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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT PROFILING OF TWO MANGROVE SPECIES OF SUNDARBANS: *Heritiera fomes* and *Sonneratia apetala*

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Abstract

In search of prevention and cure of diseases, human have relied on plant kingdom from the very beginning of civilization. Mangrove forest has become an endless source of numerous medicinal plants from the very beginning. This present study was aimed to investigate phytochemicals present in methanolic leaf extracts of two mangrove species: *Heritiera fomes* and *Sonneratia apetala*. In this study both leaf extracts showed presence of important phytoconstituents namely: carbohydrate, alkaloid, tannin, steroid and many more. Besides assessment of DPPH free radical scavenging acitivity, total phenolic content, total flavonoid content and total antioxidant activity were done to evaluate the antioxidant potential of the selected leaf extracts. This investigation proved better antioxidant potential of *Heritiera fomes* than *Sonneratia apetala* in all experiments. Both of this plants can be great sources of novel phytochemicals with medicinal value demanding more extensive research works in future.

Keywords: *Medicinal plant, mangrove forest, phytochemicals, methanolic leaf extract, antioxidant*

Introduction

Plants and natural products are used by people for century as food and medicines for cure and prevention of diseases [1]. At present, more than 80% of the world population depends on traditional and plant derived medicine. In the last century, roughly 121 pharmaceutical products were formulated from traditional herbal sources [2]. Pharmacologists, microbiologists, biochemist, botanists, and natural-products chemists all over the world are currently investigating medicinal plants for phytochemicals and lead compounds that could be developed for treatment of various diseases [3].

Mangroves are assemblages of halophytic woody covering about 75% of the world's tropical coastline [4]. Their distinctive ecological behavior, morphology, and traditional uses made researchers to work on them. Usually mangroves are rich in polyphenols and tannins. Mangrove leaves contain phenols and flavonoids that serve as ultraviolet (UV) screen compounds. Substances in mangroves have long been used in folk medicines to treat diseases as they significant activity against animal, human, and plant viruses including human immunodeficiency virus [5]

The mangrove forest Sundarbans is named after the tree *Heritiera fomes* L. (local name Sundri). It is an evergreen medium sized tree, growing up to 25m in height. This species is a well-known mangrove plant for its significant traditional use(s) by the local traditional health practitioners against various diseases in the southern areas of Bangladesh. *H. fomes* leaves, roots, and stems are used by rural people for the treatment of gastrointestinal disorders, skin diseases, and hepatic disorders. Bark is used for diabetes and goiter in rural areas. This plant is also used to cure pain and fever in local areas [5]. Mahmud et al., (2014) [5] claimed about its significant antioxidant and antidiabetic property. Due to the presence of high amount of procyanidins, *Heritiera fomes* can act both as radical scavenger and 15-lipoxygenase inhibitor.

Sonneratia apetala Buch.-Ham., abundantly grown in the coastal areas in India, Bangladesh, Malaysia, Australia, etc, is a fast growing mangrove of 7.2 m high with quadrangular branches. According to Bandaranayake (1998) [6], fruits and barks of the plants belonging to genus *Sonneratia* have remedial activity against asthma, febrifuge, ulcers, swellings, sprains, bleeding, and hemorrhages. There are few reports on the antibacterial and antioxidant activities of fruit of *S. apetala* [7] and other *Sonneratia* species [8]. Hossain et al., (2013) [7] reported that *S. apetala* fruit extracts have antioxidant, antidiabetic and antibacterial activities. Jaimini et al. (2011) [9] determined the antibacterial potential of *S. apetala* leave extracts. Also, Patra et al. (2015) [10] focused on both *S. apetala* leaf and bark extracts and they concluded that the extracts have potent antibacterial, antioxidant, antidiabetic and anticancer properties. Hossain et al., (2013) [7] found antihyperglycemic activity of seeds and pericarps of *S. apetala* fruits in STZ induced diabetic mice. So this study investigated for major phytochemicals of the crude extract and ended up with assessment of antioxidative acitivity of them.

Method

Place of study: This study was carried out at Natural Product Research Laboratory of Department of Pharmacy, Jahangirnagar University, Savar, Dhaka- 1342.

