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Investigation of the Antimicrobial Activity and *in Vivo* Cytotoxicity of *Diospyros malabarica* (Desr.) Kostel. Fruit Extracts

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ABSTRACT

Mankind is facing an unprecedented threat of existence due to the antibiotic resistance developed by bacteria. The unripe fruits of *Diospyros malabarica* (Desr.) Kostel. (family: Ebenaceae) can be considered as one of the natural sources to tackle this issue. The present study is designed to assess the antimicrobial activity of *D. malabarica* seed and flesh extracts. Herein, *D. malabarica* extracts were prepared using polar solvents (*i.e.*, water and 70% ethanol) and their antimicrobial activity as well as *in vivo* toxicity was investigated. Their antibacterial activity was investigated against gram positive (*Bacillus subtilis*) and gram negative (*Escherichia coli* DH5α, and *Salmonella typhi*) bacteria at different time points. All the extracts showed the highest antibacterial activity after 2 hours of incubation. The aqueous seed extract showed the maximum zone of inhibition (*i.e.*, ~13 mm) against *Bacillus subtilis* with a minimum inhibitory concentration (MIC) value of 2 μg/μl. The antibacterial propensity was also confirmed through trypan blue dye exclusion assay, CellTox™ Green assay, and lipid peroxidation (LPO) assay. On the other hand, the ethanolic seed extract demonstrated higher antifungal activity through inhibition of mycelial growth. All the extracts showed excellent hemocompatibility against both human and rat red blood cells (RBCs). They also did not show any toxicity to rat liver and kidneys. Taken together, this study demonstrates that the aqueous and ethanolic extracts of *D. malabarica* seed and flesh could be an effective source of natural antimicrobial agents with no cytotoxic activity.

1. INTRODUCTION

Medicinal plants are the rich source of therapeutically important compounds that provide them with the ability to show different bioactive properties [1]. Because of the immense potential of plants and plant derived products to improve the health care sector, researchers are now focusing on phytomedicine to tackle the menace of multidrug resistant bacteria. Furthermore, the toxicity of chemically synthesized drugs makes plant-based medicines (*i.e.*, phytomedicine) as suitable alternatives [2]. During the ancient times, infectious diseases caused by different pathogenic microorganisms were the leading cause of death all over the world. Mankind used plants or plant-derived medicines to cure from illness as well as to prevent themselves from diseases [3, 4]. At present, the emergence of multidrug-resistant bacteria is causing millions of casualties every year in the world [5]. In the modern medical treatment, huge consumption of chemically synthesized antibiotics is not only preventing the infectious diseases but also paving the way to generate antibiotic resistant bacteria that is one of the biggest threats to global health, agriculture, food security, and development [3]. Furthermore, the synthetic antibiotics have various side effects [6, 7] that have necessitated the pursuit of natural antibacterial agents. In addition, pathogenic fungi are highly responsible for hampering the production and quality of agricultural crops, fruits, and vegetables [7, 8] besides causing diseases to human. Recent studies revealed that fungal attack on rice plants causes 11% - 15% yield loss annually [9]. On the other hand, more than 300 fungal metabolites are known to cause allergic reactions and diseases to the consumers [8]. Different commercially available chemically synthesized fungicides are effective for fungal growth inhibition. However, they are highly toxic to human health as well as hazardous for the environment [10]. Hence, there is an urgent need to discover new antimicrobial compounds against pathogenic microbe-induced diseases. Many plants or plant-derived products having antimicrobial properties can effectively provide a wide range of protection against pathogenic bacteria or fungi as well as can be an effective alternative to the chemically synthesized antibiotics or fungicides [3, 4, 7, 8].

Since plant extracts contain various bioactive molecules including secondary metabolites and many people rely on herbal medicine as their primary healthcare needs, it is one of the major concerns to investigate the biocompatibility of plant extracts [11]. Hemolysis activity of the plant extracts is an important parameter to investigate their toxicological effects, because it provides primary information about their interaction with biological entities at the cellular level [1]. Besides blood cells, due to the diverse chemical properties of plant extracts, they can interact with other body organs (*i.e.*, liver, kidney, lung, spleen, and heart) and result in adverse effects [11]. Hence, the investigation of the biocompatibility of plant extracts is crucial, because various secondary metabolites produced by plants can be a novel agent for new drug leads [12].

Diospyros malabarica (Desr.) Kostel. (family: Ebenaceae) is a lowland rainforest species [13]. It is medicinally important, because different parts of it contain anti-diabetic, anti-diarrheal, and anti-inflammatory properties [14, 15]. For example, the seeds of the immature fruits of *D. malabarica* were traditionally used for the treatment of ulcer, diarrhea, and chronic dysentery [13-15]. Although several studies on different parts of *D. malabarica* have been performed, the extensive antimicrobial activity and *in vivo* cytotoxicity of *D. malabarica* seed and flesh are yet to be investigated.

