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TOTAL FLAVONOIDS CONTENT AND REDUCING POWER ASSAY OF TWELVE COMMON BANGLADESHI LEAFY VEGETABLES

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Abstract

Around hundred different types of vegetable are available in Bangladesh for human consumption that are the important nutritional source of dietary fibers and antioxidants. *Amaranthus gangeticus, Amaranthus viridis, Basella alba, Brassica campestris, Chenopodium album, Centella asiatica, Coriandrum sativum, Enhydra fluctuans, Ipomoea aquatica, Lagenaria siceraria, Pisum sativum* and *Spinacia oleracea* are the twelve common leafy vegetables of Bangladesh. In this study, their ethanol extracts were studied to determine total flavonoids (TF) content and reducing power (RP) to evaluate the antioxidant activity of the extracts. This study reveals the high antioxidant contents in the leaves of the vegetables. *I. aquatica* was found to highest in TF and RP followed by *P. sativum* and *L. siceraria*. The lowest TF was found in *S. oleracea* which also showed the lowest RP. A significant correlation between flavonoids content and reducing power (r^2 = 0.87) was found. The high antioxidant contents in the vegetables may the reason behind high life expectancy of people in this region although they are highly exposed to high oxidative stress, risky food habit and worst level of pollution.

Key words: Antioxidant, Polyphenolic Substances, Reactive Oxygen Species, Reducing Power, Total Flavonoid Content, Traditional Leafy Vegetables.

Introduction

Vegetables are herbaceous plant that does not have much hard fiber and its stems are green and soft with abundant leaves. Mainly the leaves and also the fruit, seeds, roots, tubers, bulbs etc. are used as food. Around 100 different types of vegetable. In Bangladesh, around hundred different types of vegetable are available in Bangladesh for human consumption that are the important nutritional source of dietary fibers and antioxidants. However, the abundancies of vegetable for human ingestion is only nearly one fifth of the recommended requirement of 200 g per individual per day. There is a huge demand of leafy vegetables in Bangladesh due to they are popular food, low production cost and price and they are an integral part of food habit of people. They are cultivated throughout the year but its production increases much more during winter.[1] Oxygen is a matter of life and death. Though oxygen plays important role in different cellular functions, it generates reactive oxygen species (ROS) and free radicals in large amount during the production of ATP in mitochondria.[2] Free radicals are biochemical molecule type which possess an unpaired electron that can be considered as fragments of molecules which are usually extremely reactive.[3] These unbound radicals and other ROS are derived either from usual essential metabolic pathways in the human body or from external sources such as exposure to air and environmental pollutants, radioactive radiation, ozone (O_3), cigarette smoking, and industrial hazardous chemicals.[4] There are lots of commonly generated free radicals that are produced in modern urban processes namely the superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydro- gen peroxide (H_2O_2) and lipid peroxide radicals. They are responsible to create a number of pathological conditions like asthma,[5] cardiovascular disease,[6] cancer,[7] eye cataracts,[8] diabetes mellitus,[9] digestive inflammatory diseases,[10,11] macular degeneration,[12] liver disease,[13] periodontal disease[14] and other pain pathways. The risk of diseases caused by free radicals can be reduced by increased consumption of antioxidants which are abundant in food.[4] Certain Polyphenolics and flavonoids have been shown in a various strong antioxidants, capable of scavenging hydroxyl and superoxide anions and also lipid peroxy molecules. Flavonoids and bioflavonoids are a universal group of polyphenolic substances containing phenyl ring, heterocyclic ring and some polyphenol groups. They are found in most plants and in plant parts- leaves, bark, flowers, seeds, fruit flesh and in fruit skin or peel.[15] Bangladesh is rich in different types of leafy vegetables using as food as well as traditional medicine. Nevertheless, most of these vegetative plants have not yet undergone any systematic study for their biochemical, pharmacological or toxicological potentials to examine their bioactive chemical groups(s). Hence, this study was outlined in order to investigate the flavonoids content and the scavenging activity towards free radical of twelve common Bangladeshi leafy vegetables and the correlate among the amount of flavonoids, ability to scavenge free radicals and their beneficial effects. *Amaranthus gangeticus* synonym *Amaranthus polygamus* is an annual, shrubby herb, often much branched, up to 1.0-1.5 m tall. The plant grows in Bangladesh, Pakistan, China, Japan, Indonesia, and New Guinea. Though it is used in Bangladesh as a vegetable it has several medicinal uses.[16] The plant is used in the treatment of Lipoma (Tumor),[16] to cure constipation, poor eye sight, inflammation, anemia menorrhagia, leucorrhea and possess radio protective activity.[17]

