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Antioxidant activities of *Artocarpus heterophyllus*, *Terminalia bellerica* and *Carica papaya* leaves in methanolic extract

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Abstract
The present study was undertaken in order to study the antioxidant properties of leaf of three medicinal plants viz. *Artocarpus heterophyllus, Terminalia bellerica* and *Carica papaya*. Methanol was used as organic solvent for extraction. The antioxidant activity was determined by DPPH free radical scavenging assay, superoxide scavenging assay, reductive ability, nitric oxide free radical scavenging assay, hydroxyl radical scavenging assay, vitamin C and vitamin E determination. The present study revealed that all the three medicinal plants have a good free radical scavenging activity and thereby act as potent antioxidant. Extract of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* could be useful for preparation of neutraceuticals as potent antioxidant to treat various diseases and its complications.

Keywords: Phytochemicals, antioxidant activity, free radical, DPPH, reductive ability, methanol, oxidative stress

1. Introduction
Reactive oxygen species (ROS) or free radicals are generated inside the cells during metabolic processes that modulate the various physiological functions of the body. Excessive generations of these free radicals disrupt the antioxidant defence system of the body that may lead to "oxidative stress". This situation brings about a variety of disorders including coronary heart disease, neurodegenerative disorders, diabetes, arthritis, inflammation, lung damage and cancer [1]. Antioxidants are capable of preventing oxidative damage. Plants are the natural source of antioxidant. The antioxidant property of the plant material is due to the presence of many active phytochemicals including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, plant sterol and etc. [2]. These natural antioxidants prevent oxidative damage caused by free radical and ROS by interfering with the oxidative processes of the cells. The use of natural antioxidants in the management of oxidative damage has gained importance throughout the world. The world health organization has also recommended and encouraged the use of natural products as antioxidant.
North eastern India has been known for its biological diversity which comprises of various types of flora and fauna. *Artocarpus heterophyllus* (Jack fruit) commonly known as Kathal is a species of mulberry family Moraceae. This plant has been used in the traditional medicinal due to its antioxidant, antibacterial, hypoglycaemic, anti-inflammatory activities.
Terminalia bellerica (Bahera) commonly known as Bhumura belongs to the family Combretaceae. It has been reported that this plant is rich source of phytochemical specially flavanoid and polyphenols and thereby act as antioxidant [3].
Carica papaya (Papaya) is ever green plant belonging to the family Caricaceae. This plant is a good source of beta carotene, flavanoid, polyphenols. It has anti-inflammatory, antiviral, antimicrobial, anthelmintic, antitumor, free radical scavenging activites [4].
Therefore, the present study was planned to investigate the antioxidant properties of methanolic leaf extract of three species using DPPH free radical scavenging assay, superoxide scavenging assay, reductive ability, nitric oxide free radical scavenging assay, hydroxyl radical scavenging activity, vitamin C, vitamin E determination.

2. Materials and Methods

2.1 Collection of plant sample

The fresh leaves of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* were collected from different parts of Assam, India. The plant materials were washed under running tape water followed by tipple glass distilled water to remove dust and cut into pieces, dried under shed and pulverized into fine powder in a grinding machine (Bajaj), the powder was kept in small plastic bags away from the light, heat, moisture with proper labelling.

2.2 Preparation of plant extract

Plant extract was prepared by dissolving 100 g of the sample in 1L of methanol and kept for three consecutive days. The plant mixture was stirred two times at an interval of 6 - 8 h with a clean glass rod. After third day the mixture was filtered through muslin cloth and filter paper. Filtrate was collected and stored in the refrigerator at 4 ^{0}C. Residue was collected and the extraction process was repeated two more times in the similar manner. Now the pooled filtrate containing methanol was finally extracted using Rotary Vacuum evaporator (EVATOR.Iwp). After that a semi solid plant extract was obtained which was kept in the refrigerator at 4 ^{0}C.

