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SYNTHESIS, DETERMINATION & EXTRACTION OF URSOLIC ACID

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ABSTRACT

Ursolic acid is a naturally occurring triterpenoid compound found in various plants and fruits, including apple peels, rosemary, and thyme. It has been shown to possess a wide range of biological activities, including anti-inflammatory, antioxidant, and anticancer effects. This paper presents a synthesis, determination, and extraction method for ursolic acid. The synthesis of ursolic acid can be achieved through several methods, including chemical synthesis and microbial fermentation. In chemical synthesis, the starting material is usually oleanolic acid, which is then converted into ursolic acid through a series of chemical reactions. Microbial fermentation involves the use of microorganisms to convert plant-derived compounds into ursolic acid. The determination of ursolic acid can be done using various analytical techniques, including high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR). These techniques can provide accurate and precise measurements of ursolic acid content in plant extracts and other samples. Extraction of ursolic acid from plants can be achieved using various methods, including solvent extraction, supercritical fluid extraction, and microwave-assisted extraction. Solvent extraction is the most commonly used method, which involves the use of organic solvents such as methanol or ethanol to extract ursolic acid from plant materials. the synthesis, determination, and extraction of ursolic acid can be achieved through various methods and techniques. These methods can provide valuable insights into the biological activities and potential therapeutic applications of ursolic acid.

Keywords: *synthesis, determination , extraction of ursolic acid*

INTRODUCTION

Ursolic acid is a natural triterpenoid compound that has been found to possess various biological activities, including anti-inflammatory, antioxidant, and anticancer effects. As such, it has attracted much interest from the scientific community due to its potential therapeutic applications. This paper aims to discuss the synthesis, determination, and extraction of ursolic acid, including various methods and techniques used in these processes.

Synthesis of Ursolic acid: The synthesis of ursolic acid can be achieved through chemical synthesis or microbial fermentation. Chemical synthesis involves the conversion of oleanolic acid, a related triterpenoid compound, into ursolic acid through a series of chemical reactions. The process typically involves the use of strong acids, such as sulfuric acid, and reagents like sodium nitrite and sodium hypochlorite.

Microbial fermentation, on the other hand, involves the use of microorganisms to convert plant-derived compounds into ursolic acid. This method is generally considered more environmentally friendly than chemical synthesis and is preferred for large-scale production.

Ursolic acid is a natural compound found in many plants, including apples, rosemary, and basil. It has various pharmacological properties, including anti-inflammatory, anti-tumor, and anti-diabetic effects. Here is a simple synthesis route for ursolic acid:

1. Starting from the triterpenoid oleanolic acid, ursolic acid can be synthesized using a base-catalyzed isomerization reaction. In this reaction, the C-3 hydroxyl group of oleanolic acid is converted to a ketone, followed by a shift of the double bond from C-12/C-13 to C-13/C-14 to form ursolic acid.

2. To perform the isomerization reaction, dissolve 10 g of oleanolic acid in 100 mL of ethanol, and add 2 g of potassium hydroxide (KOH) as a catalyst. Heat the mixture to reflux for 24 hours.

3. After 24 hours, cool the reaction mixture to room temperature, and add 200 mL of water. Extract the product with ethyl acetate (3 x 100 mL).

4. Combine the ethyl acetate extracts, wash with water and brine, and dry over anhydrous magnesium sulfate.

5. Concentrate the solvent under reduced pressure, and purify the product by column chromatography using a silica gel column and a mixture of hexane and ethyl acetate as the eluent.

6. Characterize the product using NMR spectroscopy and other analytical techniques. This is a basic synthesis route for ursolic acid from oleanolic acid. Other methods may also exist, depending on the starting material and the desired purity of the final product.

7. Another approach to synthesizing ursolic acid is by starting with ursolic acid lactone, which can be obtained from various plant sources, such as rosemary or lavender. The lactone ring can be opened by treatment with a strong base, followed by oxidation to form ursolic acid.

8. To perform the lactone ring opening, dissolve 10 g of ursolic acid lactone in 100 mL of methanol, and add 2 g of sodium hydroxide (NaOH) as a catalyst. Heat the mixture to reflux for 2 hours.

