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EFFECT OF SOXHLET AND ULTRASOUND ASSISTED EXTRACTION ON ANTIOXIDANT ACTIVITY OF POMEGRANATE PEEL EXTRACT

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ABSTRACT

Pomegranate (*Punica granatum* L.) peel of 'Bhagwa' variety were subjected to extraction using different solvent viz. MeOH and DI water by ultrasound assisted extraction (UAE) and soxhlet assisted extraction (SAE). Bioactive compound of pomegranate peel extracted by UAE show higher yield 51% (60ºC, 40 min and 40 kHz) as compare to the yield 39.4% obtained by SAE method. The total phenolic contents (TPC) of extract varied from 1.82 to 4.0 and 2.45 to 4.49 mg gallic acid equivalent (GAE) / g in SAE and UAE respectively. The DPPH scavenging activity of extract varied from 15 to 56.02% and 51.84 to 67.94% inhibition, in SAE and UAE respectively. The FRAP value varied from 693.42 to 799.82 mg ferrous sulphate equivalent (FSE) / g and 763.73 to 832.17 mg FSE / g, in SAE and UAE respectively. HPLC was used for the quantification of polyphenol in PPP extract, chlorogenic acid present in highest quantity (1444.03 ppm in UAE as compare to 399.72ppm in SAE). The present research showed that UAE can be used for highest extract yield and which showed highest antioxidant activity as compare to SAE. On the other side UAE prevent the degradation of the bioactive compound.

Keywords: Pomegranate, Ultrasound extraction, Antioxidant activity, Gallic acid equivalent, HPLC.

INTRODUCTION

Pomegranate peels constitute approximately 40% of the whole pomegranate fruit. Pomegranate peel contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid etc. It has been used in the preparation of tinctures, cosmetics, therapeutic formulae and food recipes [1]. Flavonoids extracted from pomegranate showed strong anti-oxidant activity [2, 3]. A cursory survey of the literature reveals that the tannins from the pericarp of pomegranate exhibit antiviral activity against the genital herpes virus [4]. The pomegranate rind extract is also shown to be a potent virucidal agent [5] and has been used as a constituent of antifungal and antiviral preparations [6]. There are reports of the use of a water decoction of pomegranate peel powder as a multifunctional vaginal suppository for contraception and for the prevention and cure of venereal disease [7]. Pomegranate peel is reported as a part of a preparation used for treating the infection of male or female sexual organs, mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis and scald for curing diarrhea and dysentery [8] and as part of the medicine for the treatment of oral diseases [9].

Extraction is a key step for obtaining antioxidants with an acceptable yield. Solvent extraction is more frequently used for the isolation of antioxidants and the extraction yield and economic viability is dependent on the type of solvent (mostly due to the differing polarity of these compounds) and method of extraction. Several extraction techniques have been reported for the extraction of phenolic compounds from different matrices using solvents with different polarities such as methanol, water, ethyl acetate and petroleum ether [10, 11]. In recent years, there has been an increasing interest in extraction of antioxidants from agricultural and industrial by-product by different innovative technology. The extraction of bioactive compounds under ultrasound irradiation (20–100 KHz) is one of the upcoming extraction techniques that can offer shorter operation times, reduced solvent consumption and temperature and lower energy input. Hence, ultrasound-assisted extraction (UAE) can be called an ''environment-friendly'' or ''green'' technique [12]. The main advantages of ultrasound-assisted extraction over conventional soxhlet extraction are: cavitations increase the polarity of the system and increase the extraction efficiency, allows the extraction of thermolabile analytes and operating time is invariably shorter [13].

MATERIALS AND METHODS

Pomegranate fruits of 'Bhagwa' variety were procured from Aurangabad (Maharashtra). The pomegranate fruit was washed manually. After washing, the fruit was cut into pieces. The peel was then separated manually. After separation, peel was dried in the cabinet drier at a temperature of 47±1ºC for 30 hrs. Dried pomegranate peels were separately ground in the grinder to reduce the particle size. The ground material is then passes

through a BSS sieve size 30 mesh; the retentate on the sieve was again taken for grinding and passed through the sieve. The peel powder was then packed in the air tight polyethylene pouch separately, and stored under freezing condition until used for further extraction of bioactive compounds (Figure 1).

