Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(12)60314-1

In vitro assessment of antioxidant and antibacterial properties of *Terminalia chebula* Retz. leaves

Kathirvel A , Sujatha V *

Department of Chemistry, Periyar University, Salem-636 011, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised from 5 July 2012
Accepted 17 August 2012
Available online 28 August 2012

Keywords:
Terminalia chebula Retz.
In vitro
Antioxidant
Antibacterial activity
IC_{50}
EC_{50}

ABSTRACT

Objective: To study the antioxidant activity, biochemical constituents and antibacterial property of *Terminalia chebula* Retz. (Combretaceace) (*T. chebula*) leaves. **Methods:** The present investigation comprises, estimation of total phenol, flavonoid, flavonol, tannin, ascorbic acid, protein, carbohydrate contents, *in vitro* antioxidant assays such as DPPH-radical scavenging activity, reducing power activity and antibacterial activity of *T. chebula* leaves. **Results:** Increasing order obtained for phenolic, tannin, flavonoid and flavonol content in various solvent extracts were acetone > ethylacetate > methanol > water > chloroform > petroleum ether. Parameters tested in different concentrations of crude extracts showed an excellent potential of which acetone and ethyl acetate revealed good IC_{50} values. About 0.13 mg against the standard α-tocopherol (0.197 mg) and ascorbic acid (0.18 mg) was obtained as IC_{50} value for the scavenging activity in acetone extract. EC_{50} value for reducing power was 0.0375 mg in acetone extract against the standards like α- tocopherol (0.197 mg) and ascorbic acid (0.18 mg). Acetone extract has extended its best antibacterial activity against majority of the bacteria tested, especially *Shigella bodie* which responded only for acetone. **Conclusions:** With such stronger phytochemical properties, *T. chebula* leaves can be utilized as an effective and safe source of functional food material such as natural antioxidants.

1. Introduction

Ethanopharmacological surveys conducted among the herbal practitioners of traditional medicine, related a large number of indigenous plants, used as a source of their herbal therapies [1]. They are being used along with modern medicine in many countries and this combination plays an important role in health care. The global documentation of 85 000 plants in medicinal use signifies the interest of scientists and medical professionals for their remedies as well as recognition for true benefits. A single plant contains a large number of bioactive compounds, indicating their potential as a source of new drugs. In recent years, plant-derived antioxidants have raised considerable interest among food scientists, manufactures, and consumers. Many spices and culinary herbs are common sources of phenolic compounds which have been reported to show superior antioxidant capacity to fruits, cereals, and nuts[2]. Correlation studies have demonstrated a link between antioxidant activities in plants and their phenolic content, underlining the significant contribution which phenolics can make to antioxidant activities [3]. Hence, it is common practice to measure both phenolic content and antioxidant activities when investigating the antioxidant potential of plants as various studies have shown that plants rich in phenolics are also potent antioxidants [4, 5]. It is believed that the antioxidant properties of phenolics are a result of their ability to act as reducing agents, hydrogen donors and free radical quenchers and phenolics may act as metal chelators which prevent the catalytic function of metal in the process of initiating radicals. Antioxidant compounds scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus reduce the level of oxidative stress and slow or prevent the development of complications associated with oxidative stress related diseases [6]. Many synthetic antioxidants have shown toxic and mutagenic effects, which have shifted attention towards naturally occurring antioxidants [7]. Hence, it is fair to identify and assay the active principles present in those plant extracts, which are necessary to ensure their consistency in composition

*Corresponding author: Dr. V. Sujatha, Department of Chemistry, Periyar University Salem, Tamil Nadu, India
Tel: +91-9444886804
Fax: +91427-2345124
E-mail: chemsujatha@gmail.com
Foundation Project: The study was financially supported by URF (University research fellow, F.No:PU/R/R3/URF/2009 dated 03.12. 2009) rendered by Periyar University, Salem, Tamil Nadu, India.

by employing relevant modern methods. From this point of view, efforts have been made to explore few *in vitro* activities from *Terminalia chebula*, in favour of antioxidant properties to study their inhibitory reactions.

Numerous other works have also been conducted in the parts of *T. chebula* in several aspects especially fruits. Either the dry or fresh their fruits has been reported to have strong antioxidant capacity and high content of phenolic compounds [8-10]. The compounds present in them were shown to have anti-cancer, antimicrobial, anti-inflammatory, antimutagenic, antifungal, antiviral, antidiabetic and antianaphylactic activities [11]. Also, it is a strong antioxidant, which might prove useful for treating neurodegenerative disorders by inhibiting the production of reactive oxygen species (ROS)[12]. Fruits are valuable in the prevention and treatment of several diseases of the mouth such as dental caries, spongy and bleeding gums, gingivitis and stomatitis[13]. This plant has been extensively used in Ayurveda and Siddha for constipation, dyspnea, dyspepsia, hemorrhoids, candidiasis, parasites, malabsorption syndrome, hepatomegaly, vesicular and renal calculi, urinary discharges, tumors, skin diseases, leprosy, intermittent fever, rheumatism, arthritis, gout, neuropathy, paralysis, memory loss, epilepsy, depression, diabetes, cardiovascular diseases, anorexia and wounds[14, 15]. Chemical constituents of *Terminalia* species have been identified as tannins, sterols, flavonoids, aminoacids, fructose, resin and fixed oil. Manikandan and Rijula (2008) identified allelochemicals in leaves and bark, which were found to control the weed growth in agroecosystem [16]. The fruit extracts posses antiviral activity against Herpes Simplex Virus type-1 (HSV-1), Human Immunodeficiency Virus-1 (HIV), cytomegalovirus [17] and antibacterial potential against clinically important CLSI (Clinical and Laboratory Standards Institute) reference bacterial strains.

