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ISOLATION AND CHARACTERIZATION OF *SALACIA CHINENSIS* AND ITS EVALUATION OF ANTIOXIDANT ACTIVITY

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ABSTRACT: *Salacia chinensis*, commonly known as Saptarangi in the Hindi family of Hippocrateaceae, it is a woody climbing shrub found in Africa, Vietnam, and Thailand. A large number of biologically active compounds like salacinol, kotalanol, neokotalanol, neosalacinol, salasol, and mangiferin are isolated from *S. chinensis*. Traditionally, the plant is used in the treatment of diabetes, but there are few studies that demonstrate its use as anti-inflammatory, nephroprotective, anticancer, and treatment of cardiac disorders. The present study involves extraction, isolation, structural elucidation, and prediction of antioxidant activity from the roots of *S. chinensis*. The roots were extracted with water and methanol by using a hot extraction method. The methanol extract was fractionated with ethyl acetate. The antioxidant activity of different extracts was determined by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method. The highest antioxidant activity was found in ethyl acetate extract, followed by methanol extract and water extract. Ethyl acetate extract showed maximum antioxidant activity, so the extract was used for the isolation of antioxidant compounds by column chromatography. The compound was isolated from the *Salacia chinensis* with higher yield and new technique. The compound isolated was characterized as 25, 26-oxido friedelane 1, 3-dione, and was elucidated using ¹H NMR, ¹³C NMR, and MS. The study shows that the obtained pure compound could be a good source of natural antioxidants.

INTRODUCTION: Large numbers of bioactive compounds are produced by plants that are used as an herbal medicine for the treatment of diseases since ancient times. Various phytochemicals, namely polyphenols, flavonoids, terpenes, phenolic acids, tannins, and coumarins, are present in plant and high concentrations of these phytochemicals may protect against free radical damage ^{1,2}.

The plants consist of beneficial phytochemicals, which is a need of the human body, and these phytochemicals act as natural antioxidants and source of supplementation for human diseases ^{3,4}. Antioxidants are considered a crucial chemical component, which may be responsible for preventing and delaying various types of cell damage.

The presence of antioxidants enhances the action of the immune system by producing a free radicle that is considered as one of the essential roles ⁵. It was observed that flavonoids and phenols are considered as strong antioxidants, and these are found to be distributed amongst various parts of plants.



The main aim of this study was to screen plant material extracts of finish origin with respect to antioxidant activity in order to find new potential sources of natural antioxidants. Different methods are used to evaluate the *in-vitro* antioxidant capacity of isolated compounds as well as those compounds containing different mixtures, which includes different mechanisms for the determination of antioxidant capacity of plant extract^{2, 6}. *Salacia chinensis* is an essential genus consisting of nutritional, medicinal, and pharmaceutical values belonging to the family of Hippocrateaceae. It is widely distributed across India, Sri Lanka, China, Myanmar, Thailand, Vietnam, and other Asian countries. Twenty-one species are found in India alone⁷.

In India, *S. Chinensis* is abundantly found in Karnataka, Goa, and Maharashtra. It is also called as, Saptarangi, Dimal, Modhupal, Ingli, Cherukuranti, Nisul-bondi and Eka Nayaka in Kannada. It is spread throughout the coast of the Andaman and Nicobar Islands and is a small erect or straggling tree or large, woody, climbing shrub⁸. The *S. chinensis* tree can grow up to 3-10 m in height and 16 cm in diameter in the tropical forests⁹. The species of *S. Chinensis* have medicinal value with high pharmacological significance. In the traditional system, it is used as acrid, bitter, thermogenic, urinary, and as a liver tonic. Extensive use has been reported in Ayurvedic system of medicine, traditional Indian medicine, and Unani for treating diabetes, gonorrhea, rheumatism, itching, asthma, ear diseases, leukemia, and inflammations¹⁰.