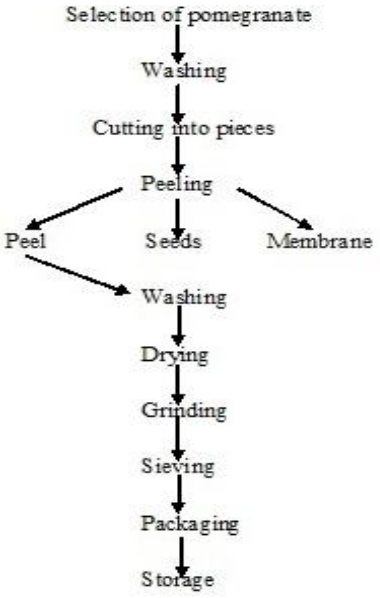

Figure 1- Process flowchart for pomegranate peel powder preparation

METHOD OF EXTRACTION OF BIOACTIVE COMPOUND

Two method of extraction were used for the extraction of bioactive compound from the pomegranate peel powder (PPP) are soxhlet assisted extraction (SAE) and ultrasound assisted extraction (UAE) by using different proportion (100, 75, 50, 25 and 0%) of methanol (MeOH) and deionized water (DI).

SOXHLET ASSISTED EXTRACTION

To prepare pomegranate peel extract 10 g of PPP were weighted and extracted for 6 hrs by soxhlet apparatus with MeOH and DI water in different concentration to extract the bioactive components. After 6 hrs, the soxhlet extraction flask containing extract and solvent mixture was removed from soxhlet apparatus. The solvent evaporated under vacuum in a rotary evaporator (40- 60ºC). The concentrate obtained after vacuum evaporation was dried in vacuum drier at 40ºC. Yield of the extract was determined and dried extract was then stored under refrigerated condition until used for further analysis.

ULTRASOUND ASSISTED EXTRACTION

The process of extraction bioactive components from pomegranate peel powder by ultrasonic was performed in an ultrasonic bath. 1 g of sample was mixed with solvent (MeOH and DI water) was added to a 100 ml beaker. The beaker was immersed into an ultrasonic clearer bath, with the liquid level in the beaker kept lower than that of the cleaner tank. The optimal extraction parameters were: extraction temperature 60ºC, extraction time 40 min, frequency 40 kHz and S/S ratio 50 ml/g. Then, the sample extract was centrifuged at 4500 rpm for 10 min. The extract was concentrated by rotary evaporator, dried at 40 ºC under vacuum to dryness and the yield of extract was determined according to the method of Tabaraki et al. (2012)[14] with slight modifications and stored under refrigerated condition until further analysis.

Total extract yield (%) = $W_e \times 100 / W_s$
Where
W_e -Weight of extract
W_s - Weight of sample

TOTAL PHENOLIC CONTENTS (TPC)

The total phenolic content in the extract was determined by the Folin-Ciocalteu method [15]. A 0.05 g of dried extracts was dissolved in 5 ml methanol or the filtrate made up to 50 ml were used directly. Aliquots of 10 µl of samples were mixed with 2.5 ml of 10-fold-diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The total volume of the mixture was adjusted to 25 ml using DI water and allowed to stand for 30 min at room temperature before the absorbance was measured at 760 nm using a spectrophotometer. The total phenolics content in the extract was calculated and expressed as gallic acid equivalents (GAE; mg/ g dry mass) using a gallic acid standard curve.

DETERMINATION OF ANTIOXIDANT ACTIVITY BY DIPHENYLPICRYLHYDRAZYL (DPPH) ASSAY

The total antioxidant activity was determined by Calculating % radical scavenging activity by using 2, 2-Diphenyl-1-picrylhydrazyl [16]. 1 mg/ml of DPPH solution was made by dissolving 1mg of DPPH in 1 ml of MeOH. 100 µl of DPPH solution was diluted to 5 ml methanol and absorbance was taken at 517nm in UV-Spectrophotometer. The absorbance was taken as control absorbance. 100 µl of (0.05 g of dried extracts was dissolved in 5 ml methanol or the filtrate made up to 50 ml were used directly) extract was taken and 100 µl of 1 mg/ml of DPPH solution was added to this extract. Then it was diluted to 5 ml MeOH and was incubated at room temperature for 30 min. Then absorbance was measured at 517nm in UV-Spectrophotometer, the absorbance was taken as sample absorbance.