The present study focused on the investigation of antimicrobial activity as well as *in vivo* biocompatibility of different parts of *D. malabarica* fruit. Herein, we prepared *D. malabarica* seed and flesh extracts using water and 70% ethanol (*i.e.*, polar solvent). The antibacterial activity in terms of zone of inhibition (ZOI) in millimeter (mm), minimum inhibitory concentration (MIC) value, trypan blue dye exclusion assay, and CellTox™ Green cytotoxicity assay of *D. malabarica* seed and flesh extracts was performed against gram negative (*i.e.*, *S. typhi* and *E. coli* DH5α*)* and gram positive (*i.e.*, *B. subtilis*) bacterial strains. The mechanism of antibacterial activity was also investigated through lipid peroxidation assay. On the other hand, antifungal activity was investigated against *Pestatotiopsis microspora*, *Botrytis cinerea*, *and Fusarium oxysporum*. The hemocompatibility of the extracts was tested using human and rat RBCs. The *in vivo* cytotoxicity of extracts was investigated using animal model (*i.e.*, rat).

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Absolute ethanol, EDTA, trichloroacetic acid (TCA), and HCl were purchased from Merck, Germany. Sodium hydroxide (NaOH) was bought from Ashland Inc., USA. Agar powder was collected from Titan Biotech Ltd., India. Peptone, yeast extract powder, dextrose, and sodium chloride were purchased from UNI-CHEM, China. Hydrogen peroxide (H_2O_2), ketamine/xylazine, and thiobarbituric acid (TBA) were bought from SIGMA ALDRICH, Germany. Potassium chloride (KCl), Na_2HPO_4, and KH_2PO_4 were obtained from Scharlab, Spain. Propiconazole was purchased from Syngenta, Australia. Ascorbic acid was purchased from VEGA, China and trypan blue was procured from Alfa Aesar, United Kingdom. CellTox™ Green dye was bought from Promega, USA. The biochemical analysis kits for serum AST, ALT, and γ-GT analysis were purchased from Vitro Scient, Egypt and the serum creatinine determination kit were bought from Crescent Diagnostics, the Kingdom of Saudi Arabia.

Escherichia coli DH5α (ATCC 25922), *Bacillus subtilis* RBW (ATCC 6051), and *Salmonella typhi* (ATCC 14028) were collected from the department of Biotechnology and Genetic Engineering, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh. The fungal strain *Pestalotiopsis microspora* (ATCC 26275), *Botrytis cinerea* (ATCC 26943), and *Fusarium oxysporum* (ATCC 62705) were collected as pure culture from the Plant Pathology Laboratory, Department of Botany, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh. Fifteen Wister female rats (weighing between 90 and 120 g) were obtained from the Department of Pharmacy, Jahangirnagar University, Dhaka, Bangladesh.

2.2. Sample Collection and Preparation

The unripe fruits of *D. malabarica* (Desr.) Kostel. were collected from the local market of Mymensingh, Bangladesh (24.7471˚N, 90.4203˚E) and brought to the laboratory. The fruits were washed properly with distilled water. Subsequently, the flesh and seed parts of the fruits were separated carefully and cut into small pieces. Both the flesh and seed parts were then dried in a hot air oven at 60˚C. Thereafter, the dried chips were ground into coarse powder and stored in sealed container for further analysis.

2.3. Preparation of Crude Extract and Fractionation

Crude extracts and their stock solutions were prepared according to our previously established protocol [16-21]. Briefly, 5 g of powdered materials of both seed and flesh were taken in clean conical flasks and soaked in 100 ml of the respective solvents. Our previously published research on the phytochemical constituents of *D. malabarica* seed and flesh indicated that *D. malabarica* fruit is a rich source of polar phytochemicals [16], that is why, we used only polar solvents (distilled water and 70% ethanol) in the present study. After that, the flasks were kept in a water bath for 72 hours upon gentle shaking at 120 rpm. The crude mixtures were then filtered by Whatman No.1 filter paper and the extracts were concentrated in a petri plate through evaporation [22]. When the evaporation was completed, the plates containing extracts were stored in a dry and cool place for further analysis. Finally, different concentrations of the extract sample were prepared using 0.9% NaCl solution.

2.4. Antibacterial Activity Assay

2.4.1. Determination of Minimum Inhibitory Concentration (MIC)

The MIC value was measured to detect the minimum amount of the extracts of *D. malabarica* seed and flesh required to inhibit the growth of bacteria. The MIC value of the extracts was determined through broth dilution method as described previously [17, 18]. Briefly, 990 µl of Luria Bertani (LB) medium was inoculated with 10 µl of overnight grown respective bacterial cultures (optical density, OD: 1.5 - 1.7). Different amounts of the extracts (*i.e.*, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 7.5, and 10 µg/µl) were added into the bacterial cultures and incubated for 2 hours at 37˚C in an incubator upon shaking at 120 rpm. The absorbance of all the cultures was taken at 600 nm using a UV-visible Spectrophotometer (Optizen POP,

Korea). The experiment was performed in triplicate ($n = 3$).

