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Anti-inflammatory activity and identification of two 'Sarpagan' indole alkaloids from the leaf methanolic extract of *Rauvolfia densiflora* (Wall.) Benth. ex Hook. f.

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ABSTRACT

The Apocynaceae plant group contains many bioactive compounds that are pharmacologically relevant and are used as potential medicine for many diseases. *Rauvolfia densiflora* Benth. ex Hook.f. (syn- *Rauvolfia verticillata* (Lour.) Baill.) coming under the same family known for its diverse medicinal properties. The current study was carried out to evaluate the bioactive compounds present in the extract using GC-MS analysis and to find out the anti-inflammatory potential of the extract using in-vitro methods. GC-MS analysis revealed the presence of 16 bioactive compounds with diverse pharmacological activities. Among them, *Oleic Acid, 17-hydroxy-, methyl ester, trans-decahydroquinoline,* and Spiro-(1,3-dioxolane-2, 3'-indolin]-2'-one possesses more important properties. Two important sarpagan indole alkaloids were also identified namely *Sarpagan-16-carboxylic acid or* (Z) *Akuammidine* and *sarpagan-17-ol or Vellosiminol*, which are pharmacologically important compounds with anti-inflammatory, anti-asthmatic activities. The *in-vitro* anti-inflammatory activity was carried out using the COX assay. COX assay showed 55.27% inhibition at a concentration of 200 µg/mL with an IC_{50} value of 155.38 µg/mL, this is an indication that the methanolic leaf extract *R. densiflora* has the potential to be developed as a non-steroidal anti-inflammatory drug (NSAID).

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INTRODUCTION

Herbal plants have a significant influence on human welfare and are successfully used in the development of novel medications (Yuan *et al.*, 2016). The high cost of synthetic drugs and their inability to act against resistant pathogens make us consider alternative medicines. Plants contain diverse chemical compounds which act against multiple pathogenic diseases. The plant belonging to Apocynaceae has been used for centuries in traditional medicine and is one of the most important medicinally varied groups in the plant kingdom (Islam & Lucky, 2019). Apocynaceae possess anti-inflammatory, antimicrobial, anti-hypertensive, anti-schizophrenic, antioxidant, and antimalarial activities (Willie *et al.*, 2021). *Rauvolfia* is a genus which is coming under Apocynaceae, presence of nitrogen-containing indole alkaloids makes it significant from a therapeutic perspective (Nair *et al.*, 2012). *Rauvolfia densiflora* Benth. ex Hook.f. (Apocynaceae) is a small tree thriving in the forests between 700 and 2200 m in Sri Lanka. The Kanikkars tribe in Tamil Nadu refers to this plant as "paarisirunila," and in Malayalam, it is called 'kattamalpori' but it is known as "Dense-flowered Snake Root." It contains a variety of physiologically active substances and is a major source of phytochemicals with pharmacological and therapeutic value (Shunmugapriya & Kalavathy, 2012; Iqbal *et al.*, 2013b; Bhavana *et al.*, 2019). This plant contains reserpine-type alkaloids (Weerakoon *et al.*, 1998).

MATERIALS AND METHODS

Plant parts of *Rauvolfia densiflora* Benth. ex Hook.f. were collected from the Shekalmudi region of Malakkapara, Kerala, India. The identification of *R. densiflora* is done by

Dr. K. H. Amitha Bachan, Research Department of Botany, MES Asmabi College, Kodungallur, Kerala, India. The herbarium specimen of the plant was deposited to the same department and the collection number of the specimen is 21616. The leaves were washed, shade-dried, and ground into coarse powder using an electric grinder. The powdered plant material was subjected to Soxhlet extraction using Methanol for 72 hours followed by solvent evaporation using a rotary evaporator. The extract was subjected to GC-MS analysis and *in vitro* anti-inflammatory screening.

