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Analysis And Relative Extraction Optimization Of Betulinic Acid Using RP-HPLC From Various Parts Of *Ziziphus jujuba* L.

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ABSTRACT

Comparison of various extraction methods as an effective extraction technique for maximum extraction of betulinic acid was done. Seeds and leaves of *Ziziphus jujuba* L. as cheaper raw material were used as preferred source. Several combinations and ratios of different organic solvents were tried to find best option for the same. Analysis of betulinic acid was done by TLC, RP-HPLC and FT-IR. The maximum amount of betulinic acid was extracted by continuous shaking with 3min. intermittent treatment of microwave i.e., 51.93ppm,57.40ppm and 61.81ppm; although after 15 min ultrasonic treatment at every 4h intervals the yield was 29.53ppm, 34.63ppm and 48.52ppm when extraction time was 8h,16h and 24h, respectively. Microwave assisted extraction with continuous shaking was found to give maximum extraction of betulinic acid from seeds and leaves of *Ziziphus jujuba* L.

Keywords: *Ziziphus jujuba* L., Extraction methods, Betulinic acid, Anti-HIV, Anti-cancer, Hepatoprotective.

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INTRODUCTION

Ziziphus is a genus with forty species from Rhamnaceae family primarily distributed in warm and subtropical regions throughout the globe. *Ziziphus jujuba* has generated notable commercial value due to its significant pharmacological functions [1-3]. Recent report enlisted China as the largest producer and exporter of *Ziziphus* fruits contributing about 90% production. Estimated total annual yield of fresh jujube fruits in 2009 was 600 million kilograms. Betulinic acid (BA) a versatile pharmaceutically valued, pentacyclic triterpene is commercially isolated from the outer bark of birch tree. Betulinic and its pentacyclic triterpenes derivatives (fig. 1) have attracted interest due to their various pharmacological activities primarily includes anti-HIV and anti-tumor [4-6]. In addition, it also has good bioactivities against fatigue, hyperhidrosis, liver complaints, urinary trouble, fever, hypertension and hyperlipidemia. The pharmaceutical quality of the bioactive molecules (as betulinic acid) is affected by a number of factors such as growing environment, processing conditions and genetic background (either hybrid or wild variety) of the plant [7]. As a vital ingredient of Indian as well Chinese traditional medicines system [8] whole fruit of *Z. jujuba* or/and its various parts specially bark and seeds have been reported containing betulinic acid as a key ingredient. The seeds of *Ziziphus jujuba* L. have abundant bioactive metabolites, including triterpene acids, unsaturated fatty acids, flavanoids, glycosides, and other wonderful chemicals involved in disease treatment and health benefits both [6]. So far extraction of betulinic acid was achieved tediously from various plants with different sources together with wide range of extraction methods such as microwave extracted soxhlet extraction solvent partition, rapid high-speed counter-current chromatographic (HSCCC) and constant extraction for several days [9] partition chromatographic isolation [10, 11]. Varied amount of betulinic acid was obtained from different parts of *Ziziphus jujuba L.* (Seeds, leaves, and stem bark) using ethanol as extracting solvent. Few reports advocated the presence of triterpenes as a bioactive molecule suggested betulinic acid in seeds [6]. The poor solubility of triterpenes in both polar and non-polar solvents as well as expensive extraction procedures have prevented large-scale isolation and quick screening of this compound. The present work deals to compare various techniques with different solvents for extraction of betulinic acid from crude extract of seeds and leaves (green and brown both). The seeds of *Ziziphus jujuba* L. was taken here as a cheaper and easily available source for extraction of betulinic acid.

