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Antimicrobial and melanin synthesis inhibitory activities of the roots *of* *Inula racemosa* Hook f.

G. Venkateswara Rao*, M. Vijaya Bhaskar Reddy, R. Pirabakaran, M. S. L. Madhavi, K. Kavitha and T. Mukhopadhyay

Cavin Kare Research Centre, 12, Poonamalle Road, Ekkattuthangal, Chennai, Tamil Nadu, India

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ABSTRACT

*Bio-assay guided isolation of air dried roots of Inula racemosa yielded two known sesquiterpene lactones, dehydrocostus lactone (**1**) and costunolide (**2**). The structure of the compounds were established based on physicochemical data and co-comparison with an authentic compounds. The crude extract, fractions and isolated compounds were examined for antimicrobial properties and pure compounds were evaluated for melanin synthesis inhibition property.*

Key words: *Inula racemosa*, roots, sesquiterpene lactones, anti-microbial studies, melanin synthesis inhibition.

INTRODUCTION

In the early 1900 BC, the pharmaceutical industry did not exist and there were no synthetic drugs or medicines for treating various skin disorders; instead, people turned to nature and looked for natural remedies. This is true even today when we have such a variety of skin care products available to us, which claim to have the latest discovery in creating a flawless complexion. Consumers have become more sophisticated and more informed about various cosmetics and toiletries. They are always searching for products that give more value for money and deliver benefits. Now a days many suppliers are identifying and selling effective ingredients that exceed even the most sophisticated consumer's expectations.[1] The pigment, melanin is responsible and deciding factor for the color of human skin and hair, which is biosynthesized by the melansomes and distributed in the basal layer of the epidermis.[2] Melanin pigment is one of the host defensive factors against ultraviolet radiation from the sun light.[3] Still people used to get abnormal hyperpigmentation, such as lentigo, melasma or chloasma and ephelide.[4] Therefore, many cosmetic industries are showing great interest and searching for melanogenesis inhibitors and antimicrobial agents from natural sources to prevent the hyperpigmenation and fungal or bacterial infections.

Inula racemosa Hook f.., belongs to Asteraceae, found in north-western Himalayas at an altitudes of 1,500-4,200m. The fresh root is brownish externally and white internally, after drying it becomes grayish. The root is aromatic and irregularly wrinkled. The fresh roots of *I. racemosa* have strong aromatic odour similar to orris and camphor where as dried roots have less odour. Some times these roots are used in Kashmir as adultrant of costum (Roots of *Saussurea lappa*).[5] The roots are being used as stimulant, antiseptic, deodorant, anti-inflammatory, digestive, carminative, stomachic, expectorant, stomachic, uterine stimulant, expectorant and tonic.[6] The aqueous extract of the roots is given orally to control rheumatic and liver problems. The root extract has been reported for anti-allergic

activity in mice at 50mg/kg. The essential oil obtained from the roots exhibited antibacterial activity against several gram negative and gram positive bacteria.[7] Earlier reports on this plant occurring in different regions yielded variety of compounds, such as sesquiterpene lactones and steroids.[8] Some of the isolated constituents showed various biological properties. Allontolactone had showed strong anthelmintic activity, antiseptic, expectorant and diuretic.[5] Four compounds, septupnolide, 11α,13-dihydro-2α-hydroxy alantolactone, 11,13-dihydroivalin and isoalantolactone were showed moderate cytoxic activity, macrophyllilactone E and isoalantolactone showed potent invitro activities against the release of β-glucouronidase in rat polymorphonuclear leukocytes induced by platelet activating factor.[9] The compound, isoalantolactone showed repellent and toxic activities against rice weevil and strong phytotoxic effects on seed germination and seedling growth of wheat.[10] Indian Scientists reported that isoalantolactone has isolated from *I. racemosa* showed moderate antibacterial activity against two organisms, *P. aeruginosa* and *Bacillus cereus* whereas dihdyroalantolactone showed poor inhibition.[11]

In continuation of our interest on the isolation of biologically active molecules from medicinal plants for personal care applications,[12-20] we have undertaken the dried roots of *Inula racemosa.* In this paper, we report the isolation and structure elucidation of two known secondary metabolites, **1-2** (Figure.1) and their biological studies. Structure of the compounds were identified by NMR spectral data, comparison with literature values and co-comparison with an authentic compounds.

