JABET
Journal of Advanced Biotechnology and Experimental Therapeutics

ORIGINAL ARTICLE

J Adv Biotechnol Exp Ther. 2024 Jan; 7(1): 118-135
eISSN: 2616-4760, https://doi.org/10.5455/jabet.2024.d11
Published by www.bsmiab.org

Hemolytic, antioxidant, and phospholipase A_2 neutralization efficacy of *Asparagus racemosus, Withania somnifera, Syzygium cumini, Psidium guajava, Basella alba, Morus indica, Morus laevigata, and Morus latifolia*

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Academic editor
Md. Abdul Hannan, PhD
Bangladesh Agricultural University, Bangladesh

Article info
Received: 01 September 2023
Accepted: 11 November 2023
Published: 16 November 2023

Keywords
Antioxidant activity, Bio-active compound, Hemolytic activity, Medicinal plant, PLA_2 enzyme

ABSTRACT

Bangladesh is abundant in medicinal plants. The bioactive compounds in medicinal plants provide novel therapeutic opportunity of improving human health. But the benefit-risk balance is usually less considered during formulation of traditional medicine. However, the plants included in this study were not studied before regarding their hemolytic and anti-venom properties. Thus, the current study aimed to evaluate and compare the hemolytic activity, antioxidant activity, and phospholipase A_2 (PLA_2) enzyme neutralization efficacy of aqueous and methanolic extracts of eight known medicinal plants including *Asparagus racemosus* (root), *Withania somnifera* (root), *Syzygium cumini* (leaf), *Psidium guajava* (leaf), *Basella alba* (leaf and seed), *Morus indica* (leaf), *M. laevigata* (leaf), and *M. latifolia* (leaf). The results of the hemolytic activity test suggested that the methanolic extracts of *S. cumini*, *P. guajava*, *B. alba* (leaf and seed), *M. indica*, *M. laevigata*, and *M. latifolia* showed significantly higher hemolytic activity compared to their aqueous extracts. The methanolic extracts of *A. racemosus*, *W. somnifera*, *P. guajava*, *M. laevigata*, and *M. latifolia* showed remarkable antioxidant activity. Moreover, the results of the PLA_2 enzyme neutralization activity test revealed that both aqueous and methanolic extracts of *P. guajava*, *S. cumini*, and *B. alba* (leaf) have significant PLA_2 neutralization capacity. Altogether, the aqueous extracts of all plants, except *A. racemosus* root, have ignorable hemolytic effect. Both of the aqueous and methanolic extracts of *M. latifolia* showed the maximum antioxidant activity. Additionally, *P. guajava* and *S. cumini* leaf aqueous extracts have promising PLA_2 inhibitory effect which may become the potential source of bioactive compounds for anti-venom drug development.

INTRODUCTION

Plants are regarded as a precious source of natural wealth in the majority of the developing nations in the world, including Bangladesh. Plants are used in a number of healthcare initiatives. Medicinal plants and plant-derived products have been utilized in traditional medicinal systems since ancient times. Traditional medicine formulation utilized more than 500 medicinal plant species for treating numerous ailments [1]. Substances found in medicinal plants are known as active principles. Plants that are rich in active compounds are of great interest in term of therapeutics [2]. Recent industrial processes and advanced scientific techniques focus on bioactive molecules. Combining different medicinal plants or plant parts deserves particular attention since such herbal mixtures are the source of increased biologically active compounds [3]. Herbal drugs were used in various formulations of numerous plant products. Approximately 80% of the drugs that are being taken in recent days are medicinal plant-derived [4]. In modern times, many medicines made from plants are used to treat

illnesses like Alzheimer's disease, cancer, diabetes, diarrhea, heart disease, Parkinson's disease, and sores. According to the report of the World Health Organization (WHO), around 80% people in developing countries are depending on their local medicinal plants, whereas one-fourth of the prescription medicines and 11% medicines that are specified as vital are plant-derived [5]. In recent years, more people are interested in plant-based medicines as a way of finding alternatives to chemical drugs that work well and have fewer side effects [6]. However, the development of medicine from a plant source requires a number of steps. The toxicity of the active molecule is considered a key factor during drug design [7]. The active components of plants used as medications have activity including antioxidant, anti-inflammatory, anti-hemolytic, anti-diarrheal, antibacterial, anti-parasitic, antiviral, etc.

The major insight into the relationship between chemicals and living things at the cellular level is revealed by hemolytic activity. Any agent that hemolyzes healthy cells is cytotoxic. It is important to figure out how well medicinal plants work and if they are dangerous. Plant extracts can have a positive effect on the membrane of red blood cells, while many plants have severe negative effects. Therefore, the commonly used medicinal plant species must be undergone for hemolytic activity evaluation. *In vitro* hemolytic assay utilizing spectroscopic techniques offers an efficient and simple method for quantifying hemolysis.

Plant-derived biologically active compounds may function as replacement of natural antioxidants in human body [8]. Antioxidants interrupt free radical chain reactions and retain proper cellular signaling in human physiology which prevents cell damage [9]. Numerous chronic and degenerative diseases, such as aging, atherosclerosis, cancer, diabetes mellitus, ischemic heart disease, and neurodegeneration are caused by oxidative cell damage arising from free radicals. Free radicals and other reactive oxygen species (ROS) are the byproducts of aerobic metabolism, produced by the body's natural processes and sometimes by environmental factors and pollutants [10, 11]. They play an important role in many physiological functions, including defense, inflammation, signal transduction, adhesion, proliferation, apoptosis, etc. Plant-based natural antioxidants are of most interest globally because they are non-toxic. Many free radical-scavenging antioxidants come from fruits, vegetables, and teas. Antioxidant-rich food plants protect against carcinogenesis. Dietary supplementation strengthens the antioxidant system and prevents chronic illness. Thus, plant species must be searched for effective pharmacological antioxidants.

Numerous active plant constituents are promising contenders for the development of anti-venom drug molecules. Traditional medicine practitioners have characterized numerous plant-based remedies based on their experiences and observations. People who are involved in agriculture and their family members are the most vulnerable people to snakebite; thus, snakebite has been defined as “disease of poverty” [12]. Serious morbidity and mortality issues are caused by snake envenomation, especially in Asia. WHO defined snakebite as a “neglected tropical disease”, but persons having snakebite seek emergency care facility in rural and tribal areas, particularly in Bangladesh, India, and Nepal [13]. Approximately 100,000 victims die among 2.7 million snakebite victims each year. According to WHO’s report, around 80% of the population throughout the world depend on traditional medicine to get rid of numerous ailments [14]. Fortunately, WHO took an initiative to lessen the snakebite-induced mortality and disabilities worldwide to 50% by 2030 [15]. Phospholipase A_2 (PLA_2), a venom enzymatic entity, is responsible for various toxic effects, such as cardiac arrest, deformations, kidney failure, and amputations [16]. Snake poison can be treated with alkaloids, acids, steroids, glycoproteins, phenols, terpenoids, and other

chemical groups that are found in plant products. Medicinal plants with anti-venom properties could be used to help people who have been bitten by snakes right away. This is especially important in rural areas. In Bangladesh, different plant species are being used for a long time to treat snakebites, but there is no in-deep study to elucidate whether they work or are safe.

