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ULTRASONICATION ASSISTED EXTRACTION OF PHENOLIC ANTIOXIDANTS FROM *CITRUS MAXIMA* LEAVES AND THEIR GC-MS PROFILE

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ABSTRACT

Citrus maxima is a medicinally important plant used traditionally for protection against many diseases. Extraction of its phenolic content has been a subject of interest due to its wide range of biological effects like anti-oxidative, anti-inflammatory, anti-tumor and antimicrobial activities. The aim of the current research was to evaluate the novel ultrasonic assisted method for efficient extraction of antioxidant phenolic compounds from this plant. The extracts obtained through this method at different extraction times (15, 30, 45, 60 and 75 minutes) were estimated for 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging activity, reduction potential and total phenolic contents. The extracts obtained through ultrasonication for 60 minutes exhibited highest anti-radical activity (79.21%), highest reduction potential (OD=1.24) and maximum total phenolic content (11.6 mg gallic acid equivalent per gram dry weight). Correlation analysis showed that there was a strong positive correlation between total phenolic content and anti-oxidant activities and the Gas Chromatography Mass Spectrometry analysis of the extracts revealed the presence of many pharmaceutically active compounds. It can therefore be concluded that UAE can be considered as a time saving method for efficient extraction of phenolic compounds from this plant.

KEY WORDS : ***Citrus maxima*, DPPH· radical, Total phenolic content, Reducing power, GC-MS.**

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INTRODUCTION

Citrus maxima, commonly called pummelo or chakotra, is a 4-12 m tall tree having compound leathery leaves with broadly winged petiole. It is native to Asia and cultivated for utilization of its medicinal properties in many countries like Japan, Vietnam, Malaysia, Indonesia and Thailand. The fruits are a rich source of vitamin C, B_1, B_2, B_{12}, protein and calcium. Decoctions of the leaves, flowers, fruits and seeds are used to treat convulsive cough, fever and gastric disorders and are also given for their sedative effects in cases of epilepsy. Citrus peels exhibit prominent antioxidant and anti-inflammatory effects. Leaves and peels contain numerous bioactive compounds such as phenolic acids, flavonoids and limonoids. Some of the compounds like limonene, citral, aldehydes, geraniol, cadinene and linalool are skin irritants which may cause dermatitis on excessive contact. Citrus bioflavonoids such as hesperidin, narirutin, naringenin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin, and nobiletin are abundant in fruits and are most prominent cancer preventing agents[1-4]. The pharmaceutical activity of *Citrus maxima* is attributed mainly to the phenolic compounds and terpenoids present in its aerial parts. The capabilities of polyphenols as free radical scavengers, complexers of pro-oxidant metals, reducing agents, quenchers and oxygen singlet formation are undeniable. Many polyphenolics containing plant sources are reported to exhibit anti-radical, anti-mutagenic, anti-bacterial and cytotoxic effects[5]. Such phenolic compounds of medicinal importance are conventionally extracted from plants using classical techniques of maceration and thermal extraction for preparation of infusion and decoction. These methods generally require long processing time and might compromise on yield efficiency due to thermal degradation of heat labile compounds[6]. In order to overcome these shortcomings, several intensification techniques are used nowadays which involve microwaves and ultrasonic waves to improve the yield and quality of extracted products[7]. In the present study, ultrasonication assisted extraction (UAE) was investigated for extraction of phenolic compounds from *Citrus maxima*, and the anti-oxidant capacity of resultant extracts was evaluated. In order to gain insight of the phytochemical profile, GC-MS analysis of these extracts was also performed.

MATERIALS AND METHODS

Plant material

Authentic plant material (leaves of *Citrus maxima*) was collected from Herbal garden, Department of Botany, Jamia Hamdard University, New Delhi. The leaves were dried under shade for 5 days and powdered in a mechanical grinder.