Collection of Plant Leaves: Fresh leaves of *Heretiera fomes* (Sundri) and *Sonneratia apetala* (Kaora) were collected from Mangrove forest Sundarban (Karamjol area) which grow prominently throughout that area. They were referenced by skilled botanist (DACB- 54665 for *H. fomes* and DACB- 54904 for *S. apetala*.

Preparation of leaf extract:

The fresh leaves were washed separately and carefully with distilled water to remove any extraneous materials. Then leaves were air dried under shade for 7 days then dried in the oven at 65 °C. And then the dried leaves were pulverized into coarse powder. About 1 kg of the each powder was extracted with 2.5 L of methanol for 48 hours using Soxhlet apparatus. Then the efficient and gentle removal of solvents from samples was done by Rotary Evaporator and then the extract left behind was stored at 4 °C in a refrigerator.

A. Preliminary photochemical screening:

It involves testing of leaf extracts of *Heretiera fomes* and *Sonneratia apetala* for their contents of different classes of compounds [11]. Phytochemicals were detected by qualitative chemical test to get general idea about their presence in crude drug. The qualitative chemical tests for various phytoconstituents were carried out for all the extracts of *Heretiera fomes* and *Sonneratia apetala* separately.

1. ***Tests for Carbohydrate:***

a) **Molisch's test (General test for Carbohydrates)**: A red or reddish violet ring will be formed at the junction of the two layers if a carbohydrate is present. On standing or shaking a dark purple solution will form.

Procedure: Two drops of molisch's reagent (10% alcoholic solution of α-naphthol) need to be added to 2ml of aqueous extract. 2ml of conc. sulfuric acid is allowed to flow down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution.

b) **Barfoed's test (General test for Monosaccharides):** Red precipitate of cuprous oxide will be formed within 2 minutes if a monosaccharide is present.

Procedure: 1 ml of an aqueous extract of the plant material is added to 1 ml of Barfoed's reagent in a test tube and heat in a beaker for boiling water.

c) **Fehling's test:** A red or brick-red precipitate will be formed if a reducing sugar is present.

Procedure: 2 ml of an aqueous extract of the plant material is added to 1ml of a mixture of equal volumes of Fehling's solutions A and B then boiled for a few minutes. ***Test for Glycosides:*** A yellow color develops in the presence of glycoside.

Procedure: A small amount of an alcoholic extract of the fresh or dried plant material is

dissolved in 1 ml of water and few drops of aqueous sodium hydroxide solution are added in it.

2. ***Test for glucosides:***

A small amount of an alcoholic extract of the plant material is dissolved in water and alcohol, solution is divided into two portions and need to be treated in the following ways:

a) One of them is boiled with a mixture of equal volume of Fehling's solution A and B is boiled. Note any brick-red precipitate.

b) Other portion is boiled with a few drops of dilute Sulphuric acid for about 5 minutes, neutralized the mixture with sodium hydroxide solution, an equal volume of mixture of Fehling's solution A and B is added and then boiled.

c) Alkaline reagent test: yellow to red precipitate within short time

4. ***Test for Alkaloids:***

A small volume of each extract is neutralized by adding 1 or 2 drops of dilute H_2SO_4. This neutralized solution is treated with a very small amount of the following reagents and the respective color and precipitate formation is observed:

a) Mayer's reagent: Alkaloids give cream color precipitate with addition of Mayer's reagent.

Procedure: Solution (a) is prepared by dissolving mercuric chloride in distilled water.

Solution (b) is prepared by dissolving potassium iodide in distilled water.

Solution (a) and (b) are mixed and the volume was adjusted to 100 ml with distilled water.

b) Wagner's reagent: Alkaloids give a reddish brown precipitate with Wagner's reagent

3. ***Test for Tannins:***

A small quantity of the extract is boiled with 5 ml of 45% solution of ethanol for 5 minutes. Each of the mixture is cooled and filtered. The different filtrates are used for the following test:

a) Lead acetate test: A yellow or red precipitate is formed

Procedure: 5 ml of aqueous extract of the plant material is taken in a test tube and a few drops of a 1% solution of lead acetate are added.

b) Ferric chloride test: Test solution gives blue green color with ferric chloride

Procedure: Test solution is treated with ferric chloride solution.
Procedure: Test solution is treated with sodium hydroxide solution .

