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Research Article

QUALITATIVE PHYTOCHEMICAL SCREENING AND EVALUATION OF *IN-VITRO* ANTIOXIDANT ACTIVITY OF *CALLICARPA ARBOREA* ROXB, AN ETHNOMEDICINAL PLANT OF MIZORAM, NORTHEAST INDIA

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ABSTRACT

Objective: The objective of this study was to evaluate the phytochemical constitution and antioxidant activity of methanolic extract of dried leaves of *Callicarpa arborea* Roxb., an ethnomedicinal plant of Mizoram, Northeast India

Methods: Qualitative analysis of phytochemical constituents was performed by the well-known tests protocol available in the literature on the methanolic leaves extract of the plant. Antioxidant activity was studied through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power using butylated hydroxy toluene (BHT) and ascorbic acid respectively as standards. The total phenolic and total flavonoid content were determined using gallic acid and quercetin respectively as standards.

Results: The phytochemical screening revealed the presence of saponin, steroids, glycosides, flavonoids, and tannins. The methanol extract exhibited inhibition concentration values of 2.393 µg/ml in DPPH assay. The reducing power of the extract was lower than that of the standard ascorbic acid. The total phenolic content and flavonoid content was 130.6 mg/g and 83.9 mg/g of gallic acid and quercetin equivalent, respectively.

Conclusion: The results suggest that methanolic leaves extract of *C. arborea* has promising antioxidant activity and could serve as a potential source of natural antioxidants.

Keywords: *Callicarpa arborea*, Phytochemicals, Antioxidant activity, 2,2-Diphenyl-1-picrylhydrazyl, Phenol and flavonoid.

INTRODUCTION

Natural products have been the single most productive source of leads for the development of drugs [1]. Numerous reviews have described the importance of compounds derived from natural products, especially plants and microorganism to treat human diseases [2-4]. According to the World Health Organization, almost 80 % of the world's population relies on traditional medicines for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body [5]. Since the middle of 19th century, numerous bioactive constituents have been isolated and characterized. Many of these are being used as active ingredients of the modern medicine or as the lead compounds for new drug discoveries. Polyphenolic substances possess many biological effects, which are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition peroxidation, and chelating transition metals [6]. They provide chemoprotective effects to combat oxidative stress in the body and maintain balance between oxidants and antioxidants to improve human health An imbalance caused by excess oxidants leads to oxidative stress, resulting in damage to DNA, lipids and protein and eventually leading to chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans [7-9]. The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors [10].

Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases, and inflammatory diseases. This activity is due to the ability of antioxidants to reduce oxidative stress by neutralizing or scavenging of reactive species by hydrogen donation [10,11]. The safety of synthetic antioxidant in the food industry has become a concern among scientist and leading current immense interest in uncovering natural antioxidant and their role in human health and nutrition [12].

Since many modern allopathic medicines have been developed from the traditional knowledge, the ethnomedicinal use and hence, the probable pharmacological and medicinal activity of *Callicarpa arborea* Roxb. cannot be ignored. *C. arborea* Roxb. is a middle-sized evergreen tree belonging to the family *Verbenaceae*. It is commonly known as Hnahkiah in Mizoram, Northeast India. Traditionally a decoction prepared by boiling the leaves is used for various ailments such as cholera, dysentery, diarrhea, internal bleeding, colic and stomach ulcer [13]. The bark juice is applied on cuts and wounds as hemostatic [14]. There are no scientific reports regarding the activity of the plant. In order to assess and hence, isolate the probable compound that may have pharmacological activity, it is necessary to extract the crude drugs using different solvents having different polarity and qualitatively determine the different phytochemical constituents of the different extracts. However, three compounds were isolated from the bark of *C. arborea* Roxb., such as β sitosterol, bauerenol, and betulinic acid [15]. Another three compounds were also isolated from the leaves such as β sitosterol, epilupeol, and ursolic acid [16]. The present investigation was carried out to investigate the phytochemical constituents and antioxidant activity on the methanolic leaves extract of *C. arborea* Roxb.

