

Seeds of Giant Dodder (*Cuscuta reflexa*) as a Function of Extract Procedure and Solvent Nature

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Abstract

Seeds of a renowned medicinal plant, giant dodder (*Cuscuta reflexa*), were assessed to appraise the effect of solvent and extraction technique variation on antioxidants potential. Dodder seed, also called cuscuta seed, has been considered superb tonic in traditional herbal medication for eyes, liver, spleen and kidney. Results show that selected solvent and procedure plays a key role in the composition and activity of extractable material. Three extraction procedures Orbital shaker, Decoction and Ultrasonic assisted extraction and five different solvents n-hexane, ethyl acetate, 100% methanol, 80% methanol and 60% methanol were used to get optimized conditions. Total phenolic and flavonoids content were found maximum in the extracts of aqueous organic system containing 80% methanol in Ultrasonic assisted extraction method but in case of tannins ethyl acetate and Orbital shaker extraction was found more suitable partner. Antioxidant estimation assays showed a little bit variation as DPPH and ABTS exhibited maximum inhibition in 80% methanol and Ultrasonic assisted extraction but 100% methanol was found better for FRAP assay. Decoction results were mostly in between the both Orbital shaker and Ultrasonic assisted extraction. Overall results indicate that coexistence of polar solvents and Ultrasonic assisted extraction gives a better choice for extractability of potent antioxidants from seeds. HPLC analysis confirmed presence of valuable phenolic acids. Pearson's correlation coefficient reveals a significant relationship between extracted components and antioxidant capacity $P < 0.05$ or 0.01 .

Keywords: antioxidants; decoction; orbital shaking; phenolic acids; ultrasonic assisted extraction

Introduction

Plants are gifted with a wide variety of natural antioxidants owning diverse composition, properties, reaction pathways and ultimate goals of action in the body (Naik, 2003). These are considered as prime line of protection against degenerative diseases including cancer, cardiovascular, neurological disorders and oxidative stress dysfunctions (Bolck, 1992; Diplock, 1995; Szóllósi and Varga, 2002). There is a growing attention towards the

extraction of these antioxidants in order to meet the concerns of professionals and consumer to maintain health and nutrition with safety and security (Sun and Ho, 2005; Suhaj, 2006; Zahin *et al.*, 2009). Extraction is the preliminary screening of bioactive moieties from plant materials and immense deal of efforts are being consumed to extract, isolate and characterize these natural resources to explore novel antioxidants. A successful process will lead to separate the maximum concentration of targeted components equipped with highest antioxidant activity (Spigno *et al.*, 2007). Investigative reports of identification

and characterization of natural products from plant matrices indicate astonishing variability in chemical composition of antioxidants and their observed activities (Dorman *et al.*, 2003). Mostly these variations are related to inherent properties of plant such as complicated genetic machinery or caused by environmental interactions (Furbank *et al.*, 2015) but these are also dependent upon technical variation and extraction parameters like sample preparation scheme, extraction methodology, solvent type, ratio of solvent to plant material, interfering compounds, detection capability, temperature, pH and time duration (Tanko *et al.*, 2005; Sultana *et al.*, 2009; Radojkovića *et al.*, 2012). Extraction is the opening move during recovery and purification of bioactive ingredients from plant constituents and crucially control properties of the final outcomes (Azmir *et al.*, 2013; Tan *et al.*, 2014). The qualitative and quantitative analysis of active principle from any plant mostly relies on the choice of appropriate extraction method (Sasidharan *et al.*, 2011). Application of a standardized extraction procedure will lead to maximum separation of therapeutically desired components safely and elimination of inert material with the help of proper solvent (Huic, 2002). Increasing demand to extract bioactive species encourages incessant search for convenient and suitable extraction methods (Azmir *et al.*, 2013). Yield and efficacy of antioxidants tremendously depends on the solvent selection because diverse chemical characteristics and remarkable differences in polarities of these compounds made a solvent either fit or unfit for extraction. Polar solvents mostly aqueous organic mixtures of methanol, ethanol, acetone and ethyl acetate are frequently applied (Peschel *et al.*, 2006). The polarities of the diverse organic solvents momentarily influence the choice of a solvent to extract a specific set of bioactive compounds. The selection of a precise extraction technique mainly depends on the simplicity and convenience of the technique (Musa *et al.*, 2011). Classical extraction methods are often time consuming and less productive as compared to fast and efficient unconventional procedures so there is need to revise these less economical choices with innovative options such as microwave assisted and ultrasonic assisted extraction to increase recovery and preserve bioactivity (Aspé and Fernández, 2011).

Giant dodder (*Cuscuta reflexa*) belonging to *Convolvulaceae* family is a reputable medicinal plant of traditional health care systems. It has been investigated for its wide variety of pharmacological attributes including antioxidant potential (Vijikumar, 2011; Patel *et al.*, 2012). Excellent antioxidants potential of aerial parts of giant dodder has been estimated by different researchers using various assays (Amol *et al.*, 2009; Anjum *et al.*, 2014). In herbal therapies, giant dodder seeds alone or in mix with different combinations are considered a superb tonic for kidney, liver, and spleen and can help the treatment of different maladies specifically alopecia, weakness, impotence, habitual abortion, visual deterioration, nocturnal emission, frequent urination, lower back pain caused by kidney deficiency. This study was performed to speculate the effect of solvent and opted methodology on the antioxidant potential of giant dodder seeds to get optimized conditions for better extraction. Hopefully the

results of this experimentation will help to review a better extraction scheme using either typical or modern method of extraction.