The plants including *Asparagus racemosus* (root), *Withania somnifera* (root), *Syzygium cumini* (leaf), *Psidium guajava* (leaf), *Basella alba* (leaf and seed), *Morus indica* (leaf), *M. laevigata* (leaf), and *M. latifolia* (leaf) were not studied for elucidating their hemolytic effect and the protective aptitude against snake venom before in Bangladesh. Thus, the present study aimed to evaluate hemolytic activity, antioxidant properties, and PLA_2 enzyme neutralization efficacy of the aqueous and methanolic extracts of the abovementioned eight medicinal plants growing in Bangladesh. Another objective of this study was comparing the effect of aqueous and methanolic extracts of the plants.

MATERIALS AND METHODS

Selection and collection of plant materials

The nine plant samples from eight medicinal plants were selected based on their medicinal properties (Table 1). Roots of *A. racemosus* and *W. somnifera,* fresh leaves and seeds of *B. alba* were collected from the Botanical Garden, University of Rajshahi, Bangladesh. Fresh leaves of *P. guajava* and *S. cumini* were collected from the Regional Fruit Research Station, Rajshahi, Bangladesh. Fresh leaves of *M. indica*, *M. laevigata*, and *M. latifolia* plants were collected from the Bangladesh Sericulture Research and Training Institute, Rajshahi, Bangladesh. Importantly, *A. racemosus*, *W. somnifera*, *S. cumini*, *P. guajava*, and *B. alba* are available under accession number RR098, RR564, RR242, RR302, and RR078, respectively in the herbarium of the Department of Botany in University of Rajshahi, Bangladesh. *M. indica*, *M. laevigata*, and *M. latifolia* are available under accession number BSRM-42, BSRM-29, and BSRM-50, respectively in the herbarium of the Bangladesh Sericulture Research and Training Institute (BSRTI), Rajshahi, Bangladesh.

Table 1. A short description of the plant samples used in this experiment.

Name of plants	Name of families	Used part	Medicinal values	References
Asparagus racemosus	Asparagaceae	Root	Anticancer	[17]
Withania somnifera	Solanaceae		Antigastric, antiulcerative	[18]
Syzygium cumini	Myrtaceae	Leaf	Antianemic, constipation preventive	[19]
Psidium guajava			Antidiarrheal, antidiabetic, anticancer	[20]
Basella alba	Basellaceae	Leaf	Anticancer	[21]
		Seed	Anticancer	[22]
Morus indica	Moraceae	Leaf	Anti-fatigue, antianemic	[23]
Morus laevigata			Anticancer	[24]
Morus latifolia			Anticancer	[25]

Preparation of plant extracts

Firstly, the collected fresh leaves, roots, and seeds were washed well with distilled water. Subsequently, they were washed with 70% ethanol and finally again with distilled water. Then the collected materials were dried at room temperature (RT). The dried materials were kept in an air incubator at 65°C for 7-10 days. After drying well, the plant materials were powdered using an electric grinder machine. The powder was stored in an airtight container and kept in a dark, cool place for further use. For

aqueous and methanolic extract preparation, 25 mL of distilled water and methanol were taken into 18 (9 for aqueous extract and 9 for methanolic extract) different test tubes and 2 g of powdered plant material was added to each corresponding test tube. They were mixed well by vortexing and then sonicated at 20 Hz for 5 min at 30 sec intervals after each 30 sec sonication at RT. Then, the extracts were incubated at RT for 1 h and filtered with a vacuum pump filtration system as described by Hasan et al. [26]. Finally, the filtrated crude extracts were stored at 4°C for further use.

Hemolytic activity test

Ex vivo hemolytic activity test was carried out according to the methods described by Yang et al. [27] with minor modification. For this assay, fresh human blood was collected in a citrated tube and mixed gently. This study was performed following the guideline set by the Institutional Animal, Medical Ethics, Biosafety, and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes, and Living Natural Sources (No. 31/320/IAMEBBC/IBSc), Institute of Biological Sciences (IBSc), University of Rajshahi, Rajshahi, Bangladesh. Of note, blood from a healthy donor was collected with consent. The tubes were centrifuged at 5000 rpm (Heraeus Multifuge X1R, Thermo Scientific, USA) for 20 min to separate the red blood cells (RBC). After that, the RBC was washed 4 times with 1% PBS. Then a total of 18 samples (9 for aqueous extracts and 9 for methanolic extracts) were taken into marked tubes to evaluate their hemolytic activity by using three different concentrations including 25 µL, 50 µL, and 100 µL. The samples were then mixed with 10% RBC and 1% PBS buffer and incubated at 37°C for 1 h. Then the supernatant was transferred into a sterilized flat bottom 96-well microtiter plate after spinning the tubes at 3000 rpm for 5 min. After that, the absorbencies were measured at 540 nm using a MultiSkan EX, UV-Vis spectrophotometer (Thermo Scientific, USA). SDS (1%) solution was used as standard. Finally, the percentage of hemolysis was calculated using the following formula:

$$\text{Percentage (\%) of hemolysis} = \frac{A_t - A_n}{A_c - A_n} \times 100$$

where, A_c is the absorbance of the control (water solution), A_n is the absorbance of the control (saline solution), and A_t is the absorbance of the extract-treated sample.

Antioxidant activity test

The antioxidant activity test was done as described by Hasan et al. [28] with minor modification. To carry out this assay, at first 6 tubes were taken for each sample, and 0 µL, 25 µL, 50 µL, 100 µL, 150 µL, and 200 µL of crude aqueous and methanolic extracts were added into the tubes. Then absolute methanol was added to make the final volume 1000 µL. Subsequently, 1.5 mL of 0.1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution was added to each tube and the tubes were left at RT for 30 min in a dark place. Then, absorbance of each sample was measured at 517 nm using a spectrophotometer. Ascorbic acid (0.2 mg/mL) was used as the standard. Finally, the percentage (%) of DPPH radical scavenging was calculated according to the equation articulated below:

$$\text{Percentage (\%) of DPPH inhibition} = [(A_c - A_t)/A_c] \times 100$$

where, A_c is the absorbance of the control and A_t is the absorbance of the extract-treated sample.

Finally, 50% inhibition concentration (IC_{50}) values were calculated by plotting the inhibition percentages against different concentrations on scatter graph for both standard and test samples.

PLA_2 neutralization efficacy test

The PLA_2 neutralization activity test was conducted according to the methods described by Cardoso et al. [29] with minor modification. For this assay, a snake venom stock solution was prepared by dissolving 1.0 mg of lyophilized *Naja naja* snake venom in 1.0 mL of ddH_2O. Besides, the egg yolk suspension was also prepared by mixing an egg yolk in 250 mL saline water. Then, experimental extracts were taken into wells of flat bottom 96-well microtiter plate. Three different concentrations (low = 1.5 µL, medium = 3 µL, and high = 6 µL) of each plant sample were used to evaluate dose-dependent effect. Thereafter, 5 µL of snake venom solution and 85 µL of egg yolk suspension were added to each well. A required amount of tris-HCl (pH = 7.4) was added to each well to reach the final volume 100 µL. Finally, OD values were measured at 740 nm after 10 min incubation to evaluate the neutralization activity of the tested plant samples. The control for this experiment was a mixture of venom, egg yolk solution, and tris-HCl buffer. The percentage of neutralization of PLA_2 enzyme by plant extracts as well as control was calculated using the following equation:

Percentage (%) of PLA_2 neutralization = $[(A_t - A_c)/A_t] \times 100\%$

where, A_c is the absorbance of the control and A_t is the absorbance of the extract-treated sample.