MATERIALS AND METHODS

Chemicals and plant materials

Betulinic acid of highest purity (98%) as reference compound was purchased from Sigma Aldrich, Bangalore (India) and other solvents viz. methanol, ethanol, butanol, acetonitrile, dichloro methane, tri-fluoro-acetic acid (TFA), DMSO, and chloroform were of HPLC grade used in the present work (SRL, Mumbai, India). Fresh seeds from mature *Ziziphus* fruits, green leaves and brown leaves were collected from trees growing as a single population from five different mature individuals at Rohtak, Haryana (India) between December to February 2015 (28.8909°N 76.5796°E).The materials were dried at 60-62°C in hot air oven for 48h and the dried material was ground to fine powder in a grinder. *Ziziphus* samples were extracted according to the previously described methods with some modifications.

Selection of appropriate solvent system for maximum extraction of betulinic acid from Ziziphus seed and leaves

Various solvents with different polarity were used to determine suitable solvent system for maximum recovery of betulinic acid from the powdered samples of seed and leaves. Five grams dried powdered sample with 75ml of different solvents were used in three replicates for different extraction strategies. Rest of the powdered samples was kept in poly-bags in freezer until the time of use. Ethanol (95%), dichloro-methane, butanol, trifluro-acetic acid (TFA), dimethyl sulfoxide (DMSO), and chloroform of HPLC grade were used in the present work (SRL, Mumbai, India). Three temperatures 30, 45 and 60°C at 125rpm for 48 hours was taken as an initial screening parameter for extraction of betulinic acid in triplicates.

Quantification of BA and effectiveness of extraction techniques using reverse phase- HPLC

Extraction through continuous shaking method (ECSM)

The continuous shaking extractions were performed on temperature controlled orbital shaker (CIS-24plus, Remi, India). A combination of stirring of 115, 130,150, 175 and 190 rpm with 20, 30, 45, 60 and 70°C for 8,18,32, 48 and 60 hours was maintained for the samples' of Ziziphus seeds suspensions under controlled condition. The samples were extracted with 95% ethanol for 8h, 18h, 32h, 48h and 60 h at 45-47°C temperature separately.

Extraction through microwave assisted extraction method (EMaeM)

In 250ml conical flasks two grams of dried ground powdered of seed and leaf (green and brown) with 50ml of 95% ethanol were individually kept. All flasks with suspension were exposed for 1, 2 and 3min in a microwave oven (Kenstar, OM34ECR) at 1000W. During exposure to microwaves heat is generated which give bumping to the sample suspension that was avoided by keeping sample in cool water bath. Above steps were repeated in order to complete 3min microwave irradiation. Then the extracts were filtered firstly through blotting paper then 0.22 micron whatman filter paper to remove maximum suspended particles. Then this filtered extract can use for further analysis. As microwave irradiation generates heat during the process so some solvent gets evaporated, which volume was made up to 50 ml with ethanol to avoid stoichiometry disturbance.

Extraction through Soxhlet method (ESM)

Finely ground 2gm dried powder of *Ziziphus* seeds, green leaves and brown leaves was extracted into 75ml Soxhlet thimble. The Soxhlet assembly was fitted with 250 ml round bottom flask containing 100ml of 95% ethanol. The flask was heated for 180, 300, and 480 min at a controlled extraction temperature of 65-67°C separately. At the end the extracts were collected separately and filtered and analyzed by RP-HPLC.

Extraction through Ultrasonic method (EUsM)

As an alternative extraction method for betulinic acid from *Ziziphus* seed ultrasonic extraction was also studied as considered by other researchers [12-14]. The samples to solvent ratio were kept as 1:25 (2grams sample in 50ml 95% ethanol) and were sonicated for 15, 30 and 45min at room temperature on ultrasonic bath (PCI analytics, Model 1.5L-50) at working amplitude of 60. After the completion of sonication the samples were filtered and later used for analysis.

All the extracts were double filtered firstly from normal blotting sheets to remove coarse particles and secondly through whatman no. 1 filter paper to avoid suspended particles then re-volumized to 50ml and the filtrates were analyzed by RP-HPLC after passing through 0.2 µm nylon filters (Axiva, Germany).

