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Research Article

Anti-oxidant effect of *Flemingia stricta* Roxb. leaves methanolic extract

Md. Shahrear Biozid[1,2], Mohammad Nazmul Alam[1,2]*, Md. Jainul Abeden[1], Ahmad Ibtehaz Chowdhury[1,2], Md. Faruk[1,2], Khandoker Usran Ferdous[2], Iffat Ara Nitul[2], Md. Masudur Rahman[1]

[1] Department of Pharmacy, International Islamic University Chittagong, Chittagong, Bangladesh

[2] Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh

*Corresponding author: Mohammad Nazmul Alam, E-mail: nazmulalam.pharm@gmail.com

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ABSTRACT

Aim of the study was to evaluate the possible anti-oxidant activity of *Flemingia stricta* leaf extract. In antioxidant study, plant crude methanol extract was evaluated for 1,1-diphenyl,2-picrylhydrazyl (DPPH) and reducing power capacity. Moreover, total phenolic and total flavonoid content of plant extracts were determined and expressed in mg of gallic acid equivalent per gram of dry sample (mg GAE/g dry weight). In the DPPH free radical scavenging assay, methanol extract showed concentration dependent inhibition of the free radicals. IC_{50} of ascorbic acid and *F. stricta* leaves were 4.25 µg/ml and 320.47 µg/ml respectively. In case of reducing capacity, the methanol extract at concentrations of 25, 50, 100, 200, 400 µg/ml, the absorbances were 0.56, 0.92, 1.41, 1.76, 2.23, respectively. Total phenolic content was estimated by gallic acid and expressed as milligrams of gallic acid equivalent (GAE). The methanol extracts contained a considerable amount of phenolic contents of 482±8.72 of GAE/g of extract and the total flavonoid content of the *F. stricta* leaf was estimated by using aluminium chloride colorimetric technique and found that the extract contained flavonoid content 340.625±4.50 of GAE/g of extract. These results suggested that the methanol extract of *F. stricta* Roxb. possess anti-oxidant activity.

Keywords: *Flemingia stricta*; Antioxidant activity; DPPH; Free radical scavenging; Phenol; Flavonoid.

1. INTRODUCTION

Flemingia stricta Roxb. (Fabaceae) is erect subshrub, distributed in southeast Asian countries: Bangladesh, Bhutan, China, India, Indonesia, Laos, Myanmar, Philippines, Thailand, and Vietnam [1-2]. The species is also used for traditional treatment such as bone fracture, cough, goiter and polio, asthma, and polio [3-5].

The phytochemical studies showed that methanol plant extract of *F. stricta* contains flavonoids, alkaloids, saponins, tannins, cardiac glycosides and phytosterols which made us inquisitive to study the antioxidant effect of this plant extract. Anti-oxidative effects of phenols, flavonoids help to eradicate free radicals which in turns protect cells from oxidative stress [6-8]. Oxidative damage generally caused by free radicals [9]. Free radicals are produced by normal physiological process which

is essential for our body such as granulomatous disease [10-11].

On the contrary, if larger amount of free radicals for instance reactive oxygen species, reactive nitrogen species are produces inside the body, then it causes great harm to the body which can results as cancer, diabetes, nephropathy, arthritis, cardiovascular diseases and various diseases [12-14]. For the prevention of this diseases created by free radicals, different types of synthetic, semi-synthetic drugs are used which is costly and also harmful for human being in some ways. On the other hand, drugs produced from natural resources are good for human being for instance easy drug excretion from the body as well as cost effective. Thus the experiment was designed to evaluate the in vitro anti-oxidant effect of *Flemingia stricta* leaves.

2. MATERIALS AND METHODS

2.1. Extract preparation

The leaves were dried under shade and ground. The ground (500 g) were soaked in methanol for one week at room temperature with occasional shaking and stirring then filtered through a cotton plug followed by Whitman filter paper No. 1. The solvent was evaporated under vacuum at room temperature to yield semisolid. The extract was then preserved in a refrigerator till further use.

2.2. Chemicals and drugs

DPPH (Sigma Aldrich CHEMIE GmbH USA), methanol was bought from Sigma-Aldrich, St Louis, USA, sodium carbonate (Na_2CO_3), ferric chloride ($FeCl_3$), aluminium chloride, potassium acetate potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), buffer and ascorbic acid were purchased from Merck (Darmstadt, Germany). Gallic acid (GmbH USA) and Folin-Ciocalteu reagent (FCR) was purchased from Merck Co. (Germany). All chemicals in this investigation were of analytical grade.

2.3. Phytochemical screening

Phytochemical screening of the crude extract was carried out employing standard procedures [15-18], to reveal the presence of various chemical constituents.