In the present communication, assessment of various contents of phytoconstituents present in *T. chebula* leaves in different solvent fractions accompanied by biochemical activities such as free radical scavenging activity, reducing power and antibacterial activities were carried out.

2. Materials and methods

2.1. Chemicals

Folin-ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, (±)-catechin, rutin, α-tocopherol, ascorbic acid, bovine serum albumin, glucose, tannic acid, solvents and other reagents used were of analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant collection and extraction

Healthy *T. chebula* leaves were collected from Vellimalai, Thumbal, Salem district, Tamil Nadu State, India. The plant materials were identified and confirmed by Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India. The voucher specimen number (BSI / SRC/5/23/2011-12/Tech.32).

The plant materials were washed with tap water, prior to distilled water, shade dried and powdered. The powdered plant materials were subjected to successive extraction with petroleum ether, chloroform, ethylacetate, acetone, methanol and water using soxhlet extractor. The extracts were dried in vacuum pump (40 ℃). The dried crude extracts were stored in freezer (0 ℃) for future use.

2.3. Phytochemical screening

The preliminary phytochemical screening tests were carried out to identify the useful constituents by standard methods [18].

2.4. Determination of total phenolic contents

The total phenolics in the extracts were estimated by spectrophotometric assay [19]. One mL of sample (concentration 1 mg/mL) was mixed with 1 mL of Folin-Ciocalteu's phenol reagent. After 3 minutes, 1 mL of saturated sodium carbonate solution was added to the mixture and made upto 10 mL with distilled water. The reaction was kept in the dark for 90 minutes, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve (20-100 μg/mL, $Y=0.025x+0.0378$, $R^2= 0.997$) and the results were expressed as μg of gallic acid equivalents/mg of extract (GAEs).

2.5. Determination of total flavonoid contents

Flavonoid contents in the extracts were determined by spectrophotometric method [19]. The (250 μL) extract (concentration 1 mg/mL) was mixed with 1.25 mL of distilled water and 75 μL of a 5% $NaNO_2$ solution. After 5 minutes, 150 μL of 10% $AlCl_3$ solution was added. After 6 minutes, 500 μL of 1 M NaOH and 275 μL of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (±)-Catechin was used to calculate the standard curve (20-160 μg/mL, $Y=0.0006x+0.0603$, $R^2=0.9997$) and the results were expressed as μg of (±)-catechin equivalents (CEs) per mg of extract.

2.6. Estimation of total flavonol contents

One mL of leaf extract (concentration 1 mg/mL) was mixed with 1 mL aluminium trichloride (5 mg/mL) and 3 mL sodium acetate (25 mg/mL). The absorbance read at 440 nm was read after 2.5 h. The absorption of standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions [20]. All determinations were carried out in duplicates. The amount of flavonols in plant extracts in rutin equivalents (RE) were calculated by the following formula. $X = (A.m_0)/(A_0.m)$, where X is the flavonol content, mg/mg plant extract in RE, A is the absorption of plant extract solution, A_0 is the absorption of standard rutin solution, m is the weight of plant extract (mg), m_0 is the weight of rutin in the solution (mg).

2.7. Estimation of tannin content

Tannin content of the extracts was measured by Folin-Denis method [21]. The various extracts (50 μL) were made upto 7.5 mL by adding double distilled water. Then 0.5 mL Folin- Denis reagent and 1mL of Na_2CO_3 were mixed with it. Again volume was made upto 10 mL by double distilled water. Absorption was recorded at 700 nm. Tannic acid was used to calculate the standard curve (20-120 μg / mL, Y=0.069x+0.0091, R^2=0.9985) and the results were expressed as μg of tannic acid equivalents (TAE) per mg of extract.

2.8. DPPH radical scavenging activity

Various concentrations of *T. chebula* leaf extracts (0.3 mL) were mixed with 2.7 mL of methanol solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and allowed to stand for 60 minutes in the dark. The reduction of the DPPH radical was determined by reading the absorbance at 517 nm [22]. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation:

% RSA= $[(A_{DPPH}-A_S)/A_{DPPH}]$ x100, where A_S is the absorbance of the solution when the sample extract is added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity (IC_{50}) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid and α -tocopherol were used as standards.

2.9. Reducing power

The reducing power of *T. chebula* leaf extracts was determined [21]. Various concentration of different solvent extract (1 mL), phosphate buffer (1 mL, 0.2M, pH=6.6) and potassium ferricyanide (1 mL, 10 mg/mL) were mixed together and incubated at 50℃ for 20 minutes. Trichloroacetic acid (1 mL, 100 mg/mL) was added to mixture and centrifuged at 8 000 rpm for 5 minutes. The supernatant (1 mL) was mixed with distilled water (1 mL) and ferric chloride (0.1 mL, 1 mg/mL) and then the absorbance was measured at 700 nm.

2.10. Estimation of carbohydrate content

Total carbohydrate contents were estimated by Anthrone method [23]. Glucose was used to calculate the standard curve (20-120 μg/mL, Y=20x, R^2=1) and the results were expressed as μg of glucose equivalents per mg of extract.

