

Ursolic extraction process from *Rosemary officinalis* leaves of Nepal Cultivar

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Abstract

Rosmarinus officinalis is a woody perennial Mediterranean shrubby herb with fragrant needle like green leaves. It's a member of mint Lamiaceae family which includes many other herbs which grows up to 1.5 m tall on altitudes above 750 feet high. From ancient times it been used to treat many illnesses and culinary purpose, thus it is cultivated worldwide. Rosemary extracts are high in demand due to high natural antioxidant property obtained from Carnosic, Carnosol and other Triterpenoids like Ursolic, betulinic and Oleanolic acids. Ursolic acid is widely used in cosmetic industry because of its broad biological activities like anticancer, anti-inflammatory, anti-microbial and hepatoprotective activities. Thus, we have carried out traditional solvent extraction and purification of Ursolic acid using ethanol and acetone as solvents. In this research work an easy and effective HPLC UV method quantification of Ursolic acid was carried out at 210 nm, flow rate of 1ml/min, injection volume of 10 µL, through C18 reverse phase column. Ursolic acid purity obtained was 50% with a retention time of 21.39 minutes. Thus, purity of 50% Ursolic acid obtained by solvent extraction method with recovery yield of above 98%. This is first time we are reporting method of analysis and extraction of Ursolic acid content from Nepal Cultivar of *Rosemary officinalis*.

Keywords: *Rosemary officinalis*, Leaves, solvent extraction, Ursolic acid, Triterpenoids, Carnosic acid. HPLC quantification, Betulinic acid, Oleanolic acid

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Introduction

Rosemary (*Rosmarinus officinalis*) is the Mediterranean shrubby herb that grows in the wild. Due its common culinary house hold flavoring application it been cultivated worldwide. Further Rosemary hydro alcoholic extracts are used in food industry as preservative and antioxidant^{2, 4, 22}. Due to its high antioxidant activity Rosemary extract has got food additive status from EU regulation as E 392. Since ages it has been used in traditional medicines as antiviral, anti-inflammatory, ant diabetic, anticancer, hepatoprotective and antimicrobial agent. Rosemary extracts used for wound healing and to treat many other illnesses. Rosemary extracts cell proliferation inhibitory activity against different cancer cell lines like breast, prostate, lung, Liver and acute myeloid leukemia cancer cells³

Different potent phytochemical biological activities of rosemary extract been attributed due to the presence of highly useful bioactive compounds like Carnosic acid, Carnosol, Rosmarinic acid, Flavonoids like genkwanin, cirsimaritin and triterpenes such as Ursolic acid, Oleanolic and betulinic acid etc²³. Rosemary leaves and stems contain 1.0 to 2.5% essential oil main components as 1,8-cineol (20-50%), α -pinen (15-25%), camphor (10-25%) along with limonene, bornyl acetate, myrcene, borneol, α -terpinol and verbenone.^{1,7,8}

Ursolic acid, Betulinic acid and Oleanolic acid Figure.2,3,4, are the three types of pentacyclic Triterpenoids with pharmacological potential of anti-inflammatory, antioxidative, antiviral, serum lipid-lowering, and antineoplastic and anticancer activities^{12,13,21}. Ursolic acid mostly used in cosmetic industry⁵ as natural antimicrobial and anti-blemish agent. It's also a potential drug intermediate to synthesize many bioactive antitumor drugs. Ursolic acid is an STAT3 activation pathway inhibitor and may also induce cell death by apoptosis. In many studies it has shown neuron protection and regeneration. In mice experiments it has shown suppressing TH17 immune cells¹. It also has shown strong hepatocellular protection from oxidative damage¹². As they can act at different stages of carcinogenesis by blocking NF- κ B activation,¹¹ induce apoptosis and inhibit proliferation, metastasis Ursolic acid and betulinic acid are considered as agents of both chemoprevention and chemotherapy.^{3, 7,8,14,19,20}

