

Formulation of Antioxidant Enriched Topical Skin Care Cream from *Sonneratia apetala* Fruit Extract.

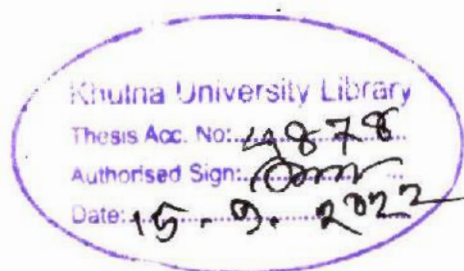
A Thesis Submitted

To

Biotechnology and Genetic Engineering Discipline, Life Science School

Khulna University, Bangladesh

**For the Partial Fulfillment of Requirements for the Degree of One and Half Years
Masters of Science in Biotechnology and Genetic Engineering**



Submitted by
Meher Nigar Shapla

MS 160707

Session # 2015-2016



Biotechnology and Genetic Engineering Discipline

Life Science School, Khulna University

Khulna-9208, Bangladesh

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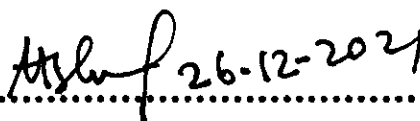
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Declaration

This thesis report is the original research work of the author, except as otherwise stated. The materials presented have not been submitted either in whole or in part for a degree for this or any other university. The findings of the research have not been/ will not be published anywhere without the concurrence of the concerned supervisor.

Meher Nigar Shapla
~~18.06.2019~~

18.06.2019

Meher Nigar Shapla

Date

Ms 160707

Dedicated To

My Parents.....

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It's my pleasure to express the greatest and deepest gratitude to the supreme of the universe, the almighty Allah, to whom all praise go for enabling me to complete my work.

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The Author

Abstract

Photo aging is a common problem that occurs in our community due to ongoing exposure to ultraviolet rays. Recently much attention has been focused on reactive oxygen species and free radicals which lead to oxidative stress and ultimately contribute to photo ageing. The use of antioxidants is an effective approach to prevent symptoms related to photo-induced ageing of the skin. Widely used synthetic antioxidants are now under question due to their adverse effects. Therefore, in response to the growing consumers concern, search for antioxidants from natural sources have gained interest. Thus the present study aims to formulate and evaluate antioxidant skin care cream comprising the fruit extract of *Sonneratia apetala* for their free radical scavenging activity. *S. apetala* is the principal planting species in the massive mangrove plantations of Bangladesh and is extensively used as food and for traditional medicinal purpose in the tropical coastal areas. The solvents used for the fractionation were methanol and water. Between the fractions, methanolic extract showed the highest polyphenols (201.702 mgGAE/g extract), total antioxidant capacity (442.947 μ M Fe(II)/g of dried extract) and lowest IC₅₀ value in DPPH free radical scavenging assay. In addition, methanolic extract of *S. apetala* fruit demonstrated antimicrobial activity by inhibiting the growth of *Staphylococcus aureus*, *Bacillus megaterium*, *Escherichia coli* and *Pseudomonas aurigonisa* with MIC values 6.25, 6.25, 6.25 and 12.5 μ g/ml, respectively. Based on the higher antioxidant potential and polyphenols content three formulations (F1= formulation from 2.5% methanolic extract, F2= formulation from 2.5% aqueous extract, F3 (placebo) = formulation without extract) were prepared. The formulations were evaluated for pH, spreadability, rheological property, homogeneity, loss on drying, stability, MIC and microbial limit test. Both the formulations (F1 and F2) showed good pH, homogeneity, appearance, ease to remove, spreadability but only F1 could successfully limit microbial growth in microbial limit test. The present result indicates that methanolic extract of *S. apetala* fruit has a good potential for the cosmeceutical product development.

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1.1 Introduction

Plants are essential for the survival of human beings and to the balance of nature. Human beings have been utilizing plants and plant parts for basic preventive and curative health care since the primitive era [Farnsworth and Soejarto 1991]. Plants not only provide essential nutrients but also other bioactive compounds for disease prevention and health promotion. *Sonneratia apetala* is a mangrove tree belonging to the family Lythraceae, abundantly grown in the coastal areas in Bangladesh, India, Malaysia, Australia etc and locally named as keora in Bangladesh. It is a fast growing mangrove tree used in the reforestation of salinity affected areas. The fruit is extensively consumed by the people of coastal Bangladesh especially adjacent to the sundarbans forest. Fruits are major source of dietary functional components such as polyphenoles, flavonoids, anthocyanins, vitamins etc [Hossain et al., 2013]. *Sonneratia apetala* is the principle planting species in the massive mangrove plantations of Bangladesh. In fact, it alone constitutes about 95% of the plantations on new accretions in coastal areas [Banglapedia]. Fruits are widely used as food and in treating various diseases in the tropical coastal areas. Despite medicinal value of the tree there are few reports on the antioxidative activity of it and other sonneratia species.

Recently much attention has been focused on reactive oxygen species and free radicals which lead to oxidative stress. Oxidative stress is an important contributor to a variety of pathological conditions. Free radicals are electrically charged molecules i.e. they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Generally, free radicals attack the nearest stable molecule, “stealing” its electron. When the “attacked” molecule loses its electron, it becomes a free radical itself, beginning a chain reaction and can be thousands of events long [Przedborski et al., 1998]. Once the process is started, it can cascade, finally resulting in the disruption of a living cell [Jesberger and Richardson 1991]. The most dangerous free radicals are the atomic and molecular varieties of oxygen which are known as reactive oxygen species (ROS). The ROS produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical (OH) and the superoxide anion (O_2^-) [Valko et al., 2007]. In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell [Baillie et al., 2009]. Almost all organisms are well protected against

free radical damage by antioxidative enzyme system such as superoxide dismutase and catalase or non-enzymatic antioxidant system such as α -tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione. However these systems are frequently insufficient to totally prevent the damage, especially under the conditions of severe oxidative stress resulting in progression of diseases [Patra et al., 2015]. For this regard supplementation of antioxidants by plant source *S. apetala* can be suggested since this plant is very much available in the coastal region of our country.

S. apetala is an enriched source of antioxidant and vitamins. Various epidemiological studies have suggested that consumption of *S. apetala* fruits is associated with reduced risk of cardiovascular diseases and cancer, neurodegenerative diseases such as parkinsons as well as inflammation and aging [Hossain et al., 2013].

Our skin can be benefited from antioxidant rich diets and also from antioxidant rich topical skin care cream since this fruit is rich in antioxidants. Antioxidants have one primary purpose and that is to neutralize the free radicals that naturally find themselves in our body. This presence of free radicals can result in damaged cell which leads to wrinkles, signs of aging, sagging skin and even thinning hair. Bangladesh has a subtropical climate; being high temperature and humidity almost throughout the year and Bangladeshi people exposes more to ultraviolet rays. Ultraviolet rays facilitate the aging process and cause the decrease in skin elasticity. So by increasing the intake of antioxidants and also by applying skin care cream supplemented with natural antioxidants from the available source of *Sonneratia apetala* can counter these negative skin changes and preserve the beauty. For this reason, the investigations of free radical scavenging activities of *Sonneratia apetala* fruit extract is done to know the ability of extract to combat or delay the photo aging process.

Besides, synthetic antioxidants are used in food industries and pharmaceuticals as additives. Widely used synthetic antioxidants are now under question due to their side effects, like carcinogenicity [Patra et al., 2015]. Therefore in response to the growing consumer concern, search for antioxidants from natural sources have gained interest and as *Sonneratia apetala* is abundantly grown in sundarbans and has known antioxidant activity, it can fulfill the growing demand of antioxidants in food, pharmaceuticals and cosmetics industries. Despite many

research is still ongoing for *Sonneratia apetala*, however so far the *Sonneratia apetala* fruits extract have not been used as an antioxidant formulation for skin care.

The study from Nair et. al., concluded that it is possible to develop creams containing herbal extracts having antioxidant property and they can be used as the alternative as a barrier to protect skin. The *Sonneratia apetala* fruits are proven to have high antioxidant due to its high in phenolic compounds content. Thus, the present study focused to develop a formulation that may scavenge free radicals and protect skin against oxidative damage.

1.2 Objectives

The objectives of the research work are mentioned below-

- Determining total antioxidant capacity of *S. apetala* fruit.
- Determining the amount of flavonoid, phenol and tannin contents of *S. apetala* fruit.
- Formulation of skin care cream with the supplementation of natural antioxidant metabolites from *S. apetala*.
- Determining the antibacterial activity and minimum inhibition concentration (MIC) of bacterial growth of *S. apetala* fruit extract and the formulated cream.



2.1 Plant Preview

Sonneratia apetala (Fruit)

Sample Name: *Sonneratia apetala* Buchanan- Hamilton.

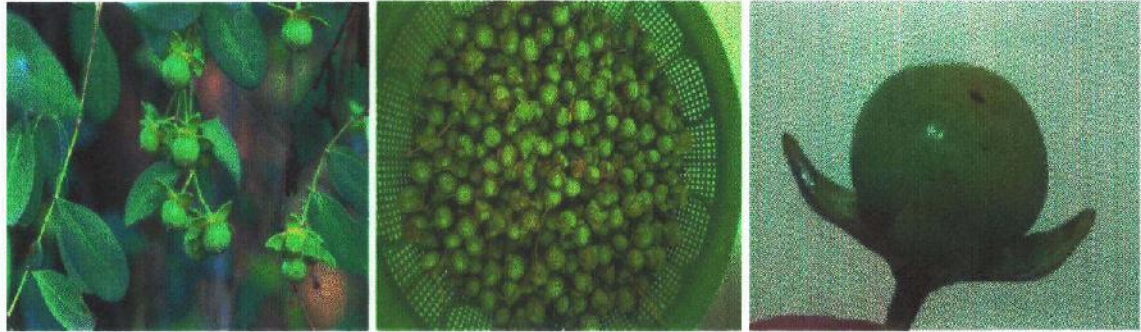


Fig 1: Fruits of *Sonneratia apetala*

2.1.1 Systemic Classification:

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Lythraceae

Genus: *Sonneratia*

Specific epithet: *apetala* Buch.-Ham.

Botanical Name: *Sonneratia apetala* Buch.-Ham

Local Name: Keora (Bengali), *Sonneratia* mangrove, Maram (Tamil), Kyalanki (Telugu), Khirwa (Oriya), Kandal (Marathi), Chipi (Marathi).

Distribution: Bangladesh, India, Sri Lanka, Myanmar, some coastal regions of Asia, Africa and Australia.

2.1.2 Plant Description

The plant has been named *Sonneratia* in honor of Pierre Sonnerat (1749-1814), who travelled to New Guinea, the East Indies and China, and introduced many new plants to the botanists of Europe. *Sonneratia* mangrove is a small to medium size tree, which can attain a height of about 20m and a girth of about 2.5m. The tree occurs on newly accreted soil in moderately to strongly saline areas and is considered as a pioneer species in ecological succession. The leaves are simple opposite, entire and leathery. Bark thin, light brown, irregularly fissured. Flowers are apetalous, cream colored, and arranged in axillary 3-flowered or 7 flowered dichasial cyme. The flowers have no petals but 4 prominent green sepals. Cream colored mass of stamens give the flowers a cream colored look. The style consists of a white, 2-3 cm long, curved, stigma, flattened like umbrella or mushroom. The upper portion is reddish in color. The fruit is globose, berrylike, leathery, indehiscent and crowned by style base. Seeds are numerous, embedded in foul-smelling pulp, irregularly angular or falcate, seed coat is thickened, roughened and somewhat corky.

Uses

The fruit of *S. apetala* is eaten in some of Bangladesh and Africa. They are also processed to produce sour sauce and marketed in many regions. The leaves are mainly used as fodder. The timber of the plant is resistant to shipworm and pests and is used for building boats, piling and posts for bridges and houses. However, the wood corrodes metal, probably because of the timber's high mineral content. The pneumatophores are made into floats for fishing nets.

Traditional Medical Uses

Sonneratia is used in poultices for cuts, bruises, sprains and swellings. The wall of an old fruit is given as a vermifuge. Ripe fruits are used to expel intestinal parasites and half-ripe fruit for coughs. Fermented fruit juice is said to be useful in arresting haemorrhage.

3.1 Literature Review

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reaction. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols. Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer, coronary heart disease and even altitude sickness. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and a large quantity in cosmetics industry.

A free radical is a compound with one or more unpaired electrons in its outer orbital [Jesberger and Richardson 1991]. Electrons strongly prefer to go around in pairs. So as a result of this lonely unpaired electrons, free radicals have a tendency to steal back an electron from neighboring molecules [Sreejayan and Rao 1997]. These neighboring molecules can be any of the foreign body's own active cellular component.

When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be "thousands of events long" [Goldfrab 1999]. Free radicals produced *in vivo* include superoxide, the hydroxyl radical, nitric oxide, oxygen-centered organic radicals such as peroxy and alkoxy radicals, and sulfur-centered thiol radicals. Other oxygen-containing reactive species that are not radicals are also formed. These include hydrogen peroxide, peroxynitrite, and hypochlorous acid. While these are not radical species, they are actually or potentially damaging oxidants. The collective term ROS is often used to describe both radical and non-radical species [Benzie and Strain 2005].

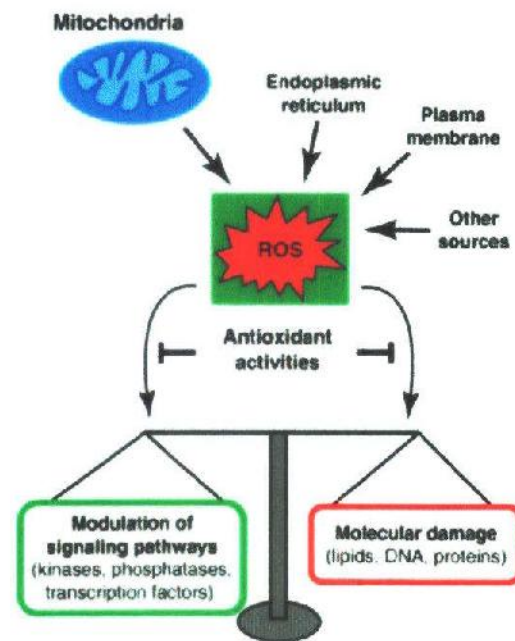


Fig 2: Cellular response to reactive oxygen species (ROS).

Free radicals are extremely harmful and can damage important molecules within the body. In our body there are many types of lipids or fat molecules. One type is responsible for maintaining the structural integrity of the cell membrane. Free radicals can cause damage to the cell membrane by oxidation thereby making them leaky [Shigenaga et al. 1994]. Oxidation of another type of lipid, the low-density-lipoprotein, is considered to play a major role in the development of atherogenesis (the thickening of arterial walls) [Witztum 1994]. Free radicals also cause damage to proteins and genes. Oxidative damage to DNA over time can cause change to both the structure and function of chromosomes. These changes in the genetic code lead to cancer and other chronic diseases [Agarwal and Sohal 1994]. Free radicals also interfere with protein functions, leading to irregular and abnormal metabolism. Free radicals spoil foods and degrade materials such as rubber, gasoline and lubricating as well [Dexter et al. 1994].

To neutralize the free radicals, antioxidant plays an important role. In the late 19th and early 20th century, extensive study was devoted to the use of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines [Matill 1947]. Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity and also in the use of daily products [German 1999]. However, it

was the identification of vitamin A, C and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms [Jacob 1996; Knight 1998].

The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized. However in oxidized state, they are not reactive itself. The human body is generally well equipped with an array of 'antioxidative' strategies to protect against the damaging effects of free radicals. Our antioxidant system is complex, and consists of various intracellular and extracellular, endogenous and exogenous, and aqueous and lipid-soluble components that act in concert to prevent ROS formation (preventive antioxidants), and terminate chains of ROS-initiated peroxidation of biological substrates (chain-breaking antioxidants). In addition, metals and minerals (such as selenium, copper, and zinc) that are the key components of antioxidant enzymes are often referred to as antioxidants.

