells on

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the other side. It also interferes with numerous enzymes, including the ones serving directly to DNA synthesis. It is also a potent acetylcholinesterase (AChE) inhibitor in Alzheimer's disease. Besides, it is significantly effective against chronic skin inflammation.

3a.4.4. Compound 4 (SSf-4)

It was obtained as colourless needles with melting point 307-311 °C. The HR-EIMS showed a molecular ion peak at $m/z$ 456.3611. From elemental analysis, HR-EIMS and other spectral data ($^1$H NMR, $^{13}$C NMR), SSf-4 was assigned the molecular formula C$_{30}$H$_{48}$O$_3$. The compound showed positive Liebermann-Burchard test indicating the presence of triterpene skeleton. The IR spectrum showed intense absorptions at 3450 cm$^{-1}$ (OH group), 1700 cm$^{-1}$ (carbonyl of carboxyl group) and 1660 and 820 cm$^{-1}$ (trisubstituted double bond).

![Figure 3a.7. Structure of SSf-4 (Oleanolic acid)](image)

The $^1$H-NMR spectrum revealed signals at $\delta$ 0.93, 0.97, 1.02, 1.02, 1.04, 1.24 and 1.30 which were assigned to protons of seven methyl groups. A doublet at $\delta$ 5.48 (1H, $J$ = 3.4 Hz) was ascribed to olefinic proton while double doublet at $\delta$ 3.58 (1H, dd, $J$ = 6.8, 9.4 Hz) was assigned to proton geminal to hydroxyl group. In the mass spectrum the prominent fragments at 248 (C$_{16}$H$_{24}$O$_2$) and 203 (C$_{15}$H$_{23}$) showed the characteristic $\Delta^{12}$-amyrin skeleton.

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The $^{13}$C NMR-DEPT spectrum of the compound indicated thirty carbon signals including six methyl, twelve methylene, six methine, and six quaternary carbon atoms. The chemical shifts at $\delta_C$ 180.4, 122.4 and 144.1 were the characteristic peaks for oleanolic type of skeleton, assigned to C-28, C-12 and C-13 respectively. The oxygen deshielded chemical shift at $\delta_C$ 78.5 was assigned to C-3.

From the spectral evidence and comparing the physical data with the literature values, the SSf-4 was identified as **Oleanolic acid** (Figure 3a.7). This compound is reported for the first time from this plant species.

Oleanolic acid is of interest to scientists because of its diverse biological activities. It has antifungal, insecticidal, diuretic and anti-diabetic activities. Oleanolic acid has been shown to act at various stages of tumor development to inhibit tumor initiation and promotion, as well as to induce tumor cell differentiation and apoptosis. Oleanolic acid is well known for its anti-HIV, hepatoprotective and anti-inflammatory effects.

### 3a.4.5. Compound 5 (SSf-5)

It was obtained as white amorphous powder with melting point 280-284 °C. The HR-EIMS showed a molecular ion peak at $m/z$ 456.3588. From elemental analysis, HR-EIMS and other spectral data ($^1$H NMR, $^{13}$C NMR), SSf-5 was assigned the molecular formula C$_{30}$H$_{48}$O$_3$. The compound was positive to Liebermann-Burchard test indicating the presence of triterpene skeleton. The IR spectrum showed a hydroxyl band at 3430 cm$^{-1}$. An absorption band at 1692 cm$^{-1}$ was also observed indicating the presence of a carbonyl (C=O) group. The absorption band at 1645 was assigned to the double bond and a sharp band at 707 cm$^{-1}$ is the characteristic absorption of =C-H bending.

The $^1$H NMR spectrum of SSf-5 displayed a pair of downfield signals centered at $\delta_H$ 4.95 (1H, s) and 4.77 (1H, s), attributable to an exo-methylene group which together with an allylic methyl singlet at $\delta_H$ 1.79 indicated an isopropenyl functionality. The presence of

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exo-methylene group was further supported by $^{13}$C NMR-DEPT spectrum showing a downfield signal at $\delta C$ 109.93 for an olefinic methylene group. A triplet at $\delta H$ 3.45 (1H, t, $J = 7.2$ Hz) was assigned to proton geminal to hydroxyl group. In addition to this, the $^1H$ NMR spectrum of this compound exhibited signals for the protons of six methyl groups at $\delta H$ 0.83, 1.01, 1.06, 1.07, 1.21 and 1.79.

![Structure of SSf-5 (Betulinic acid)](image)

The $^{13}$C NMR-DEPT spectra showed thirty carbon signals comprising of seven methyls, eleven methylenes, six methines and six quaternary carbons. The signal at $\delta C$ 178.84 indicated the presence of carbonyl of carboxylic group. The signals at $\delta C$ 151.32 and 109.93 were attributed for the presence of terminal olefinic carbons in the compound. These signals have been assigned to the isopropylene group at C-20 and C-29. The oxygen deshielded chemical shift at $\delta C$ 78.14 was assigned to C-3. The $^1H$ and $^{13}$C NMR data indicated the compound as a pentacyclic lupane triterpenoid and comparison of its physical and spectral data with published values confirmed the identity of SSf-5 as $3\beta$-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid). $^{108,109}$ This compound is reported for the first time from this plant species.

Betulinic acid is a common plant secondary metabolite which has gained a lot of attention in the recent years since it exhibits a variety of biological and medicinal properties. This pentacyclic triterpene shows a broad spectrum of activity against various cancer cell types $^{110}$ and is also known for its anti-HIV, $^{111}$ anti-inflammatory, $^{112}$ antimicrobial, $^{113}$

antimalarial,\textsuperscript{114} spasmogenic,\textsuperscript{115} antinoceceptive,\textsuperscript{116} antihelmintic,\textsuperscript{117} anti-HSV-1\textsuperscript{118} and antidiabetic activities.\textsuperscript{119}

\textbf{3a.4.6. Compound 6 (SSf-6)}

SSf-6 was isolated as a colourless crystalline solid. The HR-EIMS showed a molecular ion peak at \( m/z \) 308.2688. From elemental analysis, HR-EIMS and other spectral data (\(^1\)H NMR, \(^{13}\)C NMR), it was assigned the molecular formula \( \text{C}_{20}\text{H}_{36}\text{O}_{2} \). The IR spectrum showed a hydroxyl band at 3280 cm\(^{-1}\) and bands at 1635 and 708 cm\(^{-1}\) due to double bond and \( =\text{C-H} \) bending respectively.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{structure.png}
\caption{Structure of SSf-6 (Scareol)}
\end{figure}

In the \(^1\)H NMR spectrum of SSf-6, the signals at \( \delta 5.94 \) (IH, dd, \( J = 10.8, 17.4 \) Hz), 5.22 (IH, dd, \( J = 17.4, 1.6 \) Hz) and 4.99 (IH, dd, \( J = 10.8, 1.6 \) Hz), were considered for the protons of a terminal olefinic moiety. The four signals at \( \delta H \) 1.25 (3H, s), 1.15 (3H, s), 0.87 (3H, s) and 0.79 (6H, s) were attributable to five methyl groups. The \(^{13}\)C NMR-DEPT spectra showed twenty carbon signals comprising of five methyls, eight methylenes, three methines and four quaternary carbons. The signals at \( \delta C \) 147.6 and 110.8 indicated the presence of a terminal olefinic moiety in the molecule. These signals have been assigned to C-14 and C-15 respectively. The oxygen deshielded chemical shift at \( \delta C \) 73.8 and 73.3 were assigned to C-8 and C-13 respectively.

\begin{thebibliography}{99}
\end{thebibliography}
1H and 13C NMR gave evidence of a labdane-type diterpene containing the structural elements of an exo-methylene and two oxygen deshielded carbon atoms and comparison of its physical and spectral data with that reported in literature confirmed the identity of SSf-6 as (labd-14-ene-8,13-diol) (Sclareol).\textsuperscript{120} This compound was earlier reported from this plant species.\textsuperscript{71} Labdane-type diterpenes appear to be a main group of natural products exhibiting interesting biological activities. Sclareol among other labdanes has shown high antimicrobial activity and is used as flavoring agent and as a synthon for preparation of Ambra odorants (Ambergris) in perfumery. Sclareol is known to exhibit growth-inhibiting and cytotoxic activity against a variety of human cancer cell lines\textsuperscript{121} and has been reported to induce cell-cycle arrest and apoptosis, while downregulating the expression of the proto-oncogene, c-myc.\textsuperscript{122,123} It has been shown to enhances the activity of known anti-cancer drugs like doxorubicin, etoposide and cisplatinum.\textsuperscript{124} It has also been found to arrest cell cycle at the G1 phase and induce apoptosis in human colon cancer HCT116 cells.\textsuperscript{125} Activation of caspase-8 and 9 in early stages, followed by the activation of caspase-3 and degradation of protein poly ADP-ribose polymerase (PARP), has also been observed.\textsuperscript{125} Activation of both caspases 8 and 9 suggest that sclareol can potentially induce apoptosis by activating both the mitochondrial pathway as well as the deathreceptor pathway.\textsuperscript{126} Keeping in view the promising bioactivities associated with sclareol, it has been further synthetically modified for possible SAR studies which is discussed in the other part of this chapter.

3a.4.7. Compound 7 (SSf-7)

This compound was obtained as yellow crystalline solid with melting point 189-192 °C. The HREIMS showed a molecular ion peak at \textit{m/z} 328.1264. From HR-EIMS and other spectral data (1H NMR, 13C NMR), SSf-7 was assigned the molecular formula C\textsubscript{18}H\textsubscript{16}O\textsubscript{6}

and indicated eleven degrees of unsaturation. The compound responded positively to Shinoda test (orange colour), sulphuric acid test (yellow colour) and gave light blue colour in Gibbs test indicating the presence of flavonoid skeleton. IR spectrum exhibited a hydroxyl band at 3450 cm\(^{-1}\) and a carbonyl band at 1665 cm\(^{-1}\). The UV spectrum of \textit{SSf-7} showed absorption maxima at 330 and 277 nm characteristic of flavonoids.\(^{127}\) A bathochromic shift of 24 nm (354 nm) in band-I using AlCl\(_3\)/HCl indicated the presence of a free 5-hydroxyl group\(^{128}\) and the absence of any shift in band-II using NaOAc indicated the absence of free 7-hydroxyl group.\(^{129}\)

![Figure 3a.10. Structure of SSf-7 (Salvigenin)](image)

The \(^1\)H NMR spectrum showed signals for three methoxyls at \(\delta_H 3.95, 3.91, 3.88\) assigned to 4'-OMe, 6-OMe and 7-OMe respectively. A pair of doublets \((J = 8.8 \text{ Hz each})\) observed at \(\delta_H 7.82\) and \(7.00\) integrating for four protons were assigned to H-2', H-6' and H-3', H-5' respectively. The two other singlets at \(\delta_H 6.58\) and \(6.53\) were attributed to H-8 and H-3 respectively. The \(^13\)C NMR-DEPT spectra showed the presence of eighteen carbons comprising of six methines, nine quaternary and three methoxyl carbons. The signal at \(\delta_c 182.58\) was assigned to carbonyl carbon (C-4) and the signals at 163.91, 158.55, 154.65, 153.13 and 151.98 were assigned to oxygen deshielded carbons attributed to C-2, C-5, C-4', C-7 and C-9 respectively.

From the spectral evidence and comparing the physical data with the literature values,\(^{130}\) \textit{SSf-7} was identified as 5-hydroxy-4', 6, 7-trimethoxy flavone (\textit{Salvigenin}) (Figure 3a.10). This compound is reported for the first time from this plant species.

