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Chemical examination of three Indian medicinal plants and their hair growth evaluation studies

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ABSTRACT

*Bioassay guided isolation and characterization of hexane extract of folklore medicinal plants of India, yielded three aromatic active compounds. These are: thymol (**1**) from the seeds of Trachyspermum ammi, β-asarone (**2**) from the rhizomes of Acorus calamus and eugenol (**3**) from the flower buds of Syzygium aromaticum. Structures of the compound were established based on its physical and spectral data (UV, IR, ^{1}H & ^{13}C NMR and Mass) and co-comparison with an authentic samples. All compounds have been subjected for in-vivo hair growth studies and showed good to moderate activity when compared with control compound, minoxidil.*

Key words: Folklore medicinal plants, active compounds, hair growth, minoxidil.

INTRODUCTION

Hair growth and hair coverage are the long felt needs of every human being who suffer from hair fall/ hair loss problem. The other unmet requirements are reduction in hair fall, thinning, hair length and density. The hair fall or decrease in hair density could be due to several reasons. These includes aging, scalp infections, production of excessive sebum, hormonal imbalance, post effect of certain diseases such as typhoid, jaundice, malaria, cardiovascular, smoking, conversion of testosterone to dihydrotestosterone, reduction in anagen hair either due to anagen or telogen effluvium are the major contributing factors for hair fall /loss problems. Additionally, in the case of alopecia, an autoimmune disorders do play a major role in hair fall/loss. Further poor nutrition and other general physical reasons are also known to add to hair fall/loss problem.[1] Various groups have been made good contributions to develop products to address the hair fall /loss issue and also variety of hair growth promoters have been used to prevent and treat the physiological symptoms of various types of alopecia which may result in balding and thinning of the hair. Such hair growth promoters have been proposed to facilitate circulation of the blood, activate the hair matrix cells, inhibit the secretion of lipids from the scalp skin, and supplement nutrition to the hair. An oral administration drug, Finasteride, which inhibits the action of 5α-reductase to control the production of dihydrotestosterone, a potent male hormone was approved by USFDA and is being marketed as a hair growth stimulant.[2] A few synthetic molecules/ compositions have been developed for external applications. These are 4-pyrrolidine 2,6-dimainopyrimidine 1-oxide [2], glyceride pentadecanoate, tocopherol acetate, nicotinamide and salicylic acid,[3] minoxidil, azelaic acid and its derivatives, 4-methyl-4-azasteroid.[4] Several herbal ingredients either single compounds or combinations have been identified to address the hair growth/ fall problems. Ex. oxycanthine with saponin,[4] nardin and jatamansic acid,[5] pentadecane,[6] lupane triterpenes, derivatives of lupane, oleanane, ursane triterpenes.[7]

In continuation of our interest on bio-active secondary metabolites from medicinal plants for personal care applications,[8-21] we have undertaken three folklore medicinal plants for isolation and characterization of hair growth active compounds. These are seeds of *Trachyspermum ammi*, rhizomes of *Acorus calamus* and flower buds of *Syzygium aromaticum.* In this article, we report the isolation and structure elucidation of thymol (**1**) from the seeds of *Trachyspermum ammi*, β-asarone (**2**) from rhizomes of *Acorus calamus* and eugenol (**3**) from the flower buds of *Syzygium aromaticum.* Structure of these compounds were established based on their physical, spectral data and comparison with literature data and co-TLC with an authentic compounds. The present paper also describes of three biologically active compounds and their hair growth studies by in-vivo methods.

MATERIALS AND METHODS

General procedures

Melting point was reported uncorrected. IR spectra were recorded on a Shimadzu Prestige 21 FT IR. Optical rotation was measured on Autopol IV, serial no. 80305. UV spectra were recorded on Shimadzu UV spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX 400 with TMS as an internal standard. Mass spectra were recorded on Jeol SX 102/DA 600 mass spectrometer. GC spectra were recorded on Shimadzu GC 17A with capillary column using FID detector. Column chromatography (CC) was carried on a silica gel column (100-200 mesh). Purity of the samples was checked by TLC on pre-coated aluminum sheets, silica gel 60 F_{254} (20 X 20 cm, 0.2mm thickness, Merck) and compounds were detected under UV light (254 & 366 nm) and spraying with 5% sulphuric acid in methanol followed by heating the plates at 110^{o}C for 5 min. The chemical shift values are reported in ppm (δ) units and the coupling constants (*J*) are in Hz. The standard compounds were obtained from M/s. Sigma Aldrich, USA.