METHODS

Plant material

The leaves of *C. arborea* Roxb. was collected from the Southern part of Mizoram, Northeast India during the month of March. Herbarium sheet was prepared, and authentication was done at the Botanical Survey of India, Kolkata (Reference No: CHN/60/2012/Tech. II/942 Dated: 17-01-2013). The plants were properly documented, and the specimens were preserved in the Department of Pharmacy, RIPANS, Aizawl, Mizoram, Northeast India for future references.

Preparation of plant extract

The dried powdered leaves of the plant were subjected to successive extraction first by cold maceration process at room temperature (27±2°C) using petroleum ether and then followed by soxhlet apparatus using chloroform and methanol as solvent respectively for 48 hrs. The extracts were evaporated under vacuum in a rotary evaporator, kept in airtight container and further used for the experiment.

Preliminary phytochemical screening

The methanol extract of the plant was subjected to preliminary phytochemical analysis to identify the presence of phytoconstituents. The phytochemical analysis was performed using standard procedures to identify chemical constituents [17].

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity *of C. arborea* Roxb. and the standard were estimated on the basis of the radical scavenging effect of the stable DPPH free radical according to the method described by Blois (1958) with minor changes [18]. Butylated hydoxy toluene (BHT) was used as reference standard. 0.5 ml of DPPH solution in methanol (0.1 mM) was mixed with 3 ml of the extract and 3 ml of standard prepared in various concentrations (10, 20, 40, 60, 80, 100, and 120 µg/ml), respectively. The extract and standards were incubated for 30 minutes at 37°C. Absorbance was measured at 517 nm using ultraviolet-visible (UV-Vis) spectrophotometer. Control reading was also taken. The scavenging effect of DPPH free radical was calculated using the following equation.

$$\% \text{ DPPH radical scavenging} = (\text{Abscont.} - \text{Absext.} / \text{Abscont.}) \times 100$$

Where, Abscont. is absorbance of control and Absext is absorbance of the extract.

Determination of reducing power

The reducing power of the extract of *C. arborea* Roxb. was determined by the method of Oyaizu using ascorbic acid as standard [19]. 1 ml of the extract and 1 ml of the standard with various concentrations (10, 20, 40, 60, 80, and 100 µg/ml) were mixed with 2.5 ml of phosphate buffer (6.6 pH) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 30 minutes. The reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid and the mixture was centrifuged at 3000 rpm for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water, and 0.5 ml of 0.1% ferric chloride solution and the absorbance was taken at 700 nm using UV-Vis spectrophotometer. The higher absorbance of the reaction mixture indicated that the reducing power is increased.

Determination of total phenolic content

The total phenolic content of the leaf extract of *C. arborea* Roxb. was determined separately using the method of Mc Donald *et al.* [20] with modifications. Calibration curve was prepared by mixing 1 ml of methanolic solution of gallic acid (10, 20, 40, 60, 80, and 100 µg/ml) with 5 ml Folin–Ciocalteu reagent (diluted tenfold). After 3 minutes, 4 ml of sodium carbonate solution (0.7 M) was added, and the mixture was allowed to stand for 1 hr at room temperature. Absorbance was measured at 765 nm using UV-Vis spectrophotometer. 1 ml extract (50 µg/ml) was also mixed with the reagents above and after 1 hr the absorbance was measured to determine total plant phenolic content. From the calibration curve, the amount of phenolic compounds was determined and expressed as milligrams of gallic acid equivalent (GAE)/g of the dried extract.

Determination of total flavonoids content

The total flavonoid content of the extract of *C. arborea* Roxb. was determined by the aluminum chloride method [21]. 1 ml of the extract (50 µg/ml) was mixed with 2 ml of distilled water. After 5 minutes, 3 ml of 5% sodium nitrite ($NaNO_2$) and 0.3 ml of 10% aluminum chloride ($AlCl_3$) were added. After 6 minutes, 2 ml of NaOH (1 M) was added, and the volume was made up to 10 ml with distilled water. After 1 hr, absorbance reading was taken at 510 nm. A standard curve was prepared with quercetin at different concentrations (5, 10, 20, 40, 60, 80, and 100 µg/ml). From the calibration curve of the reference standard, the total flavonoid content was determined and expressed as milligrams of quercetin equivalent (QE/g) of dried extract.