Flavonoids represent a highly diverse class of secondary metabolites comprising of more than 9,000 structures that have been identified to date. Salvigenin is an important plant secondary metabolite which has been found to have diverse biological activities. This compound has recently been screened for its antitumor and immunomodulatory effects in vivo.\textsuperscript{131} In addition to this it possesses gastroprotective,\textsuperscript{132} analgesic, anti-inflammatory\textsuperscript{133} and smooth muscle relaxing effects.\textsuperscript{134}

**3a.4.8. Compound 8 (SSf-8)**

SSf-8 was isolated as yellow crystalline needles with melting point 167-172 °C. The HR-EIMS showed a molecular ion peak at \( m/z \) 298.0838. The molecular formula \( \text{C}_{17}\text{H}_{14}\text{O}_{5} \) was established from HREIMS, elemental analysis and other spectral data (\(^1\text{H} \text{NMR}, \text{\textsuperscript{13}C} \text{NMR}). This compound responded positively to Shinoda test (orange colour), sulphuric acid test (yellow colour) and Gibbs test indicating the presence of flavonoid skeleton. IR spectrum exhibited a prominent hydroxyl band at 3225 cm\(^{-1}\) and a carbonyl band at 1675 cm\(^{-1}\). The UV spectrum showed absorption maxima at 329 and 269 nm which is the characteristic feature of flavonoids.\textsuperscript{127} A bathochromic shift of 23 nm (352 nm) in band-I using \( \text{AlCl}_3/\text{HCl} \) (shift reagent) indicated the presence of a free 5-hydroxyl group\textsuperscript{128} and the absence of any shift in band-II using \( \text{NaOAc} \) indicated the absence of free 7-hydroxyl group.\textsuperscript{129}

\[ \text{MeO} \]
\[ 7 8 9 10 11 12 13 14 15 16 \]
\[ \text{O} \]
\[ 2 3 \]
\[ \text{MeO} \]
\[ \text{OH} \]
\[ \text{O} \]

**Figure 3a.11. Structure of SSf-8 (Acacetin)**

The \(^1\text{H} \text{NMR} \) spectrum showed two singlets at \( \delta_{\text{H}} \) 3.89 and 3.87 integrating for three protons each assigned to C-4' and C-7 methoxyls respectively. A singlet at \( \delta_{\text{H}} \) 6.57 integrating for one proton can be attributed to the flavone proton (H-3). A pair

doublets at $\delta_H$ 6.48 ($J = 2.45$ Hz) and 6.37 ($J = 2.45$ Hz) each integrating for one proton were assigned to H-8 and H-6 protons respectively. The $^1$H NMR spectrum also exhibited a pair of double doublets at $\delta_H$ 7.86 ($J = 1.96, 6.85$ Hz) and 7.04 ($J = 1.96, 6.85$ Hz) each integrating for two protons, assigned to H-2', H-6' and H-3', H-5' protons respectively. The $^{13}$C NMR-DEPT spectra showed the presence of seventeen carbons comprising of seven methines, eight quaternary and two methoxyl carbons. The signal at $\delta_C$ 182.40 was assigned to carbonyl carbon (C-4) and the signals at $\delta_C$ 164.30, 156.40, 154.60, 154.02 and 148.14 were assigned to oxygen deshielded carbons attributed to C-2, C-9, C-4', C-5 and C-7 respectively. The two up-field signals at $\delta_C$ 61.50 and 56.15 were assigned to two methoxyl carbons.

From the spectral evidence and comparison of physical data with that of literature, SSf-8 was identified as 5-hydroxy-7, 4'-dimethoxy flavone (Acacetin) (Figure 3a.11). This compound is reported for the first time from this plant species.

The importance of Acacetin lies in its potential as a broad spectrum antimicrobial agent. This compound has been reported to be active against a panel of seven standard microbial strains including B. subtilis, S. aureus, M. pyogenes, P. aeruginosa, E. coli, V. cholerae and E. faecalis. This compound also been reported to be toxic against human lymphocytes.

3a.4.9. Compound 9 (SSf-9)

This compound was obtained as a yellow amorphous powder with melting point 233-237 °C. It exhibited a molecular ion peak at m/z 286.0898 in HR-EIMS, consistent with

136 Martini ND, Katerere DRP, Eloff JN. 2004. *J. Ethnopharmacol.*, 93, 207.
molecular formula of C$_{15}$H$_{10}$O$_{6}$. This compound showed positive Shinoda test (orange colour), sulphuric acid test (yellow colour) and Gibbs test indicating the presence of flavonoid skeleton. IR spectrum exhibited a prominent hydroxyl band at 3400 cm$^{-1}$ and a chelated carbonyl band at 1665 cm$^{-1}$. The UV spectrum exhibited maxima at 251, 270, and 352 nm characteristic of flavonoids.$^{127}$

The $^1$H NMR spectrum of SSf-9 showed signals for six aromatic protons. The $^1$H NMR spectrum revealed the presence of ABX type protons at $\delta_H$ 7.75 (1H, d, $J = 8.8$ Hz), 6.50 (1H, d, $J = 2.4$ Hz) and 6.44 (1H, dd, $J = 2.4, 8.8$ Hz) assignable to H-6’, H-3’ and H-5’ protons of 2’, 4’-dihydroxy-substituted B ring of the flavone nucleus. A pair doublets for two weakly meta-coupled protons at $\delta_H$ 6.43 ($J = 2.2$ Hz) and 6.17 ($J = 2.2$ Hz) each integrating for one proton were assigned to H-8 and H-6 protons respectively. In addition to this, a singlet at $\delta_H$ 6.98 integrating for one proton can be attributed to the flavone proton (H-3). The $^1$H and $^{13}$CNMR data including DEPT experiments showed the presence of fifteen carbon atoms including six methines and nine quaternary carbons. The signal at $\delta_C$ 181.80 was assigned to the chelated carbonyl carbon (C-4) and the signals at $\delta_C$ 164.30, 161.9, 161.9, 161.5, 159.1 and 157.4 were assigned to oxygen deshielded carbons attributed to C-7, C-4’, C-2, C-5, C-9 and C-2’ respectively.

From the spectral evidence and comparison of physical data with that of literature,$^{138}$ SSf-9 was identified as 5, 7, 2’, 4’-tetrahydroxy flavone (Norartocarpetin) (Figure 3a.12). This compound is reported for the first time from this plant species.

Norartocarpetin is better known for its tyrosinase inhibitory$^{139}$ and anti-inflammatory activities.$^{140}$ It has also been reported for the inhibition of $\beta$-secretase,$^{141}$ an enzyme strongly tied to the onset of Alzheimer’s disease. The development of inhibitors of $\beta$-secretase is critical to combating this disease, which threatens an ever increasing number of the population.$^{141}$ It has recently been reported as a potent neuraminidase inhibitor a target for the prevention of influenza infection.$^{142}$

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3a.5. Conclusions

Nine natural products were isolated and characterized from the aerial parts of *Salvia sclarea*. Hexane extract yielded seven compounds i.e., β-Sitosterol (SSf-1), α-amyrin (SSf-2), Ursolic acid (SSf-3), Oleanolic acid (SSf-4), Sclareol (SSf-6), Salvigenin (SSf-7) and Acacetin (SSf-8) while as Betulinic acid (SSf-5) and Norartocarpin (SSf-9) were isolated from the acetone extract. Compounds SSf-1, SSf-2 and SSf-6 were earlier reported from this plant species (Ulubelen et al., 1994) while as SSf-3, SSf-4, SSf-5, SSf-7, SSf-8 and SSf-9 are reported for the first time.

3a.6. Experimental
3a.6.1. General experimental procedures

$^1$H NMR and $^{13}$C NMR spectra (with chemical shifts expressed in δ and coupling constants in Hertz) were recorded on Bruker DPX 500 instrument using CDCl$_3$/pyridine/DMSO as the solvents with TMS as internal standard. Infrared spectra were recorded as KBr pellets in cm$^{-1}$ on a Hitachi 270-30 spectrophotometer. Melting points were determined on a Buchi melting point apparatus. UV spectra were scanned in methanol on specord S100. HR-EIMS were recorded on an Agilent Technologies G6540-UHD LC/MS Q-TOF. Column was run using silica gel (60-120 mesh). TLC plates were visualized under UV light and after exposure to iodine vapour in iodine chamber.

3a.6.2. Plant Material

The aerial parts of *S. sclarea* at different developmental stages were collected from the IIIM gene bank (Srinagar). After proper identification, a voucher specimen (No. 2101/12) was deposited in the Herbarium of the Indian Institute of Integrative Medicine, Srinagar, India.

3a.6.3. Extraction and Isolation

Air-dried and coarsely powdered plant material (aerial part, 1.0 Kg) was extracted with hexane for 48 hours. The marc was dried and extracted with acetone for 48 hours. The hexane and acetone extracts thus obtained were concentrated under reduced pressure to give 76.0 g of hexane and 21.0 g of acetone extracts. The extracts thus obtained was
dissolved in minimum amount of respective solvents and adsorbed on silica gel to form slurry. The dried slurry of hexane extract was subjected to silica gel column chromatography leading to Fr-I (5% ethyl acetate in petroleum ether), Fr-II (10% ethyl acetate in petroleum ether) and Fr-III (50% ethyl acetate in petroleum ether). Repeated column chromatography of these fractions and acetone extract using different percentages of petroleum ether-ethyl acetate afforded seven compounds from hexane extract and two compounds from acetone extract: β-Sitosterol (SSf-1), α-amyrin (SSf-2), Ursolic acid (SSf-3), Oleanolic acid (SSf-4), Betulinic acid (SSf-5), Scareol (SSf-6), Salvigenin (SSf-7), Acacetin (SSf-8) and Norartocarpentin (SSf-9).

**Figure 3a.13.** Flow chart depicting preparation of extracts and isolation of compounds from the aerial parts of *Salvia sclarea.*
3.6.3.1. SSf-1 (β-Sitosterol)

Colourless crystalline solid; yield: 172 mg, mp. 137-139 °C; [α]D25^25: -39° (c 2.0, CHCl3)
IR (KBr) νmax cm⁻¹: 3440 (OH), 3060 (C-H), 1655, 815 (C=C); EIMS m/z: 414, 399, 396, 381, 329, 303, 273, 255, 83, 69; ^1H NMR (CDCl₃, 500 MHz) δ: 5.40 (1H, t, J = 3.1 Hz, H-6), 3.42 (1H, dd, J = 11.4, 4.5 Hz, H-3), 2.07 (2H, m, H-2), 0.97 (3H, s, H-19), 0.90 (3H, d, J = 6.5 Hz, H-21), 0.87 (6H, d, J = 5.0 Hz, H-26, H-27) 0.80 (3H, t, J = 7.0 Hz, H-29), 0.69 (3H, s, H-18); ^13C NMR(CDCl₃, 125 MHz) δ: 142.0 (C-5), 121.71 (C-6), 71.82 (C-3), 56.90 (C-17), 56.07 (C-14), 50.17 (C-9), 43.8 (C-4), 43.0 (C-13), 40.80 (C-12), 39.3 (C-22), 38.7 (C-1), 37.1 (C-10), 36.19 (C-20), 33.01 C-7), 31.95 (C-8), 29.9 (C-2), 29.7 (C-16), 29.5 (C-23), 29.2 (C-24), 25.8 (C-15), 23.7 (C-28), 23.11 (C-25), 21.5 (C-11), 21.27 (C-27), 19.82 (C-21), 19.44 (C-19), 18.03 (C-26), 12.24 (C-18), 12.1 (29); HR-EIMS: 414.3837 (calculated for C₂₉H₅₀O, 414.3861).

3.6.3.2. SSf-2 (α-Amyrin)

Colourless crystalline solid; yield: 18 mg, mp. 179-183 °C; IR (KBr) νmax cm⁻¹ : 3320 (OH), 1640, 1380, 1372; ^1H NMR (CDCl₃, 500 MHz) δ: 5.53 (1H, t, J = 3.3 Hz, H-12), 3.85 (1H, d, J = 11.2 Hz, H-3), 1.91 (2H, m, H-11), 1.81 (1H, d, J = 11.8 Hz, H-18), 1.78 (2H, d, J = 3.5 Hz, H-2), 1.58 (1H, m, H-9), 1.52 (2H, m, H-16), 1.51 (2H, m, H-7), 1.48 (2H, d, J = 3.5 Hz, H-1), 1.41 (1H, m, H-19), 1.32 (2H, d, J = 7.3 Hz, H-6), 1.27 (2H, m, H-15), 1.25 (3H, s, H-23), 1.16 (3H, s, H-27), 1.08 (3H, s, H-26), 1.03 (2H, d, J = 10.2 Hz, H-21), 0.95 (3H, s, H-25), 0.94 (2H, d, J = 10.2 Hz, H-22), 0.93 (1H, m, H-20), 0.89 (3H, m, H-28), 0.88 (3H, d, J = 6.2 Hz, H-29), 0.88 (3H, d, J = 6.2 Hz, H-29), 0.82 (1H, m, H-5), 0.80 (3H, s, H-24); ^13C NMR (CDCl₃, 125 MHz) δ: 139.6 (C-13), 124.5 (C-12), 78.9 (C-3), 59.1 (C-18), 55.3 (C-5), 47.8 (C-9), 42.1 (C-14), 41.5 (C-22), 40.1 (C-8), 39.7 (C-19), 39.6 (C-20), 38.9 (C-1), 38.7 (C-4), 36.9 (C-10), 33.7 (C-17), 33.0 (C-7), 31.2 (C-21), 28.7 (C-28), 28.1 (C-23), 28.1 (C-16), 27.3 (C-2), 26.7 (C-15), 23.4 (C-11), 23.2 (C-27), 21.3 (C-30), 18.4 (C-6), 17.4 (C-29), 16.9 (C-26), 15.6 (C-24), 15.6 (C-25); HR-EIMS: 426.3848 (calculated for C₃₀H₅₀O, 426.3862).