Statistical analysis

All data are presented as mean±SD (standard deviation) of three independent experiments. Statistical difference among the groups was evaluated using GraphPad Prism (version 8) software. One-way ANOVA following Tukey's multiple comparison test was done for calculating statistical difference. The bar and scattered line graphs were prepared using Microsoft Excel 2013.

RESULTS

Effect of extracts on hemolysis

A. racemosus (roots) showed the highest hemolytic activity for both aqueous and methanolic extracts (Figure 1, Figure 2). At 100 µL dose, the aqueous extract of *A. racemosus* (roots) showed the highest (91.411%) hemolytic activity, which is almost same as the standard solution (1% SDS, hemolytic activity 93.186%) (Figure 1); while the aqueous extract of *B. alba* (seeds) showed the lowest (3.651%) hemolytic activity. The hemolytic activity of the aqueous extract of *A. racemosus* (roots) was 87.218% at 25 µL, 89.462% at 50 µL, and 91.411% at 100 µL dose. The aqueous extract of *B. alba* (seeds) showed 1.968%, 2.575%, and 3.651% at 25 µL, 50 µL, and 100 µL dose, respectively. The aqueous extract of other plant samples showed less than 10% hemolytic activity at 100 µL dose (Figure 1).

On the other hand, the highest hemolytic activity for methanolic extract was shown in the case of roots extract of *A. racemosus* (92.561%) at 100 µL concentration, while the lowest activity was shown by the roots extract of *W. somnifera* (33.609%) in comparison to standard solution (Figure 2). The hemolytic activity of the methanolic extract of

A. racemosus (roots) was 90.335% at 25 µL, 91.402% at 50 µL, and 92.561% at 100 µL dose. The methanolic extract of *W. somnifera* (roots) showed 2.382%, 8.910%, and 33.609% hemolysis at 25 µL, 50 µL, and 100 µL dose, respectively. The methanolic extract of *B. alba* (seeds) showed 87.236%, 87.770%, and 90.786% hemolytic activity at 25 µL, 50 µL, and 100 µL dose, respectively. All of the other methanolic extracts of selected plants showed more than 85% hemolytic activity, including the leaves of *S. cumini* (91.991%), leaves of *P. guajava* (88.405%), leaves of *B. alba* (91.788%), seeds of *B. alba* (90.786%), leaves of *M. indica* (89.959%), leaves of *M. laevigata* (88.754%), and the leaves of *M. latifolia* (90.933%) (Figure 2).

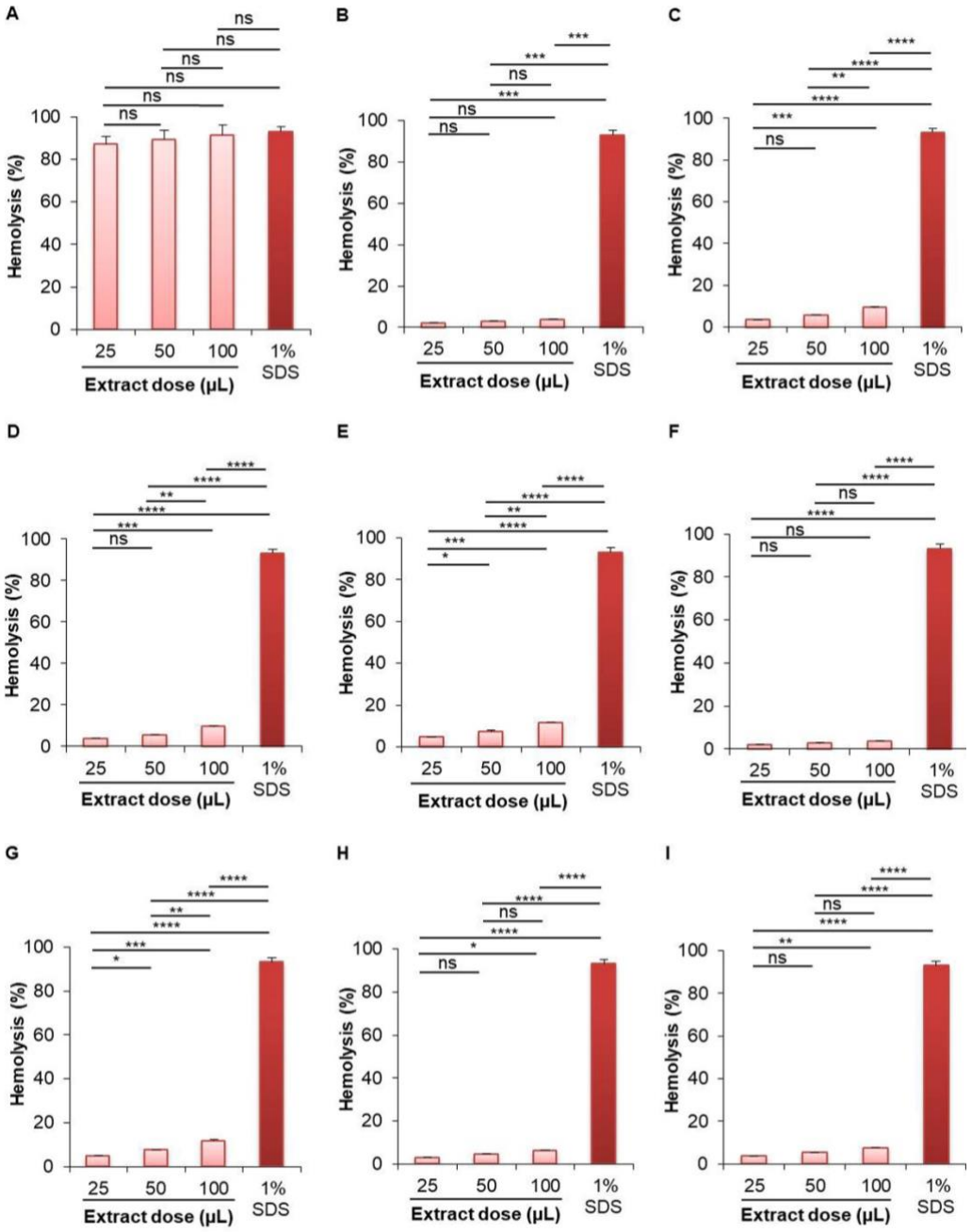

Figure 1. Hemolytic activity of aqueous extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf). SDS = *sodium dodecyl sulfate.* Data are presented as mean±SD, n = 3. ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

