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COMPARATIVE ASSESSMENT OF FREE RADICAL SCAVENGING, MEMBRANE STABILIZING AND THROMBOLYTIC POTENTIALS OF TWO *AMARANTHUS* SPECIES OF BANGLADESH

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Abstract

Amaranthus lividus and *Amaranthus tricolor* were evaluated for their primary free radical scavenging, membrane stabilizing and thrombolytic potentials. The methanol crude extracts of these two species were partitioned into petroleum ether, dichloromethane and aqueous soluble fractions. Antioxidant potential of the extracts was evaluated through DPPH and H_2O_2 free radical scavenging assays, where the aqueous soluble fraction of *A. lividus* exhibited the highest activity ($p < 0.05$). Besides, the maximum level of total phenolics was quantified in the aqueous fraction of *A. lividus*. Higher level ($p < 0.05$) of flavonoids (> 70 mg of QE/g of extract) were measured in the aqueous, dichloromethane and crude methanol extracts of *A. lividus*. The membrane stabilizing (anti-inflammatory) potential of the extracts was studied by measuring the inhibition of hemolysis of RBC induced by hypotonic solution and heat. Considering the conditions of both assays, the aqueous extract of *A. lividus* showed potent membrane stabilizing activity ($p < 0.05$) indicating their primary anti-inflammatory potential. Very mild thrombolytic activity was noticed for both plants compared to the standard. Furthermore, existence of some major phytochemicals, like alkaloids, steroids, triterpenes and quinones were also observed at various levels. This is the first time report of the comparative assessment of the above mentioned plant extracts so far.

Introduction

` Cellular oxidation is a process for generating free radicals, which can damage biochemical substances and produce oxidative stress (Clement and Luo 2020). Inflammation is an issue for many decades to make people suffer along with many other diseases. Current anti-inflammatory drugs like acetylsalicylic acid, ibuprofen, acetaminophen, naproxen, etc. are effective but they have many side effects (Colebatch *et al.* 2012). Thrombosis is one of the prominent reasons for death due to vascular blockage. Tissue plasminogen activator (TPA), streptokinase, etc. are being applied in the treatment of thrombosis but they might show anaphylactic reaction, hemorrhage, lack of specificity, etc. (Hilleman and Campbell 2011). Many plants were previously reported to have remedial potentials against numerous diseases (Alamed *et al.* 2009, Amaral *et al.* 2009, Chaity *et al.* 2016).

Two *Amaranthus* species of Bangladesh, *Amaranthus lividus* and *Amaranthus tricolor* belonging to Amaranthaceae have been selected to assess their biological potentials. *A. lividus* (wild amaranth, green amaranth in English and 'Goburanotey' in Bangla) is an annual herb found on waste lands and extensively used as vegetables. Leaves and roots of *A. lividus* were reported to treat constipation of children and apply on abscesses as emollient poultice (Rahman and Gulshana 2014). *A. tricolor* ('Lalshak' in Bangla) is an annual herb and cultivated in dry places as a vegetable and ornamental plant (Larsen *et al.* 2003). It has some local uses as stomachic, laxative, antipyretic, appetizer, etc. (Rahman and Gulshana 2014). The present investigation was designed to assess the comparative free radical scavenging, membrane stabilizing and thrombolytic potentials of both plants for the first time.

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Materials and Methods

Chemicals used in the present study such as 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, hydrogen peroxide, aluminum chloride, acetylsalicylic acid, and quercetin were procured from Sigma-Aldrich Co., St. Louis, USA. Folin-Ciocalteu's reagent and ascorbic acid were collected from Loba Chemie Pvt. Ltd., Mumbai, India.

A. lividus and *A. tricolor* were collected from Savar, Dhaka and voucher specimens are stored at Bangladesh National Herbarium (BNH), Mirpur, Dhaka for future use (Table 1). The 500 g of each of the dried powdered plant was soaked in 3l of methanol at room temperature for a week followed by filtering using Whatman filter paper (number 1) and concentrating to obtain methanol crude extracts (MCE). An aliquot (5 g) of this was partitioned into petroleum ether (PE), dichloromethane (DCM) and aqueous (AQ) soluble materials as per modified Kupchan method (VanWagenen *et al.* 1993) (Table 2).

Table 1. DACB accession no. of *A. lividus* and *A. tricolor.*

Name of plant	Family	Plant part	Voucher specimen (DACB accession No.)
Amaranthus lividus	Amaranthaceae	Whole plant	43581
Amaranthus tricolor	Amaranthaceae	Whole plant	43580

Table 2. % yield of Kupchan partitionates of *A. lividus* and *A. tricolor*.