3.6.3.3. SSf-3 (Ursolic acid)
Colourless crystalline solid; yield: 65 mg; mp. 282-286 °C; \([\alpha]_D^{25} +65^\circ\) (c 2.0, CHCl3); IR (KBr) \(\nu_{max}\) cm\(^{-1}\): 3440 (OH), 1700 (C=O), 1640, 1380, 1372; \(^1\)H NMR (CDCl3, 500 MHz) \(\delta\): 5.50 (1H, t, \(J = 3.8\) Hz, H-12), 3.46 (1H, dd, \(J = 6.4, 9.6\) Hz, H-3), 2.62 (1H, m, H-18), 2.30 (1H, m, H-15\(\beta\)), 2.13 (1H, m, H-16\(\alpha\)), 2.01 (1H, m, H-16\(\beta\)), 1.95 (2H, d, \(J = 10.5\) Hz, H-22), 1.94 (2H, m, H-11), 1.82 (2H, m, H-2), 1.62 (1H, m, H-9), 1.58 (1H, m, H-7\(\alpha\)), 1.58 (1H, m, H-6\(\alpha\)), 1.57 (1H, t, \(J = 3.8\) Hz, H-1\(\beta\)), 1.48 (1H, m, H-21\(\beta\)), 1.47 (1H, m, H-19), 1.40 (1H, d, \(J = 10.5\) Hz, H-21\(\alpha\)), 1.39 (1H, m, H-6\(\beta\)), 1.37 (1H, m, H-7\(\beta\)), 1.23 (3H, s, H-23), 1.21 (3H, s, H-27), 1.20 (1H, m, H-15\(\alpha\)), 1.04 (3H, s, H-26), 1.03 (1H, m, H-20), 1.01 (3H, s, H-24), 1.00 (1H, m, H-1\(\alpha\)), 0.98 (3H, d, \(J = 6.6\) Hz, H-29), 0.93 (3H, d, \(J = 6.6\) Hz, H-30), 0.88 (1H, m, H-5), 0.87 (3H, s, H-25); \(^1\)C NMR (CDCl3, 125 MHz) \(\delta\): 179.8 (C-28), 139.3 (C-13), 125.7 (C-12), 79.0 (C-3), 53.6 (C-18), 52.8 (C-5), 48.1 (C-17), 47.6 (C-9), 42.5 (C-14), 40.0 (C-8), 39.5 (C-4), 38.7 (C-1), 37.5 (C-19), 37.5 (C-22), 36.8 (C-10), 36.4(C-20), 34.3 (C-21), 33.3 (C-7), 28.7 (C-15), 24.9 (C-16), 23.9 (C-27), 23.8 (C-23), 23.6 (C-11), 23.5 (C-2), 23.4 (C-30), 21.1 (C-29), 18.8 (C-6), 16.5 (C-24), 16.5 (C-26), 15.7 (C-25); HR-EIMS: 456.3594 (calculated for C\(_{30}\)H\(_{48}\)O\(_3\), 456.3603).

3a.6.3.4. SSf-4 (Oleanolic acid)

Colourless needles; yield: 78 mg; mp. 307-311 °C; \([\alpha]_D^{25} +76.2^\circ\) (c 2.0, CHCl3); IR (KBr) \(\nu_{max}\) cm\(^{-1}\): 3450 (OH), 1700 (C=O), 1660, 1382, 1378, 820; \(^1\)H NMR (CDCl3, 500 MHz) \(\delta\): 5.48 (1H, d, \(J = 3.4\) Hz, H-12), 3.58 (1H, dd, \(J = 6.8, 9.4\) Hz, H-3), 3.30 (1H, dd, \(J = 13.5, 4.2\) Hz, H-18), 2.19 (1H, m, H-15\(\beta\)), 2.12 (1H, m, H-16\(\alpha\)), 2.04 (1H, m, H-22\(\beta\)), 1.97 (2H, m, H-11), 1.96 (1H, m, H-16\(\beta\)), 1.83 (1H, m, H-19\(\alpha\)), 1.82 (2H, m, H-2), 1.81 (1H, m, H-22\(\alpha\)), 1.70 (1H, m, H-9), 1.58 (1H, m, H-6\(\alpha\)), 1.56 (1H, m, H-1\(\beta\)), 1.53 (1H, m, H-7\(\alpha\)), 1.46 (1H, m, H-21\(\alpha\)), 1.39 (1H, m, H-6\(\beta\)), 1.36 (1H, m, H-7\(\beta\)), 1.32 (1H, m, H-19\(\beta\)), 1.30 (3H, s, H-27), 1.24 (3H, s, H-23), 1.23 (1H, m, H-21\(\beta\)), 1.22 (1H, m, H-15\(\alpha\)), 1.04 (3H, s, H-26), 1.03 (1H, m, H-1\(\alpha\)), 1.02 (3H, s, H-30), 1.02 (3H, s, H-24), 0.97 (3H, s, H-29), 0.93 (3H, s, H-25), 0.88 (1H, m, H-5); \(^1\)C NMR (CDCl3, 125 MHz) \(\delta\): 180.4 (C-28), 144.1 (C-13), 122.4 (C-12), 78.5 (C-3), 55.5 (C-5), 48.1 (C-9), 46.7 (C-17), 46.1 (C-19), 42.0 (C-14), 41.5 (C-18), 39.6 (C-8), 39.2 (C-4), 38.6 (C-1), 37.0 (C-10), 33.7 (C-21), 32.8 (C-29), 32.6 (C-7), 32.3 (C-22), 30.4 (C-20), 28.8 (C-23), 27.7 (C-15), 26.7 (C-}
3a.6.3.5. **SSf-5 (Betulinic acid)**

White amorphous powder; yield: 28 mg; mp. 280-284 °C; \([\alpha]_D^{25} +10.1^\circ (c 2.0, \text{CHCl}_3)\); IR (KBr) \(\nu_{\text{max}} \text{ cm}^{-1}\): 3430 (OH), 1692 (C=O), 1645 (C=C), 707; \(1H\) NMR (Py-d5, 500 MHz) \(\delta\): 4.95 (1H, s, H-29\(\alpha\)), 4.77 (1H, s, H-29\(\beta\)), 3.52 (1H, dt, \(J = 11.1, 3 \text{ Hz}, H-19\)), 3.45 (1H, t, \(J = 7.2 \text{ Hz}, H-3\)), 2.73 (1H, m, H-13), 2.63 (1H, m, H-16\(\beta\)), 2.25 (1H, m, H-22\(\beta\)), 2.24 (1H, m, H-21\(\beta\)), 1.94 (1H, m, H-12\(\beta\)), 1.88 (1H, m, H-18), 1.86 (2H, m, H-2), 1.79 (3H, s, H-30), 1.77 (1H, t, \(J = 11.5 \text{ Hz}, H-3\)), 1.67 (1H, d, \(J = 12.8 \text{ Hz}, H-1\(\beta\))), 1.57 (1H, m, H-22\(\alpha\)), 1.56 (1H, m, H-6\(\alpha\)), 1.55 (1H, m, H-16\(\alpha\)), 1.53 (1H, m, H-21\(\alpha\)), 1.45 (1H, m, H-7\(\alpha\)), 1.43 (1H, m, H-11\(\alpha\)), 1.38 (1H, m, H-6\(\beta\)), 1.38 (1H, m, H-9), 1.37 (1H, m, H-7\(\beta\)), 1.43 (1H, m, H-11\(\alpha\)), 1.26 (1H, m, H-15\(\alpha\)), 1.21 (3H, s, H-23), 1.21 (1H, m, H-12\(\beta\)), 1.07 (3H, s, H-27), 1.06 (3H, s, H-26), 1.01 (3H, s, H-24), 0.99 (1H, m, H-1\(\alpha\)), 0.83 (3H, s, H-25), 0.82 (1H, m, H-5). \(^{13}\)C NMR (Py-d5, 125 MHz) \(\delta\): 178.84 (C-28), 151.32 (C-20), 109.93 (C-29), 78.14 (C-3), 56.64 (C-17), 55.94 (C-5), 49.80 (C-9), 47.78 (C-19), 42.88 (C-14), 41.14 (C-8), 39.52 (C-4), 37.54 (C-10), 37.56 (C-22), 34.86 (C-7), 32.89 (C-16), 31.24 (C-21), 30.28 (C-15), 28.66 (C-23), 28.31 (C-2), 26.14 (C-12), 21.22 (C-11), 19.50 (C-30), 18.81 (C-6), 16.43 (C-25), 16.43 (C-26), 16.33 (C-24), 14.92 (C-27); HR-EIMS: 456.3588 (calculated for C\(_{30\text{H}_{48}\text{O}_{3}}\), 456.3603).

3a.6.3.6. **SSf-6 (Sclareol)**

White crystalline solid; yield: 3.2 g; mp. 99-100 °C; \([\alpha]_D^{25} -2.9^\circ (c 2.0, \text{CHCl}_3)\); IR (KBr) \(\nu_{\text{max}} \text{ cm}^{-1}\): 3280, 2910, 1635, 1450, 1380, 1360, 910, 890, 708; \(1H\) NMR (CDCl\(_3\), 500 MHz) \(\delta\): 5.94 (1H, dd, \(J_{\text{cis}} = 10.8 \text{ Hz}, J_{\text{trans}} = 17.4 \text{ Hz}, H-14\)), 5.22 (1H, dd, \(J_{\text{trans}} = 17.4 \text{ Hz}, J_{\text{gem}} = 1.6 \text{ Hz}, H_{\text{trans-15}}\)), 4.99 (1H, dd, \(J_{\text{cis}} = 10.8 \text{ Hz}, J_{\text{gem}} = 1.6 \text{ Hz}, H_{\text{cis-15}}\)), 1.84 (1H, dt, \(J = 3.2, 12.2 \text{ Hz}, H-7\(e\))), 1.64 (2H, H-12), 1.61 (1H, m, H-1e), 1.59 (2H, m, H-6\(a\), H-2\(a\)), 1.46 (1H, m, H-11\(a\)), 1.42 (1H, m, H-7\(a\)), 1.40 (2H, m, H-2e, H-3e), 1.30 (2H, m, H-6e, H-11e), 1.25 (3H, s, Me-16), 1.15 (3H, s, Me-17), 1.14 (1H, m, H-9), 1.12 (1H, m, H-3\(a\)), 0.99 (1H, m, H-1a), 0.94 (1H, dd, \(J = 2.2, 12.0 \text{ Hz}, H-5\)), 0.87 (3H, s, Me-19), 0.83 (3H, s, H-5)
0.79 (6H, s, Me-18, Me-20). $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 147.6 (C-14), 110.8 (C-15), 73.8 (C-8), 73.3 (C-13), 62.4 (C-9), 56.2 (C-5), 45.1 (C-12), 44.2 (C-7), 42.4 (C-3), 40.5 (C-1), 39.8 (C-10), 33.7 (C-4), 33.7 (C-19), 27.3 (C-16), 24.6 (C-17), 21.2 (C-6), 21.8 (C-18), 20.2 (C-11), 19.1 (C-2), 15.8 (C-20); HR-EIMS: 308.2688 (calculated for C$_{20}$H$_{36}$O$_2$, 308.2715).

3a.6.3.7. SSf-7 (Salvigenin)

Yellow crystalline solid; yield: 67 mg; mp. 189-192 °C; IR (KBr) $\nu_{max}$ cm$^{-1}$: 3450 (OH), 1665 (C=O), 1600, 1498, 1365, 830; UV (MeOH) $\lambda_{max}$: 330 and 277 nm; NaOMe: 375, 332 (sh), 295; AlCl$_3$: 360, 302 (sh), 235; AlCl$_3$/HCl: 354 (sh), 301, 262, 235; NaOAc: 376, 329, 277; NaOAc/H$_3$BO$_3$: 329, 276; $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.82 (2H, d, $J = 8.8$ Hz, H-2', H-6'), 7.00 (2H, d, $J = 8.8$ Hz, H-3', H-5'), 6.58 (1H, s, H-8), 6.53 (1H, s, H-3), 3.95 (3H, s, 4'-OMe), 3.91 (3H, s, 6-OMe), 3.89 (3H, s, 7-OMe); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 182.58 (C-4), 63.91 (C-2), 158.55 (C-5), 154.65 (C-4'), 153.13 (C-7), 151.98 (C-9), 132.54 (C-6), 124.9 (C-1'), 120. 62 (C-2', 6'), 112.44 (C-3', 5'), 105.4 (C-10), 103.3 (C-3), 90.5 (C-8), 60.77(C-4' OMe), 56.24 (C-6 OMe), 55.47 (C-7 OMe); HR-EIMS: 328.1264 (calculated for C$_{18}$H$_{16}$O$_6$, 328.0947).