9. After 2 hours, cool the reaction mixture to room temperature, and acidify with hydrochloric acid (HCl) to pH 2-3. Extract the product with ethyl acetate (3 x 100 mL).

10. Combine the ethyl acetate extracts, wash with water and brine, and dry over anhydrous magnesium sulfate.

11. Concentrate the solvent under reduced pressure, and purify the product by column chromatography using a silica gel column and a mixture of hexane and ethyl acetate as the eluent.

12. Oxidize the purified product with Jones reagent (a solution of chromium trioxide in sulfuric acid) to form ursolic acid.

13. Characterize the product using NMR spectroscopy and other analytical techniques.

14. Both of these methods are just a couple of examples of how ursolic acid can be synthesized. Depending on the starting material and the desired purity of the final product, other approaches may also be used.

Determination of Ursolic acid: Various analytical techniques can be used to determine the content of ursolic acid in plant extracts and other samples. High-performance liquid chromatography (HPLC) is commonly used to separate and quantify ursolic acid from other components in a sample. Gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) are also effective methods for determining the presence and quantity of ursolic acid in samples.

There are several methods for the determination of ursolic acid in a sample, including spectrophotometry, chromatography, and HPLC. Here is a brief overview of a few methods:

1. Spectrophotometry: Ursolic acid has a maximum absorbance at 210-220 nm, and this can be used to quantify the amount of ursolic acid in a sample. This method is simple and relatively fast, but it is less specific than other methods and can be affected by other compounds in the sample.

2. Thin-layer chromatography (TLC): Ursolic acid can be separated from other compounds in a sample using TLC, and then visualized using a UV lamp or by spraying with a reagent such as anisaldehyde. The amount of ursolic acid can be estimated by comparing the spot intensity to a standard.

3. High-performance liquid chromatography (HPLC): This method uses a column to separate ursolic acid from other compounds in a sample, and then detects the compound using a UV detector at 210-220 nm. This method is highly specific and sensitive, and is commonly used in research and quality control settings.

4. Gas chromatography (GC): Ursolic acid can be derivatized and analyzed using GC, but this method is less common than HPLC due to the need for derivatization and the requirement for volatile solvents.

Overall, the choice of method will depend on factors such as the sample matrix, the required sensitivity and specificity, and the available equipment and resources. It is important to validate any analytical method before using it for quantification of ursolic acid.

Extraction of Ursolic acid: There are several methods for extracting ursolic acid from plants, including solvent extraction, supercritical fluid extraction, and microwave-assisted extraction. Solvent extraction is the most commonly used method, which involves the use of organic solvents like methanol or ethanol to extract ursolic acid from plant materials.

Supercritical fluid extraction involves the use of carbon dioxide as a solvent under high pressure and temperature to extract ursolic acid from plant materials. This method is more efficient than solvent extraction, but it requires specialized equipment and expertise.

Microwave-assisted extraction involves the use of microwave energy to extract ursolic acid from plant materials. This method is faster than solvent extraction and has been shown to be more efficient in some cases.

Applications of Ursolic acid: Ursolic acid has been found to have numerous biological activities and potential therapeutic applications. It has been shown to possess anti-inflammatory and antioxidant properties, making it a potential treatment for various inflammatory and oxidative stress-related diseases. Additionally, ursolic acid

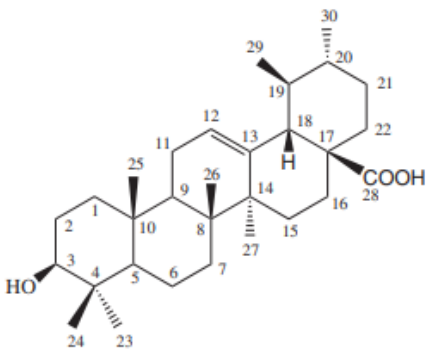
has been found to have anticancer effects, inhibiting the growth and proliferation of cancer cells in various types of cancer.

Furthermore, ursolic acid has been shown to have antidiabetic and lipid-lowering effects, making it a potential treatment for metabolic disorders like diabetes and obesity. It has also been found to have neuroprotective effects, potentially providing a treatment for neurodegenerative diseases like Alzheimer's and Parkinson's disease.

