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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF A NOVEL SAPONIN FROM *ROSMARINUS OFFICINALIS* L. (LAMIACEAE).

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Abstract

Rosemary (*Rosmarinus officinalis* L.), a spice and medicinal herb of Lamiaceae family with a characteristic aromatic smell, is widely used around the world and accepted as one of the spices with the highest antioxidant activity. The aim of this research was to identify and isolate the unknown saponins from the leaves of *R. officinalis* L. using HPTLC, HPLC, UV, FTIR and HR-LCMS techniques. The saponins were identified by High Performance Thin Layer Chromatography (HPTLC) and confirmed by HPLC. On the basis of spectral data analysis, the structure of the new saponin isolated by HPTLC from methanol extract of leaves of *R. officinalis* L. has been formulated by UV, FTIR and HR-LCMS spectral analysis as 1 α -hydroxy-18-(4-hydroxy-4-methyl-2-pentynyloxy)-23,24,25,26,27-pentanorvitamin D3/1 α -hydr. This is a new saponin isolated from *R. officinalis* L. and being reported for the first time.

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Introduction:-

Rosmarinus officinalis L. (Lamiaceae), Rosemary, is a perennial herb native to the Mediterranean region but is widely distributed in many parts of the world. It grows as a shrub or herbaceous plant with about 0.8 to 2m height (Atik bekkara *et al.*, 2007). This plant prefers dry and arid regions, hills and low mountains, calcareous, shale, clay and rocky substrates (El Amrani *et al.*, 1997). The herb *R. officinalis* L. has been used as a food spice and medicine since ancient times. The fragrance of the leaf has been said to enhance memory. Rosemary oil was applied to the skin to treat muscle and joint pain and taken internally to promote abortions. Its use since ancient times in traditional medicine is justified by its antiseptic (Bult *et al.*, 1985), antirheumatic (Makino *et al.*, 2000), anti-inflammatory, antispasmodic (Juhas *et al.*, 2009; Beninca *et al.*, 2011), antimicrobial and anti-hepatotoxic properties (Stefanovits-Banyai *et al.*, 2003). Its appreciation as a spice for seasoning and food preservation (Arnold *et al.*, 1997) is supported by a very high antioxidant activity (Wang *et al.*, 2008). This antioxidant activity of *R. officinalis* L. is due to its phenolic compounds including: carnosic acid, carnosol, rosmarinic acid and hydroxycinnamic acid ester (Inatani *et al.*, 1983). Aerial parts of *R. officinalis* L. are orally used to relieve renal colic and dysmenorrhoea (Gonzalez-Trujano *et al.*, 2007). Recent research shows that *R. officinalis* L. extracts possess strong anticancer properties (Vassiliki *et al.*, 2013).

Nowadays market demand of the plant is growing, as it is used in several medicinal products. *R. officinalis* L. is indigenous to South Europe and Asia but it is also cultivated in Mediterranean basin and India (WHO, 2007). It is

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used as carminative, rubifacient, stimulant and as flavouring agent for liniments, hair lotions, inhaler, soaps and cosmetics (Kokate *et al.*, 2010). Rosemary leaves have many traditional uses based on their antibacterial and spasmolytic actions. Used orally for the treatment of dyspeptic complaints (British Herbal, 1996), and in external applications for supportive management of rheumatic complaints and circulatory disorders (Blumenthal, 1998). It is used as a cholagogue, diaphoretic, digestant, diuretic, emmenagogue, laxative and tonic (Bedevian, 1994; Farnsworth, 2005) also used in the management of headache, menstrual disorders, nervous menstrual complaints, tiredness, defective memory, sprains and bruises (Hagers, 2003; Asia *et al.*, 2013)

It is described in cases of congestion of the liver, inflammation of the gall bladder, gastric lavage, in some cases of jaundice, fatigue, physical and intellectual weakness following the diseases debilitating to the body, migraine, dizziness, palpitations, jittering, strikes, heartburn, carminative and as an antiseptic (Antoine, 1998). Many compounds have been isolated from *R. officinalis* L. including flavones, diterpenes, steroids, and triterpenes.

