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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT POTENTIAL OF *PORTULACA OLERACEA* LINN.

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Abstract

Portulaca oleracea, an edible herb of Portulacaceae family is widely found in Bangladesh. This plant has many traditional medicinal uses and it is used to treat disorders of urinary, digestive, cardiac, hepatic, renal systems and many diseases. The goal of this research was to search antioxidant potential of crude extract of *P. oleracea* and its various solvent fragments (water, butanol, chloroform and *n*-hexane). Phytochemical screening revealed the existence of various phytochemical groups. In DPPH assay, IC_{50} value of the methanol extract, water, butanol, chloroform fractions were found to be 23, 25, 28 and 41μg/mL respectively. Total flavonoid content of methanol extract and water, butanol and chloroform fractions were found to be 137.72, 102.08, 88.93 and 67.83 mg of QE/gm of arid extract. Methanolic extract, butanol and chloroform fractions exhibited significant reducing power with absorption maximum 1.3121, 1.4231 and 1.4892, respectively. Total content of phenolic of methanolic extract, water, butanol, and chloroform fractions showed 176.70, 157.13, 122.84 and 70.61 mg of GAE/gm of arid extract, respectively. Methanolic extract, butanol and water fractions exhibited significant ferrous ion chelating ability with IC_{50} value of 25, 32 and 29 μg/mL respectively. Total tannin content of methanol extract and water, butanol, and chloroform fractions showed 140.56, 114.10, 89.14 and 41.44 mg of TAE/gm of arid extract respectively. Methanolic extract, water, butanol and chloroform fragments exhibited significant scavenging ability of nitric oxide (NO) with IC_{50} 10, 27, 24 and 36 μg/mL respectively. Based on these findings, we can say that *P. oleracea* is a possible reservoir of antioxidants and it can be applied to combat oxidative damages of our body.

Keywords: *Portulaca oleracea*, antioxidant activity, phytochemical, DPPH assay

Introduction

Oxidative stress plays a pivotal role in the evolution of age-related diseases including diabetes, dementia, atherosclerosis, arthritis, obesity, cancer, vascular diseases, osteoporosis and metabolic disorders. Biological system generated ROS (Reactive Oxygen Species) modulate the cellular activities such as cell survival, stressor responses, and inflammation (Zuo *et al.* 2015). ROS elevation progresses the process of aging. Due to their reactivity, high concentrations of ROS can cause oxidative stress by disrupting the balance of antioxidant and prooxidant levels (Zuo *et al.* 2015). Antioxidant plays a central role in the termination of oxidative chain reactions by removing the free radical intermediates. Antioxidants control the autoxidation by interrupting the propagation of free radicals or by inhibiting the formation of free radicals. These compounds help in scavenging the species that initiate the peroxidation, breaking the autoxidative chain reaction, quenching $\bullet O_{-2}$, and preventing the formation of peroxides. These reactions subsequently reduce oxidative stress, improve immune function, and increase healthy longevity. In biological system, antioxidants perform incisive functions for the defense of living systems against oxidative stress. Antioxidants are found in different food items,

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nutrients, vitamins even minerals and also in enzymes. They play vital roles in preventing the onset of various chronic diseases as heart disease, stroke, neurological disorders, arthritis, atherosclerosis, cancer and cataracts (Masuda *et al.* 2003).

Portulaca oleracea is one type of fleshy herb from Portulacaceae family. This annual herb is distributed worldwide mainly in temperate and tropical areas. It is commonly called Purslane in English and in Bangladesh, it is known as "Nuneshak". Stems of this herb are smooth, reddish in color. Green leaves are gathered at stem joints at both opposite and alternate directions. Flowers are divided in 5 parts, up to width of 6 mm. The flowers may visible at any time of the year depending on rainfall. In sunny morning, the flowers bloom singly for few hours. Tiny seeds are formed inside a pod. It has a fibrous taproot and it can tolerate drought and compacted soils (Kumar *et al.* 2008; Olusina *et al.* 2016; Zhou *et al.* 2015).

Portulaca oleracea is a powerhouse of nutrition due to its rich content of different beneficial secondary metabolites and omega-3 fatty acids. Omega-3 fatty acid performs vital roles in boosting the immunity of our body. This plant is a great source of necessary minerals like Calcium, Iron, Magnesium, Potassium, Zinc, Sodium (Aberoumand, 2009). Previous study showed that Purslane herb contains numerous compounds. Jin *et al.* 2016 reported the presence of L-malic acid, succinic acid, L-4-methyl malate, stearic acid, L-dimethyl malate, mono-methyl succinate, 5-hydroxymethyl-furoic acid, L-1-methyl malate, 4-hydroxy-5-methylfuran-3-carboxylic acid, L-6-ethyl citrate, succinimide, L-1-methyl citrate, (–)-epiloliolide, L-1, 5-dimethyl citrate, L-pyroglutamic acid, 3, 4-dihydroxyphenylethanol, and uracil. Sicari *et al.* 2018 performed HPLC-DAD analysis for phytochemical characterization and they quantified the presence of flavonoids like apigenin, kaempferol, luteolin, quercetin, isorhamnetin, kaempferol-3-O-glucoside, rutin etc. and phenolic compounds like caffeic acid, *p*-Coumaric acid, Ferulic acid etc. in the *P. oleracea* plant.

