Extraction and Purification of essential oil rich in carnosic Acid from *Rosemarinus officinalis* leaves using one-factor-at-a-time method

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ABSTRACT

Carnosic acid is a hydrophobic secondary metabolite and it was extracted from *Rosemarinus officinalis* leaves. The extract ensures a strong Anti-Microbial activity and Anti -oxidant predominantly due to the presence carnosic acid. Carnosic acid is increasingly exploited with in food, nutritional health and veterinary medicine. The extraction of carnosic acid was performed with different concentration of acetone in the varying ranges from 35% - 95% using one factor at a time method. The HPLC analysis was formed on Varian Pro star system equipped with a Microsorb-100 C18 column (Varian) of 25 cm × 4.6 mm and 5 µm particle size. The mobile phase consists of 0.2% acetonitrile and 0.1% of phosphoric acid in water. The flow rate was constant at 0.7 ml/min, Injection volume was 20 µl .The results show that 65% of concentrated acetone shows more activity in HPLC. The essential oil also separated by The Gas Chromatography was carried out with shimadzu gcms-qp-2010 ultra system. The capillary column (30 × 0.25mm Internal Diameter, film thickness 0.25µm) at a flow rate 1.53 ml/min, helium carrier gas was used. The results showed that major constituents are 1, 8 cineol (53.63%) and camphor (37.32%) .It has efficient anti-bacterial activity due to the carnosic acid.
Key words: Rosemary Leaves, HPLC, GC/MS, anti-microbial activity.

1.INTRODUCTION

Medicinal plants are an important and intense source of bioactive natural compounds or bio-nutrients that have vital role in enhancing health and preventing different diseases. Rosmarinus officinalis is one of the important bioactive medicinal plants perennial spice plant and is grown in chalky soil which has leaves with the height of 1-2 m. Aromatic oil can be and belongs to mint family and to sub family Nepetoideae. Rosemary (R. officinalis) is a woody extracted from stems, leaves and flowers of saliva *Rosmarinus*. The varieties of *Rrosmarinus officinalis* can be divided into two types according to the shape of the plant: one is the vertical type with trunk growing upward, the other are the creeping type with branches growing as lateral. This plant is characterized by its aromatic odor with needle-like leaves. Even though Its native is Mediterranean region it is now grown worldwide. The leaf and its oil are used to make medicines. Carnosic acid which is used for improving memory, indigestion (dyspepsia), arthritis-related joint pain, hair loss, and other conditions. Rosemary is a rich source of antioxidants, Anti-microbial and anti-inflammatory compounds, which are thought to boost the immune system and improve the blood circulation. Laboratory studies has shown rosemary to be rich in antioxidants, which play an important role in neutralizing harmful particles called free radicals. Antimicrobial agents are really important in reducing the global burden of various infectious diseases. In spite of this, the resistance of pathogens to antimicrobial agent increased and also the efficiency of the antibiotic drugs is diminished. All these may give rise to abnormal effect on human health. Using rosemary plant for traditional alternative medicine as a source to treat infectious diseases has been accomplished since the origin of mankind. The leaves are used as anti-inflammatory, analgesic, antispasmodic, antidepressant agents and also used for abdominal pain, carminative, arthritis, gout problems, wound healing (antiseptic), diuretic problems. The aim of this study is qualitative, quantitative analysis of carnosic acid in extracted essential oil.

2. MATERIALS AND METHODS

2.1 Collection of plant sample and reagents

Rosmarinus officinalis Leaves was collected from Indfrag Biosciences Private Limited Krishnagiri District. The plant material was washed with distilled water and dried in hot air oven at 96 ºC for 5 hours. The sample was then powdered using mixer grinder and stored at 4 ºC in aseptic container for further studies. The chemicals required for this investigation were obtained from Adhiyamaan College of Engineering, Hosur.

2.2Optimization of acetone concentration for carnosic acid extraction

The varying acetone concentration in the range from 35% - 95% for the time interval of 15 to 90 min at 60ºC was performed for carnosic acid extraction. 1g of sample is taken and added with 10 ml of varying concentration of acetone and incubated for efficient Extraction of essential oil rich in carnosic acid.