2.11. Estimation of protein content

Total proteins were estimated by Lowry's method [24]. Bovine serum albumin was used to calculate the standard curve (20-160 μg / mL, Y=0.0004x+0.032, R^2=0.9569) and the results were expressed as μg of bovine serum albumin equivalents per mg of extract.

2.12. Estimation of ascorbic acid content

One mg of various extract was treated with 4.0 mL of 10% trichloroacetic acid and centrifuged for 20 minutes at 3 500 rpm and 0.5 mL of supernatant was then, mixed with 0.1 mL DTC reagent (2, 4-Dinitrophenylhydrazine-thiourea-copper sulphate reagent). The tubes were incubated at 37 ℃ for 3 hours. Ice cold 65% H_2SO_4 (0.75 mL) was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. The colour developed was read at 520 nm [25]. Ascorbic acid was used to calculate the standard curve (20-160 μg/mL, Y=0.0043x+0.03682, R^2=0.9652) and the results were expressed as μg of ascorbic acid equivalents per mg of extract.

2.13. Antibacterial activity

Four gram-positive bacterial strains *Bacillus subtillis* (*B.subtillis*), *Enterococcus faedalis* (*E. Faedalis*), *Staphylococcus aureus* (*S. aureus*), Corynebacterium and three gram-negative bacterial strains *Salmonella typhi* (*S. typhi*) , *Klebsiella pneumonia* (*K. pneumonia*), *Shigella boydii* (*S.boydii*) were used. All the bacterial strains were obtained from clinical laboratories, Salem District, Tamil Nadu. The test organisms were prepared by inoculating a loopfull of culture in a 5ml of nutrient broth and incubated (37 ℃) for 14 hours.

The bacterial activities of the various extracts were evaluated by means of the agar well diffusion assay. The assay was carried out according to the method [26]. Approximately 25 mL of Mueller Hinton Agar (MHA) (HiMedia) were poured into sterile petri dish and allowed to solidify. About 100 μL of bacterial inoculums were poured then swabbed on the MHA media by using sterile cotton swab. In each of these plates four wells (5 mm diameter) were punched into the agar by using sterile cork borer. Then 50 μL of each extract (50 mg/mL) was separately added into wells and allowed to diffuse at room temperature. Equal volume of DMSO was served as negative control and standard antibiotic (Streptomycin) used as positive control. The plates were incubated for 24 h at 37 ℃ and the diameter (in mm) of clear zone of growth inhibition was recorded.

2.14. Statistical analysis

The results are expressed as mean values and standard error (SE) or standard deviation (SD), n=3. Data were analysed using one way analysis of variance (ANOVA) followed by Turkey's multiple comparison post hoc test using SPSS software 16.0 versions. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Qualitative phytochemical screening

The data of qualitative determination of secondary metabolites is presented in Table 1 for all solvent extracts. In particular, many constituents have shown their copious presence in acetone extract. A slight to copious presence has been observed in all the extracts for all the constituents

Table 1
Phytochemical screening of *T.chebula* leaves extracts using various solvents.

Phytochemical constituents	Tests	Various solvent extracts					
		PE	CH	EA	AC	ME	WA
Alkaloids	Mayers test	+	++	++	+++	+++	+++
	Wagners test	−	++	+	+++	++	++
	Hagers test	−	++	++	++	++	++
Phenolics / Tannins	$FeCl_3$ test	+	+++	+++	+++	+++	+++
	Lead acetate test	+	++	+++	+++	+++	+++
	$K_2Cr_2O_7$ test	−	++	++	+++	+++	++
Flavonoids	Shinoda test	+	+++	+++	+++	+++	+++
Proteins / Amino acids	Ninhydrin test	+	+	+++	+++	++	++
	Biuret test	+	+	++	+++	++	+++
Carbohydrates	Molisch's test	+	++	++	+++	++	+++
	Fehling's test	+	++	++	+++	+++	+++
	Barfoed's test	−	++	++	+++	++	+++
Fats / Oils	Sudan IV test	+++	+	−	−	−	−
Steroids	Salkowski test	−	+	+	+	+	++
	Libermann's test	−	+	+	+	+	++
Saponins	Foam test	+++	+	−	−	−	−
Terpenoids	Knollar's test	−	+	++	++	++	++
Cardiac glycoside	Keller-Killiani test	−	++	+	+	++	++
Anthraquinones	Borntrager's test	−	−	++	++	+	+

PE=Petroleum ether extract; CH=Chloroform extract; EA=Ethylacetate extract; AC=Acetone extract; ME=Methanol extract; WA=Water extract. +++ = Copiously present, ++ = Moderately present, + = Slightly present, − = Absent.

Table 2
Estimation of various content of *T. chebula* leaves using various solvents.