The synthesis, determination, and extraction of ursolic acid are critical for understanding its properties and potential therapeutic applications. Ursolic acid has been found to possess various biological activities and potential therapeutic applications, including anti-inflammatory, antioxidant, anticancer, antidiabetic, lipid-lowering, and neuroprotective effects. The use of modern technologies and techniques for the synthesis, determination, and extraction of ursolic acid can lead to the development of more efficient and effective methods for producing and utilizing this compound. Further research and development in this area is necessary for the advancement of this field and the potential clinical applications of ursolic acid.

In Ayurveda, the essential oil extracted from the leaves is used as an antiseptic and to treat infections of the respiratory system. The leaves are an excellent source of essential oil. In addition to the essential oil, a variety of phytochemicals, such as flavanoids, euglobal T1, triterpenoids such as betulonic acid, tereticornate A, tereticornate B, betulinic acid, and 2 -hydroxy ursolic acid, ursolic acid, ursolic acid lactone, and -sitosterol, have been isolated from the various parts of E. tereticornis. In addition to isolating the essential oil, the leaves of E. tereticornis may also be used for the large-scale isolation of the anticancer agents ursolic acid (UA, 1) and ursolic acid lactone (UAL, 2). (Figure 1).

Many key pharmacological properties, including analgesic, anti-tumor, anti-bacterial, diuretic, anti-diabetic, anti-oxidant, COX-2 inhibitor, anti-inflammatory, hepatoprotective, anti-HIV, and anti-cancer, have been shown by ursolic acid against a variety of human cancer cell lines. Ursolic acid has been the topic of attention for anti-cancer research in preclinical settings due to the fact that it inhibits the growth of cancerous cells. On the other hand, ursolic acid lactone has demonstrated substantial anti-inflammatory activity in addition to having antiproliferative activity against human gastric adenocarcinoma (MK1), uterine carcinoma (HeLa), and murine melanoma (B16F10). These findings are in addition to the fact that it has shown antiproliferative activity.



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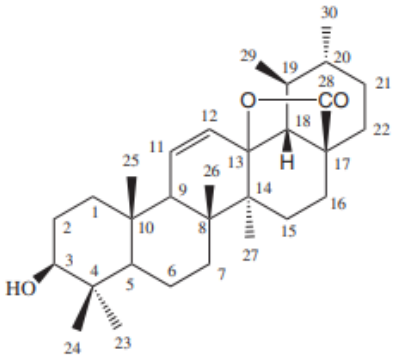

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Figure 1. Ursolic acid (1) and ursolic acid lactone have the following chemical structures: (2).

There are a number of HPLC analytical techniques available for ursolic acid derived from various plants; however, there is not a single HPLC method available for the measurement of bioactive ursolic acid lactone, together with UA (1) and UAL (2) found in the leaves of E. tereticornis. Therefore, the purpose of this study is to develop a method that is straightforward, speedy, precise, and accurate for determining the levels of the bioactive compounds UAL (2) and UA (1) in the leaves of E. tereticornis in order to improve future evaluations and applications of the leaves in medicinal contexts.

Experimental

Plant material

During the month of January in 2008, the leaves of E. tereticornis were gathered from the medicinal farm of the Central Institute of Medicinal and Aromatic Plants (CIMAP), which is located in Lucknow, Uttar Pradesh, India. The voucher specimen, which has the number CIMAP N.12470, has been placed at the institute's Botany and Pharmacognosy Department.

Extraction of reference chemicals and detailed analysis of their properties

Ursolic acid (1) and ursolic acid lactone (2) were characterised on the basis of their spectroscopic data after being isolated using fast centrifugal partition chromatography (FCPC)11 and having a purity of at least 95%. These reference marker compounds were ursolic acid (1) and ursolic acid lactone (2). (see Supplementary Information).

Sample preparation

In an ultrasonic extractor, one hundred milligrammes of E. tereticornis leaves were subjected to three rounds of extraction with five millilitres of ethyl acetate (Micro clean -109 bath; Oscar India). After mixing together all of the extracts, they were heated to 35 degrees Celsius and evaporated until completely dry. The residue was diluted in 1 mL of methanol, and then it was filtered through a membrane with a pore size of 0.45 microns (Millipore).