Triterpenoids, diterpenes and other bioactive flavonoids have been extracted using different methods like solid-liquid extractions (SLE), microwave assisted extraction (MAE), super

critical CO₂ extraction etc¹⁷. Every technique has got its own advantages and disadvantages. Such as solvent extraction with SLE technique is time consuming process with high solvent usage but sometimes with good extraction yields based on target compound needed to be isolated. SCO₂ extraction is fast and effective with less solvent consumption, with good yields but not cost effective at commercial levels. MAE is an alternative process to extract phytochemicals with less time consumption and efficient extraction process with low consumption of solvents. Previously methanol and ethanolic solvent extraction and purification were used for Rosemary Ursolic acid. In previous research it has been shown that Ursolic acid, oleanolic acid solubility increase with temperature in methanol, ethanol, isopropanol and ethyl acetate. Mole fraction solubility of Ursolic acid is high in 2-propanol than ethyl acetate higher than methanol higher than ethanol and oleanolic acid mole fraction solubility is higher in ethyl acetate than 2-propanol than ethanol and then methanol in order 5, 6

Being rich source of bioactive compounds and biological activities rosemary extracts are in demand in global market. Thus, main objective of this work was to study the Rosemary cultivar of Nepal the extraction, purification of Ursolic, betulinic and Oleanolic pentacyclic triterpenoids simultaneously and further quantification of Ursolic acid by HPLC method. In this current study demonstrates the simultaneous extraction and purification of three terpenoids which could be useful in many systems of medicine as well as natural antioxidants and cosmetic applications.⁵

For the first time we are reporting method of analysis and extraction of Ursolic acid content from Nepal Cultivar of Rosemary officinalis.

Materials and Methods

Methanol, Millipore water and phosphoric acid HPLC grade, methanol, ethanol, acetone and chloroform of analytical grades were purchased. Ursolic acid working standard: purity $\geq 98\%$ was gifted from Herbal creations India. Reagents and chemicals used throughout the experiment were analytical grade.

Dried *Rosemary officinalis* cultivar of Nepal was provided by Larke Himal Jadibuti Udhyog Kathmandu.

Extraction of total Terpenoids

Leaves were dried to residual moisture content of 10% in an oven below 50 °C temperature. 100 grams of dried leaves were soaked in 300 ml distilled water for 30 min in a percolator. Additional 1:5 ratio distilled water was added and left for 10 h. The soaked leaves were dried in oven till moisture reaches 15%. The dried leaves were loaded in Soxhlet apparatus and extracted with 96% ethanol in 1:1 ratio at 50 °C for 3-4 h. and extracted further two more times with 1:1.5, 96% ethanol for period of 3 h each. The total extractive volume was filtered and reduced to 80% of the total volume and kept at freezing temperature of 5 °C for 4-5 h in a refrigerator. The light brownish yellow precipitate thus formed should be collected through membrane filtration and washed with ice cold ethanol to get total terpenoids.¹¹ The obtained precipitate was then dried at 40 °C in an oven till moisture reaches 3%.

Simultaneous extraction of Ursolic acid, Betulinic acid and Oleanolic acid

Leaves were dried to residual moisture content of 10% in an oven below 50 °C temperature. 100 grams of dried leaves were soaked in 1:12 ratio of acetone. An additional 1:10 ratio acetone added consecutively for three times extraction for 3 h each at 50 °C in Soxhlet. The total acetone extract was collected and reduced to 80% of total volume and kept at freezing temperature of 5 °C for 4-5 h in a refrigerator. The white yellow precipitate thus formed should be collected through membrane filtration and washed with cold acetone to get mixture of Ursolic acid, Betulinic acid and Oleanolic acid. The mixture was further tested for purity by HPLC analysis of Ursolic acid. The obtained precipitate was then dried at 40 °C in an oven till moisture reaches 3%. The extracted was further subjected to column chromatography using silica of mesh size 90 in mobile phase of 2-propanol, ethanol and n-hexane in ratio of 5:3:1 with extract to solvent ratio of 1:20 and fractions of 30 ml each collected and further subjected to crystallization and HPLC analysis for quantification of Ursolic acid^{9,13,15,16}

Analysis Method of Ursolic Acid by HPLC

1. Reagents and materials

Methanol: HPLC

Ursolic acid working standard: purity $\geq 98\%$

Phosphoric Acid solution: 0.1ml Phosphoric Acid added into 100ml water

Standard stock solution: accurately weigh 25mg (accurate to 0.0001g) of Ursolic acid reference standard, dissolve in mobile phase and constant volume to 25ml, mix well, and store in the refrigerator.^{9,13,15,16}

2. Instruments, Equipment and Reference Chromatographic Condition

HPLC: equipped with UV-detector or diode-array detector

Column: C18 reverse column (Phenomenex)

Mobile phase: Methanol: Phosphoric Acid solution =85:15

Flow Rate: 1 ml/min

Column temperature: 25 °C

Injection volume: 10UL

Detection wavelength: 210nm

Retention time: 30min.