Extraction procedure

An extractor equipped with an ultrasonic horn transducer (Model 750W, Sonics & material Inc., USA) working at 20 kHz frequency and 750W input power with amplitude range was used for the extraction. The extraction was carried out as described by Laghari et al., 2011[8] with some modification. Five gram of sample was extracted with 50 ml of 70% ethanol and was kept in the ultrasonic bath for 75 minutes. The sampling was done at every 15 minutes. Ultrasonication was carried out at room temperature and the extracts were filtered and concentrated in-vacuo.

Estimation of phenolic compounds

Total phenolic content (TPC) in *Citrus maxima* leaf extracts was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method[9], calibrating against gallic acid standards and expressing the results as milligram gallic acid equivalents per gram dry weight (mg GAE/ gDW) of plant material.

DPPH· free radical scavenging activity

The antioxidant activity of the plant extracts as well as ascorbic acid (100 µg/ml) taken as the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) free radical by modified method[10]. DPPH· (0.002%) was prepared in methanol and 1 ml of this solution was mixed with 1 ml of sample solution. These mixtures were kept in dark for 30 min and optical density was measured at 517 nm at different time intervals 10, 20, 30 and 40 minutes using spectrophotometer. Methanol (1 ml) with equi-volume DPPH· solution (0.002%) was used as blank. The optical density was recorded and percentage inhibition was calculated using the formula given below[11]:

$$I\ \% = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

where,

I% is the Inhibition of DPPH· in percent,
A_{blank} is the absorbance of blank consisting of DPPH· in aqueous methanol,
A_{sample} is the absorbance of different extracts.

Reducing power assay

The reducing power of different extracts was measured according the method used by Hinneburg et al. (2006)[12]. One ml of each sample extract was mixed with 2.5 ml of phosphate buffer (200mM; pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated at room temperature for 20 minutes. It was added with 2.5 ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. An aliquot of supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%) was added. On development of colour the absorbance was measured spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated higher reductive potential.

GC-MS Analysis

The analysis of phenolic compounds in the extracts was performed using GC- MS (model GC-MS-QP-2010 plus, Shimadzu Make). Two micro-litres of sample was injected into a RTX-5 column (60 m x 0.25 mm internal diameter, film thickness 0.25 µm) of GC-MS. The carrier gas helium was used with a constant column flow of 1.21 ml/min at 95.2 kpa inlet pressure. Temperature was maintained from 100°C to 250°C at a constant rise of 5°C/minute and thereafter held isothermal at 250°C for 10 min. Further the temperature was raised up to 300°C and finally held isothermal at 300°C. The crude extract dissolved in methanol (Chromatography grade, Merck, India) was injected with a split ratio of 1:10. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and the total GC-MS running time was 35 minutes. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) and WILEY 8. The mass spectra of unknown components were compared with the spectrum of the known components stored in these libraries. The name, molecular weight and structure of the phenolic compounds of the test materials were ascertained.

Statistical analysis

Experimental results were presented as mean ± standard error (SEM) of four replicates and linear regression analysis was performed to find out the correlation coefficient and to determine the relationship between total phenolic content and antioxidant activity of phenolic extracts.

RESULTS AND DISCUSSION

Total phenolic contents

Plant-derived phenolics are well-known natural antioxidants and can contribute directly to the antioxidative action[13]. In order to study the

effect of time of ultrasonication extraction on phenolic composition, total phenolic contents (TPC) of all extracts were determined using Folin-Ciocalteu reagent and were expressed as gallic acid equivalents. This method is based on an oxidation-reduction reaction in which phenolic compounds are oxidized reducing phosphotungsten - phosphomolybdate to a blue coloured complex[14]. The results showed that extraction efficiency of ultrasonication for total phenolic contents enhanced significantly with increase in time (Figure 1). The TPC ranged from 3.4 to 11.6 mg GAE / gDW and highest yields of phenolic compounds were obtained on 60 minutes of extraction. The ultrasonic field generates local micro-cavitations in the liquid surrounding the plant tissue which cause mechanical disruption of the cell walls releasing their content. The enhanced kinetic energy improves mass transfer across the solid-liquid interface causing enhanced diffusion of extracted compounds[15]. This shows that the increase in total phenolic contents with prolonged extraction time is due to the proper disruption of the cell wall and increase in the extract diffusion. Continuing extraction beyond 60 minutes could not improve the TPC yield further. This could have happened due to maximum rupture of cells at 60 minutes enabling complete release of phenolic compounds. There are many other reports in which UAE has been used widely for the extraction of phytochmicals[16, 17]. Ultrasonication assisted extraction method has advantage of less time consumption and the efficiency of extraction can be further improved by standardizing other conditions such as material-solvent ratio, solvent concentration, temperature etc.