GC-MS Analysis

The GC-MS analysis of the sample was performed using a Shimadzu Nexis GC- 2030, fitted with a SH-I- 5Sil MS column (30 X 0.25 mm, with 0.25 cm film thickness). The oven temperature was programmed at an initial 80 °C and was held for 2 minutes. Then, the temperature was increased at a rate of 15 °C per minute until it reached 150 °C, where it was held for 1 minute. After that, the temperature was increased at a rate of 10 °C per minute until it reached 220 °C, where it was held for 5 minutes. A total of 50:00 minutes was taken for the complete run. Data handling was done using GCMS solution software. The identification of compounds was based on a comparison of their mass spectra with those of NIST 20 Libraries.

In Vitro Anti-Inflammatory Screening

Cyclooxygenase (COX) assay

RAW 264.7 cells were grown to 70% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1 µg/mL). LPS-stimulated RAW cells were exposed with different concentrations of test sample solution and the standard provided. The plates were then incubated for 24 hours. After incubation, the anti-inflammatory assays were performed using the cell lysate. The COX enzyme activity was assayed by the method of Walker and Gierse with slight modifications. The cell lysate in Tris-HCl buffer (pH 8) was incubated with glutathione 5 mM/L, and haemoglobin 20 µg/L for 1 minute at 25 °C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes of incubation at 37 °C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm (Walker & Gierse, 2010).

RESULT AND DISCUSSION

The bioactive compounds present in the methanolic leaf extract of *R. densiflora* was identified by GC-MS analysis (Figure 1). The active principles with a retention time (RT), molecular formula, molecular weight (MW), and peak area (%) of the extract are presented in Table 1. From the GC-MS analysis, sixteen compounds were identified. The first eluted compound was *Dodecanoic acid* with a retention time of 20.09 and the last eluted compound with a retention time of 47.460 was *Benzofuro[3,2-c] pyridine, 8-methoxy-3-methyl-1-(4-methylphenyl)-*. The identified compounds were *Dodecanoic acid* (20.09), *Spiro-[1,3-dioxolane-2,3'-indolin]-2'-one* (20.877), *Oleyl alcohol*(21.278), *(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxy phenol* (24.058), *2-Furancarboxylic acid 3-(3,4-dihydroxy phenyl* (26.067), *(E)-Dodec-2-enyl isobutyl carbonate*(26.323), *3,7,11,15-Tetramethyl-2-hexadecen-1-ol*(27.210), *Hexadecanoic acid, methyl ester*(28.117), n-*Hexadecanoic acid* (28.765), Oleic Acid (32.074), *Octadecanoic acid*(32.535), *Sarpagan-16-carboxylic acid, 17-oxo-, methyl ester*(42.851), Trans-Decahydroquinoline (43.121), *Sarpagan-17-ol* (45.158), 5-(3-fluoro-4-methoxyphenyl)-3-methyl-5-oxopentanoic acid (45.70), Benzofuro[2,3-c] pyridine (47.460).

Dodecanoic acid or *lauric acid* (Rt-20.09) is a medium-chain triglyceride. It is a non-toxic, digestible type of bioactive compound with pharmacological activities. It prevents various skin diseases, lowers bad cholesterol level, and possesses anti-oxidant, anti-bacterial, anti-fungal, anti-viral, and anti-cancer activities (Sandhya *et al.*, 2016; Nazir *et al.*, 2017; Borrelli *et al.*, 2021). LA will inhibit the growth of *Mycobacterium tuberculosis* and has the potential to bind with protein kinase B (Muniyan & Gurunathan, 2016). *Spiro-[1,3-dioxolane-2,3'-indolin]-2'-one* was synthesized by the condensation of isatin with glycerol (Meng & Miao, 2010). It possesses high anticonvulsant activity (Rajopadhye & Popp, 1988), and exhibits anti-proliferation properties against human cancer cells (Fawazy *et al.*, 2022). *Oleyl alcohol, chlorodifluoroacetate* is a long-chain fatty alcohol, it mainly used in cosmetic products, as it exhibits a very low order of toxicity (Orienti *et al.*, 2007). *Coniferol* or *(e)-4-(3-hydroxyprop-1-en-1-yl)-2-methoxyphenol* is a phenylpropanoid which is a monolignol produced by the reduction of the carboxy functional group in cinnamic acid. Coniferol in high concentration is toxic to plant cells, at high concentrations it causes growth inhibition (Vaisanen *et al.*, 2015) it acts as both animal and plant metabolite which functions as a pheromone and volatile oil component