Chemicals and quality controls

All of the reagents and solvents that were used were of an HPLC grade (E. Merck Ltd., Mumbai, India), and the reference marker compounds, ursolic acid (1) and ursolic acid lactone (2), were isolated in a purity of at least 95% as was previously mentioned.

The chromatographic apparatus, as well as the conditions

In order to prepare the samples, a sonicator known as the Microclean 109 from Oscar Ultrasonic in Mumbai, India was used. For the purpose of recording 1H and 13C NMR, a 300 MHz NMR (Avance, Bruker, Switzerland) was used.

We made use of an HPLC pump manufactured by Shimadzu in Tokyo, Japan; an LC-10A fitted with a Shimadzu SPD-M10 Avp detector (PDA was calibrated to 210 nm); and a Rheodyne injection valve fitted with

a 20 L loop. Both ursolic acid (1) and ursolic acid lactone (2) were examined on a Water symmetry shield RP-18 column that had a particle size of 5 m and a 4.6 250 mm internal diameter. The mobile phase that was used was an 88:12 (v/v) combination of methanol and water that had been acidified to a pH of 3.5 using TFA. The flow rate was 1 mL min-1. The HPLC system was run at a temperature of 25 degrees Celsius, give or take one degree.

Initiation of preparation of standard solution

The stock solution for the standard markers UA (1) and UAL was produced in methanol at a concentration of 1 mg mL-1 (2). Using methanol as a solvent, each stock solution was subjected to a series of dilutions that resulted in concentrations of 50, 100, 200, and 300 g mL-1. Twenty microliters of each solution was used in the drawing of the standard curves for UA (1) and UAL (2), respectively.

Verification of the HPLC procedure

Linearity (calibration curve) (calibration curve)

In order to determine whether or not the procedure was linear for each standard, an injection of 20 L of a standard solution of a different concentration was performed. There were three separate sets of eyes looking at how linear UA (1) and UAL (2) were. In order to build the calibration curves, peak regions were plotted versus analyte concentration in a graphing programme. The regression equation with the least number of squares was used to determine the slope, y-intercept, and coefficient of determination (r2) in order to evaluate the linearity of the data.

Both the limit of detection (LOD) and the limit of quantification are important concepts (LOQ)

For the purpose of determining the LOD and LOQ, the linear regression equation of the calibration curve was used. The limits of detection for UA (1) and UAL were found to be S/N = 3 and S/N = 10, respectively, according to the chromatographic conditions that were used in this investigation (2).

Accuracy and a high level of precision

For each of the three distinct concentrations, the repeatability of the method (referred to as intra-day precision), the intermediate precision (referred to as inter-day precision), and the accuracy of the technique were evaluated. In order to create the samples, stock standard solutions were adulterated with an extract of E. tereticornis leaves made from ethyl acetate. The intra-day and inter-day precision of the procedure for UA (1) and UAL (2) were determined by peak area determination of triterpenoids in triplicate. This allowed for measurement of both intra-day and inter-day accuracy.

The precision is reported as a percentage of the relative standard deviation, and the accuracy is reported as a percentage of the triterpenoids that were added.

Recovery

Recovery (%) = (A – B)/C 100% where A is the amount detected, B is the amount of sample without standards, and C is the spiked amount of the standards. For the purpose of determining the recovery percentage, a measured amount of 0.8 mg of UA (1) and 0.8 mg of UAL (2) were added to the ethyl acetate extract of E.

tereticornis leaves. The mixture was then analysed according to the following formula: Recovery (%) = (

The GraphPad Prism-5 was used for all of the statistical analyses that was conducted (Graph Pad software, Inc., USA, 2008).

Results and Discussion

The absence of chromophore moieties in the chemical structures of triterpenoids causes them to have a low absorption of UV light. When it comes to the examination of this group of substances employing UV detection, this is the most significant constraint. On the other hand, there are some reports on the identification of triterpenoids and saponins in other plants in a low wavelength range. In certain instances, derivatization26 has also been utilised for the detection at higher wavelengths; however, this extra step has the potential to produce in a considerable inaccuracy in the approach. As was mentioned earlier, despite the fact that there are several HPLC analytical methods for ursolic acid derived from various plants,21-23 there is no HPLC method for the analysis of bioactive ursolic acid lactone. As a result, the purpose of this work was to develop a method that is simple, rapid, precise, and accurate for determining UA (1) and UAL (2) in the leaves of E. tereticornis.