Pharmacologically they are used in diseased conditions like respiratory disorder, chronic fever, cold, cough, malaria, dysentery, diarrhea, arthritis, skin diseases, convulsions, diabetes, trauma, and in treatment of internal organs, hepatic, vessel and immunologic disorders, Liver tonic and back pain¹¹. *Salacia chinensis* possess phytoconstituents like alkaloids, glycosides, polyphenols, coumarins, proteins, carbohydrates, gums and mucilage, fixed oil and volatile oil. The principle constituent like salacinol, kotalanol, salaprinol and ponkoranol, mangiferin is present in roots and stems and have been shown to be vital intestinal α -glucosidase inhibitors, and it lowers the absorption of carbohydrates in gastrointestinal tract and ultimately

aims in delaying a rise in blood sugar by playing an essential role as anti-diabetic drug¹². Phytoconstituents are divided according to different parts of plant, phenolic glycosides, foliachineno-sides A1, A2, A3, B1, B2, C, and D are found to be present in leaf¹³, Friedel-1-en-3-one; friedelane-1, 3-dione; 1, 3-dioxofriedelan-24-al and 7 α - hydroxyfriedelane-1,3-dione and 25, 26-oxido-friedel-1, 3-dione are present in root bark^{14, 15}. Salasones D; salasones E; salaquinone B; salasol B are found in the stem.

Morikawa *et al.*, suggested the presence of wide variety of Triterpenoids like Friedelane-type triterpenes, salasones A, B, and C, norfriedelane-type triterpene, salaquinone A, acylated eudesmane-type sesquiterpine, salasol A, 3 β 22 β -dihydroxyolean -12-en-29-oic acid; tingenone; tingenine B; regeol A; triptocalline A are found to be present in stem¹⁶. However, not much literature is available on the antioxidant activity of isolated compounds obtained from *Salacia* species, which shows that no work has been carried out. Therefore, it is necessary to evaluate the antioxidant and biological activity of various isolated compounds. The present investigation involves isolating and characterizing antioxidant compounds from crude extract to determine their antioxidant activity.



FIG. 1: ROOT OF *SALACIA CHINENSIS*

MATERIALS AND METHODS:

Chemicals and Reagents: The solvents like hexane, ethyl acetate, methanol, chloroform, and acetone were used in this experiment obtained from Rankem Company, India. Silica gel, TLC silica gel aluminum sheets obtained by Merck Life Sciences, BHT (Butylated hydroxytoluene) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) obtained from Himedia Laboratories Pvt. Ltd., Mumbai. All glassware used in this experiment were from borosil, India. In addition, other chemicals used were of analytical grade.

Instruments: The absorbance of extracts and isolated pure compounds at different concentrations were measured by UV-visible spectroscopy (V630 JASCO, Japan) for the determination of antioxidant activity. The measurement of ^1H and ^{13}C NMR spectra were carried on Bruker (500 MHz) spectrometer (Pune University, Pune) using TMS as an internal standard. The measurement of FT-IR (4600 JASCO, Japan) was performed by using standard potassium bromide. Mass spectra were recorded on an Agilent 6460 Triple Quadrupole LC/MS system and rota evaporator (BUCHI, model I200, Switzerland) used for the concentration of different solvents.

Sample Collections: The roots of *Salacia Chinensis* were sourced from Mayurbhanj, Orissa. A voucher specimen was collected from the source populations and was identified and authenticated by taxonomist Dr. P. Santhan. The herbarium sheets are maintained at R & D center, Sava Healthcare Ltd, Pune, India.

Extraction: The dried root samples of *Salacia chinensis* were grounded into coarse powder and the powdered samples 3 kg were further extracted with methanol 3 volume by using a hot extraction 60 °C method for four hours 3 cycles.

The extraction was followed by the evaporation of the solvent by using a rotary evaporator under reduced pressure. Further, the solvent-free extract 500 g was fractionated using ethyl acetate 1500 ml. Solvent-free ethyl acetate extract 303 gm was again obtained by evaporating the solvent using a rotary evaporator.