Phytochemical constituents (μg/mg extract)	Petroleum ether	Chloroform	Ethylacetate	Acetone	Methanol	Water
Total phenolic content*	22.63 ± 2.10^{a}	45.40 ± 1.05^{b}	$102.58 \pm 0.88^{d,e}$	105.84 ± 1.08^{e}	97.62 ± 0^{d}	85.28 ± 4.75^{c}
Total flavonoid content*	17.43 ± 2.45^{a}	37.74 ± 3.37^{b}	96.50 ± 2.14^{e}	104.01 ± 3.25^{f}	82.45 ± 1.47^{d}	49.46 ± 2.21^{c}
Total flavonol content*	20.88 ± 1.39^{a}	87.01 ± 6.13^{b}	$97.19 \pm 0.80^{c,d}$	100.13 ± 0.46^{d}	$90.23 \pm 1.67^{b,c}$	$89.96 \pm 2.13^{b,c}$
Total Tannin content*	11.56 ± 2.10^{a}	39.93 ± 1.55^{b}	104.31 ± 1.84^{e}	109.78 ± 1.84^{f}	81.17 ± 1.78^{d}	72.95 ± 1.58^{c}
Total ascorbic acid content*	20.50 ± 0.57^{a}	47.17 ± 0.43^{c}	54.13 ± 0.28^{e}	60.92 ± 0.85^{f}	49.22 ± 0.57^{d}	39.24 ± 0.14^{b}
Total protein content*	22.13 ± 5.06^{a}	30.13 ± 2.13^{a}	64.84 ± 6.13^{c}	80.63 ± 5.46^{d}	62.01 ± 3.86^{c}	49.86 ± 1.40^{b}
Total carbohydrate content*	17.91 ± 0.93^{a}	39.67 ± 0.88^{b}	86.22 ± 0.93^{d}	103.73 ± 0.76^{e}	80.04 ± 1.94^{c}	101.90 ± 1.93^{e}

*Data represent the mean ± S.D (n=3). Mean values of each row followed by different superscript letter significantly differ when subject to Turkey's multiple comparison test, $P < 0.05$.

Table 3
Antibacterial activity of *T. chebula* leaves using various solvents.

Exracts used	Organisms tested (Diameter of inhibition zone in mm)						
	B.subtilis	*E.faecalis*	*S.typhi*	*K. pneumoniae*	*S.aureus*	*C.diphtheria*	*S.boydii*
Petroleum ether*	07.33 ± 0.58^{a}	08.33 ± 1.24^{a}	10.33 ± 0.58^{a}	09.67 ± 1.53^{a}	9.67 ± 0.58^{a}	08.67 ± 1.15^{a}	0^{a}
Chloroform*	11.00 ± 0.00^{b}	10.33 ± 0.58^{a}	$12.00 \pm 1.00^{a,b}$	$12.33 \pm 0.58^{a,b}$	$10.33 \pm 0.58^{a,b}$	$10.00 \pm 1.00^{a,b}$	0^{a}
Ethyl acetate*	$12.33 \pm 0.58^{b,c,d}$	$18.67 \pm 2.08^{c,d}$	18.67 ± 1.15^{c}	13.33 ± 0.58^{b}	$10.67 \pm 0.58^{a,b}$	$11.33 \pm 1.53^{a,b,c}$	0^{a}
Acetone*	14.33 ± 1.15^{d}	20.00 ± 1.73^{d}	18.67 ± 0.58^{c}	14.33 ± 0.58^{b}	13.67 ± 0.58^{c}	14.33 ± 0.58^{c}	13.33 ± 1.15^{b}
Methanol*	$12.67 \pm 0.58^{b,c,d}$	19.67 ± 2.08^{d}	13.67 ± 1.15^{b}	13.3 ± 0.58^{b}	$12.33 \pm 0.58^{b,c}$	14.00 ± 1.00^{c}	0^{a}
Water*	$11.67 \pm 1.53^{b,c}$	$11.67 \pm 0.58^{a,b}$	23.00 ± 1.00^{d}	13.00 ± 1.73^{b}	$11.67 \pm 1.15^{a,b,c}$	$13.33 \pm 0.58^{b,c}$	0^{a}
Streptomycin*	$13.50 \pm 1.05^{c,d}$	$15.00 \pm 1.26^{b,c}$	18.00 ± 1.67^{c}	14.00 ± 1.41^{b}	22.00 ± 1.10^{d}	22.17 ± 1.83^{d}	21.83 ± 1.83^{c}
DMSO*	−	−	−	−	−	−	−

*Data represent the mean ± S.D (n=3). Mean values of each column followed by different superscript letter significantly differ when subject to Turkey's multiple comparison test, $P < 0.05$.

except fats and saponins which showed their copious presence in petroleum ether extract.

3.2. Total phenolic content

A significant phenolic content was observed in acetone extract (105.84 μg GAE/mg of extract). The next highest contents were recorded by ethylacetate, methanol followed by water extract. An unsatisfactory content was produced by chloroform (45.4 μg) and petroleum ether (22.63 μg) extracts.

3.3. Total flavonoid content

Acetone extract recorded an excellent flavonoid content

of about 104.01 μg followed by ethylacetate and methanol extracts as shown in Table 2. The order obtained was acetone > ethylacetate > methanol > water > chloroform > petroleum ether extracts in the decreasing flavonoid contents.

3.4. Total flavonol content

Except petroleum ether extract almost all the solvent fractions exhibited potent flavonol content. Only slight variations were observed, among which the significant content was given by acetone (100.13 μg). For a note, close variations in the contents were observed in water (89.96 μg) and chloroform (87.01 μg) extracts.

3.5. Total tannin content

Tannins are the polyphenolics and they showed their significant content in acetone extract (109.7 μg) obviously. It was found to be the highest not only in tannin content but also among all the contents examined in this study. A suitable competence was noted in ethylacetate fraction, whose total tannin content was 104.31 μg. On the otherhand, a least content can also be inferred in this estimation i.e. petroleum ether extract of 11.56 μg and also among the other contents estimated.