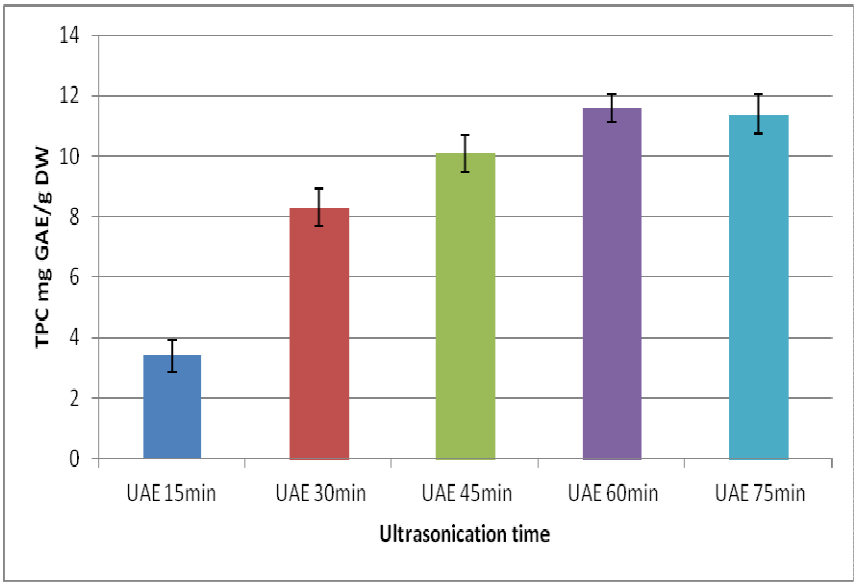

Figure 1
Effect of ultrasonication time on Total Phenolic Content (TPC) of Citrus maxima leaves. Values are presented as the mean ± SE of four replicates each.

DPPH· radical scavenging activity

The antioxidant activity of phenolic extracts of *Citrus maxima* was determined on the basis of its DPPH· radical scavenging capacity. DPPH· is a stable free radical which on dissolution in methanol shows a characteristic absorption at 515 nm. Antioxidant molecules scavenge free radicals by hydrogen donation and the color from the DPPH· assay solution becomes light yellow resulting in a decrease in absorbance. Figure 2 shows the DPPH· radical scavenging activity of the phenolic extracts of *Citrus maxima* obtained through ultrasonication. The anti-radical activity was estimated as a function

of time at 10, 20, 30 and 40 minutes and was recorded as percentage inhibition of DPPH• radical. A potential antioxidant ascorbic acid was used as reference standard.

