Comparative assessment on *in vitro* antioxidant activities of ethanol extracts of *Averrhoa bilimbi, Gymnema sylvestre* and *Capsicum frutescens*

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ABSTRACT

Background: *Averrhoa bilimbi, Gymnema sylvestre* and *Capsicum frutescens* are medicinal plants commonly used as traditional medicine for the treatment of various diseases. The present study was designed to investigate the antioxidant activities of Ethanolic extract of A. *bilimbi, G. sylvestre and C. frutescens*. **Materials and Methods:** The antioxidant activity of the extracts were evaluated using total phenolic and flavonoid contents, ferric reducing power and the free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH). **Results:** Total phenolic and flavonoid contents were higher in *G. sylvestre* (53.63636 ± 0.454545 mg/g gallic acid equivalent) and *C. frutescens* (26.66667 ± 2.081666 mg/g quercetin equivalent) respectively. Reducing power of the crude ethanol extracts increased with the concentrations of the extracts and all the extracts showed moderate free radical scavenging activity against DPPH. The plant extract displayed moderate phenolic and flavonoid contents compared to gallic acid and quercetin equivalent respectively, whereas also exhibited significant scavenging of DPPH radical and reducing power compared with ascorbic acid as standard. **Conclusion:** Our study suggests that *G. sylvestre* has significant antioxidant activity. The antioxidant compound of this plant might be a therapeutic candidate against oxidative stress related diseases. Different sub-fraction of *A. bilimbi* and *C. frutescens* should be studied further to assess the effect. Further study is necessary for isolation and characterization of the active antioxidant agents for better treatment.

Key words: Antioxidant, *G. sylvestre*, ethanolic extract, free radical, oxidative stress

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INTRODUCTION

Antioxidants in a biological system have several functions, which include protection from oxidative damage and participating in the major signaling pathways of the cells. One of the major actions of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (ROS). ROS include the superoxide anion (O_2), hydrogen peroxide, nitric acid radicals and free radicals such as the hydroxyl radical are generated in living organisms during excessive metabolism.[1-3] These molecules are highly reactive and unstable and they cause extensive oxidative cell damage by chain reactions leading to cancer promoting mutation or cell death, age related degenerative diseases and a wide range of human diseases. In order to prevent or reduce this damage, all cells invariably contain antioxidants.[4-6] ROS such as superoxide, hydroxyl and peroxyl radicals are formed in human cells by exogenousl and endogenous factors.[7]

Several synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisol and tert-butylhydroquinone are commercially available and currently in use. However, use of such antioxidants is now constrained due to their side effects. It has been revealed that they stimulate the development of cancerous cells

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in rats. These findings have strengthened the efforts for the development of alternate antioxidants from natural sources.[8]

Moreover, antioxidants from natural origin increase the shelf-life of foods.[9] Consequently, consumption of antioxidants and accumulation of antioxidant in food materials protect the body against various agents. Many researchers have reported various types of antioxidants in different kinds of higher plants.[10,11] In this regards many antioxidants have already been isolated from different kinds of natural origin, such as oilseeds, vegetables, cereal crops, spices and herbs.[12,13]

G. sylvestre commonly known as Gurmar is a traditional herb used for medicinal purpose. It is distributed in the tropical forests of southern and central India, Bangladesh and Sri Lanka.[14] It is used as a treatment for diabetes for near two millennia.[15]

Averrhoa bilimbi also called *Bilimbi* are found throughout the Indonesia, Philippines, Bangladesh, Sri Lanka, Myanmar (Burma) and Malaysia. It is also common in other Southeast Asian countries. *Bilimbi* has been used in traditional medicine to control obesity and diabetes mellitus.[16] It also possesses anti-hyperlipidemic properties.[17]

Capsicum frutescens is an aromatic plant, which is spread throughout the tropical and subtropical regions. It is not only an edible vegetable, but also used in folk remedies to inhibit the growth of gastric pathogen *Helicobacter pylori*,[18] inhibit platelet aggregation[19] and also used as anti-diabetic, GI stimulant, mildly diuretic.[20,21]

Until date, there is no detail information in the scientific literature on the antioxidant properties of aqueous extract of *Gymnema sylvestre*, *A. bilimbi* and *C. frutescens*. Therefore, the aim of this study is to investigate whether herbal preparations (aqueous extract) from *G. sylvestre*, *A. bilimbi* and *C. frutescens* possess antioxidant activity or not, which may be beneficiary for diseases caused by ROS. In addition, relationship between antioxidant activities, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, total phenolic content and flavonoid content were also analyzed.