Plant material

The seeds of *Trachyspermum ammi* Linn., rhizomes of *Acorus calamus* Linn., and flower buds of *Syzium aromaticum* were obtained from local market in Chennai, Tamil Nadu in August 2008. The taxonomical identification was done by Dr. P. Santhan, taxonomist, M/s. Durva Herbal Centre, Chennai, Tamil Nadu, India. All voucher specimens of the species were deposited in M/s. CavinKare Research Centre, Chennai, India.

Extraction and Isolation procedure

1). The dried seeds of *T. ammi* Linn., (200 g) was coarsely powdered, extracted with hexane (1.0 L) by using soxhlet apparatus. The dilute extract was concentrated by using rotary evaporator under reduced pressure at ~40^{o}C to get 2.32 g crude hexane extract. The hexane extract was showed reduction in hair growth time by 27%. Further, 500g of seeds were extracted with hexane to get 6.0g of hexane extract. Part of the extract (5 g) was dissolved in chloroform and adsorbed on silica gel (100-200 mesh, 9g). 147g of silica gel (100-200 mesh, Acme) was packed in glass column, followed by loaded the adsorbed silica gel which was eluted with chloroform, chloroform: ethyl acetate (9:1, 8:2) and finally with ethyl acetate. A total of 40 fractions were collected, combined homogeneous fractions based on TLC and divided into three major fractions, Fr. A (0.34g), Fr. B (3.57g) and Fr. C (0.57g). Fraction B was showed one major spot on TLC along with some minor impurity. It was further purified by silica column using hexane: chloroform (1:1) as an eluent to get pure compound, 1.8g which came as pale yellow colored liquid. The compound was kept at low temperature and became solid, mp: 49-50^{o}C (**1**). The compound was identified as thymol based on NMR spectral data.[22] The compound has been submitted for *in-vivo* hair growth studies and showed 37% reduction in hair growth completion time.

2). The air dried rhizomes of *Acorus calamus* Linn., (650g) were crushed into small pieces and extracted with hexane through soxhlet apparatus for about 8 hrs. The dilute extract was concentrated under reduced pressure to get 54g of crude extract. Part of the extract (5g) was adsorbed on silica gel and separated major compound, 3.75 g which came as pale yellow colored liquid (**2**).[23] The compound has been submitted for *in-vivo* hair growth studies and showed 10% reduction in hair growth completion time.

3). The air dried flower buds of *Syzygium aromaticum* Linn., (100g) were crushed and extracted with hexane through soxhlet apparatus for about 8hrs. The dilute extract was concentrated under reduced pressure through rotary evaporator to obtain 3.31g of crude hexane extract. The crude extract was submitted for *in-vivo* hair growth studies and showed reduction in hair growth time by 10%. The crude extract (3.2g) showed one major spot on TLC, which was adsorbed on silica gel and purified by small silica gel column using hexane: chloroform mixture to get pure

compound, eugenol (**3**, 2.6g).[24] It has been confirmed by comparing with an authentic compound obtained from M/s. Sigma Aldrich, USA. The compound showed 10% reduction in hair growth completion time.

Compound 1 (**Thymol**): Colorless crystals, mp:49-50^{o}C; UV ($CHCl_3$) nm: 273, IR (nujol) cm^{-1}: 3253 (hydroxyl), 2962, 1089, 806; ^{1}H NMR ($CDCl_3$, 400MHz): δ 1.15 (6H, d, J=6.9Hz), 2.18 (3H, s), 3.08 (1H, m), 4.84 (1H, OH), 6.47 (1H, s), 6.65 (1H, d, J=7.7Hz), 7.03 (1H, d, J=7.8Hz), ^{13}C NMR ($CDCl_3$, 100 MHz): δ 22.8, 22.6, 22.6, 26.6, 116.0, 121.6, 126.2, 131.3, 136.5, 152.4; EIMS (m/z) : 150 (M^+, 0.4%)