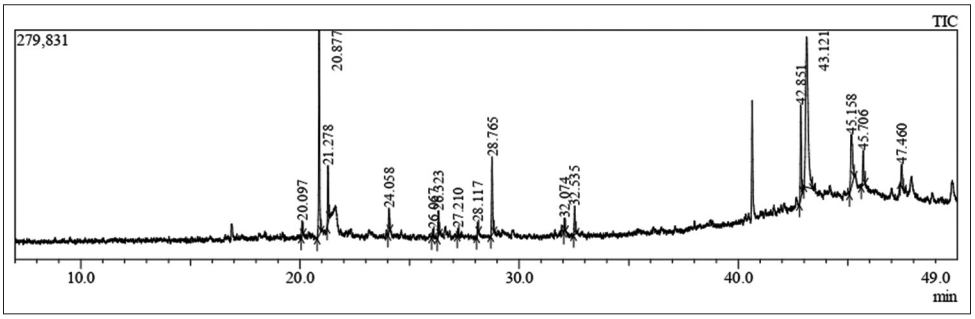

Figure 1: GC-MS chromatogram of the methanolic extract

Table 1: Phytocomponents identified in the methanolic extracts of *R. densiflora* by GC-MS

S. No.	Retention Time	Peak area %	Name of the compound	Molecular Formula	Molecular Weight
1	20.09	1.29	Dodecanoic acid	$C_{24}H_{12}O_2$	200.32
2	20.877	17.97	Spiro-(1,3-dioxolane-2, 3'-indolin]-2'-one	$C_{10}H_9NO_3$	191.18
3	21.278	4.64	Oleyl alcohol, chlorodifluoroacetate	$C_{18}H_{36}O$	268.5
4	24.058	2.28	Coniferol	$C_{10}H_{12}O_3$	180.20
5	26.067	0.59	2-Furancarboxylic acid, 3-(3,4-dihydroxy phenyl	$C_5H_5O_3$	112.08
6	26.323	2.09	(E)-Dodec-2-enyl isobutyl carbonate	$C_{17}H_{32}O_3$	284.4
7	27.210	0.57	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5
8	28.117	0.78	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45
9	28.765	6.97	n-Hexadecanoic acid	$C_{16}H_{32}O_3$	256.42
10	32.074	0.98	Oleic Acid	$C_{18}H_{34}O_2$	282.5
11	32.535	2.42	Octadecanoic acid	$C_{18}H_{34}O_2$	282.5
12	42.851	8.50	Sarpagan-16-carboxylic acid, 17-oxo-, methyl ester	$C_{21}H_{24}N_2O_3$	382.46
13	43.121	35.57	Trans-Decahydroquinoline	$C_9H_{17}N$	139.24
14	45.158	9.31	Sarpagan-17-ol	$C_{19}H_{22}N_2O$	294.4
15	45.706	3.48	5-(3-fluoro-4-methoxyphenyl)-3-methyl-5-oxopentanoic acid	$C_{13}H_{15}FO_4$	254.25
16	47.460	2.56	Benzofuro[3,2-c] pyridine, 8-methoxy-3-methyl-1-(4-methylphenyl)-	$C_{20}H_{17}NO_2$	303.36