Amaranthus viridis belonging to the botanical family Amaranthaceae, is commonly known as Notay sak, Pora notay (Bengali), Green amaranth (English) in Bangladesh[16]. This green soft but erect annual herb with deeply veined, ovate-lanceolate, green leaves and tufts of small flowers on long spikes grows as a weed all over Bangladesh.[18] This plant is also distributed throughout India, Sri Lanka and all other tropical and warmer countries of the world.[16] In Bangladesh this plant is used as leafy vegetables. The pharmacological benifits of amaranth formulations are considered of pivotal importance.[19] For reducing tissue swelling the leaves are well thought-out to be constructive, and they have a cleansing effect too. The plant has also been used curatively for diarrhea, dysentery, excessive menstrual flow, ulcers and intestinal hemorrhaging.[20]

Basella alba (family-Basellaceae) well-known as Malabar spinach or cyclone spinach and locally called pui shak. It is a fast growing vegetable, native to tropical Asia, probably originating from India or Indonesia and extremely heat tolerant. The leaves of this climbing vegetable are thick, rugose, succulent and green to purple in color and known for being rich in ß-carotene and vitamin A [21] and used to treat catarrhal affections and to hasten suppuration. Decoction of the root relieves bilious vomiting. It is also a good source of vitamins and minerals. The plant is reported to treat against laxative, inflammation, hemorrhages, skin diseases, sexual weakness, burns, ulcers, diarrhea and cancer and

used as rubefacient.[22]

Brassica campestris, commonly recognized as field mustard or turnip mustard is a plant widely cultivated as a leafy vegetable, a root vegetable and an oilseed.[23] Field mustard is an upright winter annual or biennial that is a member of the mustard family-Brassicaceae which is highly regarded for its nutritional value and proteins containing balanced amino acid profile.[24] Almost all parts of the species have been developed for food, including the root, stems, leaves and seeds. Brassinosteroids, a group of steroidal substance, has also been reported in seed of *Brassica campestris*, which were shown to have substantial anti-viral activity against pathogenic viruses including herpes simplex virus type 1, RNA viruses and measles virus.[25]

Chenopodium album of the family *Chenopodiaceae* is known as Batua shak, Chandan betu (Bengali) and cooked as a vegetable or stir-fried.[16] It is a polymorphous, mealy white and erect herb which is 3.5 m in height, and found wield in altitude of 4,700 m. The tender shoots are also eaten raw in salad or with curd. This weedy plant has various medicinal applications.[26] The plant is used in the treatment of abdominal pain, body pain, flatulence and gynecological disease.[16]

Centella asiatica, urban of Hydrocotylaceae family, commonly known as Brahmabuti, Thankuni (Bengali), Mrang khua, Gaokolibos (Marma), Shakkumu bakla (Tripura) is taken either of raw and cooked. It is a perennial herb with simple leaves and creeping stem [16] It has been used as a memory enhancing, strength promoting, wound healing, immune booster, anti-anxiety, anti-epilepsy and anti-stress substance.[27, 28]

Coriandrum sativum belongs to the family Apiaceae is commonly known as Dhunia and Dhonia in, Bengali, Coriander in Chinese and parsley in English. The plant is used in the treatment of blood dysentery, constipation, diarrhea, fever, flatulence, gastric tumour, hiccup, hook worm infestation, impotence, indigestion, leprosy, leucorrhoea, lumbago, lumps in the throat, piles and strangury.[16]

Enydra fluctuans belongs to the family Compositae commonly known as Helencha Shak, Hincha Shak and Harhach in Bengali; Water Cress and Marsh Herb in English. This tasty edible semi-aquatic herbaceous vegetable plant grows commonly in marshy and watery places all over Bangladesh. Plant is rich in protein and is a good source of beta-carotene, saponins, myricyl alcohol, kaurol, cholesterol, sitosterol glucoside and sesquiterpene lactones including germacranolide, enhydrin, fluctuanin and fluctuandin. Plant is nutritious and is used in ascites, dropsy, anasarca and snakebite. Leaves are demulcent, laxative, antibilious and cure inflammations, bronchitis, biliousness, leucoderma and other skin affections and nervous diseases. [18]