Compound 2 (β-Asarone): Pale yellow color liquid; ^{1}H NMR ($CDCl_3$, 400MHz): δ6.82 (1H, s, H-6), 6.54 (1H, s, H-3), 6.51 (1H, dd, J=15.7 Hz and 1.5 Hz, H-1′), 5.77 (1H, dq, J=6.4 Hz and 15.7 Hz, H-2′), 3.87, 3.82 and 3.80 (s, 3H, each, 3-OCH_3) and 1.84 (3H, dd, J=6.4 Hz and 1.5 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ151.3 (C-2), 148.4 (C-4), 142.3 (C-5), 125.4 (C-1′), 124.6 (C-2′), 118.1 (C-1), 114.2 (C-6), 97.5 (C-3), 56.4, 56.3 & 55.8 (3×OCH_3) and 14.4 (C-3′); EIMS m/z 208 (M^+, 100), 193 (46), 165 (24).

RESULTS AND DISCUSSION

The compound **1** was isolated as colorless crystals. It was readily recognized as aromatic compound based on its preliminary spectral data. The molecular formula has been fixed as $C_{10}H_{14}O$ based on its mass spectrum. Its UV spectrum showed a band at 273 indicating that molecule having conjugation. The IR spectrum showed the presence of hydroxyl group at 3253cm^{-1}. The ^{1}H NMR spectrum clearly showed the presence of an isopropyl group i.e., two methyls at δ1.15 as doublet (J=6.9 Hz), one benzylic methyl at δ 2.18 (3H, s), one methine proton at δ3.08 (m) and three aromatic protons at δ 6.47 (s), 6.65 (1H, d, J=7.7Hz), 7.03 (1H, d, J=7.8Hz). Further, the spectrum also showed one D_20 exchangeable protons at δ 4.84 as singlet indicating that the molecule having one phenolic hydroxyl group. The carbon spectrum showed a total of ten carbons, off which. 4 carbons belongs to aliphatic in nature (δ 22.8, 22.6, 22.6, 26.6) and six aromatic carbons at δ 116.0, 121.6, 126.2, 131.3, 136.5, 152.4. Its mass spectrum showed molecular ion peak at m/z 150. Based on the available data and literature information, by direct comparison with an authentic compound obtained from M/s. Sigma Aldrich, USA and also GC analysis, the compound has been identified as thymol which was reported from the different species of *Thymus* genus.[22] The compound has been reported for anti-fungal[25] and anti-bacterial activities.[26]

The compound **2** was isolated as pale yellow color liquid. It was readily recognized as aromatic derivative based on its spectral data. Its molecular formula has been fixed as $C_{12}H_{16}O3$ based on its mass spectrum. Its UV spectrum showed three bands at 245, 272 and 303nm indicating that molecule having an extending conjugation. The IR spectrum did not show any characteristic peaks. The ^{1}H NMR spectrum clearly showed the presence of one double bond methyl group at δ1.84 as dd (J=6.4, 1.54 Hz), three methoxyl groups at δ 3.80, 3.82, 3.87 (each as singlet), two olefinic protons at δ 6.51 (1H, dd, J=15.7 Hz and 1.5 Hz), 5.77 (1H, dq, J=6.4 Hz and 15.7 Hz,), and two aromatic protons at δ 6.54 (s) and 6.82 (s). The carbon spectrum showed a total of twelve carbons, off which one methyl carbon appeared at δ 14.4, three methoxyl carbon signals at δ 55.8, two olefinic carbons δ 124.6 and 125.4, and six aromatic carbon signals. Based on the above data, a through literature search has been done. The compound data is well agreed with literature data[23] and it was identified as β-asarone. The compound has been reported for anti-microbial, anti-bacterial, anti-oxidant and insecticidal properties[28] and also improves cognitive function.[29]

Hair growth promotion studies:
The hair growth promotion activity of the crude extract, active fractions and isolated compounds along with carvacrol (**4**) were evaluated by using *in-vivo* animal mode. The hair growth was observed by visual method. The assay method is most precise.[5,6,30]

Animals: Female Wistar rats weighing 120-150 gm were used for the study. The animals were maintained in a clean cage and were provided with food and water ad libitum. The floor mat husk in each cage was removed and laid afresh on daily basis.

