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In vitro antioxidant properties of three ethnomedicinal plants of Southern India

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Abstract

Background

Humans are exposed to several deleterious foreign agents which initiates several disease pathologies which become evident as time passes by. Herbal drugs are considered to be one of the major strategies in treatment of such diseases. Screening of drugs for common mechanisms of disease causation like quenching the oxidative stress may prove beneficial in curbing such diseases in the future. Thus, a study was designed to study the in vitro antioxidant activity of three medicinal plants with ethnobotanical references viz, Withania somnifera, Albizia lebbeck and Aristolochia indica.

Methodology: methanolic extract of the three drugs were screened for the major phytochemicals present in them along with quantitative estimation of of flavonoids and phenols. Invitro antioxidant properties of FRAP assay, Fe chelation assay, DPPH assay, superoxide, nitric oxide, hydroxyl and peroxide inhibition assays and lipid peroxidation were studied.

Results: the results showed that major groups of phytochemicals were present in the three drugs and they contained significantly higher concentrations of flavonoids and phenols. The drugs showed statistically significant inhibition of all the antioxidant assays and W, somnifera showed the highest activity followed by A. lebbeck and A. indica.

Conclusion: the study concludes that the drugs used in ethnobotanical practices have scientific basis for their action and that they exhibit activity to mitigate the common mechanism of disease causation like oxidative stress.

Key words: Withania somnifera, Albizia lebbeck, Aristolochia indica, antioxidant, free radical scavenging

Introduction

All traditional medicines including Ayurveda relied heavily on the wealth of the flora around. Thus, medicinal plants became the mainstay of treatment regimen among the civilized communities around the world.[1] Medicinal plants first came to exist among the folklore traditions which later gained their entry into the mainstay treatment systems as time passed. Thus, we can see folklore practices and textual references regarding same set of plants for the same set of diseases.[2] Tapping the rich traditions of the folklore practices is of great importance as we

see many emerging medicines emanating from them. Three commonly used medicinal plants of medicinal importance are Withania somnifera, Albizia lebbeck and Aristolochia indica. They have been used for minor ailments like fever and cold to the treatment of cancers and neurodegenerative diseases. Present study aims at studying the in vitro antioxidant activity of the methanolic extract of these three drugs. Antioxidant property is considered to be the common pathway to arrest any disease process especially in conditions of toxicity arising from exposure to xenobiotics.[3]

Materials and Methods

Preparation of drug extract

Authenticated samples of WS roots, AL bark and AI roots were collected from Raw Drugs Processing Centre of Arya Vaidya Sala, Kottakkal and were cleaned, dried and powdered and extracted with 70% methanol-water in a Soxhlet's extractor. Accurately weighed 25 g of powder was taken in a filter paper thimble and extracted with 150 ml of the solvent. Extraction was carried out till the solvent became colourless in the extractor. The extract was then evaporated to dryness in a vacuum evaporator and the solvent free extract was stored under -20^{0}C till the beginning of the study.

Qualitative phytochemical screening

Qualitative screening for major secondary metabolites were done using standard procedures.[4]

Test for alkaloids

About 2 g of the extract was heated with 10 ml of 0.1 M hydrochloric acid at 50^{0}C for 5 minutes and filtered through a Whatman filter paper No. 1. After cooling 3 drops of Dragendorff's reagent was added and mixed. Development of brick-red colour was taken as a positive reaction.

Test for Terpenoids

Methanolic extract of the drug (2 ml) were taken in a test tube and mixed with 5 drops of acetic anhydride followed by drop wise addition of concentrated sulphuric acid along the sides of the test tube. Appearance of a blue ring at the junction of the two liquids were considered as a positive end point.

Test for Flavonoids To 2 ml of the methanolic extract, 5 drops of concentrated hydrochloric acid were added. Appearance of red colour indicated the presence of flavonoids. To another portion of the extract 1 ml of dilute ammonia solution was added and gently mixed. Appearance of a greenish-yellow colour indicates the presence of flavonoids.

Test for Cardiac Glycosides

About 0.5 g of the methanolic extract of the drugs were dissolved in 2 ml of glacial acetic acid and few drops of 10 % ferric chloride solution were added. Concentrated sulphuric acid 1 ml was then layered over the mixture and observed for the development of a violet band at the boundary of the solutions.