Detection and validation of betulinic acid in extracted sample as crude product reverse phase-high performance liquid chromatographic analysis

Instrument Specification

The analyses of extracted samples were done by RP-HPLC (YL9112, South Korea). The HPLC system used throughout current study was equipped with of Yong Ling's 9112 parallel dual-plunger pumps, YL9120UV-Vis detector, and chromatographic separation was achieved on a prontosil C18-HQ105 H column (250mm X4.6 mm X 5 mm).

Chromatographic conditions for detection of betulinic acid

All the extracted samples were analyzed by using acetonitrile and water as mobile phase maintaining at a flow rate of 0.75 ml/min at room temperature. The ratio of mobile phase was used as 90:9 (v/v) in an isocratic mode with sample injection volume 20µl and the wavelength was set at 210 nm [15-17]. The calibration curve was generated by dissolving betulinic acid in methanol with dichloromethane (1:1)

diluted to various concentrations (10-50µg/ml) and kept at 4ºC in dark. The solutions were filtered through 0.22 µm millipore filter before analysis through HPLC (YL9112, South Korea). The calibration curve was constructed by plotting the peak area (AUC=Area Under Curve) versus the ratio of their corresponding concentrations (Table 3).

The system suitability

The system suitability test was assessed by injections of three replicate of the standard solutions at a particular concentration. For this 10, 20, 40, 60, 80 and 100ppm betulinic acid solution as standard were analyzed at 210nm. The peak areas were the basis for evaluating the repeatability of the proposed method, and their peaks were analyzed for resolution.

Detection and validation of extracted sample for presence of betulinic acid by TLC

The ethanolic extracts used for RP-HPLC analysis were analyzed using pre-coated, silica gel plates of UV_{254} (Merck, India. 150 mm × 50 mm). For comparative analysis the extracted samples and standards were applied on the plate on a 3mm diameter circles manually just 15 mm from the bottom and 15 mm from the sides. The space between two spots was 15 mm on the plate. The plates were developed using different combination of different polarity solvent system viz. acetonitrile: water (1:1), butanol:acetic acid: water (5:4:1), chloroform :acetic acid: water (5:4:1); n-hexane: ethyl acetate:methanol:water (10:5:2.5:1); n-hexane :ethyl-acetate : acetic acid (7:3:0.03);and detected under UV light. The average length of the chromatogram run was 8.5 ±0.5cm in different sample. Consequently the TLC plates were dried in air current using a hair dryer and evaluation of the plates were done under UV detector mode at 254 nm.

Validation and characterization of betulinic acid with FT-IR spectroscopy

Infrared spectra were taken on a Bruker Alpha, Platinum ATR spectrophotometer. FT-IR analysis was done in the mid IR region of 600-3600 cm^{-1} with 16 scan speed. All filtered extracted samples were analyzed and compared with standard betulinic acid.

RESULTS

The seed of mature fruits of *Ziziphus jujuba L.* provided betulinic acid without requiring expensive purification steps that made this process as a cost effective simpler technique for the extraction of this high therapeutically valued product. The continuous shaking with microwave treatment and ultrasonic extraction method were found suitable for seed as well leaves both but the conventional approaches as extraction through soxhlet's method was proved to be weak for extraction of betulinic acid with above mentioned combinations. Another hand green and dried brown leaves were not shown encouraging results as compared to seed. Butanol and chloroform were given good results for leaves but as compared to results obtained from seed, their results were not considered further.

Quantification of betulinic acid using reverse phase-HPLC and efficiency of extraction techniques