Saponins are natural high-molecular-weight glycosides of triterpene or steroids with a very wide distribution in the plant kingdom (Hostettmann and Marston, 1995). Saponins exhibit a range of biological activities (Oleszek and Marston, 2000) which include, anticholesterolemic (Oakenfull, 1981), anti-inflammatory, anti-parasitic and antiviral (Just *et al.*, 1998; Traore *et al.*, 2000). Saponins are also effective against drug-resistant cancer cells (Cheung *et al.*, 2005). The great structural diversity of saponins, novel bioactivities which is relevant to the pharmaceutical industry, the challenges of identification, are now opening new opportunities or newer trends for exploitation of novel saponins. Hence the aim of the current investigation was to identify and characterize active saponins from the leaves of medicinally important plant *R. officinalis* L.

Materials and Methods:-

The fresh plants of *R. officinalis* L. were collected from Ooty, Tamil Nadu, India. The plant materials were identified and authenticated by Dr. K.V. George, Emeritus Scientist (KSCSTE), Department of Botany, St. Berchmans College, Changanacherry, Kerala. The voucher specimen (Voucher No. N/PG/074) is deposited in the herbarium of New Udaya Pharmacy & Ayurvedic Laboratories, Cochin, Kerala, India.

Sample preparation:-

About 10 gm of the powdered sample was taken in a thimble and extracted with 250 ml methanol in a Soxhlet apparatus. The extract was then concentrated in a Rotary Vacuum Evaporator to a volume of 30 ml and stored in small air tight brown bottles. This methanolic extract was used for the screening and isolation of compound.

High Performance Thin Layer Chromatography (HPTLC):-

HPTLC is a flexible, reliable, and cost-efficient separation technique ideally suited for the analysis of botanicals and herbal drugs. HPTLC studies were carried out using the method described by Wagner *et al.* (1996). Methanol extract of the selected plant was subjected to HPTLC (CAMAG, Switzerland) analysis. A Camag HPTLC instrument consisting of Linomat V automatic spotter equipped with a 100 μ L syringe connected to a nitrogen cylinder, Scanner-III, twin-trough developing chambers, and viewing cabinet with dual wavelength UV lamps (Camag, Muttenz, Switzerland) were used. Before analysis, HPTLC plates were cleaned by predevelopment with methanol and activated at 110°C for 5 min for solvent removal. Plant extract were spotted on a silica gel 60F₂₅₄ (Merck, Germany) TLC plate. The plate was air dried and then developed by using the solvent system Chloroform: Acetic acid: Methanol: Water (6.4:3.2:1.2:0.8) (v/v/v/v) as mobile phase in a CAMAG- twin-trough glass chamber (20x10x4) previously saturated with mobile phase vapour for 20 minutes. After developing the plate, it was dried and scanned using Scanner 3 (CAMAG, Switzerland) at 275 nm using WinCATS software. Chromatograms were evaluated before and after spraying with Anisaldehyde – sulphuric acid reagent. After derivatization, plate was dried in hot air oven for 5 minutes at 105°C and viewed under UV at 366 nm.

High Performance Liquid Chromatography (HPLC):-

High Performance Liquid Chromatography was used to analyse the isolated fraction obtained from HPTLC. Sample was dissolved in HPLC grade methanol in concentration of about 1-10 μ g/ml, 20 μ L of the solution was injected in the column RP-C18 and analyzed by PDA detector. The wavelength range was 250 - 500 nm. Thermo HPLC system consisted of Quaternary gradient pumps (LC – 10ATvp), Photodiode Array (PDA) and detector (SPD – M10Avp) with built-in system controller. The analysis was performed on a 25 cm x 4.6 mm, 5 μ m particle size CNW, Athena C18-WP column. The data acquisition was done on Chrom Quest 5 software. The isolated compound from HPTLC was analyzed by using Methanol: Acetonitrile (95:5) as mobile phase.

Characterization of isolated compound:-

The isolated compound was characterized by UV, FTIR and HR-LCMS analysis

UV Spectroscopy:-

The absorbance of the isolated compound was read using one cm cell in a UV – Vis - NIR spectrophotometer (Varian, Cary 5000, Netherlands). The instrument have a spectral range of 175 nm to 3300 nm, wavelength accuracy of ± 0.1 nm (UV –Vis), ± 0.4 nm (NIR), wavelength reproducibility of 0.025nm and a limiting resolution of 0.05nm(UV-Vis), 0.2nm(NIR).The maximum range of absorbance of isolated compound in the methanolic solution was noted by comparing it against HPLC grade methanol as a blank. Separated components (1 mg each) were dissolved in methanol and recorded the spectrum in the range of 200 to 500 nm using a UV double beam spectrophotometer.

