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Research Article

EFFECT OF DIFFERENT SOLVENTS ON ANTIOXIDANT ACTIVITY OF LEAF EXTRACTS OF *CALOTROPIS PROCERA* AND *AZADIRACHTA INDICA*

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ABSTRACT

Objective: This study was performed to identify the phytochemicals and comparatively evaluate the antioxidant activity of *Calotropis procera* and *Azadirachta indica* by detection of total phenolics, hydrogen peroxide radical scavenging activity, and estimation of condensed tannins in different solvent systems and at different temperatures.

Methods: Leaves of *C. procera* and *A. indica* were extracted in water, methanol by soaking dried leaf powder at room temperature and also by boiling the leaf powder in water for 30 minutes. Phytochemical tests were performed in all of the extracts. The antioxidant activity was determined by hydrogen peroxide radical scavenging activity. Quantitative estimation of total phenolics and hydrolysable tannins was also performed.

Results: The total phenolics in both leaf extracts was obtained maximum in boiled extract (40.7±1.20 mg gallic acid equivalent [GAE]/g dry extract in *C. procera* and 33.66±1.45 mg GAE/g dry extract in *A. indica*). The amount of hydrolysable tannins in both leaf extracts was found to be highest in methanol (150±1.88 mg catechin equivalent/g dry extract in *C. procera* and 144.8±2.63 mg catechin equivalent/g dry extract in *A. indica*).

Conclusion: The study showed promising results indicating that these plants are a good source of antioxidants. The majority of phytochemicals were extracted in distilled water and methanol acts as a good solvent for extraction of tannins, whereas an increase in temperature leads to poor extraction of tannins.

Keywords: Antioxidant, Phytochemicals, Phenolics, Radical, Tannins.

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INTRODUCTION

Numerous scientific studies indicate that during oxidative stress, oxygen radicals such as hydroxyl radical (OH), peroxyl radicals hydrogen peroxide (H_2O_2), and superoxide anion (O^{2-}) are generated in living systems which are commonly known as reactive oxygen species. These free radicals may cause oxidative damage to biomolecules such as DNA, lipids, and proteins. They also play a significant role in many degenerative diseases such as cardiovascular diseases, cancer, ageing, Alzheimer's and several other neurodegenerative disorders [1-4].

Numerous researches indicate that the antioxidants in fruits and vegetable play a major role in reducing the incidence of chronic diseases including heart disease and some cancers [5-8]. An antioxidant is any substance that delays or stops oxidative damage [9]. Antioxidant has the ability to remove free radicals by acting as hydrogen donors and quenchers of singlet oxygen [10,11]. The purpose of using antioxidants as therapeutic agents is to lower the oxidative stress by preventing or delaying the development of disease or reversing some of the complicacies associated with oxidative stress [12].

The use of plants, plant extracts, and pure compounds isolated from natural sources has always provided a basis for modern pharmaceutical compounds [13]. Plants have been a rich source of medicines as they produce a variety of bioactive compounds, majority of which probably evolved as a chemical defense against predation or infection [14]. Phyto is the Greek word for plants. There are many "families" of phytochemicals which help the human body in many ways. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plants produce these chemicals to protect themselves, but recent research shows that many phytochemicals have the potential to provide protection against human diseases [15].

In recent decades, many researchers are interested in medicinal plants for evaluation of antioxidant phytochemicals such as phenols, flavonoids, and tannins which have got enormous attention because of their potential in preventing many human diseases [16]. *Calotropis procera* belonging to the family: Asclepiadaceae, commonly called as "aak" in India is used as an herbal medicine by human since time immemorial. It has numerous biological activities and hence can act as anti-inflammatory, analgesic, antidiabetic, antioxidant, antiarthritic, antihelminthic, wound healer, anticandidal, anticonvulsant, antiasthmatic, hepatoprotective, and antitumor agent [17]. *Azadirachta indica* popularly known as neem is evenly spread in India and its neighboring countries since ancient times as one of the most potent medicinal plants having wide spectrum biological activity [18]. Neem oil, leaves, bark, and stem products have been therapeutically used for the treatment of respiratory disorder, inflammation, constipation, skin infection [19], arthritic disorders, fever and diabetes, etc. [20]. Apart from these, there are several reports on pharmaceutical and biological activities of neem based on the scientific investigation such as antibacterial, antifungal, antiviral, and antioxidant activities [21].