Standard betulinic acid (3-Hydroxy-lup-20(29)-en-(28)-oic acid) of 10-100ppm concentrations for generating standard curve were detected at 210 nm wavelength using acetonitrile and water as mobile phase by RP-HPLC technique (Table 1). The peaks at retention time of 13.7min for standard betulinic acid were clear, sharp indicating its purity (98%) with no mixture of compounds and also ensuring no compatibility issues between the sample extraction solvent and the RP-HPLC mobile phase (fig 1). A six point standard computer generated internal linear calibration curve for betulinic acid within the concentration range of 10-100 ppm (R^2 (curve coefficient) = 0.910) was obtained. The chromatogram for quantification of betulinic acid using 06 solvents, 4 different extraction methods and sample of 3 different materials from plant (seed, green and dried brown leaves) were obtained as a final output. The results indicated in fig 2, advocates encouraging results for seed samples in ethanol (95%) and DMSO but chloroform, dichloromethane and TFA were not so notable. Another side the sample of leaves was not shown good results for the presence of betulinic acid in these solvents so further only powdered seed samples were considered for different extraction optimization studies (Table 2). Brown leaves shown response to the extraction by ultrasonic and continuous shaking methods while dichloro-methane and butanol giving 15.26 and 14.34ppm while green leaves only shown 11.13 and 10.28ppm

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betulinic acid with same solvent and extraction system respectively. Considering results with other solvent no such notable results were found that's why extraction from leaves (either green or dried brown) was not taken for next steps (as shown in fig 2).Whereas betulinic acid extraction optimization from seeds of mature fruits of *Ziziphus jujuba L.* in continuous shaking extraction method upto 60h with 3min intermittent microwave treatment at an interval of 4h shown the maximum extraction in 48h (51.93, 57.40 and 61.81ppm betulinic acid was extracted). For this continuous shaking was maintained at 150 rpm at 45°C. The amount of betulinic acid increased gradually as we increased the extraction time from 24 to 48 h but later there were no significant changes were observed in extraction after 48h (fig 3a, and Table 4). Another hand during ultrasonic assisted extraction method when 15 min intermittent ultrasonic treatment were given at every 4h intervals for upto 60h also shown encouraging results for extracted betulinic acid as 29.53, 34.63and 48.52ppm (fig 3b and Table 4).

Table: 1 Details of retention time and area under curve (AUC) of different concentration of betulinic acid obtained from RP-HPLC used for calibration and quantification for quantification of extracted betulinic acid.

Sl No.	Conc. of standard betulinic acid (ppm)	Area under Curve (AUC)*	Retention time (min.)
1	10	386.86±11.39	13.5±0.4
2	20	554.96±14.26	13.5±0.7
3	40	654.31±12.73	13.7±0.3
4	60	909.61±13.32	13.6±0.7
5	80	1162.15±9.37	13.8±0.9
6	100	1771.02±11.28	13.6±0.52

*(AUC=area under curve is denoted in voltage (mV) in Fig 1)

Table: 2 Selection of solvent system for maximum extraction of betulinic acid from seeds, green leaves and dried brown leaves obtained from RP-HPLC showing area under curev (AUC) and quantification of extracted betulinic acid

	Green Leaves		Dried brown leaves		Seed from mature fruits	
Solvent Used	AUC*	BA conc.(ppm)	AUC	BA conc.(ppm)	AUC	BA conc.(ppm)
TFA**	123.82 ±4	6.61	138.79 ±0.2	7.41	150.78 ±1.3	8.05
DCM***	162.88 ±1.5	8.7	173.27 ±1.7	9.26	359.03 ±4.4	19.18
Chloroform	238.29 ±2.6	12.73	285.62 ±0.5	15.26	192.58 ±1.6	10.29
Butanol	192.38 ±2.3	10.28	268.32 ±0.7	14.34	228.22 ±1.7	12.19
Ethanol	87.98 ±0.5	4.7	128.51 ±1.9	6.86	868.72 ±2.0	46.43
DMSO	57.38 ±0.7	3.06	103.72 ±1.2	5.54	793.36 ±2.1	42.4

*(AUC=area under curve is denoted in voltage (mV) in figure 3)

* Values are the means ± standard deviations of three measurements (n=3).