Ipomoea aquatica is a plant of Convolvulaceae family and commonly known as Kalmi shak (Bengali), Kengjoi (Marma). This plant grows all over Bangladesh and India. It's a glabrous trailer on mud or floating on water, stems usually hollow, rooting at the nodes. The leaves and young shoots are used as a vegetable. The plant is used in the treatment of chicken pox, diabetes, gastric tumour, lactomenia, paralysis, stomachache and vaginitis. [16]

Lagenaria siceraria (Family-Cucarbitaceae) a luxuriantly growing large climbing or creeping plant with large hirsute palmate leaves, white epigynous flowers and large fleshy fruits, cultivated as a vegetable crop throughout Bangladesh. This plant is commonly known as Lau, Kadu (Bengali), White Pumpkin, Bottle Gourd (English).[18] Outside Bangladesh it is also found in Pakistan, India, Nepal, Myanmar, China, Japan, Malaysia, Australia and Africa [18]. *L. siceraria* is cooling, antibilious, diuretic, emetic and purgative and is given in jaundice. Cooked pulp is used in cholera. Seeds are nutritive and diuretic. Seed oil is used to relieve headache, and warm juice of tender stem relieves earache.[18]

Pisum sativum belongs to the family Papilionaceae and commonly known as Motor (Bengali), Pea, Garden Pea (English) is cultivated as a pulse crop in most areas of Bangladesh. The leaves are used in the treatment of burning of the skin and for alleviating bile, phlegm and blood diseases. Seed oil has sterility and anti-sex hormonic effects, produces sterility in women and antagonises effect of male sex hormone.[16]

Spinacia oleracea is commonly known as "spinach" belonging to family Chenopodiaceae.[29] It has been used in the treatment of urinary calculi, cools, moistens and promotes the urine flow. Beta carotene and lutein in spinach help to maintain healthy eyes and prevent the occurrence of eye disorders like itchy eyes, dry eyes or eye ulcers. It also helps to fight diseases of the bone and skin disorders and helps to protect the health of infants and newborns.[16,29]

The life expectancy in Bangladesh is higher although the people are exposed to highly pollution of environment, adulterated and contaminated food and unhealthy food habit. Besides, Bangladeshi cuisine used to employ high temperature for longer time to cook food than the other customs which

generates more free radicals and ROS. Despite, the rate of asthma, cancer, cardiovascular disease, cataracts, diabetes, gastrointestinal diseases, liver disease, macular degeneration, periodontal disease and other inflammatory diseases in Bangladeshi population is less than many other developed countries. People here are habituated to consume lots of leafy vegetables and they are the integral part of their traditional dietary habits. [30] These leafy vegetables are cheap, comparatively easy to grow anywhere in fields or beside home. They may rich in antioxidants and this is assumed to be one of the reasons of high life expectance in this region. Therefore, the aim of this study is to determine the antioxidant activity through the total flavonoids content and reducing power of ethanolic extracts of twelve common leafy vegetables of Bangladesh- *A. gangeticus*, *A. viridis*, *B. alba*, *B. campestris*, *C. album*, *C. asiatica*, *C. sativum*, *I. aquatica*, *P. sativum* and *S. oleracea* and the correlate among flavonoids content, free radical scavenging activity and their beneficial effects.

Methods

Collection and identification of plant material

The different parts of twelve leafy vegetable plants were collected from different area of Bangladesh in different time of the year round which are listed in the table 1. The exsiccated plant samples were mounted on herbarium sheet and deposited fulfilling the institutional requirements in Bangladesh National Herbarium (BNH). The specieses were confirmed by Sarder Nasir Uddin, Principle Scientific officer, BNH, Mirpur, Dhaka and a voucher specimen of the plant has been deposited and preserved in the library of the same institution for further collection and references.