3.6. Total ascorbic acid content

In this estimation, deviating from the uniformity of results, the aqueous extract showed lesser contents compared to chloroform extract as shown in Table 2. This order was not observed in the case of other extracts. With an uniformity, highest contents were observed for acetone followed by ethylacetate and methanol extract.

3.7. Total protein content

The order obtained for the total protein content is acetone > ethylacetate > methanol > water > chloroform > petroleum ether extract in the decreasing order. Acetone extract presented a significant content being clearly distinct from all the other fractions. A close variation between the contents was given by the ethylacetate and methanol extracts.

3.8. Total carbohydrate content

The highest carbohydrate content was observed in acetone extract. Unexpectedly, aqueous extract recorded the next highest content of about 101.9 μg. Ethylacetate, methanol and chloroform extracts ranged thereafter.

Petroleum ether extracts present a least contents for all the above results which may be due to the less polar compounds which got extracted. This particular extract is expected to show lower activities for the following *in vitro* assays too.

3.9. DPPH radical scavenging activity

Figure 1 depicts the radical scavenging activity of various extracts, proving their ability to terminate the free radicals with respect to various concentrations. The efficiency of this activity can be revealed by an important factor i.e. IC_{50} value (inhibition of 50% radical scavenging activity), which was 130 μg for acetone, 137 μg for ethylacetate and 143 μg for methanol, 153 μg for water extract. They were found to be potent compared to the standards like ascorbic acid (180 μg) and α-tocopherol (197 μg). On the otherhand, the absorption values decreased as the intensity of radical colour decreased due to scavenging activity of the extracts (Figure 1).

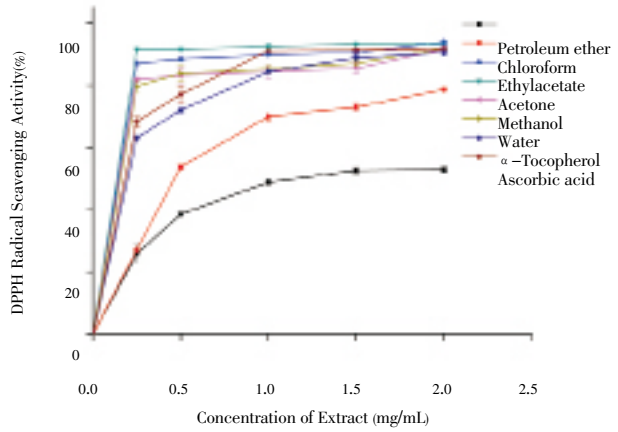

Figure 1. DPPH radical scavenging activities of *T. chebula* leaves extracts in different concentrations. Each value represents a mean ± SD. Symbols represent statistical significance from control (*P <0.05).

3.10. Reducing power

The efficiency of the extracts exhibited an excellent reducing power, whose orders of activity were in agreement with the DPPH scavenging assay. This activity also showed EC_{50} values (efficient concentration for absorption 0.5 at 700 nm) higher than the standards like ascorbic acid, α-tocopherol for acetone, ethylacetate, methanol and water extracts. Acetone extract found to be potent in this assay too, with an EC_{50} value of 375 μg. Their absorption values increased with increase in concentration, showing that as the concentration of the extract was increased, their ability to reduce Fe^{3+} to Fe^{2+} was also increased (Figure 2).

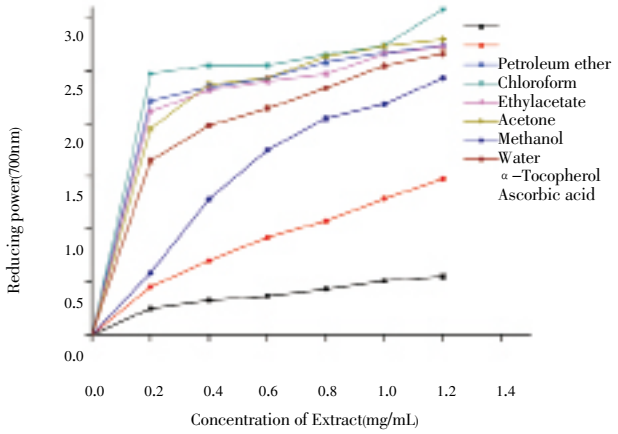

Figure 2. Reducing power of *T. chebula* leaves extracts in different concentrations. Each value represents a mean ± SD. Symbols represent statistical significance from control (*P <0.05).

3.11. Antibacterial activity

B. subtilis, *E. faecalis*, *K. pneumoniae*, *S. aureus* and *C. diphtheria* showed higher inhibitions for acetone extract. Particularly *B. subtilis*, *E. faecalis*, *K. pneumoniae* showed higher activity against the standard, Streptomycin and *S. boydii* responded only on application of acetone extract. *S. typhi* showed higher antibacterial activity for water extract. Ethylacetate and methanol extracts gave best competence with acetone on the above parameters which showed moderate antibacterial activity. These results are tabulated in Table 3.

4. Discussion

The preliminary qualitative screening has revealed the displayed constituents in Table 1, revealing the phytochemicals that got extracted in the subjected solvent fractions. As given in results, the copious presence of those constituents in acetone extract is due to the ability of the acetone to extract them much more effectively compared to other solvents. Though the soxhelation was performed successively with various solvents, it is clear from these preliminary tests that the phytochemicals being polar get dissolved in the solvents that matches with their polarity. Hence, there is not a unique solvent or mixture of solvents that can solubilise all antioxidant compounds[27]. Earlier, solvents such as methanol, ethanol, butanol, acetone, chloroform and water have been commonly used for the extraction of phenolics from brown and red seaweeds[28, 29]. Hence, the quantification of those components through some standard methods of estimation performed in this study will be highly informative to explore the maximum utilities buried in *T. chebula* leaves. The total contents of various constituents like phenolic, flavonoid, flavonol, tannin, ascorbic acid, protein, carbohydrate shall be discussed for their immense presence in various extracts in the light of antioxidant activity.

