QUALITY CONTROL: CURB COUNTERFEITING OF *ASPHALTUM* (SHILAJIT)

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Abstract: Increased demand of *Asphaltum* (Shilajit) in immunity modulation and fertility has generated intense interest among the producers and regulators to ensure its quality. For doing so, there is a need to isolate its chemical markers and analyze the same. Urolithins (Benzocoumarins) are one of the bioactive phytoconstituents in *Asphaltum*. Urolithin A and B are found to be present in Shilajit. The objective of this study was to develop a simple method for isolation of Urolithin A and B from *Asphaltum*. Elementary solvent partitioning technique was used for the extraction and isolation of Urolithins. Organic solvents like Methanol, Ethyl acetate, Petroleum ether etc were used. The isolated compounds were identified and characterized by TLC (Thin Layer Chromatography), UV (Ultra-violet), IR (Infrared) and MS (Mass) spectral analysis and purity was confirmed by HPLC (High Performance Liquid Chromatography) analysis. The isolated markers Urolithin A shows absorption maxima at 280, retention factor at 0.22, m/z at 277; Urolithin B shows absorption maxima at 277 and 303, retention factor at 0.6 and m/z at 211 nm which complies with the literature values. The purity as obtained by HPLC for Urolithin A and B is 93.1%w/w and 90.65%w/w. This isolation method developed involved easy solvent-solvent extraction technique instead of tedious column chromatography and preparative thin layer chromatographic technique.

Keywords: Urolithins; *Asphaltum*; Benzocoumarin; HPLC

I. INTRODUCTION

Ayurvedic practitioners pioneered the applications of Shilajit and used it as a panacea with all the herbal formulations used in healing practices. Thousands of years of demand of highly populated India created a situation, where once most abundant reserves of mineral pitch in the Himalayas were exhausted. The manufacturing response to this situation was an introduction of counterfeiting Shilajit to Indian market. Thus to curb the imitation, we need to study the chemistry of *Asphaltum.* In doing so, we are isolating the actives present in the core of shilajit: "Urolithins".

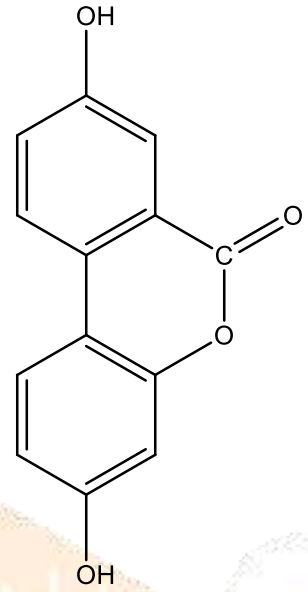
Urolithin A and B are members of a family of metabolites of the 6H-dibenzo- [b,d]pyran-6-one structure (combination of coumarin and isocoumarin) [Fig. 1,2] with different phenolic hydroxylation patterns. It has wide range of pharmacological activities like antioxidant, antimicrobial, hepatoprotective, antitumor, immune stimulating, anti-inflammatory. Urolithins are considered as bioactive markers of Shilajit for its antioxidant acitivity in mitochondrial targeting. Urolithins are metabolites of ellagic acid and its derivatives. They are normally found in human urine in the form of glucuronide. Espin et al. 2013 mentioned it to occur in *Punica granatum* (Lythraceae*), Frageria sp.* (Rosaceae), *Juglan sp.* (Juglandaceae)).

The name urolithin was first given to two metabolites isolated from the renal calculus of sheep that were named Urolithin A and Urolithin B. On removal of two lactones present in ellagic acid by gut microbiota (lactonase/decarboxylase activity), Urolithins are produced and by successive removals of hydroxyls (dehydroxylase activities). Urolithin M-5 (pentahydroxy-urolithin) is obtained after decarboxylation, and later, several tetrahydroxy-urolithin isomers are produced by removal of one hydroxyl group from different positions (Urolithin D, Urolithin M-6). On removal of a second hydroxyl group, Trihydroxy-urolithins (Urolithin C, Urolithin M-7) were produced and urolithin A and isourolithin A after the removal of a third one. Monohydroxy-Urolithin (Urolithin B) was also found to be present, particularly in cases where IsoUrolithin A was produced (Gil et al. 2000, Larrosa et al. 2010, Mertens Talcott et al. 2006, Tomas-Barberan et al. 2009).