3. Antioxidant analysis

3.1 DPPH scavenging assay

The DPPH radical scavenging activity was measured according to the method described by Cotelle *et al.*, [5], with slight modification. In brief, 3 ml reaction mixture containing 200µl of DPPH (100µM in methanol) and 2.8 ml of sample (at various concentrations 3-110µg/ml) in methanol was incubated at 37^{0}C for 30 minutes and absorbance of the test mixture was read at 517 nm using UV- visible Spectrophotometer (Thermo Fischer Scientific Model 1119300). The percentage inhibition of DPPH radical was calculated using the following formula:

% inhibition = (Absorbance of control-absorbance of test) x 100/ absorbance of control

3.2 Superoxide radical scavenging activity

Superoxide anion scavenging activity of plants was measured by the method of Robak and Gryglewki, [6]. It is based on the inhibition of the production of nitro blue tetrazolium formazon of the superoxide ion. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4) to 1 ml of Nitro blue tetrazolium (NBT) (156 mM) and 1 ml of reduced Nicotinamide adenine dinucleotide (NADH) (468 mM), 3 ml plant extract (3-110 µg/ml) was mixed. The reaction was started by adding 100µl of Phenazine methosulphate (PMS) (60µM) and the mixture was incubated at 25 ^{0}C for 5 min, measurement of absorbance was done at 560 nm by using UV- visible Spectrophotometer. The percentage inhibition of superoxide radical was calculated using the following formula**:**

% inhibition = (Absorbance of control-absorbance of test) x 100/ absorbance of control

3.3 Reductive ability

Reducing power of plant extract is based on the ability of anti-oxidants to form coloured complex with potassium ferricyanide, TCA and $FeCl_3$. The reductive ability was estimated by the method described by Jayprakash *et al.*, [7]. According to this method 1ml of different concentration of plant extract (to produce final concentration 100-600 µg/ml) was mixed with 2.5 ml of potassium ferricyanide (1 %) and 2.5 ml of phosphate buffer (pH 6.6) and the mixture was incubated at 50 ^{0}C for 20 min. Then 2.5 ml of TCA (1%) was added and centrifuged at 3000 rpm for 10 min. From the above solution, 2.5 ml of supernatant was taken. To this 2.5 ml of water, 0.5 ml of $FeCl_3$ (0.1%) was added. Measurement of absorbance was done at 700 nm. The percentage inhibition of reductive ability was calculated.

3.4 Nitric oxide scavenging activity

For nitric oxide scavenging activity of plant extract method described by Marcocci *et al.*, [8] was followed. Sodium nitroprusside (1 ml of 10 mM) was mixed with 1 ml of plant extract of different concentrations (3-110 µg/ml) in phosphate buffer (pH 7.4). The mixture was incubated at 25 ^{0} C for 150 min. To 1ml of the incubated solution, 1ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm by using UV- visible Spectrophotometer. The scavenging activity was expressed as percentage inhibition.

3.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and test extract for the hydroxyl radical generated by Fenton's reaction [9]. One ml of reaction mixture containing 500 µl of plant extract (3-110 µg/ ml) and 100 µl each of 2-Deoxy-D-ribose (278 mM), Ethylene diamine tetra acetic acid (EDTA 1.04 mM), $FeCl_3$ (0.2 mM) and Ascorbic acid (1 mM) was incubated at 37 ^{0}C for 1 h. One ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) are added and incubated at 100 ^{0}C for 20 min. After cooling, absorbance was read at 532 nm by using UV- visible Spectrophotometer. The percentage inhibition was calculated using the following formula:

% inhibition = (Absorbance of control-absorbance of test) x 100/ absorbance of control

3.6 Vitamin C

Vitamin C was estimated in plant extract according to the method of Abbasy *et al*., [10]**.** At first 1 g of the plant sample material was grinded using pestle and mortar in 25 ml of 4 % oxalic acid followed by filtration. Now, 10 ml of aliquot was transferred to a conical flask and bromine water was added drop by drop with constant mixing. The enolic hydrogen atom in ascorbic acid is removed by bromine. When the extract turns orange yellow due to excess bromine, it was expelled by blowing air and the volume was made 25 ml with 4 % oxalic acid. Similarly, 10 ml of stock ascorbic acid solution was converted into dehydro form by bromination. Now, 0.1ml of standard dehydroascorbic solution was taken in a test tube. Accordingly 1 ml of different aliquot of brominated sample extract was pipetted. Distilled water was added to make the volume 3 ml. One ml of DNPH (Dinitrophenyle hydrazine) reagent followed by 1-2 drops of thiourea (10%) to each tube. A blank was set as above but with water in place of ascorbic acid solution. Content was mixed thoroughly and incubated at 37 ^{0}C for 3 h. After incubation the orange red osazone crystals were dissolved by adding 7 ml of 80% sulphuric acid. Absorbance was measured at 540 nm using UV- visible spectrophotometer and the amount of vitamin C was expressed as mg/ 100 g sample.