**Dichloromethane, ***Tri-fluoroacetic-acid

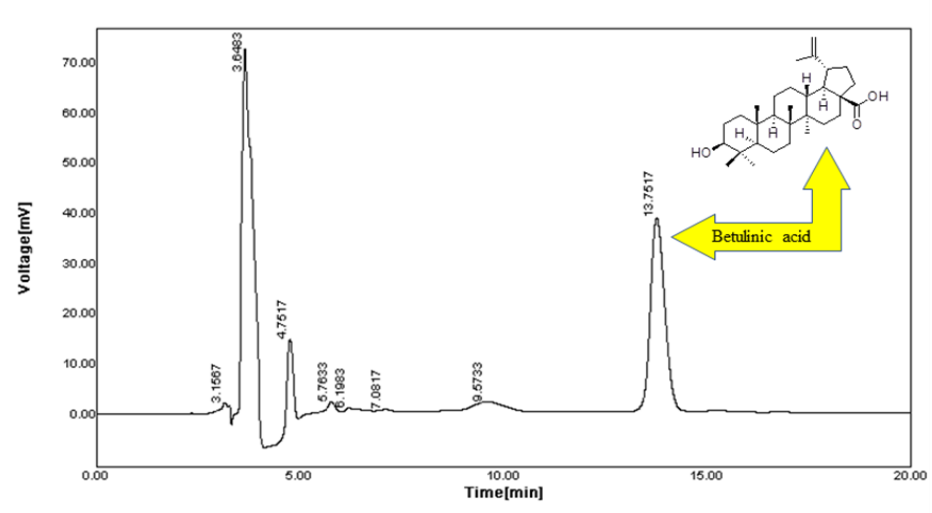

Figure 1. The RP-HPLC profile of betulinic acid as reference compound (conc.100ppm)

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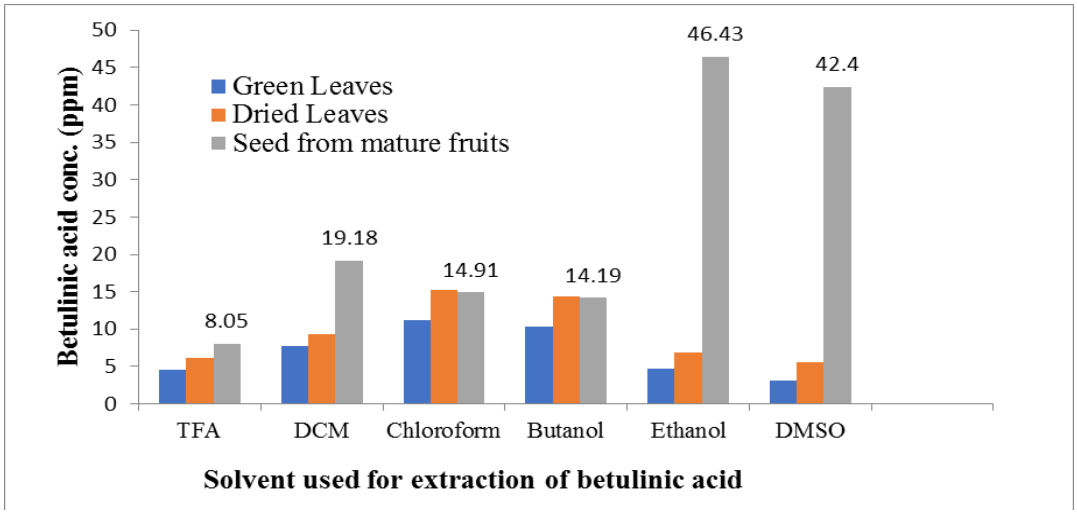

Figure 2. Selection of solvent system for maximum extraction of betulinic acid from seed, green leaves and dried brown leaves of *Ziziphus jujuba L.*