MATERIALS AND METHODS

Sample collection

C. frutescens, *G. sylvestre* and *A. bilimbi* were collected from Bangladesh Council of Scientific and Industrial Research laboratories and brought directly to our laboratory. Samples were picked up and washed through tap water first then with distilled water to remove dirt particles. Samples were cut into small pieces and then dried first in sunlight followed by electric dryer at a temperature of 60°C - 70°C. The samples were pulverized by mechanical grinder. Later on powder sample was kept into separate airtight container to preserve.

Extract preparation

C. frutescens, *G. sylvestre* and *A. bilimbi* was then subjected to ethanol extraction. Each extraction processes repeated twice. After that extracts were subjected to filtration in a cotton filter and filtrates were collected. Using a hot water bath at 60°C solvent from the extracts evaporated and kept in vacuum container for 72 h then they were concentrated using a rotary evaporator.

Procurement of chemicals and drugs

We purchased DPPH (trichloroacetic acid) and ferric chloride from Sigma Chemical Company of USA, Ascorbic acid from SD Fine Chem. Ltd. of India and ammonium molybdate from Merck, Germany.

Total phenolic compound

Amount of phenolic content of ethanol extracts were determined by widely used Folin-Ciocalteau method.[22] Aliquot of samples were mixed with Na_2CO_3. At room temperature in dark absorbance of all aliquots measured at 760 nm wave length twice for each sample at the same concentration. Total phenolic content was expressed as mg gallic acid equivalent (GAE)/g of extract.

Total flavonoid content determination

Kumaran and Karunakaran method we used to determine the flavonoid content[23] and quercetin was used as standard. The 1 mg extract was mixed with 1 ml of aluminium trichloride in ethanol (20 mg/ml) and then one drop of acetic acid added. The mixture was then diluted up to 25 ml with ethanol and 40 min later, the absorbance measured at a wave length of 415 nm. Measurement of blank samples and standard quercetin was done under the same condition.

DPPH radical scavenging activity

The DPPH radical scavenging activity of our extracts was performed by using standard protocols.[24] This assay is based on the measurement of the ability of antioxidants to scavenge the stable radical DPPH. The extract was diluted in methanol to make 10, 50, 100, 500 μg/mL dilutions. 2 mm of each dilution was mixed with 1 mL of DPPH solution (0.2 mM/mL in methanol) and mixed thoroughly. The mixture was incubated in the dark at 20°C for 40 min. Free radical DPPH is reduced to corresponding hydrazine when it reacts with hydrogen donors.[25] The decrease of solution absorbance due to proton donating activity of

components of each extract was determined at 517 nm. Lower absorbance of mixture indicates higher free radical scavenging capability.[26] Absorbance of DPPH activity was measured at 517 nm using ultraviolet-vis spectrophotometer with methanol as blank. Vitamin C was used as the positive control. Finally, the result was measured at IC_{50} value and then the percentage of scavenging of DPPH by the extracts was calculated as formula:

$$\text{Percentage DPPH radical scavenging} = ([A_c - A_t]/A_c) \times 100.$$

Here, Ac is the absorbance of the control (DPPH) and A_t is the absorbance of the test samples.

Reducing power

The reducing power of our sample extracts was determined by the method of Oyaizu.[27] Different concentration of sample extract (10, 50, 100, 500 μg) in 1 ml of distilled water was 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. Centrifugation of the mixture was done at 3000 rpm for 10 min after adding little amount of (2.5 ml) of trichloro acetic acid (10%). The upper layer of the solution was mixed with distilled water and $FeCl_3$ (0.5 ml, 0.1%) and then reading of the absorbance was taken at 700 nm. Here, ascorbic acid was reference standard and the blank solution had phosphate buffer in it.

RESULTS

TPC

Total Phenolic content followed by Folin-Ciocalteau method is showed in Table 1. Phenolic content of *A. bilimbi*, *G. sylvestre* and *C. frutescens* is 38.78788 ± 0.946212, 53.63636 ± 0.454545 and 33.0303 ± 2.049659 mg/g GAE respectively after replicating the process thrice [Table 1].