Antioxidants may counter the action of free radicals in three ways: increase in levels of enzymes that degrade free radicals, increase in levels of proteins such as transferrin that can bind the metals which stimulate the production of free radicals, chemical antioxidants such as vitamin C and E act as direct free radical scavengers [Rahimi et al. 2005].

The importance of antioxidants in preventive medicine has been recognized for several years and polyphenols have become the focus of research interest due to the perceived health benefit effects [Wollgast and Anklam 2000]. Polyphenols have the ability to interfere with cell/tissue based assays in diabetes research by causing damage to cellular proteins, membrane lipids and nucleic acid. During the past decade the interest in polyphenols including flavonoids has increased considerably with nutritionist, epidemiologist and food manufacturers taking a special interest in polyphenols this is mainly due to their various biological properties like their antioxidant effects. Polyphenols include other subclasses such as phenolic acids, stilbenes, lignins, tannins and oxidized polyphenols [George et al. 2005].

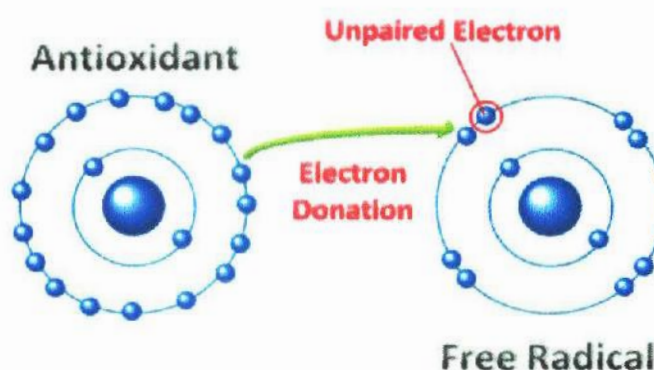


Figure 3: Role of antioxidants to neutralize free radicals.

Polyphenols constitutes one of the most numerous and distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently being identified [Wollgast and Anklam 2000]. Polyphenols are products of secondary metabolism of plants and originate from two main synthetic pathways; the shikimate pathway and the acetate pathway, both of which are derived or occur from glucose metabolism within plants [Wollgast and Anklam 2000]. Polyphenols may also exert their effect as chelators of divalent cations.

3.1.1 Reported antioxidant activity in *S. apetala*

Banerjee et al. (2008) reported the antioxidant activity of leaves, steam bark and root of *S. apetala* the amount of total phenolic content of leaves, steam bark, and roots in GAE (Galic Acid Equivalent) mg/g of dry material were 47.52 ± 2.22 , 42.68 ± 2.75 , and 42.75 ± 1.67 respectively. The reducing power was evaluated as AAE (ascorbic acid equivalent) mg/g of dry material and that for leaves, steam bark, and roots were 5.71 ± 0.24 , 7.06 ± 0.07 , and 6.87 ± 0.10 respectively. The evaluation of anti-radical properties of mangrove plant materials was performed by DPPH radical scavenging assay. The 50% inhibition of DPPH radical, IC₅₀ value for leaves, steam bark, and roots were 163.49 ± 6.32 , 193.09 ± 14.35 , and 183.04 ± 1.74 respectively.

According to Hossain et al. (2012) seeds of *S. apetala* consists of very high content of polyphenols (300 ± 8.2 mg GAE/g extract), flavonoids (30.6 ± 0.7 CE/g extract), anthocyanins (2.3 ± 0.03 μ mol/g extract) and vitamin C (4.0 ± 0.08 mg extract). The IC₅₀ values for DPPH and NO free radical scavenging were 4.3 and 49.4 μ g/ ml for seed extract and that for pericarp

extract were 59.8 and 751.6 $\mu\text{g/ml}$ respectively. Seed extract also showed very high reducing power (O.D. 1.14 at 50 $\mu\text{g/ml}$ extract) and total antioxidant capacity (280.8 GAE or 310.24 AAE/g extract). In streptozotocin (STZ) induced type 2 diabetics rats, seed extract treated group showed serum glucose decreased from 13.75 ± 2.21 mmol/L (at 30 min) to 10.3 ± 1.75 mmol/L (at 135 min) and in the pericarp treated group from 14.36 ± 2.16 to 11.32 ± 1.74 mmol/L. the area under the glucose curve was more profoundly decreased in the seed treated group than the pericarp treated group [Hossain et al. 2012].

3.1.2 Reported Antimicrobial Activity of *S. apetala*

Bobbarala et al. (2009) identified the antimicrobial activity of the mangrove plant extract of *S. apetala* on the various test microorganisms, including clinical multiple antibiotic resistant bacteria and phytopathogens. In vitro screening of *S. apetala* on the various test microorganisms, including clinical multiple antibiotic resistant bacteria and phytopathogens. *In vitro* Screening of *S. apetala* mangrove plant extracts showed species specific activity in inhibiting the growth of bacteria and fungi. Hexane, chloroform and methanolic extracts showed good activity against all the pathogens, whereas methanolic extracts were active against most of the pathogens.

According to Hossain et al. (2012) susceptibility test showed that methanolic seed extract of *S. apetala* inhibited the growth of both gram positive and gram-negative pathogenic bacteria. Methanolic seed extract inhibited the growth of all tested bacteria whereas methanolic pericarp extract showed no inhibition of *E. coli*, *E. faecalis*, *Pseudomonas sp.*, *S. flexneri* and *S. epidermidis*.



3.2 Antioxidant Enriched Topical Cream:

Skin is the largest and outermost organ of the body also known as cutaneous membrane or the integumentary system. The skin constitutes 7% of total body weight and about 0.02" to 0.16" in thickness in adults. Human skin acts as a frontier between interior and exterior of the body and performs various important functions. It regulates the transport of water and other substances

with environment and is responsible for defensive mechanisms such as protection from environmental hazards i.e., chemical, physical, biological and microbial [Arshad et al. 2016]. It also protects the body from lethal effects of solar radiations and reduces the free radical production and overall oxidative stress in the body. It is also responsible for thermoregulation during extreme temperature conditions (8). Skin consists of many layers among which stratum corneum (SC) is the super facial, tough and water repellant layer and act as main barrier by regulating the selective permeability of substances in and out of the skin [Pereira and Mallya 2015].

Photo aging is a common problem that occurs in our community due to ongoing exposure to ultraviolet rays. Long term exposure of human skin to ultraviolet rays produces reactive free radicals and induces oxidative stress on skin leading to the development of erythema, sunburn, hyperplasia, immunosuppression, photoaging, melanogenesis and DNA damage that can lead to skin cancer.

The use of chemical sunscreens as photoprotectives is less desirable now-a-days because it can cause photo contact dermatitis, skin tumors and estrogen like effects on prolonged use. Hence the use of photoprotectives from natural origin is gaining attention to prevent the deleterious effects of UV rays. The polyphenolic compounds such as flavonoids, phenolic acids, tannins and their hydrolysable and condensed polymers due to their antioxidant property can be used as photoprotectives [Pereira and Mallya 2015].

Most of cosmetic products are emulsions that function as drug delivery vehicle to the skin and are classified according to different body parts to be applied i.e., skin, teeth, hair and mouth. Emulsions are heterogeneous systems and consist of mixture of two immiscible liquids i.e., aqueous phase and oil phase and system is stabilized by the presence of third substance known as emulsifying agent or emulsifier. Main ingredients of these formulations are emulsifier, viscosity modifiers and some active ingredients having auspicious skin care properties. Peculiarly the use of botanical extracts with antioxidant activity provides most valuable cosmetic products. There is exorbitant curiosity about the use of herbal extracts containing phytochemicals with high antioxidant potential and play an important role in eviction of various degenerative diseases and production of modern phytocosmetic preparations. Most important bioactive phytoconstituents of plants include polyphenols, flavonoids, tannins and essential oils. The topically applied cosmetic

preparations are more beneficial in respect to solubility, spreadability, enhanced bioavailability and topical delivery of hydrophilic and hydrophobic ingredients [Arshad et al. 2016].

Due to these grounds there is more attention towards investigation of the plants and phytoconstituents having moisturizing and anti-aging properties. Phenolics are reported antioxidant compounds due to excellent free radical scavenging activity and provide emollient, moisturizing and anti-aging effects in various skin care products. *S. apetala* fruits are abundant in phenolics flavones and tanins. It has reported free radical scavenging activity. Thus, it should be considered valuable for maintaining proper health conditions and provides the basis for the selection of this fruit for skin care formulations.

4.1 Materials and Methods

4.1.1 Plant materials

Collection of the sample:

S. apetala fruit was selected for present research work. This fruit was collected from the local market (gollamari bazar) Khulna, Bangladesh on 27th july, 2018.

4.1.2 Preparation of Extract

Drying and Grinding:

The fruits were carefully cleaned and separated from undesired materials and cut into small pieces. The sliced plants materials were shed dried for several weeks until all the water evaporated and a biscuit like fragile comes. After completion of drying they were ground into powder form with a grinder. The powder of plants were weighted and stored in airtight plastic bags in a dark cool and dry place for further use.

Extraction with solvent

The powder of *S. apetala* fruits were taken into 2 different clean, flat-bottomed glass jars and soaked into 99% methanol and distilled water respectively. They were then sealed and kept for a period of 8 days in a dark room accompanying occasional shaking and stirring. The mixtures filtered coarsely using white cotton material.

Evaporation and storage

Filtrates obtained from two different solvent systems were evaporated at room temperature with an electric fan until they were completely dried. The crude extracts were weighed and kept in refrigerator for further experiments.

4.1.3 Methods

4.1.3.1 DPPH Free Radical Scavenging Assay:

Principle

A simple, rapid and inexpensive method to measure antioxidant capacity of sample involves the use of free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [Sharma & Bhat 2009]. It is a dark-

colored crystalline powder composed of stable free-radical molecules and a trap ("scavenger") for other radicals. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. Antioxidants reduce 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured (Figure 4) [Cuvelier et al. 1992].

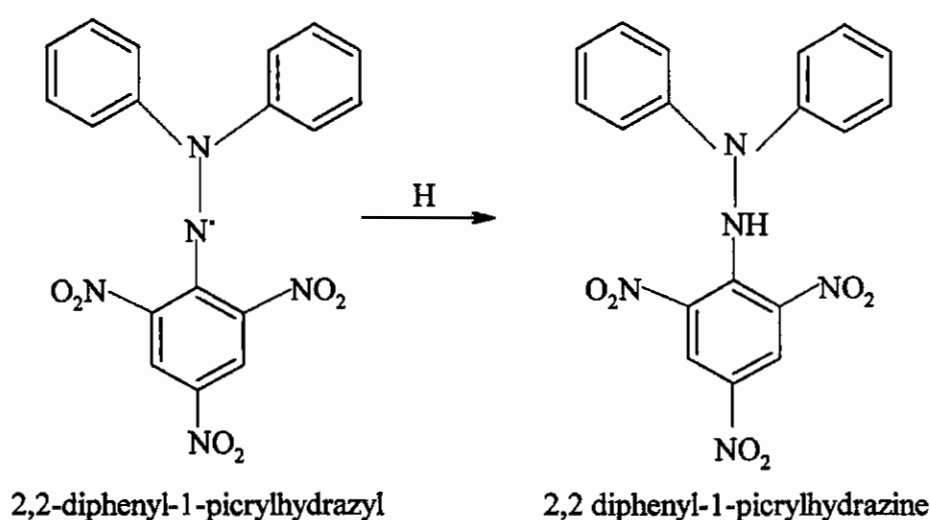


Figure 4: Reaction between DPPH and antioxidant.

Chemical Requirements

The chemicals were ethanol, distilled water, 0.004% DPPH ethanolic solution, ascorbic acid (as positive control).

Equipments

The equipments used for this experiment were test tubes, beakers, digital balance, vortex machine, refrigerator, marker, measuring cylinder, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), spoon, test tube stand, aluminum foil and UV spectrophotometer.

Methodology of DPPH free radical scavenging assay:

1. At first 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and another 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for ascorbic acid which was taken as positive control.
2. Sample extract and ascorbic acid were weighed and dissolved in ethanol to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex.
3. DPPH was also weighed and dissolved in ethanol to make 0.004% (w/v) solution.
4. After making the desired concentrations, 2ml of 0.004% DPPH solution was applied on each test tube containing 1ml of solution by pipette. The final volume of the solution was 3ml. This was done for both the ascorbic acid and sample extract.
5. Then the test tubes were kept for 30 minutes in dark to complete the reaction.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
7. After 30 minutes, absorbance of each test tube was determined by UV sepectrophotometer at 517 nm.
8. Percentage of inhibition was calculated as-

$$\% \text{ Inhibition} = [(\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Sample}}) / \text{Absorbance}_{\text{Blank}}] \times 100$$
9. IC₅₀ value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plot of % inhibition against the log concentration of sample extract.

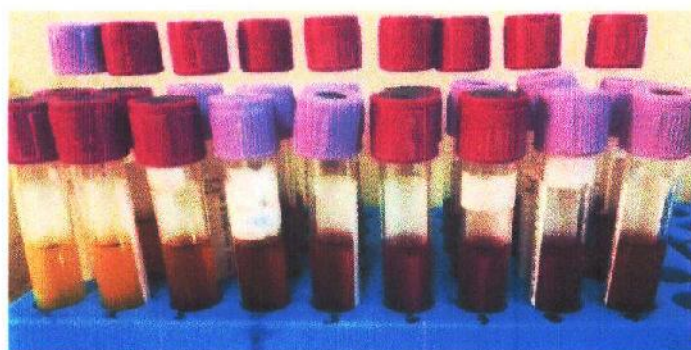
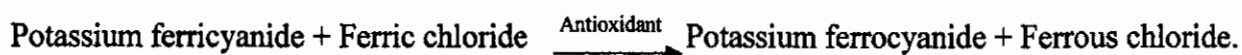


Figure 5: DPPH free radical scavenging assay of *S. apetala* Fruit Extract.

4.1.3.2 Reducing Power Assay

Principle

Reducing power assay measures the electron-donating capacity of an antioxidant [Yen & Chen 1995]. In this assay, the yellow color of the test solution changes to various shades of green color depending on the reducing power of each compound. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then react with ferric chloride to form ferric ferrous complex. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [Chen et al. 2010].



The reducing power was determined according to the method of Oyaizu (1986) with some modification [Oyaizu 1986].

Chemicals required

The chemicals for the experiment were phosphate buffer (0.2M, pH 6.6), potassium ferricyanide (1% w/v), trichloro acetic acid (10%), ferric chloride (0.1%) and ascorbic acid (1%) as positive control.

Equipments

Test tubes, beakers, digital balance, vortex machine, centrifuge machine, water bath, measuring cylinder, micropipette (20 μl , 200 μl and 1000 μl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

Phosphate Buffer preparation

Dibasic sodium phosphate (37.50ml of 0.2M) is mixed with monobasic sodium phosphate (62.5ml of 0.2M) and diluted to 100ml of distilled water.

Protocol for Reducing Power

1. Sample extract and ascorbic acid were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex.
2. Four test tubes were taken to make aliquots of 4 concentrations (50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and 4 test tubes were taken to make aliquots of 4 concentrations (50, 100, 200 and 400 µg/ml) by serial dilution technique for ascorbic acid which was taken as positive control.
3. After making the desired concentrations, 2.5ml of phosphate buffer and 2.5ml of potassium ferricyanide was applied on each test tube. The solution was mixed by vortex.
4. The mixture was kept at 50°C in water bath for 20 minutes.
5. After cooling, 2.5ml of 10% trichloro-acetic acid was added and centrifuged at 1400rpm for 10 minutes.
6. Then 2.5ml of the upper layer of solution was taken in each test tube and mixed with 2.5ml of distilled water.
7. Finally, freshly prepared 0.1% ferric chloride solution was added and mixed by vortex.
8. The absorbance was measured at 700nm.

A blank tube was prepared in similar manner excluding test sample or standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

The percent increase in reducing power was calculated using the following equation: Increase in

reducing power (%) = $\frac{A_{test} - A_{blank}}{A_{blank}} \times 100$ [Where A test is absorbance of test solution; A blank is absorbance of blank].