2.3 Quantification of carnosic acid by HPLC

Carnosic acid was identified and quantified in the extracted samples using an HPLC (Varian Pro star) equipped with a Microsorb-100 C18 column (Varian) of 25 cm × 4.6 mm and 5 µm particle size. The mobile phase consists of 0.2% acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23% A, 8-25 min, 75% A, 25-40 min 75% A and the 40-45 min 23% A. Initial conditions were gained in 5 min. The flow rate was constant at 0.66 ml/min. Injection volume was 20 µl and the detection were accomplished by using a diode array detection system (Varian) storing the signal at a wavelength of 230, 280 and 350 nm. HPLC was performed for all the five samples.

2.4 Analysis of essential oil by GC/MS

Gas Chromatography-Mass analysis was carried out with SHIMADZU GCMS-QP-2010 ULTRA at Indfrag Bioscience. The capillary column (30 × 0.25mm Internal Diameter, film thickness 0.25µm) at a flow rate 1.53 ml/min, helium carrier gas was used. Injection mode was split and injection temperature was 240°C, the oven temperature was programmed at 70 °C for 3

min, then raised to 150 °C with-hold time for 2 min and raised to 240° C, the ionization mode was electronic impact mode (SEI) at 70e.The composition of the extracted oil was obtained by means of GC-MS analysis using a gas chromatography (Shimadzu GC-2010) coupled to a mass spectrophotometer (Shimadzu MS QP-2010), equipped with an auto injector AOC-20i series, and using a column AT-5ms (30 m, 0.25 mm, 0.25 mm). The column temperature was initially set at 60ºC (held for 1 min), then increased up to 250ºC at a rate of 2ºC/min (held for 5 min). The mass spectrometer was operated with an injected volume of 2 ml and Helium as the carrier gas at an inlet pressure of 37.0 kPa, a velocity of 32.4 cm/s and ionization energy of 70 eV. The identification of oil components were based on matching their recorded retention indices and mass spectra with those in National Institute of Standards and Technology (NIST) general library (Standard reference Data Program Gaithersburg, MD 20899).

2.5 Antibacterial activity of carnosic acid

The antimicrobial activity of the rosemary leaves extract was done and preliminary antibacterial activity was performed using well diffusion method. The rosemary extract has been studied for their antimicrobial activity in vitro against four tested bacteria. Two Gram positive (Staphylococcus aureus&Streptococcus pyougenes) and two Gram negative bacteria (Klebsiella pneumoniae & Escherichia coli) were used for the antimicrobial activity test. The stock solutions 100mg/mL were prepared by dissolving 100 mg from the extract of rosemary leaves in 1mL of dimethyl sulfoxide (DMSO). The dilution serials of 20, 30, 40, and 50 mL were prepared for determination of antibacterial assay by agar well diffusion assay and carried out by using pure culture for all species of bacteria. Inoculum of bacteria was first sub cultured in brain heart infusion broth & incubated at 37°C for 18-24 hour. After incubation a loopful of each species transferred to tube containing 3 mL normal saline and vortex well. The concentration (1.5×108 CFU/mL) was obtained by using McFarland turbidity standard of each bacteria inoculated by using glass spreader on the surface of Mueller Hinton Agar (MHA) plates previously prepared. The plate was allowed to dry and punched five wells in diameter of 6 mm into agar. Subsequently, in each agar plate of tested bacteria five wells were made and 100µl of dilutions of the extracts (10, 20, 30, 40, and 50 mg/ mL) introduced. It was incubated at 37ºC for 24 hours and looked for zone formation.