It is well-recognized that different solvents have the ability to extract different types of phytochemicals on the basis of their polarity; henceforth, the biological activity of the extracts can be varied [22-24]. Alcohol extracts are more preferable over water extracts in many pharmacological assays, since lipophilic bioactive secondary metabolites; usually phenolics are more easily extracted with alcohol than with water [25]. In addition to the phytochemicals found in alcoholic extracts, hot water or boiled water also tends to extract other biological components [26,27].

This study was done to investigate the phytochemicals present in leaf extracts of *C. procera* and *A. indica*, effect of different solvents on the extraction of phytochemicals as well as on its antioxidant activity.

METHODS

Sample collection

Fresh leaves of *C. procera* and *A. indica* were collected from Amity University campus, Madhya Pradesh. The plants were authenticated by botany professor, Dr. Sushil Kumar Sharma, Assistant Professor, Amity University, Gwalior, Madhya Pradesh, India. The leaves were washed thoroughly with tap water to remove the dust particles. Then, the leaves were kept in shade and spread evenly for drying until they become crispy. Dried leaves were ground to fine powder in pestle and mortar. The powder was stored in air tight polythene bags until further use.

Preparation of extract

About 1 g of powdered leaf (*C. procera* and *A. indica*) was soaked separately in 10 ml of distilled water and methanol and kept for 72 hrs at room temperature. Then, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and stored in refrigerator at 4°C for further activity analysis.

Aqueous boiled extract was also prepared by boiling 5 g of dried leaf powder in 100 ml of distilled water for 30 minutes. Then, it was filtered using cheese cloth and stored at 4°C for further analysis.

Phytochemical screening

The plant extracts were evaluated qualitatively for different phytochemicals by protocol given by Harborne in 1984 with certain modifications [28].

Tri-terpenoids

About 500 µl of leaf extracts (*C. procera* and *A. indica*) prepared in all solvent system was pipetted in three different test-tubes and was dissolved in 200 µl of chloroform, thereafter equal volume of concentrated sulfuric acid was added slowly along the wall of the test-tube. A brown/red/purple ring at the interface indicates the presence of steroids.

Tannins

About 100 µl of leaf extracts (*C. procera* and *A. indica*) prepared in all solvent system was pipetted in three different test-tubes and few drops of 1% lead acetate were added to each of the test-tubes. A yellowish precipitate indicates the presence of tannins.

Saponins

About 100 µl of leaf extracts (*C. procera* and *A. indica*) prepared in all solvent system was pipetted in three different test-tubes and 4 ml of distilled water was added in each test-tubes. The mixture was then shaken vigorously for 30 seconds. Then, it was left undisturbed for few minutes. The presence of intense foam indicated that saponins are present.

Leucoanthocyanins

About 100 µl of leaf extracts (*C. procera* and *A. indica*) prepared in all solvent system was pipetted in three different test-tubes and 100 µl of iso amyl alcohol was added to each test-tubes. The appearance of red color in the upper layer indicates the presence of leucoanthocyanins.

Coumarins

About 100 µl of leaf extracts (*C. procera* and *A. indica*) prepared in all solvent system was pipetted in three different test-tubes and then to each of the test-tubes 150 µl of 10% NaOH was added. The appearance of yellow color indicates the presence of coumarins.

Flavonoids

About 100 µl of prepared leaf extracts (*C. procera* and *A. indica*) in all solvent system was pipetted in three different test-tubes, and then, 10 µl of 1% NaOH was added to each of the test-tubes. On appearance of intense yellow color, 10 µl of concentrated HCl was further added. Reversion to original color of the extract indicated the presence of flavonoids.