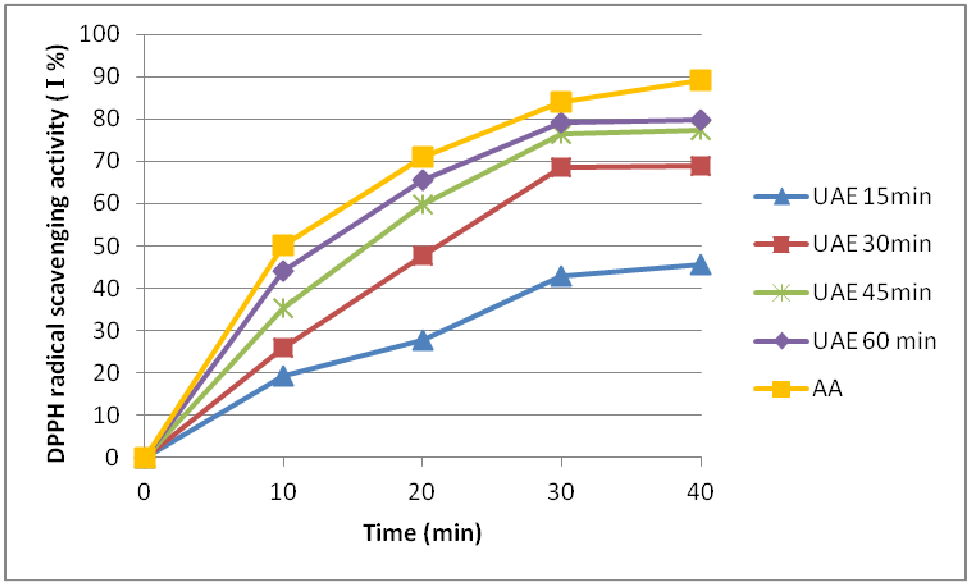

Figure 2
Time response curve for DPPH• radical scavenging activity of Ascorbic acid and Citrus maxima leaf extracts obtained by ultrasonication assisted extraction (UAE) for different periods.

All the phenolic extracts obtained by ultrasonication for different time periods were found to actively scavenge the DPPH• radicals. The anti-radical activity increased successively with increase in the extraction time and highest DPPH• radical scavenging activity was exhibited by extracts obtained at 45 and 60 minutes of extraction (I % = 76.92 and 79.21 % respectively). The percentage inhibition achieved by these extracts was also quite comparable to that of a strong antioxidant compound ascorbic acid (100 µg/ml), indicating potential antiradical activity of these extracts (Figure 2). Extract - DPPH• radical reaction time also has a considerable impact on the radical scavenging activity of different extracts. Percentage inhibition by all the extracts increased with increasing reaction time and attained its maximum at 30 minutes, leading to a steady state thereafter. Free radicals react promptly with biological macro molecules such as enzymes, nucleic acids, membrane lipids etc., and cause cellular damage. Being active free radical scavengers, phenolic compounds are mainly involved in the cell defense system[18]. Phenolics like flavonoids are remarkable free radical scavengers, while tannins and saponins also exhibit good antioxidant activity[19]. Antioxidant potential of *Citrus maxima* can be seen as a strong contributor to its pharmaceutical properties.

Reducing power assay

Phenolic extracts of *Citrus maxima* were assayed for their reducing power in which the change in yellow colour of the test solution to various shades of green and blue was observed which depend upon the reducing power of each compound. Reducing compounds react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex and gives a prussian blue colour showing absorption maximum at 700 nm. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron which is an important mechanism of phenolic antioxidant action[20]. In

this study, a linear increase in the absorbance was seen with increase in the ultrasonication time (Figure 3) and extract obtained at 60 minutes showed the highest absorbance (OD =1.24).

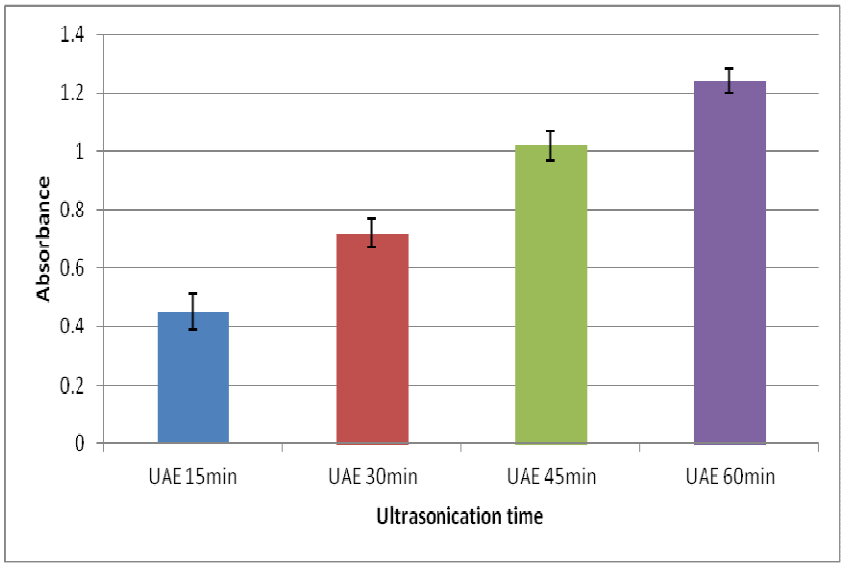

Figure 3
Effect of different ultrasonication time periods on reducing power of Citrus maxima leaf extracts. Values are presented as the mean ± SE of four replicates each.