A clear understanding about the various contents shall be grasped from Table 2. From the above results it can be observed that acetone extract exhibited higher content for almost all the estimations. Phenolic content of any plant are highly responsible for the antioxidant activity and the acetone extract presented a promising phenolic content and was also given by all other extracts which provides a certain μg amount of phenolics present in each gram of the extracts. Phenolic compounds, one of the most widely occurring groups of phytochemicals, are of considerable interest as they are reported to exhibit anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects [30]. Such contents when consumed as diet will largely participate in cell promotion against harmful action of ROS, mainly oxygen free radicals, produced in response to environmental stresses such as salinity, drought, high light intensity or mineral nutrient deficiency [31]. With this support in this study, it has been worked on the various solvents to extract the maximum amount of phenolics, flavonoids, tannins etc. Flavonoid and flavonoid related compounds like flavonol, flavonones, isoflavones show a strong biological effects, reducing the risk of free radicals. Flavonoids are known as important secondary plant products in many foods. They have attracted much attention in relation to their wide range of activities in the prevention of cancer, inflammation and coronary heart disorders [32]. Hidalgo found that flavonoids present in a mixture could interact, and their interactions could have synergistic or antagonistic effects on total antioxidant capacity [33]. On the otherhand, a similar proportion of the content can be seen for both these constituents in various extracts. It should be highly appreciable that the contents estimated were found to be above the level of expectation especially for acetone and ethylacetate extracts. For a deeper investigation, methanol and water being highly polar than the former solvents, they have exhibited lesser contents. Thus it can be assumed that higher content of particular solvent depends on the amount of constituents present in the polarity matching with them, thereby allowing them to get dissolved at that range of polarity. It was reported that the different solvent compositions and extraction temperature can lead to differences in the profile of phenolic compounds, resulting in varied antioxidant activity of the extracts [34]. This concept was found to be followed by phenolic and tannin contents too. Both phenolic and tannin contents have roughly shown richer contents compared to flavonoid and flavonol contents among the extracts. This has been a credit to the present study showing a richer amount of phenolic or polyphenolic (tannin) will be capable of different antioxidant mechanisms, infact, to counteract the deleterious action of ROS [35, 36].

The total ascorbic acid, protein and carbohydrate contents attributes to the nutritional value of *T. chebula* leaves which gives support to the antioxidant activity, bearing in mind, the phytochemicals responsible for those estimations are inter-related with this antioxidant activity. Among the extracts, acetone extract showed significant ascorbic acid content. But were not upto the level of other contents like tannin and phenolic. The reason behind the chloroform fraction being potent than water fraction may be due to the above discussed criteria regards their polarity. Moreover, there was close approximation between the methanolic and chloroform extracts for ascorbic acid content and a wide variation was observed in water extract. A very least contents recorded by petroleum ether extract prevailed here, alike the other contents. It showed a slight presence of all these constituents (phenolic, flavonoid, protein, carbohydrate) and hence they were able to display a considerable content despite their low polarity range.

Protein content showed similar order as obtained for flavonoid, flavonol, tannin and phenolic contents. It was surprising to record such satisfactory contents of protein in leaves especially which are likely to contribute to the antioxidative index. Coming to the carbohydrate content, a second highest content observed in water extract will be noteworthy to be discussed. It can be clearly correlated that a neat competence has raised between water extract with highest acetone content of flavonoid and flavonol. There was not much difference between the acetone and water

carbohydrate contents. Carbohydrates being water soluble have got extracted efficiently in water and thus provided a highly significant content. Good carbohydrate contents were seen to be present in methanol and ethylacetate extracts and a satisfactory content in chloroform and petroleum ether extracts.

Additionally, it has been determined for the *in vitro* antioxidant activity through two important parameters: DPPH radical scavenging activity and reducing power activity. First in DPPH scavenging activity, ascorbic acid and α-tocopherol were chosen as the reference antioxidants. It was reported by Katalinic *et al.* that antioxidants can deactivate or scavenge stable free DPPH radical by two major mechanisms: by reduction via electron transfer or by hydrogen atom transfer that may occur also in parallel and steric accessibility is one of the major determinants of the reaction [37]. From the results displayed, it can be inferred that all the extracts have acted as radical scavengers, when exposed to the most stable free radical, DPPH. This is infact an concentration dependent assay, accompanied with colour difference and hence an activity change at each and every step along the various concentrations were figured out for reference (Figure 1), giving the % radical scavenging activity. This interaction of extracts with DPPH depends on the structural conformation of the bioactive compounds present in the plant extracts, among which the hydroxyl groups of flavonoids are highly favourable [38]. From a mechanistic standpoint, the DPPH radical scavenging assay could reflect the capacity of the extract transferring electrons or hydrogen atoms [39]. Acetone extract possessing highest amount of phenolics, flavonoids etc., was found to be the most active scavenger. IC_{50} values calculated from those neat plots provided interesting evidence to rate the scavenging activity of various extracts. A striking evidence for these leaf extracts to be potent is given by the relative low inhibitions of the standards. Except chloroform and petroleum ether fractions others were no-doubtedly potent, stronger than standards against free radicals. Second the reducing power activity works with the principle of reducing Fe^{3+} to Fe^{2+} by donating electrons. This was actively/readily provided by the phenolic compounds to donate their hydrogen atoms to prevent the onset or propagation of oxidative diseases. Thus, attention of the antioxidant activity of any plant is paid on the role of the phenolic compounds to neutralize the free radicals by their reducing property. The strong positive correlation between the TPC and FRAP, and the low or lack of correlation between the TPC and ORAC have been observed by others [40, 41]. To quantify this reducing activity, EC50 values have been calculated to present the reducing ability of 0.5 absorption concentration to reducing the ferric ions at 700 nm. This activity is in agreement with the phenolic and tannin contents. All the extracts demonstrated high antioxidant activity compared to the standards as seen in the case of DPPH activity.