3a.6.3.8. SSf-8 (Acacetin)

Yellow crystalline solid; yield: 16 mg; mp. 167-172 °C; IR (KBr) $\nu_{max}$ cm$^{-1}$: 3325 (OH), 1675 (C=O), 1620, 1510, 1385, 832; UV (MeOH) $\lambda_{max}$: 329 and 269 nm; NaOMe: 341, 289; AlCl$_3$: 381, 278; AlCl$_3$/HCl: 352 (sh), 278; NaOAc: 330, 269; NaOAc/H$_3$BO$_3$: 333, 269; $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.86 (2H, dd, $J = 1.96$, 6.85 Hz, H-2', H-6'), 7.04 (2H, dd, $J = 1.96$, 6.85 Hz, H-3', H-5'), 6.57 (1H, s, H-3), 6.48 (1H, d, $J = 2.45$ Hz, H-8), 6.37 (1H, d, $J = 2.45$ Hz, H-6), 3.89 (3H, s, C-4' OMe), 3.87 (3H, s, C-7 OMe); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 182.40 (C-4), 164.30 (C-2), 156.40 (C-9), 154.60 (C-4'), 154.02 (C-5), 148.14 (C-7), 124.02 (C-1'), 120.33 (C-2', 6'), 112.44 (C-3', 5'), 105.4 (C-10), 103.3 (C-3), 90.5 (C-8), 60.77(C-4' OMe), 56.24 (C-6 OMe), 55.47 (C-7 OMe); HR-EIMS: 298.0838 (calculated for C$_{17}$H$_{14}$O$_6$, 298.0841).
3a.6.3.9. SSf-9 (Norartocarpentin)

Yellow Powder; yield: 10 mg; mp. 233-237 °C; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3400 (OH), 1665 (C=O), 1622, 1515, 1392, 835; UV (MeOH) $\lambda_{\text{max}}$: 352, 270 and 251 nm; $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.75 (1H, d, $J = 8.8$ Hz, H-6$'$), 6.98 (1H, s, H-3), 6.50 (1H, d, $J = 2.4$ Hz, H-3$'$), 6.44 (1H, dd, $J = 2.4$, 8.8 Hz, H-5$'$), 6.43 (1H, d, $J = 2.2$ Hz, H-8), 6.17 (1H, d, $J = 2.2$ Hz, H-6); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 181.8 (C-4), 164.3 (C-7), 161.9 (C-4$'$), 161.9 (C-2), 161.5 (C-5), 159.1 (C-9), 157.4 (C-2$'$), 129.7 (C-6$'$), 108.4 (C-1$'$), 108.1 (C-3), 106.8 (C-5$'$), 103.7 (C-10), 103.1 (C-3$'$), 98.7 (C-6), 93.7 (C-8); HR-EIMS: 286.0898 (calculated for C$_{18}$H$_{10}$O$_6$, 286.0477).
Representative Graphs

$^1$H & $^{13}$C NMR spectra of SSf-1 ($\beta$-Sitosterol)
$^1\text{H} \& ^{13}\text{C} \text{NMR spectra of SSf-5 (Betulinic acid)}$
\textbf{\textsuperscript{1}H \& \textsuperscript{13}C NMR spectra of SSf-7 (Salvigenin)}
$^1$H & $^{13}$C NMR spectra of SSf-8 (Acacetin)
Synthesis of novel cytotoxic analogs of Sclareol using Palladium(II)-catalysed Oxidative Heck coupling reaction

Graphical Abstract

Abstract

A novel series of diverse aryl derivatives of sclareol (SS-1 to SS-15) were synthesized using palladium(II)-catalysed Oxidative Heck coupling reaction. All the synthesised analogs were subjected to 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) cytotoxicity screening against a panel of four different human cancer cell lines viz. prostate (PC-3 and DU-145), lung (A549) and cervical (HeLa) to check their cytotoxic potential. Of the synthesised derivatives, SS-1, SS-2, SS-3, SS-12 and SS-14 bearing phenyl, 3-nitrophenyl, 2,5-dimethylphenyl, 4-fluorophenyl and 4-carboxyphenyl moieties respectively, displayed the best activity with the IC50 of 81.0, 77.0, 76.0, 82.0, and 78.0 nM against prostate (PC-3) cancer cell line. In addition to this 4-Chloropyridin-3-yl (SS-7) and 4-fluorophenyl (SS-12) moieties improved the cytotoxic activity of sclareol by 11-fold against DU-145 (prostate) cancer cell line. The present study resulted in identification of SS-12 as a potent anti-metastatic agent, besides promoting the autophagy by induction of autophagosomes and acidic vesicular organelles in PC-3 cells at nanomolar concentrations.

Keywords: Sclareol, Heck coupling, cytotoxic, anti-metastatic, autophagy.
3b.1. Introduction

Terpenoids are the most structurally varied class of plant natural products. The name terpenoid, or terpene, derives from the fact that the first members of the class were isolated from turpentine ("terpentin" in German). Turpentine, the so-called "resin of pine trees", is the viscous pleasantly smelling balsam which flows upon cutting or carving the bark and the new wood of several pine tree species (Pinaceae). Turpentine contains the "resin acids" and some hydrocarbons, which were originally referred to as terpenes. Traditionally, all natural compounds built up from isoprene subunits and for the most part originating from plants are denoted as terpenes. Depending on the number of 2-methyl-1,3-butadiene (isoprene) subunits one differentiates between hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), tetraterpenes (C40) and polyterpenes (C5)n with n > 8.

The biological and ecochemical functions of terpenes have not yet been fully investigated. Many plants produce volatile terpenes in order to attract specific insects for pollination or otherwise to expel certain animals using these plants as food. Less volatile but strongly bitter-tasting or toxic terpenes also protect some plants from being eaten by animals (antifeedants). Last, but not the least, terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants, as shown by preliminary investigations. Many insects metabolize terpenes they have received with their plant food to growth hormones and pheromones. Harmless to the environment, pheromones may replace conventional insecticides to trap harmful and damaging insects such as bark beetles.

The diterpenes, which contain 20 carbons (four C5 units), include phytol (the hydrophobic side chain of chlorophyll), the gibberellin hormones, the resin acids of conifer and legume species, phytoalexins, and a host of pharmacologically important metabolites, including the powerful cytostatic compound paclitaxel (Taxol), an anticancer agent found at very low concentrations (0.01% dry weight) in yew bark, and forskolin, a compound used to treat glaucoma. Some gibberellins have only 19 carbon atoms and are

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2 Breitmaier E. Terpenes. 2006. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
considered norditerpenoids since they are supposed to have lost one carbon through a metabolic pathway. A very large number of diterpenoids possessing a labdane skeleton, occur in nature. The interest in studying labdanes is heightened due to the wide range of biological activities including antibacterial, antifungal, antiprotozoal, enzyme induction, anti-inflammatory, modulation of immune cell functions, as well as cytotoxic and cytostatic effects against human leukemic cell lines.

3b.2. Review of Literature

Without exception, the most complex organic architectures have been produced by natural organisms. Despite the considerable wealth of methods at the disposal of today’s chemist, it is arguable that nature still provides its fair share of new clinical candidates and drugs. Terpenoids are probably the most divergent secondary metabolites in the chemical structure. Among terpenes, labdanes constitute an important class of diterpenes with varied biological activities. A number of labdane type diterpenes have been reported to exhibited remarkable antiproliferative and cytotoxic activities. Labdanes from the rhizomes of Hedychium coronarium have been reported to possess potent cytotoxic activity against epidermoid carcinoma. Labdane diterpenes isolated from the leaves of Renealmia alpinia have also been reported as significant cytotoxic agents against the yeast Sc-7 and MI09 (Madison Lung Carcinoma) murine cell lines. Aulacocarpinolide and aulacocarpines A and B are the other labdane diterpenes isolated from Aframonum aulacocarpus, which have been found to exhibit moderate growth inhibiting effect against the L1210 murine lymphocytic leukemia cell line. Labdane diterpenes including sclareol isomers isolated from various species of Cistus exhibited strong cytotoxic effects

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against various cancer cell lines using MTT and thymidine methods.\textsuperscript{9,10} Labd-13 (E)-en-8α, 15-diol has been found to have an antiproliferative effect, in some of the leukemic cell lines affecting DNA synthesis in a dose and time dependent manner. This diterpene has further been tested by the National Cancer Institute (NCI), U.S.A using the sulforhodamine B (SRB) method against NCI-H460 (Lung), MCF-7 (Breast) and SF-268 (CNS) cell lines.\textsuperscript{11} Furthermore, the diterpene has also been tested in an \textit{in vitro} model by the NCI, consisting of 60 human tumor cell lines, showing appreciable results.\textsuperscript{12} Several studies concerning the cancer-related properties of forskolin have also been performed. The combined effect of forskolin with mitomycin C, a well-established anticancer drug, has been reported to significantly enhance the cytotoxicity of mitomycin C, increasing in parallel the uptake of the drug and of the intracellular c-AMP in AH66 cell line.\textsuperscript{13} It is suggested that forskolin may be suitable for antitumour combination chemotherapy. Additionally, forskolin and its derivatives have been reported to induce apoptosis in human ovarian carcinoma, cerebellar granule, RP-11 (rat periosteal), PC 12, and A-172 cancer cell lines.\textsuperscript{14,15}

\textbf{3b.2.1. Sclareol}

Sclareol (Labd-14-ene-8, 13-diol) is a well known labdane diterpene, which was first isolated from the plant \textit{Salvia sclarea}.\textsuperscript{16} This diteriary alcohol also occurs in many conifers and has also been isolated from the genus \textit{Cistus}.\textsuperscript{17} Sclareol among other labdanes is widely distributed in nature, and is used in cosmetics, flavoring additive in food and beverage industry, and as a folk medicine.\textsuperscript{18} This molecule has shown high antimicrobial activity and is used as a synthon for preparation of Ambra odorants (Ambergris) in perfumery.\textsuperscript{19} The Flavor and Extract Manufactures Association of the U.S.A generally recognize sclareol as a safe material.\textsuperscript{5} Besides its use in cosmetic and food industries, sclareol has recently been testified to displayed appreciable anti-

\begin{thebibliography}{99}
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inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and the λ-carrageenan induced edema mouse paw model by inhibition of cell growth, nitric oxide (NO) production, and the expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins in LPS-stimulated macrophages. This compound has also been reported to display its anti-inflammatory effects by reducing paw edema, the tissue content of NO, tumor necrosis factor-alpha (TNF-α), malondialdehyde (MDA), iNOS and COX-2 protein expression, and neutrophil infiltration within the tissues after λ-carrageenan stimulation. It is believed that the anti-inflammatory mechanisms of sclareol might be related to a decrease of inflammatory cytokines and an increase of antioxidant enzyme activity.20 It has also been demonstrated that sclareol possesses in vitro antioxidant activity21 and modulates the immune response through affecting the cytokine pattern in the splenocytes of intratumorally injected mice.22

Sclareol is generally considered to be inactive and non-toxic.23 However, this compound has been reported to exhibit strong cytotoxic activity against P-388, KB, and NSCLC-N6 cell lines.24 In addition to this, sclareol has been tested for cytotoxic effect against a panel of established B and T lineage (human leukemic) cell lines wherein it displayed IC₅₀ lower than 20 µg/ml in most of the cell lines tested. Furthermore, this compound has also been reported to show a significant cytostatic effect against certain leukemic cell lines. The same authors report that sclareol is the first labdane type diterpene studied which kills tumour cells by induction of apoptosis in G0/G1 phase of mitosis.25 Dimas et al., has demonstrated that sclareol induces apoptosis in human leukemic cell lines by down-regulating the expression of proto-oncogene c-myc without affecting the expression of the anti-apoptotic protein, Bcl-2,26 and in cells derived from solid tumours by a mechanism that appears to be independent of p53-expression.27

In most cases, anticancer drug treatment results in the activation of caspases, enzymes which effectively execute various forms of cell death by apoptosis. The death receptor-dependent apoptotic pathway is triggered at the cell surface and requires activation of caspase-8, whereas the mitochondrion-dependent pathway is initiated with release of

mitochondrial cytochrome c into the cytoplasm and requires activation of caspase-9. Activation of caspase-8 and -9 in early stages, followed by the activation of caspase-3 and degradation of protein poly ADP-ribose polymerase (PARP) has also been observed suggesting that sclareol can potentially induce apoptosis by activating both the mitochondrial pathway as well as the death-receptor pathway. Wild-type p53 has often been regarded as a critical factor for the apoptotic response to DNA damaging agents, and therefore, loss of p53 function is a major cause of tumour resistance to chemotherapeutic agents. Consequently, a huge effort is currently been taking place to discover drugs, whose action is p53 independent. Sclareol has recently been reported to enhance the activity of known anticancer drugs like doxorubicin, etoposide and cisplatinum against human breast cancer cells independent of p53. Sclareol is the first representative of labdanes that has been introduced into liposomal technology. Liposomes modulate the physicochemical properties of the bioactive compounds (lipophilic) and improve their pharmacokinetic properties, which influence their pharmacological efficiency. The cellular delivery of cytotoxic molecules is important aspect in the area of anticancer therapy and several delivery systems have been used as adequate carriers for improving the delivery of biologically active molecules. Liposome-incorporated sclareol has been shown to reduce the growth rate of human colon cancer tumours (HCT116) developed in SCID mice, exhibiting superior properties than that of the free sclareol, without any significant side effects. The same research group reported that liposomes have the ability to modify the subcellular distribution of sclareol uptake by HCT-116 cancer cell lines, wherein the liposome-incorporated sclareol retains its growth inhibitory activity while its cytotoxic action is diminished. Recently, it has been demonstrated that mitochondria-targeted liposomes significantly improve the apoptotic and cytotoxic action of sclareol. Sclareol has also been found to induce apoptosis in HCT116 cells in vitro and suppresses the HCT116 tumour growth in immunodeficient mice.