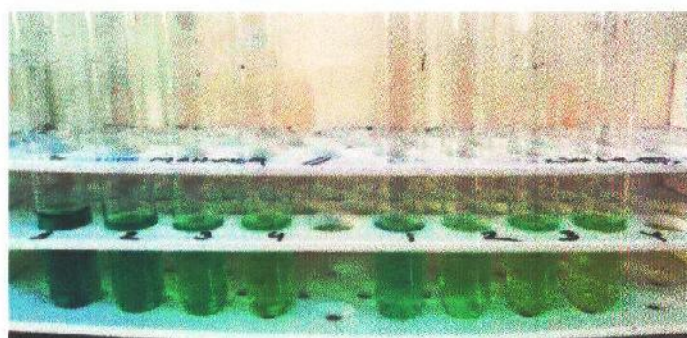


Figure 6: Reducing Power Assay of *S. apetala* Fruit Extract.

4.1.3.3 FRAP (Ferric ion Reducing Antioxidant Power) Assay

Ferric ion reducing antioxidant power (FRAP, also Ferric reducing ability of plasma) is a simple test to determine the total antioxidant power of sample. The FRAP assay was first performed by Iris Benzie and J. J. Strain of the Human Nutrition Research Group at the University of Ulster, Coleraine. It is often used to measure the antioxidant capacity of plant extract, foods, beverages and nutritional supplements containing polyphenols [Benzie et al. 1996].

Principle

The FRAP assay (ferric ion reducing antioxidant power) evaluates total antioxidant power and is chosen to assess the presumable effects of medicinal plants [Szollosi et al. 2002]. FRAP assay depends upon the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by the action of electron donating antioxidants at low pH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm. The FRAP assay was determined according to the method of Benzie and Strain (1996) [Benzie et al. 1996].

Chemicals required

The chemicals were acetate buffer (300 mM; pH 3.6), TPTZ (2, 4, 6-tripyridyl-s- triazine), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$).

Equipments

Test tubes, beakers, digital balance, vortex machine, water bath, micropipette (20 μl , 200 μl and 1000 μl), pipettes (5ml), UV spectrophotometer, marker and aluminum foil.

Acetate buffer (300 mM; pH 3.6) preparation

3.1gm sodium acetate trihydrate was weighed and added into 16 ml of glacial acetic acid and make the volume to 1L with distilled water.

TPTZ (2, 4, 6-tripyridyl-s- triazine) preparation

0.031gm TPTZ dissolve in 10ml of 40mM HCl and kept at 50°C in water bath for 20minutes.

Ferric Chloride $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM) preparation

0.054gm $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolves in 10ml distilled water.

FRAP reagent preparation

The working FRAP reagent was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution, then warmed at 37°C before using.

Protocol for FRAP assay

1. Sample extract, ascorbic acid and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. $400 \mu\text{g/ml}$, with the help of vortex.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and $400 \mu\text{g/ml}$) by serial dilution technique for plant extract, 5 concentrations (25, 50, 100, 200 and $400 \mu\text{g/ml}$) by serial dilution technique for ascorbic acid another and 5 test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and $400 \mu\text{g/ml}$) by serial dilution technique for $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ which was taken as positive control.
3. Another five test tubes were taken with 0.2ml extract, ascorbic acid and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution.
4. Then 3ml FRAP reagent was applied on each test tube.
5. The mixture was kept at 37°C in water bath for 30minutes.
6. After cooling, the absorbance was measured at 593nm.

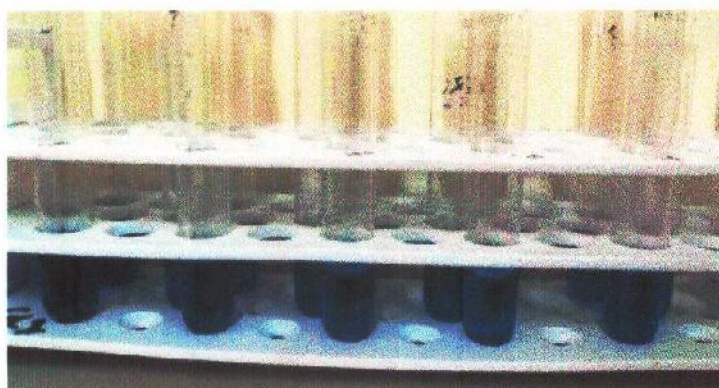


Figure 7: FRAP Assay of *S. apetala* Fruit Extract.

4.1.3.4 Total Phenolic Content Assay

Phenolic compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators and plant pigmentation, antioxidants and protective agents against UV light [Gottlieb et al. 2000]. Studies have indicated that the phenolic compounds are a major source of natural antioxidants because their structure allows them to donate hydrogen atoms or electrons for free radical scavenging.

Some of the commonly used methods to determine the content of phenolics are approximately 100 years old, initially were developed by Folin and Colleagues at Harvard Medicinal School to study the metabolism of proteins in humans. Then Folin and Denis (1915) reported a method which applied to the determination of phenolics in sample. Due to the limitation of this method Folin and Ciocalteu (1927) was reported a modified method, mainly the modification was occurred on reagent, which referred to as the Folin-Ciocalteu reagent, was used to determine the content of phenolics from a wide range of sources. The Folin-Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotungstate, clear bright yellow solution. Total phenolic content was estimated by the Folin-Ciocalteu method described by Singleton and Rossi [Singleton & Rossi 1965].

Principle

Polyphenols in plant extracts react with redox reagents (Folin-Ciocalteu reagent) to form blue complex chromogens that can be detected by visible-light spectrophotometry. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens [Katzung et al. 2009].

Chemicals requirement

Sodium Carbonate (7% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

Equipments

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

Methodology for Total Phenolic Content

1. Gallic acid was used as standard. 250 μ g/ml stock standard solution of gallic acid was prepared by dissolving 0.0025 gm in 10 ml distilled water and then diluted to six concentrations (25, 50, 100, 150, 200 and 250 μ g/ml).
2. The extract was prepared at concentration of 100 μ g/ml.
3. 9 ml distilled water and 1 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 10 ml of 7% sodium carbonate was added to the mixture and mixed gently.
6. 25 ml adjust with distilled water.
7. After standing at room temperature for 60 minutes, the absorbance was measured at 750nm using spectrophotometer.

The standard calibration curve of gallic acid was plotted.

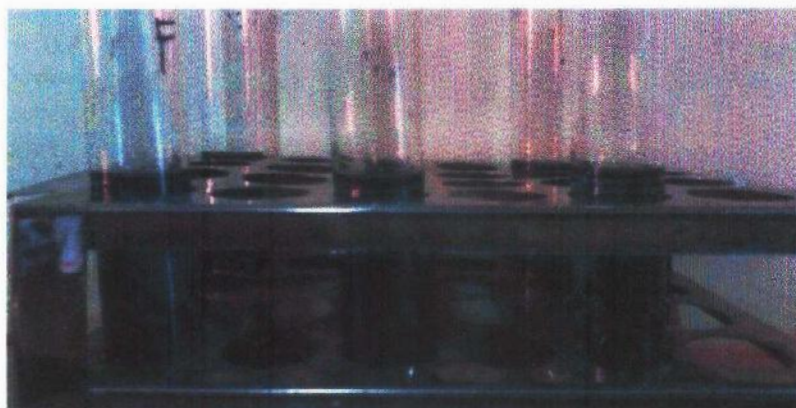
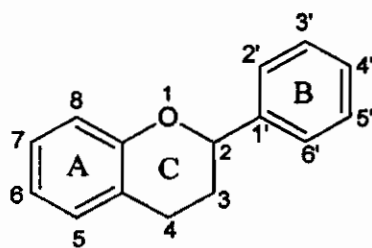


Figure 8: Phenol Content of *S. apetala* Fruit Extract.

4.1.3.5 Total Flavonoid Content

Flavonoids are bioactive polyphenols which play a vital role in photosynthesising cells [Cushnie et al. 2005]. They are secondary metabolites characterised by flavan nucleus and C6-C8-C6 carbon-skeleton (Figure 5) [Heim et al. 2002; Tsuchiya et al. 2010]. The basic structural feature of flavonoid is 2-phenyl-benzo- γ -pyrane nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) as shown in following figure [Cushnie et al. 2005]



(Basic structure of flavonoids)

Flavonoids differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings [Heim et al. 2002]. A variation in C ring provides division of subclasses. According to their molecular structure, they are divided into eight classes [Tsuchiya et al. 2010]

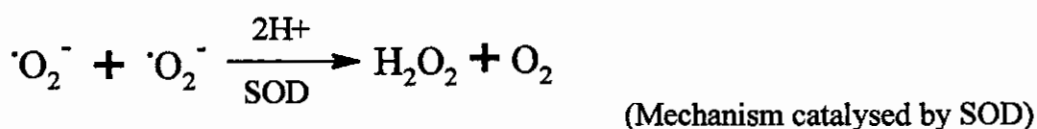
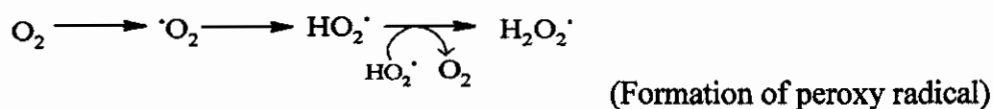
In plants, flavonoids are often present as O-glycosides or C-glycosides. The O-glycosides possess sugar substituent bound to $-OH$ of aglycone, usually at position 3 or 7, whereas, C-glycosides possess sugar groups bound to Carbon of aglycone usually 6-C or 8-C [Buhler & Miranda 2000].

Flavonoids are phenolic substances isolated from a wide range of plants, with over 8000 individual compounds known. They act in plants as photoreceptors, visual attractors, feeding repellants and for light screening [Pietta 2000]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Many studies have suggested that flavonoids exhibit biological activities, including antibacterial, antiallergenic, antiviral, anti-inflammatory [Cushnie et al. 2005, Cook et al. 1996, Murray 1998], cytotoxic, antitumour, treatment of neurodegenerative diseases and vasodilating actions [Murray 1998, Williams et al. 2004, Tsuchiya et al. 2010, Chebil et al. 2006]. In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclooxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation [Cook et al. 1996, Chebil et al. 2006, Middleton et al. 2000]. These are also reported to inhibit variety of enzymes like hydrolases, hyaluronidase, alkaline phosphatase, arylsulphatase, cAMP phosphodiesterase, lipase, α -glucosidase, kinase [Narayana et al. 2001]. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The position of hydroxyl groups and other features in the chemical

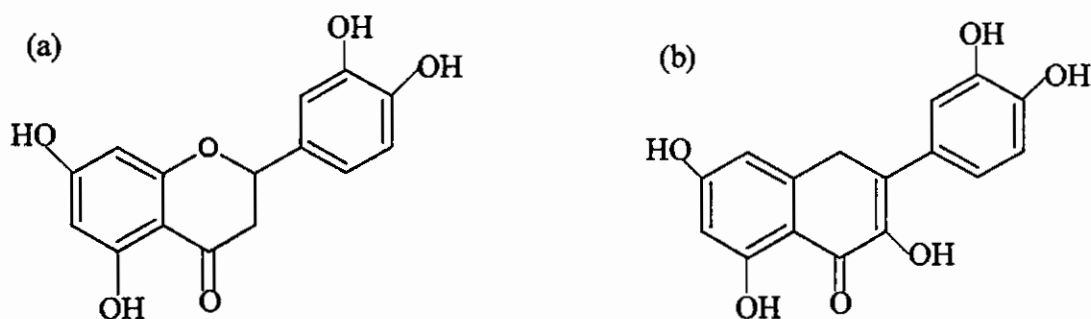
structure of flavonoids are important for their antioxidant and free radical scavenging activities. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal et al. 2009]. This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available “H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure [Tripoli et al. 2007]. Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction [Heim et al. 2002] as shown:

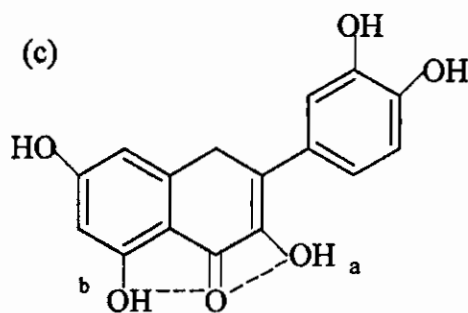


Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical as in fig (V), thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [Cook et al. 1996, Ferreira et al. 2010].



According to kinetic studies of aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is linked to its three structural groups as shown in fig.





(Structural groups responsible for antioxidant activity) [Tripoli et al. 2007]

1. The ortho-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in electron dislocation.
2. The 2,3-double bond, in conjugation with a 4-oxo function, responsible for electron dislocation from the B-ring.
3. The presence of both 3-(a)-and 5-(b)-hydroxyl groups (Fig.VII(c)).

3',4'-catechol structure in B-ring strongly enhances lipid peroxide inhibition and this arrangement is an important characteristic of most potent scavengers of peroxy, superoxide and peroxynitrite radicals [Heim et al. 2002] and its absence decreases antioxidant activity. The absence of the hydroxyl group at position 3 in flavanones and flavones decreases their antioxidant ability [Tripoli et al. 2007]. The content of flavonoids in the examined plant extracts was determined using spectrophotometric method. Total flavonoids were determined following the procedure by Dewanto, Wu, Adom, and Liu, (2002) [Dewanto et al. 2002].

Principle

Flavonoids in the sample extracts react with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [Tsuchiya et al. 2010].

Chemicals requirement

5% Sodium nitrite (5% NaNO_2): 5gm in 100ml distilled water. 10% Aluminum chloride (10% AlCl_3): 10gm in 100ml distilled water.

1 M Sodium hydroxide (1 M NaOH): 4gm NaOH in 100ml distilled water.

Equipments

Test tubes, beakers, volumetric flask, digital balance, vortex machine, micropipette (200 μl and 1000 μl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

Methodology for Total Flavonoid Content

1. Quercetin was used as standard and were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 $\mu\text{g/ml}$, with the help of vortex and sonicator.
 2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and 400 $\mu\text{g/ml}$) by serial dilution technique for Quercetin.
 3. The extract was prepared at concentration of 100 $\mu\text{g/ml}$.
 4. 5 ml distilled water and 0.3 ml of 5% NaNO_2 was added to each diluted Quercetin and 1ml of extract solution.
 5. After 5 minutes 0.6 ml of 10% AlCl_3 was added.
 6. After 5 minutes 2 ml of 1M NaOH .
 7. Then the mixture was allowed to stand at room temperature for 5 minutes.
 8. After standing at room temperature, the absorbance was measured at 510 nm using spectrophotometer.
- The standard calibration curve of Quercetin was plotted.

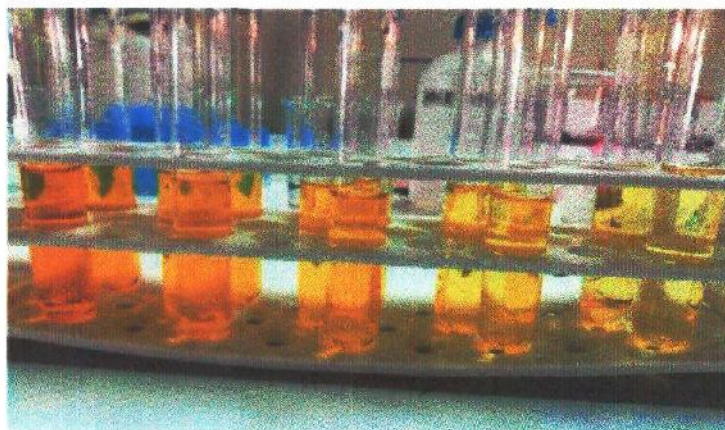


Figure 9: Flavonoid Content of *S. apetala* Fruit Extract.