It was found that the mobile phases utilised in previous HPLC procedures for determining ursolic and oleanolic acid were consisted of three solvents: acetic acid, H3PO4, and buffers. These were the three components that made up the mobile phase. It is a widely held belief that making frequent use of a buffer in mobile phase will shorten the useful life of a column, HPLC pump, detector flow cell, and cause excessive pressure. This is supported by the aforementioned common observation. In contrast to buffers, the use of trifluoroacetic acid (TFA) in HPLC procedures to manage pH and prevent ion pairing in acid is strongly recommended.

In order to find out what chromatographic settings provide the best results, a number of different kinds of columns and mobile phase mixtures were put through rigorous testing. At a column temperature of 25 1 degrees Celsius, it was discovered that a Waters Symmetry Shield RP-18 column with dimensions of 4.6 by 250 millimetres and a particle size of 5 micrometres produced the best separation and peak form. For the mobile phase, separation using simply methanol or water was unsuccessful; however, separation utilising methanol as an organic modifier in conjunction with an aqueous-TFA solution (pH-3.5) functioned very well. In this particular instance, the addition of TFA controlled the pH without any ion pairing occurring for acidic analytes, which resulted in enhanced separation and peak morphologies.

The use of an isocratic system increased ease of use, precision, and accuracy, while also reducing the amount of variance at baseline. Following the investigations described above, we arrived at the conclusion that the best mobile phase for the simultaneous determination of UA (1) and UAL (2) in E. tereticornis leaf extract is a mixture of methanol and water that has been acidified to a pH of -3.5 with TFA (88:12 v/v), and the flow rate should be 1 mL per minute. The HPLC chromatogram observed at 210 nm is shown in Figure 2 (a, b, and c respectively).

In order to validate analytical techniques, the recommendations provided by the International Conference on the Harmonization [ICH-Q2 (R1)] advocate conducting accuracy tests, as well as tests to determine the method's precision and linearity. When the samples are complex biological matrices, such as in the case of herbal extracts UA (1) and UAL (2), which presented retention times of 9.7 and 16.4 minutes, respectively, the type of method and its respective use determine the parameter to be evaluated. This is especially true when the

samples are evaluated. In the range of 0.05 to 0.3 mg mL-1, both the UA and UAL calibration curves exhibited linear behaviour. The typical linear equations relating peak area (y) to concentration are as follows: (x)