Materials and Methods

Plant material

Plant material was selected based on impressive medicinal background and present attention of researchers towards the exploration of its pharmacological attributes. Seeds of giant dodder were purchased from the local market of Sargodha and further authenticated by a taxonomist of department of Botany, University of Sargodha, Sargodha, Pakistan. Seeds were rinsed with fresh running water and successively deionized water was used to make them free of dust. Visible impurities like stones were removed by hand picking. Shadow dried seeds were ground to fine powder by using electrical stainless-steel grinder. Ready to use sample was stored in air tight glass jar at room temperature.

Chemicals

Methanol (MeOH), n-hexane, acetone, ethyl acetate (EtOAc), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sodium phosphate buffer, ferric chloride, potassium ferricyanide, trichloro acetic acid, ascorbic acid, gallic acid, aluminum chloride, sodium carbonate, ethanol, hydrochloric acid, sodium hydroxide, manganese dioxide, Folin-Ciocalteu reagent, acetic acid, Streptokinase, Muller Hinton agar media and acetonitrile. All the chemicals and reagents used were of analytical grade and purchased from E. Merck or Sigma-Aldrich otherwise specified.

Extraction methodologies

Orbital Shaker Extraction (OSE), Decoction Extraction (DE) and Ultrasonic Assisted Extraction (UAE) were applied for extraction of valuable phytoconstituents. Three groups of extracts were prepared by using three organic solvents and their companionship with water to create different level of polarity.

In shaking 10 g of seeds powder was taken in 100 mL (solid liquid ratio of 1:10 w/v) of solvent. Optima orbital shaker OS-752 was used and three successive extractions of six hours were applied. In decoction, 10 g of sample was boiled in 200 mL of solvents for half an hour then cooled. UAE extraction was performed at medium speed of sound wave that was 17 kHz for 3 h. This extraction procedure was also repeated thrice. Isolations from all methods were filtered through Whatman filter paper No. 1. Filtrates were concentrated by rotary evaporator (Buchi Rotavapor R-200) at 45 °C under vacuum. Electrical water bath was used to get sticky mass free of solvent.

Determination of Total Phenolic Content (TPC)

Total phenolic content was estimated using Folin-Ciocalteu reagent in extract. Gallic acid was used as standard for all determinations. TPC values were articulated in mg g⁻¹ of Gallic Acid Equivalents (GAE). Various concentrations of gallic acid like 10, 20, 30, 40, 50, 100, 150, 200 parts per million (ppm) in methanol were prepared. Then 10 mg of extract (1.0 mg mL⁻¹) was dissolved in methanol. In test tubes 2.0 mL of each sample solution was introduced along

with 2.5 mL of 0.5 N Folin-Ciocalteu reagents and incubated for 5.0 minutes. After adding 2.0 mL of sodium carbonate (7.5%) whole mixture was again incubated for 90 minutes in dark. The absorbance was noted at 765 nm with UV-visible spectrophotometer (Perkin-Elmer Lambda-2 Spectrophotometer). All readings were taken in triplicate (Singleton and Rossi, 1965).

Determination of Total Flavonoids Content (TFC)

Total Flavonoids Content was determined by colorimetric assay. In a 10 mL volumetric flask 1.00 mL of extract was shaken with 4.00 mL of distilled water. Then 0.3 mL of 5% NaNO₂ solution was added and incubated for 5 minutes. After pouring 0.3 mL of AlCl₃ (10%) solution mixture was kept for 6 minutes and 2.00 mL of 1M NaOH was added in each sample solution. Flask was filled up to the mark with distilled water. Absorbance was measured at 510 nm after shaking. Samples were analyzed in triplicate (Zhishen et al., 1999).

Determination of Total Tannins Content (TTC)

Tannins were determined by diluting 10 mg extract in 4.00 mL distilled water. Solution was partitioned in two sets of 2.00 mL each. Then 1.00 mL distilled water and 3.00 mL conc. HCl was mixed to both sets. One of these two was incubated at 100 °C for 30 minutes and to the second 0.5 mL ethanol was added. Absorbance was measured at three different wavelengths (470 nm, 520 nm and 570 nm). Following formula was used to get result (Hosu et al., 2014).

$$\Delta A_{520} = 1.1 \times \Delta A_{470}$$

$$\Delta A_{520} = 1.57 \times \Delta A_{570}$$

$$TTC (g L^{-1}) = 15.7 \times \text{minimum } (\Delta A_{520})$$

Determination of Total Carotenoids Content (TCC)

About 10.00 g of seeds powder was soaked in 100 mL of 80% acetone for a period of three days in refrigerator and filtered through Whatman filter paper No. 1. Filtrate was centrifuged to get clear solution for 15 minutes at 3000 revolutions per minutes (rpm). Supernatant was separated and absorbance was taken at 660 nm, 642 nm and 470 nm. Total carotenoids were estimated by the following formula (Lichtenthaler, 1987).