Extract or Fractions	Weight (g)	% Yield of fractions
Methanol crude extract (MCE)	5	--
Petroleum ether fraction (PE)	2.2	44
Dichloromethane fraction (DCM)	1.2	24
Aqueous fraction (AQ)	1.4	28

For DPPH (2,2'-diphenyl-1-picrylhydrazyl radical) free radical scavenging assay, 2 mg of each extract was serially diluted by methanol at a concentration rage 500-0.977 µg/ml and 2 ml of each diluted solution of extract was mixed separately to 3 ml of DPPH (20 µg/ml methanol), incubated at dark for 20 min followed by measurement of absorbance at 517 nm. The % inhibition of radical was plotted against extract concentrations to calculate the concentration required to inhibit 50% of radical (IC_{50}) (Parvin *et al.* 2009).

In case of H_2O_2 free radical scavenging assay, the 3.4 ml of extract solutions (500-0.977 µg/ml) was transferred separately to 0.6 ml of 40 mM H_2O_2, incubated for 10 min at room temperature and absorbance was measured at 230 nm. IC_{50} value was calculated by plotting the percent inhibition of radicals against extract concentrations (Ahmed and Rahman 2016).

For phenolic content determination, the 500 µl of the sample (500 µg/ml water), 2 ml of Na_2CO_3 (7.5% w/v) and 2.5 ml of ten times diluted Folin-Ciocalteu reagent were mixed and incubated (room temperature, 20 min). The absorbance was taken at 760 nm. Total phenolics were measured from a calibration curve of gallic acid (0-100 µg/ml methanol) and stated as mg of gallic acid equivalent (GAE)/g of the extract (Sikder *et al.* 2012).

In case of flavonoid content determination, 1 ml of 2% (w/v) $AlCl_3$ was mixed with an extract (0.5 mg/ml methanol), incubated at room temperature (15 min) and the absorbance was measured

at 430 nm. Total flavonoids were stated as mg of quercetin equivalents (QE)/ g of the extract by generating a calibration curve of quercetin (0-100 µg/ml methanol) (Ahmed and Rahman 2016).

The membrane stabilizing activity was assessed by hypotonic solution- and heat- induced hemolysis assays (Shinde *et al.* 1999). In case of hypotonic solution- induced hemolysis, 0.5 ml of RBC suspension was put together with 5 ml of hypotonic solution (50 mM NaCl) along with the plant sample (1 mg/ml) or acetylsalicylic acid (reference drug) (0.1 mg/ml). The mixture was kept at room temperature (10 min), centrifuged (3000 g for 10 min) and the optical density (OD) of the supernatants were estimated at 540 nm. % inhibition of hemolysis was calculated as $\{(OD_{control} - OD_{test})/OD_{control}\} \times 100$. In case of heat- induced hemolysis, 5 ml of isotonic buffer containing extract (1 mg/ml) or acetylsalicylic acid (0.1 mg/ml) (reference drug) along with erythrocyte suspension (30 µl) was put into a tube. One set of the tubes along with control samples were kept at 54°C (water bath) for 20 min, while another group of the tubes were kept at 0-5°C (ice bath). It was then centrifuged (1300 g, 3 min) and the optical density (OD) of the supernatants were measured at 540 nm. % Inhibition of hemolysis was $\{1 - (OD_{heated\ test} - OD_{unheated\ test}) / (OD_{heated\ control} - OD_{heated\ test})\} \times 100$.

In case of the determination of thrombolytic activity, clot was formed by dispensing human venous blood (0.5 ml/tube) and removing the serum. Each extract (1 mg/100 µl water) was added separately in the tube and kept at 37°C (90 min). 100 µl of streptokinase (corresponding to 30,000 IU) (Beacon pharmaceuticals Limited, Bangladesh) was taken as positive control (Prasad *et al.* 2006).

A primary phytochemical screening was also conducted and the required tests for this purpose (Joshi *et al.* 2013) are explained in Table 4.

Table 3. Preliminary phytochemical tests of *A. lividus* and *A. tricolor*.

Test group	Process	Confirmation color
Alkaloids	2 mg of extract was mixed with 0.5 ml of 1 % HCl and a small amount of Mayer's reagent was added.	Yellow or cream precipitate
Triterpenoids and steroids	2 mg of extract, 1 ml of acetic anhydride and 1 ml of concentrated H_3PO_4 were mixed properly.	Red (triterpenoids) and green (steroids)
Quinones	2 mg of extract was added with 2 ml of 0.1 M NaOH.	Pink or red or violet color

Table 4. Preliminary phytochemical screening of *A. lividus* and *A. tricolor*.