MATERIALS AND METHODS

General Procedures:
IR: Prestige 21 FT IR (Shimadzu); UV: Shimadzu UV spectrophotometer; NMR: ^{1}H and ^{13}C NMR (Bruker AMX 400); Mass spectrum: Jeol SX 102/DA 600 mass spectrometer. 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.

Plant material
The dried roots of *Inula racemosa* Hook f., (1.01 kg) were obtained from Bazar in December 2007 and authenticated by Dr. P. Santhan, Toxanamist, Durva Herbal Centre, Chennai, Tamil Nadu, India. A voucher specimen of the species was deposited in M/s. CavinKare Research Centre, Chennai, India.

Extraction and Isolation:
The dried roots of *Inula racemosa* Hook f., (1.0 g) were exhaustively extracted with methanol (3.0 L) by using soxhlet apparatus. The solvent was removed by rotary evaporator under reduced pressure at ~ 40^{o}C to get 55 g crude methanolic extract. The methanolic extract showed melanin inhibition activity in cell lines. The crude methanolic extract (55 g) was suspended in water, fractionated with hexane, chloroform, ethyl acetate and aq. residue to get corresponding fractions, 37.4g, 7.42g, 4.73g, and 3.2g, respectively. All four fractions were submitted for anti-microbial studies and hexane fraction showed good antimicrobial activity against the following organisms: *Malassizia furfur* (MIC:156.25µg/ml), *Staphylococus aureus* (MIC:156.25µg/ml) *Streptococus* mutans (MIC:156.25µg/ml), *Propyonibacterium acne* (MIC:250µg/ml) and *Cornybacterium xerosis* (MIC 312.5µg/ml). The other fractions showed weak activity. The hexane fraction (37g) was purified by using Vacuum Liquid Chromatography technique by using hexane, mixture of hexane:chloroform (80:20, 60:40, 40:60) and chloroform. A total of sixteen fractions were collected (250 ml each) and the fractions were analyzed by thin layer chromatography. Homogeneous fractions (similar TLC profile) were combined to obtain four major fractions, Fr. 1 (2.50 g), Fr. 2 (4.0 g), Fr.3 (6.0 g), Fr.4 (12.8g) and Fr.5 (2.5g) All fractions were submitted for biological activity and fractions, 1, Fr.3 and Fr.6 showed potent activity on melanin inhibition and moderate antimicrobial activity on various organisms. Fraction 1 was further purified by another small column of silica gel using hexane as an eluent to obtain colorless liquid. Its spot showed single on TLC but NMR spectrum showed mixture of two compounds, one major and another minor. Further, the mixture has been purified by another impregnated silica gel column to get pure one compound as colorless oil (900 mg). Initially its structure has been tentatively established and compound has been kept in refrigerator about two months. After two months entire compound has been decomposed and not fully characterized. Fr.3 was purified by silica gel column eluted with chloroform: ethyl acetate (9:1), obtained three sub-fractions. The sub-fraction 2 (2.01g), which was further purified by another impregnated silica gel column using hexane: ethyl acetate (9:1) to get colorless oil, which was kept in refrigerator for 2 days.. The compound has

been solidified and came as colorless solid (**1,** 1.50g). It has been identified as dehydrocostus lactone.[21, 22] Fraction 6 (2.5g) was obtained as almost pure by TLC, the fraction was dissolved in hexane, left at room temp and compound was separated as colorless crystals, costunolide (**2**, 1.2g). [21, 22] Compounds (**1, 2**) were submitted for melanin inhibition and antimicrobial studies. The hexane fraction was showed much better anti-microbial activity than the isolated compounds. Interestingly, the isolated compounds were showed good melanin inhibition activity at lower concentration. At higher concentration, the compounds are found to be toxic. The biological study results were mentioned in the Table 1 and 2.