$$Ca = 12.25A_{660} - 2.79A_{642}$$

$$Cb = 21.50A_{642} - 5.10A_{660}$$

$$Cx+c = 1000A_{470} - 1.82 Ca - 85.02Cb/198$$

Determination of free radical scavenging by DPPH, FRAP and ABTS assays

Free radical scavenging activity of extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured. About 2.00 mL of DPPH solution in methanol (0.1 mM) was mixed in 200 µL of samples and 0.8 mL methanol. After thorough agitation it was incubated for an hour in dark. Control was prepared by mixing 2.00 mL of DPPH in only 1.00 mL of methanol. The absorbance was taken at 517 nm in triplicates using following formula in terms of percentage inhibition of DPPH radical (Akowuah et al., 2005).

$$\text{Percent inhibition of DPPH} = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right] \times 100$$

In case of Ferric Reducing Antioxidant Power assay (FRAP) the mixture of 10 mg of extract and 3.5 mL of

phosphate buffer (0.2 M, pH 6.6) was taken in a test tube and 2.00 mL of potassium ferricyanide (1%) was added. After twenty minutes incubation at normal temperature 2.5 mL (10%) trichloroacetic acid was added. Centrifugation was done at 3000 rpm for 10 minutes and 2.5 mL of supernatant and 2.5 mL of distilled water were mixed. Finally, it was treated with 0.5 mL of 0.1% ferric chloride. Absorbance was measured at 700 nm and results were obtained using ascorbic acid standard curve (Wang et al., 2012).

ABTS cation scavenging activity was determined by using 7.00 mM ABTS reagent and 2.45 mM MnO₂ solutions of 1:1 ratio. Mixture was incubated in dark for 24-48 hours. ABTS solution was diluted with methanol (1:25). Then 2.00 mL of ABTS solution was shaken with 200 µL aqueous methanolic plant extracts (1:10). Control was prepared without extract in similar way and absorbance was measured at 734 nm. Percentage scavenging activity of ABTS cation was calculated by the formula (Proestos et al., 2013):

$$\% \text{ Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100.$$

Identification and Quantification of Phenolic Acids by High Performance Liquid Chromatography (HPLC)

High Pressure Liquid Chromatography was used to quantify phenolic acids in best optimized extract 80% methanolic extract. Concisely 50 mg of all extract from three techniques were dissolved taking 15 mL of distilled water and successively shaken for 5 minutes after adding 24 mL methanol (Merck, HPLC grade). Then 10 mL of 6.00 M HCl (Merck, Analytical grade) was poured and whole reaction mixture was kept in oven at 95 °C for 2 hours. Filtration of samples was carried out using 0.2 micron syringe filter paper and 5 mL of both extracts were injected on a Shim-Pack CLC-ODS (C-18), (25 cm x 4.6 mm, 5.00 µm) for gradient elution. Solvents employed for analysis were A (H₂O: acetic Acid 94:6, pH=2.27), B (acetonitrile 100%). The gradient profile was 0-15 min=45% B, 30-45=100% B with flow rate of 1.0 mL min⁻¹. UV/Visible detector was adjusted at 280 nm for quantification. The temperature of column was set at 25 °C. Concentrations of phenolic acids were determined by means of calibration curve (Seal, 2016).

Biological activities

Optimized extract in 80% methanol was subjected to biological tests.

Antithrombotic activity

Commercially sold lyophilized Streptokinase vial of 15, 00,000 I.U. was taken and 5.00 mL of distilled water was mixed in it. This solution was set aside as a stock for further use. All 80% methanolic extracts were soaked in distilled water for 12 hours. Supernatants were filtered by 0.22 micron syringe filter paper. Whole blood was drawn from a 25 years old healthy human volunteer without a history of anticoagulant therapy. Samples (5 mL) were shifted in pre-weighted labelled and sterilized vials (n=5) and incubated for 45 minutes at 37 °C for proper clotting. Clot in vials was weighted after removing serum carefully. Then 100 µL of extract, Streptokinase (positive control) and distilled water

(negative control) was added in clot. All vials were incubated at 37 °C for 90 minutes. Then fluid was removed and clot was weighted again. Percentage lysis was estimated by the following formula (Prasad *et al.*, 2006):

Percentage clot lysis = weight of clot after lysis / initial weight of clot × 100.

Antibacterial activity

The antimicrobial potential was appraised by disk diffusion method (Barbour *et al.*, 2004). The four selected bacterial strains *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhi* were cultured in Muller Hinton agar medium. One litre of agar solution in distilled water was prepared using 34.00 g of it. Whole parts of experiment assembly including petri plates, agar solution and filter paper disk were sterilized for 30 minutes in autoclave at 121 °C. Agar solution was pour and spread evenly in petri plates instantly to circumvent solidification and for some time to cool. After setting petri plates were inverted and kept at 3 °C by inverting to stop water droplets. These droplets may cause hindrance in microorganism intensification. The 10.00 µL of bacterial suspension was spread onto medium by using sterilized micropipette. Petri plates were spun gently to distribute bacteria evenly. Diluted samples and Ciprofloxacin as standard antibacterial drug were loaded on sterilized discs of filter paper. These discs were put on the agar surface and whole apparatus was incubated for 24 hours at 37 °C. Antibacterial activity was calculated in terms of inhibition zones in mm.