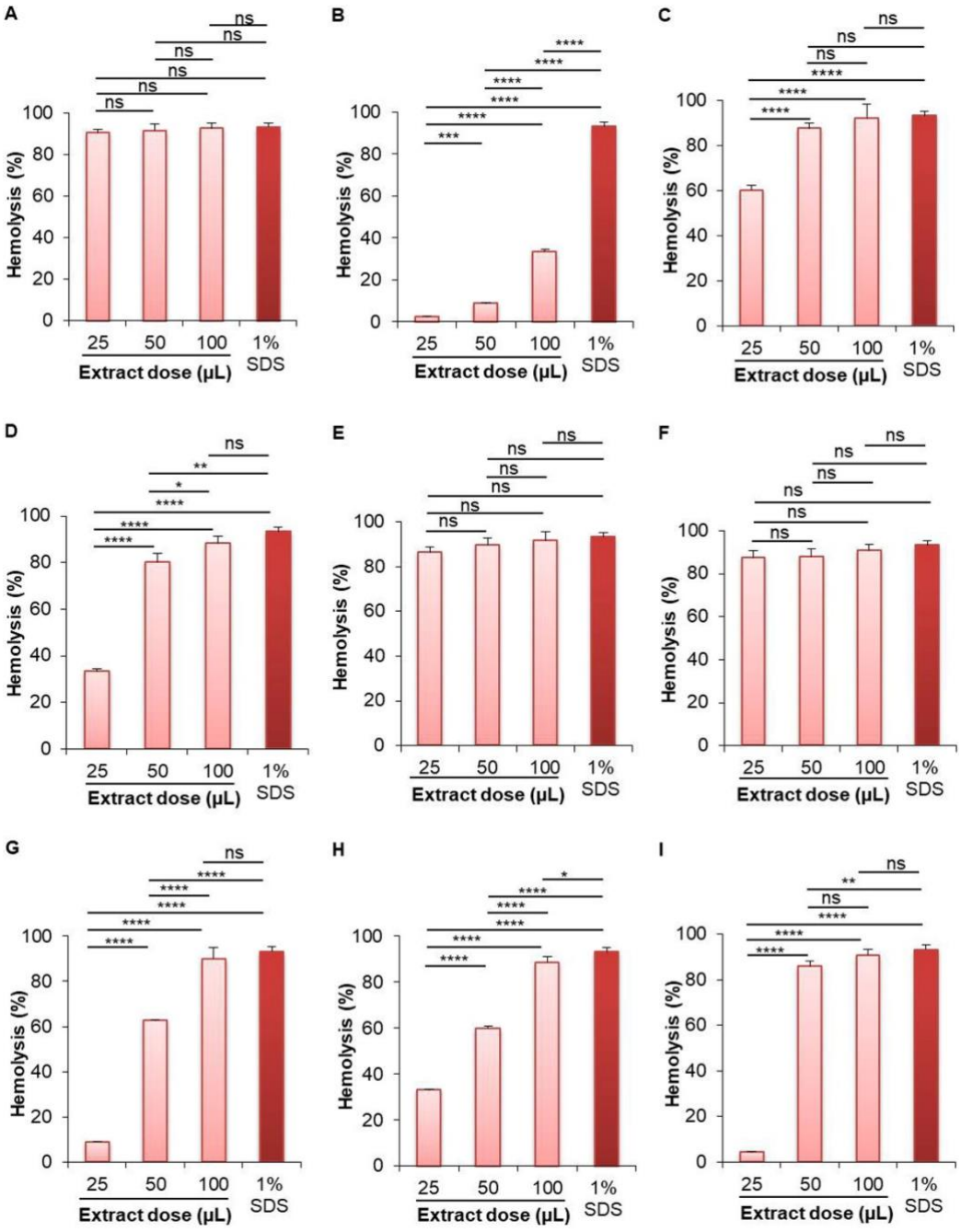

Figure 2. Hemolytic activity of methanolic extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf). SDS = *sodium dodecyl sulfate.* Data are presented as mean±SD, n = 3. ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Effect of extracts on DPPH free radical inhibition

According to the results of the antioxidant activity test, the 50% DPPH radical inhibition concentration (IC_{50}) values of aqueous extract for nine samples were calculated as roots of *A. racemosus* (167.556 µL), *W. somnifera* (138.821 µL), leaves of *S. cumini* (188.788 µL), *P. guajava* (162.196 µL), *B. alba* (278.599 µL), seeds of *B. alba* (135.387 µL), leaves of *M. indica* (157.203 µL), *M. laevigata* (142.553 µL) and *M. latifolia* (91.714 µL) (Figure 3).

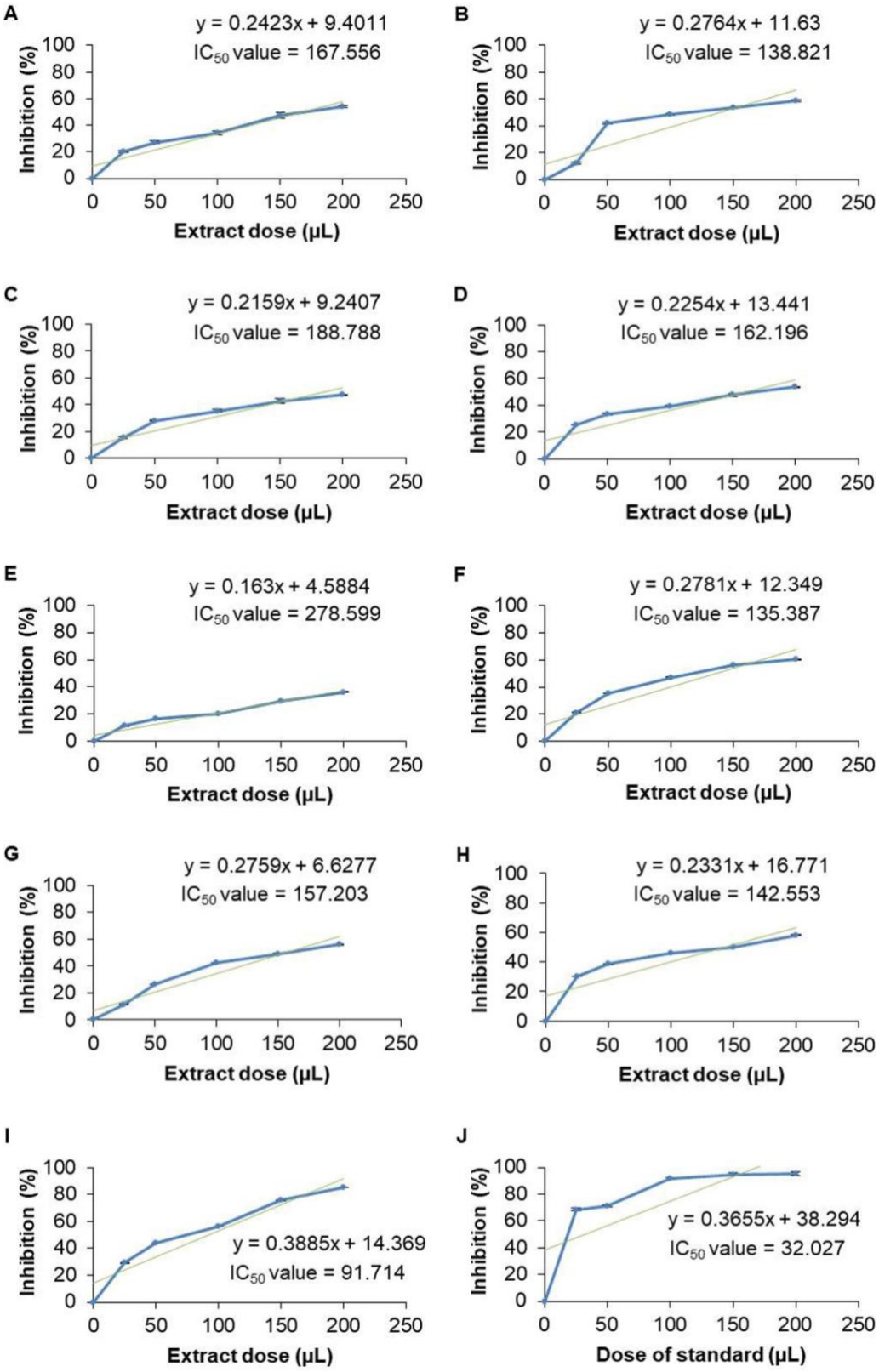

Figure 3. Antioxidant activity of aqueous extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf); (J) Ascorbic acid (standard). Data are presented as mean±SD, n = 3.