3. Analysis Procedures

3.1 Preparation of sample solution

Accurately weigh 10mg of sample (accurate to 0.0001g), dissolve it with mobile phase and constant volume to 50ml, and filter it with 0.22um Millipore filter, obtain the sample solution.

3.2 Drawing of standard curve

Accurately absorb the standard stock solution and prepare it as Standard solution series 0mg / ml, 0.25mg/ml, 0.5mg/ml, 1.0mg/ml. The standard solution series were analyzed by

Chromatography under the reference Chromatographic condition. According to the content of Ursolic acid and the corresponding Chromatographic peak area, the Chromatographic peak area was used as the ordinate and the content of Ursolic acid was used as the abscissic coordinate to draw the standard curve.

3.3 Determination

Accurately absorb 10ul of sample solution, conduct the Chromatographic Analysis under the specified chromatographic conditions, determine the quality with retention time and determine the quantity with peak area external standard method.

3.4 Result calculation

The mass fraction of Ursolic acid W_2 , calculation

$$w_2 = C \times V \times 100\% / 1000 \times m$$

C-The concentration % of Ursolic acid in the sample solution obtained from the standard curve, unit: (UG / ml)

V-Constant volume of sample solution, unit: (ml)

1000-Mass conversion factor

M-Sample quality, unit: (mg)

The test results shall be based on the arithmetic mean of the parallel test results, not more than 2% of the arithmetic mean

Results and Discussion

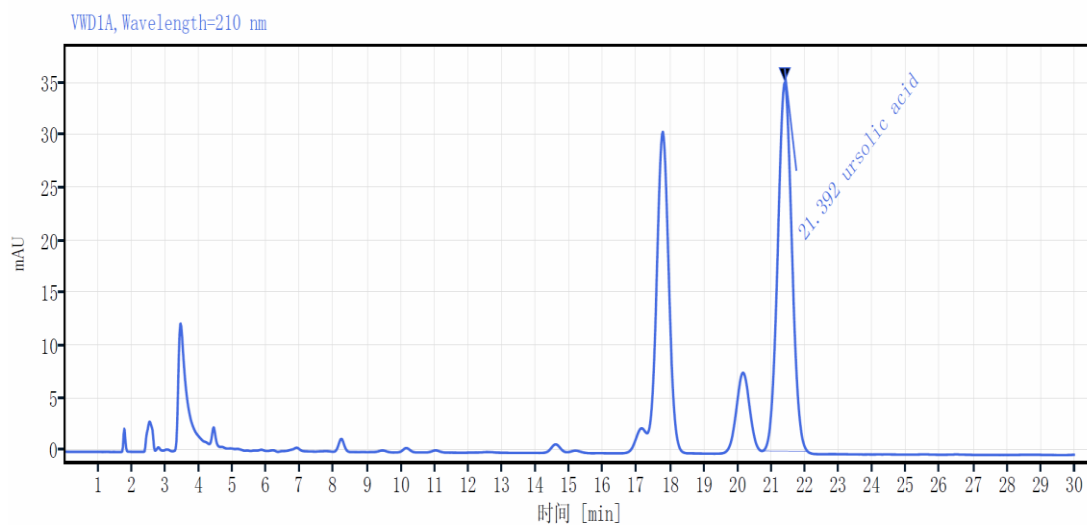
Extraction of Total Terpenoids and Ursolic acid extraction, purification and quantification.

The highest total terpenoid content of 3.4% obtained by ethanol extraction and purification. Further microporous resin purification increased the content of terpenic acids up to 98%. Acetone extraction had yielded 2.3% white colored Ursolic acid enriched content with more than 50% purity, Chromatogram Fig.1 next 40% betulinic acid and 10% Oleanolic acid by HPLC analysis method. In this paper as we are studying Ursolic acid, thus we mentioned analysis of Ursolic acid. Ursolic acid quantification was carried out on Agilent HPLC machine equipped with UV-detector, Column C18 reverse column, Mobile phase: Methanol:

Phosphoric Acid solution =85:15 Flow Rate: 1 ml/min Column temperature: 25 °C Injection volume: 10 µL, Detection wavelength: 210nm Retention time: 30min^{9,13,15,16}. The Retention time of Ursolic acid by above method was 21.39 min and purity of 50% obtained. The method was simple, robust and precise for quantification of Ursolic acid. The same method can be employed for quantification of Betulinic and Oleanolic acids.