Further ethyl acetate fraction (300 gm) was washed with (25% ethyl acetate: 75% Hexane) 900 ml. The following resulted in two parts; one was soluble 199 g, and the other obtained was insoluble 95 g. Then, the obtained soluble part was washed with hexane for reducing its non-polar impurity, after washing it with hexane the insoluble fraction 150 g was used for column chromatography.

Also, the fresh sample was extracted with water 3 volume by using a hot extraction of 60°C methods for four hours 3 cycles. The resulting extracts of water, methanol, and ethyl acetate from root powder of *S. chinensis* were then subjected to their antioxidant activity.

Antioxidant Activity: The scavenging activity on α , α -diphenyl- β -picrylhydrazyl (DPPH) free radical was evaluated by the method of Shimada *et al.*,¹⁷ with slight modification. 1 ml of extract (at concentrations of 100, 200, 300, 400 or 500 $\mu\text{g}/\text{mL}$) was taken and to that, DPPH solution (1 mL, 0.1 mM in 95% ethanol) was added. The mixture was shaken and subjected at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. 1 ml of distilled water was used instead of sample for blank and sample control was prepared for each fraction by mixing 1 ml of sample with 1ml of 95% ethanol. BHT was used as a reference at a concentration of 200 $\mu\text{g}/\text{mL}$. Further, the radicle scavenging activity was calculated¹⁸:

DPPH radical scavenging capacity (%) = $1 - (\text{Abs. of sample} - \text{Abs. of sample control}) \times 100 / \text{Abs. blank}$

Isolation and Characterization Antioxidant Compounds:

A different organic solvent like ethyl acetate and hexane was used as a mobile phase for the separation of ethyl acetate extract by using column chromatography. As per the same R_f value, the fractions obtained from column chromatography were mixed together, and the mother solvent was evaporated using a rota evaporator. According to TLC, the similar TLC pattern was integrated to give Fraction 1, Fraction 2, Fraction 3, Fraction 4, Fraction 5, and Fraction 6. Different polar solvents were used for the separation of ethyl acetate extract by using chromatographic techniques. The fractions were subjected to rotary evaporation at ambient temperature. From all the mentioned fractions, only Fraction 5 depicts a single band in TLC. The structural elucidation of the compound is carried out by using MS, IR, and 2D NMR^{19, 20}.

Fraction 5: The Fraction 5 obtained from ethyl acetate extract by column chromatography in which the stationary phase was silica gel (60-120 mesh) hexane and ethyl acetate of different polarities were used as mobile phase. The obtained fraction was found to be colorless crystals (4 g) R_f value 0.64 (ethyl acetate-hexane; 7:3); M.P 287 °C. The IR spectrum showed the presence of two carbonyl groups at 1732 and 1705 cm^{-1} characteristic diketone system. ^1H NMR 500 MHz spectrum indicated three AB coupling doublets. The pair at

3.49 (1H, J 16), 3.27 (1H, J 15.9) is assigned to the methylene (C-2) of the β -diketone system. C-4 methine gives rise to a quartet at 2.57(1H, J 6.6) and a doublet due to the methyl at C-23 at 1.07 (3H, J 6.9). Five singlets due to five methyl's are between 0.66-1.05. The C-10 methine gives rise to a singlet at 2.45. The doublet pairs at 4.57(1H, J 11.45), 4.02 (1H, J 11.25), 4.31 (1H, J 12), and

3.35 (1H, J12) are assigned to protons of the group $-\text{CH}_2-\text{O}-\text{CH}_2-$ *i.e.*, H-26a, H-26b, H25a, and H-25b respectively. The ^{13}C NMR spectrum of compound indicated the presence of 30 carbons and suggested a triterpenoid structure. (M^+ , 454); On the basis of ^1H and ^{13}C and MS spectral data, it was characterized 25, 26-oxido friedelane 1, 3-dione **Fig. 3**^{19, 20}.

