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ORIGINAL ARTICLE

Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*

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KEYWORDS

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Abstract *Withnaia somnifera* (L.) is a wonder herb with multiple medicinal properties. Its root is used in the preparation of many *Ayurvedic* medicines. Withanolides, steroidal lactones, present in the root are active chemical markers, however, phenolics, and flavonoids have also been reported in the root of this plant. In most of the herbal preparations, water extraction is carried out using the infusion or decoction preparation process. In the present study, extract yield, phytochemical constituents such as total phenol and withanolide content of water and water-alcohol extracts prepared using two most commonly used extraction techniques, also known as "Green Extraction" techniques, ultrasound and microwave assisted solvent extraction were compared with the conventional extraction method. Antioxidant activity of the extracts was also determined using DPPH and ABTS methods of antioxidant assay. Extract yield, chemical composition of the extracts (total phenol and withanolide content) and antioxidant activity of the extracts varied with the extraction process as well as solvent composition.

1. Introduction

Plants are an important source of bioactive molecules for drug discovery. Isolated bioactive molecules serve as starting materials for laboratory synthesis of drugs as well as a model for the production of biologically active compounds. Phytochemical processing of raw plant materials is essentially required to optimize the concentration of known constituents and also to maintain their activities (Aziz et al., 2003). Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials. Selection of a suitable extraction technique is also important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents. Further, selection of suitable extraction process and optimization of various parameters are critical for upscaling purposes i.e. from bench scale to pilot plant level. Various extraction techniques most commonly used include conventional techniques such as maceration, percolation, infusion, decoction, hot continuous extraction etc. Recently, alternative methods like ultrasound assisted solvent extraction (UASE), microwave assisted solvent extraction (MASE) and supercritical fluid extractions (SFE) have gained increasing interest during the last three decades (Co et al., 2012). Use of green extraction

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techniques such as UASE (Wu et al., 2001; Ahh et al., 2007), MASE (Ganzler et al., 1986a,b) and SFE (Ollanketo et al., 2002) has been rapidly and continuously increasing globally for phytochemical processing of medicinal plants as these techniques are fast as compared to traditional methods. Also these techniques are environmentally friendly in terms of solvent and energy consumption. Yield is also comparable to conventional extraction and in some cases it is even higher. However, extract yield as well as the bioactivities of the extract prepared using different extraction methods have been reported to vary in several studies (Hayouni et al., 2007).

Withania somnifera (L.) Dunal., *(Solanaceae)* commonly known as Ashwagandha, is a green shrub found throughout the drier parts of India, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa and Egypt. In India, it is cultivated widely in Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat and Haryana (Bhatia et al., 1987). It is categorized as a *rasayana* in *Ayurveda* and traditional Indian systems of medicine. The *rasayana*s, apart from their use for promoting physical and mental health also provide defence against diseases and arrest ageing process (Singh et al., 2001; Bhattacharya et al., 2002). *W. somnifera* is used as dietary supplement and the decoction of its root is used as nutrient and health restorative to pregnant and old people. The extract of *W. somnifera* is a complex mixture of a large number of phytochemicals including phenolic compounds and flavonoids. However, the pharmacological effect of the roots of *W. somnifera* is attributed to withanolides (Rahman et al., 1988; Chen et al., 1987; Udayakumar et al., 2010). Withanolides are a series of naturally occurring steroids containing a lactone with a side chain of nine carbons, generally attached to C-17. Variation of lactone moiety is found in various classes of withanolides. Although, withanolides also have been reported from the plants of families such as *Solanaceae*, *Taccaceae* and *Leguminose* as well as from some marine organisms, genus *withania* is among the richest sources of steroidal lactones in nature (Srivastava et al., 1992; Ksebati and Schmitz, 1988). *W. somnifera* extract promoted learning and memory in animal models (Dhuley, 2001), reversed the memory loss induced by oxidative damage caused by streptozotocin (Parihar et al., 2004). *In vitro* studies have also established that an aqueous extract of W. *somnifera* root inhibited amyloid β (A β) fibril formation. Cerebral extracellular deposition of protein fibrils formed by A β, mainly from the 40 or 42 amino acid A β peptide has been described as the characteristics of Alzheimer's disease in elderly people (Kumar et al., 2012). Keeping in view to find correlation between the extraction methods, yield, total phenolics and withanolides content, the present investigation was carried out. Extraction was done with water and water-alcohol as solvent using UASE and MASE since in most of the herbal preparations water or water-alcohol is used as a solvent. Conventional extraction using refluxing was also carried out for the comparison of extract yield and phytochemical qualities of the extract.