3b.2.2. Palladium catalysed carbon-carbon bond formation

The development of short synthetic routes for the synthesis of complex natural products remains a major topic in synthetic organic chemistry, because they offer the best ways to achieve overall efficiency. Ideally, the synthetic routes should also be amenable to the preparation of diverse natural product analogs, which are important for the discovery of new lead compounds and for the study of complex biological systems. Organometallic reactions leading to the formation of a new carbon-carbon bond have secured an important place in synthetic organic chemistry. Over the past decade the quest for palladium-catalyzed C-H activation/C-C bond-forming reactions have become a matter of increasing importance among both industrial and academic research units for the production of important organic-based molecules. Among the myriad of important transition metal catalysed synthetic transformations, palladium-catalysed Heck coupling, cross-coupling (Kumada, Stille, Negishi, Suzuki-Miyaura, Hiyama), Tsuji-Trost allylation, and Buchwald-Hartwig amination reactions using organohalides and other surrogates are particularly valuable tools in synthetic chemistry. Palladium-catalyzed reactions for the formation of C-C<sub>ar</sub> bonds are widely used for the synthesis of novel natural product analogs for drug development. The vast majority of these transformations (e.g., Stille, Suzuki-Miyaura, Sonogashira, Hiyama, and Negishi reactions) involve coupling of an aryl halide with an organometallic fragment. The disadvantage of this approach is that it requires the use of two functionalized starting materials, which can be challenging and/or expensive to access in the context of complex molecule synthesis. An alternative strategy for C-C<sub>ar</sub> bond construction would involve Pd-mediated C-H activation followed by functionalization of the resulting Pd-aryl/alkyl species with an appropriate arylation reagent. The development of such C-H activation/arylation reactions, particularly with broad scope, high functional group tolerance, and mild reaction conditions, represents an area of significant interest, as such transformations

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promise to facilitate selective construction of carbon-carbon bonds at late stages in the synthesis of drug molecules and/or natural products.\textsuperscript{38}

The Heck reaction is one of the fundamental palladium (0) catalyzed C-C bond-forming reactions, and its impact on the way molecules are built today was recognized with the Nobel Prize in Chemistry in 2010.\textsuperscript{39} This reaction is remarkably chemoselective and amenable to a large variety of starting materials, it has become one of the true power tools of contemporary organic synthesis.\textsuperscript{40} This reaction has successfully been used in the facile synthesis of biologically important polyhydroxy stilbenes, including resveratrol and its derivatives.\textsuperscript{41} The breadth and depth of research dedicated to its various facets over the past decades leaves the erroneous impression that Heck chemistry is now a mature area.\textsuperscript{42} The mechanistic understanding of how the countless parameters of a Heck reaction affect its outcome is still premature, and that includes the subtle factors that govern the regioselectivity of the migratory insertion as well as the $\beta$-hydride elimination. These issues are particularly evident in intermolecular scenarios with internal (sterically and electronically nonbiased) alkenes. The inability to control these steps in a predictable manner is likely to account for the limited progress in the asymmetric variant. Enantioselective intermolecular Heck reactions usually require cyclic alkenes where conformational rigidity and, hence, restricted rotation around C-C bonds steers $\beta$-hydride elimination away from the newly formed C-C bond.\textsuperscript{39} Besides the tremendous applications of Heck reaction, it requires reactive functional group in both the reactants. Thus, it seems logical to reduce the number of required functionalities in coupling processes.\textsuperscript{43} This would represent a powerful synthetic approach in which only one component of the coupling reaction needs to possess a reactive group.\textsuperscript{44} Research in this field has largely focused on the discovery of new modes of catalysis and the expansion of substrate scope. The substrates which contain a reactive C-H bond would provide very interesting alternatives for such synthetic purposes, provided that these reactions are


achievable under acceptable thermodynamic conditions such as mild temperatures and low pressures.45

One of the earliest reports concerning the C-H activation of benzene by Pd(OAc)$_2$ and subsequent carbopalladation and $\beta$-hydride elimination to afford olefinated arenes.46 However, two major drawbacks largely hampered the application of this catalytic reaction i.e. a large excess of the arene was required and there was a lack of control of the regioselectivity when monosubstituted benzene was used as the substrate. Interestingly, Heck had observed in 1975 that boronic acids are competent cross-coupling partners when stoichiometric quantities of palladium are employed.47,48 This reaction involving palladium-catalyzed cross-coupling of organoboronic acids and olefins, known as the oxidative Heck reaction,49 has emerged as a promising new catalytic transformation and is now growing rapidly as one of the alternative tools for constructing C-C bonds, mainly because boronic acids are stable to air and moisture, less toxic and commercially available.50 Furthermore, the palladium catalysed oxidative coupling of organo-boron compounds with olefins has received much attention,51 because the reaction conditions are halide free and milder than the original Heck reaction, which employs aryl halides instead of arylboronic acid.

3b.3. Objectives of Present Work

Natural products have made an enormous contribution in the field of cancer chemotherapy, and over half of the current anticancer agents in clinical use are natural products or their derivatives. In view of the broad spectrum anticancer activity of labdane diterpenes in general and sclareol in particular, it seems logical to develop this phytomolecule through structural manoeuvring into a high therapeutic agent. The general aim of the current study is to investigate sclareol and its derivatives for cytotoxic potential. The specific objectives are:

- Isolation of sclareol from *S. sclarea* in quantitative amounts.
- Preparation of novel semi-synthetic analogs of sclareol using palladium catalysed oxidative Heck coupling reaction.
- *In vitro* screening of all the synthesised novel analogs against different human cancer cell lines.
- Identification of lead molecules and detailed investigations related to structure activity relationship (SAR).

As mentioned in aforesaid section, sclareol has been reported for its strong anticancer activity against various human cancer cell lines. In a bid to upscale its activity, chemical modifications of this phyto-constituent were envisaged. At the very outset, the important susceptible site (C-15) of sclareol (*SCL-0*) was identified for the structural modification as shown in Figure-3b.1. The idea behind the selection was to study the effect of extra substituted phenyl groups towards anticancer activity, which would help in the establishment of structure activity relationship (SAR). Thus, oxidative Heck reaction was performed to get a series of diverse analogs at C-15 site of sclareol.

![Figure 3b.1. Structure of Sclareol](image)

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123
3b.4. Results and discussion
3b.4.1. Chemistry

In order to investigate the effect of aryl moieties at C-15 site, sclareol was subjected to palladium(II)-catalysed oxidative Heck coupling reaction with arylboronic acids of varied functionalities. The first step of this reaction involves transmetalation, wherein, Pd(OAc)$_2$ reacts with arylboronic acid to form arylated complex of Pd(II) (Figure 3b.2). This step is followed by olefin insertion to generate arylated olefin complex of palladium (II), which subsequently involves β-hydride elimination to produce the required coupled product and AcO-Pd$^{II}$-H. Under basic conditions, AcO-Pd$^{II}$-H undergoes reductive elimination in presence of a base (NaOAc), ending up with inactive Pd(0). An oxidant is needed to regenerate Pd(II) to continue the catalytic cycle. Some commonly used reoxidants are metal salts, such as copper$^{52}$ and silver salts$^{53}$ MnO$_2$$^{54}$ p-BQ$^{55}$ and other quinones$^{56}$ TEMPO$^{57}$ desyl chloride$^{58}$ and peroxides$^{57}$. Recently, the introduction of oxygen as the sole reoxidant has resulted in more sustainable Pd(II) protocols$^{59}$. The mechanism behind the oxidative Heck reaction has largely been adopted from the Pd(0)-catalyzed classical Heck reaction. These two reactions differ in the steps providing the arylpalladium intermediate and the regeneration of the active catalyst.

Figure 3b.2. Proposed mechanism of palladium(II)-catalyzed oxidative Heck arylation

Scheme-3b.1: Preparation of aryl derivatives of sclareol using oxidative Heck arylation

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% Yield\textsuperscript{a}</th>
<th>% Yield\textsuperscript{b}</th>
<th>Entry</th>
<th>R</th>
<th>% Yield\textsuperscript{a}</th>
<th>% Yield\textsuperscript{b}</th>
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<td>82</td>
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\textsuperscript{a} Yield in presence of Cu(OAc)\textsubscript{2}; \textsuperscript{b} Yield in presence of molecular O₂
The reaction of sclareol (1 mmol) with arylboronic acids (1 mmol) was carried out under the oxidative Heck conditions using 10 mol % of Pd(OAc)$_2$, 2.0 eq of Cu(OAc)$_2$, and 3.5 eq NaOAc in DMF (5.0 ml) at 80 °C (Scheme-3b.1). The product was obtained in 40-45% yield after stirring for only 3-4 hours. Use of a slightly excess amount (1.5 eq) of arylboronic acid improved the yield to 60-70% after stirring. Use of molecular oxygen (O$_2$) enhanced the yield to 70-85%. Having successfully optimized the synthetic route, the synthesis of sclareol analogs using boronic acids with different electronic and steric properties were examined. The results indicated that the reaction proceeded smoothly yielding $E$ isomers exclusively. Under Heck conditions a series of such analogs was synthesized to look for the possible structure-activity relationship. The C-C double bonds conjugated to electron-withdrawing groups such as CO$_n$R ($n = 1$ or 2, R = H or alkyl) generally serve as good acceptors of aryl nucleophiles. This methodology enables us to synthesize diverse regioselective analogs, wherein, the parent molecule retains its structural identity. The regiochemical outcome using Pd(II) catalysis is governed by the steric and electronic properties of the olefin, the aryl-palladium species and the ligand. In general, the electronic properties of the olefin influence the regiochemistry. The electron-poor Pd(II) center coordinates most strongly to the carbon with the highest electron density, while the aryl group migrate to the carbon with the lowest charge density. As a result, electron-poor alkenes will favor the linear product, and electron-rich alkenes will favor the branched product. When olefins with intermediate electronic properties are used, it is often difficult to predict the regioselectivity.

The structures of all the synthesized derivatives (SS-1 to SS-15) were characterized by spectral data analysis. Formation of products could easily be confirmed by the appearance of two downfield doublets ($J = 16.0$ Hz) for two trans coupled protons (almost around 6.2 to 6.7 ppm) and other proton signals in the aliphatic (sclareol signals) and aromatic region (aryl moieties). Further characterization of all the products was done using $^{13}$C NMR-DEPT and HR-ESIMS.

3b.4.2. Biology
3b.4.2.1. In vitro screening of the novel analogs against human cancer cell lines

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The variety of biological activities exhibited by labdane diterpenes indicate that such molecules hold the key to new drug discovery. Therefore the preparation of new analogs of this skeleton could be carried out to get new leads in drug development. Sclareol induces cell cycle perturbations and apoptosis in leukemic cell lines,25 and regulates the expression of c-myc.26 Taken together, the results described in this and previous studies demonstrate that sclareol possesses a significant antitumor activity. Further, other members of the labdane diterpenes have also been reported to demonstrate significant cytotoxic activity,26, but in general, very little is known for this group of natural compounds with the exception of forskolin. Because of its unique biochemical properties, sclareol could be further investigated in the field of oncology. The preparation of natural product derivatives and their biological screening is an important aspect of synthetic and medicinal chemistry as it may lead to the discovery of new therapeutic agents. The significance increases when the results are compared with standard references or drugs. While analysing the literature on analog synthesis of sclareol, we noticed that there are no such reports highlighting the structure-activity relationship (SAR) linking the effect of an aryl substituent in the parent molecule to the cytotoxic properties of the resultant molecule.

**Table-3b.1:** % Growth inhibition (GI) data of sclareol analogs against prostate (PC-3 and DU-145), lung (A549) and cervical (HeLa) cancer cell lines at 50 µM using MTT assay.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Entry</th>
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<th>A549</th>
<th>HeLa</th>
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<td></td>
<td>(% GI)</td>
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<tr>
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<tr>
<td>17</td>
<td>Staurosporine</td>
<td>100</td>
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*Staurosporine* was used as positive control
Motivated by the well-documented cytotoxic activity of labdanes in general and sclareol in particular, sclareol and its aryl derivatives synthesized using oxidative Heck coupling reaction were tested for cytotoxic activity against a panel of four different human cancer cell lines viz. prostate (PC-3 and DU-145), lung (A549) and cervical (HeLa) using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] cytotoxicity screening assay. These cell lines were chosen because they represent cancer types with great incidence in the human population. Preliminary cytotoxicity screening of the analogs was carried out at 50.0 µM concentration and cell death was determined (Table 3b.1). Staurosporine was used as positive control in this assay. All the synthesized analogs displayed broad spectrum cytotoxic effect in a dose dependent manner. The analogs which exhibited significant cytotoxic effect, greater than 50% growth inhibition at the preliminary screening concentration were further assayed at different concentrations (0.05-50 µM) to generate the IC₅₀ values (Table 3b.2). The values are the average of triplicate analysis.