RESULTS AND DISCUSSION

The phytochemical screening in this study has revealed the presence of secondary metabolites such as saponin, steroids, glycosides, flavonoids, and tannins in the leaves extract. These secondary metabolites contribute significantly toward the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy activities, etc. [22]. The phytochemical constituents present may be liable for the antioxidative efficacy as these phytochemicals act as antioxidants [23].

There are several mechanisms by which antioxidants can act. One of them is by scavenging of reactive oxygen and nitrogen free radicals. There are many different experimental methods by which the free radical scavenging activity can be estimated. One such method, by which total free radical scavenging can be evaluated, is by determining their efficiency to scavenge DPPH radicals [24]. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. In general, DPPH scavenging activities increased with increasing phenolic components such as flavonoids, phenolic acids, and phenolic diterpenes. These phenolic components possess many hydroxyl groups including o-dihydroxy group which have very strong radical scavenging effect and antioxidant power [10]. In the DPPH assay, the DPPH radical is scavenged by antioxidants through the donation of electrons forming the reduced DPPH. The color changes from purple to yellow after reduction, and the accompanying decrease in absorbance can be quantified at wavelength 517 nm. Inhibition concentration (IC_{50}) parameter was used for the interpretation of the results from DPPH method. The discoloration of the sample was plotted against the sample concentration in order to calculate the IC_{50} value [25]. It is defined as the concentration in µg/ml of the extract to scavenge 50 % of the DPPH radical, and lower IC_{50} values indicate higher antiradical activity. The dose response curve of DPPH radical scavenging activity of crude extract of the plant was observed, when compared with standard butylated hydroxytoluene (BHT) and shown in Fig. 1. Antioxidant activity in the form of IC_{50} values of the extract were calculated as 2.393 µg/ml compared to standard BHT, which exhibited 1.962 µg/ml (Fig. 1) and the scavenging effect can be attributed to the presence of active phytoconstituents in them.

The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity [26]. Compounds with reducing power indicate that they are electron donors and can reduce the

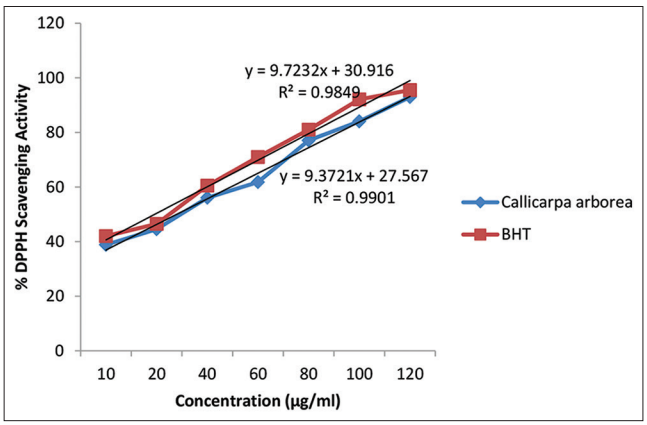

Fig. 1: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity of *Callicarpa arborea* Roxb. extract