The antioxidant activity of Shilajit is found to be because of Urolithins which target the mitochondrial cells for its action (Bhattacharyya et al. 2009). The antiulcerogenic effect, mast cell protecting action, antistress activity, adaptogenic effect of shilajit is a because of the presence of Urolithins in Fulvic acids in *Asphaltum* (Gallardo et al. 2012, Wilson et al. 2011, Ghosal 2006a, Stohs et al. 2014, Agarwal et al. 2007). Thus, the isolation of Urolithins would open door in the studying the chemistry of Shilajit.

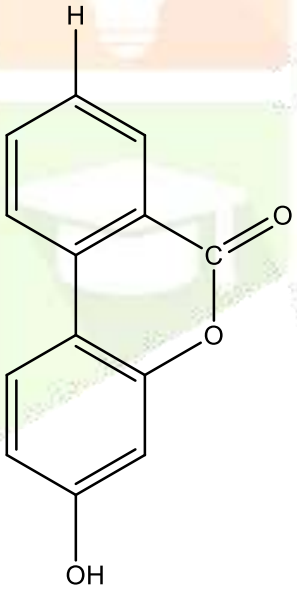
Urolithins were often isolated by column chromatography which is tedious and time consuming method (Wu et al. 2009). Another patented method is reported in which preparative thin layer chromatographic technique is used, but this method is not possible on large scale and not correct in case of plant material having very low concentrations of urolithins (Ghosal 2006b). Thus, the aim of our work was to develop a simple yet efficient method for the extraction and isolation of urolithins from *Asphaltum*. The solvent fractionations of

Shilajit first with Petroleum ether lead to the removal of all highly non polar impurities (Ghosal et al. 1989). The dried residue was then extracted with Ethyl acetate to separate Urolithins from the core structure of humic components present in shilajit. Further partitioning the ethyl acetate extract with Petroleum ether lead to precipitation of Urolithins.



3,8-Dihydroxydibenzo-alpha-pyrone

Figure 1: Structure of Urolithin A



3-Hydroxydibenzo-alpha-pyrone

Figure 2: Structure of Urolithin B

II. MATERIALS AND METHOD

2.1 Raw material: Raw Shilajit was procured from National Herbs Company (Delhi) which was further authenticated and ICT/MNPRL/NV/4001 voucher specimen was deposited in Medicinal Natural products laboratory, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga, Mumbai.

2.2 Chemicals: All the chemicals used for extraction and isolation were of Laboratory reagent grade and obtained from S.D. fine, India. All the solvents used for HPLC (High Performance Liquid Chromatography) analysis were of Analytical reagent grade and obtained from Rankem Limited, India.

2.3 Instrumentation: UV (Ultra-violet) spectrum was recorded on Jasco V-530 spectrophotometer. HPLC analysis was performed with a Jasco (C18 HiQ sil column-250 mmX4.6 mm i.d) Detector: Jasco MD-40. Mass spectrum was recorded on Micromass Q-TOF (Time of Flight) MS Mass spectrometer.

III. EXPERIMENTAL

3.1Method of Isolation:

Raw shilajit (100gm) was powdered and extracted with petroleum ether (500 ml) for 6 hours under reflux. The residue after extraction was dried and again refluxed with Ethyl acetate (500ml) for 6 hours. The ethyl acetate extract was concentrated and partitioned with petroleum ether for three times. Precipitates of Urolithin B (0.41gm) were obtained on partitioning. Precipitates were separated on filtration. This precipitates were recrystallized using alcohol to give pure Urolithin B (0.36gm). Petroleum ether filtrate was concentrated, precipitating Urolithin A (0.3gm). This precipitate obtained was recrystallized with ethanol to give pure Urolithin A (0.21gm) TLC (Thin Layer Chromatography) and HPLC studies were carried out to determine the purity of the sample while the structure was elucidated and confirmed by UV and MS spectral analysis.

3.2 Identification of Isolated Urolithins:

Thin layer chromatography was performed on precoated silica gel plates using Chloroform: Methanol (90:10 v/v) as mobile phase and detected under UV 254 nm and UV 360 nm. 0.01% w/v solution of isolated Urolithins in methanol was prepared in a test tube. A 10 ppm solution of Urolithins in methanol was scanned for a UV/Vis spectrum for its specific absorption maxima. IR (Infrared) spectrum was determined of the isolated solid samples of Urolithins for its functional group identification. Mass Spectrometry was performed to find the molecular weight of isolated Urolithins.

3.3 Determination of purity of isolated Urolithins by HPLC:

3.3.1 Urolithin A and B solution preparation:

Stock solution of Urolithin A and B was prepared in HPLC grade methanol at a concentration of 10 mg/ml. Test solution of 100µg/ml was prepared by appropriate dilutions from stock solution.