3. RESULT AND DISCUSSION

3.1 Collection of plant sample

The plant sample rosemary Leaves was collected from Indfrag Biosciences Private Limited, KrishnagiriDistrict, Hosur.Fig.2 The plant material is washed with distilled water and dried in hot air oven at 96ºC for 5 hours. Sample was then powdered using mixer grinder and stored in aseptic container

Fig:1 Rosemary Leaves sample

Fig: 2 powder sample

3.2 Optimization of acetone for extraction by using One-Factor-at-a-Time (OFAT)

The varying acetone concentration in the range from 35% - 95% for the time interval of 15 to 90 min at 60ºC was performed for carnosic acid extraction. 1g of sample is taken and added with 10 ml of varying concentration of acetone and incubated for efficient Extraction of essential oil rich in carnosic acid.

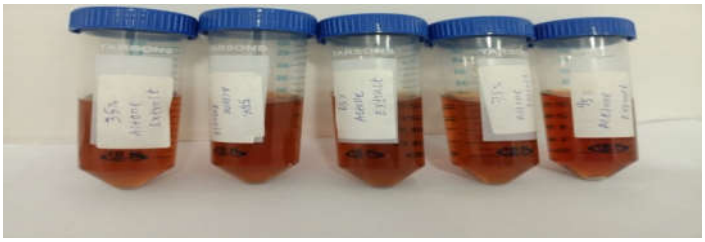

Fig:3 varies acetone concentration

3.2.1 Quantification of carnosic acid by HPLC

The acetone was optimized to extract carnosic acid from the rosemary leaves using five different concentration and are subjected to HPLC analysis for quantification. The results obtained and compared for the best extraction and 65% acetone shows maximum extraction of 1.45 ml/100 g of dry leaves as shown in table 1.

Table.1 Amount of carnosic acid

S.no	Concentration of Acetone	Amount of carnosic acid (%)

1.	35%	0.82
2.	55%	1.19
3.	65%	1.45
4.	75%	1.28
5.	95%	1.03

This investigation had carried out on the chemical composition of dried *R.officinalis* leaves cultivated in IndFrag Biosciences Private Limited, Hosur. The quantity of essential oil isolated by hydro-distillation method was found 1.45% (1.45ml/100g of leaves), revealed a good percentage in comparisons with the percentage in the European monograph (2011:1846) that provides the following definition: whole, dried leaf of Rosmarinus officinalis L. contain minimum 12 ml/kg (1.2%) of essential oil obtained by steam distillation.

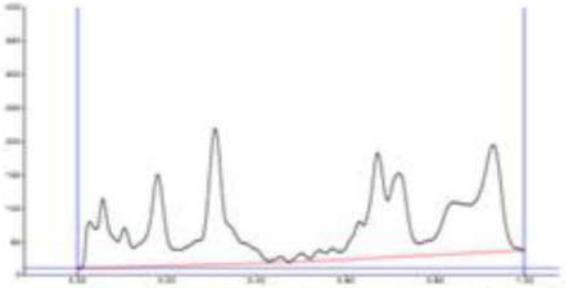

Fig:5 chromatogram of extract from leaves of R.officinalis

3.3 Analysis of essential oil components by Gas Chromatography-Mass

The GC-MS analysis of the components of the oil was performed as shown in Figure. The chromatogram reveals thirteen peaks; only six volatile compounds were identified from the separated components in comparison with National Institute of Standard and Technology (NISTA08) library data base; representing 96.05% as oxygenated monoterpenes from the total oil, as seen in Table 2, while

unidentified compound represents the remaining contents. On other hand, the results showed that major constituents are 1, 8

Peak no.	R.T	Area%	Mass Peak (m/z)	Name of compound
1	**5.12**	**53.63**	**400**	(1,8 cineol)
4	**9.58**	**37.32**	**410**	Camphor
5	**9.72**	**0.84**	**357**	β –Linalool
7	**10.46**	**0.23**	**330**	β- terpineol
10	**11.96**	**3.66**	**423**	Boraneol
11	**12.12**	**0.37**	**361**	Verbenone