2.4. Test for tannins

Ferric chloride test: 5 ml solution of the extract was taken in a test tube. Then 1 ml of 5% ferric chloride solution was added. Greenish black precipitate was formed and indicated the presence of tannins.

Potassium dichromate test: 5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

2.5. Test for terpenoids

2.0 ml of chloroform was added with the 5 ml plant extract and evaporated on the water path and then boiled with 3 ml of H_2SO_4 concentrated. A grey color formed which showed the entity of terpenoids.

2.6. Tests for glycosides

Keller-Kiliani test: a solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with the 10 ml methanol plant extract and 1 ml H_2SO_4 concentrated. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.

Liebermann's test: 2.0 ml of acetic acid was added with 2 ml of chloroform with plant extract. The mixture was then cooled and we added H_2SO_4 concentrated. Green color showed the entity of aglycone, steroidal part of glycosides.

2.7. Test for steroids

2 ml of chloroform and concentrated H_2SO_4 were added with the 5 ml plant extract. In the lower chloroform layer red color appeared that indicated the presence of steroids.

2.8. Test for alkaloids

Mayer's test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a

test tube. Then 1 ml of Mayer's reagent was added. Yellowish buff color precipitate was formed and that was indicated as the presence of alkaloids.

Dragendorff's test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed and that was indicated as the presence of alkaloids.

2.9. Tests for reducing sugar

Fehling's test: 2 ml of plant extract was added 1 ml of a mixture of equal volumes of Fehling's solutions A and B. Boiled for few minutes. A red or brick red color precipitate was formed in the presence of a reducing sugar.

2.10. Test for saponins

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimeter layer of foam indicates the presence of saponins.

2.11. Test for phytosterols

Libermann-Burchard's test: extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

2.12. Test for flavonoids

Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Immediate development of a red color indicates the presence of flavonoids.

2.13. Antioxidant activity

Antioxidant activity was evaluated by DPPH, reducing power, total phenolic and total flavonoid content assay.

2.14. DPPH radical scavenging assay

Free radical scavenging ability of the samples, based on the scavenging activity of 1,1diphenyl,2-picrylhydrazyl (DPPH) free radical, was evaluated using the procedure described previously [19]. Different dilutions (31.3, 62.5, 125, 250, 500 and 1000 μg/ml) of plant extract (0.1 ml) were added to 0.004% methanol solution of DPPH. After 30 minutes, absorbance was determined at 517 nm using UV spectrophotometer. Ascorbic acid was used as positive control, percent scavenging activity was calculated as:

$[(A_0 - A_1)/A_0] \times 100$

where A_0 represents absorbance of control and A_1 is the absorbance of the plant extracts.

2.15. Reducing power capacity

The reducing power of *F. stricta* was determined according to the method previously described by Oyaizu [20]. Different concentrations of *F. stricta* extract (25-400 μg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml. 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean ± standard deviation.

2.16. Estimation of total phenolic content

Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method [21]. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent there by producing blue colored complex. The phenolic concentration of extracts was evaluated from a gallic acid calibration curve. To prepare a calibration curve, 0.5 ml aliquots of 15.6, 31.3, 62.5, 125, 250, and 500 μg/ml

gallic acid solutions were mixed with 2.5 ml Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 ml (75 g/l) sodium carbonate. After incubation at 25°C for 30 min, the quantative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer 1650 Shimadzu, Japan. The calibration curve was constructed by putting the value of absorbance vs. concentration. A similar procedure was adopted for the extracts as above described in the preparation of calibration curve. All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

2.17. Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination [22]. About 1 ml of the plant extracts/standard of different concentration solution (50, 100, 200, 400, 800 µg/ml) was mixed with 3 ml of methanol, 0.2 ml of aluminum chloride, 0.2 ml of 1 mol/l potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with spectrophotometer against blank. Methanol served as blank. The total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following equation:

$C=(c\times V)/m$

where C is total content of flavonoid compounds, mg/g plant extract, in gallic acid equivalent; c is the concentration of gallic acid established from the calibration curve in mg/ml, V is the volume of extract in ml, and m is the weight of crude plant extract in g.

2.18. Statistical analysis

The data was analyzed by Microsoft Excel 2010 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Statistical comparisons were performed using Student's t-test with the SPSS program (SPSS 20.0, USA). The values obtained were compared with the standard and were considered statistically significant when ($P<0.05$).

3. RESULTS

3.1. Phytochemical screening

The *Flemingia stricta* leaf extract was confirmed to contain chemical constituents such as such as tannins, glycosides, cardiac glycosides, alkaloids, saponins, phytosterols and flavonoids (Table 1).

Table 1. Chemical constituents of *Flemingia stricta*.