4.1.3.6 Total Tannin Content

Tannins are a complex group of polyphenolic compounds found in a wide range of plant species as secondary metabolites which have been known and used by man for centuries. Tannins are a heterogeneous group of high molecular weight phenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals etc [Mueller et al. 1992]. Tannins play an important role in plant function and evolution. Tannins found in plants are divided into two major classes: the condensed and the hydrolysable tannins. The tannins of different plant species have different physical and chemical properties [Mangan, 1988] and therefore they have very diverse biological properties [Zucker, 1983].

Principle

The method is based on the oxidation of molecule containing a $-OH$ groups. The tannin and tannin like compound reduce Phosphotungstomolybdic acid in alkaline solution to produce a highly blue colored solution [Cetkovic et al. 2008].

Chemicals requirement

Sodium Carbonate (35% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

Equipment's

Test tubes, beakers, digital balance, vortex machine, measuring cylinder, micropipette (20 μ l, 200 μ l and 1000 μ l), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

Methodology for Total Tannin Content

1. Gallic acid was used as standard. 100 μ g/ml stock standard solution of gallic acid was prepared by dissolving 0.001 gm in 10 ml distilled water and then diluted to five concentrations (6.25, 12.5, 25, 50 and 100 μ g/ml).
2. The extract was prepared at concentration of 100 μ g/ml.
3. 7.5 ml distilled water and 0.5 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 1 ml of 35% sodium carbonate was added to the mixture and mixed gently.
6. 10 ml adjust with distilled water.
7. After standing at room temperature for 30 minutes, the absorbance was measured at 725nm using spectrophotometer.

The standard calibration curve of gallic acid is plotted.



Figure 10: Tannin Content of Methanolic and Aqueous Extract of *S. apetala* Fruit Extract.

4.2 Preparation of Cream Formulation

Principle:

Most of cosmetic products are emulsions that function as drug delivery vehicle to the skin and are classified according to different body parts to be applied i.e., skin, teeth, hair and mouth. This experiment focus on the topical application of emulsions and their effect on topical absorption.

Emulsions are heterogeneous systems and consist of mixture of two immiscible liquids i.e., aqueous phase and oil phase and system is stabilized by the presence of third substance known as emulsifying agent or emulsifier. Main ingredients of these formulations are emulsifier, viscosity modifiers and some active ingredients having auspicious skin care properties. Peculiarly the use of botanical extracts with antioxidant activity provides most valuable cosmetic products. There is exorbitant curiosity about the use of herbal extracts containing phytochemicals with high antioxidant potential and play an important role in eviction of various degenerative diseases and production of modern phytocosmetic preparations. Most important bioactive phytoconstituents of plants include polyphenols, flavonoids, alkaloids, tannins and essential oils. The topically applied cosmetic preparations are more beneficial in respect to solubility, spreadability, enhanced bioavailability and topical delivery of hydrophilic and hydrophobic ingredients.

[Otto et.al. 2009].



Chemicals requirement:

Table 1: Composition of Cream

Components	Amount (%)		
Active ingredient	Formulation 1 (F1) (ME)	Formulation 2 (F2) (AE)	Formulation 3 (F3) (Placebo)
<i>S. apetala</i> fruit extract	3.00%	3.00%	-
Oily Phase			
Stearic acid	7.00%	7.00%	7.00%
Cetyl alcohol	2.00%	2.00%	2.00%
Mineral oil	20.00%	20.00	20.00%
Aqueous Phase			
Glycerin	9.00%	9.00%	9.00%
Methyl paraben	0.05%	0.05%	0.05%
Triethanolamine	2.00%	2.00%	2.00%
Distilled Water	100%	100%	100%
ME= Methanolic Extract; AE= Aqueous Extract; "-" indicates Nil or no Extract			

Equipment:

Electronic balancer, beakers, vortex machine, measuring cylinder, Water bath, pH meter, Homogenizer, Microwave oven, micropipette, marker and aluminum foil.

4.2.1 Formulation of Cream Bases:

Listed ingredients are accurately weighed and placed in separate beakers (for oil phase and water phase). Three different formulations F1, F2 and F3 are formed. F1 contains *S. apetala* methanolic fruit extract whereas F2 contains *S. apetala* aqueous fruit extract and F3 contains all active ingredients except *S. apetala* fruit extract and this formulation is called placebo. The composition of the cream is shown in the table above. Formulation F1, F2 and F3 adopts the same method to prepare the creams.

1. The oily phase components and the aqueous phase components are measured and taken to separate beaker.
2. Oily phase and aqueous phase components are heated separately up to 70°C in the water bath till the proper melting of the components.
3. They are then mixed properly by using homogenizer.
4. After this methyl paraben, *S. apetala* fruit extract and perfume are added.
5. Continuous stirring until the mixture cools and formed as cream.
6. Placebo (base cream) is prepared in the same method as above without extract.

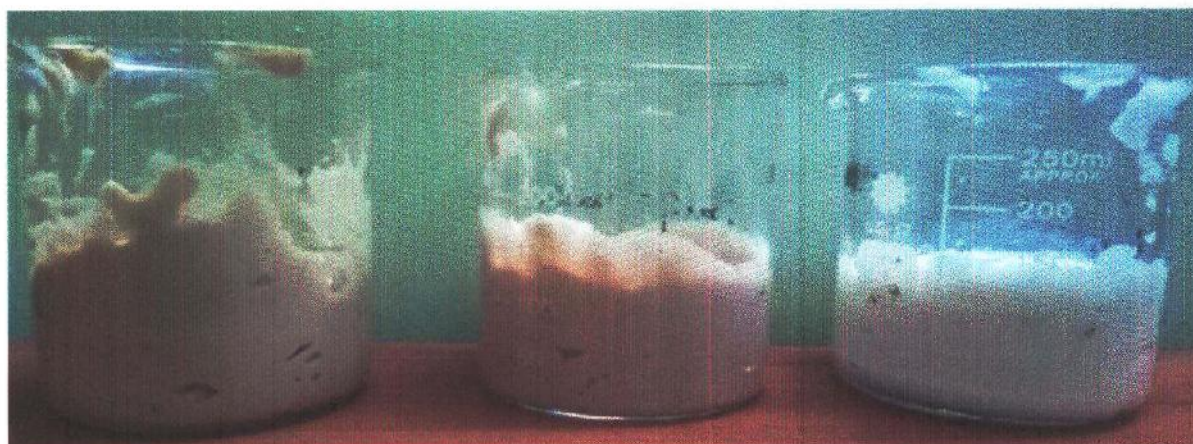


Figure 11: Formulation 1 (F1), Formulation 2 (F2) and Formulation 3 (F3 or placebo) Cream.

4.2.2 Evaluation of Antioxidant Cream

The following parameters are used to evaluate the antioxidant cream. The standard procedure is followed to evaluate all the parameters.

Physical Properties:

The cream is observed for color, odor and appearance.

Determination of pH:

The pH meter is calibrated using standard buffer solution. About 0.5 g of the cream is weighed and dissolved in 50 ml of distilled water and its pH is measured.

Homogeneity:

The formulations are tested for the homogeneity by visual appearance and touch.

After Feel Effect:

Emolliency, slipperiness and amount of residue left after the application of fixed amount of cream is checked.

Loss on Drying:

1g of cream is taken in petri dish and kept in an oven at 105°C for 2 hours.

Stability:

To assess the formulation stability, the formulations are kept at room temperature for three months and also the cream filled beakers were kept in refrigerator at 8°C and in incubator at 40°C for two week. The samples were then analyzed for physical property.

Rheological Study:

The formulated cream is found to be non-newtonian. A fixed quantity of 10 g of cream is placed in a 10 ml beaker. Kept it impact for 1 hr. the beaker is inclined to one side see whether consistency has changed or not. The beaker is again tilted and checked for pourability of the cream.

4.3 Evaluation of Antibacterial Activity *S. apetala* Fruit Extract and formulated cream:

There is constant interaction between microbes and humans in all walk of human life. Microbes produce disease by killing host cells or by liberating toxins that can cause tissue damage and functional derangements even without extensive colonization of host tissues. So, we need to

protect ourselves from these to lead a healthy life. The capacity of the substances to kill or inhibit microbial growth is called antimicrobial activity and the substances that possess this activity are called antimicrobials. Antimicrobial properties of medicinal plants are being increasingly reported from different part of the world. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant material effects of plant materials typically results from the combination of secondary products present in the plant which are capable of producing definite physiological action on body.

Mangrove plant extracts have been used for centuries as a popular method for treating several health disorders. Plant-derived substances have recently become of great interest owing to their versatile applications. Mangroves are biochemically unique, producing a wide array of novel natural products. Mangrove and mangrove associates contain biologically active antibacterial, antiviral and antifungal compounds.

4.3.1 Test of Antimicrobial Activity by Disc Diffusion Assay

Principle:

In this method measured amount of test sample were dissolved in definite amount of volatile solvent to get a solution of known concentration ($\mu\text{g/ml}$). Then sterile filter paper discs were impregnated with known amount of test substances using micropipette and dried. Standard antibiotic discs were used as positive control and disc impregnated with solvent was used as negative control. These discs were then placed in Petri dishes containing agar medium seeded with the test organisms of log phase. The test material diffuses from the discs to the surrounding medium. The seeded Petri dishes containing test and control discs were then placed into the incubator for 24 hours at 37°C to allow the growth of microorganisms. If the test material had any antimicrobial activity, it would inhibit the growth of microorganism giving a clear distinct zone called "Zone of Inhibition". The antimicrobial activity of the sample was determined by measuring the diameter of the zone of inhibition in terms of millimeter.

4.3.1.1 Materials for test

Test material:

Methanolic and aqueous extract of the fruit of *S. apetala* and the antioxidant cream formulated from those extracts.

Positive control:

Antibiotic disc Ciprofloxacin (For *S. apetala* fruit extract)

Equipment:

Aluminum foil, autoclave, cotton, digital slid calipers, electronic balance, filter paper discs, laminar air flow cabinet, marker, micropipette, nose mask, apron and gloves, Petri dishes, pipette, refrigerator, spirit lamp, sterile forceps, sterile inoculating loop and test tubes.

Reagents:

Distilled water, ethanol, nutrient agar medium and nutrient broth.

Bacteria used for the activity test

Both Gram-positive and Gram-negative bacterial strains are taken for the test. The bacterial strains used for this investigation are listed in following table. These organisms are preserved in Microbiology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh.

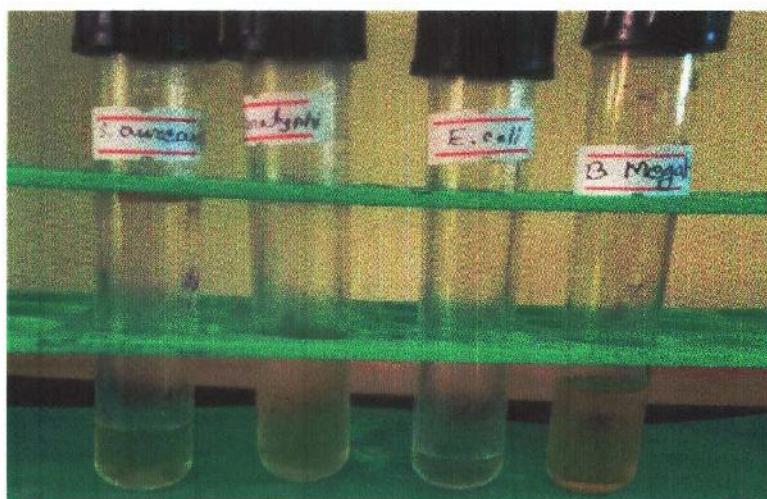


Figure 12: Gram +ve and Gram -ve Bacterial Strains Used in Antimicrobial Test.

Table 2: List of bacteria with diseases caused by them

Gram-positive	Diseases	Gram-negative	Diseases
<i>Staphylococcus aureus</i>	Skin infections, respiratory disease and food poisoning.	<i>Escherichia coli</i>	Gastroenteritis, Urinary tract infections, Neonatal meningitis and Food poisoning
<i>Bacillus megaterium</i>	Non-pathogenic	<i>Pseudomona aurigonisa</i>	Infection in pulmonary tract, urinary tract, burns and wounds

Culture Media

A mixture of nutrient used in the laboratory to support growth and multiplication of a culture (a population of microorganisms) is called culture medium [Pelczar et al. 1996]. The nutritional requirements of bacteria vary widely so there are great differences in the chemical compositions of the media used in the laboratory. Some microorganism can grow in medium containing only inorganic compounds, whereas others require a medium containing organic compounds (amino acids, sugars, purines or pyrimidines, vitamins or coenzymes). Some require complex natural substances (peptone, autolysate, blood cells or blood serum) and some cannot as yet be grown in an artificial laboratory medium and can be propagated only in a living host or living cells. Each kind of organism also requires specific physical conditions for growth. For example, some bacteria cannot grow below 40°C, some cannot grow above 20°C and some require a temperature close to that of the human body (i.e., 37°C). Light may be another important physical condition. The successful cultivation of bacteria requires an awareness of all of these factors. Each kind of microorganism grows in a characteristic manner. Certain complex raw materials such as peptones, beef extract and yeast extract are used and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired [Pelczar et al. 1993]. The following media are used normally to demonstrate the antibacterial activity and to make subculture of the test organisms:

- Nutrient Agar Media
- Nutrient Broth Media
- Mueller-Hinton Agar Media
- Tryptic Soy Broth (TSP)

Among these, the first one is most frequently used which is also used for the present antibacterial screening.

Media Composition:

For 100ml

Ingredients	Amount
Distilled water	100ml
Peptone	0.5gm
Beef extract	0.3gm
Agar	1.5gm

4.3.1.2 Methodology

Preparation of media:

Nutrient agar media:

Nutrient agar media is prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps are done to do this:

- 1) For 100ml nutrient agar media, 0.5gm peptone, 0.3gm beef extract and 1.5gm agar are taken in the screw capped conical flask.
- 2) So 100ml distill water was added to that amount of powder.
- 3) Then the media is subjected to sterilization in the autoclave.

Nutrient broth media:

Nutrient broth media is prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps were done to do this:

- 1) For 100ml nutrient agar media, 0.5gm peptone and 0.3gm beef extract were taken in the screw capped conical flask.
- 2) So 100ml distill water was added to that amount of powder.
- 3) Then the mixture was shaken well and then it was subjected to sterilization in the autoclave.

Sterilization:

Both the culture media, Petri dishes, micropipette, test tubes, forceps, screw capped vial, screw capped conical flasks, pipette were sterilized by autoclaving at a temperature of 121°C and a pressure of 15lbs/sq inch for 15 minutes. Blank disc were subjected to dry heat sterilization at 110°C for 20 minutes. The Laminar air flow cabinet was disinfected by ethanol with spray bottle. The UV light in the laminar air flow cabinet was switched on before one hour to avoid accidental contamination.

Preparation of Subculture:

This was done by culturing pure microbial population in the agar media. To obtain better growth of bacterial colony tow generation of subculture were prepared. Following steps was performed for preparation of first generation of subculture:

- 1) At first prepared culture media was poured into Petri dishes which were marked for desired strains of microorganisms.
- 2) At least two subculture plates were made for each strain.
- 3) The Petri dishes were allowed to cool in refrigerator for 15 minutes.
- 4) Then the inoculating loop (sterilized by spirit lamp) was dipped into the pure culture of bacteria and streaking was done in the Petri dishes marked for those strains of bacteria.
- 5) Then the Petri dishes were allowed to incubate for 12-18 hours at 37°C in the incubator.
- 6) After 24 hours bacterial colony was observed in the culture media.
- 7) Then the prepared plates of first generation subculture were kept in refrigerator which can be used for 2 months.

Preparation of Seeded Test Plates:

For the evaluation of antimicrobial activity by disc diffusion assay seeded test plate of microbes is necessary to made. It was done by two steps:

In the first step-

- 2 ml of nutrient broth was taken in each screw capped vial where all of them were marked according to their strains. Two vials were taken for each strain.
- Then each vials containing nutrient broth were inoculated by the inoculating loop which looped single bacterial colony from the sub culture. These tasks were done in the laminar air flow cabinet.
- Then all the vials were allowed to incubate at 3-4 hours at 37°C. This was done to get the bacteria in their log phase at which the growth rate is very high.