cineol (53.63%) and camphor (37.32%), followed by minor components as boraneol (3.66%), linalool (0.84%), verbenone (0.37%) and β-terpineol (0.23%). So, the chemotypes of Karbala plant are: 1, 8 cin-eol /camphor/ boraneol. The volatile oil of rosemary leaves grown in Karbala had particularly high levels of 1,8-cineole (53.63%) and sometimes higher than literature values from previous studies such as: The Lebanese essential oils of rosemary collected from three locations were determined by GC/MS. The three oil samples were revealed to be rich in a-pinene (18.8-38.5%) and 1, 8-cineole (19.1-25.1%). Also, a high quantity of α-terpineol (2.9-11.2%) and geraniol (1.8-9.3%). In Graz, Austria the mass analy-sis detected the essential oil constituents of the dried leaves were 1,8-cineole (41.6%), α-terpineol (4.9%), α-pinene (9.9%), borneol (4.8%) and camphor (17.0%). The oil samples of R.officinalis L. native to India subjected to GC and GC-MS detection showed the presence of α-pinene (6.7-15.6%), camphor (23.1-35.8%) and 1,8-cineole (21.4-31.6%) as major constituents in the oils.

Table.2 Analysis of essential oil components

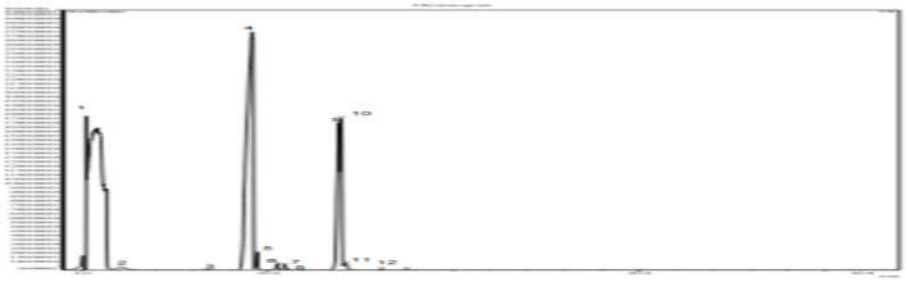

Fig:6 Identification of essential oil contents in rosemary plant by using GC/MS

3.4 Antibacterial activity of carnosic acid

Table 3 illustrates the inhibition zone in (mm) for each concentration of the extract. The results of antibacterial activity of rosemary ex-tracts under study against shows good activity of crude extract.In this study crude extract of rosemary showed an efficient dose dependent -antibacterial activity against different species of bacteria. *Escherichia coli* with inhibition zone 23 mm at high concentration Similar to our results, studies showed that extract of rosemary plant had a good antibacterial action and as a source of natural antibiotics against different species of pathogenic bacteria, this antibacterial effect belongs to the presence of diterpeneoids and phenolic acid compounds

Fig:7F.Staphylococcusaureu

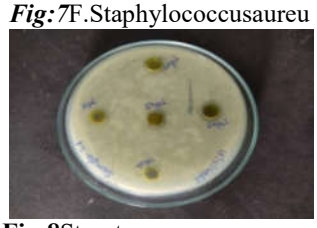

Fig:8 Streptococcuspyougenes

Fig:9 Klebsiellapneumoniae

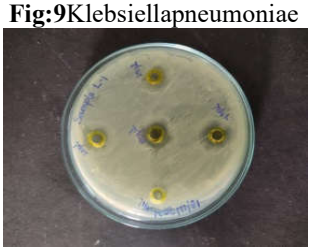

Fig :10Escherichia coli

Table:3 Antibacterial activity zone formation

Bacterial specie	Concentrations of extractsmg/ml				
	10	20	30	40	50
	Inhibition zone (mm)				
Staphylococcus aureus	-	10	14	17	20
reptococcuspyougenes	-	-	-	16	-
Klebsiella pneumoniae	-	-	-	-	-
Escherichia coli	-	15	17	23	20

CONCLUTION

Carnosic acid is recognized as one of the major antioxidant substances present in rosemary leaves. From this study we conclude the leaves of rosemary plant cultivated in Indfrag biosciences has good content of essential oil (1.5%). carnosic acid was detected as a major component in aqueous–Acetone extract of rosemary leaves. Biologically, rosemary plant has a good anti-bacterial activity against some of gram negative and gram-positive bacteria.

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