Test for steroids

Roughly 0.5 g of the extract was dissolved in chloroform (2 ml) followed by drop wise addition of Liebermann-Burchard reagent and gently mixed. The development of reddish-purple colour indicated the presence of steroids.

Test for Phenols

About 0.5 g of the extracts were boiled with 70% ethanol in a water bath for 5 minutes and filtered through a Whatman filter paper No. 1. Subsequent to cooling, 5 drops of 5% ferric chloride was added and observed for the development of a green precipitate, which indicated the presence of phenols.

Determination of in vitro antioxidant properties

Ferric Reducing Antioxidant Power Assay (FRAP Assay)[5]

Extracts were taken at graded doses of 0.2, 0.4, 0.6, 0.8 and 1 µg/ml concentrations and the same concentrations of L-ascorbic acid dissolved in phosphate buffer (0.2 M, pH 6.6, 2 ml) and an equal volume of potassium ferricyanide solution (1%) were added. At the end of incubation for 20 minutes at 50^0C, 2 ml of trichloroacetic acid (TCA) at 10% concentration was added and centrifuged at 1000 rpm and the supernatant collected. 2 ml of the supernatant was mixed with an equal quantity of distilled water and half the volume of 0.1% ferric chloride and the absorbance of the mixture was recorded in a spectrophotometer at 700 nm wavelength. Median effective concentration (EC_{50}) of the extract that gave an absorbance of 0.5 was determined from the standard graph (Oyaizu).

Determination of Fe^{2+} chelating activity assay[6]

Graded concentration of the extract was prepared ranging from 20 to 100 µg/ml and to it 0.1 ml pf $FeCl_2$ (2mM) in 1.6 ml distilled water. 0.2 ml of ferrozine solution (5mM) was added to the mixture and incubated for 10 minutes at 30^0C. the absorbance of the Fe2 + - ferrozine complex was then measured spectroscopically at 563 nm. The chelating activity of the extracts on Fe2+ was compared with that of EDTA (0.01 mM) and citric acid (0.025 M) and expressed as percentage of chelating activity.

Determination of 1,1, diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity[7]

Five concentrations of the extract (0.0625, 0.125, 0.25, 0.5, and 1 µg/ml) were prepared in methanol along with L-ascorbic acid at the same concentrations. 1 ml of the extract was treated with 0.5 ml of DPPH in methanol (0.3 mM). the mixture was vortexed and allowed to stand in dark at room temperature for 15 minutes. Blank solutions of extracts (2.5 ml) in methanol (1 ml) were used as baseline. 2.5 ml DPPH solution in methanol was used as negative control and L-ascorbic acid solutions were used as positive control. After incubation, the absorbance of the solutions was read at 517 nm in a spectrophotometer. (Brand-Williams)

Nitric oxide radical scavenging activity[8]

Sodium nitroprusside solution (10mM) solution (1 ml) was mixed with 1 ml of extract at different concentrations in phosphate buffer (0.2 M pH 7) and incubated for 160 minutes at 30^0C. after incubation, the solution was mixed with 1ml of Griess reagent prepared by mixing 1% sulphanilamide, 0.1% naphthyl-ethylenediamine dichloride and 2% phosphoric acid. The absorbance of the resultant solution was read at 546 nm and the percentage inhibition was calculated.

$ABTS^{\cdot+}$ Radical Cation Decolourisation Assay[9]

$ABTS^{\cdot+}$ was generated by oxidation of ABTS with potassium persulfate. 2 ml of the resultant solution was mixed with 20 ml of the extract solution at various concentrations. Decrease in absorbance of the solution was determined after 6 min interval at 734 nm using a spectrophotometer.

Superoxide anion scavenging activity[10]

Various concentrations of the extracts of the drugs were taken in 1 ml quantity and to which phenazine methosulphate solution (60 mm PMS in 0.1 M phosphate buffer) and 1 ml of nitro blue tetrazolium (NBT) solution (150 mM NBT in phosphate buffer 0.1M). the mixture was incubated at 270C for 10 minutes and absorbance read at 560 nm. L ascorbic acid was used as standard. Superoxide scavenging activity was reported as percentage inhibition.