Plant	Test	Methanol crude extract	Petroleum ether fraction	Dichloromethane fraction	Aqueous fraction
A. lividus	Alkaloids	(+)	(-)	(-)	(+)
	Triterpenes	(+)	(++)	(+)	(+)
	Steroids	(+)	(+ + +)	(+)	(-)
	Quinones	(+)	(-)	(+ +)	(-)
A. tricolor	Alkaloids	(+)	(-)	(+)	(+)
	Triterpenes	(+)	(+)	(+ +)	(+)
	Steroids	(+ +)	(+ + +)	(++)	(-)
	Quinones	(+)	(-)	(+)	(+)

Here, (+++) specifies abundant, (++) specifies moderate, (+) specifies trace; (−) specifies absent.

Three replicates (n = 3) of each sample were taken for statistical analysis and the values are described as mean ± standard deviation (SD). Data were evaluated by statistical package for social science (SPSS) software (Version 20, IBM Corporation, USA) using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. The values were considered significantly different at $p < 0.05$.

Results and Discussion

In case of DPPH free radical scavenging assay, the aqueous soluble fraction of *A. lividus* exhibited the highest radical scavenging activity ($p < 0.05$) having IC_{50} 54.23 µg/ml among the extracts. The moderate activity was shown by the dichloromethane and crude methanol extracts of both the plants as well as aqueous fraction of *A. tricolor* having IC_{50} less than 85 µg/ml (Fig. 1A). During the H_2O_2 free radical scavenging assay, the maximum level of scavenging activity ($p < 0.05$) was also displayed by the aqueous fraction of *A. lividus* having IC_{50} 63.25 µg/ml. Besides, methanol crude extract and aqueous fraction of *A. tricolor* as well as dichloromethane fraction and methanol crude extract of *A. lividus* displayed moderate scavenging activity (IC_{50} less than 100 µg/ml) (Fig. 1B). Up-regulation of free radicals triggers the oxidative stress causing inflammatory damages, neurological disorders, aging, cancers, etc. (Young and Woodside 2001, Schetter *et al.* 2010), which might be counteracted by the free radical scavengers. In this aspect, the aqueous fraction of *A. lividus* was appeared as a very promising extract to perform free radical scavenging.

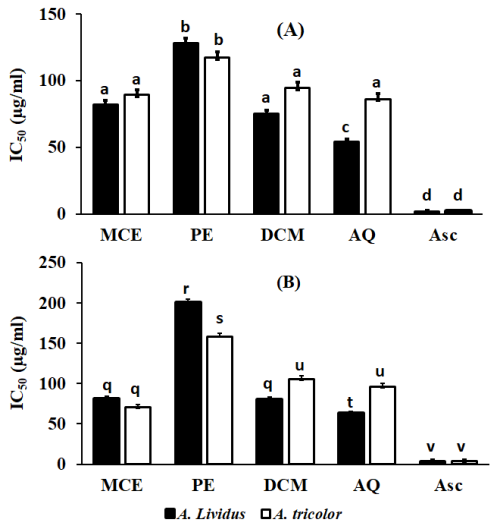

Fig. 1. IC_{50} values of the extracts of *A. lividus* and *A. tricolor* in (A) DPPH free radical scavenging and (B) H_2O_2 free radical scavenging assays. MCE, Methanol crude extract; PE, Petroleum ether fraction; DCM, Dichloromethane fraction; AQ, Aqueous fraction; Asc, Ascorbic acid (standard); Bars with different letters are significantly different ($p < 0.05$).

In case of phenolic content determination, the upper most level ($p < 0.05$) of phenolics was found in the aqueous part of the *A. lividus* (20.25 mg of GAE/g of extract). The methanol crude extract and dichloromethane fraction of *A. lividus* as well as aqueous fraction of *A. tricolor* were found to have phenolics more than 15 mg of GAE/g of extract (Fig. 2A). In case of flavonoid content determination assay, higher level ($p < 0.05$) of flavonoids (> 70 mg of QE/g of extract)

were found in the aqueous, dichloromethane and crude methanol extracts of *A. lividus*. Besides, >50 mg of QE/g of extract flavonoids were possessed by the aqueous, dichloromethane and methanol crude extracts of *A. tricolor* (Fig. 2B). Phenolic and flavonoid compounds are known as antioxidants as they can scavenge free radicals and slow down lipid peroxidation (Baharfar *et al.* 2015). Based on the result of this investigation, it might be assumed that the free radical scavenging potentials of these plants might be linked with phenolics and flavonoids to an extent.