Procedure: Iodine and Potassium iodide are dissolved in water and the volume is made 100ml with distilled water.

c) Dragendroff's Reagent: Alkaloids give reddish brown precipitate with Dragendorff's reagent.

Procedure: Basic bismuth nitrate and Tartaric acid are dissolved in water. This solution is mixed with a solution containing Potassium iodide and water.

d) Hager's reagent: Alkaloids give yellow color precipitate with Hager's reagent

Procedure: Neutralized solution of extract is mixed with 1% solution of picric acid.

e) Tannic acid test: Alkaloids give buff color precipitate with
Procedure: Neutralized solution of extract is mixed with 10% Tannic acid solution.

5. ***Test for Flavonoids:***

A small quantity of the extract is heated with 10 ml of ethyl acetate in boiling water

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for 3 minutes. The mixture is filtered and the filtrates are used for the following test.

a) **Ammonium Test:** A yellow coloration in ammonia layer indicates the presence of the flavonoid.
Procedure: The filtrate is shaken with 1 ml of dilute ammonia solution (1%). The layers were allowed to separate.

b) **Aluminum Chloride Test:** The light yellow colour indicates the presence of flavonoid and when dilute NaOH and HCl is added the yellow solution turns colorless.
Procedure: The filtrates are shaken with 1 ml of 1% aluminum chloride solution and observed for light yellow color.

6. ***Test for Saponin:***

a) **Frothing test**: Production of a persistent frothing (which remains stable in heating)
Procedure: About 0.5 ml of extract is shaken vigorously with water in a test tube

b) **Haemolysis Test:** Appearance of Hemolytic zone.
Procedure: Add leaves extract to one drop of blood placed on a glass slide.

7. ***Test for Triterpenoids:***

Salkowski test: A reddish brown coloration in the interface.

B. Determination of antioxidant activities

1. ***DPPH Free Radical Scavenging Assay*** [12]

DPPH is a reactive free radical that acts as an electron acceptor (oxidant/ oxidizing agent) and causes oxidation other substances. On the other hand, antioxidants act as electron donors (reductant/ reducing agent). Antioxidants neutralize DPPH by being oxidized themselves. DPPH is found as dark-colored crystalline powder composed of stable free-radical molecules and forms deep violet color in solution. The scavenging of DPPH free radical (neutralization) is indicated by the deep violet color being turned into pale yellow or colorless.

The potential antioxidant activity of plant extracts was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Aliquots of 30 µL of a methanolic solution containing leaf extract were added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent

Procedure: The extract is mixed with 2 ml of chloroform and concentrated H_2SO_4 (3ml) carefully to form a layer.

8. ***Test for Steroids:***

Liebermann- Burchard's Test: A greenish color is produced which turns blue on standing if a steroid is present

Procedure: A small amount of a petroleum ether extract of the plant material is dissolved in 1 ml of chloroform then 2 ml of acetic anhydride is added with 1 ml of conc. sulphuric acid.

9. ***Fats & Fixed Oils***

a) **Stain test:** The stain on 1 filter paper indicates the presence of fixed oils.

Procedure: The small quantity of extract is pressed between two filter papers.

b) **Saponification test:** The formation of soap or partial neutralization of alkali indicates the presence of Fixed oils and Fats.

Procedure: A few drops of 0.5 N of alcoholic potassium hydroxide is added to small quantities of various extracts along with a drop of Phenolphthalein separately and heated on a water bath for 1-2 hrs.

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inhibition activity was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