Many parts of the plant had been utilized in traditional medicine system since many years. The plant is helpful in the prevention and treatment of cancer, autoimmune diseases, hypertension, coronary artery disease and other inflammatory diseases. (Palaniswamy *et al.* 2001). It is used as a febrifuge, antiseptic, vermifuge in folk medicine. It possesses various types of pharmacological effects like anti-inflammatory, antioxidant, antiulcerogenic, antibacterial, and wound-healing properties. World Health Organization has recorded this plant as "Global Panacea" because it is one of the most used medicinal plant. It tastes sour and cold in nature. It is applied to reduce bleeding, resolve toxins, and decrease the body temperature. The dried leaves are used to treat dysentery, diarrhea, carbuncle, eczema, fever and hematochezia (Zhou *et al.* 2015).

Alam *et al.* 2014 and Fernández-Poyatos *et al.* 2021 reported their experiments on ethanol extract of *P. oleracea* of various locations and Sicari *et al.* 2018 conducted antioxidant tests of methanol and water, ethanol fraction of *P. oleracea*. From previous studies we found that all experiments were done on polar solvent extract of *P. oleracea*. From their observation, we learned that *P. oleracea* is rich in antioxidant. We conducted our experiment on different polarity solvent fractions as well as crude methanol extract of *P. oleracea* plants in order to investigate its different antioxidant content determination as well as radical scavenging activity. This experiment was designed to explore comparison of antioxidant properties of *P. oleracea* crude methanol extract and its different solvent fractions (water, butanol, Chloroform and *n*-Hexane) according to their difference in polarity.

Materials and Methods

Plant collection and drying

Aerial parts of *P. oleracea* was compiled from surrounding areas of Khulna University, Bangladesh. After collection, it was identified by Professor Fazlul Haque, Forestry and Wood Technology Discipline, Khulna University, Bangladesh and a specimen were preserved for future reference. During collection, all types of undesirable and admixtures were discarded. Plant parts were cut into small pieces and kept shade drying for 50 days. The dried materials were crushed to have refined powder utilizing a suitable mill (Capacitor start motor, Wuhu Motor Factory, China).

Preparation of extract

The extraction of powdered material (250 gm) was done using 95% methanol (700 mL) in a hermetic glass vessel to impede alcohol evaporation at laboratory temperature. Filter paper (Whatman) was used to filter the slurry and the liquid extract was concentrated in reduced pressure utilizing rotary evaporator using ambient temperature. Then, 7.25 gm extract was obtained and the yield was 2.9%.

Chemicals and reagents

All reagents used to carry out the experiments were analytical grade. The reagents such as Folin-Ciocalteu reagent (Sigma, USA), quercetin (Sigma, USA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, USA), gallic acid (Sigma, USA), ascorbic acid (Merck, Germany), EDTA (Loba, India), BHA (Merck, Germany) were used. Solvents like methanol, n-hexane, Ethylacetate, butanol, chloroform and ethanol were from Merck, Germany were used.

Phytochemical test

Primary phytochemical analysis was conducted based on the specific standard protocols found in Ayoola *et al.* 2008. This phytochemical experiment was carried out to investigate the presence or absence of different phytochemical groups such as flavonoid, reducing sugar, alkaloid, glycoside, tannin, saponin, terpenoid etc.

Qualitative Antioxidant Assay using DPPH

In vitro qualitative antioxidant experiment was conducted based on the protocol of Sadhu *et. al.* 2003. This study was done according to radical scavenging activity of DPPH. Ascorbic acid *P. oleracea* extract diluted with solvents were dotted on TLC plates pre-coated with silica gel. Chromatogram was amplified by soaring manner using three different solvent systems i.e., non-polar (*n*-hexane: Acetone = 3:1), medium polar (CH_3OH: $CHCl_3$ = 1:5) and polar (H_2O: CH_3OH: $CHCl_3$ = 1:10:40). The mobile phase solvents were permitted to shift upto the designated line. Then the chromatogram developed TLC plates were evaporated naturally. The TLC plates were then examined beneath the UV light both in long (360 nm) and short (254 nm) wavelength. 0.02% DPPH solution was prepared in ethanol and it showed deep pink color. When DPPH splashed over the chromatogram developed TLC plate, it produces yellow or pale-yellow color that implied the existence of antioxidant compounds.

Quantitative Antioxidant Assay using DPPH

DPPH possesses a pair of electrons in its structure that act as free radical. DPPH is used to detect scavenging capability of free radical in various chemical analysis (Gupta *et al.* 2003). Various conentrations (5-100 µg/mL) of methanolic extract and three different solvent (Butanol, Chloroform and Water) fractions were made using stock solution (1 mg/mL) by serial dilution. 0.004% concentration of DPPH solution was produced with methanol and 3 mL of it was admixed with 2 mL of samples solution at various concentrations and the mixtures were shaken vigorously for 15 seconds. At room temperature the mixtures were placed at dark area for 30 minutes. Finally, UV/Visible spectrophotometer (Analykjena Model 205, Germany) was to measure the absorbance at 517 nm against a blank. The radical-scavenging capability of DPPH was estimated as:

$$\textit{DPPH free radical-scavenging capability (\%)} = [(A_0 - A) / A_0] \times 100 \qquad (1)$$

Where A_0 = absorbance of blank solution (only reagents); A = absorbance of DPPH solution with sample or standard.