As displayed in results, acetone extracts has revealed higher antibacterial activity followed by methanol, water and ethylacetate extracts. Compared to standard, acetone extract has showed their excellent effects on inhibiting *E. faecalis, B. sabtilis* and *K. pneumoniae* bacterias. Such significant antibacterial activity of acetone may due to following reasons (i) The bioactive compounds like phenolics, flavonoids, flavonols, tannins, ascorbic acid etc., being present in greater amounts compared to other extracts. (ii) Solubility of constituents in acetone being higher than other extracts. A striking evidence for these reasons was provided by the inhibition on the bacteria *Shgella boydii*. None other extracts showed antibacterial activity than acetone extract on that particular bacteriae. As reported by Ghosh *et al.*, methanolic leaf extracts of *T. chebula* has showed best antibacterial effects against *B. subtilis* and *S. aureus* [42]. In the present study, acetone extract revealed its potency.

In this study, the antioxidant potential of *T. chebula* leaves, evaluated using *in vitro* DPPH-radical scavenging activity and reducing power has proved its crucial role in scavenging activity. Our study provides evidence that the estimated contents for the secondary metabolites exhibit interesting antioxidant properties. Antibacterial study on various microrganisms was the highlighting assay that has presented the potency of the acetone extract comparatively. For all the contents and *in vitro* antioxidant assays, acetone extract has provided a gateway for the promising pharmacological properties. In line with the efforts to balance the conservation of biodiversity and encouraging controlled exploitation of plant resources for economic gains, especially in biopharming, waste of valuable resources should be minimising. The information presented here could be used as preliminary data and biologically more relevant experiments that will examine the therapeutic potential of *Terminalia chebula* isolated compounds will be designed.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

One of the author, Mr. A. Kathirvel gratefully acknowledges the financial assistance, URF (University research fellow, F.No:PU/R/R3/URF/2009 dated 03.12. 2009) rendered by Periyar University, Salem, Tamil Nadu, India.

References

[1] Said EA, Krust B, Nisole S. The anti-HIV cytokine midkine binds the cell surface expressed nucleolin as a low affinity receptor. *J Biol Chem* 2002; **277** (40): 37492-3502.

[2] Carlsen MH, Halvorsen BL, Holte K, Bohn SK, Dragland S, Sampson L, et al. The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. *Nutri J* 2010; **9**(3): 1-11.

[3] Razab R, Abdul Aziz A. Antioxidants from tropical herbs. *Nat Prod Comm* 2010; **5**(3): 441-445.

[4] Maisuthisakul P, Suttajit M, Pongsawatmanit R. Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chem* 2007; **100**: 1409-1418.