2. Materials and methods

2.1. Plant material and extract preparation

Roots of *W. somnifera* were collected from the research farm of the Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand, Gujarat, India in the year 2011 and air dried in shade. The air dried roots were finely powdered using an electric grinder. For conventional extraction (refluxing), 5 g of powdered plant material was mixed with 50 ml of distilled water in a round bottom flask and refluxed for about 5 h at 100 °C. Similarly, 5 g of powder was mixed with ethanol and water–ethanol (9:1) separately in round bottom flasks and refluxed for 5 h. Liquid extracts obtained were separated from the solid residue by vacuum filtration, concentrated using a rotary evaporator.

For UASE, 5 g of powdered plant material was mixed with 50 ml of distilled water, ethanol and water–ethanol (9:1) separately in beakers. Extraction was carried out by placing the three beakers in an ultrasonic bath (Bandelin Sonorex, Germany, 480 W, 35 kHz) for 5, 10 and 20 min. Water in the ultrasonic bath was circulated at room temperature (25 °C) to avoid overheating caused by ultrasound. The supernatant was similarly processed as described in conventional extraction to get dried UASE extract of *Withania somnifera*. Similarly, for MASE, 5 g of powdered plant material was mixed with 50 ml of distilled water, ethanol and water–ethanol (9:1) separately in Pyrex beakers. Extraction was carried out by placing the beakers in the middle of the oven over a rotating dish and exposed to microwave radiation (LG Electronics, India, Model MG-556 P, 1350 W, 2450 MH_Z) for 5, 10 and 20 min. At the end of heating, the beaker was left for temperature stabilization (1 min).The supernatants were re-centrifuged and concentrated as described earlier. Dried extract samples were kept in an airtight container at 4 °C.

2.2. Determination of total phenolics in extracts

Total phenolic contents in the extracts were determined spectrophotometrically by Folin–Ciocalteau method (Singleton et al. 1999). Dried extracts were reconstituted in distilled water (1 mg/ml). Folin–Ciocalteau reagent (0.5 ml) was added to the extract solution (0.5 ml) and the total volume was adjusted to 8.5 ml with distilled water. The tubes were kept at room temperature for 10 min and thereafter 1.5 ml of sodium carbonate (20%) was added. The tubes were incubated in a water bath at 40 °C for 20 min The intensity of the blue colour developed was measured by recording the absorbance at 755 nm using a UV–visible spectrophotometer (Varian, CARY-300 Bio).The reagent blank was also prepared using distilled water. For quantification of the total phenolic in the extract, a standard calibration curve was prepared using gallic acid. Total phenolic content of the extract samples was expressed as gallic acid equivalent (GAE) milligrams per gram of the extract.

2.3. Identification and quantification of withanolides and withaferin in extracts using high performance liquid chromatography (HPLC)

Withanolides (withanolide A and 12-deoxy withastramonolide) and withaferin A were quantified in the extracts using HPLC (Shimazdu Prominence UFLC) on RP- C_{18} column (Lichrocart, Merck, 250 × 4.6 mm, 5 μm). Mobile phase consisted of acetonitrile (40%) and 0.1% acetic acid in water (60%) in an isocratic elution mode with the flow rate of 1.0 ml/min. Injection volume was 20 μl. The solvents were degassed using vacuum. Samples for HPLC analysis were also filtered through a 0.45 μm membrane filter. The peaks were monitored at 230 nm using a UV–visible detector (UV–visible

SPD −20 A).The peaks were identified on the basis of retention time with the comparison of the retention time of standard withanolide A, 12-deoxy withastramonolide and withaferin A, their contents in the extracts were quantified on the basis of area of the peak.