Preparation of ethanol extract

The collected leafy vegetables were washed by water to remove undesirable materials. Then excess of water drained off. They were dried in open air protecting from direct sunlight for about two weeks. The shade dried plant parts were then ground into a coarse powder with the help of a suitable grinder (capacitor start motor).The obtained powders were stored in individual airtight container with proper labeling and kept in a cool, dark, and dry place until the analysis commenced. Extraction was conceded following the method depicted by Khatun et al. (2016).[31] For the preparation of ethanol extract 100 g of each powdered materials were taken in individual clean, flat-bottomed glass containers and soaked in about 250 mL ethanol. Then it was homogenized with a suitable homogenizer. The containers along with their contents were sealed and kept in dark place for a period of 10 days with daily gentle hand shaking. The mixtures then underwent a coarse filtration by cotton and Whatman filter paper (Whatman International Ltd., Maid stone England). Each filtrate were collected into individual amber glass container and concentrated under air. Different amounts of concentrate extracts were obtained which were designated as crude ethanol extracts.

Chemicals and reagents

Sodium nitrite (PubChem CID:23668193), aluminum chloride hexahydrate (PubChem CID:24564), sodium hydroxide (PubChem CID: 14798) and catechin (PubChem CID: 73160) purchased from Sigma Aldrich, GmbH were used for flavonoid content test. L-Ascorbic acid (PubChem CID: 54670067) was used as standard for reducing power test. Sodium phosphate (Na_2HPO_4, PubChem CID: 24203) purchased from Scharlab S.L., Spain, potassium ferricyanide (PubChem CID:26250) was purchased from Sigma Aldrich, GmbH, trichloroacetic acid (PubChem CID:6421) purchased from RCI Labscan, Thailand were used in reducing power test. Ethanol (PubChem CID:702) purchased from Merck, Germany was used as solvent.

Instruments and equipment

Electronic balance, test tubes, beakers, micropipettes magnetic stirrer, vortex mixer, conical flask, shaking water bath, pH meter, UV Spectrophotometer (Shimadzu, model: UV-1800), centrifuge, homogenizer, screw capped amber bottle etc were used during different steps of the study.

Test for total flavonoids content

Total flavonoids content was determined spectrophotometrically using catechin as a standard according to the method of Khatun et al. (2016). [31] 2 mg/mL stock solution of sample extract was prepared. Then 1 mL of stock solution was taken into a 10 mL volumetric flask. 4 mL of distilled water was added to make the volume 5 mL. Further 0.3 mL of $NaNO_2$ solution (5% w/v) was added to the flask. After 5 min, 3 mL of $AlCl_3$ solution (10% w/v) was added. After another 6 min, 2 mL of 1M NaOH solution was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well again and the absorbance was measured against a blank preparation (without sample) at 510 nm with a UV-visible spectrophotometer. Catechin solution of 20 µg/mL, 40 µg/mL, 60 µg/mL and 80

µg/mL were used for the preparation of calibration curve.

Test for reducing power

The reducing power of the extracts was quantified according to the method of Ferreira (2007).[32] Extracts of 400 µg/mL concentrations were prepared and taken into individual test tubes. To all the extracts in test tubes 2.5 mL of sodium phosphate buffer (200mM Na_2HPO_4, pH 6.6) followed by 2.5 mL of potassium ferricyanide solution (1% w/v) were added. The contents were vortexed well and then incubated at 50°C for 20 minutes. After incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added to all the tubes and centrifugation was carried out at 3000 rpm for 10 minutes. Afterwards, to 5 mL of the supernatant, 5 ml of deionized water was added. Then about 1 mL of ferric chloride (1% w/v) was added to each test tube and incubated at 35°C for 10 minutes. The absorbance was read at 700 nm. Mean values were obtained from triplicate experiments. The reducing power of the extract was linearly proportional to the concentration of the sample. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical Analysis

All values obtained were calculated as mean ± standard deviation of minimum three replicates, not shown in figures. Data analysis was conceded using Microsoft Excel, Microsoft Corporation, USA, to attain the descriptive statistics. The different levels of significances within the separate treated groups were analyzed using one-way analysis of variance followed by Dunnett's test to determine statistical significance. Statistical differences between control and treated groups were tested by Student's t-test. Variances with $p<0.05$ were considered statistically significant.