In contrast, the IC_{50} values of methanolic extracts were determined as follows- roots of *A. racemosus* (53.338 µL) and *W. somnifera* (61.064 µL), leaves of *S. cumini* (78.04 µL), *P. guajava* (50.547 µL), and *B. alba* (143.074 µL), seeds of *B. alba* (71.285 µL), leaves of *M. indica* (80.981 µL), *M. laevigata* (49.897 µL), and *M. latifolia* (32.598 µL) (Figure 4). But the IC_{50} value of the standard solution (ascorbic acid) was 32.027 µL.

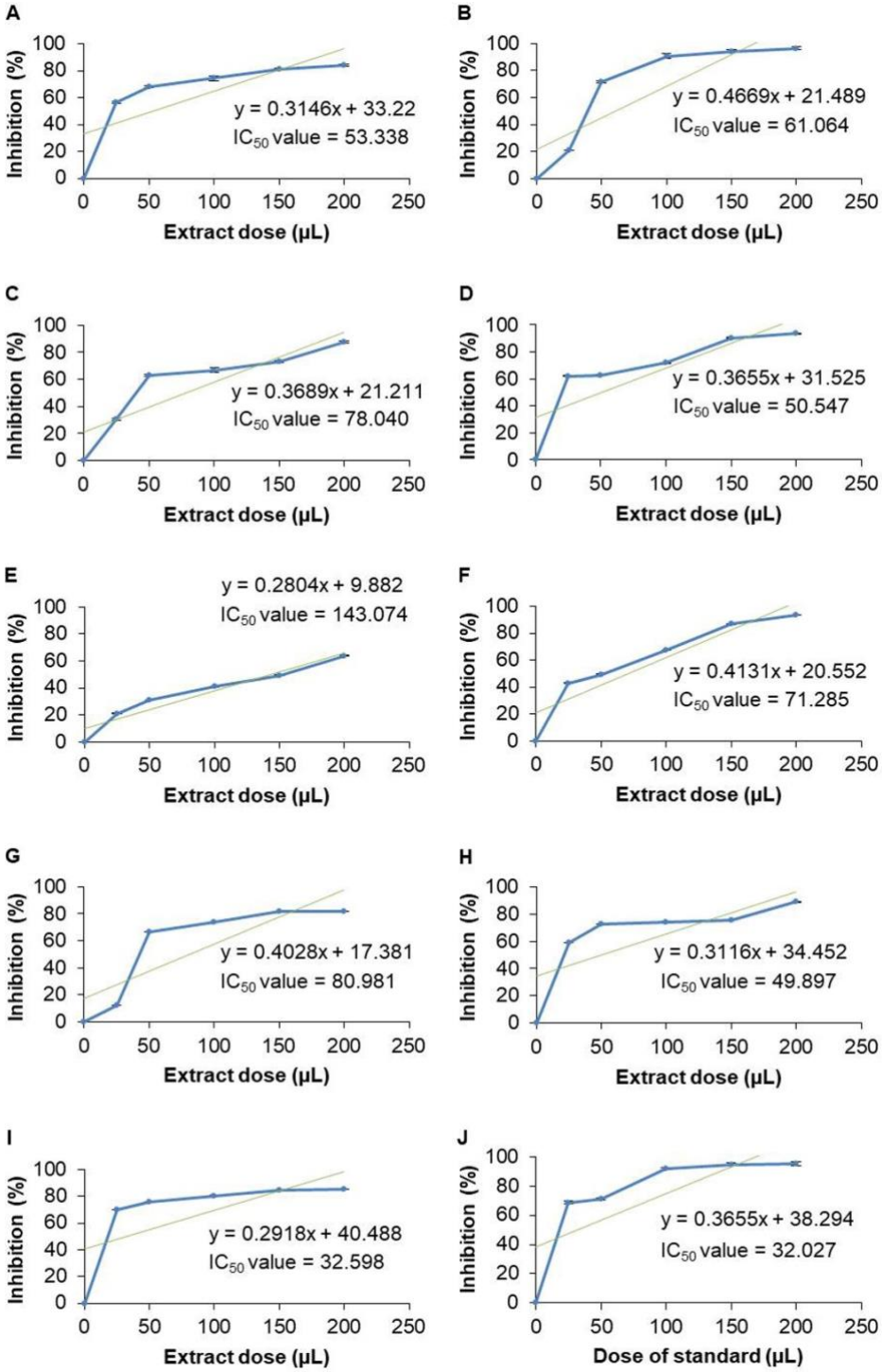

Figure 4. Antioxidant activity of methanolic extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf); (J) Ascorbic acid (standard). Data are presented as mean±SD, n = 3.

Effect of extracts on PLA_2 enzyme neutralization

The results of the PLA_2 neutralization activity test presented that the aqueous extract of leaves of *S. cumini* (88.814%) and leaves of *P. guajava* (88.662%) showed the best activity, while the roots of *W. somnifera* (25.207%) showed the lowest activity at high dose compared to their respective control (5.525%) (Figure 5). The aqueous extract of leaves of *S. cumini* showed 84.305%, 87.823%, and 88.814% PLA_2 neutralization at low, medium, and high dose, respectively. The aqueous extract of leaves of *P. guajava* showed 84.260%, 87.891%, and 88.662% PLA_2 neutralization at low, medium, and high dose, respectively. The roots of *W. somnifera* neutralized 18.598%, 19.958%, and 25.207% PLA_2 at low, medium, and high dose, respectively. Besides, the aqueous extract of leaves of *B. alba* (71.370%), *M. indica* (69.582%), *M. laevigata* (52.614%), and *M. latifolia* (68.271%) showed more than 50% PLA_2 neutralization activity, whereas the roots of *A. racemosus* (41.535%) and seeds of *B. alba* (28.687%) showed less than 50% PLA_2 neutralization activity (Figure 5).

On the contrary, in the case of methanolic extract of selected plant samples, the highest PLA_2 neutralization activity was found by the leaves of *P. guajava* (89.421%) and the lowest activity was found by the roots of *W. somnifera* (30.861%) at even high concentration in comparison to their corresponding controls (5.525%) (Figure 6). The methanolic extract of *P. guajava* leaves neutralized 87.589%, 88.354%, and 89.421% PLA_2 at low, medium, and high dose, respectively. The methanolic extract of leaves of *S. cumini* showed 86.341%, 88.668%, and 89.399% PLA_2 neutralization activity at low, medium, and high dose, respectively. The methanolic extract of the roots of *W. somnifera* inhibited 23.984%, 27.741%, and 30.861% PLA_2 activity at low, medium, and high dose, respectively. Other methanolic extracts of selected plant samples showed more than 50% PLA_2 neutralization activity, including the leaves of *S. cumini* (89.399%), leaves of *B. alba* (78.765%), seeds of *B. alba* (56.373%), leaves of *M. indica* (77.919%), *M. laevigata* (64.593%), and *M. latifolia* (69.820%) (Figure 6).