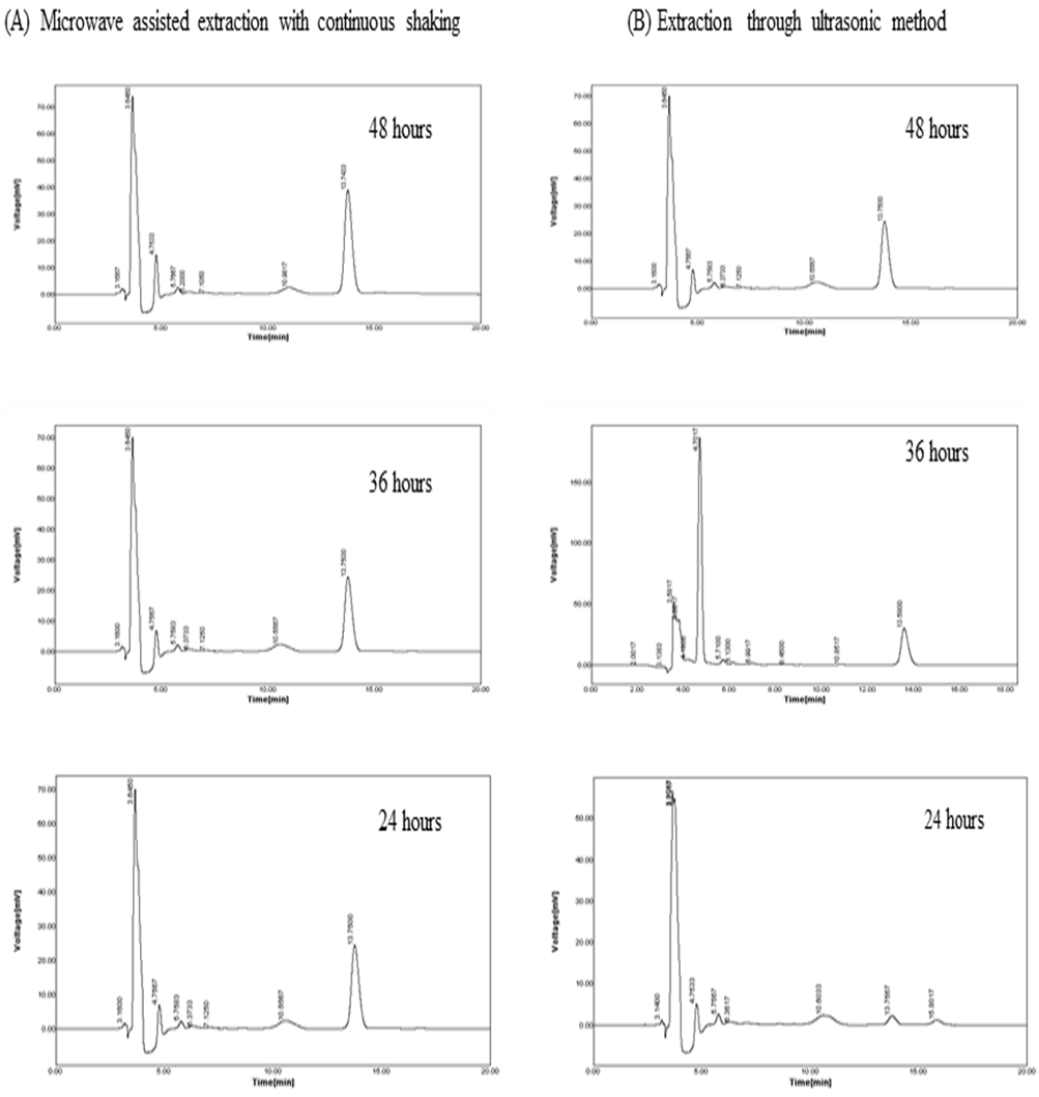

Figure 3. Comparative RP-HPLC profile of seed sample extracts (at 13.5 to 13.8min retention time) obtained from (A) microwave assisted extraction with continuous shaking (B) extraction by ultrasonic method.