TABLE 1: ^{13}C NMR DATA OF 25, 26-OXIDO FRIEDELANE 1, 3-DIONE

Carbon no	Chemical shift	Carbon No	Chemical shift	Carbon no	Chemical shift
1	202.8	11	35.8	21	32.5
2	60.5	12	26.5	22	38.9
3	230.7	13	38.1	23	7.5
4	59.7	14	40.0	24	15.7
5	37.5	15	34.7	25	67.1
6	38.5	16	36.8	26	69.9
7	17.0	17	30.8	27	19.3
8	45.2	18	44.0	28	30.0
9	37.1	19	35.4	29	31.4
10	69.0	20	28.3	30	35.1

RESULT: Different solvents like water, methanol, and ethyl acetate were used for the preparation of crude extracts of *Salacia* root, and the yield obtained is given in **Table 2**.

Antioxidant Activity: The antioxidant activity of different fractions of *Salacia chinensis* was evaluated by the DPPH method with modification. The highest antioxidant activity was found in ethyl acetate fraction with a percentage of inhibition value of 96.4, methanol fraction with percentage inhibition of 95.7, followed by water with percentage inhibition of 93.8 **Table 3**. The percentage inhibition of standard BHT 200 ug/ml was found to be 90%, which shows that the fractions of the selected plant species also show significant inhibition compared to standard BHT.



FIG. 2: TLC OF ISOLATED COMPOUND

Isolation and Characterization of Antioxidant Compounds: The highest antioxidant activity was found in ethyl acetate extract; the separation of ethyl acetate extract was done by column

chromatography by the different polarity of solvents. The Fraction 5 obtained from the column chromatography gives a single band in TLC **Fig. 2**, which indicates the fraction is pure. The structure of the pure compound was elucidated by using IR, MS, and NMR.

Antioxidant Activity of Pure Compounds: The antioxidant activity of the isolated pure compound at different concentrations was determined by the same modified DPPH method. The pure compounds showed a significant percentage of inhibition at all applied concentrations against the DPPH method presented in **Table 4**.

DISCUSSION: There is no proven data available on the antioxidant activity of different solvent extracts like water, methanol, and ethyl acetate of the selected plant. The antioxidant activity of the crude extracts was determined by the DPPH method, and the results are presented in **Table 3**. The highest antioxidant activity was found in ethyl acetate extract, followed by methanol and water. Due to the presence of remarkable antioxidant activity, ethyl acetate crude extract was selected for isolation and separation of antioxidant compounds.

TABLE 2: YIELD OF DIFFERENT POLARITY OF CRUDE EXTRACTS OF SALACIA CHINENSIS

Crude extract	Yield (g)
Methanol	501
Water	523
Ethyl acetate	303

TABLE 3: ANTIOXIDANT POTENTIAL OF METHANOL, WATER AND ETHYL ACETATE CRUDE EXTRACTS OF *S. CHINENSIS*

Fraction	Concentration ($\mu\text{g/ml}$)	Absorbance of sample control	Absorbance of Sample	% Inhibition
Methanol fraction	100	0.008	0.470	53.8 \pm 0.02
	200	0.031	0.382	64.9 \pm 0.02
	300	0.052	0.254	79.8 \pm 0.01
	400	0.063	0.123	94 \pm 0.02
	500	0.069	0.112	95.7 \pm 0.03
Water	100	0.008	0.501	50.7 \pm 0.18
	200	0.031	0.410	62.1 \pm 0.16
	300	0.052	0.303	74.9 \pm 0.02
	400	0.063	0.189	87.4 \pm 0.002
	500	0.069	0.125	93.8 \pm 0.01
Ethyl acetate fraction	100	0.008	0.455	55.3 \pm 0.04
	200	0.031	0.363	66.8 \pm 0.03
	300	0.052	0.205	84.7 \pm 0.01
	400	0.063	0.110	95.3 \pm 0.02
	500	0.069	0.105	96.4 \pm 0.02