Correlation analysis and data presentation

All analytes were evaluated by antioxidant assays in triplicate to confirm the reproducibility of the results. Statistical interpretations of whole experimental data are expressed as mean ± standard deviation. Liability of the outcomes acquired by antioxidants extracted by all solvents and methods was checked by analysing results. Experimental data was executed graphically via GraphPad Prism version 5.00 for Windows, San Diego California USA. Pearson's correlation coefficient (r) was used to correlate the results of extraction schemes and various applied assays by using SPSS statistics 21 version.

Results and Discussion

Percentage yield

Usually solid/liquid extractions are considered most efficient but it is observed that yield of any applied extraction methodology depends upon several factors like nature of solvents and sample, their quantity and physical parameters (Ayyanar and Subash-Babu, 2012). Detail of yields obtained by different solvents and three different extraction procedures is summarized in Fig. 1. Keen analysis of percentage yield unveils the facts that polar solvents are much better choice than non polar counterparts for extraction of phytochemicals from giant dodder seeds. Yield varies from 3.77% (n-hexane + OSE) to 36.3% (80% methanol + UAE). Maximum yield revealed by 80% methanolic extracts ascribes that addition of water causes intensification of extraction efficiency. It shows that polarity index plays a key role in extraction of constituents from

plants. This trend is compatible with previously reported results (Khan *et al.*, 2012). It is perceived from percentage of different extracts that UAE is an efficient method of extraction as compare to OSE and DE for giant dodder seeds because extracts obtained by UAE exhibited high yield values.

In both procedures OSE and UAE 80% methanol gave highest values 26.38% in OSE and 36.3% in UAE but DE extracted maximum components 26.28% in 60% methanol. These results fortify the perception that polar solvents are superior candidates for extraction of phytoconstituents from giant dodder seeds.

Total Phenolics Content (TPC)

Folin-Ciocalteu reagent assay (FCR) is a widely accepted method for the determination of total phenolic contents as it efficiently correlates total phenolics of a sample with antioxidant potential (Jatunov *et al.*, 2010). The FCR on exposure to polyphenols that are effective reducing metabolites turns to blue. Intensity of the colour is measured by absorbance (Kalpna and Mital, 2011). Quantitative analysis of TPC for all extracts is expressed in mg g⁻¹ of GAE dry weight for each sample in Fig. 2. A range of TPC from 16.43 to 52.59 mg g⁻¹ of GAE was obtained.

These results are quite comparable with previously reported results (Anjum *et al.*, 2014). Maximum phenolic content was found in 80% methanol extract in case of OSE

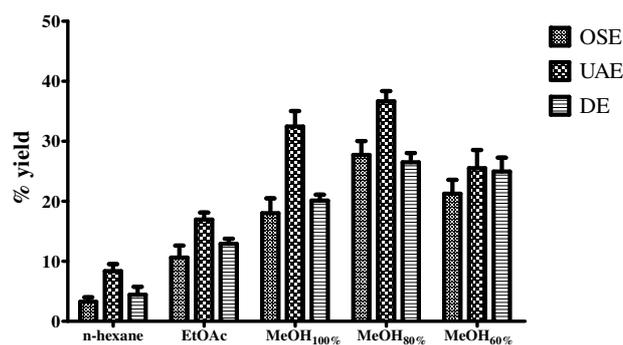


Fig. 1. Percentage yield of seed extracts of giant dodder prepared by three techniques and different solvents. UAE: Ultrasonic assisted extraction, OSE: orbital shaker assisted extraction, DE: Decoction extraction

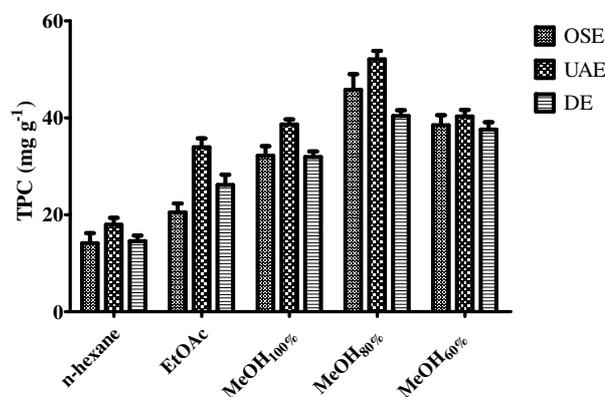


Fig. 2 Total Phenolic Content (mg g⁻¹ GAE) of seed extracts of giant dodder. OSE: Orbital shaker assisted extraction, UAE: Ultrasonic assisted extraction, DE: Decoction extraction

and UAE and minimum in n-hexane in all adopted techniques. In OSE TPC ranges from 16.43 to 40.47 mg g⁻¹ GAE, in DE values are from 16.21 to 39.00 mg g⁻¹ GAE (maximum in 60% methanol) while UAE has a range from 19.44 to 52.59 mg g⁻¹ GAE. It is evident from these results that aqueous methanol extracted more phenolic compounds. It is in accordance with the fact that polar solvents are a better choice for extraction of these compounds (Ghimire et al., 2011). It also indicates that aqueous methanol and UAE is a suitable combination for extraction of phenols from this plant material.

Total Flavonoids Content (TFC)

Flavonoids are non-uniformly distributed in plant kingdom despite their wide occurrence (Gu et al., 2004). Antioxidant potential of these compounds has been proved over the last few years (Yang et al., 2001). Total flavonoids content of giant dodder using various solvents and techniques is given in Fig. 3 and range from 10.37 to 33.86 mg of CE g⁻¹ of DW of sample. In OSE TFC values range from 10.37 to 29.78 mg of CE g⁻¹, in DE flavonoids are present from 11.40 to 28.23 mg of CE g⁻¹ and UAE gave 12.24 to 33.86 mg of CE g⁻¹. According to these results UAE and 80% methanol are better to separate flavonoids.