2.4.2. Determination of Zone of Inhibition (ZOI)

The antimicrobial activity of *D. malabarica* seed and flesh extracts was determined by disk diffusion method as described previously [23, 24]. Briefly, bacterial strains (*i.e.*, *E. coli* DH5α, *B. subtilis* RBW, and *S. typhi*) were cultured in LB medium at 37˚C and 120 rpm overnight. Then 100 µl of the respective bacterial culture (OD: 1.8 - 1.9) was taken and spread uniformly on LB agar plates. Metrical filter paper disks (diameter 5.55 mm) containing different amounts of plant extracts (*i.e.*, 0.2, 1, 2, 3, and 4 mg) were placed on the LB agar plates containing uniformly spread bacteria. The same experiment was performed using 0.9% NaCl as negative control. The LB agar plates were then incubated overnight at 37˚C for the optimum growth of bacteria. Finally, the antibacterial activity of *D. malabarica* seed and flesh extracts was determined by the presence of clear zones surrounding the disks which confirmed the inhibition of bacterial growth. The diameter of the clear zones was measured using slide calipers at different time points.

2.4.3. Trypan Blue Dye Exclusion Assay

Trypan blue dye exclusion assay was performed for the bacteria treated with *D. malabarica* seed and flesh extracts according to our previously published protocol with little modifications [23-25]. Briefly, 4 mg of plant extracts was mixed separately with 50 µl of diluted overnight grown culture (OD: 1.3 - 1.4) of the respective bacterial strains (*i.e.*, *E. coli* DH5α, *B. subtilis* RBW, and *S. typhi*) and incubated at 37˚C and 120 rpm for 2 hours. The plant extracts treated bacterial cultures were then mixed with freshly prepared 0.4% trypan blue solution at a ratio of 1:1 and incubated for 15 min at room temperature. Twenty microliter (20 µl) of the mixture was taken on glass slides and imaged for live and dead bacterial cells using a phase contrast microscope (Olympus BX50 Fluorescence Microscope, Olympus, Japan) at 40× magnification. The experiments were performed in triplicate ($n = 3$).

2.4.4. CellTox™ Green Assay

CellTox™ Green assay was accomplished according to our previously published paper [23]. Briefly, overnight grown bacterial culture was diluted with LB broth and the concentration was set to 1×10^7 CFU/ml. 4 mg of the extracts of *D. malabarica* seed and flesh was mixed separately with 100 µl of the respective bacterial cultures. Then 800 µl of fresh LB broth was added to mixtures and the tubes were incubated at 37˚C and 120 rpm for 2 hours. After the incubation, 2 µl of CellTox™ Green reagent was mixed properly with plant extracts treated bacterial culture and incubated for another 30 min at room temperature in the dark. Twenty microliter (20 µl) of the CellTox™ Green labelled bacterial culture was kept aside to observe the dead cells under fluorescence microscope (Olympus BX50 Fluorescence Microscope, Olympus, Japan). The remaining bacterial suspension was used to measure the fluorescence intensity of the dead bacterial cells using Spectrofluorophotometer (SHIMADZU RF-6000, Japan) at 490 nm. Control tubes containing plant extracts, cells, and media were run without CellTox™ Green alongside the treatment groups. All experiments were carried out in triplicate ($n = 3$).

2.4.5. Lipid Peroxidation Assay

The lipid peroxidation (LPO) potential of *D. malabarica* seed and flesh extracts was investigated according to our previously published protocol [23-25]. Briefly, 200 µl of the overnight grown bacterial cultures (OD: 1.4 - 1.6) was mixed separately with 4 mg of each of the plant extracts. After that, 500 µl of 10% trichloroacetic acid (TCA) was added and the mixtures were incubated for 30 min at room temperature before centrifuging at 10,416 ×g for 50 minutes to separate the dead cells and insoluble cellular components. The supernatant containing malondialdehyde was then collected in a fresh tube and mixed with freshly prepared 1 ml of 0.67% thiobarbituric acid (TBA) solution (pre-warmed) and incubated in a hot water bath for 10 minutes to facilitate the formation of malondialdehyde-TBA adduct. After cooling down to room temperature, the absorbance of the malondialdehyde-TBA adduct was measured by UV-vis spectrophotometer (Specord® 205, Analytik Jena, Germany) at 532 nm. The whole experiment was performed

in triplicate ($n = 3$).

2.5. Antifungal Activity Assay

Antifungal activity of *D. malabarica* seed and flesh extracts against *Pestalotiopsis microspora*, *Botrytis cinerea*, *and Fusarium oxysporum* was investigated through poisoned food method [8]. Briefly, autoclaved potato dextrose agar (PDA) medium (60˚C) containing 1 ml of each of the different extracts and lactic acid (as antibacterial agent) were poured onto separate petri dishes. PDA medium with fungicide propiconazole (500 ppm) was used as positive control and PDA medium with 0.9% NaCl was used as negative control. Lactic acid was used as antibacterial agent to prevent fungal-borne bacteria. After solidification, mycelia of the tested fungi were placed on the center of the dishes. The petri dishes were sealed with parafilm to prevent cross contamination with other fungi and incubated at 30˚C ± 2˚C for 72 hours. After incubation, the diameters of fungal growth in control and treated plates were measured. The experiment was performed in triplicate ($n = 3$). The percentage mycelial inhibition (PMI) for all extracts of *D. malabarica* seed and flesh was determined using the following formula:

$$\mathrm{PMI}(\%) = \frac{C - A}{C} \times 100 \tag{1}$$

where, C = mycelial growth in millimeter in negative control plate;

A = mycelial growth in millimeter in plant extract treated plate.