Quantification of Total flavonoid content

The total content of flavonoid in methanol extract and different solvent fractions were estimated flowing to Moreno *et al.* 2000. At first, 1 mg/mL concentration 0.5 mL sample was admixed with 0.1 mL potassium acetate (1 M), 0.1 mL 10% aluminum nitrate and 4.3 mL 80% ethanol was mixed to adjust the volume 5 mL. After vortexing, the solution was kept standing for reaction at laboratory temperature for 40 min. The UV/Visible spectrophotometer was then used to take the absorbance at 415 nm. All measurements were done three times. The quercetin calibration curve was drawn using the concentrations of solution at 1.00, 0.75, 0.50, 0.25, 0 mg/mL in ethanol. Total content of flavonoid was estimated as milligram of quercetin equivalent per gram of arid extract using a standard calibration curve of quercetin, $y = 4.7386x + 0.0355$; $R^2 = 0.9993$. Finally, Total flavonoid content is calculated as quercetin equivalents (QE) per gram of arid extract.

Reducing Power Assay

The reducing capacity of sample and standard were measured using reduction potential of potassium ferricyanide (Fe^{3+}) to produce potassium ferrocyanide (Fe^{2+}). It then reacts with ferric chloride to produce ferric ferrous conglomerate that showed absorption at 700 nm (Okolie *et al.* 2014). Different concentrations (100, 80, 60, 40 20, 10 & 5 µg/mL) of the methanol extract of *P. oleracea* Linn. and different solvent fractions (Water, Chloroform, Butanol and *n*-Hexane) were added with potassium ferricyanide (2.5 mL) and phosphate buffer (2.5 mL). The mixture was put within water bath at 50ºC

for 20 minutes and then placed to cool at room temperature. Then, 2.5 mL 10% trichloro acetic acid was mixed. This content was centrifuged for 10 min at 3000 rpm. 2.5 mL solution was separated from the above of the mixture and it was admixed with 2.5 mL distilled water and a newly produced 0.5 mL ferric chloride solution. After waiting for 10 minutes, absorbance was estimated at 700 nm. Blank solution was made in same way without samples. Butylated Hydroxyanisole (BHA) and Ascorbic acid at different concentrations were utilized as standard. Higher absorbance indicates the higher reducing power. Experiments were done three times for every test sample.

Estimation of Total Phenolic Content

The total content of phenolic compounds in the methanolic extract and different fractions (Water, Butanol, Chloroform and *n*-Hexane) of *P. oleracea* Linn. were calculated flowing the Folin-Ciocalteu method (Wolfe *et al.* 2003). All sample solutions were made 1 mg/mL concentration and 0.5 mL of it was admixed to 4 mL 7.5% Sodium carbonate and 5 mL (1:10 v/v in distilled water) Folin-Ciocalteu reagent. The content was shaken using vortex for 15 second and placed to stand at 40°C for 30 min for color development. Finally, double beam spectrophotometer was used to take absorbance at 765 nm (Analykjena UV/Visible Model 205, Germany). The standard calibration curve of gallic acid was made using solutions 1.00, 0.75, 0.50, 0.25 and 0 mg/mL concentration. Total content of phenolic compounds was estimated as milligram of gallic acid equivalent per gram of dry extract derived from a standard calibration curve of gallic acid using the equation, $y = 6.9104x - 0.0937$, $R^2=0.9972$. Finally, total content of phenolics values is revealed as milligram of gallic acid equivalent (GAE) per gm of arid extract.

Ferrous (Fe^{++}) ion Chelating Ability Assay

The Fe^{++} chelating capability of the samples and standard were examined by estimating the formation of the ferrous ion and ferrozine complex (Dinis *et al.* 1994). 5 mL of sample of various concentrations (100, 80, 60, 40, 20, 10 and 5 µg/mL) in different test tubes were mixed with 3.7 mL methanol and 0.1 mL 2 mM $FeCl_2$ solution. 0.2 mL 5 mM Ferrozine was admixed in each test tube to start the reaction and the content was vortexed and then kept for 10 minutes at laboratory temperature. Then, the mixture absorbance was determined at 562 nm using spectrophotometer. The percentage of ferrozine-Fe^{2+} complex formation inhibition was calculated using the formula:

$$\textit{Percentage of } Fe^{++} \textit{ chelating ability (\%)} = [(A_0 - A) / A_0] \times 100 \quad (2)$$

Here, A_0 = absorbance of control (only reagents), A = absorbance of sample

The control solution possesses $FeCl_2$ and ferrozine forming complex molecules. The IC_{50} values were compared with EDTA.

Total Tannin Content Assay

The total content tannin of standard and samples were measured utilizing the Folin-Ciocalteu Phenol reagent (Amorim *et al.* 2008). 0.1 mL sample was mixed with 1 mL sodium carbonate solution (35%), 0.5 mL Folin-Ciocalteu reagent, 7.5 mL of distilled water and finally the volume was adjusted adding 10 mL distilled water. The mixture content was shaken vigorously and was kept for 30 minutes at laboratory temperature. Finally, absorbance was measured at 725 nm using a UV/Visible spectrophotometer (Analykjena, Model 205, Germany). The standard calibration curve of tannic acid was made using 1.00, 0.75, 0.50, 0.25 and 0 mg/mL tannic acid solutions in water. Total content of tannin was estimated as milligram of tannic acid equivalent per gram of arid extract obtained from a calibration curve of tannic acid using the following equation, $y = 5.3692x - 0.0404$, $R^2 = 0.9996$.

Nitric Oxide (NO) Scavenging Activity Assay

scavenging capacity of NO of standard and samples were calculated flowing the protocol of Govindarajan *et al.* 2003. The standard used for this study was ascorbic acid. Various extract concentrations (5–100 µg/mL) were admixed with sodium nitroprusside (5 mmol) in phosphate buffered saline dissolved in suitable solvent system and heated for 30 min at 25 °C. After incubation of 30 min, 1.5 mL mixture was picked up from the intermixture and diluted with 1.5 mL Griess reagent (2% phosphoric acid, 0.1% naphthyl ethylenediamine dihydrochloride and 1% sulphanilamide). The chromophore was produced by diazotization sulphanilamide and nitrite and then bonding with naphthyl ethylene diamine dihydrochloride. The chromophore absorbance was taken at 546 nm using a double beam spectrophotometer (Analykjena Model 205, Jena, Germany).