Total Tannins Content (TTC)

Total tannins content has been described in tabular form in Fig. 4 and shows a range of 1.02 to 6.53 g L⁻¹. Maximum values in OSE and UAE 5.21 and 6.53 g L⁻¹ are obtained in 80% methanol but in case of DE 60% have been found most efficient 5.83 g L⁻¹. On the whole evaluation of all extracts gives a suggestion that UAE technique and polar solvents are better for tannins isolation from plant material.

Total Carotenoids Content (TCC)

Fruits, vegetables and green plants are blessed with unrivalled variety of carotenoids, which decorate them with colours (Olson, 1996). These are anticipated as effective natural defence against several degenerative diseases (Russell, 2006). Giant dodder parasitizing some specific hosts have bright orange stem indicating carotenoids presence (Mukherjee et al., 2008). Total carotenoids content, chlorophyll a and chlorophyll b values have been found 7.26 µg g⁻¹, 2.8 µg g⁻¹ and 3.32 µg g⁻¹ respectively.

Free radical scavenging by DPPH, FRAP and ABTS assays

Medicinal plants have a versatile assemblage of antioxidants along with carotenoids, vitamin C and vitamin E (Sinha et al., 2001; Kumar et al., 2003). Since these antioxidants follow a diverse mechanism of action so their activity must be estimated by different assays. Results of a given assessment method depend on many factors like the nature of solvent and substrate considered, purity level of substrate and kinship of substrate-antioxidant. Therefore, a system having different components can be described more comprehensively by using different assays working in different media and following diverse mechanistic approach (Moure et al., 2006). Estimation of *in vitro* antioxidant potential of giant dodder has been testified in previous experiments (Ali et al., 2008). Results of this study will give a comparative account of solvents and selected techniques.

DPPH is a better contestant for determination of antioxidant activity of various natural materials because of its stability as free radical (Öztürk et al., 2007). The antioxidant potential of any polyphenol is because of its hydrogen/electron donating ability or the free radical's predation (Stoilova et al., 2007). Thus, the purple colour of 2, 2-diphenyl-1-picryl hydrazyl will change to yellow colour of α, α-diphenyl-β-picrylhydrazine (Akowuah et al., 2005). Scavenging ability of stable DPPH radical is considered as a convincing and appropriate assay to evaluate antioxidant capacity of a sample (Suhaj, 2006). DPPH free radical scavenging ability in terms of percentage inhibition for all extracts was measured and given in Table 1. Results of this assay are found in agreement with inferences of experimentation reported by examination of aerial parts of giant dodder (Amol et al., 2009; Anjum et al., 2013). All extracts exhibited free radical scavenging potential but to variable degrees ranging from 18.55% to 89.38% both in OSE. This varying array of results is an indication of large variety of bioactive constituents such as flavonoids, phenolics, tannins and carotenoids in different extracts (Ghimire et al., 2011). It is evident from these results that 80% methanolic extract is most efficient in this regard by showing 89.38%, 86.29% and 88.30% inhibition by OSE, DE and UAE respectively. n-hexane was found at bottom line ranging from 18.55% to 24.18%. Generally, all methanol extracts either aqueous or pure were

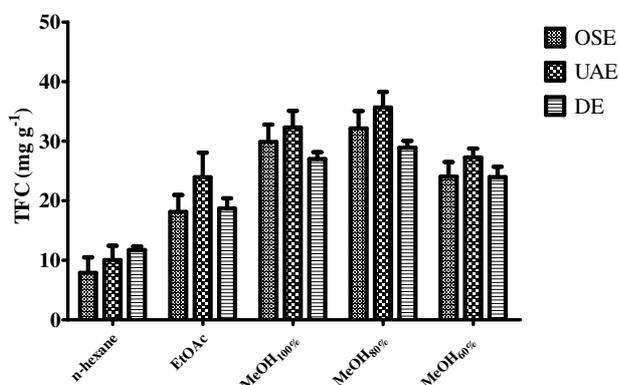


Fig. 3. Total Flavonoids Content (mg CE g⁻¹) of seed extracts of giant dodder. OSE: Orbital shaker assisted extraction, UAE: Ultrasonic assisted extraction, DE: Decoction extraction

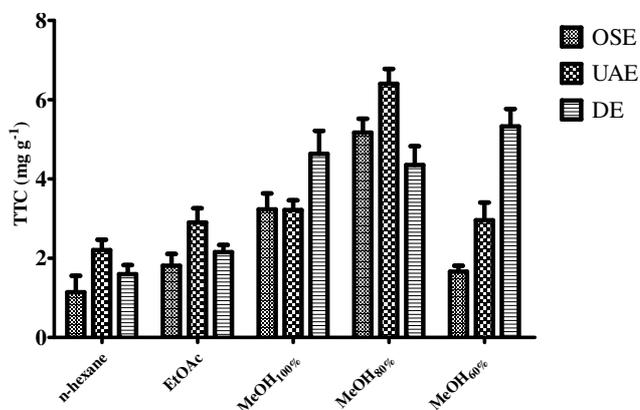


Fig. 4. Total Tannins Content (g L⁻¹) in of seed extracts of giant dodder. OSE: Orbital shaker assisted extraction, UAE: Ultrasonic assisted extraction, DE: Decoction extraction