2. ***Determination of Total Phenolics:*** [13]
13 Folin- Ciocalteu reagent was used for this determination. 2 mL of 80% methanol containing 1% hydrochloric acid was used for extraction of 200 mg of sample at room temperature on an orbital shaker set at 200 rpm for about 2 h. After centrifugation for 15 min at 1000g, the supernatant was decanted into 4 mL vials. The pellets were also extracted at same conditions. Total phenolics assay was done after combination of supernatant. One hundred microliters of extract was mixed with 0.75 mL of Folin-Ciocalteu reagent which was previously diluted 10-fold with distilled water. This mixture was allowed to stand at 22 °C for 5 min. After that, 0.75 mL of sodium bicarbonate (60 g/L) solution was added to the mixture. Absorbance was measured at 725 nm after keeping the mixture for 90 min at 22 °C. Results are expressed as gallic acid acid equivalents.
3. ***Determination of Total Flavonoid Content:*** [14]
An aliquot of the stock solution of the extract was transferred to a 10.0 mL volumetric flask and made to volume with methanol, resulting in the blank solution. A second aliquot of the stock solution was transferred to another 10.0 mL volumetric flask, a volume of the 2% $AlCl_3$ was added and made to volume with methanol, which was named test solution. After 25 min the absorbance of the test solution was measured at 430 nm against blank solution. The results were expressed as the amount of flavonoid (mg)/g of plant extract.
4. ***Determination of Total Antioxidant Capacity:*** [15]
An aliquot of 0.1 ml of sample solution containing a reducing species (in water, methanol, ethanol, dimethyl sulfoxide or hexane) was combined in an Eppendroff tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95°C for 90 min after capping tightly. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A blank solution was prepared using 1 ml of reagent solution and same solvent as the sample and it was incubated under the same conditions as the the samples. Antioxidant capacities was expressed as equivalents of ascorbic acid.

Results

A. Phytochemical Screening of Plant Extracts:

Findings of phytochemical screenings are presented in Table 01

B. Antioxidant Profiling of Plant Extracts:

1. DPPH Free Radical Scavenging Capacity of Extracts

Comparison of IC_{50} values between HF and SA leaf extract in DPPH free radical scavenging assay extract has been shown in Figure 02.

2. **Total Phenol Content of Extracts**

Total phenolic content of the *Heritiera fomes* (HF) and *Sonneratia apetala* (SA) leaf extract and was determined by using the Folin-Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the

test fractions were calculated using the standard curve of Gallic acid (y= 0.009x + 0.058; R^2 = 0.999 for HF and y= -0.0016x+ 0.4482; R^2= 0.6773). Methanol extract of *Heritiera fomes* leaf was found to

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of methanolic leaf extracts of *Heritiera fomes* (HF) and *Sonneratia apetala* (SA). The total flavonoid content was calculated using the standard curve of quercetin (y = 0.005x - 0.005; R^2 = 0.996

4.Total antioxidant capacity of extracts

Result: Total antioxidant capacity of methanolic leaf extracts of *Heritiera fomes* (HF) and *Sonneratia apetala* (SA) were evaluated by the phosphomolybdenum method and were expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.006x + 0.101; R^2= 0.991 for HF and y = -0.001x + 1.574;
R^2 = 0.8852 for SA) (Figure 4.28 and 4.29). Methanolic leaf extract of *Heritiera fomes* was found to possess the higher total antioxidant capacity than *Sonneratia apetala*. Total antioxidant capacity of the extracts was found to decrease in the following order: HF> SA (Figure 05).

Discussion

The leaves of *Heritiera fomes, Sonneratia apetala* contain phytoconstituets like

contain the greater amount of phenols than methanolic leaf extract of *Sonneratia apetala* (Figure 03).

3. Total Flavonoid Content of Extracts

for HF and y = 0.0048x + 0.1055 R^2 = 0.9977 for SA) and was expressed as quercetin equivalents (QE) per gram of the plant extract. Methanol extract of leaves of *Heritiera fomes* was found to contain the highest amount of flavonoid (Figure 04)

alkaloids, steroids, fats & fixed oil, flavonoids, tannins and carbohydrates. These phytoconstituents have their own medicinal values. Antioxidant profile of this two plants is considerable and can be good choice to reduce oxidative stress in different disease states of our body. More detailed study must be done for farther isolation leading to the pure compounds and quantitation of phytoconstituents leading to different intensive *in vivo* studies.