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Table: 4 The FT-IR data of standard betulinic acid and seed sample of mature fruits of Ziziphus *jujuba L.*

Standard betulinic acid λ_{max} (cm−1)	Assignments for Specific band	Seed of *Ziziphus jujuba L* λ_{max} (cm−1)
3322	O–H stretching vibration	3442
2944	C–H stretching vibration	2913
1670	C=O stretching vibration	1663
1449	C–H deformation of CH2 group	1436
1021	C–OH stretching vibration in CH_2OH group	1042
-----	C–H deformation of aromatic ring with two adjacent H atoms	896

Detection and validation of betulinic acid by thin layer chromatography

The quantification of betulinic acid in crude extracts was done by HPLC, and the same extracts were taken for validation through thin layer chromatography. Various combination of mobile phase was tested for resolution of betulinic acid and a combination of water: acetic acid: chloroform in 1:4:5 v/v ratios were found suitable for detection. Prior to loading the samples a 25 min saturation of chamber was done using mobile phase. A yellowish green spot of crude sample with bluish color spot of standard betulinic acid (Rf: 0.64) were developed on the TLC plate under UV light. Comparison of the characteristics of the spots developed for standard BA and that of the sample revealed the identity of betulinic acid presence in the sample.

Detection, validation and characterization of betulinic acid with FT-IR

Now for a final detection and validation for the presence of betulinic acid in extracts of *Ziziphus jujuba L.* seed sample the fraction collected from HPLC were used for FT-IR analysis. A comparison and similarities found in the sample and standard pattern recognized presence of betulinic acid in the sample (fig 4). The FTIR spectrum of standard betulinic acid showed the absorption peaks (λ max cm^{-1}) at 3322 cm^{-1} O-H stretching vibrations, 2944 cm^{-1} appears due to asymmetrical CH_2 stretching from the CH_2OH group, 1670 cm^{-1} band can be assigned to C=C stretching and CH_2 bending in the terminal methyl group, 1449 cm^{-1} appears because of bending vibrations of the methyl and of the CH_2 groups in the rings, 1021 cm^{-1} can be assigned to C-O stretching vibrations in the CH_2OH group. The crude sample showed the absorption peak at 3442, 2913, 1663, 1436 cm^{-1}, and 1042 cm^{-1}, which was more or less similar to absorption peaks of standard betulinic acid signifying presence of betulinic acid in the crude sample. The differences in the band positions and relative intensities are due to the complex composition in the sample (Table 3).

Table: 3 Representing duration for maximum extraction and quantification of betulinic acid from *Ziziphus jujuba L.* seed using RP-HPLC

Extraction Duration (Hours)	Continuous extraction+MaeM		Ultrasonic Extraction method	
	AUC	BA conc.(ppm)	AUC	BA conc.(ppm)
08	971.66 ±2.86	51.93	552.60 ± 3.8	29.53
16	1074.00 ± 7.50	57.40	648.00 ± 3.8	34.63
24	1156.60 ± 1.88	61.81	906.00 ±4.69	48.42
32	1154.30±2.64	61.69	906.00 ± 4.12	48.42
40	1155.00 ±1.63	61.73	905.60 ±3.09	48.40
48	1155.00 ±1.63	61.71	907.30±2.86	48.49
60	1153.60 ± 3.3	61.65	908.30 ±3.68	48.54