Results

Total flavonoids (TF) content

Total flavonoids (TF) content of the ethanol extracts of twelve leafy vegetables was expressed as mg catechin equivalent (CE) per g of dried extract (Figure 1). From the present experiment it was seen that *I. aquatica* contain the highest total flavonoids (23.2 mg CE/g) followed by *P. sativum* (17.64 mg CE/g) and *L. siceraria* (17.22 mg CE/g). TF was lowest in the extract of *S. oleracea* (0.91 mg CE/ g).

Reducing power (RP)

The more antioxidant compounds reduce more oxidized form of ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}). In the present study, reducing power of ethanolic extracts of twelve leafy vegetables was measured at 700 nm and expressed as optical density (OD). Reducing powers of the extracts was determined using the potassium ferricyanide. From the experimental data (Figure 2) it was seen that *I. aquatica* has the highest reducing power (OD 1.79) followed by *P. sativum* (OD 1.11), *L. siceraria* (OD 0.86) and *B. alba* (OD 0.72). The reducing power was lowest in *S. olerecea* (OD 0.27). Dose-dependent increase of reducing power of *I. aquatica*, *P. sativum, B. alba* and *C. sativum* (Figure 3).

Flavonoids content vs. reducing power

In the present study correlation between total flavonoids content and reducing power was observed by plotting reducing power against the total flavonoids content of certain plant extract. In this study, a significant correlation between flavonoids content and reducing power ($r^2 = 0.87$) was found as shown on the figure 4. It also indicates the presence of other polyphenolic compounds which may contribute to the reducing power.

Discussion

Flavonols such as quercetin, anthocyanins, myricetin, kaempherol and flavones like apigenin, luteolin in plant materials are closely associated with their antioxidant function mainly due to their redox properties exerted by various possible mechanisms namely free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity.[33, 34] In this current study, it was found that among twelve leafy vegetables *I. aquatica* had the highest content of total flavonoids (28.11 mg CE/g) followed by *P. sativum* (17.64 mg CE/g), *L. siceraria* (17.22 mg CE/g) and *B. alba* (6.54 mg CE/g). It is generally observed from the present study that vegetables with higher flavonoids contents exhibited higher reducing power. A correlation between total flavonoids content and reducing power was found 0.87. It seems that there is a strong correlation between flavonoids content and reducing power. Besides this components other than flavonoids present in the extracts of leafy vegetables might contribute in part to their reducing power. In fact, due to complexity of oxidation-anti-oxidation process, no single testing method is capable of providing a complete anti-oxidative profile. [35] Therefore, a multi-method approaches like determination of total phenolic content, DPPH

radical-scavenging assay, determination of bioactive polyphenolics are required to asses a complete anti-oxidative activity for all the leafy vegetables in this study. [36-41] Further in vivo and in vitro studies are necessary to know their mechanism of action and potential active compound(s). It is necessary to elucidate potential cytotoxic effects when they are used for the preparation of dietary supplements, as some Polyphenols perturb the membrane structure. [42] Moreover, fractionation of potential extracts is essential to know the phenolic or nonphenolic compounds responsible for the anti-oxidative activities.

Conclusions

Bangladeshi peoples cannot provide themselves with expensive anti-oxidative drugs or fruits. But plenty of cheap fruits and vegetables are grown here all the year round. Reportedly, many of the health-promoting activities of fruits and vegetables, e.g. anti-cancer, anti-microbial, anti-diabetic, anti-mutagenic, anti-inflammatory, anti-allergic, anti-amylase, anti-glycosidase activities, are related to anti-oxidative activity. In the present study, twelve most common Bangladeshi leafy vegetables were investigated to know the existence of flavonoids as a representative beneficial bioactive components and their reducing power. The results of the experiments revealed that most of the leafy vegetables are potential source of natural antioxidants. This current study supports the assumption that one of the key factors of high life expectancy and wellbeing of people of Bangladeshi population is the consumption of high amount of leafy vegetables and mandates more investigation to explore complete antioxidant profiling of the leafy vegetabls.