This process was done for methanol extract and different *P. oleracea* Linn. fractions. Scavenging capacity of nitric oxide (NO) was estimated using the equation:

$$\textit{Nitric Oxide radical scavenging activity (\%)} = (A_b - A_s / A_b) \times 100 \quad (3)$$

Here, A_b = Absorbance of control (only reagents), A_s = Absorbance of samples.

IC_{50} value means the sample concentration needed to scavenge 50% NO free radical estimated from the inhibition plot (%) against the sample concentration.

Statistical analysis

Triplicate analyses for all measurement were conducted for all samples. All analytical datas were showed as mean ± (SEM) standard error of mean.

Results

Phytochemical screening

In phytochemical analysis, methanolic extract and its various fractions showed the existence of different categories secondary metabolites such as flavonoids, alkaloids, gums, reducing sugar, glycosides, steroid and tannins (Table 1).

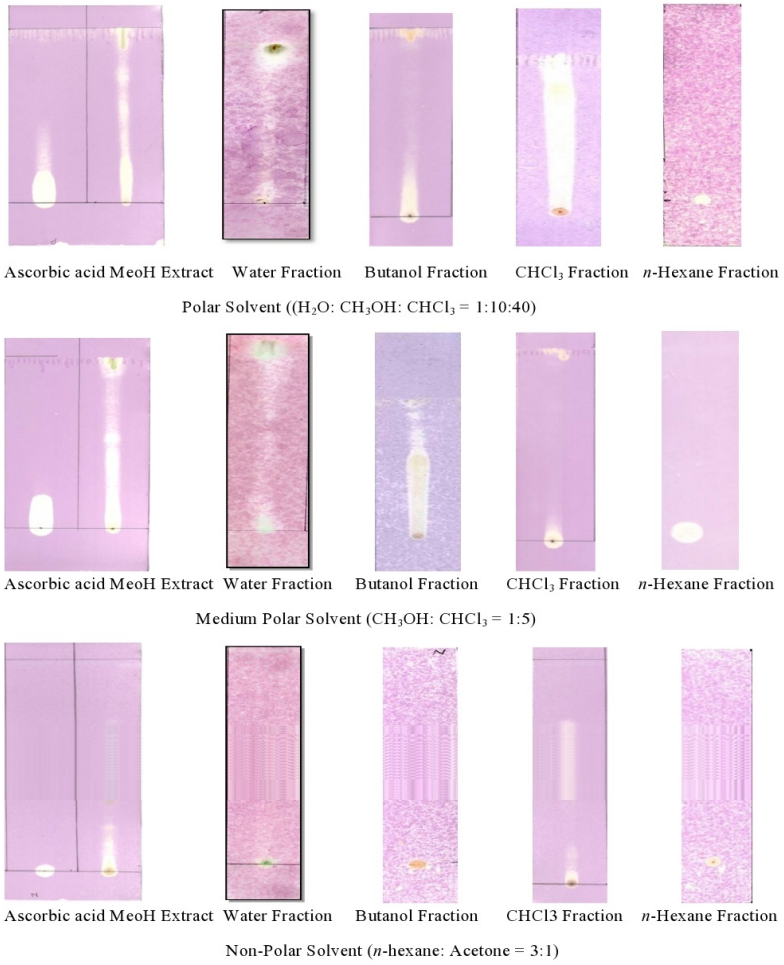

Figure 1. TLC plate comparison of methanol extract and different fractions of *Portulaca oleracea* Linn. and Ascorbic acid after DPPH spray

Table 1. Chemical Groups Present in Methanol Extract and Different Fractions of *Portulaca oleracea* Linn.

Phytoconstituents	Method of Experiments	Methanol Extract	Butanol fraction	Water fraction	Chloroform fraction	*n*-Hexane fraction
Alkaloids	Dragendroff's Test	+	+	+	+	-
Steroids	Sulphuric Acid Test	+	-	-	+	+
Tannins	Ferric Chloride Test	+	+	+	+	-
Glycosides	NaOH Test	+	-	+	-	+
Flavonoids	Lead Acetate Test	+	+	+	+	-
Saponins	Foam Test	+	+	+	-	+
Reducing Sugar	Benedict's Test	+	+	+	+	+

+: Presence & -: Absence

Qualitative Antioxidant Assay using DPPH

Methanol extract, water, butanol and chloroform fractions showed the presence of antioxidant compounds but *n*-hexane fraction did not exhibit the presence of antioxidant compound (Figure1).

Quantitative Antioxidant Assay using DPPH

In DPPH radical scavenging assay, crude methanol extract, water, butanol and chloroform fractions showed IC_{50} values 23, 25, 28 and 41 µg/mL respectively where ascorbic acid IC_{50} value was 11 µg/mL (Figure 2). *n*-hexane fraction did not exhibit the existence of antioxidant compounds.