Table 1. Free radical scavenging power of antioxidants of giant dodder seed extracts obtained by different extraction scheme by DPPH, ABTS and FRAP assay

Extracts	DPPH assay			ABTS assay			FRAP assay		
	OSE	DE	UAE	OSE	DE	UAE	OSE	DE	UAE
n-hex	18.55±1.09	22.17±0.67	24.18 ±0.93	8.45 ±0.78	17.42±1.25	43.62±1.01	12.66±0.38	15.17±0.89	16.25±0.41
EtOAc	21.41±0.71	37.65±0.96	47.57 ±0.89	11.70±1.13	40.14±0.87	64.21±0.88	15.81±0.70	14.48±0.76	18.80±0.96
Met _{100%}	85.67±1.15	75.12±0.43	86.29 ±1.12	25.28±0.88	52.37±0.54	73.93±1.18	22.69±0.49	18.34±0.98	24.31±0.93
Met _{80%}	89.38±0.84	86.29±0.45	88.30±0.85	45.44±0.91	67.16±0.78	83.77±0.63	25.89±0.57	15.25±0.65	29.67±0.33
Met _{60%}	62.81±0.74	61.75±1.12	65.65±1.00	17.50±0.81	38.27±0.67	42.56±0.78	17.47±0.58	16.68±0.67	19.47±0.48

OSE: Orbital shaker assisted extraction, DE: Decoction extraction, UAE: Ultrasonic assisted extraction, n-Hex: n-hexane, EtOAc: Ethyl acetate, Met_{100%}: 100% methanol, Met_{80%}: 80% Methanol, Met_{60%}: 60% Methanol. *All the values in table are average of three values obtained after the analysis of sample in triplicate (n=1×3) and represented as (mean ± SD).

comparatively more potent. Results of methanolic extract are nearly same as previously reported (Amol et al., 2009).

Reducing power of a component serves as an authenticated reflection of antioxidant ability by donating electrons to fix the intermediates species of lipid peroxidation (Gülçin et al., 2008; Chanda and Nagani, 2010). It is an appropriate, facile and reproducible procedure that was initially developed to appraise the plasma antioxidant ability but now widely accepted method for antioxidant assessment of various biological systems including plant extracts (Ordonez et al., 2006). Yellow coloration changed to different shades of blue/green depending upon reducing ability of compound by converting Fe³⁺ into Fe²⁺ complex. Resulting Pearl's Prussian blue colour is estimated at 700 nm to give

concentration of Fe³⁺ ion (Jayanthi and Lalitha, 2011). All results of FRAP assay are summarized in Table 1. Various extracts showed a range from 12.66 mg g⁻¹ to 29.67 mg g⁻¹ of AAE. UAE using 80% methanol was found most effective (29.67 mg g⁻¹). Over all comparison indicate polar solvents and UAE most suitable for FRAP assay except DE which shows 100% methanol as better solvent.

ABTS^{•+} radical assay is also a widely accepted spectrophotometric approach for approximation of the antioxidant ability of vegetables, food material and beverages (Cerretani et al., 2006). Blue green ABTS radical cation is simply applicable, remarkably sensitive and quickly analyses antioxidant powers of several samples at a time. ABTS cation scavenging ability is enlisted in Table 1 in terms of percentage. It can be quantified after a quick review