In the second step-

- The sterilized agar medium was poured into sterilized Petri dishes in such a way as to give a uniform layer of depth of approximately 4 mm. After the medium became cooled at room temperature, it was stored in a refrigerator (4°C).
- Incubated vials log phase were removed from the incubation.
- Test tubes were shaken by rotation to get a uniform suspension of bacteria.
- Then 5µl nutrient broth containing bacteria was taken into corresponding solidified medium containing Petri dishes.
- The Petri dishes were rotated several times, first clock wise then anticlockwise to assure homogenous distribution of the test organisms. Thus the seeded plates were ready for disc diffusion assay. These tasks were done in the laminar air flow cabinet.

All tasks were done in the laminar air flow cabinet.

Preparation of Test Sample

0.2 gm each of methanolic and aqueous extract of *S. apetala* were accurately measured by using the electronic balance and taken into screw capped vials (vials were nullified on the electronic balance by auto zero). Then 2ml of water is added and dissolved by vortex. Same procedure is also maintained for cream sample.

Preparation of Discs:

Three types of discs were used for the antimicrobial screening:

- a) Sample discs
- b) Standard discs and
- c) Blank discs

Sample discs:

Filter papers previously sterilized were perforated by punch machine thus tiny discs of 5 mm diameter were obtained. Then the discs were taken in a blank Petri dish. 10 μ l and 20 μ l of sample solution were applied on the discs with the help of micropipette in an aseptic condition from the stock solution. Then the discs were allowed to dry for a few minutes under the laminar to remove the solvent as it has some antimicrobial activity which can bias the evaluation.

Standard discs:

These were used as positive control to ensure the activity of standard against the test organisms as well as for comparison of the response produced by the known antibacterial agent with that produced by test samples. In this investigation antibiotic ciprofloxacin standard disc were used for the test of extract and L'OREAL revitalift day cream as standard for formulated cream as the reference.

Blank discs:

Blank discs were used as a negative control. They ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves. Placebo is used as the negative control in case of antimicrobial test of cream.

Application of Discs

Sample impregnated discs, standard discs and negative control discs are placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were not closer than 15 mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. Finally the plates are incubated upside down at 37°C for 24 hours.

4.3.1.3 Determination of Antimicrobial Activity by Disc Diffusion Method:

After proper incubation, the antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition in term of millimeter with a transparent scale.

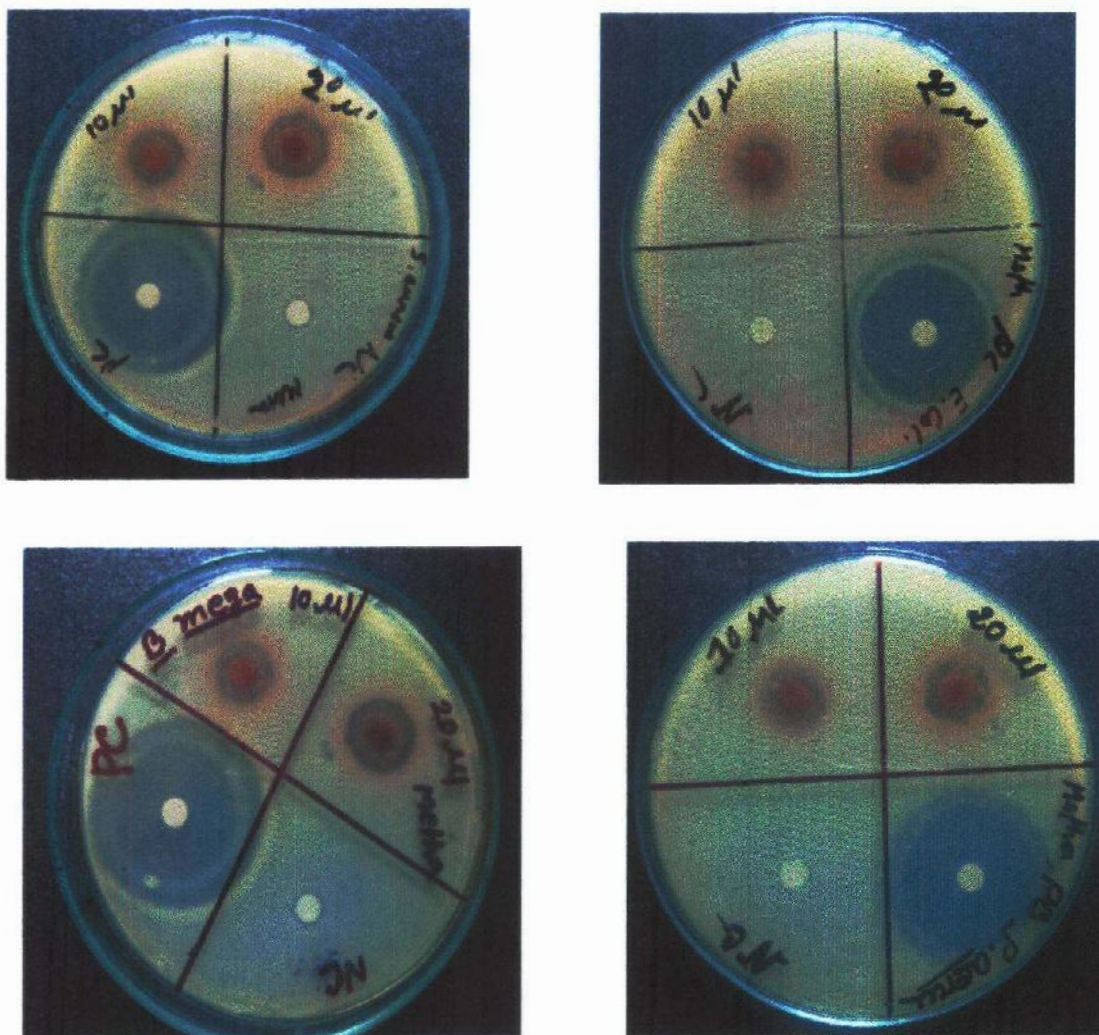


Figure 13: Bacterial Zone of Inhibition of Methanolic Extract against Gram +ve and Gram - ve Bacteria.

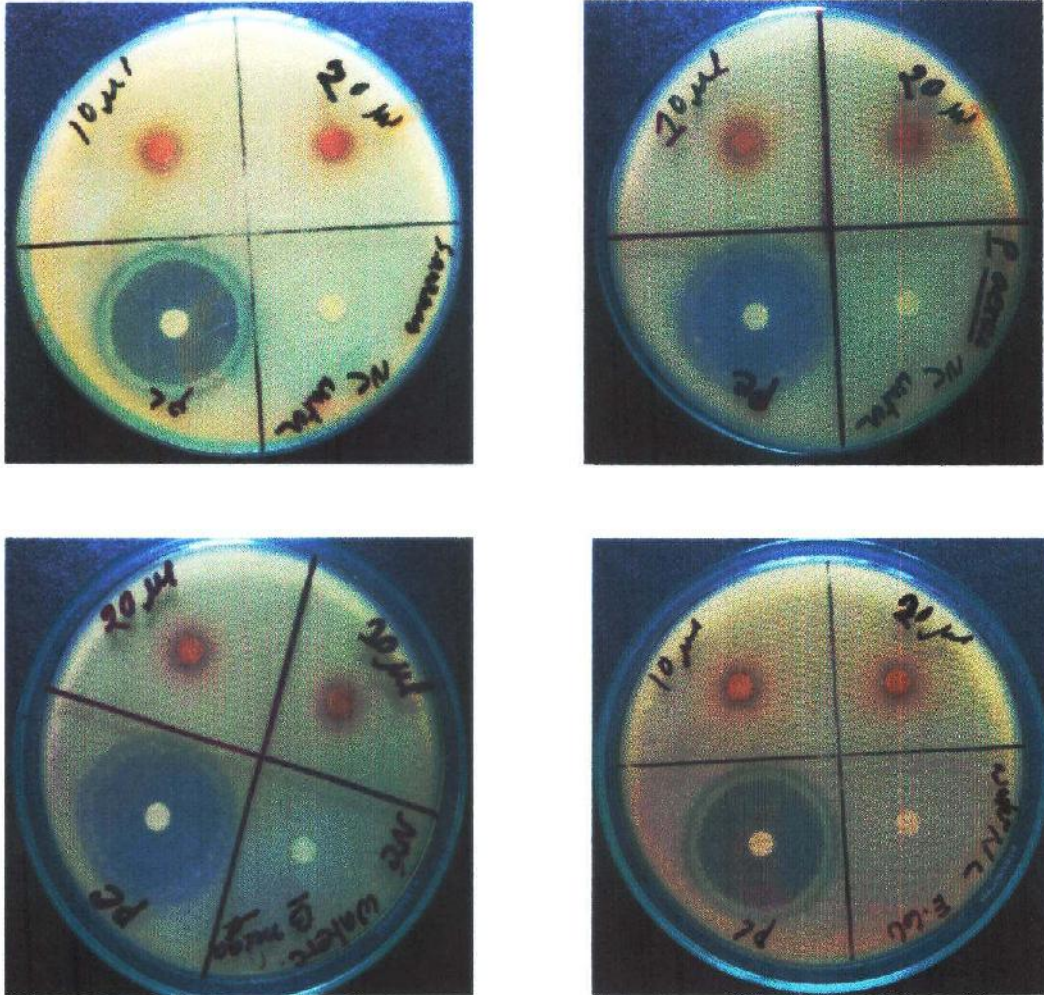


Figure 14: Bacterial Zone of Inhibition of Aqueous Extract against Gram +ve and Gram - ve Bacteria.

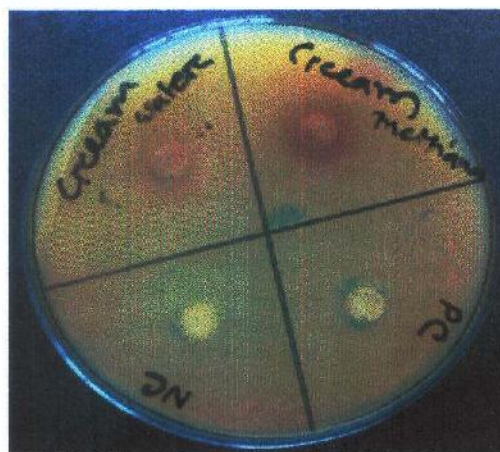
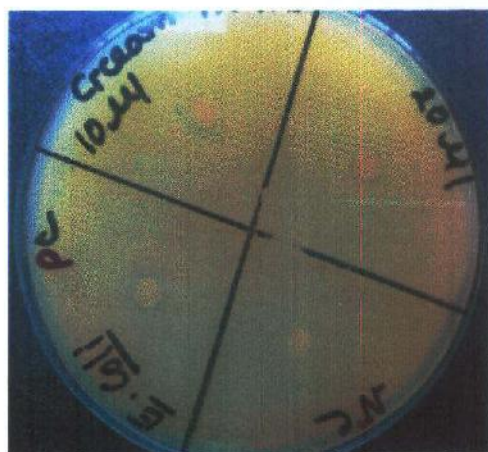
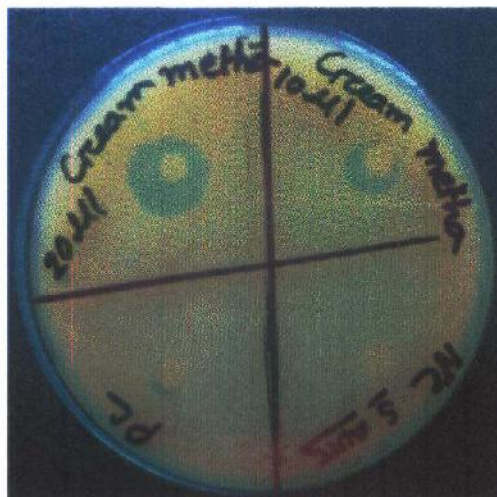


Figure 15: Bacterial Zone of inhibition of Cream Formulated from *S. apetala* Fruit Extract against Gram +ve and Gram - ve Bacteria.

4.3.2 Determination of Minimum Inhibitory Concentration (MIC) by Broth Dilution Method:

Principle:

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a chemical, usually a drug, which prevents visible growth of bacterium. MIC depends on the microorganism, the affected human being (in vivo only), and the antibiotic itself.

The MIC is determined by preparing solutions of the chemical in vitro at increasing concentrations, incubating the solutions with the separate batches of cultured bacteria, and measuring the results using broth micro-dilution. Results have been graded into susceptible

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(often called sensitive), intermediate, or resistant to a particular antimicrobial by using a breakpoint.

4.3.2.1 Materials for test

Test material:

Methanolic and aqueous extract of the fruit of *S. apetala* fruit and the antioxidant cream formulated from those extracts.

Positive control:

Antibiotic disc Ciprofloxacin (For *S. apetala* fruit extract)

L'OREAL revitalift day cream (For formulated cream)

Equipment:

Aluminum foil, autoclave, cotton, electronic balance, filter paper discs, laminar air flow cabinet, marker, micropipette, nose mask, apron and gloves, pipette, refrigerator, spirit lamp, snap cap centrifuge tubes.

Reagents:

Distilled water, ethanol, phenol red, nutrient broth.

Bacteria used for the activity test

Both Gram-positive and Gram-negative bacterial strains are taken for the test. The bacterial strains used for this investigation are listed in following table. These organisms are preserved in Microbiology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh.

Table 3: List of bacteria with diseases caused by them

Gram-positive	Diseases	Gram-negative	Diseases
<i>Staphylococcus aureus</i>	Skin infections, respiratory disease and food poisoning.	<i>Escherichia coli</i>	Gastroenteritis, Urinary tract infections, Neonatal meningitis and Food poisoning
<i>Bacillus megaterium</i>	Non-pathogenic	<i>Pseudomona aurigonisa</i>	Infection in pulmonary tract, urinary tract, burns and wounds

Procedure:

1. Marking the empty test tubes 1, 2, 3, 4, 5, 6, +ve and -ve control.
2. Preparation of 2 fold serial dilution for extract.
3. Transferring 2 ml of phenol red into all the empty test tubes.
4. Transferring 1 ml of extract (for MIC of formulated cream 1 ml of cream is used) from tube 1 to tube 2, mixing well and from 2 to 3, mixing well and repeating this for 4, 5 and 6. Then rejecting 1 ml from test tube 6.
5. Adding 5 μ l of bacterial culture to all tubes.
6. Adding 5 μ l of antibiotic (17mg/ml) to positive control tube (for MIC of formulated cream 5 μ l L'OREAL revitalift day cream is used).
7. Incubation of the tubes at 37°C for 24 hours and determination of the bacterial growth.

Calculating the MIC by determining the color change at lowest concentration of extract inhibit the bacterial growth.



Figure 16 : MIC Test of *S. apetala* (Methanolic) Fruit Extract against Gram +ve and Gram -ve Bacteria.