Chemical tests	*F. stricta*
Tannins	positive
Terpenoids	negative
Glycosides	positive
Cardiac glycosides	positive
Steroids	negative
Alkaloids	positive
Reducing sugar	negative
Saponins	positive
Phytosterols	positive
Flavonoids	positive

3.2. Antioxidant activity

3.2.1. DPPH free radical scavenging potential

In the DPPH free radical scavenging assay, methanol extract showed concentration dependent inhibition of the free radicals as shown in Figure 1. Here the methanol extract of *F. stricta* showed significant activity.

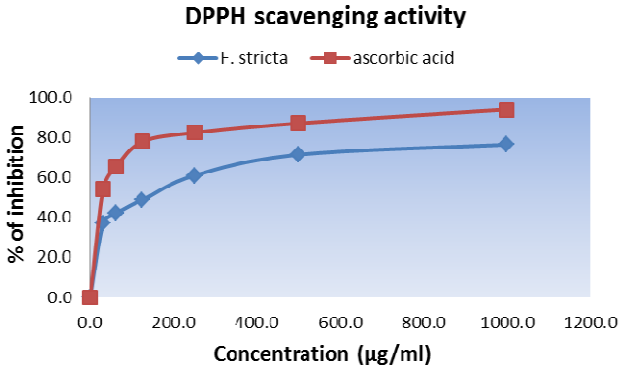

Figure 1. Antioxidant assay of plant extracts using DPPH assay.

At conc. 31.3, 62.5, 125, 250, 500, 1000 µg/ml, in the case of *F. stricta* leaves, the percentages of inhibition were 36.79, 41.85, 48.59, 60.67, 71.07 and 76.12 respectively. IC_{50} of ascorbic acid and *F. stricta* leaves were 4.25 µg/ml and 320.47 µg/ml respectively.

3.2.2. Ferric reducing capacity

The reducing capacity of a compound indicates its potential antioxidant activity. Figure 2 shows the dose response curves for the reducing power of methanol extract of *F. stricta* (25-400 µg/ml). At conc. 25, 50, 100, 200, 400 µg/ml, the absorbances were 0.56, 0.92, 1.41, 1.76, and 2.23, respectively. The extract displayed a concentration dependent increase in reducing power. The reducing power increased with increasing amount of the extracts. Higher absorbance of the reaction mixture indicates a higher reducing power. Thus, the present results showed that higher reducing power was evident in methanol extract of *F. stricta*.

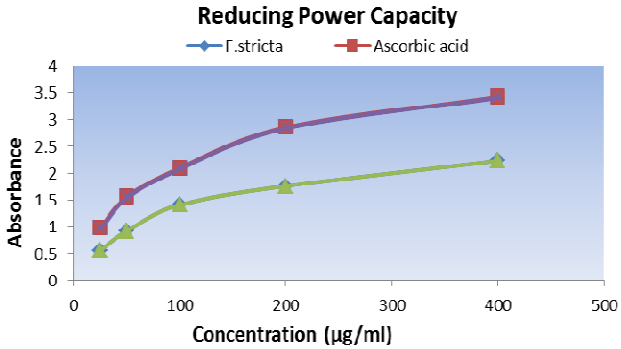

Figure 2. Antioxidant assay of plant extracts using reducing power capacity.

3.3. Determination of total phenolic content

Total phenolic content was estimated by gallic acid and expressed as milligrams of gallic acid equivalent (GAE). The methanol extracts contained a considerable amount of phenolic contents of 482 ± 8.72 of GAE/g of extract (Fig. 3).

3.4. Determination of total flavonoid content

The total flavonoid content of the *F. stricta* leaf was estimated by using aluminium chloride colorimetric technique and found that the extract contained amount of flavonoid content of 340.625± 4.50 of GAE/g of extract (Fig. 4).

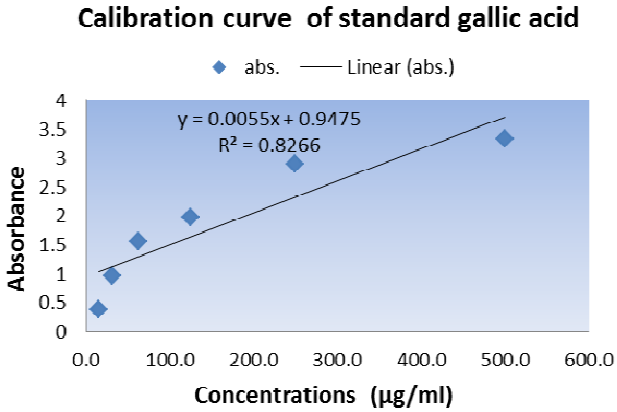

Figure 3. Calibration curve of gallic acid (total phenolic content).