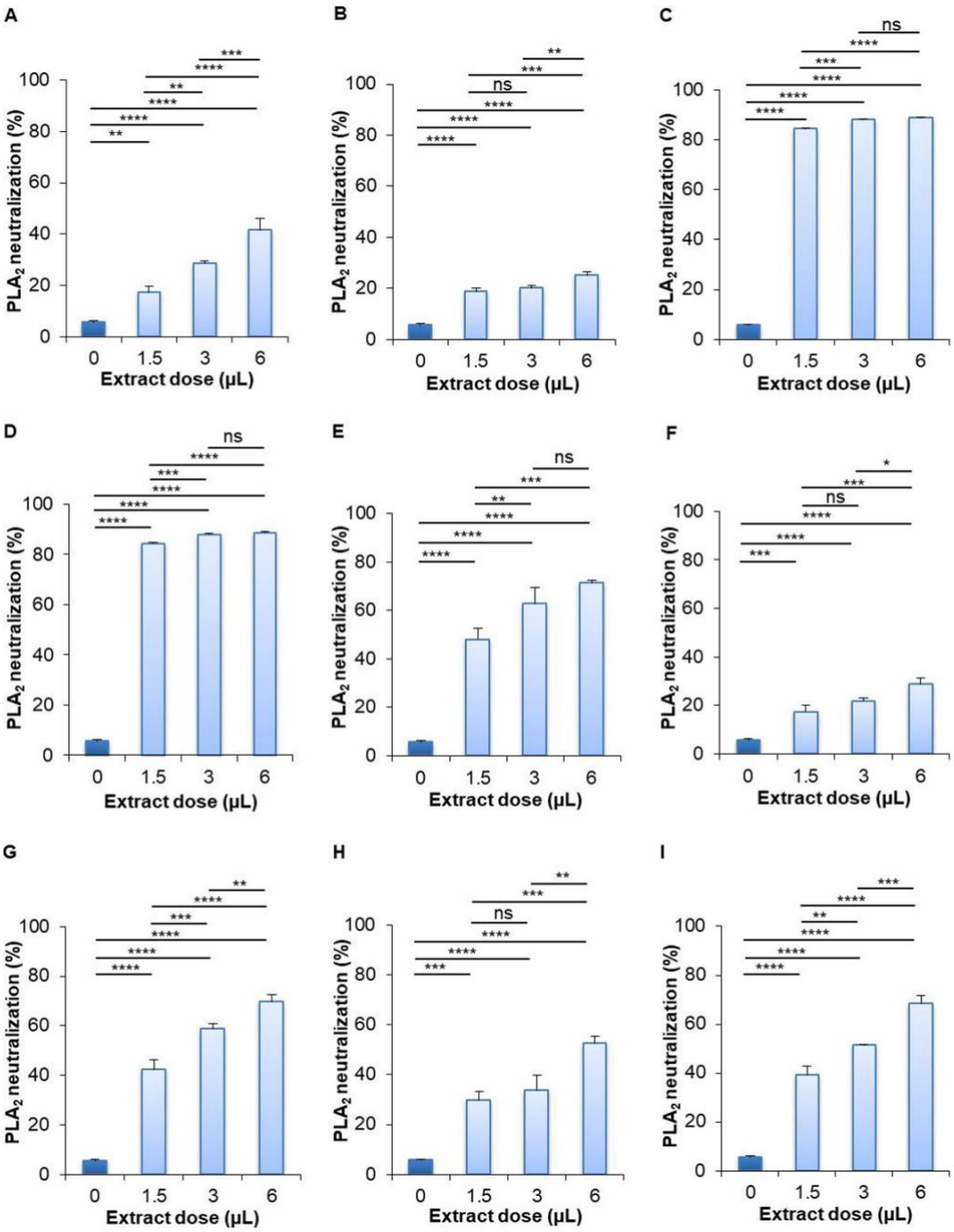

Figure 5. PLA_2 enzyme neutralization efficacy of aqueous extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf). Data are presented as mean±SD, n = 3. ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