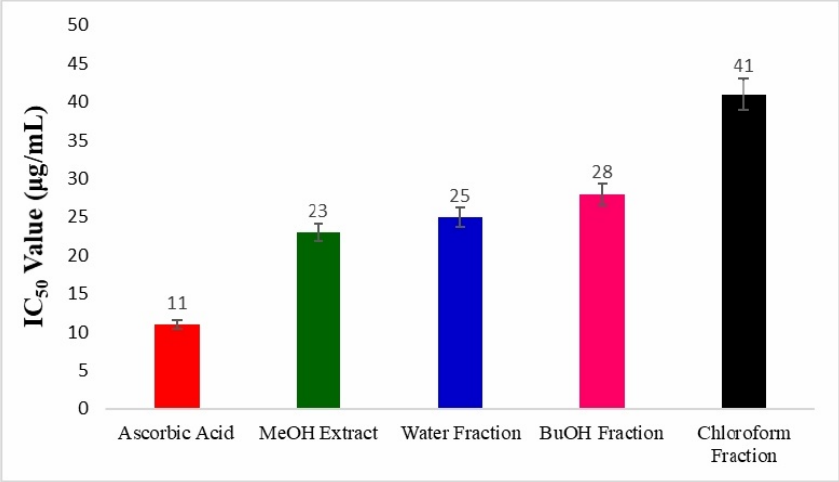

Figure 2. Comparative DPPH Radical Scavenging Assay of Methanol Extract and Various Fractions of *Portulaca oleracea* Linn. and Standard Ascorbic Acid (*Values are expressed as mean ± SD, where n=3)*

Quantification of Total flavonoid content

In total content of flavonoid test, methanolic extract and water fraction showed significant total flavonoid content of 137.72 and 102.08 mg of QE/gm of dry extract. The butanol and chloroform fractions showed moderate total flavonoid content of 88.93 and 67.83 mg of QE/gm of dry extract.

n-Hexane fraction showed negligible amount of total content of flavonoid 8.74 mg of QE/gm of arid extract (Figure 3). The above values were calculated from standard quercetin calibration curve.

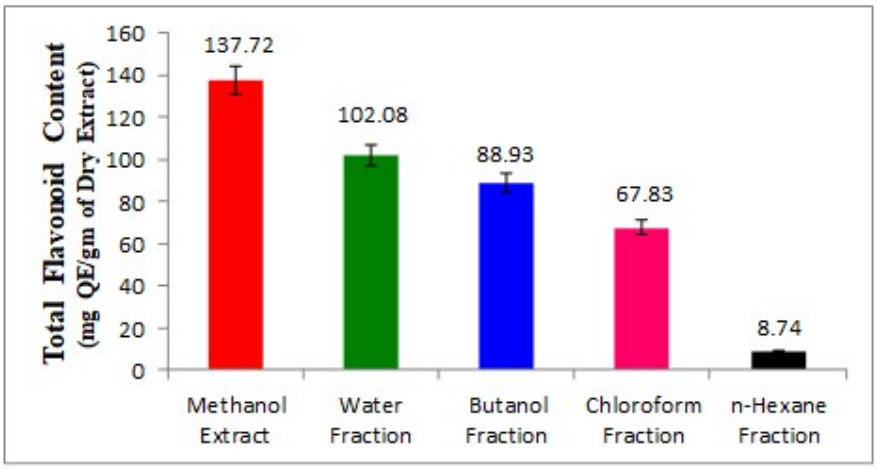

Figure 3. Total Flavonoid Content of Different Fractions of *Portulaca oleracea* Linn. ((*Values are expressed as mean ± SD, where n=3)*

Reducing Power Assay

It is an important characteristic of antioxidant molecules that they can also act as reducing agent in redox reaction. For example, Fe^{3+} reduction means electron donating ability and phenolic compounds show this antioxidant effect (Nabavi *et al.* 2009). Higher absorbance indicates the more the reductive potential of sample. Methanol extract, water, butanol, chloroform and *n*-hexane fractions showed absorbance 1.3121, 0.9532, 1.4231, 1.4892 and 0.3875 respectively that are comparable to standard BHA and ascorbic acid are 1.2153 and 1.2353 (Figure 4). So, chloroform fraction exhibited highest reducing power and *n*-hexane fraction showed negligible reducing power.

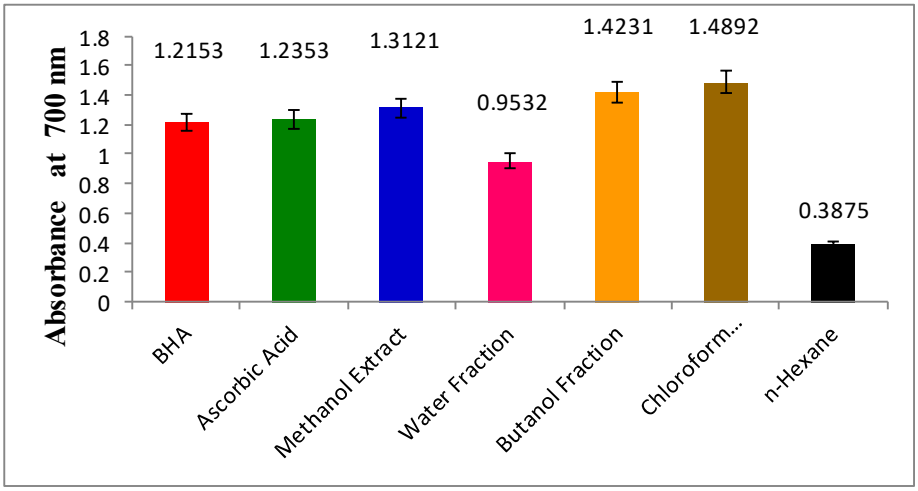

Figure 4. Maximum Absorbance Comparison of Standard, Methanolic Extract and Various Fractions of *P. oleracea* Linn.(*Values are expressed as mean ± SD, where n=3)*

Estimation of Total Phenolic Content

Total content of phenolic of samples was calculated utilizing the equation, y = 6.9104x + 0.0937; R^2 = 0.9972, attained from gallic acid calibration curve. Crude methanol extract, water, butanol, and chloroform fractions showed total phenolic content values 176.70, 157.13, 122.84 and 70.61 milligram of Gallic Acid Equivalent per gram of arid extract (Figure 5). *n*-Hexane fraction showed negligible amount of total phenolic content.