2.6. Hemocompatibility Assay

Hemocompatibiliity assay of *D. malabarica* seed and flesh extracts was performed according to our previously published paper with few modifications [5, 23, 25]. Briefly, fresh blood was collected from healthy human as well as rat in separate tubes containing EDTA (10%). The tubes were centrifuged at 775 ×g for 10 min to separate the RBCs from serum. The serum was discarded, and the RBCs pellet was resuspended in phosphate buffered saline (PBS) and centrifuged again at 775 ×g for 10 min. After discarding the supernatant, the RBCs pellet was washed trice with NaCl (150 mM) solution at 775 ×g for 5 minutes. Finally, Different concentrations of plant extracts (*i.e.*, 1, 2, and 4 mg) and 100 µl of RBCs were mixed and incubated at 37˚C for 30 minutes with gentle shaking. The tubes were then centrifuged at 1377 ×g for 10 min. The supernatant was separated, and the absorbance was measured at 570 nm. RBCs incubated with PBS, and water were used as negative and positive control, respectively. The percentage of hemolysis was calculated using the following formula:

$$\mathrm{Hemolysis}(\%) = \left(\frac{\text{Absorbance of the sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \right) \times 100 \tag{2}$$

To calculate the amount of extracts required to lyse 50% of RBCs (*i.e.*, HC_{50} value), the percentage of hemolysis was plotted against the concentration of extracts to draw a regression line. The concentration of the extracts that brought about 50% hemolysis (*i.e.*, HC_{50} value) was calculated from the following equation:

$$Y = a * X + b \tag{3}$$

Here, Y = 50%, X = the concentration of the extracts at which 50% hemolysis occurred, a = coefficient, and b = constant.

2.7. Inhibition of H_2O_2 Mediated Hemolysis

Erythrocyte hemolysis inhibition assay was performed according to Pareja *et al.* with little modifications [26]. Briefly, 6 ml of blood was collected from a healthy individual and centrifuged at 538 ×g for 10 min. The supernatant was discarded, and the pellet was washed thrice with 0.2 M before resuspending in PBS. The pellet of erythrocytes (200 µl) was diluted with 4 ml of PBS to prepare 5% suspension. Plant extracts was also diluted with PBS. In this experiment, ascorbic acid was used as standard negative control.

Different concentrations of plant extracts (*i.e.*, 0.25, 0.5, 1, 2, and 4 mg) were mixed with H_2O_2 (200 µl; 1 M), and RBC suspension (400 µl) and incubated at 37˚C for 240 min with gentle shaking. After incubation, the tubes were centrifuged at 1377 ×g for 10 min. The supernatant was then separated, and the absorbance was measured at 540 nm. Here, RBCs incubated with only H_2O_2 (1 M) was used as positive control. The percentage of inhibition of H_2O_2 induced hemolysis was calculated using the following formula:

$$\text{Inhibition}(\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of the sample}}{\text{Absorbance of control}}\right) \times 100 \quad (4)$$

The percentage of the inhibition of H_2O_2 induced hemolysis was plotted against the concentration of extracts which provided a regression line. The concentration of the extract that inhibited 50% of H_2O_2 induced hemolysis (*i.e.*, IC_{50} value) was calculated from the following equation:

$$Y = a * X + b \quad (5)$$

Here, Y = 50%, X = the concentration of the extract that inhibits 50% of H_2O_2 induced hemolysis, a = coefficient, and b = constant.

2.8. *In Vivo* Cytotoxicity Assay

2.8.1. Acute Oral Toxicity Testing

Fifteen Wister female rats were divided into 5 groups (n = 3). The rats were housed in a hygienic environment under controlled temperature (23˚C ± 2˚C) and humidity (55% ± 7%) and allowed to acclimatize for a week before oral administration. According to OECD test guideline (up and down procedure), the rats were administered 200 mg/kg (*i.e.*, small dose) and 400 mg/kg (*i.e.*, high dose) of the aqueous seed and aqueous flesh extracts, respectively, of *D. malabarica* prepared in PBS every 24 h for 14 days [27]. The control rat group was administered with the PBS only without plant extracts. Food and water intake, physical appearance, disease occurrence, and body weight were monitored during the period of administration. After 14 days, all the rats were anaesthetized with 0.3 ml/250 gm ketamine/xylazine (100 mg/ml Ketamine + 20 mg/ml xylazine). Blood (~3 ml) was drawn from all the rats and centrifuged at 538 ×g for 10 min at room temperature to separate the serum. The serum samples were stored at −20˚C for further biochemical analysis.