Total phenols

Total phenols were determined by Folin–Ciocalteu reagent method [29]. 500 µl of extract (*C. procera* and *A. indica*, concentration: 1:10 g/ml) in different solvent systems was added in separate test-tubes. Then, 5 ml of Folin–ciocalteu reagent (1:10 diluted with distilled water) was added to each of the test-tubes followed by addition of 4 ml of 1 M Na_2CO_3. The mixture was allowed to incubate at 37°C for 30 minutes. Then, the absorbance was measured at 710 nm against blank. A standard graph of gallic acid was plotted (Concentration [µg/ml] vs. optical density) from a stock solution of 500 µg/ml to determine the concentration of total phenolics. The total phenolics content was expressed as mg gallic acid equivalent (GAE)/g dry weight. The equation from standard graph of gallic acid is as follow:

Absorbance=0.0098 µg gallic acid+0.0104; R^2=0.9903.

Hydrolysable tannins

Hydrolysable tannins were quantitatively estimated by the method devised by Bate-Smith in 1977 with slight modifications [30]. 1.5 ml of plant extract (*C. procera* and *A. indica*) was mixed with 500 µl of saturated potassium iodide. The mixture was allowed to stand at room temperature for 40 minutes. Then, the absorbance was read at 550 nm against saturated potassium iodide as blank. The hydrolysable tannins were expressed as mg equivalent catechin/g dry weight. The quantity in mg equivalent of catechin was obtained by the standard equation:

Absorbance=0.8264 mg catechin+0.0392; R^2=0.9155.

H_2O_2 radical (% H_2O_2) scavenging activity

The radical scavenging activity of H_2O_2 in plant extracts of different solvent system was determined by protocol devised by Ruch *et al*. in 1989 with certain modifications [31]. 1 ml of extract was mixed with 2 ml of 20 mM H_2O_2 prepared in phosphate buffer saline (pH 7.4). The mixture was incubated at 37°C for 10 minutes. The absorbance was read at 230 nm against blank. % H_2O_2 activity was determined by:

$$\%\ H_2O_2 \text{ scavenging activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

RESULTS AND DISCUSSION

The results of this study are summarized under the following sub headings.

PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening of tri-terpenoids, tannins, saponins, leucoanthocyanins, coumarins, and flavonoids for both *A. indica* and *C. procera* were performed, and the results are shown in Tables 1 and 2, respectively.

Total phenolics (mg GAE/g extract)

The total phenolics were obtained maximum in boiled extract (40.7±1.20 mg GAE/g dry extract), then in methanol (26.43±1.41 mg GAE/g dry extract), and 21.39±0.83 mg GAE/g dry extract in leaf extracts of distilled water of *C. procera* (Fig. 1). The total phenolics in leaf extracts of *A. indica* was found to be maximum in boiled extract (33.66±1.45 mg GAE/g dry extract), then in methanol (25.44±0.7 mg GAE/g dry extract) and 15.48±1.65 mg GAE/g dry extract in distilled water (Fig. 2). The results are in accordance with a study conducted by Susanti *et al*. in 2015 [32] and they concluded that as temperature increases water polarity decreases, making the conditions favorable for extraction of phenol. This indicates that treating the leaf with distilled water at elevated temperature might result in easy extraction of phenolics which may eliminate the use of other toxic organic solvents.

Table 1: Phytochemical evaluation of *A. indica*

Tests	Tri-terpenoids	Tannins	Saponins	Leuco-anthocyanins	Coumarins	Flavonoids
Solvents						
Distilled water	+	+	-	+	+	+
Methanol	-	+	-	-	+	-
Boiled extract	-	+	-	-	+	+

+ indicates presence and - indicates absence. *A. indica: Azadirachta indica*

Table 2: Phytochemical evaluation of *C. procera*

Tests	Tri-terpenoids	Tannins	Saponins	Leuco-anthocyanins	Coumarins	Flavonoids
Solvents						
Distilled water	+	+	+	-	+	-
Methanol	-	-	-	-	-	-
Boiled extract	-	+	+	-	+	-