Table 1: Total phenolic and flavonoid content

	Total phenolic content	Total flavonoid content
A. bilimbi	38.78788±0.946212*	1.666667±0.872334*
G. sylvestre	53.63636±0.454545*	18.66667±7.767453*
C. frutescens	33.0303±2.049659*	26.66667±2.081666*

*Denotes P<0.05. *A. bilimbi=Averrhoa bilimbi*; *G. Sylvestre=Gymnema sylvestre*; *C. frutescens=Capsicum frutescens*

TFC

Using Kumaran and Karunakaran method and quercetin as standard TFC was determined in the present study. It was found that *A. bilimbi*, *G. sylvestre* and *C. frutescens* has 1.666667 ± 0.872334, 18.66667 ± 7.767453 and 26.66667 ± 2.081666 mg/g quercetin equivalents. The result we got here is statistically significant ($P < 0.05$) [Table 1]. This step was replicated thrice.

DPPH scavenging activity

The DPPH radical scavenging activity of *A. bilimbi*, *G. sylvestre* and *C. frutescens* is shown in Table 2. With the increase of the concentration of the extract, DPPH activity was found to increase. The inhibitory capacity of the plant extract was compared with the ascorbic acid standard. The DPPH activity in extracts from A. *bilimbi, G. sylvestre* and *C. frutescens* extracts was 635.066 ± 8.4102, 303.8639 ± 0.971and 423.066 ± 8.4102 respectively; whereas the standard has a value of 98.56 ± 0.754 μg/ml. Figure 1 shows the percentage of scavenging activity for different concentration of extracts and compared with the vitamin C standard. From the Figure IC_{50} value was measured which is shown in Table 2 with P value. When the values were compared for three extracts. The most effective radical scavenging activity was shown by *G. sylvestre*, while the least effective was the extract of *A. bilimbi*. *C. frutescens* also showed significant scavenging activity.

Table 2: DPPH assay

	IC_{50} value	P value
A. bilimbi	635.066±8.4102	>0.05
G. Sylvestre	303.8639±0.971	<0.05
C. frutescens	423.26±107.2199	<0.05
Ascorbic acid	98.56±0.754	<0.05

A. bilimbi=Averrhoa bilimbi; *G. Sylvestre=Gymnema sylvestre*; *C. frutescens=Capsicum frutescens*; DPPH=1,1-diphenyl-2-picrylhydrazyl

Reducing power

By using the $K_3Fe\ (CN)_6$ reduction method the reducing capacity of the plant extracts was identified in comparison with ascorbic acid, which demonstrated in Figure 2. The reducing power of the extracts was moderately strong while increasing concentration shows a gradual increase in reducing power.

DISCUSSION

Plant produces a large number of phenolic compounds with several biological activities.[28] Phenolics such as phenolic acids, flavonoids and tannins are considered to be the major contributor to the antioxidant ability of plants. These antioxidants of the plant also possess diverse biological activities, such as anti-carcinogenic, free radical scavenger, anti-atherosclerotic and anti-inflammatory, activities. These all activities are related to their antioxidant capacity.[29] Phenolic compounds contribute evidently to antioxidative action and they constitute the major class of natural antioxidants present in plants;[30] therefore, it is necessary to calculate total phenolic content in plant species. In present study, using Folin-Ciocalteau method we found different response from three different samples in various concentration for each which were quantitively

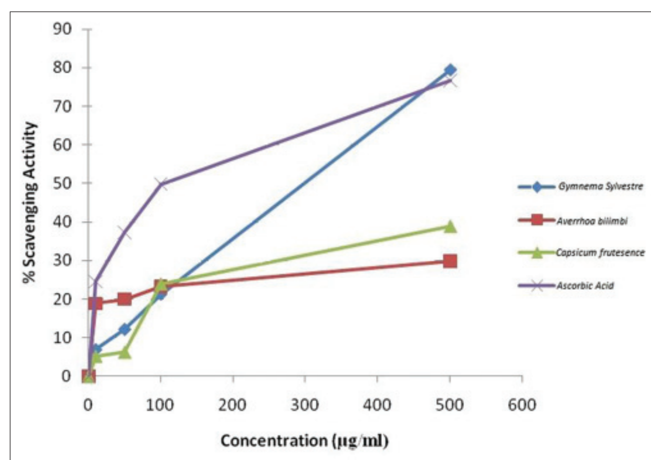

Figure 1: Scavenging activity

expressed as mg GAE/g of extract. Here, sodium carbonate produces blue color of phosphormolybdicphosphor tungstic phenol complex. Thus, most concentrated blue color contains highest total phenolic content.[31] According to results [Table 1] *G. sylvestre* has the highest amount of phenolic content than *C. frutescens* and *A. bilimbi*. Present studies indicate the presence of polyphenolic compound in *G. sylvestre*, *C. frutescens* and *A. bilimbi*.