TABLE 4: ANTIOXIDANT POTENTIAL OF PURE COMPOUND

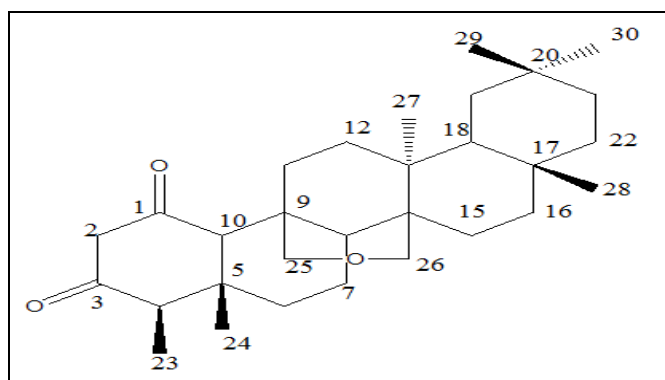
Fraction	Concentration ($\mu\text{g/ml}$)	Absorbance of Standard	Absorbance of Sample	% Inhibition
25,26-oxido friedelane 1,3-dione	100	0.008	0.365	65.2 \pm 0.03
	200	0.031	0.199	83.2 \pm 0.01
	300	0.052	0.116	95.3 \pm 0.02
	400	0.063	0.099	96.4 \pm 0.02
	500	0.069	0.093	97.6 \pm 0.02

Various separation techniques such as thin-layer chromatography (TLC), column chromatography (CC) were used for the separation of compounds from the plant extract. In column chromatography, the mobile phase of different polarity was used for separation of compounds, and silica gel (120-60 mesh) was used as a stationary phase. Firstly, in the performed experiment mobile phase, hexane-ethyl acetate (95:5) was used, and a gradual increase in polarity of the mobile phase was done by the addition of ethyl acetate. In the isolation of pure compounds from crude extracts, polarity plays an important role. The TLC behavior shows that fraction 5 contains a single band as compared to the other fractions of ethyl acetate.

The pure compound obtained had colorless crystals. It had the molecular ion peak at $[M]^+$ m/z 454, which corresponds to molecular formula $C_{30}H_{46}O_3$. In the 1H NMR spectrum, the isolated compound shows three doublets, first doublet at δ 3.49 and δ 3.27, which indicates 2 protons at the position of C-2. Another doublet pairs at δ 4.56, δ 4.02, δ 4.30, and δ 3.35 are assigned to protons of the group $-CH_2-O-CH_2-$ i.e., H-26a, H-26b, H25a, and H-25b respectively. C-4 methine gives rise to a quartet at δ 2.57. Five singlets due to five methyls are seen between δ 0.66- δ 1.05.

The C-10 methine gives rise to a singlet at δ 2.45 and a doublet due to the methyl at C-23 at δ 1.07. Based on the above spectral data, the structure of the compound was established as 25, 26-oxido friedelane 1, 3-dione. The presence of two carbonyl groups at 1732 and 1705 cm^{-1} characteristic diketone system was shown by the IR spectrum.

The IR spectrum showed the presence of 2 carbonyl groups at 1732 and 1705 cm^{-1} characteristic diketone system. The compound was isolated with a new technique for the first time from the roots of *Salacia chinensis* with a higher yield. The TLC was carried out, and according to the TLC behavior, the compound was found to be almost pure.