2.8.2. Investigation of Liver and Kidney Function Biomarkers

To determine whether the aqueous extracts of *D. malabarica* seed and flesh (as they have maximum antibacterial activity) have any toxic effects to rat tissues, the concentration of several enzymes whose level usually get hiked once there is any damage in the liver and kidneys were measured. The level of liver function biomarkers (serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (γ-GT)), and kidney function biomarker (creatinine) of the experimental and control rats were quantified according to the manual provided with the commercial reagents by using spectrophotometer (Shimadzu, Kyoto, Japan). Herein, the protocol has been provided in the following sections:

1) Liver function biomarkers

a) Determination of serum alanine transaminases (ALT) activity

The level of serum ALT activity was measured according to previously established protocol [28]. Briefly, 100 µl serum sample was mixed with 1000 µl reagent in a quartz cuvette. The absorbance of the sample and the standard against the reagent blank were measured at 340 nm at different time points (*i.e.*, 1, 2, and 3 min). The change in the absorbance per minute (ΔA/min) was determined from the linear portion of the reaction curve and the concentration of ALT was calculated by using the following formula:

$$\text{ALT activity}\left(\frac{\text{U}}{\text{L}}\right) = \frac{\Delta A}{\text{min}} \times 1375\,(30^\circ\text{C}) \quad (6)$$

Here, ΔA = Average absorbance of the sample in different time points (*i.e.* 1, 2, and 3 minutes).

b) Estimation of serum aspartate transaminase (AST) activity

The level of serum AST activity was measured according to previously established protocol [28, 29]. Briefly, 100 µl serum sample was mixed with 1000 µl reagent in a quartz cuvette. The absorbance of the sample and the standard against the reagent blank were measured in 340 nm at different time points (*i.e.*, 1, 2, and 3 min). The change in absorbance per minute (ΔA/min) was determined from the linear portion of the reaction curve and the AST concentration was calculated by using the following formula:

$$\text{AST activity}\left(\frac{\text{U}}{\text{L}}\right)=\frac{\Delta A}{\text{min}}\times 1750\left(30^{\circ}\text{C}\right) \tag{7}$$

Here, ΔA = Average absorbance of the sample in different time points (*i.e.* 1, 2, and 3 minutes).

c) Measurement of serum γ-glutamyltransferase (γ-GT) activity

The level of serum γ-GT activity was measured according to previously established protocol [30]. Briefly, 50 µl serum sample was mixed with 1000 µl reagent in a quartz cuvette. The absorbance of the sample and the standard against the reagent blank were measured at 405 nm at different time points (*i.e.*, 1, 2, and 3 min). The change in absorbance per minute (ΔA/min) was determined from the linear portion of the reaction curve and the γ-GT concentration was calculated by using the following formula:

$$\gamma\text{-GT activity}\left(\frac{\text{U}}{\text{L}}\right)=\Delta A\ 405\frac{\text{nm}}{\text{min}}\times 400 \tag{8}$$

Here, ΔA = Average absorbance of the sample in different time points (*i.e.*, 1, 2, and 3 min). One international unit (U) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

2) Kidney function biomarker

a) Estimation of serum creatinine concentration

The level of serum creatinine concentration was measured according to previously established protocol [31, 32]. Briefly, 20 µl serum sample or standard was mixed with 200 µl reagent in a quartz cuvette. The absorbance of the sample and the standard against the reagent blank were measured at 510 nm at different time points (*i.e.*, 30 and 120 sec). The concentration of creatinine in the sample was calculated by using software program with the following formula:

$$\begin{aligned}&\text{Creatinine concentration}\left(\frac{\text{mg}}{\text{dl}}\right)\\&=\left\{\left(A2-A1\right)\frac{\text{sample}}{A2-A1}\text{standard}\right\}\times C\ \text{standard}\end{aligned} \tag{9}$$

Here, $A1$ = Absorbance of sample at 30 seconds, $A2$ = Absorbance of sample at 120 seconds, C = Creatinine standard. All experiments were carried out in triplicate and the results were presented in the average measurements with standard deviation.

2.9. Statistical Analysis

The results were presented as the average measurements of the runs ± Standard error mean (SEM). Data were subjected to statistical analysis using one-way analysis of variance (ANOVA) with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). *Post hoc* test was determined by Dunnett test. The mean values were statistically significant at $p < 0.05$ [33]. All the analysis was carried out in triplicate (*i.e.*, $n = 3$).

3. RESULTS AND DISCUSSION

3.1. Antibacterial Activity Assay

3.1.1. Determination of MIC and ZOI

The lowest concentration of an antibacterial agent that inhibits the visible growth of bacteria is

known as minimum inhibitory concentration (MIC). Among all extracts, the lowest MIC value, 2 μg/μl, was obtained for aqueous seed extract against *Bacillus subtilis*. For other extracts, the MIC value was within 2.5 - 5 μg/μl of concentration against the tested bacteria (Table 1 and Figure 1).

Table 1. Zone of inhibition (ZOI) and minimum inhibitory concentration (MIC) of different *D. malabarica* seed and flesh extracts after 2 hours of incubation.