2.4. Antioxidant assay

2.4.1. Radical scavenging activity using DPPH method

The radical scavenging activity of extracts of *W. somnifera* prepared by refluxing, UASE and MASE was evaluated using DPPH assay. Different concentrations (equivalent to 200, 400, 600, 800 and 1000 ppm) of the extracts were taken in test tubes. The total volume was adjusted to 8.5 ml by the addition of methanol. 5.0 ml of 0.1 mM methanolic solution of DPPH was added to these tubes and mixed well with a vortex mixer. The tubes were kept at room temperature for 20 min. The blank was prepared as above without the extract and methanol was used for the baseline correction. Changes in the absorbance of the extract samples were measured at 517 nm using the UV–visible spectrophotometer. Radical scavenging activity (RSA) was expressed as the inhibition percentage and was calculated using the following formula

$$\%\text{Radical scavenging activity} = \frac{(\text{Absorbance of blank} - \text{absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

2.4.2. Free radical scavenging ability by the use of a stable ABTS radical cation

Free radical scavenging activity was determined by ABTS radical cation decolorization assay (Re et al., 1999). ABTS was dissolved in water to a 7 μM concentration and radical cation ($ABTS^+$) was produced by reacting ABTS solution with 2.45 μM potassium persulphate at room temperature in dark (12–16 h) before use. For assay, $ABTS^+$ solution was diluted with water to an absorbance value of 0.700 ± 0.02 at 734 nm. After addition of 3.0 ml of diluted $ABTS^+$ solution to 100 μl of extracts solutions, absorbance was recorded after 6 min.

2.4.3. Calculation of IC_{50} concentration

The extract concentration corresponding to 50 percent inhibition (IC_{50}) was calculated from the curve of RSA percentage against extract concentration. Ascorbic acid and trolox were used as standards. Each sample was assayed in triplicate for each concentration.

3. Results and discussion

Biologically active compounds usually occur in low concentration in plants. An extraction technique is that which is able to obtain extracts with high yield and with minimal changes to the functional properties of the extract required (Quispe Candori et al., 2008). Several studies have reported variations in the biological activities of extracts prepared using different extraction techniques. Therefore, it is necessary to select the suitable extraction method as well as solvent based on sample matrix properties, chemical properties of the analytes, matrix-analyte interaction, efficiency and desired properties (Hayouni et al., 2007; Ishida et al., 2001). In conventional extraction, heat is transferred through convection and conduction from the surface, here, the extractability of solvents depends mainly

Table 1 Yield, total phenolics, withanolide A, 12-deoxy withastramonolide, and IC_{50} values of the extracts of *W. somnifera* root prepared using different extraction methods[+].

Extraction method	Treatment	Yield (%)	Total phenolics (mg/g GAE)	Withanolide A (μg/mg)	12 deoxy withastramonolide (μg/mg)	Total withanolides (μg/mg)	IC50 (mg/ml)	
							DPPH	ABTS
Reflux (Soxhlet)	Ethanol	9.08	35.93	3.57	1.22	4.79	0.18	1.05
	Water: ethanol	9.43	21.15	1.25	0.40	1.65	0.39	2.04
	Water	9.51	17.63	1.14	0.36	1.50	0.40	2.14
UASE	Ethanol*	2.85	24.72	6.04	2.04	8.09	0.21	1.24
	Ethanol**	2.96	25.27	6.58	2.08	8.66	0.20	1.21
	Ethanol***	3.17	29.15	6.08	1.94	8.02	0.18	1.20
	Water: ethanol*	9.74	21.15	1.21	0.41	1.62	1.03	2.54
	Water: ethanol**	8.96	21.39	1.48	0.47	1.95	0.99	2.53
	Water:ethanol***	9.08	22.12	1.84	0.62	2.46	0.97	2.51
	Water*	9.90	14.90	0.91	0.26	1.16	1.20	2.68
	Water**	11.85	15.81	0.81	0.23	1.04	1.19	2.64
	Water***	10.27	18.18	0.67	0.20	0.87	1.17	2.62
MASE	Ethanol*	10.01	40.96	4.35	1.39	5.73	0.15	0.83
	Water: ethanol*	11.39	20.84	1.09	0.28	1.36	0.73	2.11
	Water: ethanol**	12.81	21.81	0.97	0.29	1.26	0.66	2.09
	Water:ethanol***	13.75	22.90	1.00	0.30	1.31	0.62	2.07
	Water*	11.18	17.15	0.59	0.20	0.79	0.70	2.42
	Water**	12.74	17.99	0.67	0.23	0.90	0.69	2.39
	Water***	13.02	18.72	0.69	0.19	0.89	0.68	2.36

[+] Mean of three replications.
* 5 min.
** 10 min.
*** 20 min.