Correlation Analysis

Phenolic compounds are known to possess strong antioxidant activity which renders these compounds their therapeutic properties. Correlation analysis was performed to determine relationship between total phenolic content (TPC) and DPPH˙ radical scavenging activity as well as TPC and reducing power for phenolic extracts of *Citrus maxima* (Figure 4 & 5). Results showed that a strong positive correlation exists between TPC and antioxidant activities and phenolic compounds contribute to 94.2% of the DPPH˙ radical scavenging activity in extracts from *Citrus maxima* leaves. Correlation analysis between TPC and reducing power shows that 81.6% of reduction potential was contributed by phenolic compounds. Antioxidant activity of these plant extracts is not limited to phenolic compounds but may also be related to the presence of other secondary metabolites, which was evident by the broad spectrum of compounds obtained in GC-MS analysis of these extracts.

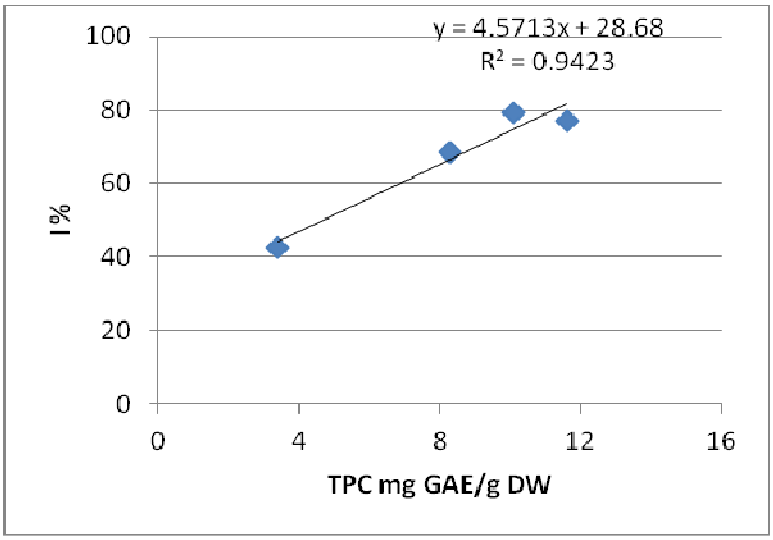

Figure 4
Correlation between TPC and DPPH˙ scavenging activity of Citrus maxima leaf extracts.

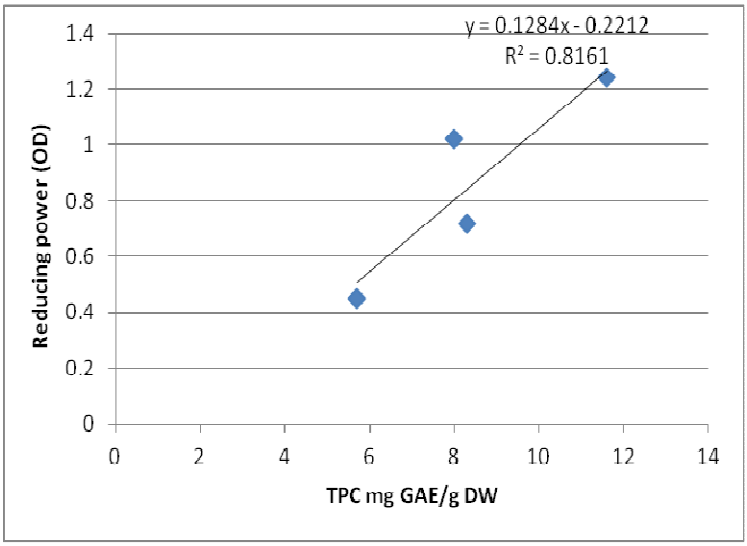

Figure 5
Correlation between TPC and Reducing power of Citrus maxima leaf extracts.