The quantitative estimation of flavonoid content of *C. frutescens*, *G. sylvestre* and *A. bilimbi* shows that they are also rich with this compound. Thus our result of flavonoid content determination test shows [Table 1] that *C. frutescens* is rich in flavonoid than *G. sylvestre*, *A. bilimbi* but all are with a considerable amount. Flavonoids are the naturally occurring compounds in plants and thought to have positive influences on human health. Several studies on flavonoids derivatives showed that they have a wide range of anti-inflammatory antibacterial, antiviral, anti-inflammatory, antiviral, anticancer and anti-allergic activities.[32,33] It is well-established that plant flavonoids are highly effective, free radical scavenging and antioxidants. Flavonoids are used for the prevention and cure of different diseases.[34] Recent interest in these substances has been stimulated by the potential health benefits arising from their antioxidant activities and free radical scavenging capacities in coronary heart disease and cancer.[35]

Phenolic compounds are commonly found in both edible and inedible plants and they have been noticed to have wider biological effects, including antioxidant activity. It has been reported that compounds such as phenolics, flavonoids, which contain hydroxils, are responsible for the radical scavenging activity of most plant.[34] The result of DPPH scavenging activity of ethanolic extracts [Table 2] indicates they are fairly significant scavenger of free radical when compared with standard ascorbic acid measured at IC_{50} value. IC_{50} value is a parameter able to inhibit 50% of the DPPH. IC_{50} value of

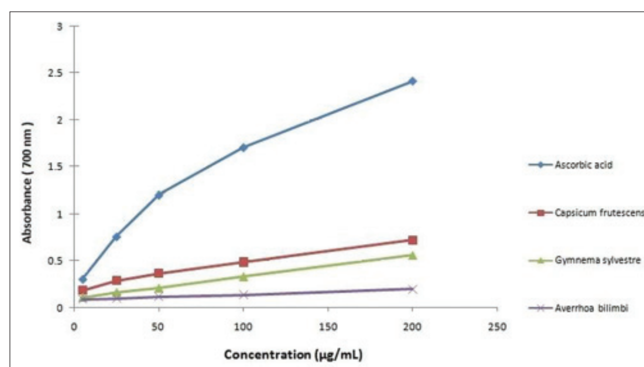

Figure 2: Reducing power

extracts was compared to the IC_{50} of the standard obtained by the same procedure. In comparative analysis, *G. sylvestre* is the most active and significant ($P < 0.05$) scavenger than other two, whereas *A. bilimbi* extract exhibited the lowest activity among three samples of different species [Table 2]. Extracts show a gradual increase in activity with increase of concentration. DPPH is a commonly used substrate for rapid assessment of antioxidant activity because of its stability and simplicity of the assay.[36] DPPH scavenging capacity of antioxidants is due to hydrogen donating ability. DPPH is stable nitrogen centered free radical, which produces violet color in ethanol solution[37] and accepts an electron or hydrogen radical to become more stable diamagnetic molecule. When a DPPH solution is mixed with a hydrogen atom donor, a stable non-radical form of DPPH is found with simultaneous change in color from violet to pale yellow.[38] This assay gives reliable information about the antioxidant activity of the tested compounds.[36,39,40] It is possible to correlate the reduction in the number of DPPH molecules with the number hydroxyl groups.[41]

Reducing power is a good pointer of antioxidant activity. The plant having high reducing power generally reported to carry high antioxidant potential too.[34] Reduction of Fe(III) by electron-donating activity of the compounds reflects the antioxidant mechanism of the compound. In this experiment, Ferric ions are reduced to ferrous with the color of the reaction mixture changes from yellow to bluish green. The ferric reducing power activity of *C. frutescens*, *G. sylvestre* and *A. bilimbi* extract with compared to ascorbic acid are reported in Figure 2. Extract exhibited dose dependent reducing power potential. However, the efficacy was found to be lower than that of ascorbic acid.

This study indicates that *G. sylvestre* has a significant antioxidant activity and the ethanolic extract of *C. frutescens* has moderate antioxidant activity. These plants can be further assessed for active antioxidant compound and future therapeutic potential.

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