RESULTS AND DISCUSSION

Compound 1 (Dehydrocostus lactone): Colorless solid,, mp: 55-57^{o}C, IR (υ cm^{-1}): 2927, 1765, 1267, 1145, 999; ^{1}H NMR ($CDCl_3$, 400MHz) : δ 1.46 (1H, m, H-8α), 1.93 (2H, m, H-2), 2.27 (2H, m, H-8β, 9β), 2.42 (3H, m, H-3, 9α), 2.87 (3H, m, H-1,5,7), 3.97 (1H, t, J=9.2Hz, H-6), 4.82 (1H, s, H-15β), 4.90 (1H, s, H-15α), 5.07 (1H, d, J=1.6 Hz, H-14β), 5.26 (1H, d, J=1.6 Hz, H-14α) 5.49 (1H, d, J=3.6 Hz, H-13β), 6.22 (1H, d, J=3.6 Hz, H-13α); ^{13}C NMR ($CDCl_3$, 100MHz) : δ 30.3 (C-3), 30.9 (C-8), 32.6 (C-2), 36.3 (C-9), 45.1 (C-1), 47.6 (C-1), 52.0 (C-5), 85.3 (C-6), 109.6 (C-14), 112.6 (C-15), 120.2 (C-13), 139.7 (C-11), 149.2 (C-10), 151.2 (C-4), 170.3(C-12); EIMS (rel. int): m/z 230 (M^+, 98%), 217 (20%), 201 (43%), 173 (23%), 132 (17%).

Compound 2 (Costunolide): Colorless crystals, mp: 105-106^{o}C, IR (υ cm^{-1}): 3448, 1691, 1458, 1031, 758; ^{1}H NMR ($CDCl_3$, 400MHz) : δ 1.43 (3H, s,H-14), 1.70 (3H, s, H-15), 4.58 (1H, t, J=9.6Hz, H-6), 4.75 (1H, d, J=9.6Hz, H-5), 4.86 (1H, dd, J=3.2, 11.6 Hz, H-1), 5.54 (1H, d, J=3.2Hz, H-13α), 6.28 (1H, d, J=3.2Hz, H-13β); ^{13}C NMR ($CDCl_3$, 100MHz) : δ 16.1 (C-14), 17.3 (C-15), 26.2 (C-8), 28.0 (C-2), 39.4 (C-9), 41.0 (C-3), 50.4 (C-7), 81.9 (C-6), 119.6 (C-13), 127.0 (C-1), 127.2 (C-5), 136.9 (C-10), 140.1 (C-4), 141.5 (C-11), 170.5 (C-12); EIMS (rel. int): m/z 232 (M^+, 8%), 217 (20%), 149 (43%), 121 (33%), 81 (100%).

Anti-microbial assay:
The assay was performed based on the tube dilution or agar dilution method reported in the literature. The cultures, *Malassezia furfur* – MTCC 1374, *Staphylococus aures* –ATCC 6538P, *Streptococus mutans* – MTCC 497, *Propionibacterium acnse*- MTCC 1951, *E. coli* –ATCC 8739, *Aspergillus niger* –ATCC 16404, *Candida albicans*-ATCC 10231 were obtained from MTCC, Chandigarh, India and *Corynebacterium xerosus*-ATCC 373 was obtained from Microbiologics, USA.

Estimation of Minimum Inhibitory concentration (MIC values)
The Minimum inhibitory concentration (MIC) values were determined against all organisms. MIC is the minimum amount of an antimicrobial that will inhibit visible growth of the organism after a suitable period of incubation. MIC was determined by macro broth double dilution method as per NCCLS guidelines.[23, 24]

Preparation of stock solution
Stock solution of the test samples were prepared by dissolving the neat samples in solvent containing DMSO and mixed thoroughly. The neat sample so dissolved in the solvent stock was further diluted with appropriate volumes of Tryptic Soy Broth (TSB) or Sabouraud Dextrose Broth (SDB) in order to give a final concentration of 20,000 µg/ml. All test solutions were prepared afresh prior to determination of MIC.