	Plant extracts	Bacterial strains		
		Bacillus subtilis RBW (Gram positive)	*Escherichia coli* DH5α (Gram negative)	*Salmonella typhi* (Gram negative)
Minimum inhibitory concentration (μg/μl)	Aqueous seed	2	2.5	2.5
	Aqueous flesh	3.5	4	4.5
	Ethanolic seed	4	4	4.5
	Ethanolic flesh	4	4	5
Zone of Inhibition (mm) Mean ± SEM	Aqueous seed (4 mg)	12.72 ± 0.19	11.41 ± 0.16	11.42 ± 0.17
	Aqueous flesh (4 mg)	9.74 ± 0.11	10.47 ± 0.4	10.48 ± 0.4
	Ethanolic seed (4 mg)	8.44 ± 0.39	8.25 ± 0.05	8.22 ± 0.06
	Ethanolic flesh (4 mg)	9.10 ± 0.21	8.05 ± 0.17	8.02 ± 0.16
	Negative control (0.9% NaCl)	5.58 ± 0.00	5.56 ± 0.002	5.56 ± 0.00

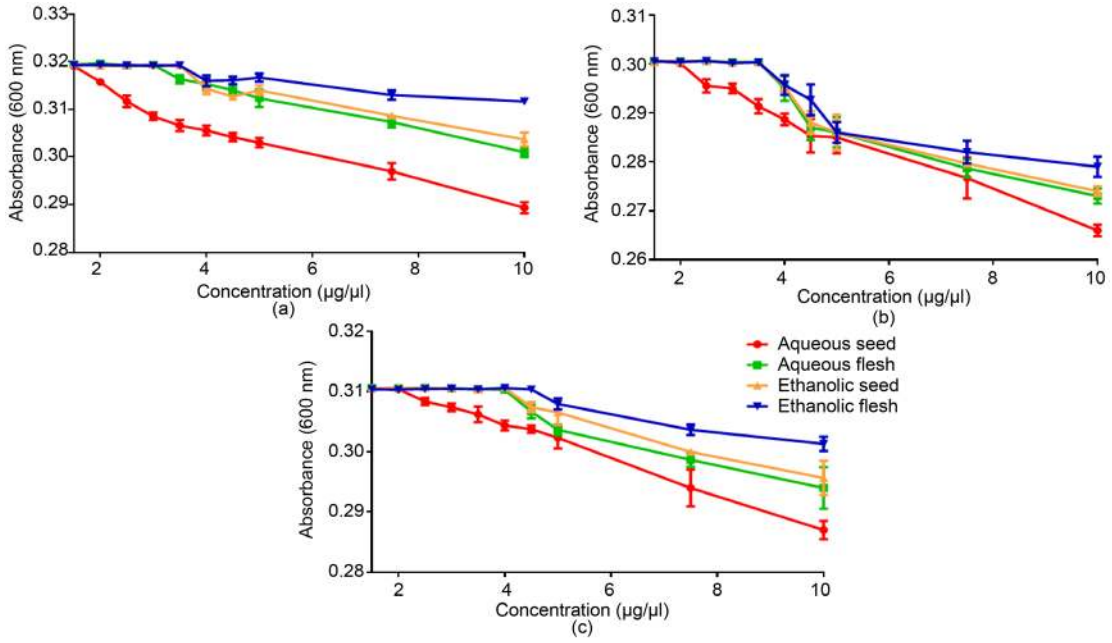

Figure 1. Absorbance of bacterial culture incubated for 2 hours in the presence of different amounts of *D. malabarica* seed and flesh extracts. The figures represent minimum inhibitory concentration (MIC) of *D. malabarica* seed and flesh extracts against *B. subtilis* (a), *E. coli* DH5*α* (b), and *S. typhi* (c). All the values are mean ± SEM of three determinations (*n* = 3).

The antibacterial activity of *D. malabarica* extracts in terms of zone of inhibition (ZOI) was also determined through measuring the diameter (in millimeter) of the clear zone surrounding the metrical filter paper disks at different time points (*i.e.*, 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours) as well as different doses (*i.e.*, 0.2, 1, 2, 3, and 4 mg). The ZOI in the presence of 0.9 % NaCl (*i.e.*, negative control) was also measured at the same time points. The highest ZOI was observed for all the extracts against all the tested bacterial strains after 2 h of the treatment and, thereafter, decreases gradually. More specifically, after 2 hours of incubation, the maximum antibacterial activity was observed for aqueous seed extract (4 mg) against *B. subtilis* (*i.e.*, 12.72 mm), *E. coli* (*i.e.*, 11.41 mm), and *S. typhi* (*i.e.*, 11.42 mm) (**Table 1**). The aqueous flesh, ethanolic seed, and ethanolic flesh extracts showed mediocre ZOI (*i.e.*, 8 - 11 mm) against the tested bacteria (**Table 1**).

Studies on *D. malabarica* plant revealed that different parts of *D. malabarica* are the rich source of phytochemicals [13-15]. Recently, we have reported that *D. malabarica* seed and flesh extracts contain various phytochemicals including phenol, tannin, flavonoid, alkaloid, proteins, reducing sugar, glycosides, terpenoids, quinones and so on [16]. From the previous studies, it was found that these phytochemicals are responsible for preventing bacterial infection through different physiological functions [34]. For example: plant polyphenols, tannin, and flavonoids prevent bacteria through binding to adhesins, enzyme inhibition or inactivation, substrate deprivation, complexing with cell wall, membrane disruption, metal ion complexation and so on [12, 34, 35]. Alkaloids intercalate into cell wall and DNA of microbes [34]. Terpenoids, proteins, glycosides, and quinones are responsible for the disruption of membrane through binding with the cell surface adhesins which result in the formation of pores, and inactivation of enzymes [36]. Hence, the presence of these phytochemicals may provide *D. malabarica* extracts with their potent antibacterial activity.