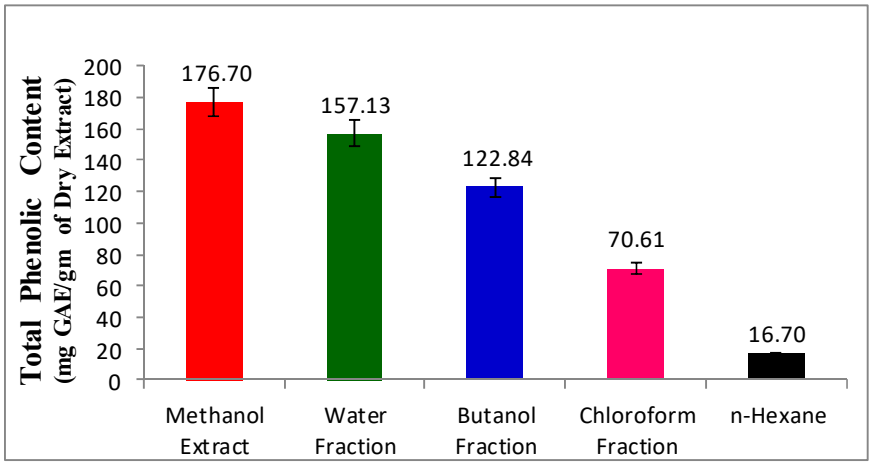

Figure 5. Total Content of Phenolic Crude Methanolic Extract and Various Fractions of *Portulaca oleracea* Linn. (*Values are expressed as mean ± SD, where n=3).*

Ferrous (Fe^{++}) ion Chelating Ability Assay

IC_{50} value of Fe^{++} ion chelating ability of crude methanol extract and water, butanol, *n*-hexane and chloroform fractions were found to be 25, 29, 32, 46 and 42 µg/mL respectively where EDTA showed IC_{50} 17 µg/mL which indicated the significant Fe^{++} chelating capability (Figure 6).

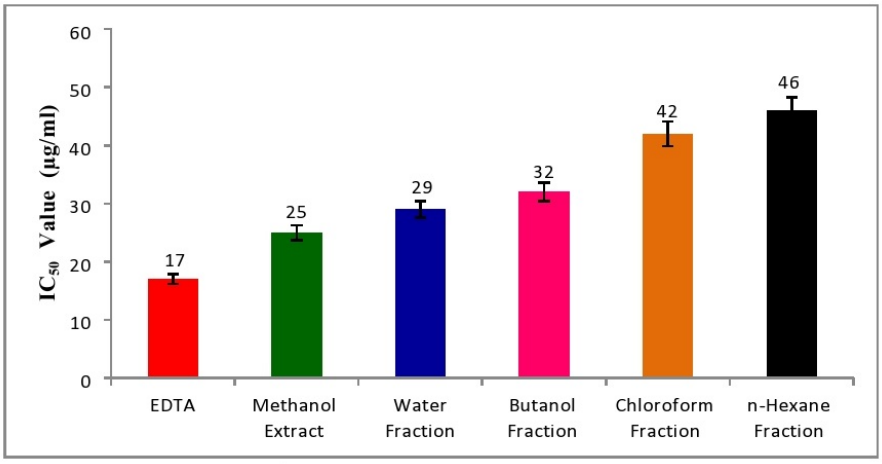

Figure 6: Comparative Fe^{++} Chelating Ability of Methanol Extract, Different Fractions of *Portulaca oleracea* Linn. and Standard EDTA(*Values are expressed as mean ± SD, where n=3).*

Total Tannin Content Assay

Total content of tannin of samples was calculated using the equation, $y = 5.3692x - 0.0404$ ($R^2 = 0.9996$) obtained from a tannic acid calibration curve. Total content of tannin of crude methanolic extract and water, butanol, chloroform and *n*-hexane fractions showed 140.56, 114.10, 89.14, 41.44 and 9.98 mg of TAE/gm of dry extract respectively (Figure 7).

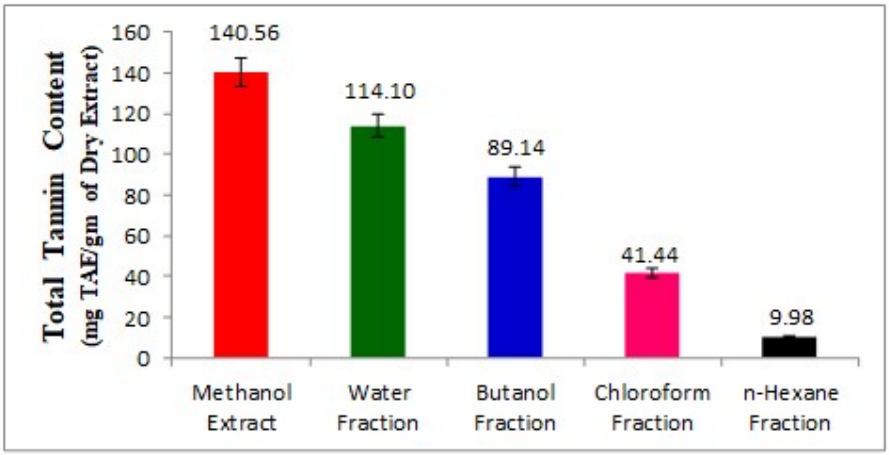

Figure 7. Total Tannin Content of Crude Methanol Extract and various Fractions of *Portulaca oleracea* Linn. (*Values are expressed as mean ± SD, where n=3).*