Preparation of Inoculum:
Inoculum was prepared by growing all bacterial strains on Tryptic Soy Agar and all yeast & mould strains were on Sabouraud Dextrose Agar with 1% Tween–80 for 48 hrs and 72 hrs respectively. Colonies were suspended in SDB with 1% Tween–80 and absorbance at 600nm were determined spectrophotometrically.[24]

Serial dilutions for the assay: A series of 50% dilutions of each test sample solution was made in test tubes with SDB + 1% Tween-80 as diluents. Each tube was inoculated with all organisms (0.5ml) of about $2X10^6$ CFU/ml for bacterial strains and $2X10^4$ CFU/ml for yeast and moulds.

Tubes were incubated at appropriate temperatures 37^{o}C +/- 1^{o}C for 48 hrs for Bacteria and 25^{o}C +/- 1^{o}C for 72 hrs for yeast and mould & then 10 µl aliquot from each tube were spot inoculated onto solid agar medium.. The resultant subcultures were incubated further as per incubation period mentioned above. The lowest concentration of the test sample inhibiting growth of organisms on the subculture plate was taken as MIC of the test material. Assays

were repeated thrice to confirm reproducibility of results. MIC is the minimum inhibitory concentration expressed as µg/ml, when assayed via the double dilution protocol described earlier probing only two fold dilutions.

Table:1 - MIC values of Fractions / pure compounds

Fraction/ compound	*M. furfur*	*S. mutans*	*P. acnes*	*C. xerosus*	*S. aureus*	*A. niger*	*C. albicans*	*E. coli*
Hexane fr.	156.25	156.25	250	312.5	156.25	>1000	>1000	>1000
Chloroform fr.	1250	1250	1000	625	625	>1000	>1000	>1000
EtOAc fr.	>2500	>2500	>1000	2500	>1000	>1000	>1000	>1000
Compound **1**	250	250	100	250	250	NA	NA	NA
Compound **2**	250	>500	200	250	>500	NA	NA	NA

Melanin inhibition activity:

The melanin inhibition activity[25] of isolated compounds along with control (IBMX) were studied in cell lines (B16F10 melanoma). The assay method is most precise and reliable. The isolated compounds, dehydrocostuslactone, **1** and costunolide, **2** were showed potent activity by inhibiting the melanin production in cell lines at very lower concentration. At higher concentration, the cells are dying indicating that these compounds are toxic to cell lines and results were mentioned in the table 2.

Table:2 – In vitro melanin modulation activity

Compound	Concentration (ug/ml)	Melanin Inhibition (%)	Melanin promotion (%)
Dehydrocostus lactone, **1**	1	10	-
	2	52	-
	3	75	-
	4	Toxic	-
Costunolide, **2**	1	17	-
	2	42	-
	3	74	-
	4	Toxic	-
IBMX (Control)	15	-	70

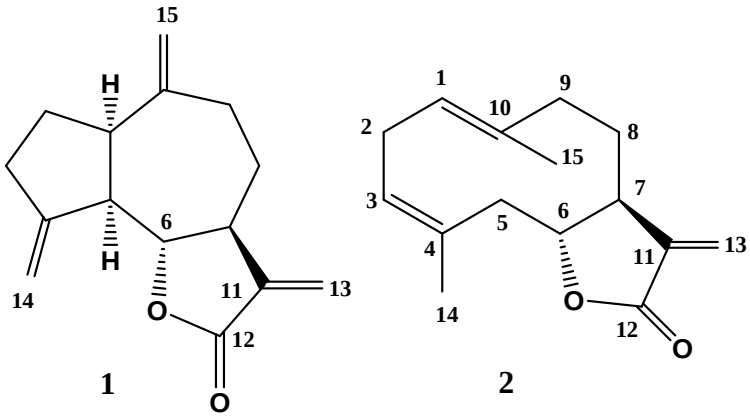

Fig. 1. Compounds isolated from roots of *Inula racemosa*

CONCLUSION

The compounds, dehydrocostus lactone and costunolides are reported first time from this plant. Earlier few antimicrobial studies have been done on this plant but studies on other organisms, like., *M. furfur*, *S. mutans*, *C. xerosus* and *P. acne* are the first report for this plant.

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