CALCULATION

Radical scavenging activity was expressed as the inhibition percentage (I %) and was calculated using the following formula:

$$\mathbf{I\% = [(A_c - A_s)/ A_c] \times 100}$$

Where, A_s: the absorbance of sample
A_c: the absorbance of control

FREE REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content [17]. An aliquots 150µl of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium

acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution in 40m HCl and 1 part of 20.0 mM $FeCl_3$. $6H_2O$ solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mg $FeSO_4$ equivalents per gram of sample.

HPLC ANALYSIS

The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon auto sampler, a K-1001 pump and a UV–VIS detector (K-2600). Analysis of gallic acid, catechin, chlorogenic acid and caffeic acid was performed according to specification given below. Before injection, each sample was centrifuged in an eppendorf tube (4 min at 5000 rpm) and the centrifuged supernatant was allowed to pass through a 0.45 µm PTFE filter (Chromafil CA-45/25 S, Duren, Germany) [18]. An RP C18 Nucleosil (5µm; 4.6×250mm) column was used for the separation of sample components. Mobile phase consisted of solvent A (Methanol: Actonitrile: Distilled water) for gallic Acid, solvent B (Methanol: Actonitrile: Distilled water) for catechin and solvent C (Acetonitrile: Acetic acid) for chlorogenic acid and caffeic acid. Flow rate for each sample was 1 ml/min and column temperature was 25ºC. Chromatograms were recorded at 271 nm, 280 nm, 335 nm and 335 nm for gallic acid, catechin, chlorogenic acid and caffeic acid respectively.

RESULT AND DISCUSSION

For the extraction of polyphenol, SAE and UAE with different ratio of MeOH and DI water were used as solvent. The total extract yields reported as percentage of g of extract per 100 g PPP on dry basis. Based on the solvent concentrations in different ratio, the percentage yield from pomegranate peel ranged from 13.70% to 39.40% in SAE method and 40% to 51% in UAE method. As shown (Figure 2), 75% MeOH afforded the highest yield of extract 39.40%, in SAE and 51% in UAE method.

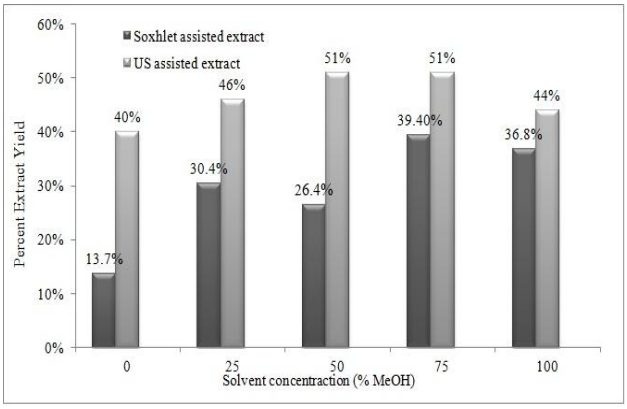

Figure 2- Percent yield of pomegranate peel extract by SAE and UAE methods

The extract yields from the PPP, obtained in this research were higher than reported by Wang et al. (2011) [19] i.e. the total extract yields reported as percentage of g of extract per 100g pomegranate peel on dry basis indicated that the pomegranate peel extracted with MeOH gave the highest total extract yield (46.51±0.86), followed by water (43.19±2.24), when the extractions were done with the ratio of solvent/sample of 15:1 (w/w) at 40 °C for 4 h.

POLYPHENOL CONTENT DETERMINATION

Total phenolic contents of pomegranate peel extracts (MeOH and DI water) were determined using Folin-Ciocalteu method. In the SAE and UAE methods the amount of total phenolic yields 1.82 to 4.0 mg GAE / g and 2.45 to 4.49 mg GAE /g respectively (Figure 3). In SAE 100% MeOH extract showed the highest total phenolic yield, 4.0 mg GAE / g while in UAE 75% MeOH extract shows the highest total phenolic yield 4.49 mg GAE / g.