Inhibition of β-carotene bleaching[11]

β-carotene solution was prepared by dissolving 2 mg in 10 ml of chloroform. 2 ml of this solution was volumetrically transferred in to a 100 ml round-bottom flask and the solvent was removed under vacuum at 40^0C. 45 µl of linoleic acid, 400 µl of Tween 80 emulsifier and 100 ml of distilled water were then added to the flask and shaken vigorously. 4.8 ml aliquots of the emulsion were then transferred into test tubes previously added

with 0.2 ml of various concentrations of the extracts. The tubes were mixed well and incubated at 50^0C in a water bath. Absorbance was measured soon after the addition of the emulsion at 470 nm in a spectrophotometer followed by 20 min intervals until the control sample changed colour. A blank sample devoid of carotene was also prepared for zeroing the instrument. LPO inhibition was calculated from the change in absorbance noted.

Determination of Hydroxyl Radical Scavenging Activity[12]

The reaction mixture consisting of 2.4 ml phosphate buffer (pH 7.8), 10-Phenanthroline (90 µl, 1 mM) hydrogen peroxide (150 µl, 0.1 mM), iron III chloride (60 ml, 1 mM) and phytexponent (1.5 µl) and L-ascorbic acid at 100%, 10%, 1% and 0.01% were prepared and incubated at room temperature for 5 minutes. Increase in absorbance at 560 nm was measured. (Klein)

Hydrogen peroxide radical scavenging activity[13]

Various concentrations of the drug extract were added to 0.6 ml hydrogen peroxide (40mM) solution prepared in phosphate buffer (pH 7.4). The tubes were then incubated for 15 min at room temperature and the resultant solution's absorbance was read at 230 nm against phosphate buffer taken as blank. The results were expressed a percentage inhibition.

Determination of Total Phenolic Content[14]

Methanolic extracts of the plants (1 ml) was mixed with 2 ml of Folin-Ciocalteu reagent (prepared by mixing Folin-Ciocalteu reagent with distilled water in 1:10 v/v ratio followed by adding 1 ml of 20% sodium carbonate). The mixture was vortexed for 20 seconds and incubated at 40^0C for 30 minutes and the absorbance of the resultant mixture was done at 765 nm. Standard curve prepared using Gallic acid was used for the calculation of total phenolic content and the results were expressed as mg of gallic acid equivalents per gram of the extracts. (Do et.al.,)

Determination of Total Flavonoid Content[15]

A 10 ml test tube was filled with 0.3 ml extract, 3.4 ml of methanol (30 %), 0.15 ml of 0.5 M $NaNO_2$ and 0.15 ml of 0.3 M $AlCl_3.6H_2O$. after 5 minutes of incubation at room temperature, 1 ml of 1 M NaOH was added and the absorbance of the mixture was read at 510 nm in a spectrophotometer. Total flavonoid content was calculated from a standard curve drawn using quercetin standard solution and the result was expressed as mg of quercetin equivalents per g of sample.

Statistical analysis

Statistical analysis was performed using SPSS version 21. Between group analysis was performed using one way ANOVA and 5% was fixed as the level of significance.

Results

Phytochemical screening

The methanolic extract of W. somnifera, A. lebbeck and A. indica showed the presence of flavonoids, phenols and alkaloids in all the specimens while steroids, saponins, glycosides and terpenoids were variedly distributed among the plants (Table 1)

Table 1 Qualitative estimation of phytochemicals

Phytochemicals	WSME	ALME	AIME
Flavonoids	±	±	±
Phenols	±	±	±
Steroids	-	±	±
Saponins	±	±	-
Alkaloids	±	±	±
Cardiac Glycosides	±	±	-
Terpenoids	±	±	-

In vitro antioxidant activity

Invitro antioxidant activity was highly exhibited by W. somnifera followed by A. lebbeck and A. indica showed the least activity. The results are shown in Table 2.