3.7 Vitamin E

Vitamin E was estimated according to the method described by Rosenberg, [11]. According to this method, 0.5 g of plant leaves was taken into a stopper tube and slowly 10 ml of 0.1N sulphuric acid was added without shaking. Now, the content was allowed to stand for overnight. In the next morning, content was shaken vigorously and filtered through Whatman no. 1 filter paper. Aliquot of the filtrate was used for estimation. Now in three centrifuge tubes (test, standard and blank), 1.5 ml of the plant extract, 1.5 ml of standard and 1.5 ml of water was pipetted out, respectively. To the test and blank 1.5 ml of ethanol and to the standard 1.5 ml of water was added and centrifuged. Then 1 ml of xylene was transferred to each tube and centrifuged. Now, 1 ml of xylene layer was transferred into another stopper tube taking care not to include any ethanol or protein. To each tube, 1 ml of 2, 2-dipyridyl reagent was added and mixed. In a cuvette, 1.5 ml of the mixture was pipetted out and absorbance was read at 460 nm against the blank**.** Beginning with the blank in turn 0.33 ml of $FeCl_3$ solution was added and mixed well. Now, exactly after 15 min absorbance was read against blank at 520 nm.

4. Calculation

Amount of Vitamin E in µg/ g sample =

$$\frac{\text{OD of test at 520 nm- OD of test at 460 nm X 0.29 X 15 X total volume of sample}}{\text{OD of standard at 520nm volume used X weight of the sample}}$$

4.1 Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean values ± standard error (SE). Data obtained from the experiment were analysed by using standard method.

5. Result and Discussion

5.1 DPPH scavenging assay

DPPH i.e 1, 1- dipheny l-2-picrylhydrazyl hydrate is used commonly to determine the *in vitro* antioxidant activity of a compound. When treated with substances or samples that are hydrogen donors, the DPPH radical is converted into a stable DPPH radical, indicated by a colour change from purple to yellow. The reaction capability of DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. At 3-110 µg, the antioxidant activities of methanolic extract of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* and the standard ascorbic acid were 61.21 -72.21 %, 55.16 - 62.99 %, 32.19 - 66.85 % and 23.03 - 63.19 % respectively. The extract exhibited concentration-dependent radical scavenging activity, that is the higher the concentration, the scavenging potential. Also polar solvent extracts shows more antioxidant activities as compared to non polar solvents [12].

5.2. Superoxide radical scavenging activity

Superoxide radical reduced NBT to a blue-coloured formation that was measured at 560 nm. At 3-110 µg, the superoxide scavenging activity of methanolic extract of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* was 64.96 - 83.6 %, 68.16 -75.88 %, 71.96 - 81.2 %, respectively and that of the standard ascorbic acid was 17.11-54.06 %. The methanolic extracts exhibited concentration dependent radical scavenging activity that is percentage inhibition increased with sample concentration.

5.3 Reductive ability

Methanolic extract of the three plants displayed considerable reducing power, primarily due to its effect as an electron donor and thereby halting radical chain reactions by converting free radicals to more stable products [13]. Increasing absorbance at 700 nm indicated an increase in reductive ability. At 100-600 µg/ ml concentration, the reductive ability of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* was 2.86 - 31.43 %, 5.00 - 27.86 %, 6.43 - 57.14 %, respectively. The extracts showed good reducing power that was comparable with that of ascorbic acid. The antioxidant activity confirmed the medicinal importance of plants as naturally occurring antioxidants.

5.4 Nitric oxide scavenging activity

The nitric oxide scavenging activity of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* was 19.19 - 38.79 %, 24.23% - 40.99 % and 17.91 - 29.34 % respectively at a concentration of 3-110 µg/ml.

5.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* was 20.82-70.99 %, 25.71-70.99 % and 53.30 - 84.76 % respectively at a concentration of 3-110 µg/ml at 532 nm. The hydroxyl radical can induce oxidative damage to DNA, lipids and proteins [14].

5.6 Vitamin C

Vitamin C or ascorbic acid is a water soluble antioxidant. It can capable of scavenging oxygen-derived free radicals. After analysis it was observed that the level of vitamin C in the three medicinal plants were 10.937, 31.25 and 17.18 mg /100 g of the sample of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya*, respectively.

5.7 Vitamin E

The level of vitamin E in the three medicinal plants was found as 40.05, 53.98 and 35.54 mg /100 g of the sample of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya*, respectively.

6. Conclusions

The present study indicates that leaves of *Artocarpus heterophyllus, Terminalia bellerica and Carica papaya* possesses antioxidant properties and could serve as free radical scavengers. The activity observed may be attributed to the presence of phenolic and flavaniod contents in the methanolic extract. Thus, these plants may be used in place of chemical antioxidants in the treatment of oxidative stress.

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