.* MaeM=Microwave assisted extraction method

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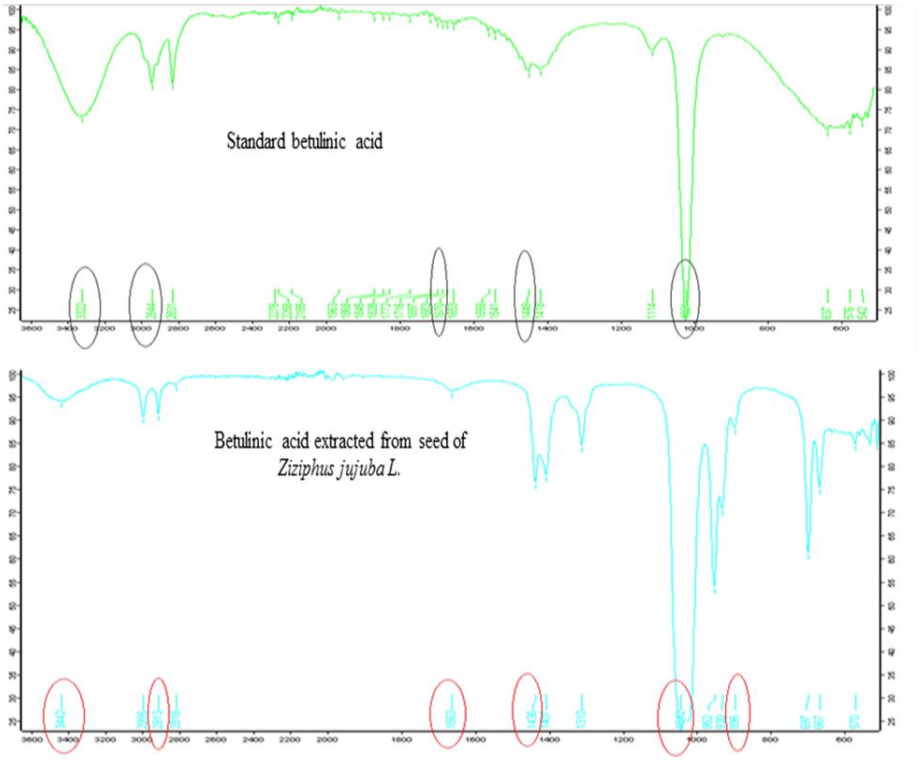

Figure 4. Comparative FT-IR profile of (A) standard betulinic acid shown in green color (B) betulinic acid extracted from seed of mature fruits of *Ziziphus jujuba L. shown in blue color.* The circles represent similar bond stretches in standard betulinic acid and extracted sample from seed of mature fruits of *Ziziphus jujuba L.*

DISCUSSION

Presently, about twenty five known anti-HIV drugs are available and almost all are obtained from chemical synthesis which has side effects on patients. Betulinic acids with its derivatives have shown encouraging results as anti-HIV and anti-tumor activity on many cell lines. Large scale extraction and production of betulinic acid is done from outer bark of *Betula* species trees. The existing procedure requires a multi-step extraction and purification as the stem bark is very hard and also contains various other impurities as tannins and gum. Various extraction methods was tried to recover maximum amount of betulinic acid and found microwave assisted with continuous shaking was the efficient method for betulinic acid extraction. Supernatant of reaction mixture was analyzed for betulinic acid concentrations by comparison of standard curve of HPLC at 210 nm; the peak at retention time of 13.7min for standard betulinic acid was overlay with the extracted sample (betulinic acid) from *Zizyphus jujuba*.

The FTIR analysis was performed for the leaves of *Orthosiphon stamineus* betulinic acid and spectra showed frequencies at 3446, and 3035-2717, indicating the presence of hydroxyl group and C-H bond, absorption peaks at 1665, 1452 and 1369 shown the presence of asymmetrical ethylene double bond, aromatic rings and aromatic -CH_3 group, respectively. In another work where IR spectra of extracted sample of birch bark was studied where the spectrum of sample was dominated by broad band's at 3400, 2935, 1621, 1048 which were the only 4 bands naturally matching the 8 bands (2943, 1693, 1642, 1451, 1377, 1190, 1043, 885) in standard spectrum. In the present work five common bands in extracted samples and standard betulinic acid were found as summarized in Table 4.

In our current findings we strongly advocates that (i) sample is cheaper and easily available around the globe especially in tropical climate zone (ii) sample does not needed specific purification steps because the plant material (i.e. seeds of *Ziziphus jujuba* L.) is easily settle down in the solvent and the supernatant contains betulinic acid i.e. analyzed by RP-HPLC and finally characterized by FT-IR. In addition this study presented a specific solvent selection with the feasibility of microwave assisted extraction and ultra-sonic extraction technique compared to traditional approaches.

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