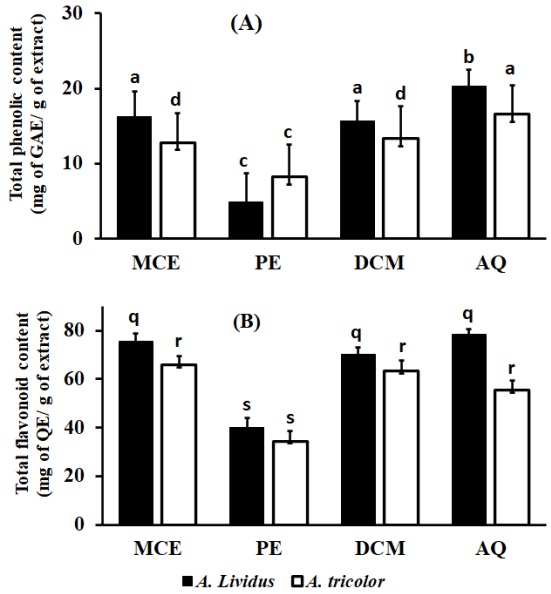

Fig. 2. Total phenolic (A) and total flavonoid (B) contents of different extracts of *A. lividus* and *A. tricolor*. Here, MCE, Methanol crude extract; PE, Petroleum ether fraction; DCM, Dichloromethane fraction; AQ, Aqueous fraction; Bars with different letters are significantly different ($p < 0.05$).

During the determination of membrane stabilizing activity, *A. lividus* and *A. tricolor* displayed potential protective activities on the isolated human RBC against hypotonic solution- and heat-induced hemolysis. The highest level of membrane stabilizing activity ($p < 0.05$) was displayed by the aqueous extract of *A. lividus* in both hypotonic solution- and heat- induced conditions (Figs 3A and 4B). Besides, dichloromethane and crude methanol extracts of *A. lividus* along with aqueous and crude methanol extracts of *A. tricolor* showed noticeable inhibition of hemolysis in hypotonic solution induced condition. In addition, methanol crude, dicholoromethane and petroleum ether extracts of *A. tricolor* along with petroleum ether extract of *A. lividus* showed moderate inhibition of hemolysis in heat induced assay. RBC membrane stabilization is a sign of anti-inflammatory effect as they mimic the protection for lysosomal membrane for preventing the release of enzyme into cytoplasm responsible for stimulating the inflammatory mediators like oxygen radicals, prostaglandins, etc. (Chou 1997).

The assay for the determination of thrombolytic activity was aimed to find some elements effective against stroke, myocardial infarction, etc. which are related to arterial and venous thrombosis (Furie and Furie 2008). *A. lividus* and *A. tricolor* were screened for this purpose but the plants showed very mild thrombolytic activity (Fig. 4).

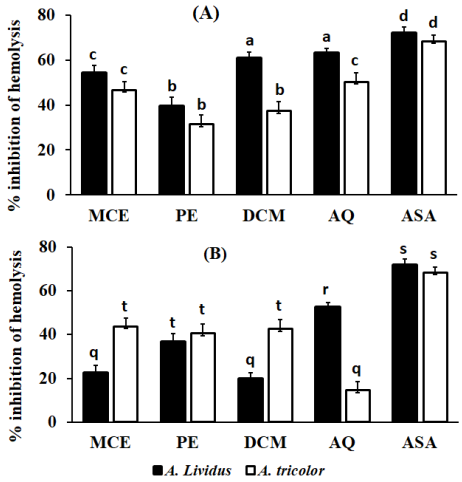

Fig. 3. % inhibition of hemolysis of RBC by different extracts of *A. lividus* and *A. tricolor* in hypotonic solution- (A) and heat- (B) induced conditions. MCE, Methanol crude extract; PE, Petroleum ether fraction; DCM, Dichloromethane fraction; AQ, Aqueous fraction; ASA, Acetylsalicylic acid (standard); Bars with different letters are significantly different ($p < 0.05$).

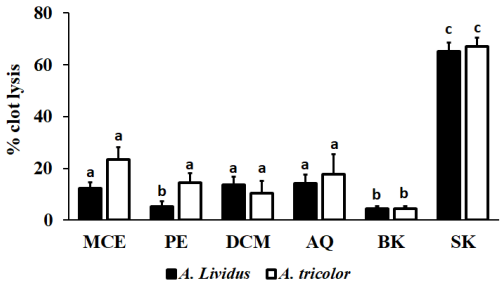

Fig. 4. % clot lysis of different extracts of *A. lividus* and *A. tricolor*. Here, BK, Blank (water); SK, Streptokinase; MCE, Crude methanol extract; PE, Petroleum ether fraction; DCM, Dichloromethane fraction; AQ, Aqueous fraction; Bars with different letters are significantly different ($p < 0.05$).

A preliminary phytochemical study on alkaloids, triterpenes, steroids and quinones was conducted and it indicated the presence of various secondary metabolites. Further detail studies are required to identify the bioactive molecules.

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