Nitric Oxide (NO) Scavenging Activity Assay

Methanol extract, water, butanol, chloroform and *n*-hexane fractions exhibited significant nitric oxide (NO) scavenging ability with IC_{50} value 10, 27, 24, 36 and 74 µg/mL respectively where ascorbic acid showed IC_{50} value 17 µg/mL (Figure 8).

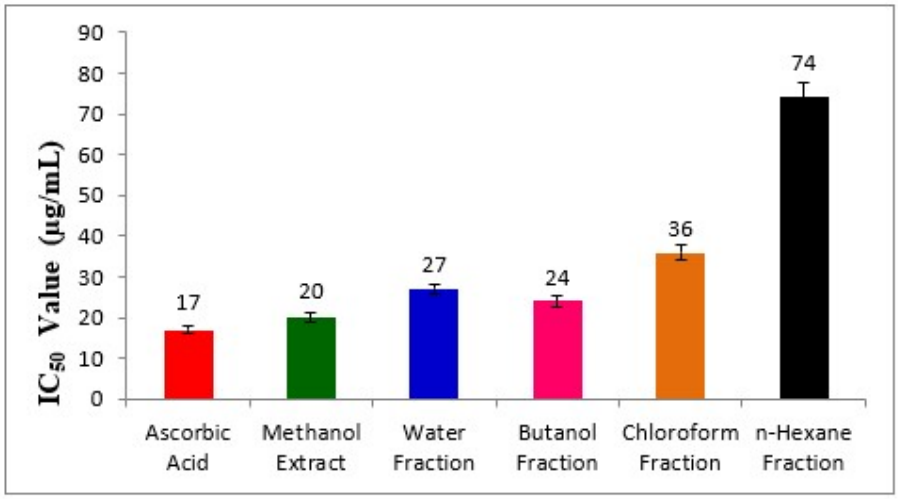

Figure 8. Comparative NO Scavenging Ability of Crude Methanolic Extract and Different Fractions of *Portulaca Oleracea* Linn. and Ascorbic Acid (*Values are expressed as mean ± SD, where n=3).*

Discussion

Different types of secondary metabolites are produced and stored by plants daily as a part of their own defensive process. These phytochemicals not only serve to facilitate the growth of the plant itself but also possess some lifesaving components and so plants are used as medicine from the primitive time (Hartmann, 2007). Phytochemical screening conducted in this study of methanol extract and different solvent fractions showed that *P. oleracea* possesses some bioactive secondary metabolites (flavonoids, steroid, reducing sugar, glycosides, tannins, alkaloids and gums). These phytochemicals assured the medicinal value of this plant.

Reactive oxygen species (ROS) are produced naturally as by-product in human metabolic process that are involved in some crucial biological activities like immune response alteration, signaling transduction and gene transcription. These ROS often integrate hydroxyl radical (•OH), superoxide (O2•–) and hydrogen peroxide (H_2O_2). When excess ROS is deposited in our body, then these can induce oxidative harm to biomolecules (lipids, and DNA proteins) and severely affect them. These results in different biological difficulties including cancer, neurodegenerative disorders, respiratory problems, gastrointestinal diseases, inflammatory symptoms, renal obstacle, pulmonary and cardiovascular diseases. This oxidative damage finally leads to cellular necrosis (Zuo *et al.* 2015; Rogers *et al.* 2014). The deleterious action of oxidative damage can be reimbursed by either biological mechanisms or by taking antioxidant food supplements. Antioxidants either endogenic or supplied externally, can neutralize the ROS and reduce oxidation of biological molecules and renew the cellular mechanism (Gilgun-Sherki *et al.* 2001).

Antioxidant compounds remained in methanol extract and different solvent fractions was identified and their quantitative measurement were also conducted. Besides these, their DPPH free radical scavenging capacity were also performed. DPPH showed deep violet color owing to movement of excess electrons in the molecule. This can be identified by taking absorption at 517 nm (Alam *et al.* 2013). Antioxidants can change the DPPH deep violet color either by supplying electrons or hydrogen atoms and this is reversely proportional to certain concentration (Alam *et al.* 2013; Rahman *et al.* 2006). Thus, enhancing the concentration of both extracts and standard ascorbic acid lower the absorbance that expressed the antioxidant capacity of the samples. (Figure 1 & 2). In our experiments, we found that polar fractions contain more antioxidant compounds that medium polar fractions and non-polar *n*-hexane fraction did not possess the antioxidant compounds.

Flavonoids and tannins are phenols have significant antioxidant capacity in plants due to their hydrogen conferring activity to scavenge free radicals (Amorim *et al.* 2008). Both the extent and class of polyphenolics demarcate the antioxidant capacity of sample. So, we have tried to measure the quantity of the flavonoid contents, phenolic contents and tannin contents. The methanol extract of plant exhibited significant amount of total flavonoid content than other fractions. Polar water fraction contains more flavonoid compounds than medium-polar and non-polar fractions. Since flavonoids are phenolic compounds and contain hydroxyl groups and so they are soluble in polar solvent. For this reason, *n*-hexane fraction contains negligible quantity of flavonoid compounds.