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Table 01: Phytochemical test results for *Heritiera fomes* and *Sonneratia apetala*

Sl. no.	Chemical Constituents	Test	Result of *H. fomes*	Result of *S. apetala*
01	Carbohydrates	Fehling's Test	+	+
		Molisch's Test	++	+
		Barfoed Test	++	+
02	Glycoside	Sodium hydroxide Test	-	-
03	Glucoside	Fehling's Test	+	+
		Dilute H_2SO_4 Test	+	+
04	Tannin	Lead acetate test	+	+
		Ferric chloride test	+	+
		Alkaline reagent test	+	+
05	Alkaloids	Mayer's Test	++	+
		Wagner's Test	++	+
		Hager's Test	+	+
		Dragendroff's Test	+	+
		Tannic Acid Test	++	+
06	Flavonoids	Ammonium Test	++	-
		Aluminium Chloride Test	++	-
07	Saponin	Foam Test	+	-
		Haemolysis Test	-	-
08	Triterpenoids	Salkowski Test	+	++
09	Steroids	Liebermann- Burchard's Test	++	+
10	Fats and Fixed Oils	Stain Test	++	+
		Saponification Test	+	+

[++ = strongly present, + = present, - =absent]

Figure 01: Leaves of *Heritiera fomes* (left) and *Sonneratia apetala* (right)

Figure 02: Comparison of IC_{50} values between HF and SA leaf extract in DPPH free radical scavenging assay (Values are the mean of experiments)

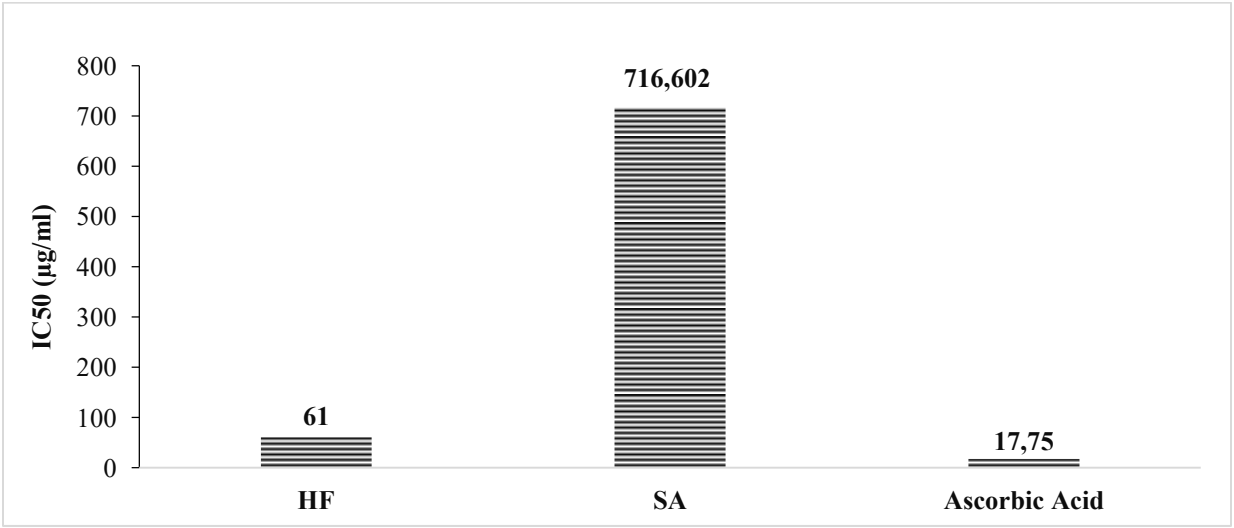

Figure 03: Comparison of total phenol content (Gallic acid equivalents, mg/gm) between HF and SA leaf extracts (Values are the mean of experiments and represented as mean ± SD)