3.3.2 Chromatographic conditions (Ghosal 2006c)

HPLC analysis of the extracts was performed on HPLC Jasco, PU-1580, UV detector Jasco UV -1575, column C18 HiQ sil column-250 mm X4.6 mm i.d. Samples were injected by using a Rheodyne injector fitted with a 20 µL fixed loop. Solutions were filtered before injection. Determination of purity of isolated Urolithins was carried out with the mobile phase composed of acetonitrile: water with 0.01% OPA (32:67) at wavelength 240 nm at a flow rate of 1.0 mL/min.

IV. RESULTS AND DISCUSSION

Studies of Urolithins have reported that its structure plays an important role in antioxidant activity of Shilajit. The objective of the study was to develop rapid and convenient method for isolation of Urolithin A and B from Asphaltum (Shilajit). Literature survey have revealed that efforts have been made previously to isolate Urolithins from Shilajit which involved Column Chromatography and preparative Thin layer Chromatography, however the yield obtained was very low and it was tedious and time consuming. The advantage of current method to isolate Urolithins is to obtain better yield, purity and in shorter time. For extraction and isolation process, a simple solvent partitioning method was used. Partitioning with petroleum ether, removed all non-polar and fatty substances from the extract, whereas ethyl acetate being semipolar extracted Urolithins leaving the polar impurities in the residue. Urolithin B precipitates first due to one less hydroxyl group. After concentrating the petroleum ether layer, precipitation of crude Urolithin A was done. For further purification, recrystallization was done with ethanol since it is less soluble in absolute alcohol.

4.1 Characterization of Urolithin A and B:

4.1.1 Urolithin A:

A single band of Urolithin A was seen at Rf 0.22 at 360 nm on track 1 as compared to the ethyl acetate extract of shilajit on track 2 [Fig. 3].The melting point was found to be in the range of 360º C. The UV/Vis maximum was found to be at 280 nm in methanol which is identical with reported literature. IR spectrum of isolated compound showed characteristic peaks at 1619 cm-1 (C=C), 1712 cm-1 (Carbonyl), 3356cm-1(Hydroxyl). The HPLC Chromatogram of the isolated Urolithin A shows a purity of 93.1%w/w [Fig. 4]. M/Z was found to be 227. The fragmentation pattern of mass spectroscopy of isolated compound was found in accordance with reported values in literature (Ghosal 2006b).

Figure 3: TLC analysis of isolated urolithin A and Ethyl acetate extract of shilajit at 360 nm
Track 1: Isolated Urolithin A
Track 2: Ethyl acetate extract of Shilajit