3.1.2. Trypan Blue Dye Exclusion Assay

Trypan blue is a negatively charged dye that can't pass through the intact cell wall of live bacteria. Therefore, it is used to differentiate cell wall compromised dead bacteria from cell wall intact live bacteria [37]. Trypan blue dye exclusion assay was performed to confirm the antibacterial activity of *D. malabarica* fruit extracts against pathogenic as well as nonpathogenic bacteria. As the extracts contain phytochemicals (*i.e.*, phenol, tannin, quinones, glycosides and so on) that are responsible for making complex with cell wall through hydrophobic interaction and membrane disruption [12, 35, 36], they help to create pores on the lipid layer of membrane. As a result, the blue dye gets inside the bacteria treated with *D. malabarica* fruit extracts and appears blue under the phase contrast microscope (**Figures 2-4**) [25].

3.1.3. CellTox™ Green Assay

CellTox™ Green is a fluorescent dye that binds to the DNA of cell wall compromised bacteria and emits green fluorescence. Therefore, it is used to distinguish live and dead bacteria [23, 38]. The fluorescence intensity of CellTox™ Green dye labelled dead bacteria was quantified using Spectrofluorophotometer (SHIMADZU RF-6000, Japan) at 490 nm (**Figure 5**) and the dead cells were also observed under a fluorescence microscope (Olympus BX50 Fluorescence Microscope, Olympus, Japan) (**Figure 6**). The fluorescence intensity of aqueous seed extract treated *B. subtilis*, *E. coli*, and *S. typhi* was significantly higher than that of the other extracts (**Figure 5**). Furthermore, the fluorescence intensity of aqueous seed extract treated *B. subtilis* and *S. typhi* was ~6 and ~8 folds, respectively, higher than that of the control (*i.e.*, untreated bacteria).

3.1.4. Lipid Peroxidation (LPO) Assay

LPO assay was performed to determine the oxidation ability of *D. malabarica* seed and flesh extracts to bacterial cell membrane fatty acids [23, 25]. As the oxidation of fatty acids brings about the disruption of cell membrane integrity, it can be an effective way to kill the bacteria [23, 24]. Hence, the cell membrane fatty acid oxidation potential of the *D. malabarica* extracts was investigated through measuring the absorbance of malondialdehyde-thiobarbituric acid (MDA-TBA) pink adduct at 532 nm (**Figure 7**). The higher

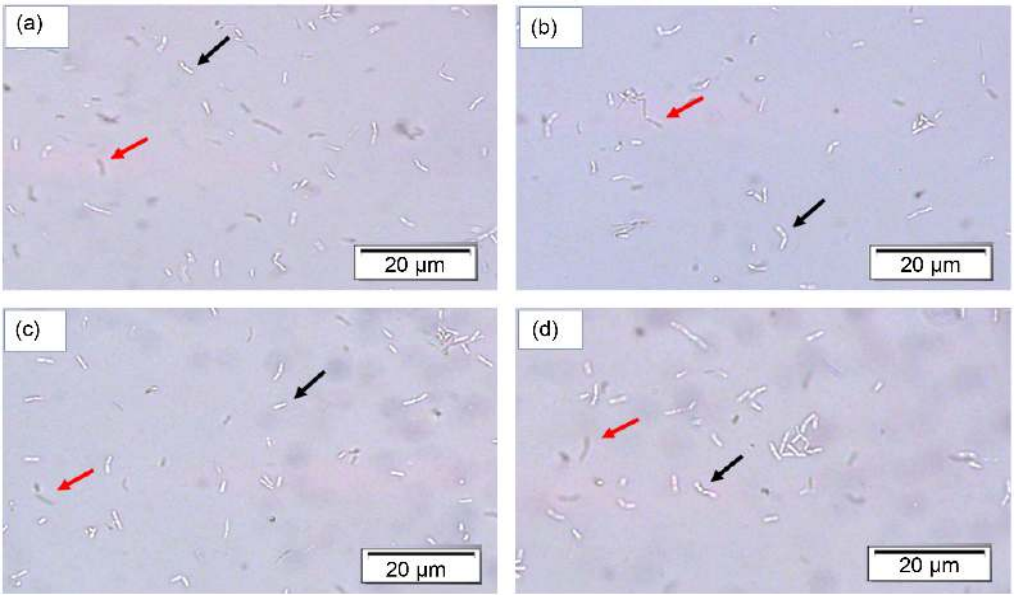

Figure 2. Trypan blue dye exclusion assay to investigate the bactericidal effect of *D. malabarica* extracts against *B. subtilis* using aqueous extract of seed (a), aqueous extract of flesh (b), ethanolic extract of seed (c), and ethanolic extract of flesh (d). Dead and live cells have been indicated with red and black colored arrows, respectively. The images were taken using a Phase Contrast Light Microscope at (40 × 1.25)× magnification.