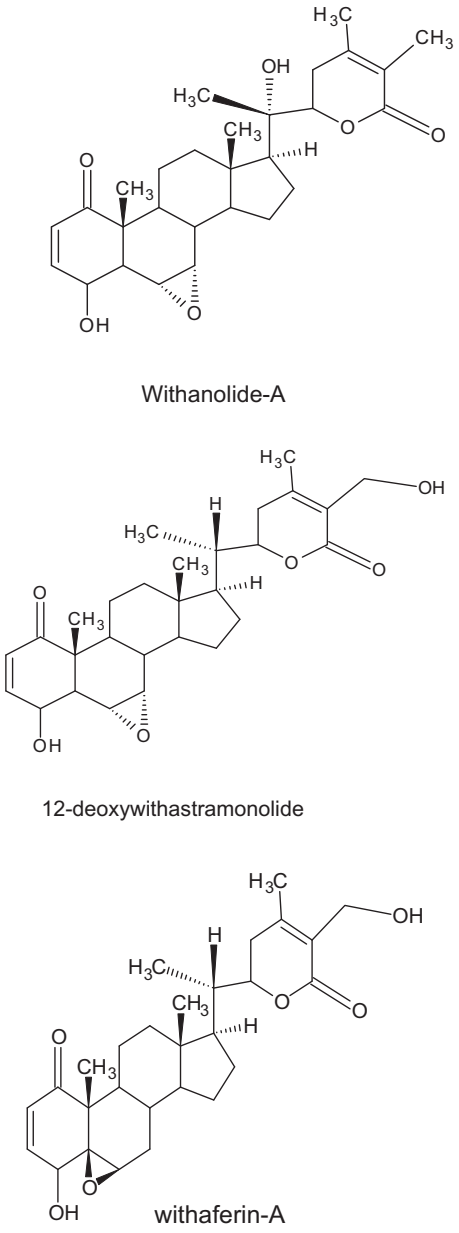

Figure 1 Structure of withanolide A, 12-deoxywithastromonolide and withaferin A.

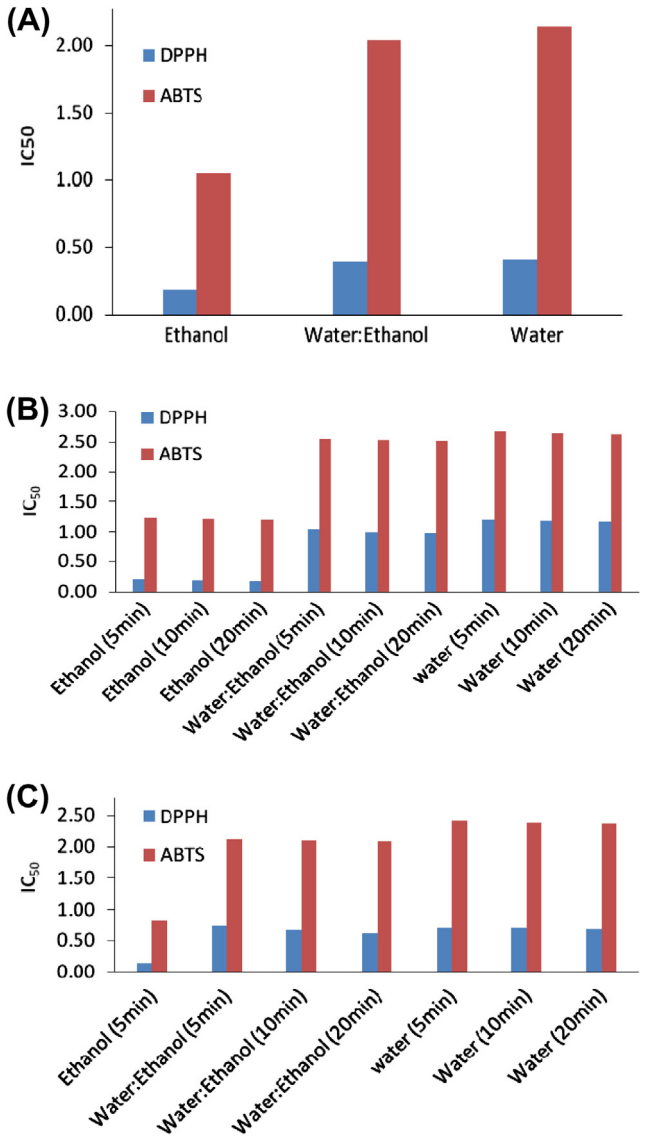

Figure 2 IC_{50} values of DPPH and ABTS assays for free radical scavenging activity of extracts prepared using (A) refluxing (B) UASE (C) MASE.