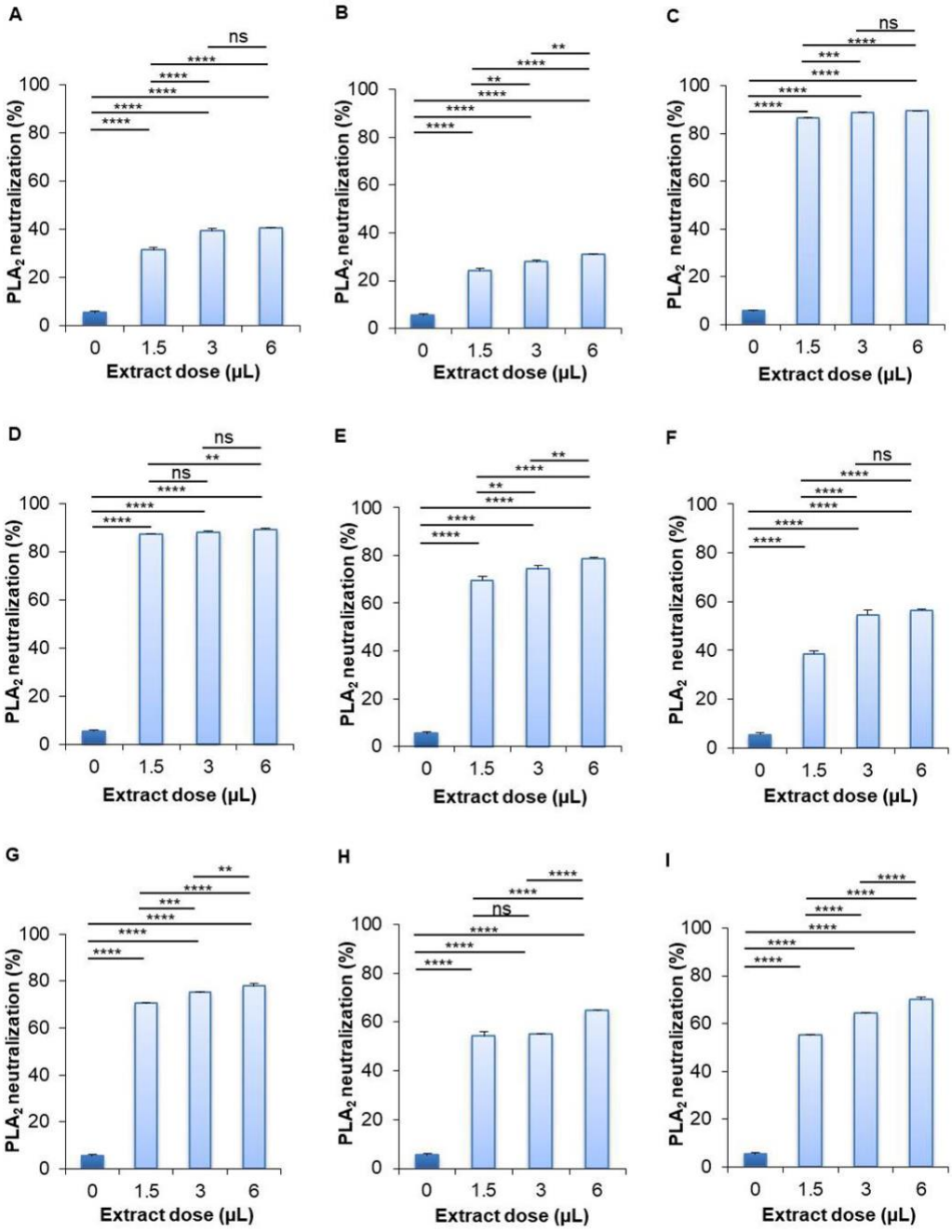

Figure 6. PLA_2 enzyme neutralization efficacy of methanolic extracts of experimental samples. (A) *A. racemosus* (root); (B) *W. somnifera* (root); (C) *S. cumini* (leaf); (D) *P. guajava* (leaf); (E) *B. alba* (leaf); (F) *B. alba* (seed); (G) *M. indica* (leaf); (H) *M. laevigata* (leaf); (I) *M. latifolia* (leaf). Data are presented as mean±SD, n = 3. ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

DISCUSSION

Researchers have been interested in herbal phytomedicines due to their possible biological activity. Since the beginning of time, there have been many investigations carried out. The red blood cell (RBC) model has been extensively utilized because it provides a direct indication of toxicity and is readily available. The demise of RBC is known as hemolysis and is caused by the lysis of the membrane lipid bilayer of RBC. This hemolysis relates to concentration, potency, and the chemical composition of plant extracts [30]. The present study evaluated the hemolytic activity of both aqueous and

methanolic extracts and the extracts induced hemolysis to different extent. Based on percentage values of hemolytic activity, aqueous extracts of *A. racemosus* roots exhibited the maximum hemolytic activity without showing any significant difference among groups and compared to the SDS, while aqueous extracts of *B. alba* seeds exhibited the lowest (3.651%) hemolysis even at high dose extract treatment and there was no statistical difference among extract treated groups. *B. alba* leaf aqueous extract also has ignorable hemolytic effect. Previous research showed protective effect of *B. alba* leaf aqueous extract against osmotic fragility of RBC and suggested regular consumption as a protectant against hemolytic anemia [31]. In our study, aqueous extract of *W. somnifera* exhibited 3.807% hemolysis at even high dose. Previous studies reported that aqueous extract of *W. somnifera* has no hemolytic activity [32, 33]. To the best of knowledge, the current study is the first evaluating hemolytic activity of *M. indica*, *M. laevigata*, and *M. latifolia* leaf extract. Before us, a group showed that flavonoids from *Morus sp.* leaves protect from 2, 2 0-azobis (2-amidinopropane) dihy-drochloride (AAPH)-induced hemolysis in a dose-dependent manner but considered harmless [34]. In opposition to the aqueous extract, methanolic extracts of all plant samples, except *W. somnifera* roots (33.609%), showed approximately 90% hemolytic activity at high dose. Based on percentage values of hemolytic activity of methanolic extract, *A. racemosus* root exhibited the maximum (92.561%) hemolytic activity at high dose. In the case of *A. racemosus*, extract at all doses showed dose-dependent but statistically insignificant hemolysis. In addition to that, there was no statistical difference between the extract-treated groups and the 1% SDS standard group, which is indicative to elevated enrichment of compounds having hemolytic potential in methanolic extract of *A. racemosus* root. Though there is no prior report regarding hemolysis induction by *W. somnifera* and *A. racemosus*, methanolic extract of these two plants have thrombolytic potential [35]. According to the comparative analysis of hemolytic activity between aqueous and methalonic extracts, methanolic extracts of *S. cumini* (leaf), *P. guajava* (leaf), *B. alba* (seed), *B. alba* (leaf), *M. indica* (leaf), *M. laevigata* (leaf), and *M. latifolia* (leaf) showed higher hemolytic activity than their aqueous counterpart. To the best of our knowledge, there is no prior report regarding hemolytic effect of *S. cumini* (leaf), *B. alba* (seed), *B. alba* (leaf), *M. indica* (leaf), *M. laevigata* (leaf), and *M. latifolia* (leaf) methanolic extract. However, prior research showed that the methanolic extract of *P. guajava* leaf induced 50% hemolysis at 10.26 mg/mL dose [36]. Our data regarding hemolysis by methanolic extract of *P. guajava* leaf is not comparable with previous report [36] because of difference in extract preparation procedure but is corroborated by data of Purba et al. [36]. According to the *in vitro* hemolytic experiment conducted on isolated rat erythrocytes, the seed extract of *Nigella sativa* and *Lepidium sativum* induced 53% and ~33% hemolysis, respectively at 100 mg/mL dose [37]. On the other hand, aqueous extract of *Putoria calabrica* (leaf) is able to protect erythrocytes from damage caused by oxidation [38]. The hemolytic effect of *Cymbopogon citratus* and *Azadirachta indica* aqueous and ethanolic extract was <5% and found to be non-toxic [39]. Plant samples having >10% hemolytic effect should be avoided for medicinal purpuse [39], therefore, the aqueous extract of *A. racemosus* root at any amount may affect the RBC and should be aware of its medicinal formulation. Besides, methanolic extracts of all plant sample also are the matter of concern in case of traditional medicinal formulation. However, the methanolic extract of *W. somnifera* (root), *M. indica* (leaf), and *M. latifolia* (leaf) may retain the possibilities of their use in medicinal formulation at low dose.

A number of methods are used to determine the free radical scavenging activity of antioxidant compounds, and the DPPH free radical scavenging assay is a widely used method because it is a fast, easy, and reliable technique that does not require special and sophisticated devices [40, 41]. The therapeutic effects of methanolic extract of two