(Hiap *et al.*, 2019). *2-Furancarboxylic acid, 3-(3,4-dihydroxy phenyl)* or *2-Furoic acid* is an organic compound consisting of furan ring and carboxylic acid side group. It is effective in lowering serum cholesterol and triglyceride. It exhibits acute toxicity (Hall *et al.*, 1992) and nematocidal activity (Lei *et al.*, 2023). *Phytol* or *3,7,11,15-Tetramethyl-2-hexadecen-1-ol* is a chlorophyll derive diterpene of long chain unsaturated acyclic alcohol with diverse biological properties. It exhibits anxiolytic, metabolism-modulating, cytotoxic, anti-oxidant, apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, anti-microbial, and antipyretic activities (Santos *et al.*, 2013; Islam *et al.*, 2018; Rahaman *et al.*, 2020; Saha & Bandyopadhyay, 2020).

Hexadecenoic acid is a fatty acid with biological activities such as anti-oxidant, hypocholesterolemic, nematicide, and pesticide (Siswadi & Saragih, 2021). According to Willie *et al.* (2021) it also exhibits anti-microbial, anti-inflammatory, and hemolytic properties. *n-hexadecanoic acid* is a saturated fatty acid that functions as an anti-inflammatory agent (Aparna *et al.*, 2012) and it also possesses antioxidant, hypocholesterolemic, and nematocidal properties (Tyagi & Agarwal, 2017). *Oleic acid* enhances mitochondrial oxidation of SFA by increasing triacylglycerol and reducing diacylglycerol and ceramide production, thus protecting the cells from inflammation and possessing antioxidant activity. It is proved to be a good inhibitor of fungi as well as termites repellent (Wei *et al.*, 2016; Alabi *et al.*, 2018; Piccinin *et al.*, 2019). *Octadecanoic acid* or *steric acid* is a straight-chain saturated fatty acid. It is mainly used in the production of cosmetics, it has the role of a plant metabolite and exhibits antioxidant and anti-inflammatory properties (Ganesh & Mohankumar, 2017). *Dl-trans-decahydroquinoline* or *2,9-Dimethyl-4-ethyl-trans-decahydroquinoline* is an alkaloid that is used as an anesthetic agent and has toxic and irritating properties (Fishchuk *et al.*, 1987).

Indole Alkaloid

The source of the amino acid can be used to name alkaloids. As a result, they can be divided into several groups according to related moieties, such as purine, β-carboline, indolinics, quinolizidine, quinolone, isoquinoline, indole, quinolizidine, pyrrolizidine, tropane, benzylisoquinoline, and quinolizidine (Adizov & Tashkhodjaev, 2019; Mohammed *et al.*, 2021). A class of alkaloids known as indole alkaloids shares structural similarities with the neurotransmitter serotonin and the important amino acid tryptophan. Indole alkaloids derived from plants have important pharmacological and biological properties. The genera *Rauwolfia* and *Alstonia* in the family Apocynaceae are the principal sources of isolation for this type of alkaloid (Namjoshi & Cook, 2016; Wu *et al.*, 2016).

Sarpagan-16-carboxylic acid, 17-oxo-, methyl ester or (Z) *Akuammidine*

Akuammidine is a sarpagine type of indole alkaloid with chemical formula $C_{21}H_{24}N_2O_3$. Its GC-MS chromatogram and the chemical structure are shown in the Figure 2. Akuammidine possesses opioid agonist action in the guinea pig myenteric plexus, primarily at -opioid receptors since they were antagonized by a low concentration of the non-selective opioid receptor (Menzies *et al.*, 1998; Hamdiani *et al.*, 2018). It possesses anti-inflammatory activities (Mohammed *et al.*, 2021), and anti-asthmatic properties (Hou *et al.*, 2012).