Antioxidant activity of flavonoids depends on their chemical structure. Free radical scavenging capability of flavonoids rely on the location of hydroxyl groups with other features in the molecular structure. Quercetin is the naturally inexhaustible dietary flavonoid and acts as effective antioxidant that has prerequisite structural formula to scavenge free radicals. Flavonoids are a class of polyphenolics and these are extensively distributed secondary metabolites of the plants. Various epidemiological studies reported that a regular intake of plant flavonoids (consumption of color fruits and vegetables) consequently reduces the risk of developing some cancers, different cardiovascular diseases, stroke, respiratory and gastrointestinal disorders (Galati and O'brien, 2004).

Total contents of phenols, flavonoids and tannins obtained from this study revealed that this *P. oleracea* possesses significant quantity of antioxidant compounds. Although there are differences between our results and reported by Sicari *et al.* 2018, Fernández-Poyatos *et al.* 2021 and Alam *et al*, 2014, these differences may be due to the difference in location of the plants as soil condition, environment and geographical condition influence the biosynthetic pathway and metabolic activities of plants.

Reducing assay is mainly based on a principle that any compound possessing reduction capability can convert potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). Fe^{2+} then

reacts with $FeCl_3$ to produce ferric–ferrous complex. Plants possess numerous reducing compounds and these compounds exhibit redox features acting as reducing agents, hydrogen donors, chelating metals and singlet oxygen quenchers (Bhalodia *et al.* 2013). In our experiment, the Chloroform, Butanol and Methanol fractions showed the higher maximum absorbance comparing to the standard BHA and Ascorbic acid. On the other hand, water fraction showed less reducing potential than the above fractions and standards. *n*-Hexane fraction was found to be the lower reducing ability.

Transition metals in our biological system combine with antioxidants to form chelate. Then it inhibits the formation of OH^- that finally suppresses the lipid peroxidation in biomolecules (Samydurai and Thangapandian, 2012). In our Ferrous (Fe^{++}) ion chelating ability test we observed that, crude methanol extract exhibited best Fe^{++} chelating capability (IC_{50} = 25 µg/mL) comparing to standard, EDTA (IC_{50} = 25 µg/mL) and *n*-Hexane fraction showed the less Fe^{++} chelating ability (IC_{50} = 46 µg/mL). Thus, it can be suggested that non polar fraction of *P. oleracea* contained few antioxidant components comparing to the polar fractions. Nowadays, Fe^{++} chelation therapy is a common therapy to neutralize excess iron in cases of treating anemia and thalassemia. Moreover, this chelation therapy is also recommended in different oxidative diseases as well as Parkinson's and Alzheimer's diseases (Sudan *et al.* 2014). Therefore, chelation of trace metals by natural phytochemicals from *P. oleracea* extract polar fractions can be a potential source in treating the oxidative damages of our biomolecules.

NO is a known free radical which constantly formed in our biological system. It plays important role in our body such as smooth muscle relaxation, platelet aggregation inhibition, neuronal signaling, dilation of blood vessels etc. But, long term to nitric oxide radical exposure may lead to different inflammatory and carcinogenic conditions including rheumatoid arthritis, ulcerative colitis, juvenile diabetes and atherosclerosis. When superoxide radicals react with NO then it increases the toxicity severely in biological system. Plant derived antioxidants specially flavonoids can directly scavenge the NO (Boora *et al.* 2014). In this experiment, all polar fractions of *P. oleracea* exhibited good NO radical scavenging ability comparing to the standard ascorbic acid (IC_{50} =17 µg/mL) where *n*-Hexane fraction showed very poor NO radical scavenging activity. As flavonoids show best effect in Nitric Oxide (NO) radical scavenging properties and considering our previous total flavonoid content determination test, it can be suggested that, using of *P. oleracea* extract may be a very potential discovery in treating and preventing many diseases associated with Nitric Oxide (NO) radical.

Conclusion

From our above experiment, it can be concluded that the aerial part of *P. oleracea* possesses significant amount of antioxidant secondary metabolites. Polar fractions showed higher antioxidant potential compared to non-polar fractions. Antioxidants prevent unstable molecules called free radicals that try to mutate DNA, damage cell membranes and other parts of cells. This oxidative stress enhances the risk of age-related diseases like stroke, cancer, Parkinson's and Alzheimer's diseases. Antioxidant supplements reduce the chance of oxidative damage and ensure healthy life. This study results are scientific evidences and could justify the use of aerial parts *P. oleracea* as food supplement to prevent age-related health risk.

Abbreviations

TLC: Thin Layer Chromatography; DPPH: 1,1-diphenyl-2-picrylhydrazyl; QA: Quercetin Equivalents; BHA: Butylated Hydroxyanisole; GAE: Gallic Acid Equivalent; EDTA: Ethylenediaminetetraacetic acid; IC_{50}: 50% Inhibitory Concentration; R2: Coefficient of Determination; ROS: Reactive Oxygen Species.

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Authors' contributions

Utpal Kumar Karmakar had done the laboratory experiments, collection of plant parts, interpretation of results, data acquisition and report writing. Pritam Kundu did statistical analysis and partial report writing. Md. Hemayet Hossain gave the partial Laboratory support in the BCSIR. Experiment design, concept development, final approval and overall monitoring of the manuscript was performed by Utpal Kumar Karmakar and Jamil Ahmad Shilpi.

Competing interests

The authors express no conflict of interest.

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