Fourier Transform Infra Red spectrometer (FTIR):-

FTIR analysis was carried out using Thermo Nicolet, Avatar 370 spectrophotometer system, which was used to detect the characteristic peaks and their functional groups. The spectral range was between $4000\text{--}400\text{ cm}^{-1}$ and resolution was 4 cm^{-1} with KBr beam splitter, DTGS Detector and HATR Assembly for convenience of measurement. The finger print region extended between $400\text{--}1600\text{ cm}^{-1}$. The spectrum of the isolated compound was elucidated against a blank of HPLC grade methanol.

High Resolution Liquid Chromatograph Mass Spectrometer (HR–LCMS):-

Further structural analysis was aided with HR-LCMS (High Resolution liquid chromatography-mass spectrometry) with a mass spectrometer using High Resolution. Liquid chromatography coupled with mass spectrometry (LC/MS) is a powerful technique for the analysis of complex botanical extracts. HPLC is efficient in separating chemical compounds in a mixture, and MS provides abundant information for structural elucidation of the compounds. The LCMS analysis provides the molecular weight information for the components of the extract. MS dissociations give further structural information on the target compounds (Chen *et al.*, 2007). Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable. The HR–LCMS analysis was performed using Agilent Technologies, USA, model 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs. The mass range is between 50–3200 amu, resolution is 40000 FWHM, high mass accuracy typically less than 1ppm, sensitivity 1 pg. reserpine S/N 100:1, direct infusion for mass analysis (MS, MS /MS), binary nano HP- LC system with mass as detector. The analytical column was an octadecylsilane C18, 250 x 4.6 mm ID, 5 μm particle size protected by a compatible guard column. For the characterization of isolated compound the HPLC method was same as that used in HPLC with CNW, Athena C18-WP column.

Results:-**High Performance Thin Layer Chromatography (HPTLC):-**

HPTLC of methanol extract of *R.officinalis* L. was carried out to confirm its nature by analyzing TLC chromatograms and to isolate active saponin ingredients from the extract. TLC of methanol extract of *R. officinalis* L. revealed the presence of 5 compounds (corresponding to 5 spots) having R_f values of 0.26, 0.42, 0.50, 0.59 and 0.68 respectively when a solvent phase of Chloroform: Acetic acid: Methanol: Water (6.4:3.2:1.2:0.8) was used (Fig:1). Compound having R_f of 0.59 was most prominent and showed clear spot (orange spot) when sprayed using Anisaldehyde – sulphuric acid reagent. Hence, this particular spot was selected for further identification and purification.

High Performance Liquid Chromatography (HPLC):-

HPLC of isolated compound from the methanol extract obtained by HPTLC was carried out to confirm its nature by analyzing HPLC chromatograms. The sharpness of peak, its retention time (R_t min), height and percent area were recorded. The HPLC chromatogram of isolated compound shown only one peak with prominent significant height 303889 and percent area ($> 100\%$) at the retention time 3.372 (R_t min) (Fig 2).

Characterization of the isolated compound:-**UV & Fourier Transform Infra red Spectrophotometry (FTIR):-**

UV spectra displayed characteristic absorption band at 260 nm. Data of IR spectrum (KBr, cm^{-1}) exhibited absorption in the range from 3374.61 cm^{-1} to 662.59 cm^{-1} . The functional group and the chemical bond

corresponding to each peak are tabulated in Table 1. The FTIR spectrum indicated the presence of C-H, O-H and C=O bonds in the isolated compound (Fig 3).

High Resolution Liquid Chromatograph Mass Spectrometer (HR-LCMS) :-

Thus from the FTIR and HR-LCMS chromatogram obtained, the chemical compound isolated from the aerial parts of *R. officinalis* L. is identified as a saponin compound with molecular formula:

1alpha-hydroxy-18-(4-hydroxy-4-methyl-2-pentynyloxy)-23,24,25,26,27-pentanorvitaminD3/1alpha-hydr.

The molecular structure of the chemical compound in Fig.4.