The hair on the dorsal portion of the body of each animal was depilated using a standard, commercially available depilatory cream. After removal of the hair, the skin was cleaned with distilled water and wiped with surgical spirit. Four centimeter square area in the depilated dorsal skin was marked with permanent ink marker. The animals which showed any skin irritant response to the depilatory were removed from the experiment and new animal was replaced.

The experimental animals were divided into three groups of 6 animals each. Group 1 animals were served as negative control without any treatment. The negative control comprised of the vehicle for application (only) without having any active compound or extract. To the Group 2 animals, 50 micro liters of 2 % Minoxidil (commercially available) was applied in the pre defined area. To the Group 3 animals, the Test samples (extract/ fractions/ pure compound/ s) prepared in liquid paraffin at 2 % was applied. The quantity of the extract used for the experiment was 50 micro liters per 4 cm sq area per animal. The application of the minoxidil and the test samples were continued for 30 days. The observations such as hair growth initiation time in days and hair growth completion time in days were recorded for all the animals on daily basis. The hair growth initiation time was defined as the presence of new hair in the treated site of 4 cm sq area. The hair growth completion time was defined as completing filling of hair in the treated site of 4 cm sq area in each animal which become indistinguishable from the adjacent untreated portion of the body. The average of hair growth initiation time and hair growth completion time was calculated for each group along with control animals. The untreated control for hair growth initiation time (HGIT) is 10 days and hair growth completion time (HGCT) is 30 days. The percentage reduction in hair growth completion time (% Reduction in HGCT) for the treatment is calculated by the formula given below and the results were recorded in Table 1.

$$\text{Calculation} = \frac{\text{HGCT in untreated control} - \text{HGCT in test sample}}{\text{HGCT in untreated control}} \times 100$$

Table 1: Comparison of % reduction in hair growth completion time

Sl. No.	Crude extract/ Active compound	Hair growth initiation time (in days)	Hair growth completion time (in days)	% Reduction in HGCT
1	Hexane crude extract of *Trachyspemum ammi*	8	22	27
2	Methanol crude extract of *Trachyspermum ammi*	10	26	13
3	Compound **1**	9	19	37
4	Compound **2**	10	27	10
5	Compound **3**	10	27	10
6	Compound **4**	8	19	37
7	Minoxidil	6	16	47
8	Untreated control	10	30	0

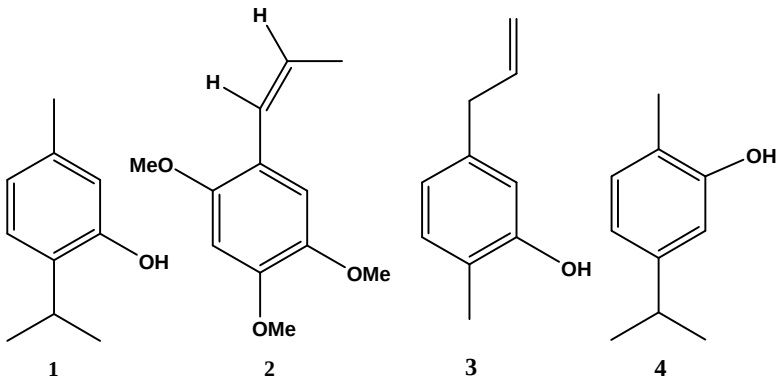

Figure 1: Hair growth active compounds

CONCLUSION

The present article describes the isolation of active compounds from folklore Indian medicinal plants. One of the compound, Thymol showed good hair growth promotion activity where as other two natural compounds showed marginal hair growth activity compared with untreated control. The compound, carvacrol (**4**), an isomer of thymol also showed similar activity with thymol. Structures of the compounds have been confirmed based NMR spectral data and also direct comparison with an authentic compound. In animal models, the compound, thymol showed hair growth promotion activity at 2% level, contradictory to Japanese scientists patent, which showed hair growth

inhibition in cell lines followed by mouse hair growth test.[31] Needs to be studied further to conclude the dose response.

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