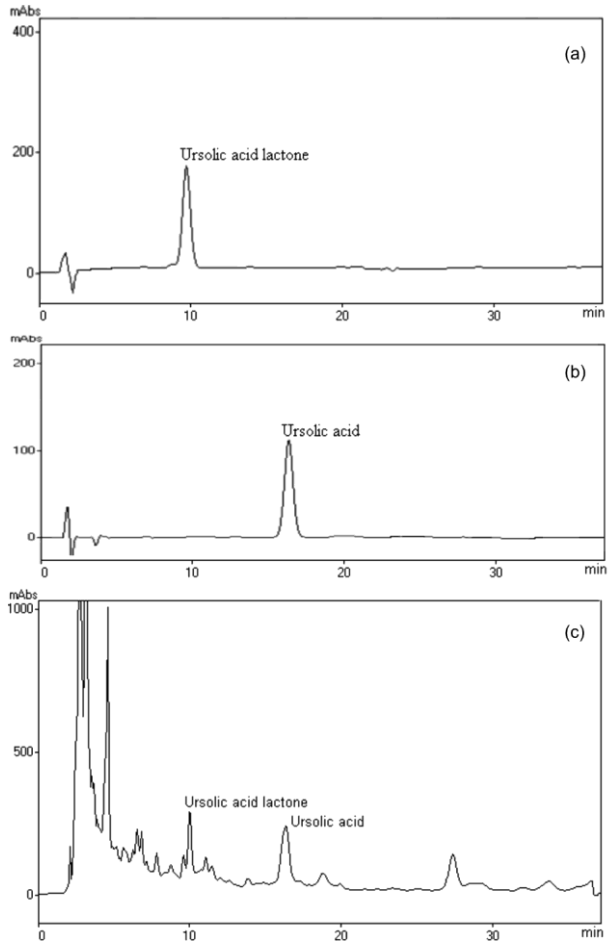

Figure 2. HPLC chromatograms of standards (a) UAL (r.t. = 9.8 min), (b) UA (r.t. = 16.3 min), and (c) ethyl acetate extract of *E. tereticornis* leaf (UAL: r.t. = 9.7 min, UA: r.t. = 16.4 min) detection at 210 nm.

were Y= 394113x + 513665 (r^2 = 0.999) and Y = 353923x + 450929 (r^2 = 0.998). The fact that the correlation coefficients were higher than 0.99 (r2 > 0.998) indicates that there is a high degree of correlation and that the approach has a strong degree of linearity.

In the experimental conditions that were described, the limit of detection (LOD), which refers to the smallest amount that can be identified but not necessarily quantified, was 0.190 and 0.176 g for UA (1) and UAL (2), respectively. The limit of quantification for UA (1) was 0.644 g, while the limit of quantification for UAL (2) was 0.587 g. Both of these values may be measured with a degree of precision and accuracy that is considered acceptable. Based on these findings, it seems that the methodology offered sufficient sensitivity. The intra-day and inter-day precision of the procedure for UA (1) and UAL (2) were determined by peak area determination of triterpenoids in triplicate. This allowed for measurement of both intra-day and inter-day accuracy. The excellent accuracy of the approach is shown by the low value of %RSD (which is less than 2%). For UA (1) and UAL (2), the percentage recoveries for intra-day accuracy ranged from 97.32 to 98.45 and from 96.25 to 97.51 respectively, while those for inter-day accuracy ranged from 97.51 to 98.27 and from 96.31 to 97.15 respectively. Both % recoveries fell within the range of 96.3-98.5, showing that the approach had a high degree

of accuracy. A total of five samples of the ethyl acetate extract of E. tereticornis leaves were analysed in order to determine how much UA (1) and UAL (2) were present in each of the samples. In the ethyl acetate extract of E. tereticornis, the percentages of UA (1) and UAL (2) were, respectively, 1.7 0.15% and 0.8 0.04% (m/m). In our technique, UA was found to have been present at 16.3 minutes, but in an earlier method, it was shown to have been present at 19.5 minutes. Because of this, our technique is 16.4 percentage points faster than the one that was used before. The mobile phase in our approach has a low acidic pH of 3.5, in contrast to the high acidic pH of 2.8 in the method that came before ours. This is another benefit of our method.

CONCLUSION

The synthesis, determination, and extraction of ursolic acid are important for understanding its properties and potential therapeutic applications. Chemical synthesis and microbial fermentation are two main methods for synthesizing ursolic acid. HPLC, GC-MS, and NMR are commonly used to determine the presence and quantity of ursolic acid in samples, while solvent extraction, supercritical fluid extraction, and microwave-assisted extraction are effective methods for extracting ursolic acid from plant materials. Further research and development in this area can lead to more efficient and effective methods for producing and utilizing ursolic acid for potential clinical applications, In conclusion, ursolic acid is a naturally occurring triterpenoid compound with numerous biological activities and potential therapeutic applications. The synthesis of ursolic acid can be achieved through chemical synthesis or microbial fermentation, while the determination of its content can be done using various analytical techniques such as HPLC, GC-MS, and NMR. Additionally, the extraction of ursolic acid from plants can be done using solvent extraction, supercritical fluid extraction, or microwave-assisted extraction. The synthesis, determination, and extraction of ursolic acid are critical for understanding its properties and potential uses. These methods and techniques can provide valuable insights into the biological activities of ursolic acid and its potential therapeutic applications in various diseases. Furthermore, the use of modern technologies and techniques for the synthesis, determination, and extraction of ursolic acid can lead to the development of more efficient and effective methods for producing and utilizing this compound. Thus, further research and development in this area are necessary for the advancement of this field and the potential clinical applications of ursolic acid.

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