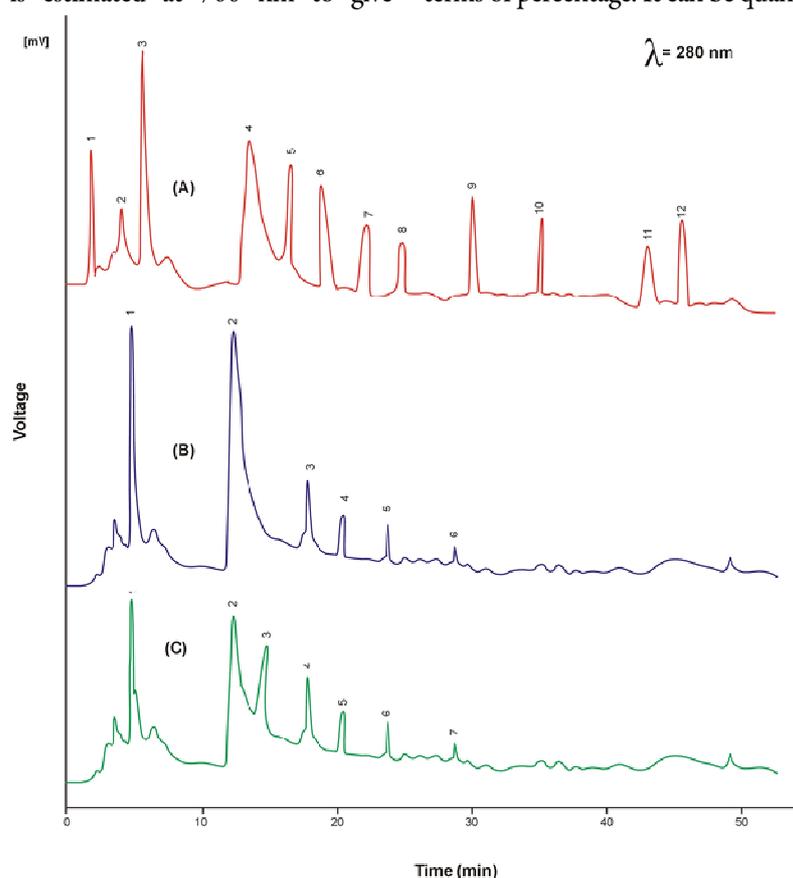


Fig. 5. Identification and quantification of phenolic acids in (ppm) by HPLC. (A) HPLC chromatogram of phenolic acid standards. Standard phenolic acid used are denoted by numbers on peak as follow: 1. Ascorbic acid, 2. Gallic acid, 3. Caffeic acid, 4. Syringic acid, 5. p-coumaric acid, 6. Sinapic acid, 7. Ferulic acid, 8. Cinamic acid, 9. Myricetin, 10. Quercetin, 11. Apigenin, 12. Kaempferol. (B) & (C) HPLC chromatogram for identification and quantification of phenolic acids in methanolic extract of dodder seeds obtained by Orbital shaker and Ultrasonic assisted extraction schemes

of these results that seed extracts of giant dodder are wonderful scavenger of ABTS cation. All extracts exhibited considerable potential towards ABTS. Results are in the range of 8.45 to 87.93% and overall comparison proves that 80% menthol gave best results. UAE has been found much better than OSE and DE for abstraction of active compounds against ABTS in this assay.

Identification and Quantification of Phenolic acids by HPLC

Five different solvent and three techniques were used for total phenolic content estimation and it was found that 80% methanol was most efficient solvent and UAE a better extraction procedure. In the light of previous results, further analysis of 80% methanolic extracts was carried out for phenolic acid by HPLC with UV/DVD that was based on retention time, compared with standard curves at 280 nm. Twelve authentic standard polyphenolics compounds (apigenin, ascorbic acid, caffeic acid, cinamic acid, ferulic acid, gallic acid, kaempferol, myricetin, syringic acid, p-coumaric acid, quercetin, and sinapic acid) were used. The phenolic acid appraised by HPLC in OSE treated methanolic extract of giant dodder are gallic acid 9.22 ppm, ferulic acid 3.96 ppm, cinnamic acid 1.57 ppm, syringic acid 1.28 ppm, m-coumeric acid 1.23 ppm, quercetin 0.97 ppm, caffeic acid 3.02 ppm, sinapic acid 0.53 ppm. UAE has separated quercetin 0.67 ppm, caffeic acid 49.49 ppm, p-coumaric acid 4.42 ppm, ferulic acid 5.18 ppm, cinnamic acid 2.25 ppm, sinapic acid 0.71 ppm. The common phenolic acid in both samples are quercetin, cinnamic acid, ferulic acid, caffeic acid and sinapic acid. Results for HPLC are presented in Fig. 5. These phenolic acids have been previously reported in various parts of giant dodder e.g quercetin, p-coumaric and caffeic acid (Löffler et al., 1995; Vijikumar, 2011). These results suggest that antioxidant capacity of dodder seeds may credit to its distinctive pharmacological properties like anticancer, antimicrobial, anti-inflammatory and so on. These are helpful to some degree for plants itself because distinctive procedures like plant cell signalling, fertilization and plant immunity.

Antithrombotic activity

Any mismanagement in haemostasis may cause a thrombus (blood clot) leading to vascular blockage and ultimately many serious diseases. Plasminogen activator, urokinase and streptokinase are most widely used thrombolytic agents against these diseases but these are not

risk free options. To avoid shortcomings associated with available drugs efforts are underway to explore new horizons of better ones. Extensive efforts have focused to discover and upgrad natural products separated from plant sources that have been consider to have antiplatelet, anticoagulant, antithrombotic and thrombolytic activity in traditional system (Prasad et al., 2007). Findings of antithrombic test show that streptokinase as positive control was found effective antithrombotic agent up to 75.88% against clot where water as negative control was negligibly active like 4.29%. Optimized 80% methanolic extract from all three methods exhibited significant antithrombotic activity as compared to standard. Results are described in Table 2.

Antibacterial activity

Growing bacterial resistance against presently available synthetic antibiotics has forced the scientists to search for novel antibacterial agents. Different antibiotics in practice inhibit the diverse population of pathogens (Davies and Davies, 2010). Unselective use of commercially available drugs is becoming the major cause of multiple drug resistance in pathogenic microorganisms. These circumstances are favouring the exploration of new antimicrobial agents of variable origins as novel chemotherapeutic agents. Manufacturing cost for these synthetic drugs is very high and they may lead to adverse effects as compared to herbal antimicrobial drugs (Priya et al., 2011). This entire scenario has made medicinal plants as a safe and cheap source of such type of medicines. Antibacterial activities of three 80% methanol extracts of giant dodder were tested against four bacterial strains. Zones of inhibition for these extracts and Ciprofloxacin used as standard were measured in mm. Results of activity shows a significant value as compared to standard. Highest value of zone in mm was shown by extract prepared by UAE and DE against *S. aureus* 18.33 mm and 18.67 mm and *B. subtilis* 18.00 mm and 16.33 mm. These results are parallel as reported by Pal and his co-workers during testing of antibacterial activity of giant dodder stem (Pal et al., 2006).