mangrove species, *Bruguiera gymnorrhiza* and *Heritiera littoralis*, were studied [5] and high radical scavenging ability against DPPH, H_2O_2, and Fe^{2+} radicals was found in both species. *B. gymnorrhiza* and *H. littoralis* extracts prevented H_2O_2-induced lysis of human RBC and their reductive capacity rose dose-dependently [5]. The Iranian people utilize 13 historically popular plant species in folk treatment because of their excellent DPPH radical scavenging capacity with IC_{50} value ranging from 1.03 µg/mL by *Salix sp.* to 187.88 µg/mL by *Allium hirtifolium*, the lowest and the highest, respectively among all species [42]. Interestingly, the DPPH scavenging capacity of *Salix* sp., *Mentha spicata*, *Salvia hydrangea*, *Zataria multiflora*, *Achillea tenuifolia*, and *Polypodium vulgare* was lower than that of quercetin (IC_{50} value = 5.22 µg/mL), a strong and well used natural antioxidant [42]. *Bruguiera gymnorrhiza* is a rich source of compounds with DPPH scavenging activity with the lowest IC_{50} value by ethanol extract of leaf and bark compared to methanol and chloroform extracts [43]. In our experiment, we evaluated the antioxidant activity of extracts utilizing DPPH radical scavenging assay by calculating the IC_{50} values using the regression equations. According to the results of the antioxidant bioassay, all of the aqueous extracts, except the leaves of *M. latifolia* (IC_{50} value = 91.714) showed IC_{50} values greater than 100 µL; while all of the methanolic extracts, except the leaves of *B. alba* (IC_{50} value = 143.074) showed IC_{50} values less than 100 µL. According to an earlier study, *A. racemosus* root ethanolic extract's IC_{50} value was 468.57±3.002 µg/mL [44]. The methanolic extract of *S. cumini* leaves scavenged 50% DPPH at 125.39±7.55 µg/mL concentration [45], while the IC_{50} value of of *S. cumini* fruit methanolic extract was 81.4±0.6 µg/mL [46]. The *P. guajava* leaf methanolic extract's IC_{50} value was 85.7±1.8 µg/mL [47]. The antioxidant activity of *B. alba* leaf and seed was determined with an IC_{50} value of 112.96±4.87 µg/mL and 145.34±4.38 µg/mL, respectively [22]. *M. leavigata* leaf aqueous extract showed antioxidant property with an IC_{50} value 186.76 µg/mL [24]. Another study reported the IC_{50} value 104.86 µg/mL of *M. laevigata* methanol extract [48]. *M. indica* leaves methanol extract scavenged 50% DPPH at 79.53 ± 0.87 µg/mL concentration [49]. The IC_{50} value of *M. latifolia* leaf methanolic extracts was 98.58 ± 7.54 µg/mL [25]. However, in our study, the standard solution (ascorbic acid) showed IC_{50} values of 32.027 µL. The comparison of IC_{50} values between methanolic extracts and standard solution showed that the methanolic extracts of roots of *W. somnifera*, leaves of *S. cumini*, leaves of *B. alba*, seeds of *B. alba*, and leaves of *M. indica* showed noticeable higher IC_{50} values than standard solution which indicated that they have less antioxidant activity. But the methanolic extracts of roots of *A. racemosus*, leaves of *P. guajava*, leaves of *M. laevigata*, and leaves of *M. latifolia* showed IC_{50} values that were almost close to the IC_{50} value of the standard solution which is indicative to high antioxidant capacity of these extracts. However, the IC_{50} values of aqueous extracts are significantly higher than that of methanolic extracts, which represents less antioxidant potential of the aqueous extracts.

Plants as well as plant parts are used as traditional medicine to treat the fatal effects of venomous snake bites. Pharmacological studies have reported anti-inflammatory, anti-viral, and anti-venom properties of plant extracts by neutralizing different toxic proteins [50]. Rosmarinic acid is an excellent plant-derived compound that can neutralize PLA_2 enzyme in snake venom [50]. Seventeen extracts from Pakistani medicinal plants were examined for evaluating their ability to inhibit PLA_2 activity in *Echis carinatus* venom [51]. Though all plant extracts were able to neutralize PLA_2, *Curcuma longa*, *Citrullus colocynthis*, and *Rubia cordifolia* showed the highest (78%) inhibitory aptitude [51]. Another study evaluated venom-antagonizing characteristics of aqueous extracts of five plants including *Sapindus laurifolius*, *Spondias pinnata*, *Plumeria lutea*, *Woodfordia fruticosa*, and *Croton roxburghii* and found entire PLA_2 and acetylcholinesterase neutralization by *Spondias pinnata* and *W. fruticosa* [52]. In our

current experiment, we evaluated the efficacy of neutralizing PLA_2 protein of *N. naja* venom. Interestingly, all plant extracts showed dramatic PLA_2 neutralizing efficacy. Both of the aqueous and methanolic extracts of *S. cumini* (leaves) and *P. guajava* (leaves) showed the highest (>80%) PLA_2 neutralization capacity at all experimental doses. Prior research showed that methanolic extract of *S. cumini* L. neutralized 50% PLA_2 in the venom of Pakistani *Echis carinatus* [39, 51]. Extract from leaves of guava cultivars including Paluma, Pedro Sato, and Roxa inhibited PLA_2 produced by *B. alternatus* venom [53]. The maximum inhibition was 63.16% by the Paluma cultivar, followed by 57.89% inhibition by Pedro Sato, and 52.63% by Roxa [53]. *W. somnifera* inhibited 30% of the PLA_2 in *N. naja* venom [54]. However, comparative PLA_2 neutralization activity between aqueous and methanolic extracts revealed better PLA_2 neutralization activity by the methanolic extracts.

Altogether, the study found extreme hemolytic activity by the aqueous extract of *A. racemosus* (root). Consumption of fresh root of *A. racemosus* as traditional medicine is common and this might be a contributing factor to hemolytic anemia. Therefore, keeping this effect in consideration during eating fresh *A. racemosus* (root) or herbal medicine formulation is suggested. However, it found dramatic PLA_2 neutralization by both aqueous and methanolic extracts of *P. guajava* (leaf) and *S. cumini* (leaf). Chewing fresh leaves of *P. guajava* and *S. cumini* after snake biting may exert protection against the fatal effects of PLA_2 enzyme in snake venom. In addition to that, the aqueous extract of *P. guajava* (leaf) and *S. cumini* (leaf) can be considered safe in terms of hemolysis since the percentage of hemolysis by these two extracts is less than 10% at even high dose [39]. Moreover, the methanolic extract of *W. somnifera* scavenged 50% DPPH at 61.064 µL dose. But the methanolic extract of *W. somnifera* at medium dose (50 µL) induced <10% hemolysis and considered less toxic compared to other methanolic extracts. This ignorable hemolytic effect of *W. somnifera* methanolic extract might be the result of high antioxidant capacity of this extract [5, 39]. However, the limitation of this study is that the concentration (weight/volume) of extracts was not optimized after drying the filtrate.

CONCLUSION

The present study revealed the *ex vivo* hemolytic activity, *in vitro* antioxidant activity, and *in vitro* PLA_2 enzyme neutralization efficacy of *A. racemosus* (root), *W. somnifera* (root), *S. cumini* (leaf), *P. guajava* (leaf), *B. alba* (leaf), *B. alba* (seed), *M. indica* (leaf), *M. laevigata* (leaf), and *M. latifolia* (leaf). The overall results of this study present *P. guajava* (leaf) and *S. cumini* (leaf) as potent sources of anti-venom drug candidates which could be used in traditional medicine to combat against snake-biting. The severe hemolytic effect of *A. racemosus* (root) aqueous extract represents it toxic and using it directly in folk medicine may become a cause of hemolytic anemia. On the other hand, the methanolic extract of *W. somnifera* may become an attractive source of antioxidant molecules.

ACKNOWLEDGMENTS

The authors are very grateful to Dr. Ariful Haque (Associate Professor, Molecular Pathology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh) for providing us lab facilities to carry out this experiment. The authors are also thankful to the director of the Botanical Garden (University of Rajshahi,

Bangladesh), the Regional Fruit Research Station (Rajshahi, Bangladesh), and the Bangladesh Sericulture Research and Training Institute (Rajshahi, Bangladesh).

AUTHOR CONTRIBUTIONS

Conceptualization: ZF, MAR, and FAJ; Data curation: FAJ and MMH; Formal analysis: FAJ and MMH; Investigation: FAJ, MMH, MAR, and ZF; Methodology: FAJ and MMH; Project administration: ZF and MAR; Resources: MAR and ZF; Supervision: ZF and MAR; Validation: ZF and MAR; Visualization: FAJ and MMH; Writing - original draft: FAJ, MSRS, and MJA; Writing – review & editing: ZF and MMH. All authors have read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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