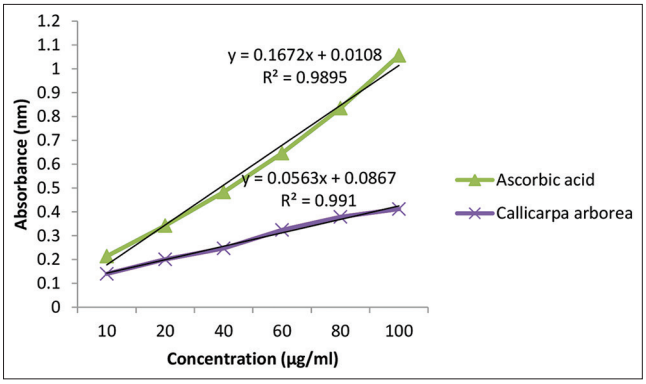

Fig. 2: Reducing power activity of *Callicarpa arborea* Roxb. extract

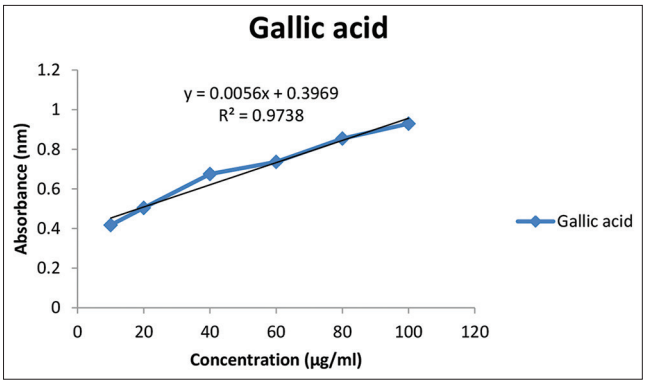

Fig. 3: Standard calibration curve for total phenolic contents

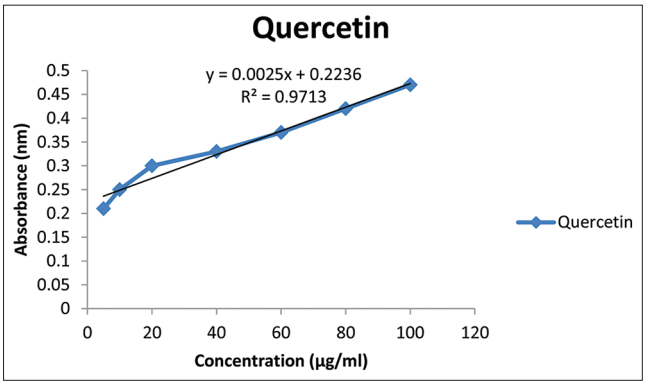

Fig. 4: Standard calibration curve for total flavonoids contents

oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. In this assay, the presence of an antioxidant in the extract resulted in reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). Concentration of ferrous ions can be determined by absorbance measurement at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power of the extracts. In this assay, the reducing power of the extract increased with the increase in their concentration suggesting that some compound in the extract may be able to donate hydrogen atom to break the free radical chain reaction [10,27]. The antioxidants present in the extract of *C. arborea* Roxb. caused their reduction of Fe^{3+}/ferricyanide complex to the ferrous form, and thus proved the reducing power even though the reducing power of the extract were lower than the standard ascorbic acid (Fig. 2).

Phenolics and flavonoids have been reported to be the main phytochemical responsible for antioxidant capacity of fruits and vegetables. Plant-derived polyphenols display characteristics inhibitory pattern towards oxidative reaction *in vitro* and *in vivo*. Phenolics or polyphenols are plant secondary metabolites and are very important by virtue of their antioxidant activity by chelating redox active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversation into reactive oxyradicals [28,29]. In this study, a total phenolics concentration equivalent of gallic acid was estimated according to Folin–Ciocalteu method. Gallic acid being the most important polyphenol in natural products was used to determine the phenolics of tested plant extract which are found to be 130.6 mg GAE/g (Fig. 3)

Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. They are a potent water-soluble antioxidant and highly effective scavenger of most oxidizing molecule including singlet oxygen and various free radical implicated in various disease, which prevent oxidative cell damage and also have strong anticancer activity The rich flavonoid plants could be a good source of antioxidant that would help to increase overall antioxidant capacity of an organism and protect it against lipid peroxidation [30]. The total flavonol content was expressed as mg of quercetin equivalent per gram of dry weight. The total flavonoid compounds in the extract of *C. arborea* Roxb. was found to be 83.9 mg/g dry weight of the sample (Fig. 4).

The results obtained in this work have considerable value with respect to the antioxidant activities of methanol extract of *C. arborea* Roxb. leaves. The activity of this extract may be attributed to the phenolic and flavonoid contents. Consequently, our results suggested that the extract can be utilized as an effective and safe antioxidant source. Although the antioxidant activities of methanol extracts were lower than that of the standard compounds. Indeed, there is a current need for availability of new plant-derived bioactive molecules; thus *C. arborea* Roxb. may be a great natural source for the development of new drugs. Further investigation on the isolation and characterization of the antioxidant constituents is however required.

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