Negi et al. (2003) [20] reported that the phenolic contents of ethyl acetate, acetone, methanol and water extracts were found to be 16.5, 52, 46.2 and 4.8% respectively, when they use 5 mg of each dried pomegranate peel extract and results were expressed as catechin equivalents at 765 nm. [19] comparing methanol with water as the solvent in pomegranate antioxidant extraction the yield of total phenolics was 5.90% and 8.26%.

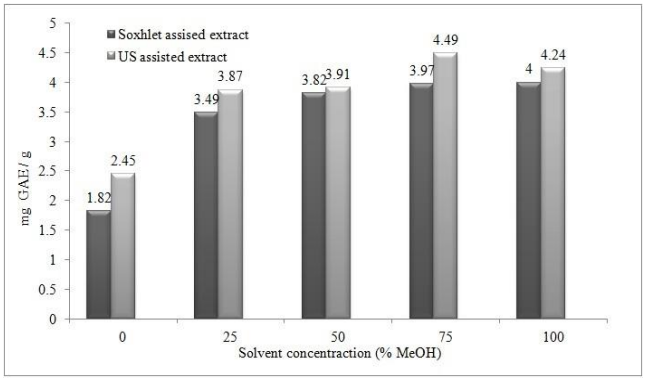

Figure 3- Polyphenol content (mg GAE/g) of PPP extract by SAE and UAE methods

DPPH SCAVENGING ACTIVITY OF EXTRACTS

In present research antioxidant activities of the extracts were carried out to investigate the correlations between the antioxidant activity of extract from both methods SAE and UAE. The radical scavenging activity ranges from 15 to 56.02% in SAE and 51.84 to 67.94% in UAE (Figure 4). In present research the UAE by 75% MeOH shows highest 67.94% radical scavenging activity while in SAE 100% MeOH extract shows highest 56.02% radical scavenging activity.

Wang et al. (2011) [19] compared methanol with water as the solvent in pomegranate antioxidant extraction, the DPPH antioxidant activities were 53.74% and 65.30%, respectively. Eghdami et al. (2011) [21] showed that antioxidant activity values of pomegranate juice and peel extract 32±0.52 and 75±2.15 respectively.

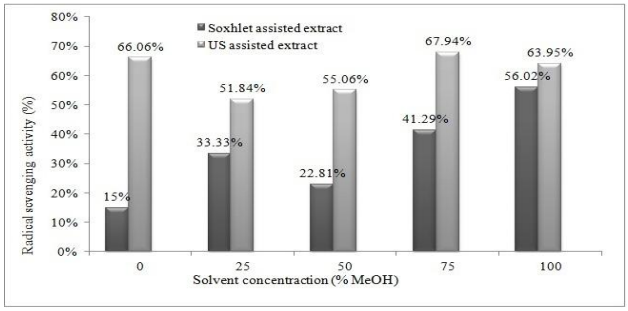

Figure 4 -Free radical scavenging activity (%) by DPPH of pomegranate peel extract by SAE and UAE methods

ANTIOXIDANT ACTIVITY BY FRAP METHOD

Ferric Reducing Antioxidant Power (FRAP) assay was determined in term of ferrous sulphate equivalents (FSE) in both the cases SAE and UAE methods from pomegranate peel (mg Fe^{II} / g extract). The total antioxidant activity of PPP extract in both SAE and UAE varied from 693.42 to 799.82 mg FSE / g and 763.73 to 832.17 mg FSE / g extract respectively (Figure 5). In SAE, 100% MeOH extract showed the highest antioxidant activity 799.82 mg FSE / g extract while in UAE, 75% MeOH extract showed the highest antioxidant activity 832.17 mg FSE / g extract.

Eghdami et al. (2011) [21] showed that antioxidant activity values of pomegranate juice and peel extract were 98.02 ± 0.5319 and 171.08 ± 0.53 mmol $FeSO_4$ equivalents per g of sample. Tabaraki et al. (2012) [14] found in their study of UAE of pomegranate (*Punica granatum* L.) peel antioxidants and showed that antioxidant activity values of pomegranate peel extract varied from 24.30 to 63.37 mmol Fe^{2}+/100 g of dry weight, for FRAP assays.