Sarpagan-17-ol or *Tombozine or Vellosiminol*

An indole alkaloid that is sarpagan bearing a hydroxy group at position 17 with molecular formula $C_{19}H_{22}N_2O$. Its GC-MS chromatogram and chemical structure are shown in the Figure 3. It is a primary alcohol and a tertiary amino compound. It is a sarpagan-type intermediate; it is functionally related to a sarpagan. It is the reduced form of the known sarpagine alkaloid vellosimine. Sarpagan-17-ol reduced PE- and serotonin-induced contractions in rat aortic rings, with a molar antagonist potency and non-competitively inhibits 5-HT-induced contractions (Iwu & Court, 1978; Omar *et al.*, 2021).

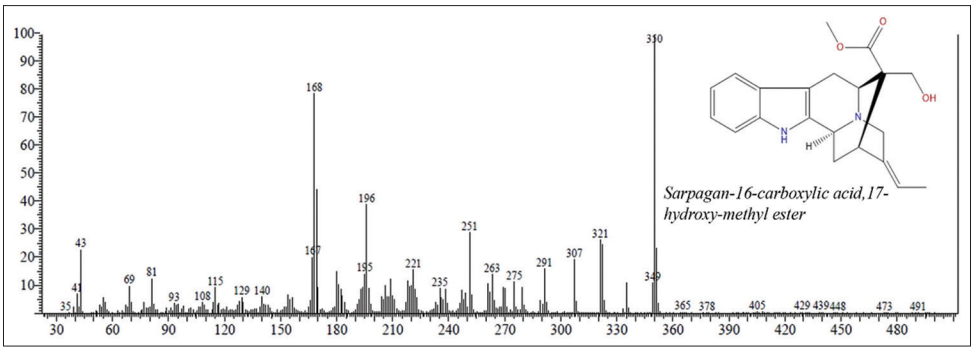

Figure 2: GC-MS chromatogram and chemical structure of Sarpagan-16-carboxylic acid, 17-oxo-, methyl ester