+ indicates presence and - indicates absence. *C. procera: Calotropis procera*

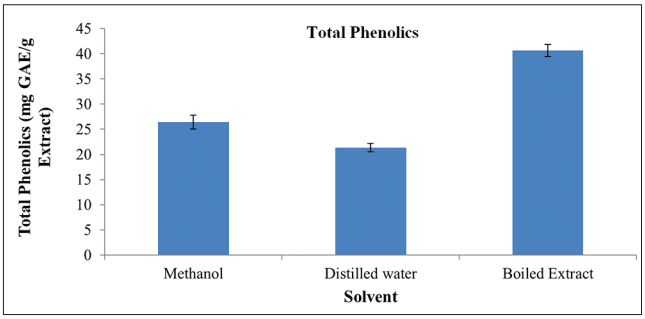

Fig. 1: Comparative analysis of total phenolics (mg gallic acid equivalent/g extract) in *Calotropis procera* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation

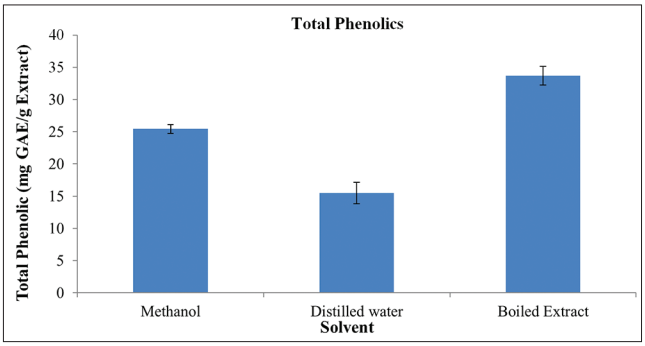

Fig. 2: Comparative analysis of total phenolics (mg gallic acid equivalent/g extract) in *Azadirachta indica* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation

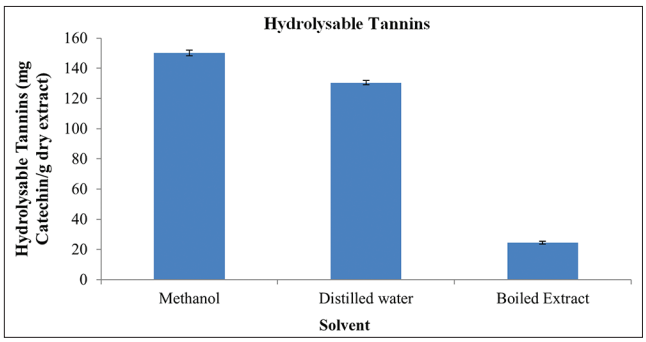

Fig. 3: Comparative analysis of hydrolysable tannins in *Calotropis procera* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation

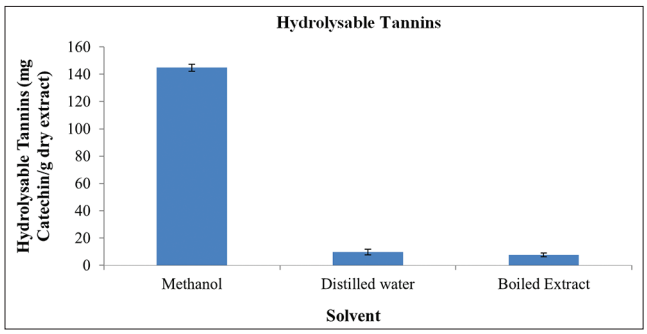

Fig. 4: Comparative analysis of hydrolysable tannins in *Azadirachta indica* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation.

Hydrolysable tannins

Tannins are complex secondary metabolites and pose many medicinal properties [33]. The amount of hydrolysable tannins in leaf extracts of *C. procera* was found to be maximum in methanol (150±1.88 mg catechin equivalent/g dry extract) followed by distilled water (130.46±1.48 mg catechin equivalent/g dry extract) and boiled extract (24.46±0.98 mg catechin equivalent/g dry extract) (Fig. 3). The hydrolysable tannin content in leaf extracts of *A. indica* was found to be highest in methanol (144.8±2.63 mg catechin equivalent/g dry extract) and almost in similar amount in distilled water (9.67±2.05 mg catechin equivalent/g dry extract) and boiled extract (7.66±1.25 mg catechin equivalent/g dry extract) (Fig. 4). This indicates that methanol acts as a good solvent for extraction of hydrolysable tannins, whereas treating the leaf powder at elevated temperature may degrade tannins and hence the amount of hydrolysable tannins was found to be minimum in boiled extracts.