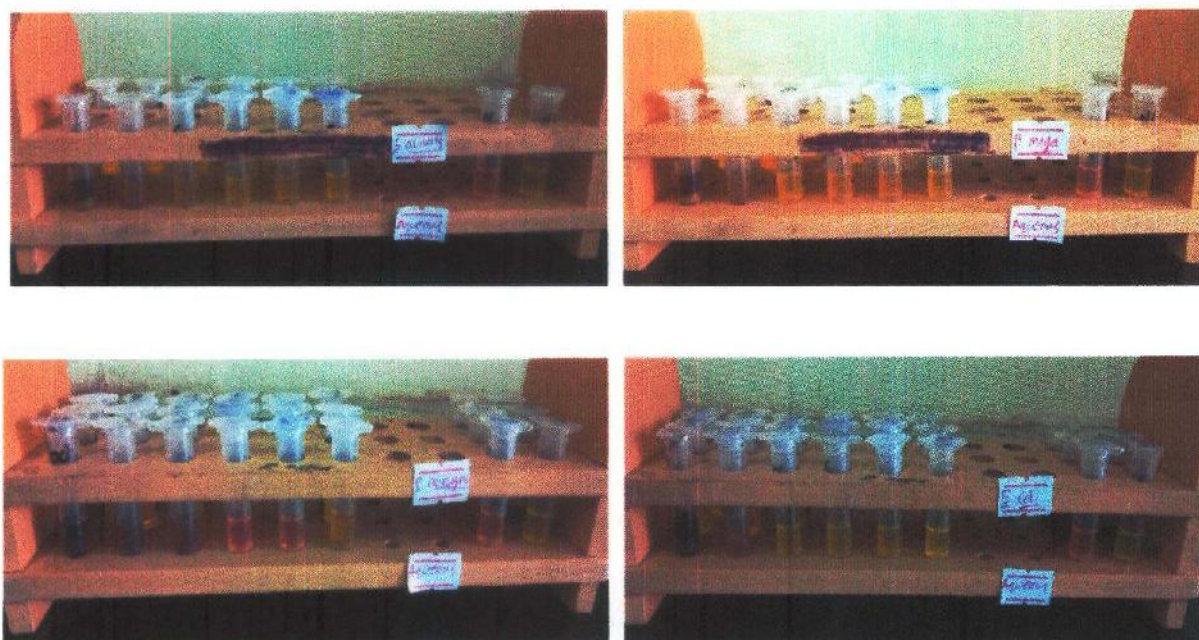


Figure 17: MIC Test of *S. apetala* (Aqueous) Fruit Extract against Gram +ve and Gram -ve Bacteria.



Figure 18: MIC Test of Formulated Cream from *S. apetala* (Methanolic) Fruit Extract against Gram +ve and Gram -ve Bacteria.



Figure 19: MIC Test of Formulated Cream from *S. apetala* (Aqueous) Fruit Extract against Gram +ve and Gram -ve Bacteria.

4.4 Skin Testing

15 human volunteers were selected whose ages between 20-60 years. Both male and female volunteers were included in this work. The volunteers were free from skin disease or damage. Before examination, every volunteers were well informed about the study. The volunteers were not informed about the contents of formulations. One the first day, patch test were performed on the forearms of each volunteer to determine any possible reactions. On the second day, each volunteer were provided with two creams. One cream was the basic formulation and the other

was the formulation containing the active ingredients. Each cream was marked with 'right' and 'left' indicating application of that cream to the respective place. The creams were applied by all the volunteers themselves as instructed for 30 days. After 30 days physical property and after feel effects and any type of side effects were noted by the volunteers.

4.5 Statistical analysis:

- One way ANOVA was carried out using graph pad prism software version 6.01 for phenol, flavonoid, tannin and FRAP.
- One way ANOVA was carried out using graph pad prism software version 6.01 for Antibacterial test.
- Two way ANOVA was carried out to analyze DPPH, reducing power and the variation between two preparations.
- $p < 0.05$ values were considered statistically significant because there were significant difference among the test samples and the control group.

5.1 Results and Discussion

5.1.1 DPPH Free Radical Scavenging Assay:

The DPPH antioxidant assay is a rapid, direct and reliable method for the antioxidant screening of plant extracts. A number of methods are available for the determination of free radical scavenging activity but the assay employing the stable 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience. This test is based on the ability of DPPH, a stable nitrogen-centered free radical, to decolorize in the presence of antioxidants.

5.1.1.1 Methanolic extract of *S. apetala*

Graphical analysis of Absorbance vs. Log Concentration, the absorbance of the extract decreased gradually as the log concentration increased. In figure 20 showed the absorbance of methanolic extract of *S. apetala* and standard ascorbic acid at different log concentration.

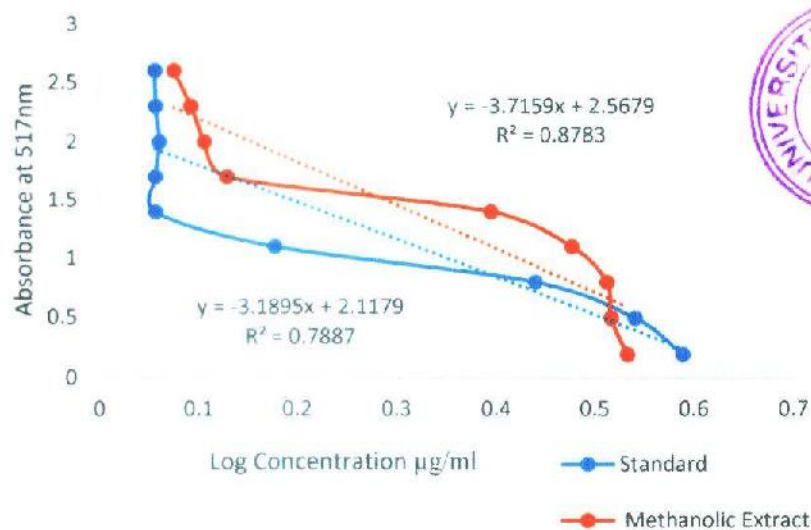


Figure 20: DPPH Scavenging assay of *S.apetala* methanolic extract (Absorbance vs Log Concentration).

Figure 21 showed that the comparison of percentage inhibition of methanolic extract of *S. apetala* and standard quercetin at different concentrations. The concentration at which 50%

inhibition (IC_{50}) of DPPH occurred were obtained by extrapolation. By using the equation $y = mx + c$ (where 'c' is intercept and 'm' is slope); IC_{50} value of extract was found at the concentration of 115.286 $\mu\text{g/ml}$ and standard ascorbic acid was 6.86 $\mu\text{g/ml}$. Here the concentration of the extract required for 50% inhibition is much greater than that of control.

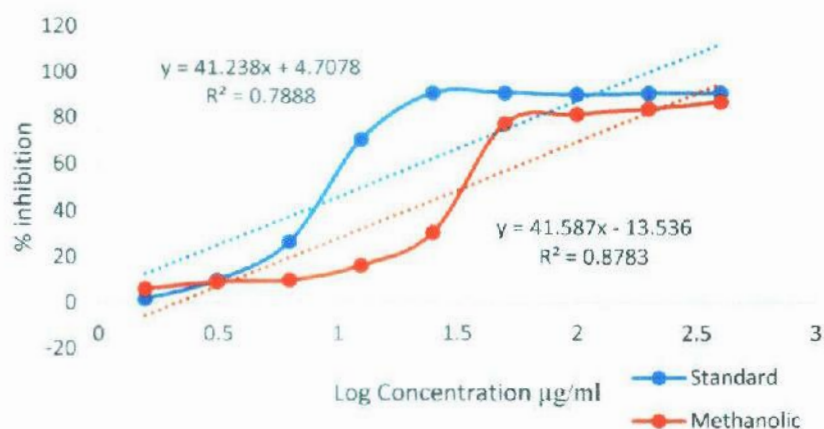


Figure 21: DPPH Scavenging assay of *S.apetala* methanolic extract (% inhibition vs Log Concentration).

5.1.1.2 Aqueous extract of *S. apetala*

Analysis of *S. apetala* aqueous extract, the absorbance of the extract decreased gradually as the log concentration increased. Absorbance at different log concentration of extract and standard ascorbic acid is given in figure 22.

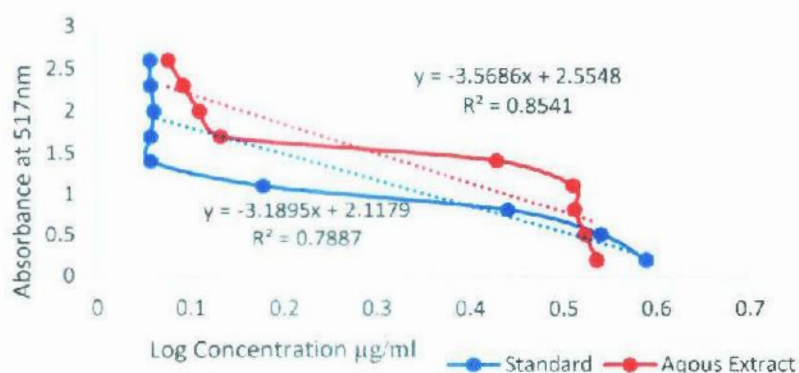


Figure 22: DPPH Scavenging assay of *S.apetala* aqueous extract (Absorbance vs Log Concentration).

Comparison of percentage inhibition of aqueous extract of *S. apetala* and standard quercetin at different log concentrations is given in fig. 23. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by using the equation $y = mx + c$. IC_{50} value of extract was found at the concentration of 122.257 $\mu\text{g/ml}$ and standard quercetin was 6.86 $\mu\text{g/ml}$. So we observed that the concentration of the extract required for 50% inhibition is greater than that of control.

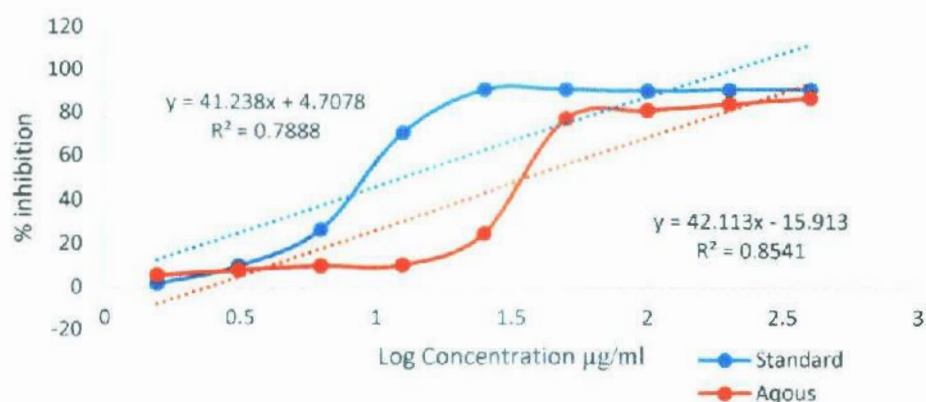


Figure 23: DPPH Scavenging assay of *S. apetala* aqueous extract (% inhibition vs Log Concentration).

5.1.2 Reducing power assay

Reducing power of the extracts is determined by direct electron donation in the reduction of ferri cyanide $[\text{Fe}(\text{CN})_6]^{3-}$ to ferro cyanide $[\text{Fe}(\text{CN})_6]^{4-}$. The product is visualized by addition of free Fe^{3+} ions after the reduction reaction, by forming the intense prussian blue color complex, $(\text{Fe}^{3+})_4[\text{Fe}^{2+}(\text{CN})_6]^{3-}$ and quantified by absorbance measurement at 700 nm. The reducing power of the extracts increases with the increase in amount of sample. Increased absorbance of the reaction mixture indicates increased reducing power.

Table 4: Reducing power assay of Standard Ascorbic Acid

Concentration ($\mu\text{g/ml}$)	Absorbance $\pm$ SD	% Increase in reducing power
Blank	0.1483 $\pm$ 0.0028	
25	0.3893 $\pm$ 0.0025	162.471
50	0.5473 $\pm$ 0.0020	268.988
100	0.7676 $\pm$ 0.0025	417.528
200	1.3756 $\pm$ 0.0020	827.415
400	2.3153 $\pm$ 0.00351	1460.898

5.1.2.1 Methanolic extract of *S. apetala*

In the case of *S. apetala* methanolic extract, table 5 shows that the methanolic extract exhibited the ranges from 56.867 to 751.225 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 24 shows the comparison between methanolic extract of *S. apetala* and standard ascorbic acid.

Table 5: Reducing power assay of methanol extract of *S.apetala*

Concentration ($\mu\text{g/ml}$)	Absorbance $\pm$ SD	% Increase in reducing power
Blank	0.136 $\pm$ 0.0017	
25	0.213 $\pm$ 0.0015	56.862
50	0.313 $\pm$ 0.0144	130.147
100	0.434 $\pm$ 0.0058	219.362
200	0.661 $\pm$ 0.0320	386.274
400	1.157 $\pm$ 0.1447	751.225

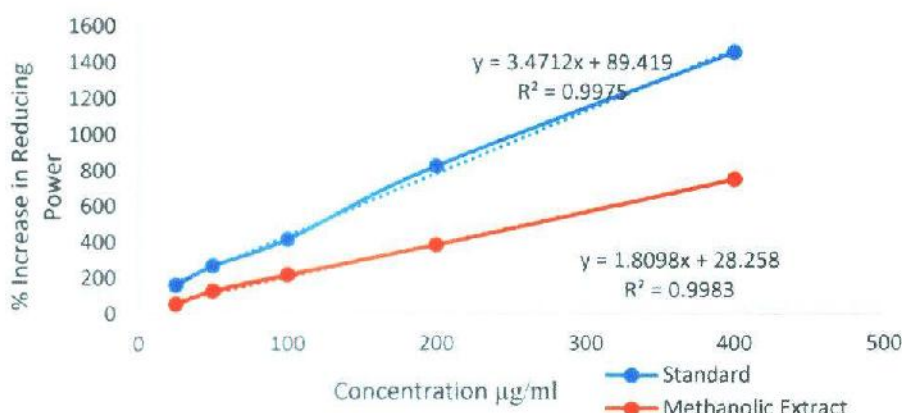


Figure 24: Reducing ability of methanolic extract of *S. apetala* and standard Ascorbic Acid at various concentrations.

5.1.2.2 Aqueous extract of *S. apetala*

Aqueous extract of *S. apetala* displayed least values of percent increase in reducing power, table 6 shows that the aqueous extract exhibited the ranges from 18.689 to 185.436 whereas standard ascorbic acid exhibited the ranges from 162.471 to 1460.898. Figure 25 shows the comparison between aqueous extract of *S. apetala* and standard ascorbic acid.

Table 6: Reducing power assay of aqueous extract of *S. apetala*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.137±0.0025	
25	0.163 ±0.0026	18.689
50	0.2056 ± 0.0132	49.757
100	0.2373 ± 0.0005	72.815
200	0.316 ± 0.0052	130.097
400	1.392 ± 0.0072	185.436

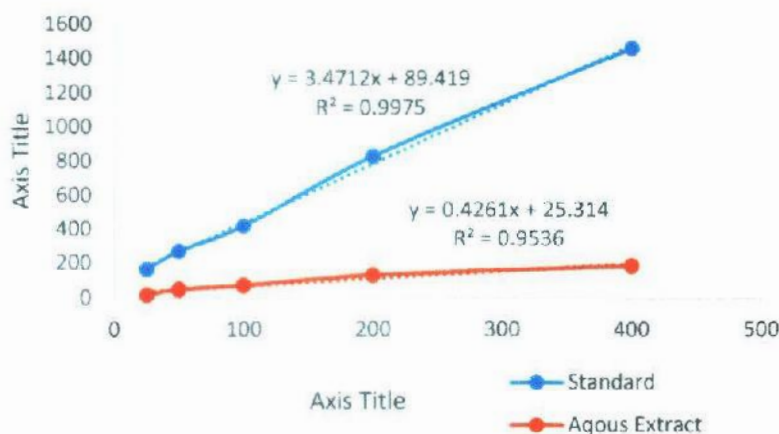


Figure 25: Reducing ability of aqueous extract of *S. apetala* and standard Ascorbic Acid at various concentrations.

Discussion

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [Jung et al. 2008]. This assay has proven the effectiveness of two types of *S. apetala* extracts compared to the standard antioxidant, Ascorbic Acid. Literature reports are evident that the reducing power of bioactive compounds is associated with antioxidant activity. The presence of reductants (i.e. antioxidants) in extracts causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration. In case of methanolic extract of *S. apetala*, the maximum absorbance is found (1.157 ± 0.144) at 400 $\mu\text{g/ml}$ concentration; whereas (2.315 ± 0.0035) is found for standard ascorbic acid. Figure 24 showed percentage increase in reducing power of extract in comparison to the standard. Analyzing this figure, aqueous extract of *S. apetala* had not much reductive capability with compare to the standard.

Methanolic extract of *S. apetala* possess moderate reducing potential in compared to standard ascorbic acid. Figure 25 showed the comparison between percentage increase in reducing power of methanolic extract of *S. apetala* and standard ascorbic acid.