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Table 1. Details of sample collection

Plant Name	Collected Part(s)	Collection Time	Collection Area	Amount (kg)
Amaranthus gangeticus	Leaf and stem	December	Baburhat Bazar, Bagerhat	10.5
Amaranthus viridis	Leaf and soft stem	September	Koia Bazar, Khulna	8.0
Basella alba	Leaf and soft stem	May	Baburhat Bazar, Bagerhat	18.2
Brassica campestris	Leaf and soft stem	February	Gopalgonj	11.0
Chenopodium album	Leaf	March	Koia Bazar, Khulna	10.0
Centella asiatica	Whole plant	June	Gollamari Bazar, Khulna	5.0
Coriandrum sativum	Leaf and soft stem	May	Gollamari Bazar, Khulna	4.2
Enydra fluctuans	Leaf and soft stem	March	Koia Bazar, Khulna	6.0
Ipomoea aquatica	Leaf and soft stem	June	Koia Bazar, Khulna	8.5
Lagenaria siceraria	Leaf and soft stem	May	Baburhat Bazar, Bagerhat	9.5
Pisum sativum	Leaf and soft stem	December	Koia Bazar, Khulna	8.0
Spinacia oleracea	Leaf and stem	January	Koia Bazar, Khulna	15

Table 2: Details of sample preparation and output

Plant Name	**Amount of fresh sample (kg) (After cleaning and screening)**	**Powder obtained (kg)**	**Extract obtained in mg/100 g of dried sample**
Amaranthus gangeticus	9.5	2.4	980
Amaranthus viridis	6.2	1.6	760
Basella alba	17.5	3.1	689
Brassica campestris	9.5	2.2	810
Chenopodium album	7.9	1.8	785
Centella asiatica	4.6	0.6	699
Coriandrum sativum	3.0	0.5	784
Enydra fluctuans	4.5	1.1	678
Ipomoea aquatica	6.8	1.7	860
Lagenaria siceraria	7.5	2.3	901
Pisum sativum	6.5	1.9	806
Spinacia oleracea	10.5	2.3	788