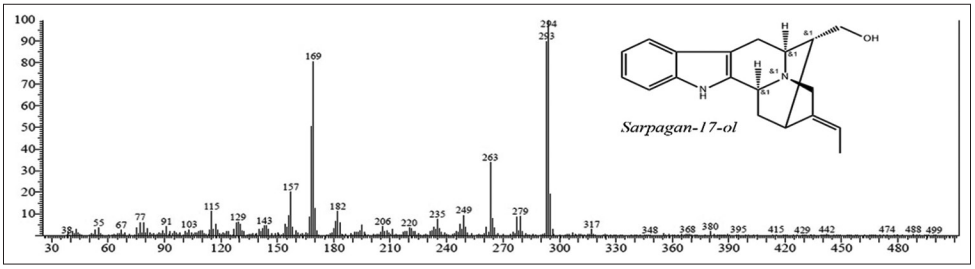

Figure 3: GC-MS chromatogram and chemical structure of Sarpagan-17-ol

Inhibition of Cyclooxygenase Enzyme Activity

Inflammation is a pathophysiological reaction that induces injury to living tissue to cause a localized build-up of plasmatic fluid and blood cells (Bonheur *et al.*, 2015). NSAIDs, or non-steroidal anti-inflammatory medicines, are used to treat and manage inflammation (Naz *et al.*, 2017). Current anti-inflammatory medications have side effects that increase with continued usage. Antioxidant-rich plants have been proposed as possible sources of chemicals with anti-inflammatory properties with the least side effects (Borquaye *et al.*, 2020). Among pro-inflammatory mediators, cyclooxygenase (COX), often referred to as prostaglandin-endoperoxide synthase, is the most significant enzyme. Non-steroidal anti-inflammatory medications directly suppress the COX enzymes (Ramadhan, 2020). The standard used in this evaluation of the cyclooxygenase (COX) inhibitory activity was diclofenac, an anti-inflammatory medication with very high COX inhibition. The percentage of inhibition and IC_{50} values of the standard and the extract are shown in Tables 2 and 3 and its graphical representation is in Figures 4 and 5. In the COX analysis, the Diclofenac exhibited 63.37 percentage of inhibition at a concentration of 100 µg/mL with an IC_{50} value of 82.98. The methanolic extract exhibited 55.27 percentage of inhibition at a concentration of 200 µg/mL and the IC_{50} value was obtained as 155.38 µg/mL. The *Rauvolfia* genus coming under the Apocynaceae family shows a wide range of biological activities including anti-inflammatory activity (Zhan *et al.*, 2020). *R. tetraphylla* possesses anti-inflammatory activity due to the synergistic activity of the phytochemicals. This is a novel finding that *R. tetraphylla* roots have the potential to be developed into an efficacious anti-inflammatory drug (Iqbal *et al.*, 2013a; Merlin *et al.*, 2020). According to Kumari *et al.* (2013) *R. serpentina* exhibits anti-inflammatory properties. *R. vomitoria* possesses bioactive substances acting in the 1st, 2nd, and 3rd phases of inflammation. It inhibits the production of histamine, serotonin, prostaglandin, kinin, and bradykinin (Bonheur *et al.*, 2015). According to the investigations, *Rauvolfia* species appear to have anti-inflammatory properties. When compared to the IC_{50} value of the standard, the *R. densiflora* leaf methanolic extract displayed a good IC_{50} value, which indicates that the methanolic leaf extract of *R. densiflora* has the potential to be developed as a non-steroidal anti-inflammatory drug (NSAID).

Table 2: Percentage inhibition of COX enzyme activity by Diclofenac standard

Standard	Concentration (µg/mL)	Percentage of Inhibition
Diclofenac	6.25	13.48
	12.5	22.65
	25	32.77
	50	46.85
	100	63.37
IC_{50}	82.98	

Table 3: Percentage inhibition of COX enzyme activity by methanolic extract

Concentration (µg/mL)	Percentage of Inhibition
6.25	8.10
12.5	17.64
25	25.92
50	38.63
100	41.47
200	55.27
IC_{50}	155.38

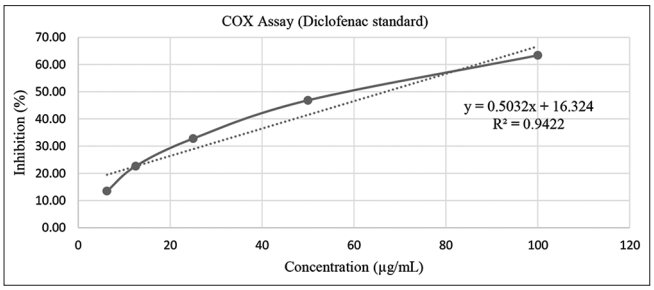

Figure 4: Percentage inhibition of COX enzyme activity by Diclofenac

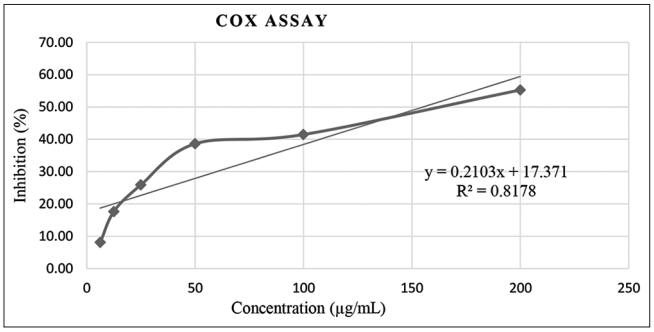

Figure 5: Graphical representation of percentage inhibition of COX enzyme activity by methanolic extract

CONCLUSION

The leaf extracts from the plant *R. densiflora* contains a variety of bioactive compounds with a broad spectrum of pharmacological characteristics. The sarpagan indole alkaloids of the plants have anti-inflammatory and anti-asthmatic qualities. The *in vitro* anti-inflammatory investigation, which shows a very good inhibitory concentration, suggests that the methanolic leaf extract of *R. densiflora* has the potential to be used to create a non-steroidal anti-inflammatory drug.

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