Between the two extracts methanolic extract of *S. apetala* possess highest reducing potential in compared to standard.

In the present investigation, methanolic extract of *S. apetala* possess highest reducing potentiality and highest scavenging activity in DPPH assay in compared to all extracts. The reducing power of the extract might be due to their hydrogen donating ability. Possibly, methanolic extract of *S. apetala* contain high amounts of reductive, which could react with radicals to stabilize and terminate radical chain reactions.

5.1.3 FRAP (ferric reducing antioxidant power) Assay:

The total antioxidant potential of sample is determined using FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from colorless oxidized FeIII form by the action of electron donating antioxidants. In this research work, the total antioxidant potential of *S. apetala* is determined by using FRAP reagent with standard $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Standard curve is prepared using different concentrations (400-25) $\mu\text{g/ml}$. In the FRAP assay the antioxidant efficiency of the antioxidant under the test is calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results are expressed in μM Fe (II)/100g dried plant material using the equation $y = 0.0044x + 0.1307$, coefficient $R^2 = 0.9974$, where y is absorbance at 593 nm and x is the total antioxidant potential of different extracts. Absorbance of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in different concentration is given in table below and from which calibration curve plotted in the figure.

Table 7: Absorbance of standard $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at different concentration

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (593 nm)	Absorbance $\pm$ SD
Blank	0.0955	
400	1.8673	1.8673 ± 0.0025
200	1.067	1.067 ± 0.011
100	0.5697	0.5697 ± 0.0040
50	0.353	0.353 ± 0.0072
25	0.2107	0.2107 ± 0.0035

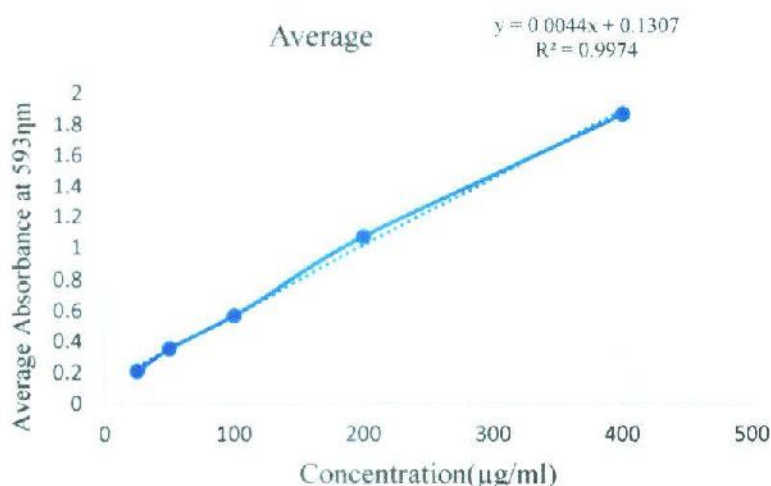


Figure 26: Calibration curve of standard $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ for determination of FRAP assay.

5.1.3.1 Total antioxidant capacity of *S. apetala*:

In this research; using standard calibration curve, total antioxidant power of methanolic and aqueous extract of *S. apetala* is determined. Table 8 shows the absorbance and antioxidant power of methanolic and aqueous extract of *S. apetala*. Total antioxidant power of methanolic extract exhibited 442.947 ± 0.040129 $\mu\text{M Fe (II)}/100\text{g}$ of dried plant material and aqueous extract showed 410.75 ± 0.006245 $\mu\text{M Fe (II)}/100\text{g}$ of dried plant material.

Table 8: Antioxidant power of *S. apetala*

Extract	Conc. ($\mu\text{g/ml}$)	Absorbance	Ferric reducing antioxidant power ($\mu\text{M Fe (II)}/\text{g}$) $\pm$ SD
Methanolic	100	2.079667	442.947 ± 0.040129
Aqueous	100	1.938	410.75 ± 0.006245

5.1.3.2 Total antioxidant capacity of Ascorbic acid:

Ascorbic acid is very popular for its antioxidant properties. For this reason total antioxidant capacity of ascorbic acid is also measured by FRAP assay to compare it with total antioxidant capacity of *S. apetala* fruit extract. From quantitative estimation of total antioxidant capacity of ascorbic acid showed 583.1742 $\mu\text{M Fe (II)}/100\text{g}$. Absorbance and ferric reducing antioxidant power of ascorbic acid is furnished in table 9.

Table 9: Antioxidant power of Ascorbic acid

Sample	Conc. ($\mu\text{g/ml}$)	Absorbance	Ferric reducing antioxidant power ($\mu\text{M Fe (II)/g}$) $\pm$ SD
Ascorbic acid	100	2.696667	583.1742 $\pm$ 0.073962

Discussion

FRAP assay measure the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+} -TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe^{2+} -TPTZ] [Guo et al. 2003]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction [Ercan et al. 2011].

The reducing capacity of an extracts may serve as a significant indicator of its potential antioxidant activity. FRAP assay showed variability between the two extracts. In this research using FRAP assay from the quantitative estimation of total antioxidant potential of *S. apetala* methanolic and aqueous extract exhibited 442.947 $\pm$ 0.040129 and 410.75 $\pm$ 0.006245 $\mu\text{M Fe (II)/100g}$ of dried plant material respectively. Methanolic extract of *S. apetala* showed significant antioxidant power, whereas ascorbic acid which has potential antioxidant potential showed 583.1742 $\pm$ 0.073962. Investigating the results of the present study, *S. apetala* methanolic extract showed the slightest antioxidant capacity which is closest to ascorbic acid.

In this research work, previous assays also showed that the methanolic extract had a greater antioxidant potentiality than the aqueous extract.

5.1.4 Total Phenolic Content

The amount of total phenol was determined with the Folin-Ciocalteu assay using Gallic acid as a standard phenolic compound. This method is a fast and simple method and can be useful in characterizing and standardizing botanical samples. Folin-Ciocalteu method is based on

oxidation of phenolics by a molybdotungstate in Folin-Ciocalteu reagent to yield a colored product with max 745 – 750 nm [Javanmardi et al .2003].

A linear calibration curve of Gallic acid, in the range of 25–250 µg/ml with coefficient of determination (R^2) value of 0.9661 was obtained. Absorbance of gallic acid in different concentration is given in table 9 and from which calibration curve plotted in figure 27. Using this calibration curve, the total amount of phenolic content of plant extract was determined using the equation $y = 0.0019x + 0.0413$, where y is the absorbance at 750 nm and x is total phenolic content in the different plant extracts.

Table 10: Absorbance of standard gallic acid at different concentration

Concentration (µg/ml)	Avg. of Absorbance (750 nm)	Absorbance $\pm$ SD
250	0.481333	0.481333 ± 0.0065
200	0.436667	0.436667 ± 0.0073
150	0.365	0.365 ± 0.0060
100	0.256333	0.256333 ± 0.0011
50	0.139	0.139 ± 0.009539
25	0.052467	0.052467 ± 0.00023

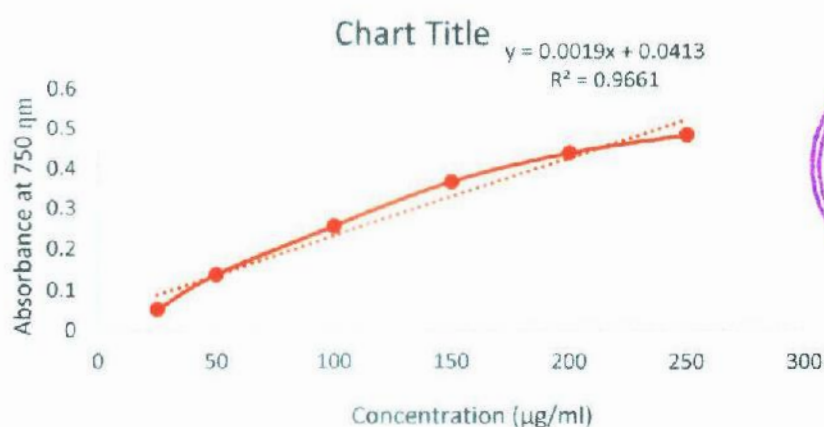


Figure 27: Calibration curve of standard Gallic acid for determination of total phenolics content.

5.1.4.1 Total phenolic content of *S. apetala*

In this work, total phenolic content of *S. apetala* fruit extract is determined using the standard calibration curve. Table 11 represents the amount of total phenolic content in methanolic and aqueous extract of *S. apetala*. Methanolic extract shows 201.7017 ± 0.0005773 mg GAE/g of

dried sample and aqueous extract shows 76.43859 ± 0.008736 mg GAE/g of dried sample respectively.

Table 11: Total phenolic content of *S. apetala*

Extract	Conc. (µg/ml)	Absorbance (750 nm)	Total Phenolic Content (mgGAE/g of extract) $\pm$ SD
Methanolic	100	0.426333	201.70175 ± 0.000577
Aqueous	100	0.188333	76.438596 ± 0.008736

Discussion

Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities [Razali et al. 2008].

Investigating the results of the present study, the quantitative estimation of total phenolic content of aqueous extract of *C. crista* exhibited 76.438596 ± 0.008736 mg GAE/g of extract. Methanolic extract showed the amount of total phenolic content is 201.70175 ± 0.008736 mg GAE/g of extract. In this research work, we found a quite good amount of polyphenolic content. The amount of total phenol in methanolic extract of *S. apetala* is the highest compared to all the other extracts in this research work. Amazingly, methanolic extract was found as the strongest scavengers (IC_{50} 115.2863 µg/ml) in DPPH assay, strongest reductant capability in reducing power assay and also possess highest antioxidant capacity in FRAP assay.

5.1.5 Total Flavonoid Content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo- γ -pyrone structure. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Total flavonoid contents can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [Bakar et al. 2009]. A linear calibration curve of Quercetin, in the range of 25–400 µg/ml

with coefficient of determination (R^2) value of 0.9862 was obtained. Absorbance of quercetin in different concentration is given in table 11 and from which calibration curve plotted in figure 28. Using this calibration curve, the total amount of flavonoid content of plant extract is determined using the equation $y = 0.0018x + 0.0233$, where y is the absorbance at 510 nm and x is total flavonoid content in the different plant extracts.

Table 12: Absorbance of standard quercetin at different concentration

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (510 nm)	Absorbance $\pm$ SD
400	0.734667	0.734667 ± 0.037541
200	0.384667	0.384667 ± 0.015044
100	0.259	0.259 ± 0.002
50	0.092333	0.092333 ± 0.0023
25	0.043333	0.043333 ± 0.0057

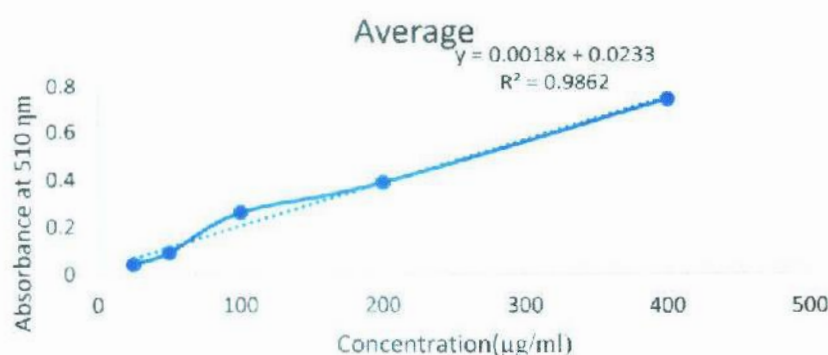


Figure 28: Calibration curve of standard Quercetin for determination of total flavonoid content.

5.1.5.1 Total flavonoid content of *S. apetala*

In this work, total flavonoid content of different plant extract of *S. apetala* is determined using the standard calibration curve. Table 13 represents the amount of total flavonoid content in methanolic and aqueous extract of *C. crista*. Methanolic extract shows 92.61111 ± 0.002 mg QE/g of dried sample and aqueous extract shows 51.12963 ± 0.001155 mg QE/g of extract respectively.

Table 13: Total flavonoid content of *S. apetala*

Extract	Conc. ($\mu\text{g/ml}$)	Absorbance (510 nm)	Total Flavonoid Content (mg QE/g of extract) $\pm$ SD
Methanolic	100	0.19	92.61111 ± 0.002
Aqueous	100	0.115333	51.12963 ± 0.001155

Discussion

Flavonoids constitute a wide array of biological active compounds that are found abundantly in plant kingdom and dietary intake [Cushnie et al. 2005]. They are gaining interest due to their wide variants and number of members. These are reported to be effective in pathogenesis of majority of diseases. Antioxidant activity is the foundation of many actions which lead to its beneficial effects in majority of the diseases. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal et al. 2009]. Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [Cook et al. 1996; Ferreira et al. 2010]. A positive correlation between the content of flavonoids and the antioxidant capacity in plant extract has been found [Nagarajan et al. 2008]. For that reason, determination of the quantity of flavonoids compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total flavonoid content of methanolic extract exhibited 92.61111 ± 0.002 mg QE/g of dried sample whereas aqueous extract showed 51.12963 ± 0.001155 mg QE/g of dried sample. In this research work, we found methanolic extract of *S. apetala* showed a quite good amount of flavonoid content, good reducing capability in reducing power assay and also showed a good antioxidant potentiality in FRAP assay, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of $115.28 \mu\text{g/ml}$ in DPPH assay.

5.1.6 Total Tannin Content

Tannin compounds are polyphenolic compounds that are very complex and widely distributed in almost all plant species. These complex polyphenolic compounds are soluble in water and organic solvents. In this research work, Tannin content in each sample is determined using Folin-Denis method and Gallic acid as a standard [Polshettiwar et al. 2007]. The tannin and tannin like compound reduce Phosphotungstomolybdic acid in alkaline solution to produce a highly blue colored solution with λ_{max} 725 nm [Cetkovic et al. 2008]. A linear calibration curve of Gallic acid, in the range of 6.25–100 $\mu\text{g/ml}$ with coefficient of determination (R^2) value of 0.988 was obtained. Absorbance of gallic acid in different concentration is given in table 13 and from which calibration curve plotted in figure 29. Using this calibration curve, the total amount of tannin content of plant extract was determined using the equation $y = 0.0057x + 0.037$, where y is the absorbance at 725 nm and x is total tannin content in the different plant extracts.

Table 14: Absorbance of standard gallic acid at different concentration

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (725 nm)	Absorbance $\pm$ SD
Blank	0.058	
100	0.586	0.586 ± 0.016
50	0.354	0.354 ± 0.038
25	0.196	0.196 ± 0.002
12.5	0.095	0.095 ± 0.004
6.25	0.057	0.057 ± 0.002

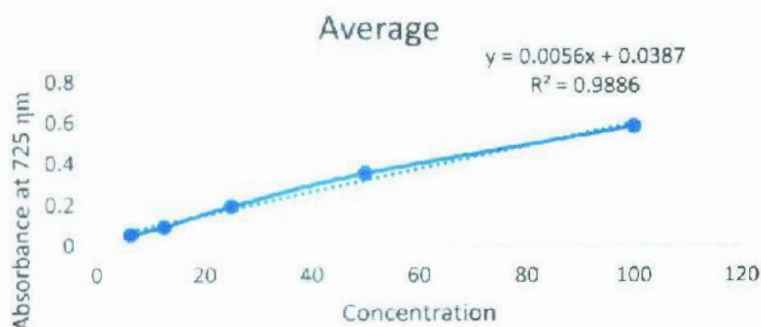


Figure 29: Calibration curve of standard Gallic acid for determination of total tannin content.

5.1.6.1 Total tannin content of *S. apetala*

In this work, total tannin content of two different type of plant extract of *S. apetala* is determined using the standard calibration curve. Table 15 represents the amount of total tannin content in methanolic and aqueous extract of *S. apetala*. methanolic extract shows 40.1345 ± 0.016503 mg GAE/g of extract and aqueous 11.77193 ± 0.003 mg GAE/g of extract respectively.