Table 3b.2: IC₅₀ (µM) values of sclareol analogs against prostate (PC-3 and DU-145), lung (A549) and cervical (HeLa) cancer cell lines using MTT assay

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Entry</th>
<th>PC-3</th>
<th>DU-145</th>
<th>A549</th>
<th>HeLa</th>
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<tbody>
<tr>
<td>1</td>
<td>Sclareol</td>
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<td>2.92 ± 0.076</td>
<td>3.21 ± 0.091</td>
<td>3.35 ± 0.078</td>
</tr>
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<td>2</td>
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<tr>
<td>3</td>
<td>SS-2</td>
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<tr>
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<td>SS-3</td>
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<td>2.95 ± 0.096</td>
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<td>3.38 ± 0.133</td>
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<td>5</td>
<td>SS-4</td>
<td>3.90 ± 0.023</td>
<td>44.0 ± 0.133</td>
<td>45.70 ± 0.098</td>
<td>46.0 ± 0.231</td>
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<td>6</td>
<td>SS-5</td>
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<td>48.20 ± 0.224</td>
</tr>
<tr>
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<td>SS-6</td>
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<td>SS-7</td>
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<td>SS-8</td>
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<td>45.0 ± 0.185</td>
<td>45.0 ± 0.153</td>
<td>48.50 ± 0.182</td>
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<td>2.80 ± 0.143</td>
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<td>3.88 ± 0.066</td>
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<td>0.029 ± 0.0016</td>
<td>0.020 ± 0.005</td>
<td>0.025 ± 0.0081</td>
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</table>

IC₅₀ values are indicated as the mean ± SD of three independent experiments.  
Staurosporine was used as positive control.

In the present studies, sclareol and its aryl derivatives (SS-1 to SS-15) displayed interesting cytotoxic effects against all the tested human cancer cell lines. Among these
molecules, SS-3, SS-2, SS-14, SS-1 and SS-12 showed the best cytotoxic effects against prostate (PC-3) cancer cell line with IC\textsubscript{50} of 76.0, 77.0, 78.0, 81.0 and 82.0 nM respectively, while as, sclareol showed the IC\textsubscript{50} of 85.0 nM against the same cancer cell line. This observation revealed that some of these derivatives induced more potent cytotoxicity in the prostate (PC-3) cancer cell line than the parent molecule, highlighting the beneficial impact of differently substituted aryl moieties (2,5-dimethylphenyl, 3-nitrophenyl, 4-carboxyphenyl, phenyl and 4-fluorophenyl) on the cytotoxic activity. Additionally, SS-7 and SS-12 exhibited strong cytotoxic effects against DU-145 (prostate) cancer cell line with IC\textsubscript{50} of 0.266 and 0.267 µM respectively. This observation demonstrates that 4-chloropyridin-3-yl and 4-fluorophenyl groups enhance the cytotoxic potential of sclareol by about 11-fold against DU-145 cancer cell line. The other synthesized derivatives displayed moderate to good cytotoxic activity against all the tested cancer cell lines. In such studies, modification of natural product scaffolds facilitate the expansion of existing structural requirements consistent with improved biological activity.

In view of structure activity relationship (SAR), these in vitro cytotoxic results suggest that simple modifications of the parent molecule at C-15 position can produce new potentially interesting derivatives. Introduction of a simple phenyl moiety (SS-1) as substituent in sclareol resulted in the fractional increase in its cytotoxic potential against all the tested cancer cell lines. The analog with 3-nitophenyl substitution (SS-2) enhanced the cytotoxicity against PC-3 cell line only, while as, against the other cell lines, it displayed more or less the similar cytotoxic effects as that of the parent sclareol. Among the dimethylphenyl analogs of sclareol, 2,5-dimethylphenyl analog (SS-3) displayed best cytotoxic activity with IC\textsubscript{50} of 76.0 nM against PC-3 cancer cell line, while as, 2,3-dimethylphenyl (SS-6) and 2,6-dimethylphenyl (SS-10) analogs demonstrated similar cytotoxic activity as that of the parent molecule. Introduction of a 4-t-butylphenyl moiety (SS-8) in the parent natural product appreciably reduced its activity, while as, 4-phenoxyphenyl substituent (SS-15) had inconsequential effect on the cytotoxic activity of the parent molecule. Among the heterocyclic derivatives, SS-7 with 4-chloropyridin-3-yl moiety improved the cytotoxic activity of sclareol by 11-fold against DU-145 cancer cell line, while as, the incorporation of isoquinolin-4-yl moiety (SS-4) greatly reduced the activity against all the tested cancer cell lines. Similarly, the incorporation of a
dimethoxyphenyl moieties (SS-5 and SS-9) resulted in the loss of cytotoxicity of sclareol by many folds. SS-14 (4-carboxyphenyl moiety on the C-15 of sclareol) displayed selective enhanced cytotoxic effect (78.0 nM) against PC-3 cancer cell line, besides, showing reduced activity against the other tested cell lines. Among the analogs bearing halogenated aryl moieties as R groups, SS-12 with 4-fluorophenyl moiety demonstrated the enhanced cytotoxic effects against the two prostate cancer cell lines (PC-3 and DU-145) with IC$_{50}$ of 82.0 nM and 267 nM respectively, while as, SS-11 and SS-13 bearing 4-bromophenyl and 10-Bromoanthracen-9-yl R moieties reduced the activity of sclareol by an appreciable margin. A comparison of the activities of the synthesized analogs revealed the significant contribution of 2,5-dimethylphenyl (SS-3), 4-chloropyridin-3-yl (SS-7) and 4-fluorophenyl moieties in achieving the better cytotoxic activity than the parent natural product. All these observations reveal that the structural features have profound influence on the biological profile of a compound. From the results, it may be summarized that the compounds in general were more specifically active towards prostate cancer cell lines (PC-3 and DU-145) followed by lung (A549) and cervical (HeLa) cancer cell lines.

3b.4.2.2. Anti-metastatic activity of SS-12

One of the hallmark traits of cancer cells is their ability to undergo isolated clonal growth followed by metastasis. Wound healing assay has been carried out in tissue culture for many years to estimate the cell migration and proliferation rates in vitro. This method is based on observation of cell migration into a “wound” that is created on a cell monolayer. Although not an exact duplication of cell migration in vivo, this method mimics to some extent migration of cells in wound healing.64 This assay generally involves the growing of a confluent cell monolayer. A small area is then disrupted and a group of cells destroyed or displaced by scratching a line through the layer. The open gap is then inspected microscopically over time as the cells move in and fill the damaged area. This healing can take from several hours to over a day depending on the cell type, conditions, and the extent of the wounded region. To check the anti-metastatic activity, SS-12 displaying the most impressive cytotoxic effects with IC$_{50}$ of 0.082, 0.267, 3.10 and 3.46

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µM against PC-3, DU-145, A549 and HeLa cancer lines respectively, was further evaluated by scratch motility (wound healing) assay against PC-3 cell line (Figure 3b.3). Herein, the dishes were incubated at 37 °C until cells reached 100% confluence to form a monolayer. After 48 h, cell monolayers were wounded, the vehicle DMSO treated cells had almost completely filled in the cleared area, whereas, treatment with 100 and 200 nM of compound SS-12 significantly abrogates motility and invasion potential of PC-3 cells.

Figure 3b.3. (A) PC-3 cells (0.5 x 10^5 cells/well) were grown to confluence in six well tissue culture plate and scratched with sterile tip, compound SS-12 was added to cultures as indicated. Scratched areas were photographed (magnification 100x) at zero hour and then subsequently again 24 h later to assess the degree of wound healing. (B) The scratched areas were quantified in three random fields in each treatment, and data were calculated from three independent experiments. Columns mean; bars SD of three independent experiments. ***P < 0.01 compared with untreated control.

**3b.4.2.3. Induction of Autophagy by SS-12**

Autophagy is one of the major degradative pathways that involves the sequestration of cytoplasmic portions and intracellular organelles in a double-membrane structure, known as autophagosome, which then delivers the enclosed material to a lysosome for degradation. It is a physiological intracellular process that can be induced by several conditions, including starvation, hormone treatment and stress.65 Initially believed to be a system dedicated tool to the recycling of macromolecular material within the cell, autophagy is now known to be involved in a multitude of cellular processes including

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immunity, tumorigenesis, programmed cell death, the selective degradation of organelles, aging and numerous neurodegenerative conditions.66 Presently there are limited therapies available for the treatment of malignant cancers. It becomes customary to explore new molecules for tumour treatment by modulating autophagy. For example, rapamycin, an anticancer autophagy inducer, promotes autophagy by inhibiting mTOR.67 Therefore, in this study, we set out to investigate SS-12 induced autophagy against PC-3 cancer line.

In our studies, we have used the autofluorescent compound monodansylcadaverine (MDC), a specific autophagolysosome marker to analyse autophagic process. In addition, rapamycin, the best characterized drug that enhances autophagy, was used as a positive control. The accumulation of vesicular MDC into PC-3 cells following SR-12 treatment was studied using fluorescence microscopy. As shown in Figure 3b.4, PC-3 cells treated with SS-12 for 24 h demonstrated a punctate pattern of MDC-labeled fluorescence. By contrast, untreated cells (vehicle) exhibited a diffused distribution of MDC-labeled fluorescence. SS-12 induced autophagy of PC-3 cells in a concentration-dependent manner which is visible in figure 3b.4 where an increased number of autophagosomes is observed upon treatment with higher doses of SS-12.

Figure 3b.4. Visualization of autophagy activation with monodansylcadaverine (MDC) in PC-3 cells. PC-3 cells were treated with SS-12 (100, 200 and 300 nM) and were incubated for 24 h. Cells were stained with MDC. (a) Vehicle; (b) 100 nM rapamycin (positive control); (c) 100, 200 and 300 nM SS-12; (d) The data were calculated from three independent experiments. Columns mean; bars SD of three independent experiments. ***P < 0.01 compared with untreated control.

3b.4.2.4. Development of acidic vesicular organelles (AVOs) in SS-12 treated PC-3 cells

Although autophagy can serve as a protective mechanism against apoptosis and starvation by recycling macromolecules and removing damaged mitochondria and other organelles, excessive autophagy results in cell death with appearance of excessive autophagic vesicles.\textsuperscript{68} While apoptosis is called type-I programmed cell death, autophagic cell death is named as type-II programmed cell death.\textsuperscript{69}

![Image of autophagy activation with acridine orange (AO) in PC-3 cells.](image)

**Figure 3b.5.** Visualization of autophagy activation with acridine orange (AO) in PC-3 cells. PC-3 cells were treated with SS-12 (100, 200 and 300 nM) and were incubated for 24 h. Cells were stained with MDC. (a) Vehicle; (b) 100 nM rapamycin (positive control); (c) 100, 200 and 300 nM SS-12; (d) The data were calculated from three independent experiments. Columns mean; bars SD of three independent experiments. ***P < 0.01 compared with untreated control.

In a complimentary assay for the assessment of autophagy, SS-12 cells was subjected to acridine orange (AO) assay for the detection of AVOs in PC-3 cell line. To identify the development of AVOs, which is characteristic of autophagy, we used the lysosomotrophic agent, acridine orange (AO) that moves freely across biological membranes when uncharged. AO is a fluorescent weak base whose protonated form accumulates in acidic compartments, such as autolysosomes and lysosomes, which are called AVOs, where it forms aggregates that fluoresce bright red, whereas the cytoplasm fluoresce bright green.\textsuperscript{70} To detect and quantify the SR-12 induced increase in acidity of AVOs, a flow cytometric analysis was performed. As shown in Figure 3b.5, SR-12 increased the

strength of the bright red fluorescence in PC-3 cells from 33.0 to 62.0% at 100 and 300 nM concentrations respectively, indicating the development of AVOs. Rapamycin (100 nM) was used as a positive control to induce autophagy in the tested cancer cell line. The experiment was repeated three times and a similar trend was detected and the effect was statistically significant (P < 0.01). These results indicate that the SR-12 induced increase of the bright red fluorescence is attributable to the development of AVOs associated with autophagy.