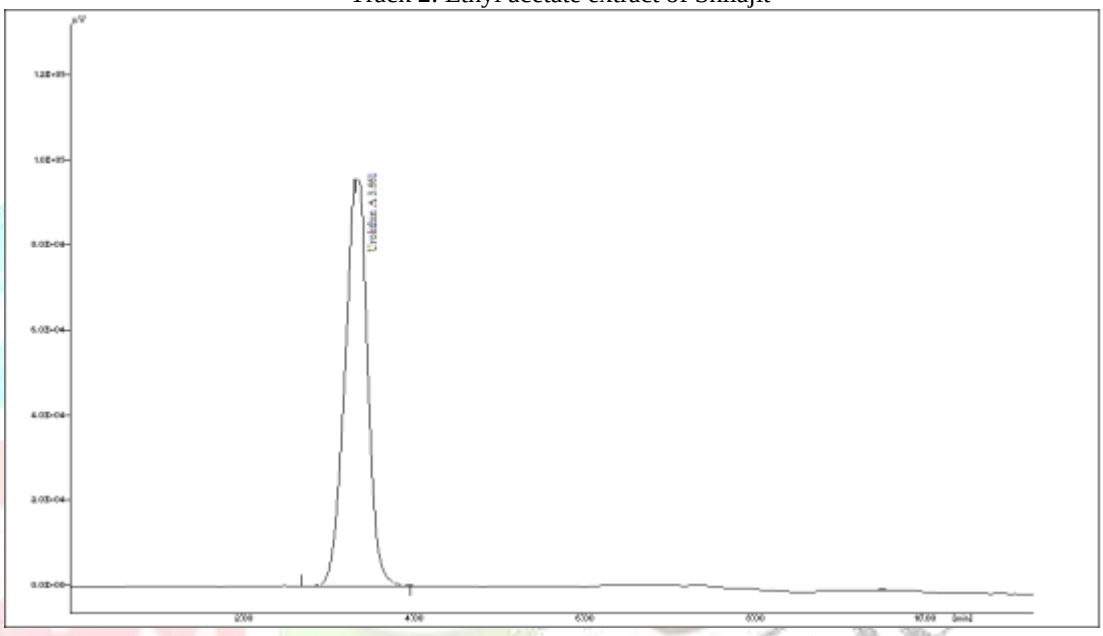


Figure 4: HPLC Chromatogram of isolated Urolithin A

4.1.2 Urolithin B:

A single band of Urolithin B was seen at Rf 0.6 at 254nm on track 1 as compared to ethyl acetate extract of Shilajit on track 2 [Fig. 5].The melting point was found to be in the range of 232º C. The UV/Vis maximum was found to be at 277 nm and 303nm in methanol which is identical with reported literature. IR spectrum of isolated compound showed characteristic peaks at 1619 cm-1 (C=C), 1712 cm-1 (Carbonyl), 3356cm-1(Hydroxyl). The purity was found to be 90.65%w/w by HPLC [Fig. 6]. M/Z was found to be 211. The fragmentation pattern of mass spectroscopy of isolated compound was found in accordance with reported values in literature (Ghosal 2006b).

Figure 5: TLC Analysis of isolated Urolithin B and Ethyl acetate extract of shilajit at 254 nm
Track 1: Isolated Urolithin B

Track 2: Ethyl acetate extract of shilajit

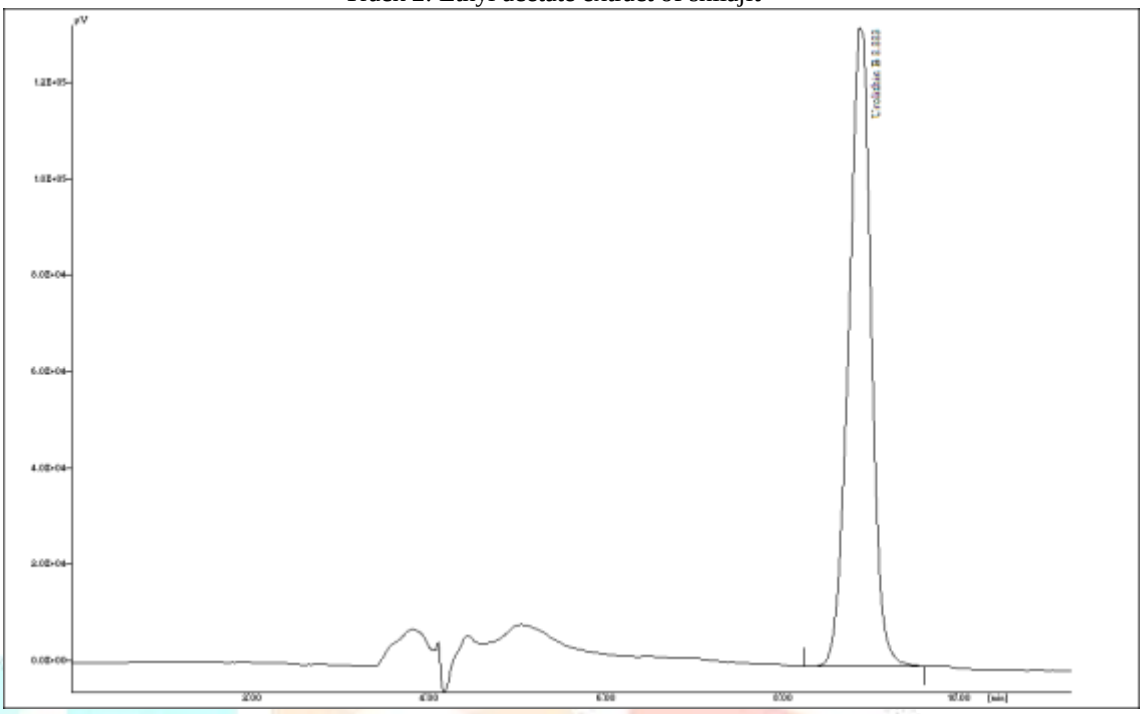


Figure 6: HPLC Chromatogram of isolated Urolithin B

V. CONCLUSION

This isolation method developed involved easy solvent-solvent extraction technique followed by recrystallization to purify the compound. The reported method of isolation has shown good reproducibility in terms of purity and can be used to study the chemical markers required to curb counterfeit.

VI. ACKNOWLEDGMENT

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