Tabel 2. In vitro antioxidant activity of Withania sominifera, Albizia lebbeck and Aristolochia indica methanolic extracts

Antioxidant Assay	WSME	ALME	AIME
FRAP (mmol Fe/Kg)	4.64 ± 9.63^{c}	2.48 ± 3.66^{a}	3.98 ± 2.49^{b}
Fe2 ± _ Chelation IC_{50} (mg/ml)	0.64 ± 0.04^{c}	0.18 ± 0.06^{a}	0.47 ± 0.11^{b}
DPPH Assay	846.73 ± 6.84^{c}	474.85 ± 7.29^{b}	266.74 ± 9.52^{a}
Nitric Oxide scavenging assay	74.59 ± 3.84^{a}	558.73 ± 6.86^{c}	118.15 ± 8.65^{b}
ABTS scavenging assay	38.68 ± 4.95^{a}	64.52 ± 2.13^{c}	146.22 ± 7.62^{b}
Superoxide scavenging assay	123.86 ± 8.69a	274.25 ± 3.84^{c}	139.83 ± 9.26^{b}
Lipid peroxidation	74.68 ± 5.44^{c}	71.48 ± 8.67^{b}	65.52 ± 6.28^{a}
Hydroxyl radical scavenging assay	684.25 ± 14.62^{c}	408.62 ± 8.61^{b}	216.73 ± 11.22^{c}
Hydrogen peroxide scavenging assay	169.42 ± 2.82^{c}	72.91 ± 5.88^{a}	96.43 ± 4.87^{b}

(c significant at $p < 0.001$, b – significant at $p < 0.01$ and a – significant at $p < 0.05$)

Quantitative phytochemical screening

Quantitative estimation of phenols and flavonoids in the methanolic extracts of the three drugs showed significantly higher amount of phenols and flavonoids in them. The antioxidant activity exhibited by them are highly attributed to the presence of these compounds (Table 3).

Table 3. Quantitative phytochemical estimation

	WSME	ALME	AIME
Total Phenolic content (mg/ml)	0.52 ± 0.03	0.28 ± 0.02	0.15 ± 0.08
Total Flavonoid content (mg/ml)	0.50 ± 0.04	0.104 ± 0.01	0.16 ± 0.04

Discussion

Medicinal plants are the store house of several secondary metabolites that help in their utilization as sources of medicine. Ethnobotanical practices prevailing in several indigenous populations have contributed significantly in the propagation and identification of medicinal sources of drugs even to the modern system of medicine. Qualitative screening of secondary metabolites forms an essential step in the standardization of a drug and is considered to be the preliminary step in drug discovery. Preliminary phytochemical screening of the methanolic extracts of *Withania somnifera, Albizia lebbeck* and *Aristolochia indica* are shown in Table no: 1. All the three drugs showed the presence of flavonoids, phenols and alkaloids which are considered to be the most popular drug candidates in a plant. Flavonoids and phenols are predominantly the molecules responsible for the antioxidant property in plants. Antioxidant defence system is considered to be one of the most prominent mechanisms of curbing the disease-causing mechanisms in the body. Sufficiently large amounts of flavonoids and phenolics present in these three medicinal plants contributes much to their pharmacological activity in diverse disease pathologies. Flavonoids and phenolics are abundantly distributed among the plant kingdom and they are widely distributed among the plant parts including leaf, fruits, flowers, stem, bark and roots.

The invitro antioxidant properties measured by different methods showed significant activity in W. somnifera treated samples followed by A. lebbeck and the least activity was shown by A. indica. The study proved the efficacy of W. somnifera as a potent antioxidant drug among the three and several other studies have also shown a similar result with respect to the drug[16,17,18]. The antioxidant activity exhibited by this drug is fairly attributed to the presence of falvonoids and phenolics (Table 3) which are the widely acclaimed secondary metabolites with antioxidant property. The predominant distribution of flavonoids and phenolics in the three drugs studies shows the high rate of antioxidant activity exhibited by them and also shows their popularity among the ethnobotanical practices in using them as drug.[19,20] The presence of pharmacologically active phytochemicals in the three drugs also contributed to the wide literatures that are available in the public domain regarding their varied activities. The study shows that the major mechanism involved in using these three drugs in diseases is probably due to their high antioxidant activity through multiple pathways.

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