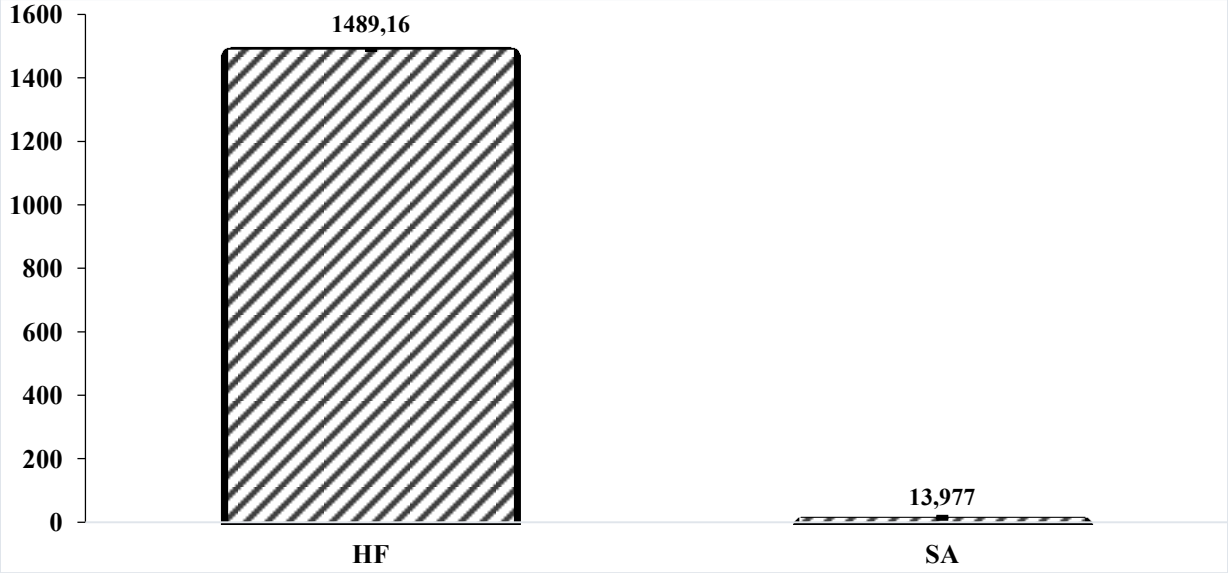

Figure 04: Comparison of flavonoid content between HF and SA leaf extracts (Values are the mean of experiments and represented as mean ± SD)

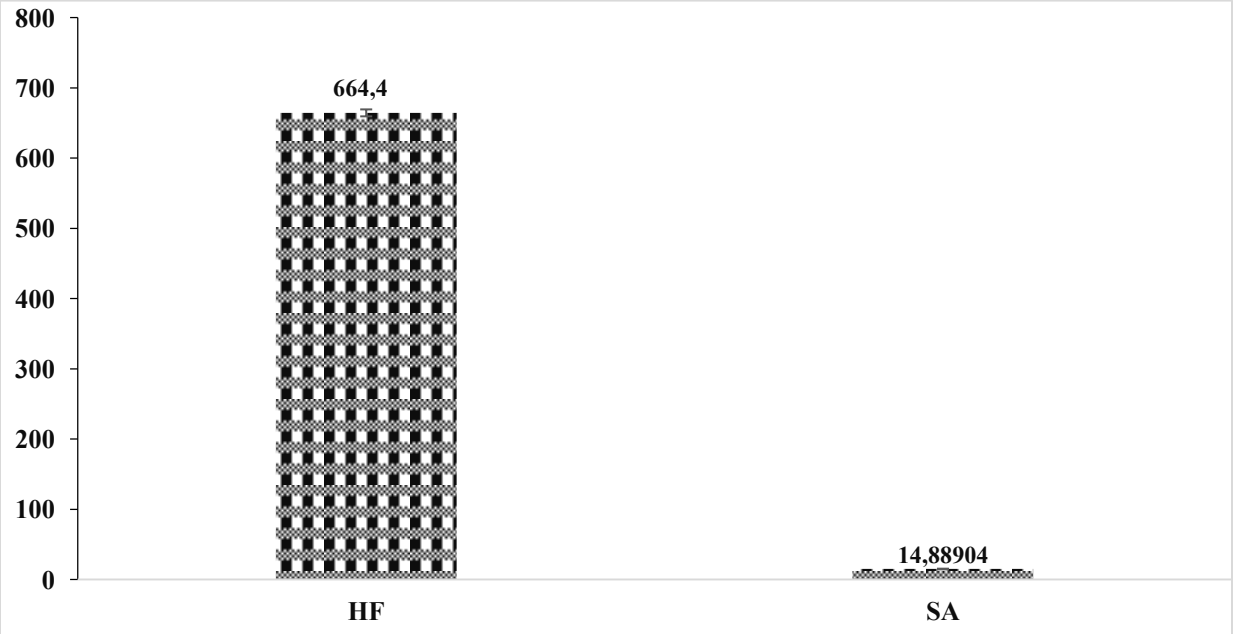

Figure 05: Comparison of total antioxidant capacity (mg/gm, Ascorbic Acid Equivalent) between HF and SA leaf extracts (Values are the mean of experiments and represented as mean ± SD)