**FIG. 3: 25, 26-OXIDO FRIEDELANE 1, 3-DIONE**

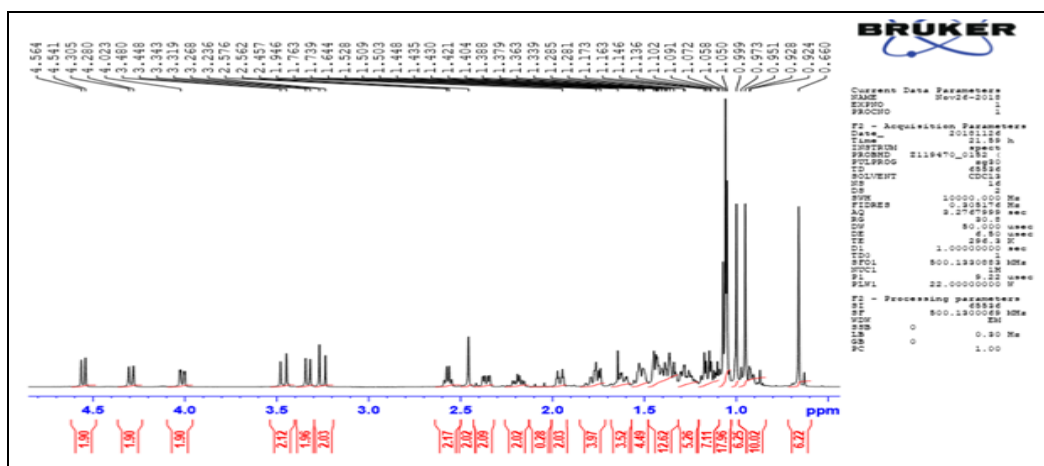


FIG. 4: ¹H NMR SPECTRUM OF PURE COMPOUND

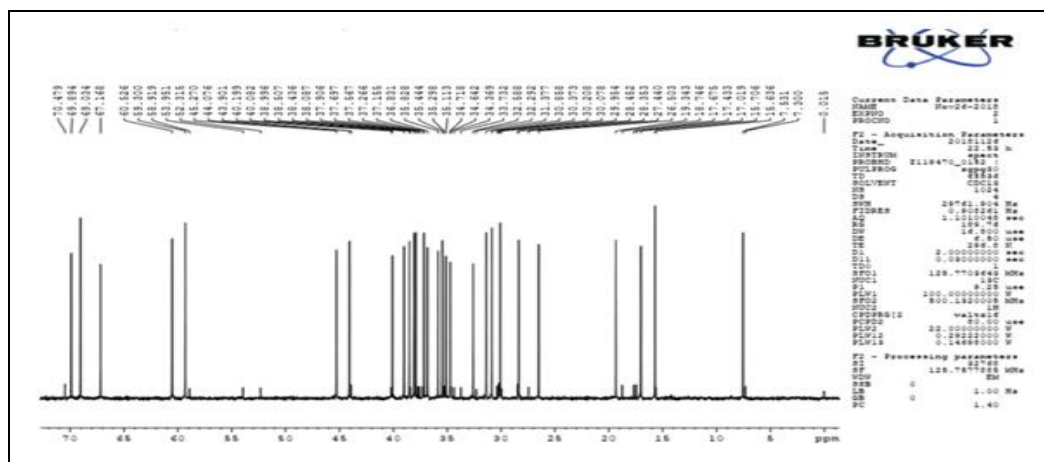


FIG. 5: ¹³C NMR SPECTRUM OF PURE COMPOUND

Antioxidant Activity of Pure Compound: The evaluation of antioxidant activity of the isolated pure compound 25, 26-oxido friedelane 1, 3-dione showed higher antioxidant activity against DPPH as compared to standard Butylated hydroxytoluene. The pure isolated compound shows the highest antioxidant activity as compared to water, methanol, and ethyl acetate extracts of *Salacia chinensis*.

CONCLUSION: Waste substances called free radicals are produced by cells as the body processes food and reacts to the environment. If the body is unable to process and remove free radicals, it will result in oxidative stress. This oxidative stress is directly connected to heart disease, cancer, arthritis, stroke, respiratory diseases, immune deficiency, emphysema, Parkinson's disease, and other inflammatory or ischemic conditions.

Role of antioxidants is to neutralize free radicals in our body, which boosts overall health. Therefore, the risks of certain diseases are lowered due to

antioxidants. *Salacia chinensis* is a medicinal plant, which contains high levels of antioxidant activity^{21, 22}. The ethyl acetate extract has the highest antioxidant activity so it was selected for isolation and separation of antioxidant compounds. The antioxidant activity of extracts was found in the order of ethyl acetate > methanol > water. The isolated compound 25, 26-oxido friedelane 1, 3-dione showed maximum antioxidant activity against DPPH as compared to standard BHT.

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CONFLICTS OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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