H_2O_2 radical scavenging (% H_2O_2) activity

% H_2O_2 scavenging activity in leaf extracts of *C. procera* was observed maximum in distilled water (17.99±0.57%), then almost similar amount of activity was observed in boiled extract (17.93±0.23%) and in methanol (17.44±1.01%) (Fig. 5). % H_2O_2 scavenging activity in leaf extracts of *A. indica* was observed in very less amount in boiled extract (8.83±1.20%), and no scavenging activity was present in distilled water

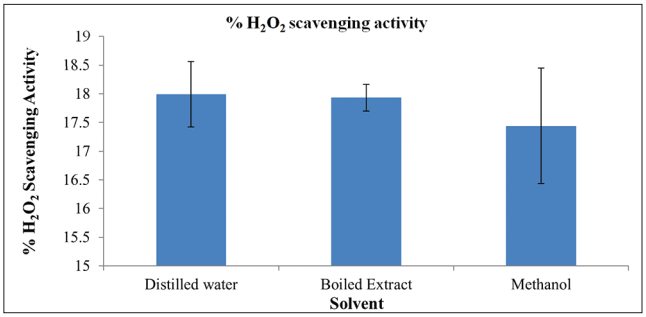

Fig. 5: Comparative analysis of % hydrogen peroxide scavenging activity in *Calotropis procera* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation

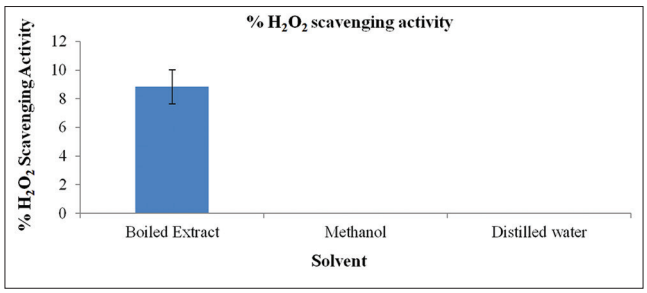

Fig. 6: Comparative analysis of % hydrogen peroxide scavenging activity in *Azadirachta indica* in different solvent system. All experiments were performed in triplicates and the results are expressed in mean values±standard deviation

and methanol (Fig. 6). H_2O_2 as such is not that reactive but it may cause toxicity to cells because of production of hydroxyl radicals [34]. Thus, removal of H_2O_2 is very much essential for antioxidant defense in cell or food systems [35]. Thus, it could be inferred that scavenging activity of H_2O_2 in leaf extracts may be due to the presence of antioxidants.

CONCLUSION

This study showed the presence of antioxidant compounds (phenolic acids and tannins) and demonstrated some level of antioxidant activities in *A. indica* and *C. procera*. Total amount of hydrolysable tannins was found to be maximum in methanol extracts followed by distilled water and minimum in boiled extract in leaf extracts of both the plant (*A. indica* and *C. procera*). Thus, it could be inferred that methanol acts as a better solvent for extraction of hydrolysable tannins and minimum amount of tannins in boiled extract may be due to degradation of hydrolysable tannins at elevated temperature. Total phenolics were obtained in maximum amount in boiled extracts of *A. indica* and *C. procera*. This indicates that treating the leaf powder with solvents at elevated temperature might result in easy extraction of phenolics. % H_2O_2 radical scavenging activity was observed maximum in distilled water in *C. procera* and in boiled extract in *A. indica*. Hence, we could conclude that maximum extraction of total phenolics could be achieved either in boiled extract or aqueous solvents and also it could be a better source of antioxidant. The leaf extracts showed antioxidant activities quantitatively comparable to that of gallic acid. It may be concluded from the phytochemical and quantitative tests that both the leaf extracts of *A. indica* and *C. procera* has certain bioactive components that pose antioxidant activity and the extraction of these components depends on the type of the solvent used for extraction and also the temperature of extraction.

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