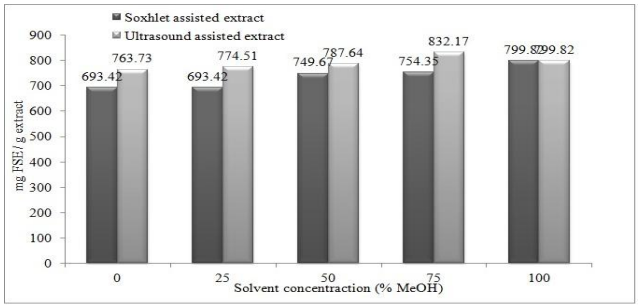

Figure 5- Antioxidant activities (mg FSE/g) by FRAP of pomegranate peel extract by SAE and UAE methods

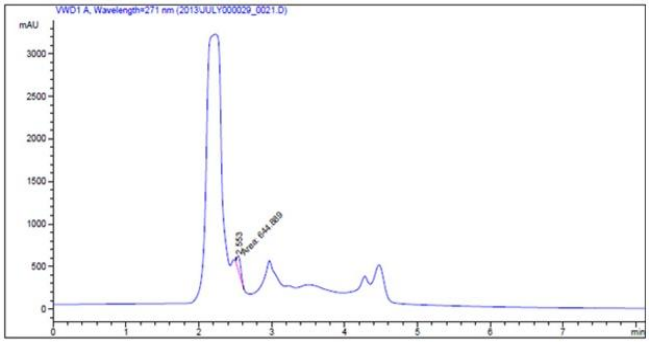

Figure 6- Gallic acid curve of pomegranate peel extract by SAE

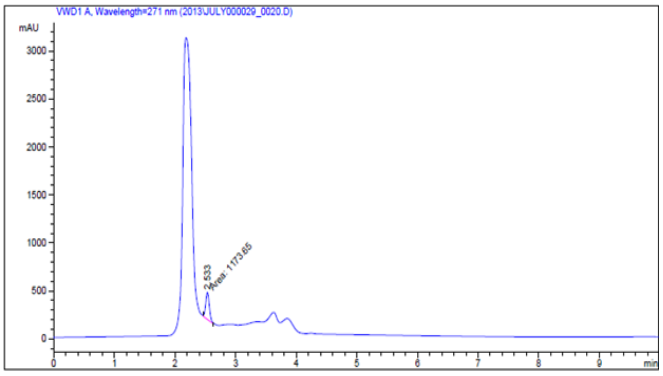

Figure 7- Gallic acid curve of pomegranate peel extract by UAE

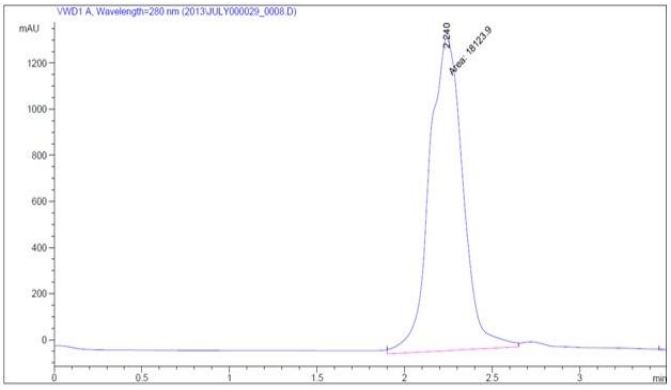

Figure 8- Catechin curve of pomegranate peel extract by SAE

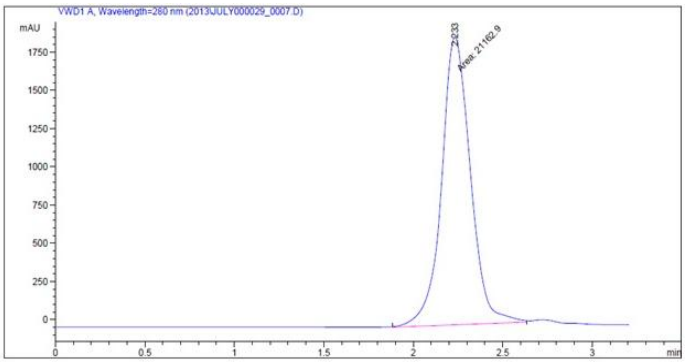

Figure 9- Catechin curve of pomegranate peel extract by UAE

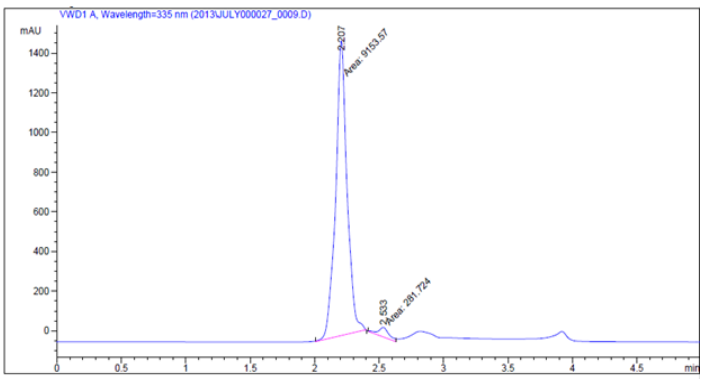

Figure 10- Chlorogenic acid and Caffeic acid curve of pomegranate peel extract by SAE

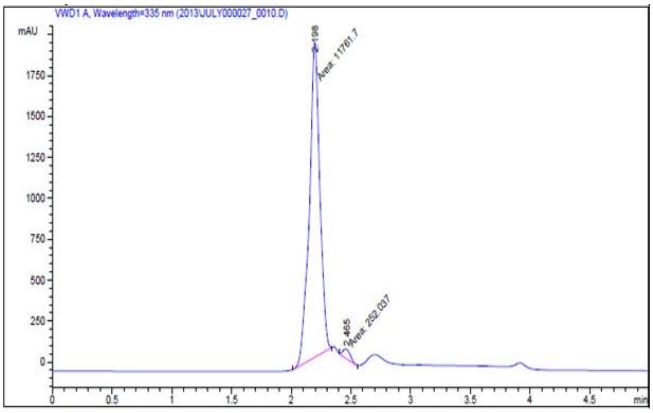

Figure 11- Chlorogenic acid and Caffeic acid curve of pomegranate peel extract by UAE

IDENTIFICATION AND QUANTIFICATION OF BIOACTIVE COMPOUNDS BY HPLC

HPLC is a chromatographic technique which was used to separate the components of a mixture, to identify and quantify each component. Gallic acid, catechin, chlorogenic acid and caffeic acid are the antioxidants which was identified and quantified through this chromatographic technique. The main chemical constituents isolated from pomegranate peel are: gallic acid, ellagic acid, caffeic acid, chlorogenic acid, p-coumaric acid