GC-MS analysis

The ethanolic leaf extracts of *Citrus maxima* obtained by ultrasonication assisted extraction method at 60 minutes which showed highest TPC and antiradical activity, were quantitatively analyzed through GC-MS analysis. The GC-MS profile revealed the presence of 57 compounds (Figure 6) belonging to different classes of secondary metabolites. Most of the compounds were essential oils, carboxylic acids, fatty acids or their esters. Many other compounds of pharmaceutical importance were identified such as chalcones, cyclic alkanes, carboxylic acids and terpenoids. The peak report of the chromatogram obtained with details of peak number, retention time, area percentage, name of the identified phytocomponent, molecular formula, molecular weight and their pharmaceutical properties are presented in Table 1. Ultrasonication method is considered to be an efficient and time saving method for extraction of secondary metabolites. Schinor et al. (2004)[21] found that as compared to the traditional extraction methods, ultrasonic

waves are more effective and about three times faster in the extraction of active compounds such as saponins, steroids and triterpenoids from *Chresta spp*. Several other reports comply with the suitability of ultrasonic extraction for large number of compounds from plant material[23-24].

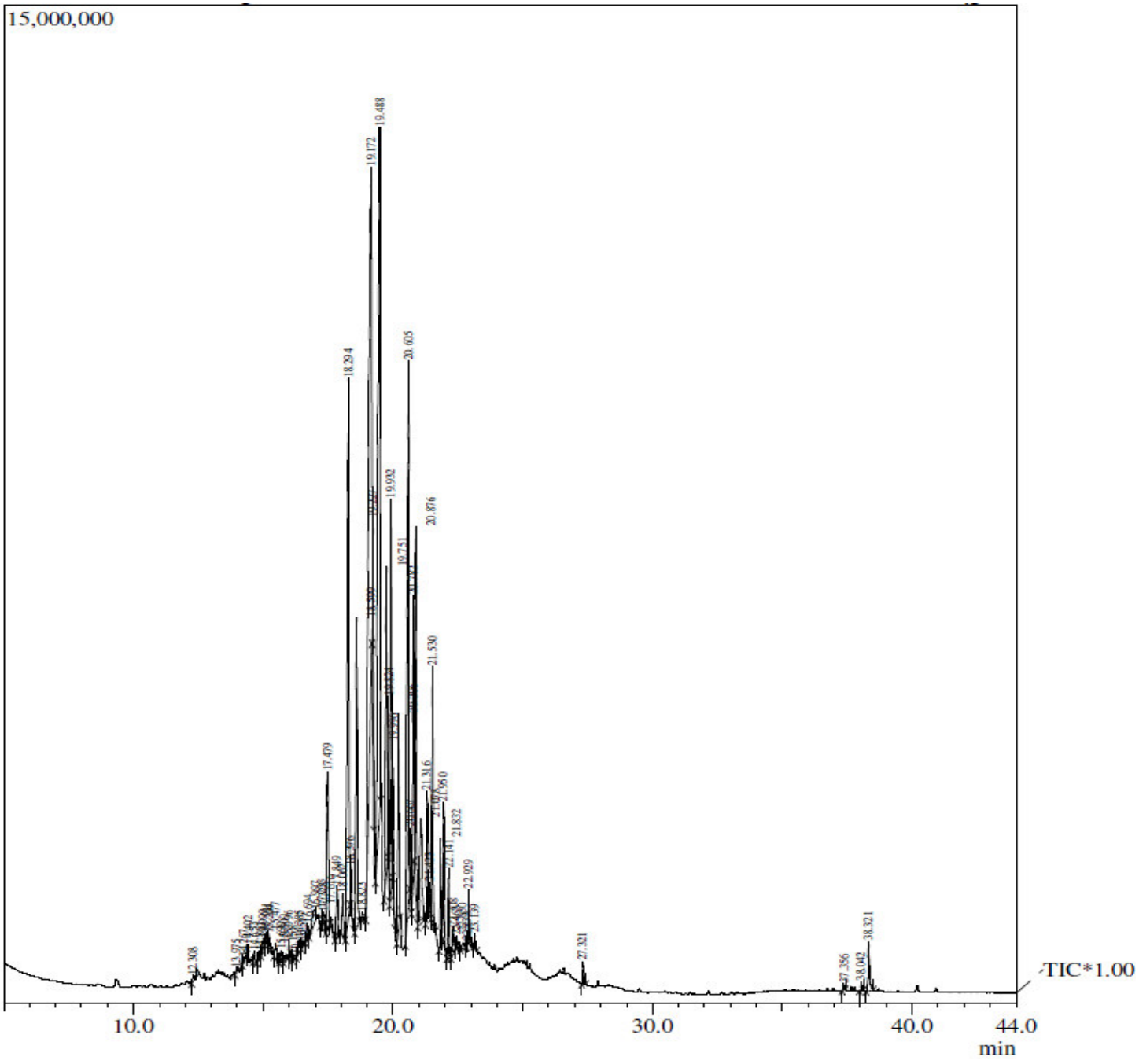

Figure 6
The GC-MS chromatogram of ethanolic extract of Citrus maxima leaves obtained by ultrasonication for 60 minutes.

Table 1
Peak report of some bioactive compounds identified in the Citrus maxima leaf extract obtained by Ultrasonication Assisted Extraction for 60 minutes.

Peak no.	Retention Time	Area%	Name of the compound	Molecular formula	M.W	Pharmaceutical property
1	12.308	0.09	Citral	$C_{10}H_{16}O$	152	Antimicrobial, anti-inflammatory,diuretic activities[25,26]