Table 15: Total tannin content of *S. apetala*

Extract	Conc. (μ g/ml)	Absorbance(725 nm)	Total Tannin Content (mgGAE/g of extract) $\pm$ SD
Methanolic	100	0.26667	40.1345 ± 0.016503
Aqueous	100	0.105	11.77193 ± 0.003

Discussion

Tannins are very complex polyphenolic compounds and are widely distributed in almost all plant species. Tannins have been reported to exhibit a wide range of activities and prevent the attack of free radicals on the human body. Therefore, determination of the quantity of tannin compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total tannin content of methanolic extract of *S. apetala* exhibited 40.1345 ± 0.016503 mg GAE/g of extract. . In this research work, we found methanolic extract of showed a quite good amount of tannin content, moderate reducing capability in reducing power assay, a good antioxidant potentiality in FRAP assay and quite good flavonoid content. We also found in the literature, polyphenols or tannins are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

6.1 Antimicrobial Activity of *S. apetala* Extract

Table 16: Antimicrobial activity of methanolic and aqueous extract of *S. apetala*, positive control Ciprofloxacin and negative control

Bacterial strain	Diameter of Zone of Inhibition(mm)					
	Methanolic		Aqueous		Positive Control	Negative Control
	20µl/disc	10µl/disc	20µl/disc	10 µl/disc	Ciprofloxacin 5µl/disc	Nil
Gram-positive						
<i>Staphylococcus aureus</i>	15.5	10	6	-	28	-
<i>Bacillus megaterium</i>	16	12		-	25	-
Gram-negative						
<i>Escherichia coli</i>	16	10	-	-	20	-
<i>Pseudomonas aurigonisa</i>	15	11	8	6	25.5	-

Number indicates the mean diameter of inhibition of experiments; Control disc= 6mm; "-" means no inhibition.

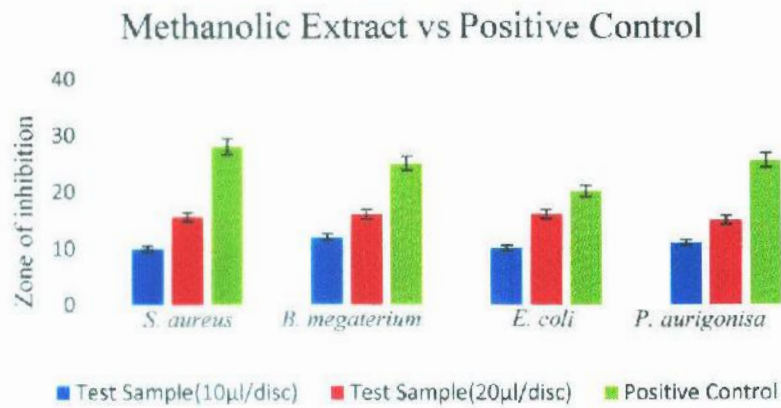


Figure 30: Evaluation of antimicrobial activity of *S.apetala* methanolic extract (Microorganism vs zone of inhibition)

6.2 Antimicrobial Activity of Formulated Cream

Table 17: Antimicrobial activity of formulated cream from methanolic and aqueous extract of *S. apetala*, positive control L'OREAL revitalift day cream and negative control

Bacterial strain	Diameter of Zone of Inhibition(mm)					
	Cream (Methanolic)		Cream (Aqueous)		Positive Control	Negative Control
	20µl/disc	10µl/disc	20µl/disc	10 µl/disc	L'OREAL 20 µl/disc	Placebo
Gram-positive						
<i>Staphylococcus aureus</i>	8.5	6	5.5	-	5.5	-
<i>Bacillus megaterium</i>	8	6		-	-	-
Gram-negative						
<i>Escherichia coli</i>	6.5	-	-	-	6.5	-
<i>Pseudomonas aurigonisa</i>	7	5.5	-	-	6	-

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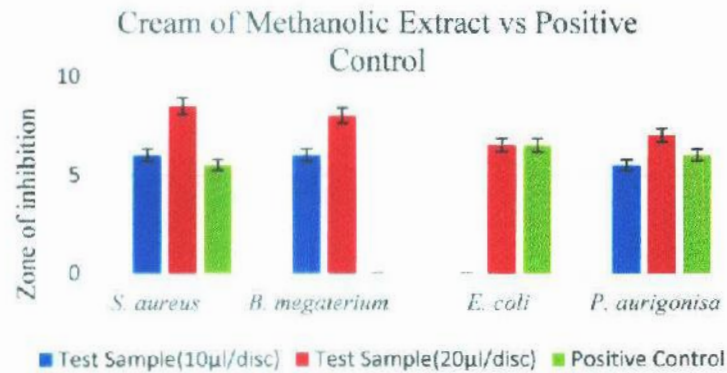


Figure 31: Evaluation of antimicrobial activity of formulated cream of *S. apetala* methanolic extract (Microorganism vs zone of inhibition)

6.3 Determination MIC of *S. apetala* Fruit Extract

Table 18: Minimum Inhibitory Concentration (MIC) of Bacterial Growth (Broth Dilution Method) by *S. apetala* Fruit Extract and Formulated Cream.

Bacterial Strain	MIC of Extract and Cream (µg/ml)			
	Extract (methanolic)	Extract (aqueous)	Cream (methanolic)	Cream (aqueous)
<i>Staphylococcus aureus</i>	6.25	50	25	100
<i>Bacillus megaterium</i>	6.25	100	25	100
<i>Escherichia coli</i>	6.25	200	50	200
<i>Pseudomonas aurigonisa</i>	12.5	12.5	50	200

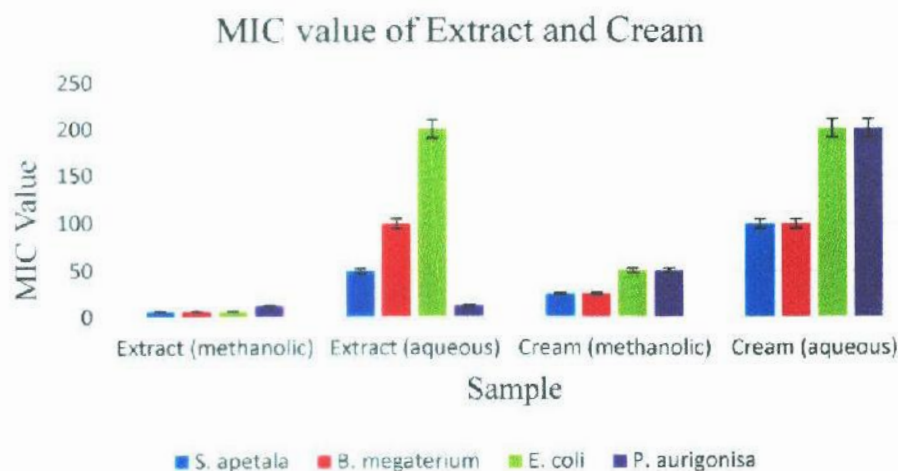


Figure 32: Evaluation of Minimum Inhibitory Concentration Bacterial Growth (Broth Dilution Method) by *S. apetala* Fruit Extract and Formulated Cream.

6.4 Evaluation of Antioxidant Cream

The methanolic and aqueous extract of *S. apetala* fruit were used to formulate the cream but because of higher antioxidant and overall activity as well as antimicrobial activity of methanolic extract it was chosen to accept as final active cream.

Stability Assessment:

Accelerated conditions of temperature and centrifugation test are important parameters to access the physical stability of creams. Stability of creams (actives and placebo) were evaluated at high temperature (105°C for 2 hours). Similarly, no phase separation was observed in active creams and placebo control kept at various temperatures with centrifugation test performed immediately after preparation and at various time intervals (24 h and on days 7, 14, 30, 60 and 90) (Table 18). The formulated F1 and F2 creams showed pleasant odour and light brownish color. Formulated creams were not greasy after application to the skin and they were easily removable by washing with tap water. Homogenous distribution of extract in the cream was confirmed by visual examination. There was no change in color of formulated cream upon keeping for long time. The loss on drying of the formulated creams were found to be within the limit to standard procedure. Accelerated stability studies at temperatures 8°C in refrigerator and 40°C in incubator for 2 weeks for F1,

F2 and placebo. However, the formulations showed good stability in color no dispersed phase and separation between oil and water phase until the end of accelerated study period. After feel test showed that the creams were emollient and slipperiness. All the physicochemical parameters were well maintained during the period of accelerated stability study. It was obvious from the literature that the stability of emulsion under stress conditions could be enhanced by maintaining proper homogenization speed during preparation of emulsion [Abdurahman et al., 2012].

pH analysis

The pH analysis of creams is an important parameter regarding stability and efficacy. The normal range of skin pH is 4.5-6, so formulations intended to be applied to the skin should have pH closer to this range [Arshad et al., 2016]. pH analysis were performed of freshly prepared creams and after (24h, 48h, 7d, 14d, 50d, 60d and 90d) and average change in pH values are reported in Table 18. It was evident from results that no significant variation was observed in pH values throughout the study period.

Table 19: Physiological Evaluation of the Formulated cream

Parameter	F1	F2	Placebo
Homogeneity	Homogenous	Homogenous	Homogenous
Appearance	Light brown semisolid	Light brown semisolid	White semisolid
Odour	Good	Good	Good
Loss on Drying	0.10%	0.12%	0.18%
Spreadability	Good	Good	Good
After Feel	Emollient, Slipperiness	Emollient, Slipperiness	Emollient, Slipperiness
Removal	Easy to remove	Easy to remove	Easy to remove
Stability (room temp)	Stable for 3 months	Stable for 3 months	Stable for 3 months
Stability (at 8°C)	Stable (No cracking)	Stable (No cracking)	Stable (No cracking)
Stability (at 40°C)	Stable (No phase separation)	Stable (No phase separation)	Stable (No phase separation)
Greasy	Non- greasy	Non- greasy	Non- greasy
Sticky	Non-sticky	Non-sticky	Non-sticky
Microbial Limit	6-8.5mm zone of inhibition against different bacteria	Almost no zone of inhibition	No zone of inhibition at all

F1= Formulation 1, F2= Formulation 2.

Table 20: Stability study profile of F1, F2 creams and placebo with time

Physical Parameter	Fresh			24h			48h			7d			14d			30d			60d			90d		
	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P	F 1	F 2	P
Color	LB	LB	W	LB	LB	W	LB	LB	W	LB	LB	W	LB	LB	W	LB	LB	W	LB	LB	W	LB	LB	W
pH	6.18	6.20	6.52	6.18	6.19	6.48	6.15	6.18	6.20	5.88	5.79	5.92	5.56	5.51	5.55	5.25	5.19	5.39	5.10	4.70	4.89	4.86	4.57	4.30
Centrifugation	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	U
Liquefaction	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	U	U	U	U

P= Placebo, h= Hour, d= Day, LB= Light Brown, W= White, S= Stable, US=Unstable.

6.5 Panel Test

Sensory evaluation of the two creams by the volunteers are presented in table 19. A questionnaire containing seven questions was prepared and the copy of this form was given to each volunteer for sensory evaluation of the two cream formulations and the placebo. Average marking for the first question, ease of application of creams were found to be 'Good' for the active formulations and the placebo or basic formulation. This indicates that both of the active formulations and placebo can be easily applied on the skin. Average marking regarding spreadability for active formulations F1 and F2 were 'Good' but 'Normal' for the basic formulation which means that the active formulations spreads on skin better than the basic formulation. Average values for feel on application were same for active formulations and also for the basic formulation just after application but in long term active formulations produce more pleasant feeling on application to skin than the basic formulation. There was no irritation on the skin for both the active formulations and placebo marked by all the volunteers. Shine on skin was normal for the active formulations and also for the placebo or basic formulation. Actually the preparations seem to be similar upon sensorial evaluation.

Table 21: Average quality of formulations F1, F2 and Placebo.

Considerations	Average quality of F1	Average quality of F2	Average quality of Placebo
Ease of application	Good	Good	Good
Spreadability	Good	Good	Normal
Sense just after application	Good	Good	Good
Sense in long term	Good	Good	Normal
Irritation	No	No	No
Shine on skin	Normal	Normal	Normal
Sense of softness	Good	Good	Good

6.6 Statistical Analysis

Table 22: Level of Significance of Antioxidant test of *S. apetala* Fruit Extract.

Sample	DPPH assay (µg/ml)	FRAP assay (µM Fe(II)/g of dried plant material)	Reducing power assay (µg/ml)
<i>S. apetala</i> (methanolic extract)	115.286****	442.947****	132.275****
<i>S. apetala</i> (aqueous extract)	122.257****	410.75****	560.345****

****= P value is <0.0001

Table 23: Level of Significance of Secondary Metabolites test of *S. apetala* Fruit Extract.

Sample	Total Phenolic content(mgGAE//g of extract)	Total Flavonoid content (mgQE/g of extract)	Total Tannin content (mgGAE/g of extract)
<i>S. apetala</i> (methanolic extract)	201.701****	92.611****	40.708****
<i>S. apetala</i> (aqueous extract)	76.438****	51.129****	11.839****

****= P value is <0.0001

Table 24: Correlation Analysis

Compared Parameters for Correlation Analysis	Level of Significance at $p < 0.05$
Flavonoid vs DPPH	Significant
Flavonoid vs Reducing Power	Significant
Flavonoid vs Phenol	Significant
Flavonoid vs FRAP	Non-Significant
Flavonoid vs Tannin	Non-Significant

Table 25: Correlation Analysis

Compared Parameters for Correlation Analysis	Level of Significance at $p < 0.05$
Phenol vs DPPH	Significant
Phenol vs Reducing Power	Non-Significant
Phenol vs Flavonoid	Significant
Phenol vs FRAP	Significant
Phenol vs Tannin	Non-Significant

Table 26: Level of Significance of Antimicrobial Test for Methanolic and Aqueous Extract of *S. apetala*

Bacteria	Statistical Analysis			
	Extract	Significance Level	Extract	Significance Level
<i>S. aureus</i>	Methanolic	***	Aqueous	***
<i>B. megaterium</i>				
<i>E. coli</i>				
<i>P. aurigonisa</i>				

Table 27: Level of Significance of Antimicrobial Test for Creams Formulated from Methanolic and Aqueous Extract of *S. apetala*.

Bacteria	Statistical Analysis			
	Cream	Significance Level	Cream	Significance Level
<i>S. aureus</i>	From	NS	From	NS
<i>B. megaterium</i>	Methanolic		Aqueous	
<i>E. coli</i>	Extract		Extract	
<i>P. aurigonisa</i>				

NS= non-significant



7. Conclusion

S. apetala is commonly known as Keora in Bangladesh and widely used by the people of coastal region for its medicinal value in the traditional system of medicine. In this research work, extracts of *S. apetala* fruit were investigated to know the existence of beneficial functional bioactive components, antioxidant and antibacterial activities and formulation of antioxidant skin care cream by addition of *S. apetala* fruit extract were done. The study showed that *S. apetala* fruit extract especially methanolic extract is a potential source of polyphenols, antioxidants and antibacterial bioactive compounds and therefore have beneficial activities to human use. The antioxidant creams are widely used today as it appears to be an interesting way to safeguard the skin against oxidative stress caused by various extrinsic sources. To maintain the effectiveness of antioxidant against free radicals, it is important to stabilize the final formulation on its antioxidant properties. The current practice moves towards in the formulation of cream from methanolic and aqueous extract and the study revealed that the cream from methanolic extract having higher antibacterial activity compared to that of aqueous and placebo. The evaluation test reveals that the formulated cream from methanolic extract showed that it is safe to be used in the skin to protect from extrinsic oxidation sources. The research work suggests that, to ensure the quality and purity of the cream it must have the consistency and uniformity in the ingredients of the herbal antioxidant cream. The trend of using herbal skin cream is becoming in demand since it is proven that topical application of antioxidant cream is effective against UV radiation and protect the skin from major consequence of UV damage. In conclusion, the topical application of the formulated cream from *S. apetala* methanolic extract will help in reducing oxidative damage and give the antioxidant effect to our skin due to its high antioxidant values.

8. Reference

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