on the solubility of the compound in the solvent, the mass transfer kinetics of the product and the strength of solute/matrix interaction with corresponding limitations on heat and mass diffusion rate. Ultrasound assisted solvent extraction is a process that uses high intensity, high frequency sound waves and solvents to extract targeted compounds from various matrices. Physical and chemical properties of the materials subjected to ultrasound are altered due to the propagation and interaction of sound waves as they disrupt the plant cell walls, thereby, facilitating release of extractable compounds and enhancing mass transport of solvent from the continuous phase into plant cells. Microwave energy and solvents are used for the extraction of targeted compounds from plant matrices in the case of microwave assisted solvent extraction. Highly localized temperature and pressure can cause selective migration of targeted compounds from the matrices to the surroundings at a more rapid rate. Recoveries are similar or better in both UASE and MASE as compared to conventional extraction. However, reduced extraction time and solvent consumption are the main advantages of UASE and MASE. Although, extraction of bioactive compounds from the plants has been extensively investigated using conventional solvent extraction, the present investigation was undertaken to study the effect of extraction methods on yield and phytochemical qualities of *W. somnifera*.

Extract yield of *W. somnifera* root prepared by refluxing, UASE and MASE methods using water, ethanol and water–ethanol is summarized in Table 1. Extraction yield (mass of extract/mass of dry matter) was used as an indicator of the effects of the extraction conditions. In reflux, water extract yield (9.51%) was maximum followed by water–ethanol and ethanol. For UASE and MASE, three time periods 5, 10