3b.5. Conclusion

In summary, all the analogs, synthesized in a single C-C bond-forming step using palladium-catalyzed Heck coupling between sclareol and arylboronic acids, were evaluated for cytotoxic activity for establishing SAR. The analogs 3-nitrophenyl (SS-2), 2,5-dimethylphenyl (SS-3), 4-fluorophenyl (SS-12) and 4-carboxyphenyl (SS-14) substitutions displayed the best cytotoxic effects against PC-3 cancer cell line in nanomolar (nM) concentrations. 15-(4-Chloropyridin-3-yl)-sclareol (SS-7) and 15-(4-fluorophenyl)-sclareol (SS-12) exhibited 11-fold better activity than sclareol against DU-145 cancer cell line. Among the tested cancer cell lines, A549 and HeLa cells were sensitized ineffectively by the synthesized sclareol derivatives. The results suggest that these novel sclareol derivatives can inhibit the growth of various cancer cell lines at nano and micromolar concentrations and may be promising new experimental anticancer agents. Moreover the cytotoxicity of these compounds appears to be selective as A549 and HeLa cells tolerated substantially higher doses of these compounds than PC-3 and DU-145 cancer cell lines. Additionally, SS-12 exhibiting the most effective cytotoxicity against all the tested cancer lines displayed significant anti-metastatic activity by abrogating the motility and invasion potential of PC-3 cancer cell line. Our results also demonstrate that SS-12 appreciably promotes autophagy by induction of autophagosomes and acidic vesicular organelles in PC-3 cells. Work is currently in progress in our laboratory in order to elucidate the exact mechanism of the anticancer action of these analogs and results will be reported in due course of time. However, more studies are needed to establish sclareol and its analogs as a potential class of natural products/analogs for prospective antitumour use.
3b.6. Experimental

All reagents for chemical synthesis were obtained from Sigma Aldrich and the solvents used in reactions were distilled and dried prior to use. All the chemical reactions were monitored by TLC on 0.25 mm silica gel 60 F254 plates (E. Merck) and the spots were visualized at 366 and 254 nm in a UV chamber. Purification of compounds was carried out by column chromatography using silica gel 60-120 mesh stationary phase. \(^1\)H NMR and \(^{13}\)C NMR spectra (with chemical shifts expressed in \(\delta\) and coupling constants in Hertz) were recorded on Bruker DPX 400 instrument using CDCl\(_3\) as the solvent with TMS as internal standard. High resolution mass spectra (HRMS) were recorded on Agilent Technologies 6540 instrument.

RPMI-1640 medium, Penicillin, streptomycin, rapamycin, staurosporine, fetal calf serum, sodium bicarbonate, phosphate buffer saline, trypsin, gentamycin sulphate, tryphan blue, ethanol, DMSO, paraformaldehyde, monodansylcadaverine and acridine orange were purchased from Sigma Chemicals Co. Glacial acetic acid from Fischer scientific, PBS and trichloroactetic acid (TCA) from Merck specialties private limited. All the human cancer cell lines were obtained from National Center for Cell Science, Ganeshkhind, Pune-4111007 (India) and National Cancer Institute, Biological Testing Branch DTP/DCTD/NCI, Frederick Cancer Research and Development Center, Fairview Center, Suite 205, 1003 West 7th Street, Frederick, MD 21701-8527 (USA).

3b.6.1. General procedure for the synthesis of aryl analogs of Sclareol (SS-1 to SS-15)

Under inert atmosphere, arylboronic acid (1.5 eq), Pd(OAc)\(_2\) (10 mol%), Cu(OAc)\(_2\) (2.0 eq) and NaOAc (3.0 eq) were combined with scclareol (1.0 eq) in a 25 ml round-bottom flask. Dimethyl formamide (DMF, 5.0 ml) was added and the flask was sealed with a septum. The resulting mixture was placed in an oil bath at 80 °C for 3-6 h. Progress of reaction was monitored using TLC at regular intervals. After the completion of reaction, the product was extracted with (3 \(\times\) 20 ml) EtOAc. The combined organic layer was dried over sodium sulphate and concentrated under vacuum. The resulting crude product was purified by column chromatography on silica gel to give the required cross-coupled derivative (SS-1 - SR-15) in 80-85 % yield.
3b.6.1.1. 15-(Phenyl)-sclareol SS-1

Colourless liquid; \( ^1H \) NMR (400 MHz, CDCl\(_3\)) \( \delta \): 7.38 (1H, d, \( J = 8.0 \) Hz), 7.29 (2H, t, \( J = 8.0 \) Hz), 7.22 (2H, m), 6.61 (1H, d, \( J = 16.0 \) Hz), 6.29 (1H, d, \( J = 16.0 \) Hz), 1.82 (1H, m), 1.76 (1H, m), 1.60 (m, 6H), 1.42 (1H, m), 1.41 (3H, s), 1.38 (1H, m), 1.28 (4H, m), 1.16 (3H, s), 0.97 (2H, m), 0.85 (3H, s), 0.78 (6H, s); \( ^{13}C \) NMR (126 MHz, CDCl\(_3\)) \( \delta \): 137.85, 137.23, 129.63, 128.55 (2C), 127.22, 126.39(2C), 75.00, 73.62, 61.39, 56.05, 45.18, 44.36, 41.98, 39.70, 39.25, 33.39, 33.24, 27.54, 24.29, 21.49, 20.51, 19.13, 18.43, 15.36; HR-ESIMS: 385.3102 (Calculated for C\(_{26}H_{40}O_2\), 384.3028).

3b.6.1.2. 15-(3-Nitrophenyl)-sclareol SS-2

Yellowish liquid; \( ^1H \) NMR (400 MHz, CDCl\(_3\)) \( \delta \): 8.24 (1H, s), 8.06 (1H, d, \( J = 8.0 \) Hz), 7.66 (1H, d, \( J = 8.0 \) Hz), 7.47 (1H, t, \( J = 8.0 \) Hz), 6.69 (1H, d, \( J = 16.0 \) Hz), 6.43 (1H, d, \( J = 16.0 \) Hz), 1.80 (2H, m), 1.59 (4H, m), 1.43 (1H, m), 1.39 (3H, s), 1.36 (1H, m), 1.26 (2H, m), 1.21 (2H, m), 1.17 (3H, s), 1.14 (1H, m), 0.96 (2H, m), 0.88 (1H, m), 0.85 (3H, s), 0.79 (3H, s), 0.78 (3H, s); \( ^{13}C \) NMR (101 MHz, CDCl\(_3\)) \( \delta \): 148.59, 141.52, 139.25, 132.34, 129.37, 124.30, 121.68, 120.76, 75.12, 73.44, 61.38, 56.11, 44.94, 44.44, 41.95, 39.76, 39.25, 33.36, 33.21, 27.13, 24.29, 21.47, 20.50, 18.96, 18.40, 15.32; HR-ESIMS: 430.2942 (Calculated for C\(_{26}H_{39}O_4\), 429.2879).

3b.6.1.3. 15-(2,5-Dimethylphenyl)-sclareol SS-3

Cream coloured liquid; \( ^1H \) NMR (400 MHz, CDCl\(_3\)) \( \delta \): 7.23 (1H, s), 7.03 (1H, d, \( J = 8.0 \) Hz), 6.96 (1H, d, \( J = 8.0 \) Hz), 6.77 (1H, d, \( J = 16.0 \) Hz), 6.15 (1H, d, \( J = 16.0 \) Hz), 2.31 (6H, s), 1.80 (3H, m), 1.64 (4H, m), 1.44 (2H, m), 1.38 (3H, s), 1.32 (1H, m), 1.25 (4H, m), 1.16 (3H, s), 0.98 (2H, m), 0.86 (3H, s), 0.79 (3H, s), 0.78(3H, s); \( ^{13}C \) NMR (126 MHz, CDCl\(_3\)) \( \delta \): 139.08, 136.21, 135.35, 132.43, 130.13, 127.90, 126.35, 124.50, 74.87, 73.76, 61.25, 56.09, 45.31, 44.41, 41.99, 39.74, 39.28, 33.40, 33.25, 28.03, 24.30, 21.51, 21.08, 20.52, 19.48, 19.31, 18.43, 15.37; HR-ESIMS: 413.2678 (Calculated for C\(_{28}H_{44}O_2\), 412.3341).
3b.6.1.4. 15-(Isoquinolin-4-yl)-sclareol SS-4

Brownish liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 8.52 (1H, s), 8.27 (1H, s), 8.02 (1H, d, $J = 8.0$ Hz), 7.96 (1H, d, $J = 8.0$ Hz), 7.55 (2H, m), 6.77 (1H, d, $J = 16.0$ Hz), 6.56 (1H, d, $J = 16.0$ Hz), 1.82 (3H, m), 1.61 (5H, m), 1.40 (1H, m), 1.32 (3H, s), 1.25 (2H, m), 1.18 (5H, m), 0.98 (3H, m), 0.88 (3H, s), 0.79 (6H, s); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 152.87, 143.22, 134.68, 132.10, 130.08, 128.88, 129.98, 128.03, 125.94, 123.08, 121.22, 75.10, 73.42, 61.31, 56.13, 44.94, 44.41, 41.90, 39.72, 39.27, 33.30, 33.21, 27.13, 24.29, 21.47, 21.04, 18.92, 18.30, 15.33; HR-ESIMS: 436.3207 (Calculated for C$_{29}$H$_{41}$NO$_2$, 435.3137).

3b.6.1.5. 15-(2,6-Dimethoxyphenyl)-sclareol SS-5

Yellowish liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.22 (1H, m), 7.10 (1H, m), 6.92 (1H, m), 6.80 (1H, d, $J = 16.0$ Hz), 6.53 (1H, d, $J = 16.0$ Hz), 3.82 (6H, s), 1.89 (2H, m), 1.66 (4H, m), 1.52 (2H, m), 1.48 (3H, s), 1.36 (2H, m), 1.33 (4H, m), 1.26 (3H, s), 1.12 (2H, m), 0.92 (3H, s), 0.88 (3H, s), 0.84(3H, s); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 156.90, 156.86, 125.97, 125.85, 119.02, 114.71, 102.59 (2C), 75.12, 73.44, 61.38, 56.11, 54.94, 44.94, 44.44, 41.95, 39.76, 39.25, 33.36, 33.21, 29.69, 27.13, 24.29, 21.47, 20.50, 18.96, 18.40, 15.32; HR-ESIMS: 445.3324 (Calculated for C$_{28}$H$_{44}$O$_4$, 444.3240).

3b.6.1.6. 15-(2,3-Dimethylphenyl)-sclareol SS-6

Colourless liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.23 (1H, m), 7.04 (2H, m), 6.86 (1H, d, $J = 16.0$ Hz), 6.08 (1H, d, $J = 16.0$ Hz), 2.28 (3H, s), 2.24 (3H, s), 1.76 (3H, m), 1.59 (5H, m), 1.41 (1H, m), 1.38 (3H, s), 1.26 (3H, m), 1.16 (3H, s), 1.13 (2H, m), 0.90 (2H, m), 0.85 (3H, s), 0.78 (6H, s); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 139.76, 136.98, 136.68, 134.04, 129.56, 128.82, 125.47, 124.19, 74.90, 73.77, 61.39, 56.08, 45.37, 44.35, 42.00, 39.74, 39.28, 33.40, 33.24, 27.81, 24.25, 21.50, 20.61, 20.51, 19.28, 18.44, 15.54, 15.37; HR-ESIMS: 413.2683 (Calculated for C$_{28}$H$_{44}$O$_2$, 412.3341).
3b.6.1.7. 15-(6-Chloropyridin-3-yl)-sclareol \(\text{SS-7}\)

Brown liquid; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta:\) 8.36 (1H, d, \(J = 2.0\) Hz), 7.66 (1H, dd, \(J = 2.0, 8.0\) Hz), 7.25 (1H, d, \(J = 8.0\) Hz), 6.59 (1H, d, \(J = 16.0\) Hz), 6.33 (1H, d, \(J = 16.0\) Hz), 1.83 (2H, m), 1.75 (2H, m), 1.56 (4H, m), 1.42 (1H, m), 1.37 (3H, s), 1.24 (4H, m), 1.17 (3H, s), 1.10 (1H, m) 0.93 (2H, m), 0.85 (3H, s), 0.78 (6H, s); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta:\) 149.58, 147.89, 141.22, 135.57, 132.04, 124.04, 121.66, 75.14, 73.49, 61.30, 56.09, 44.89, 44.44, 41.96, 39.73, 39.24, 33.37, 33.22, 27.22, 24.32, 21.46, 20.49, 18.99, 18.40, 15.31; HR-ESIMS: 420.2683 (Calculated for C\(_{25}\)H\(_{38}\)ClNO\(_2\), 419.2591).