Fig 1:- HPTLC chromatogram of *Rosmarinus officinalis* L. After dervatization UV366nm

A. Crude methanolic extract

B. Isolated compound

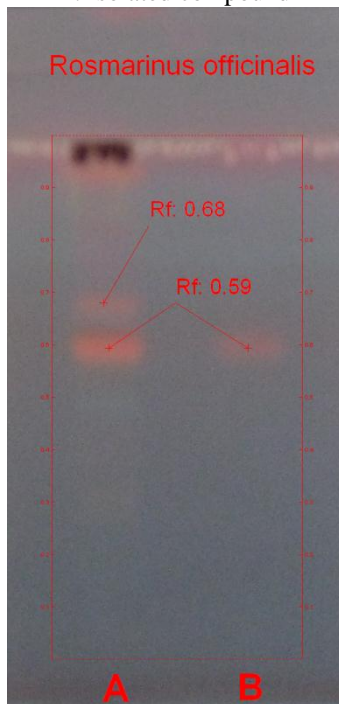


Fig 2:- HPLC chromatogram of the isolated compound.

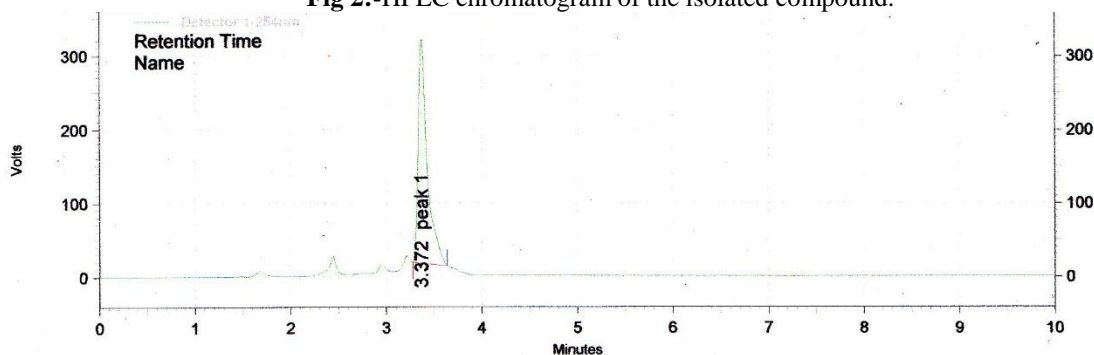
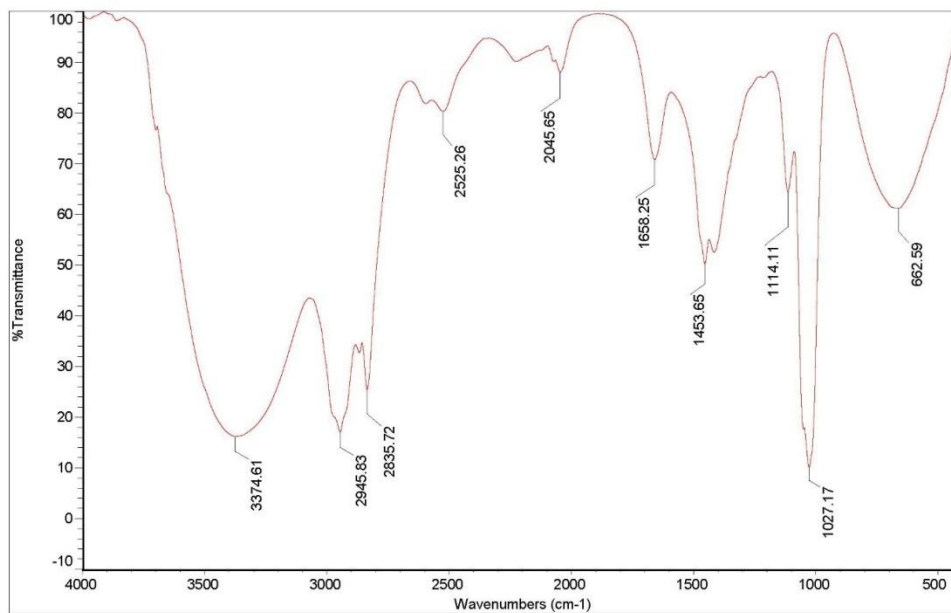
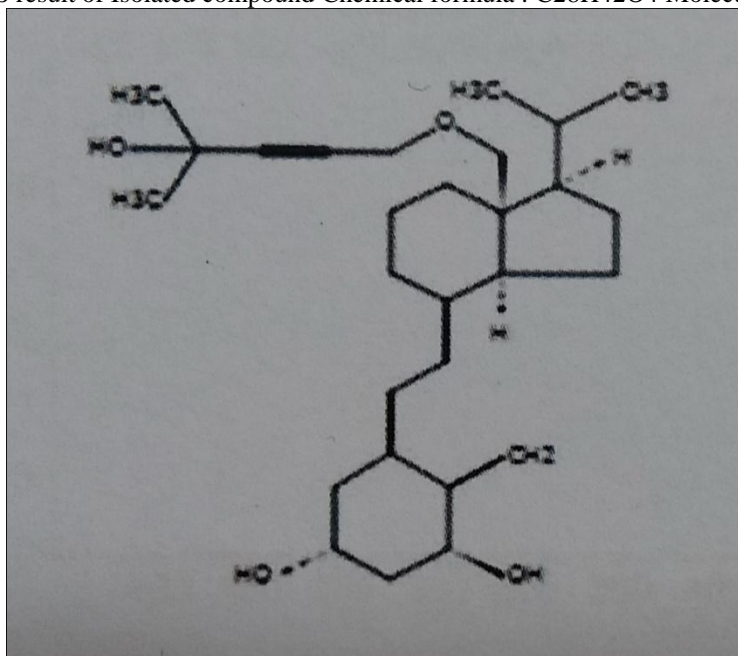