Correlation analysis

Generally, method variation to determine the antioxidant potential gives a variable range of results because each of the followed protocol is based on a different mechanism of action. Additionally response of phenolic to an oxidizing agent estimated by various procedures is structure dependent (Zhao et al., 2008). So, correlating

Table 2. Antithrombotic activity (percent) of seeds extracts of giant dodder and standard

Material	OSE	DE	UAE
Streptokinase	75.88±0.29	-	-
80% methanol	26.55±0.51	32.43±0.74	29.91±0.59
Water	04.29±0.44	-	-

Table 3. Antibacterial activity of seeds extract of giant dodder inhibition zones are expressed in mm

Solvents	Technique	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>S. typhi</i>
80% Methanol	OSE	7.67±2.51	11.00±1.73	10.67±1.55	5.33±2.51
	DE	10.67±1.55	18.67±2.08	16.33±2.08	11.00±1.73
	UAE	5.33±1.52	18.33±1.52	18.00±2.00	5.00±1.00
Standard		29.67±0.34	28±2.74	30.44±269	28±2.18

Table 4. Pearson correlation coefficient (r) of different solvents in OSE scheme, total phenolics, total flavonoids and total tannins with different antioxidant indicators

Trait	Yield	TPC	TFC	Tannin	DPPH	ABTS	FRAP
TPC	0.989**						
TFC	0.936*	0.880*					
Tannin	0.828	0.747	0.867				
DPPH	0.880*	0.826	0.977**	0.816			
ABTS	0.881*	0.801	0.964**	0.961**	0.932*		
FRAP	0.904*	0.849	0.894*	0.979**	0.853	0.951*	

TPC= Total Phenolic Contents, TFC= Total Flavonoid Contents, DPPH= %age scavenging ability, ABTS= %age scavenging ability values of extract, FRAP=mg/g of AAE *,**= significant at P < 0.05 or 0.01, respectively.

Table 5. Pearson correlation coefficient (r) of different solvents in DE scheme, total phenolics, total flavonoids and total tannins with different antioxidant indicators

Trait	Yield	TPC	TFC	Tannin	DPPH	ABTS	FRAP
TPC	0.977**						
TFC	0.894*	0.787					
Tannin	0.892*	0.882*	0.781				
DPPH	0.880*	0.760	0.991**	0.761			
ABTS	0.408	0.334	0.508	0.735	0.503		
FRAP	0.752	0.625	0.934*	0.513	0.925*	0.232	

TPC = Total Phenolic Contents, TFC = Total Flavonoid Contents, DPPH = %age scavenging ability, ABTS = %age scavenging ability values of extract, FRAP =mg/g of AAE *,**= significant at P < 0.05 or 0.01, respectively.

Table 6. Pearson correlation coefficient (r) of different solvents in UAE extraction scheme, total phenolics, total flavonoids with different antioxidant indicators

Trait	Yield	TPC	TFC	Tannin	DPPH	ABTS	FRAP
TPC	0.960**						
TFC	0.996**	0.964*					
Tannin	0.804	0.848	0.776				
DPPH	0.981**	0.900*	0.983**	0.688			
ABTS	0.933*	0.873	0.907*	0.912*	0.889*		
FRAP	0.779	0.676	0.723	0.791	0.736	0.899*	

TPC = Total Phenolic Contents, TFC = Total Flavonoid Contents, DPPH = %age scavenging ability, ABTS = %age scavenging ability, FRAP =mg g⁻¹ of AAE. **,*= significant at P < 0.05 or 0.01, respectively.

various results may give valuable information. Analysis of Pearson correlation results of OSE shown in Table 4 reveals that yield showed a strong positive correlation with TPC, TFC, DPPH, FRAP and ABTS with P < 0.01 or 0.05 while TTC have a poor correlation P > 0.05. Significantly high positive correlation between yield and TPC reinforce the fact that due to hydrophilic nature phenolic are the most abundant extractable secondary metabolites (Thaipong *et al.*, 2006). TPC correlation with DPPH assay specifies the fact that phenolic components are generally responsible for antioxidants potential in DPPH method (Clarke *et al.*, 2013). Analysis of correlation values of DE and UAE given in Table 5 and 6 respectively also gives parallel relations with slight differences. All these high correlation coefficient gives an indication that increase in yield extract more constituents and in turns move effective population against damaging oxidizing threats.

Conclusions

Overall, results of all applied test signpost that giant dodder has wide array of potent antioxidants whose concentration is notably affected by nature of solvents and extraction methodology. Two sets of five different solvents were investigated but there was some inconsistency in results. UAE and aqueous organic combination of solvents was found more effective for total phenolic and flavonoids

separation as well DPPH and ABTS assays but OSE and ethyl acetate was found suitable for tannins and FRAP assay. Such type of variations regarding antioxidant attributes of giant dodder forced to make a concept that successful assessment of antioxidant activity need multiple type of analysis assays. The correlation of results specifies that phenolic, flavonoids and tannins act as major contributor of antioxidant capacity of giant dodder seeds. The growing interest on plant derived phenolics is supporting the rapid research for versatile, economical, easy and green contemporary extraction technologies to triumph over the technical limitations of conventional schemes. Therefore, UAE has appeared as a promising method to combat the vital standard of perfect extraction.

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