3b.6.1.8. 15-[4-(t-Butyl)phenyl]-sclareol \(\text{SS-8}\)

Creamish liquid; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta:\) 7.34 (2H, d, \(J = 8.0\) Hz), 7.25 (2H, d, \(J = 8.0\) Hz), 6.58 (1H, d, \(J = 16.0\) Hz), 6.26 (1H, d, \(J = 16.0\) Hz), 1.82 (3H, m), 1.65 (4H, m), 1.42 (1H, m), 1.37 (3H, s), 1.31 (9H, s), 1.25 (3H, m), 1.21 (2H, m), 1.15 (3H, s), 1.10 (1H, m), 0.93 (2H, m), 0.85 (3H, s), 0.78 (6H, m); \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta:\) 150.28, 137.01, 134.43, 126.40, 126.27, 126.09, 125.47, 114.79, 74.92, 73.65, 61.44, 56.04, 45.28, 44.37, 41.98, 39.70, 39.25, 34.55, 33.39, 33.24, 31.33 (3C), 27.71, 24.30, 21.49, 20.51, 19.19, 18.43, 15.37; HR-ESIMS: 441.3703 (Calculated for C\(_{30}\)H\(_{48}\)O\(_2\), 440.3654).

3b.6.1.9. 15-(2,3-Dimethoxyphenyl)-sclareol \(\text{SS-9}\)

Yellowish liquid; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta:\) 7.06 (1H, m), 7.00 (1H, t, \(J = 8.0\) Hz), 6.88 (1H, d, \(J = 16.0\) Hz), 6.80 (1H, dd, \(J = 2.0, 8.0\) Hz), 6.32 (1H, d, \(J = 16.0\) Hz), 3.86 (3H, s), 3.81 (3H, s), 1.79 (2H, m), 1.59 (4H, m), 1.42 (2H, m), 1.38 (3H, s), 1.25 (3H, m), 1.19 (2H, m), 1.15 (3H, s), 1.10 (1H, m), 0.92 (2H, s), 0.85 (3H, s), 0.77 (6H, s); \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta:\) 152.98, 146.57, 139.56, 131.51, 124.00, 120.75, 118.30, 110.93, 74.87, 73.76, 61.44, 60.85, 56.02, 55.79, 45.25, 44.19,

3b.6.1.10. 15-(2,6-Dimethylphenyl)-sclareol SS-10

Colourless liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.13 (1H, t, $J$ = 8.0 Hz), 6.97 (2H, t, $d$ = 8.0 Hz), 6.52 (1H, d, $J$ = 16.0 Hz), 5.75 (1H, d, $J$ = 16.0 Hz), 2.29 (6H, s), 1.74 (2H, m), 1.62 (4H, m), 1.42 (2H, m), 1.38 (3H, s), 1.26 (4H, m), 1.15 (3H, s), 1.13 (1H, m), 0.95 (3H, m), 0.86 (3H, s), 0.79 (3H, s), 0.78 (3H, s); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 137.05, 135.91 (2C), 128.60, 127.64 (2C), 126.34, 124.14, 74.88, 73.85, 61.57, 56.02, 45.47, 44.22, 41.98, 39.69, 39.27, 33.40, 33.25, 27.65, 24.20, 21.52, 21.07 (2C), 20.49, 19.38, 18.43, 15.43; HR-ESIMS: 413.2698 (Calculated for C$_{28}$H$_{44}$O$_2$, 412.3341).

3b.6.1.11. 15-(4-Bromophenyl)-sclareol SS-11

Yellow liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.55 (2H, d, $J$ = 8.0 Hz), 7.50 (2H, d, $J$ = 8.0 Hz), 6.64 (1H, d, $J$ = 16.0 Hz), 6.34 (1H, d, $J$ = 16.0 Hz), 1.80 (4H, m), 1.62 (3H, m), 1.39 (3H, s), 1.36 (1H, m), 1.25 (4H, m), 1.16 (3H, s), 1.10 (1H, m), 0.94 (3H, m), 0.85 (3H, s), 0.78 (6H, s); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 136.76, 131.88 (2C), 128.47, 126.93 (2C), 125.83, 121.44, 75.03, 73.61, 61.50, 56.07, 45.20, 44.33, 41.99, 39.71, 39.26, 33.40, 29.74, 27.31, 24.25, 21.50, 20.51, 19.09, 18.45, 15.39; HR-ESIMS: 463.2197 (Calculated for C$_{28}$H$_{39}$BrO$_2$, 462.2133).

3b.6.1.12. 15-(4-Fluorophenyl)-sclareol SS-12

Colourless liquid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.33 (2H, t, $J$ = 8.0 Hz), 6.99 (2H, t, $J$ = 8.0 Hz), 6.57 (1H, d, $J$ = 16.0 Hz), 6.20 (1H, d, $J$ = 16.0 Hz), 1.77 (3H, m), 1.61 (3H, m), 1.42 (1H, m), 1.37 (3H, s), 1.26 (4H, m), 1.16 (3H, s), 1.10 (1H, m), 0.93 (3H, m), 0.85 (3H, s), 0.78 (6H, m); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 161.11, 137.63, 133.37, 127.83, 127.77, 125.28, 115.47, 115.30, 75.10, 73.53, 61.45, 56.07,
45.14, 44.31, 41.97, 39.69, 39.25, 33.38, 33.23, 29.73, 24.22, 21.48, 20.49, 19.04, 18.42, 15.36; HR-ESIMS: 403.2996 (Calculated for C₂₇H₃₉FO₂, 402.2934).

3b.6.1.13. 15-(10-Bromoanthracen-9-yl)-sclareol SS-13

Yellow liquid; ¹H NMR (400 MHz, CDCl₃) δ: 8.56 (2H, d, J = 8.0 Hz), 8.33 (2H, d, J = 8.0 Hz), 7.59 (2H, t, J = 8.0 Hz), 7.26 (2H, t, J = 8.0 Hz), 6.85 (2H, d, J = 16.0 Hz), 6.08 (2H, d, J = 16.0 Hz); 1.82 (2H, m), 1.76 (2H, m), 1.57 (4H, m), 1.44 (1H, m), 1.38 (3H, s), 1.24 (4H, m), 1.18 (3H, s), 1.10 (1H, m) 0.93 (2H, m), 0.85 (3H, s), 0.78 (6H, s); ¹³C NMR (126 MHz, CDCl₃) δ: 133.96, 130.30, 130.24, 129.57 (2C), 128.03 (2C), 126.91 (2C), 126.64 (2C), 125.41 (2C), 122.06, 115.46 (2C), 75.02, 73.74, 61.61, 56.02, 44.88, 44.19, 41.96, 39.64, 39.25, 33.38, 33.23, 26.84, 24.15, 21.50, 20.48, 19.02, 18.43, 15.40; HR-ESIMS: 563.2508 (Calculated for C₃₄H₄₄BrO₂, 562.2446).

3b.6.1.14. 15-(4-Carboxyphenyl)-sclareol SS-14

Colourless liquid; ¹H NMR (400 MHz, CDCl₃) δ: 7.93 (2H, d, J = 8.0 Hz), 7.34 (2H, d, J = 8.0 Hz), 6.61 (1H, d, J = 16.0 Hz), 6.39 (1H, d, J = 16.0 Hz), 1.77 (3H, m), 1.60 (3H, m), 1.42 (2H, m), 1.37 (3H, s), 1.25 (4H, m), 1.19 (3H, s), 0.94 (3H, m), 0.85 (3H, s), 0.79 (3H, s), 0.78 (3H, s); ¹³C NMR (126 MHz, CDCl₃) δ: 170.45, 142.35, 140.99, 130.39 (2C), 128.06, 126.25 (2C), 125.58, 75.65, 73.67, 61.52, 56.01, 44.97, 43.90, 41.95, 39.66, 39.27, 33.36, 33.22, 29.72, 26.48, 24.00, 21.48, 20.43, 18.41, 15.46; HR-ESIMS: 429.2987 (Calculated for C₂₇H₄₀O₄, 428.2927).

3b.6.1.15. 15-(4-Phenoxyphenyl)-sclareol SS-15

Colourless liquid; ¹H NMR (400 MHz, CDCl₃) δ: 7.23 (4H, m), 6.97 (4H, m), 6.57 (1H, d, J = 16.0 Hz), 6.20 (1H, d, J = 16.0 Hz), 1.77 (2H, m), 1.58 (4H, m), 1.40 (5H, m), 1.36 (3H, s), 1.24 (4H, m), 1.10 (3H, m), 0.87 (3H, s), 0.80 (3H, s), 0.79 (3H, s); ¹³C NMR (126 MHz, CDCl₃) δ: 156.55, 155.98, 135.3,
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132.43, 130.29 (2C), 130.06 (2C), 128.68, 124.55, 121.25 (2C), 120.41 (2C), 75.85, 72.97, 61.55, 56.20, 44.88, 43.85, 41.75, 40.06, 39.45, 33.44, 33.32, 29.78, 26.56, 24.12, 20.89, 20.03, 18.26, 15.88; HR-ESIMS: 477.3364 (Calculated for C$_{32}$H$_{44}$O$_{3}$, 476.3290).

3b.6.2. MTT Cytotoxicity assay

All the compounds were evaluated against a panel of four different human cancer cell lines viz. prostate (PC-3 and DU-145), lung (A549) and cervical (HeLa) using MTT assay in a 96 well plate. Cells were routinely maintained in RPMI 1640 (Sigma Aldrich) supplemented with 10% FBS (Merck) and 1% penicillin G and streptomycin (Sigma Aldrich) at 37 °C in a humidified incubator with 5% CO$_{2}$ and were subcultured at 1:5 ratio once a week. For antiproliferative activity, compounds were dissolved in cell culture grade DMSO. Briefly, cells (10$^{4}$ cells/well) were cultured in 96 well tissue culture plates and treated with different concentrations of compounds for 48 h. At the end of incubation, 20 µL of MTT (2.5 mg/mL) was added to the wells and incubated for 4 h. Absorbance was recorded at 570 nm using Eliza Plate Reader. Inhibition of formation of coloured MTT formazan was taken as an index of cytotoxicity activity. The IC$_{50}$ values on the cancer cells of different tissue origin used for screening were determined by non-linear regression analysis using graph pad software.$^{71}$

3b.6.2. Scratch motility (wound healing) assay

PC-3 cells were seeded in a 6 well plate at a concentration of (5.5 x 10$^{5}$ cells/well) and allowed to form a confluent monolayer for 24 h, it was then serum starved for 24 h. After that the monolayer was scratched with a sterile pipette tip (200 µL), washed with serum free medium to remove floated and detached cells and photographed (time 0 h). Cells were successively treated in medium containing low serum (1.0%) in presence of different concentrations of SS-12 (100, 200, and 300 nM) along with vehicle DMSO for 24 h. Wounded areas were progressively photographed with Olympus C-7070 with 700M camera (100x magnification). The percentage of wound closure was estimated by the following equation:

\[
\text{Wound closure } \% = [1-(\text{wound area at } t_{1}/\text{wound area at } t_{0}) \times 100]
\]

where $t_1$ is the time after wounding and $t_0$ is the time immediately after wounding.

3b.6.3. Detection of Autophagic vacuoles with Monodansylcadaverine (MDC)

PC-3 cells were cultured in 8-well chamber slides (155411 LabTek, NUNC) for 12 h with or without 3-AWA and positive control Rapamycin. Following treatments, autophagic vacuoles were detected by incubating the cells at 37 °C for 10 min in PBS containing 50 µM MDC, a widely used special tracer for autophagic vacuoles. After incubation, cells were washed four times with PBS and immediately analysed at 380 nm excitation and 525 nm emission by inverted fluorescence microscope (Zeiss LSM-510). Cells having 15-20 bright dots/cell were considered MDC positive. Graphs represent quantification of three independent experiments and a total of 100 cells were counted per treatment.

3b.6.4. Detection of acid vesicular organelles (AVOs) with acridine orange (AO)

Acridine orange was used to detect and quantify the formation of acid vesicular organelles (AVOs), by fluorescent microscopy and flow cytometry. AO is an acidotropic fluorescent dye that stain DNA and cytoplasm bright green (AO⁻) and when protonated in the presence of acid compartments it fluoresces bright red (AO⁺). PC-3 cells were cultured in presence or absence of 3-AWA for 12 h and after incubation cells were stained with AO at (1 µg/ml) for 15 min at 25°C followed by thorough washing with PBS. The traces of AVOs were detected by bright red fluorescence, analyzed in the fluorescence microscope equipped with a 490 nm band-pass blue excitation filters and a 515 nm long pass-barrier filter (Zeiss LSM-510, Carl Zeiss, Germany). Graphs represent quantification of three independent experiments and a total of 100 cells were counted per treatment.
Representative Graphs

$^1$H & $^{13}$C NMR spectra of SS-1
$^1$H & $^{13}$C NMR spectra of SS-2
$^1\text{H}$ & $^{13}\text{C}$ NMR spectra of SS-6
\[ ^1H \text{ } \& \text{ } ^{13}C \text{ NMR spectra of SS-7} \]
1H & 13C NMR spectra of SS-12