etc [22]. Chromatograph of gallic acid (Fig 6 and Fig 7), catechin (Fig 8 and Fig 9), chlorogenic and caffeic acid (Fig 10 and Fig 11) of pomegranate peel extract by SAE (using 100% MeOH) and UAE (using 75% MeOH) are shown respectively. Polyphenol content was found to be highest in UAE, chlorogenic acid was found in the highest quantity i.e. 1444.03ppm followed by catechin 1365.36ppm. The quantities of each component are shown in Table 1.

Table 1- Quantification of polyphenol compound through HPLC analysis

Bioactive compound	Sample/Method of extraction	Quantity (ppm)
Gallic acid	PPP/SAE	265.40
	PP/UAE	304
Catechin	PPP/SAE	628.05
	PP/UAE	1365.36
Chologenic acid	PPP/SAE	399.72
	PP/UAE	1444.03
Caffieic acid	PPP/SAE	7.24
	PP/UAE	6.03

CONCLUSION

Ultrasound assisted extraction method is the innovative and the most promising technique, which can be used as a better option for extraction of polyphenol from PPP at industrial level because of its low extraction time, lower temperature and a significant difference in the yield of the extract. UAE method helps to prevent the thermal degradation, increase the bioavailability of the bioactive components which causes the increase in the antioxidant activity and overcome the disadvantage of the SAE method.

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