[5] Razali N, Razab R, Mat Junit S, Abdul Aziz A. Radical scavenging and reducing properties of extracts of cashew shoots (*Anacardium occidentale*). *Food Chem* 2008; **111**: 38-44.
[6] Wu XJ, Hansen C. Antioxidant capacity, phenolic content, polysaccharide content of Lentinus edodes grown in Whey permeate based submerged culture. *J Food Sci* 2008; **73**: 434-438.
[7] Vijayabaskar P, Shiyamala V. Antioxidant properties of Seaweed polyphenols from *Turbinaria ornate* (Turner) J. Agardh, 1848. *Asian Pac J Trop Med* 2012; 1-9.
[8] Madlener S, Illmer C, Horvath Z. Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukaemia cells. *Cancer Left* 2007; **245**: 156-162.
[9] Yoshioka K, Kataoka T, Hayashi T, Hasegawa M, Ishi Y, Hibasami H. Induction of apoptosis by gallic acid in human stomach cancer KATO 111 and colon adenocrcinoma COLO 205 cell lines. *Oncol Rep* 2000; **7**: 1221-1223.
[10] Masamune A, Satosh M, Kikuta K, Suzuki N, Satoh K, Shimosegawa T. *Biochem Pharmacol* 2005; **70**: 869-878.
[11] Chattopadhyay RR, Bhattacharyya SK. Plant review *Terminalia chebula*: An uptate. *Pharmacog Rev* 2007; **1**(1): 151-156.
[12] Cheng HY, Lin TC, Yuk H, Yang CM, Lin CC. Antioxidant and free radical scavenging activities of *Terminalia chebula*. *Biopharm Bull* 2005; **26**: 355.
[13] Date BB, Kulkarni PH. Assessment of rasandanti in various oral disorders. *Ayurveda Res Pap* 1995; **2**: 167-175.
[14] Nadakarni KM. *Indian metria medica*. Bombay:Popular prakasan Pvt Ltd; 1976, p.1202-1211.
[15] Kirtikar KR, Basu BD. *Terminalia chebula* In: Kirtikar KR, Basu BD. (eds.) *Indian medicinal plants*. 2nd ed. Allahabad: Lalit Mohan Basu Publications; 1935,p.1020-1023.
[16] Manikandan M, Rejula M. Identification of allelochemicals from *Terminalia chebula*. *African Res Rev* 2008; **2**(3): 306-314.
[17] Yukawa TA, Kurokawa M, Sato H, Yoshida Y, Kageyama S, Hasegawa T, et al. Prophylatic treatment of cytomegalovirus infection with traditional herbs. *Antiviral Res* 1996; **32**(2): 63-70.
[18] Onwukeame DM, Ikuegbvweha TB, Asonye CC. Evaluation of phytochemical constituents, antibacterial activities and effects of exudates of *Pycanthus angolensis* weld warb (mysristicaeceae) on corneal ulcers in rabbit. *Trop J Pharm Res* 2007; **6**(2): 725-730.
[19] Barreira JCM, Ferreira ICFR, Oliveira MBPP, Pereira JA. Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. *Food chem* 2008; **107**: 1106-1113.
[20] Grubesic RJ, Vukovic J, Kremer D, Vladimir-knezevic S. Spectroscopic method for polyphenols analysis. Prevalidation and application on plantogo L. species. *J Pharmaceut Biomed* 2005; **39**: 837-842.
[21] Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Natur* 1986; **44**: 307-315.
[22] Barros L, Baptista P, Ferreira ICFR. Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays. *Food Chem Toxicol* 2007; **45**: 1731-1737.
[23] Hedge JE, Hofreiter BT, Whistler RL, Be Miller JN. *Carbohydrate chemistry*. 17th ed. New York: Academic Press;1962,p. 11-12.
[24] Lowery OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275.
[25] Omaye KA, Reddy Cross CE. Enhanced lung. Dystrophy in vitamin-E deficient rabbits. *J Boil Chem* 1962; 237.
[26] Natarajan D, John Britto S, Srinivasan K, Nagamurugan N, Mohanasundari C, Perumal G. Anti-bacterial activity of *Euphorbia fusiformis* -A rare medicinal herb. *J Ethnopharmacol* 2005; **102**(1): 123-126.
[27] Gokmen V, Serpen A, Fogliano V. Direct measurement of the total antioxidant activity of foods: the 'QUENCHER' approach. *Trends in Food Sci and Tech* 2009: **20**(6-7): 278-288.
[28] Chandini SK, Ganesan P, Bhaskar N. *In vitro* antioxidant activities of three selected brown seaweeds of India. *Food Chem* 2008; **107**(2): 707-713.
[29] Ganesan P, Kumar CS, Bhaskar N. Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresource Tech* 2008; **99**: 2717-2723.
[30] Lazos ES, Lafka TI, Lazou AE, Sinanoglou VJ. Phenolic and antioxidant potential of olive oil mill wastes. *Food Chem* 2011; **125**: 92-98.
[31] Ashraf M, Harris PJC. Potential biochemical indicators of salinity tolerance in plants. *Plant sci* 2004; **166**:3-16.
[32] Garcia-Mediavilla V, Crespo I, Collado PS, Esteller A, Sanchez-Campos S, Tunon MJ, et al. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in chang liver cells. *Eur J Pharmacol* 2007; **557**: 221-229.
[33] Hidalgo M, Sánchez-Moreno C, Pascual-Teresa S. Flavonoid-flavonoid interaction and its effect on their antioxidant activity. *Food Chem* 2010; **121**: 691-696.
[34] Karacabey E, Mazza G. Optimisation of antioxidant activity of grape cane extracts using response surface methodology. *Food Chem* 2010; **119**(1): 343-348.
[35] Pereira JA, Pereira APG, Ferreira ICFR, Valentao P, Andrade PB, Seabra R, et al. Table olives from potential and antimicrobial activity. *J Agri and food chem* 2006; **54**: 8425-8431.
[36] Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. *Food chem* 1996; **66**: 401-436.
[37] Katalinic V, Mozina SS, Skroza D, Generalic I, Abramovic H, Milos M, et al. Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chem* 2010; **119**: 715-723.
[38] El-Sayed SA. Total phenolic contents and free radical scavenging activity of certain Egyptian Ficus species leaf samples. *Food Chem* 2010; **114**: 1271-1277.
[39] Perez-Jimenez J, Saura-Calixto F. Literature data may underestimate the actual antioxidant capacity of cereals. *J Agri Food Chem* 2005; **53**: 5036-5040.
[40] Samec D, Gruz J, Strnad M, Kremer D, Kosalec I, Grubesic RJ, et al. Antioxidant and antimicrobial properties of Teucrium arduini L. (Lamiaceae) flower and leaf infusions (*Teucrium arduini* L. antioxidant capacity). *Food Chem Toxicol* 2010; **48**(1): 113-119.
[41] Razab R, Abdul Aziz A. Antioxidants from tropical herbs. *Natural Product Comm* 2010; 5(3): 441-445.
[42] Ghosh A, Dass BK, Roy A, Mandal B, Chandra G. Antibacterial activity of some medicinal plant extracts. *J Nat Med* 2008; **62**: 259-262.