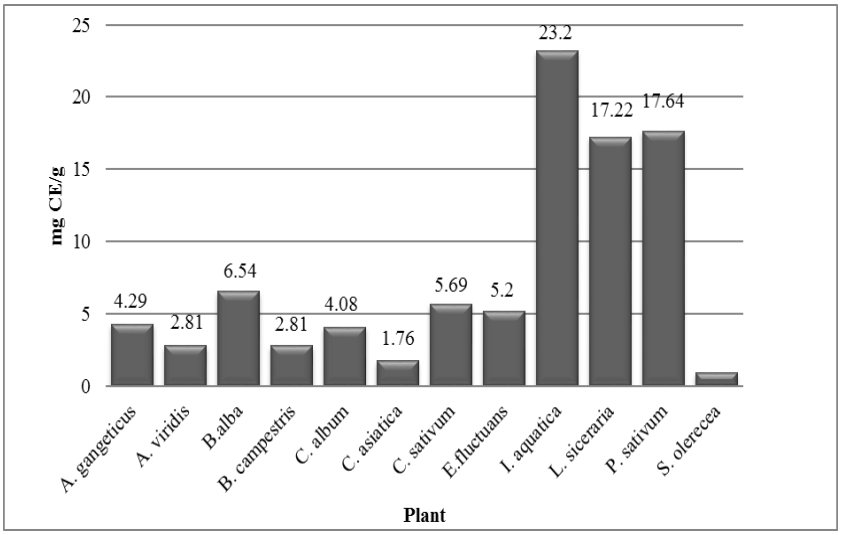

Figure 1. Total flavonoids content in mg CE/g of plant extracts

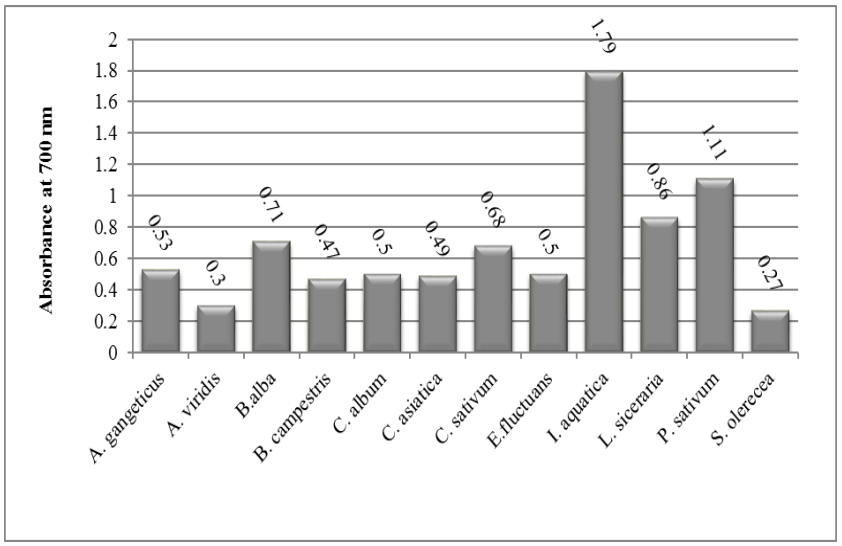

Figure 2. Optical density of twelve sample preparation representing reducing power.

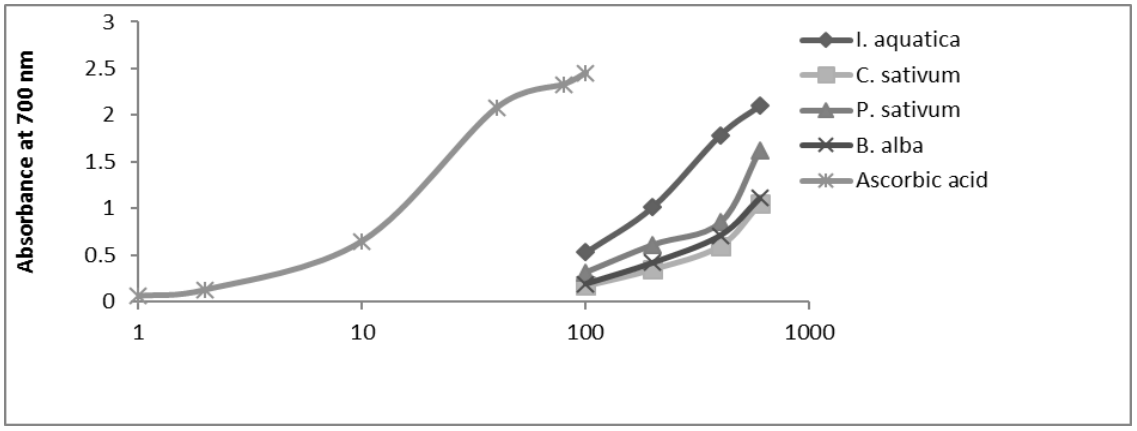

Figure 3. Dose dependent increase of reducing power.

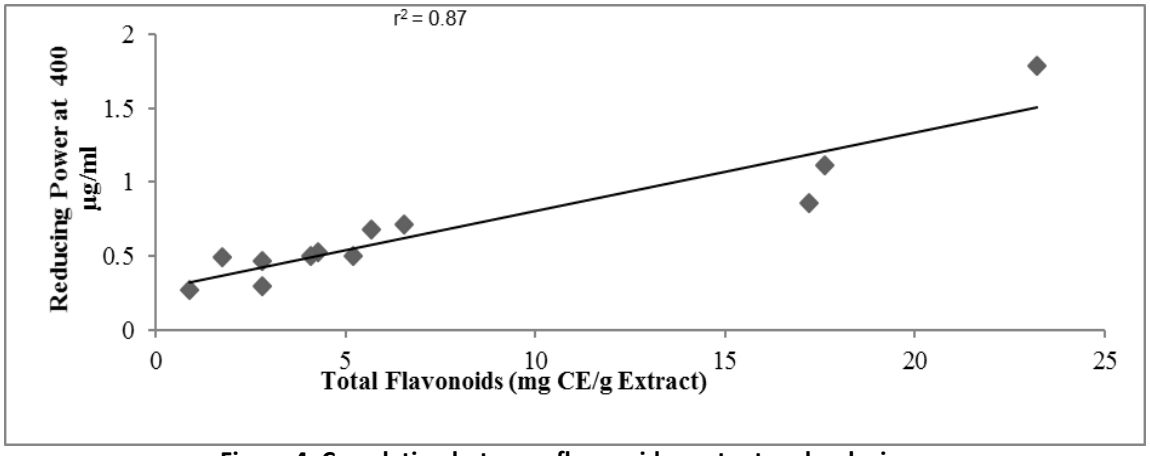

Figure 4. Correlation between flavonoids content and reducing power.