2	13.975	0.03	Cedran-8-en-13-ol	$C_{15}H_{24}O$	220	Insecticidal[27]
5	14.65	0.11	Adamantane	$C_{10}H_{16}O$	136	Subunit in synthetic cannabimetic drugs. Derivatives used as antiviral drugs[28]
16	16.517	0.10	Hydrocinnamic acid	$C_9H_{10}O_{21}$	150	Used in Preparation of pharmaceuticals used in treatment of HIV[29]
19	17.233	0.04	Chalcone, 4'-chloro-	$C_{15}H_{11}ClO$	242	Anti inflammatory[30]
42	21.32	2.05	Citronellol	$C_{10}H_{20}O$	156	Anti cancerous, anti inflammatory, wound healing[31]
45	21.816	0.96	Phytol	$C_{20}H_{40}O$	296	Treatment of Rheumatoid arthritis[32] Adjuvant in vaccine formulation[33].
52	22.929	0.51	β-Caryophyllene	$C_{15}H_{24}$	204	Antitumor, anti-inflammatory[34] .
55	37.356	0.08	β-Sitosterol	$C_{29}H_{50}O$	414	Treatment of cancer and pulmonary tuberculosis[35].
56	38.042	0.10	Lup-20-(29)en-3ol	$C_{30}H_{50}O$	426	Antiprotozoal, antimicrobial, inflammatory,antitumor and chemoprotective[36].

CONCLUSION

Selection of an appropriate extraction method is crucial in order to increase process productivity and yield of phenolic compounds. The purpose of the experiment was to evaluate the modern ultrasonication extraction technique for its efficiency in extracting antioxidant phenolic compounds and studying the influence of extraction time on the total phenolic content and antioxidant activities. UAE emerged as a time efficient method for extraction of potential antioxidant compounds from *Citrus maxima* leaves. It can be therefore concluded that UAE with further optimization can be reiterated as a worthy method to improve on time and energy consumption and can be far more useful for phenolic extraction than the conventional methods.

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Conflict of Interest: Conflict of interest declared none.

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