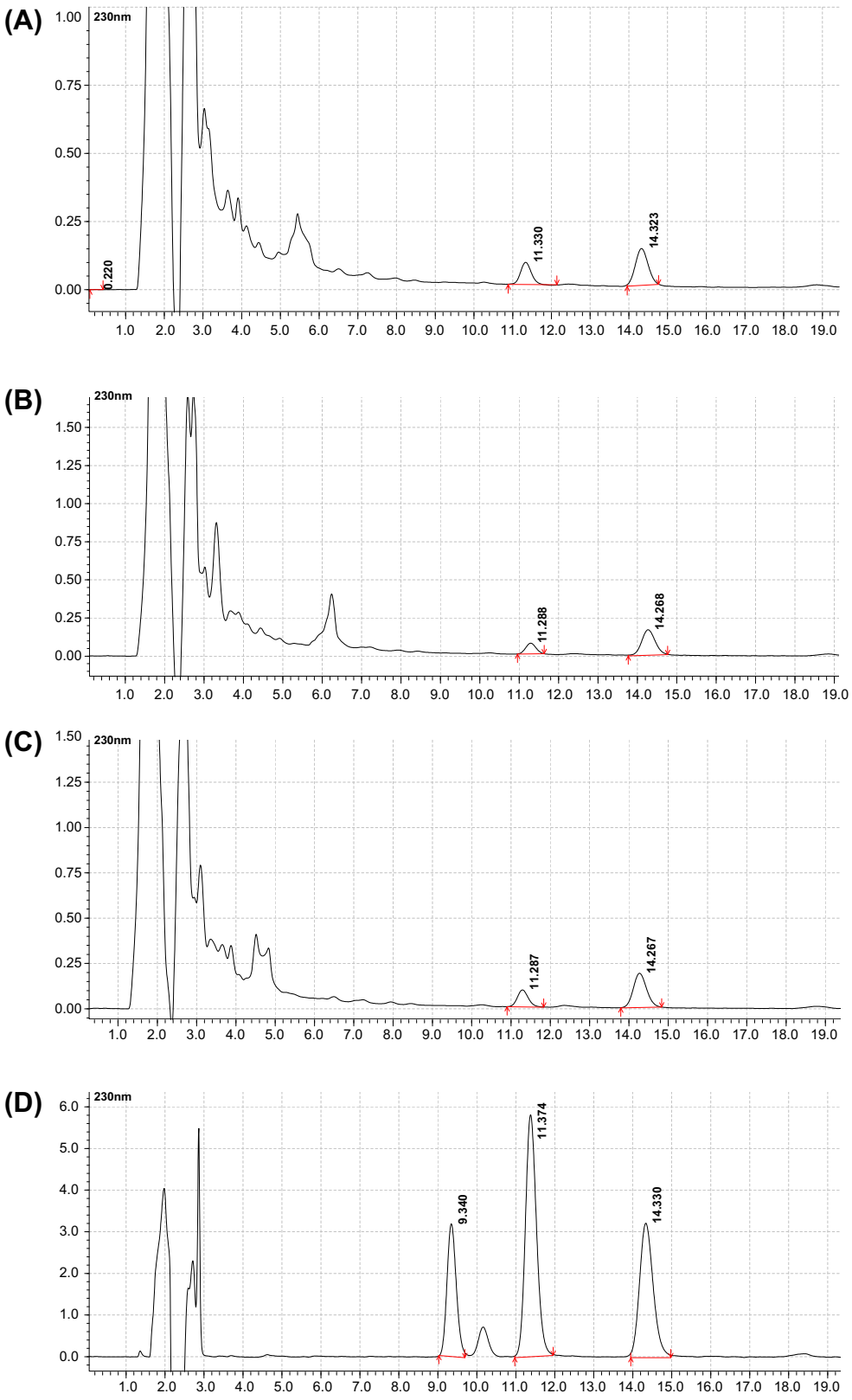

Figure 3 HPLC chromatogram of extracts of *W. somnifera* root prepared using (A) refluxing (B) UASE and (C) MASE extraction (D) standard withaferin A, (R_t = 9.32 min) 12-deoxy withastramonoidie (R_t = 11.46 min), withanolide A (R_t = 14.26 min).

and 20 min were used. For UASE also, the trend was similar as observed in the case of refluxing. But extract yield with ethanol (3.17%, 20 min) was about 3.74 times lower than the maximum extract yield (11.85%) obtained with water at 15 min. Possibly, this could be due to the higher polarity of water and also at elevated extraction temperature as in the case of

UASE and MASE, water has a similar dielectric constant as organic solvents like methanol and acetonitrile. In case of MASE, the extract yield increased with the time of exposure to microwave radiation and water (13.02%, 20 min) and water–ethanol (13.75%, 20 min) were found to be comparable. Maximum yield of UASE and MASE was higher than the maximum extract yield using refluxing. Direct heat generation within the volume with a significant impact on heating kinetics and also on the pressure effects on the cell wall membrane structure resulting into the higher and faster diffusion or partition rate of the solute from the solid matrix into solvent may be the probable reason for the highest yield in MASE (Spigno and De, 2009; Terigar et al. 2011). Also improved extract yield in the case of UASE, may be explained in terms of the cavitational effects caused by high intensity ultrasound (Li et al., 2004).

Total phenol content was maximum in the extract prepared with ethanol (35.93 GAE mg/g) followed by water–ethanol (21.15 GAE mg/g) and water (17.63 GAE mg/g). In the case of UASE and UASE, it increased with the exposure time, however, a similar trend as in the case of the extract prepared using refluxing was observed.

Withanolide A, 12-deoxy withastramonolide and withaferin A (Fig. 1) contents in theextracts were quantified using reverse phase HPLC and the chromatogram of standard mixture has been shown in Fig. 3. Withaferin A, 12-deoxy withastramonolide and Withanolide A were detected at the retention time of 9.32, 11.46 and 14.26 min respectively. However, withaferin A was not detected in the extracts. Total withanolide content (sum of withanolide A and 12-deoxy withastramonolide) of the extract prepared using refluxing varied in the following order: ethanol > water–ethanol > water. For UASE and MASE also, total withanolide content was higher in the ethanol extract as compared to water–ethanol and water extracts.

DPPH and ABTS assays were used for the determination of antioxidant activity of the different extracts prepared using refluxing, UASE and MASE methods. Antioxidant capacities of the extracts were expressed in terms of IC_{50} value of the extracts and low IC_{50} value corresponds to a high antioxidant capacity (Fig. 2). In both DPPH and ABTS assays, ethanol extracts had lowest IC_{50} values and they varied in the following order: ethanol < water–ethanol < water. Further, antiradical power of the extracts ($1/IC_{50}$) was found to be highly correlated in DPPH ($r^2 = 0.73$) and ABTS assays ($r^2 = 0.86$).

4. Conclusion

These results indicate that the ultrasound and microwave assisted solvent extraction methods can be a viable alternative for traditional extraction methods. However, for industrial application purposes, further investigations are required to develop a mathematical model to control and predict the optimization parameters of the extraction process. Green extraction techniques besides improving the yield and quality would be able to save energy and time.

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