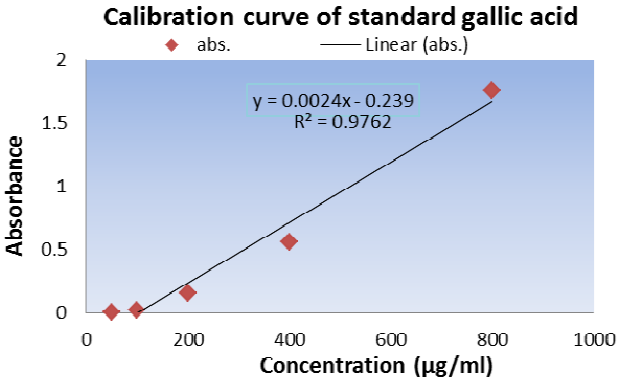

Figure 4. Calibration curve of gallic acid (total flavonoid content).

4. DISCUSSION

DPPH is a widely used system to assess the free radical scavenging potential of drugs [23]. Scavenging of DPPH radicals by antioxidants occurs through the donation of hydrogen, thus producing reduced DPPH-H that change the color from purple to yellow following reduction. DPPH is quantified by analyzing absorbance at wavelength of 517 nm [24]. The ability of electron donation of natural products can be measured by 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) purple-colored solution bleaching [25]. This method is based on scavenging of DPPH through the inclusion of a radical species or antioxidant which decolorize the DPPH solution. The concentration and potency of antioxidants are

proportional to the degree of color change. A considerable decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [26]. In the current study of methanol extract of *F. stricta* has significant higher inhibition percentage and correlated with total phenolic content.

In this study, methanol extract of *F. stricta* showed significant activity. IC_{50} of ascorbic acid and *F. stricta* leaves were 4.25 µg/ml and 320.47 µg/ml respectively. Results of this study suggest that the plant extract contain phytochemical elements that are capable of donating hydrogen to a free radical for the scavenging of the potential damage. In case of reducing power assay, the yellow color of the test solution changes to green which depends on the reducing power of the test specimen. The reduction of the Fe^{3+}/ferricyanide complex to the ferrous form is caused by the presence of the reductants in the solution. Therefore, Fe^{2+} can be observed by absorbance measurement at 700 nm. Previous reports indicated that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain [27]. At the concentration of 25, 50, 100, 200, 400 µg/ml, the absorbance were 0.56, 0.92, 1.41, 1.76, and 2.23 respectively. Increasing absorbance at 700 nm indicates an increase in reducing ability. Reduction of Fe^{3+}/ferricyanide complex to the ferrous from is caused by the antioxidants present in the methanol extract of *F. stricta*, and thus proved the reducing power. Phenolics are a class of antioxidant compounds which function as free radical terminators [28]. Previous reports indicate that the free radicals scavenging ability of phenolics is dependent on their molecular weight, presence of aromatic rings and nature of OH group's substitution [29].

Phenolic compounds of plants fall into several categories; among these are the flavonoids which have potent antioxidant activities [25]. In this test, Total phenolic content was estimated by gallic acid and expressed as milligrams of gallic acid equivalent (GAE). The methanol extracts contained a substantial amount of phenolic contents of 482±8.72 of GAE/g of extract. Flavonoids are naturally developing in plants and are thought to have positive effects on human health. Studies on flavonoid derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities [30, 31]. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [32] implicated in several diseases in the reduction of cholesterol and fat and also in the reduction of the risk of coronary heart disease. The total flavonoid content of the *F. stricta* leaf was measured by using aluminium chloride colorimetric technique and found that the extract contained considerable amount of flavonoid content of 340.625±4.50 of GAE/g of extract.

Based on the findings in the literature for this plant product, our results suggested that phenolic acids and flavonoids may be the major contributors for the antioxidant activity as the methanol of *F. stricta* and the contents of phenolics or flavonoids exhibited significant activity.

5. CONCLUSION

Even in this modern era, traditional medicines are used to treat several diseases because of its effective, safe and cost-effective profile. Our experiment was designed to evaluate the antioxidant effect of *F. stricta* plant extract which is generally used as traditional medicine for treating several diseases. Hence, the results obtained from this study indicated that crude methanol extract of *F. stricta* leaf possess considerable amount of anti-oxidant activity which can boost immune system of body by destroying free radicals. These results further support the use of this plant as traditional medicine. Further investigations are needed to decode the exact mechanism of action of this plant extract.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

MMR, MSB and MNA designed the whole study. MSB, MNA, AIC and MF collected the plant and arranged all the materials for laboratory experiments. MSB, MNA, MJA, AIC and MF performed antioxidant tests. MSB, KUF and IAN performed phytochemical screenings. MNA and MSB wrote the whole manuscript. All authors read and appro-

ved the final manuscript.

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