Conclusion

Rosmarinus officinalis (Rosemary) a Mediterranean shrubby herb well known for its culinary and medicinal properties from ancient times. Recent advancements in phytochemical and ethnopharmacology research studies had shown rosemary extracts has antioxidant, anticancer, antiviral, anti-inflammatory and anti-microbial activities. Rosemary leaves has different phytochemicals like Carnosic acid, Carnosol, Rosmarinic acid, Ursolic acid, betulinic acid, Oleanolic acid and many other flavonoids and terpenes Ursolic acid²⁰ had a high commercial demand in cosmeceutical industry. Many other herbs contain Ursolic acid like Holy basil species. Rosemary Ursolic acid is easy to isolate and present upto 2-3 %. Many extraction techniques like MAE, SFE (Supercritical fluid extraction), UAE (Ultrasonic assisted extraction) and Solid –Liquid solvent extractions techniques are used for isolation and purification of Ursolic acid. For the first time we had used simple traditional solid-liquid solvent extraction technique using ethanol and acetone as solvents for extraction of Ursolic acid and proceeding with column elution to with silica of mesh size 90 in mobile phase of 2-propanol, ethanol and n-hexane in ratio of 5:3:1 with extract to solvent ratio of 1:20 and fractions of 30 ml each collected and further subjected to crystallization and HPLC analysis for quantification of Ursolic acid. Isocratic reverse phase HPLC chromatography was carried using Methanol: Phosphoric Acid solution 85:15 as mobile phase, flow rate 1ml/min, injection volume of 10UL at 210 nm wavelength, at 25 °C column temperature for 30 minutes. Ursolic acid detected at 21.39 minutes retention time consistently with comparison against working standard with purity of 50%. Thus we suggest that this method extracting Ursolic acid from dried leaves of Rosemary is simple, effective and also our HPLC method of detection is simple, specific, fast and effective to quantify Ursolic acid content.



Signal: VWD1A, Wavelength=210 nm

Name	keep time [min]	Response factor	Peak area	content [mg/ml]	concentration [%]	set
ursolic acid	21.39	4281.091	1018.784	0.238	50.365	
			sum	0.238		

Figure 1: HPLC chromatogram of Ursolic acid Rt 21.39 min at 210 nm wavelength in UV detector.

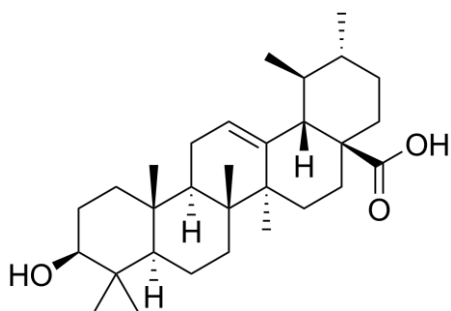


Fig.2. Ursolic acid

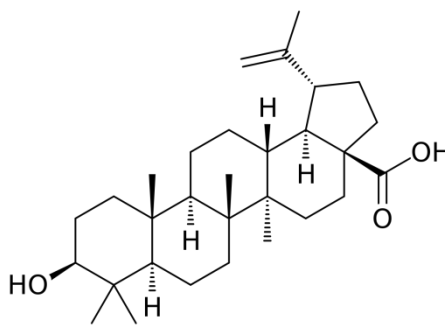


Fig.3. Betulinic acid

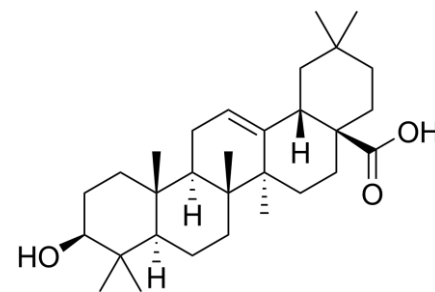


Fig.4.Oleanolic acid

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