Fig 3:-FTIR spectra of the isolated compound.**Table 1:-** Interpretation of IR spectra.

Frequency (cm ⁻¹)	Functional Group
662.59	-C≡C-H: C-H bend
1027.17	=C-H stretch
1453.65	C-H bend
1658.25	C=O stretch
2945.83	C-H stretch
3374.61	O-H stretch, H-bonded

Fig 4:- HR- LCMS result of Isolated compound Chemical formula : C₂₈H₄₂O₄ Molecular weight : 442.31

Discussion:-

In recent years, although technology and medicine have developed extensively due to decrease in natural richness and other drawbacks, some countries have made it obligatory to use natural products for many goals (Erturk *et al.*, 2003). For this reason we have chosen an important medicinal plant *R. officinalis* L., which is a herb and spice with incredible medicinal properties. In the above studies, saponins were extracted from the plant by HPTLC and HPLC and UV, FTIR and HR-LCMS techniques were carried out to investigate unknown saponins present in the methanol extract.

Renukappa *et al.* (1999) have applied LC-NMR (liquid chromatography-nuclear magnetic resonance) and LC-mass and LC-coupled bioassay to determine two anthelmintic dammarane-type triterpenoidal saponins, significantly active against *Caenorhabditis elegans* from a crude fraction of *Bacopa monniera*. Earlier studies on the biological activities of saponins were limited to crude extracts containing saponins as well as other polar constituents. The advent of modern sophisticated methods of isolation and structure elucidation has attracted great interest of scientific community to study structure activity relationships (Garai, 2014). Nyberg *et al.* (2003) also applied solid phase extraction followed by NMR and MALDI-TOF mass spectrometry on chromatographic fractions QH-A and QH-B of immuno adjuvant active saponins to identify 28 different saponins of *Quillaja saponaria*. Three new olean type triterpenoid saponins were isolated by 1D and 2D NMR and MS spectroscopic data from the aerial parts of *Eclipta prostrata* (L.) by Xi *et al.* (2014). Zhang *et al.* (2002) identified a new saponin as a ginsenoside-Ro derivative containing a polyacetylene side chain by spectroscopic means including 1D and 2D NMR.

In conclusion, we can state that the present study revealed the presence of saponins in *R. officinalis* L. leaves which were confirmed by various characterization studies. Chemical markers are now applicable in many research areas, which include authentication of species, identification of adulterants, structure elucidation and purity determination of medicinal plants. So this isolated saponin can be used as a chemical marker in *R. officinalis* L. which can be exploited more in future. Hence an attempt was made to isolate, purify and characterize the unknown saponins which can be used as markers and can serve as a powerful tool for the standardization of the extracts.

Conclusion:-

The methanol extract of *Rosmarinus officinalis* L. (Rosemary) revealed the structure of compound as 1 α -hydroxy-18-(4-hydroxy-4-methyl-2-pentynyloxy)-23,24,25,26,27-pentanorvitamin D₃/1 α -hydr. and is found to be a Saponin moiety which can be used as marker compound. Further studies need to be conducted for its pharmacological activity.

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