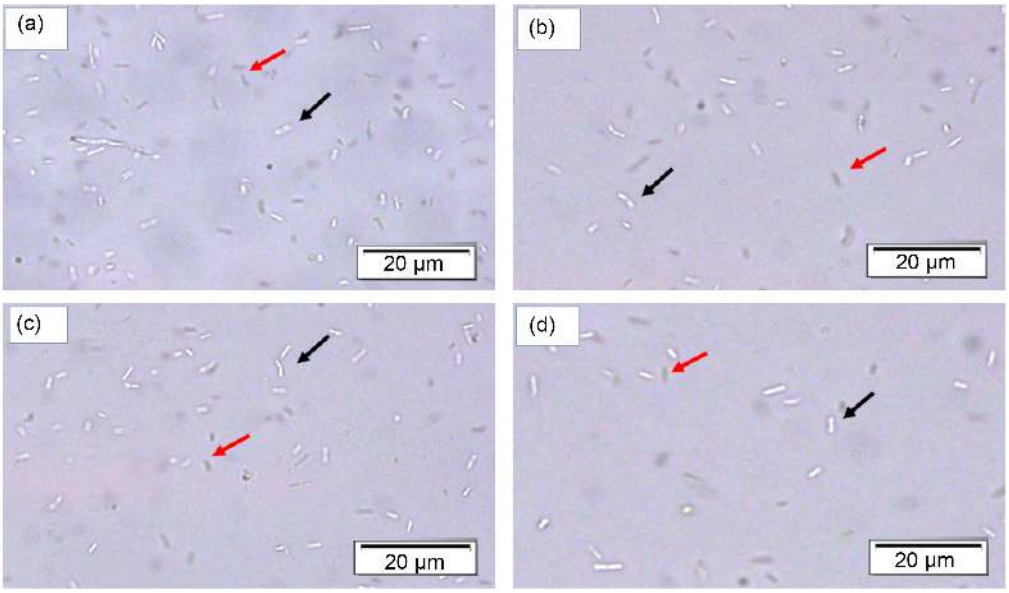

Figure 3. Trypan blue dye exclusion assay to investigate the bactericidal effect of *D. malabarica* extracts against *E. coli* DH5α using aqueous extract of seed (a), aqueous extract of flesh (b), ethanolic extract of seed (c), and ethanolic extract of flesh (d). Dead and live cells have been indicated with red and black colored arrows, respectively. The images were taken using a Phase Contrast Light Microscope at (40 × 1.25)× magnification.

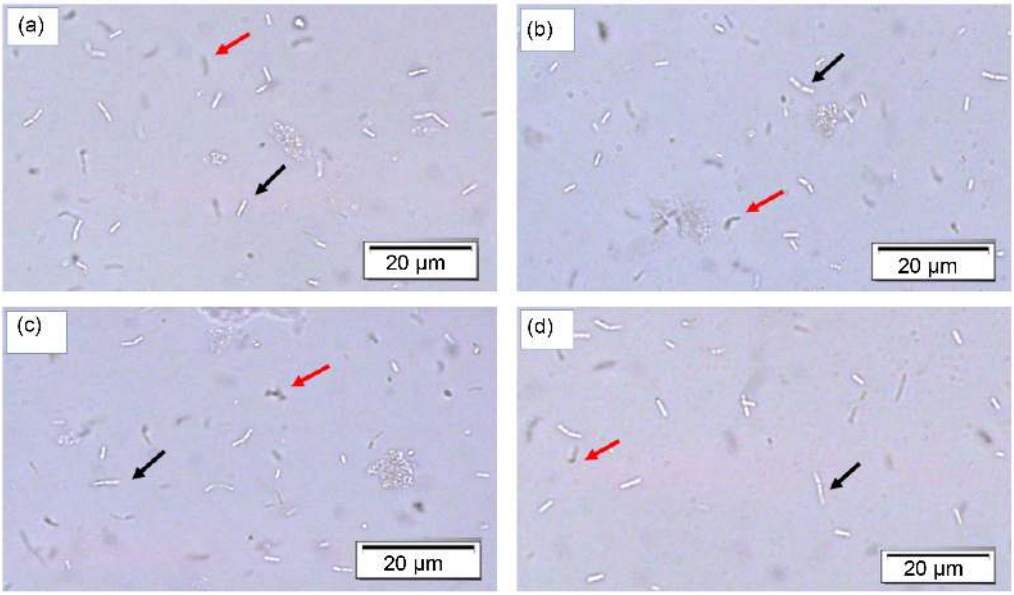

Figure 4. Trypan blue dye exclusion assay to investigate the bactericidal effect of *D. malabarica* extracts against *S. typhi* using aqueous extract of seed (a), aqueous extract of flesh (b), ethanolic extract of seed (c), and ethanolic extract of flesh (d). Dead and live cells have been indicated with red and black colored arrows, respectively. The images were taken using a Phase Contrast Light Microscope at (40 × 1.25)× magnification.

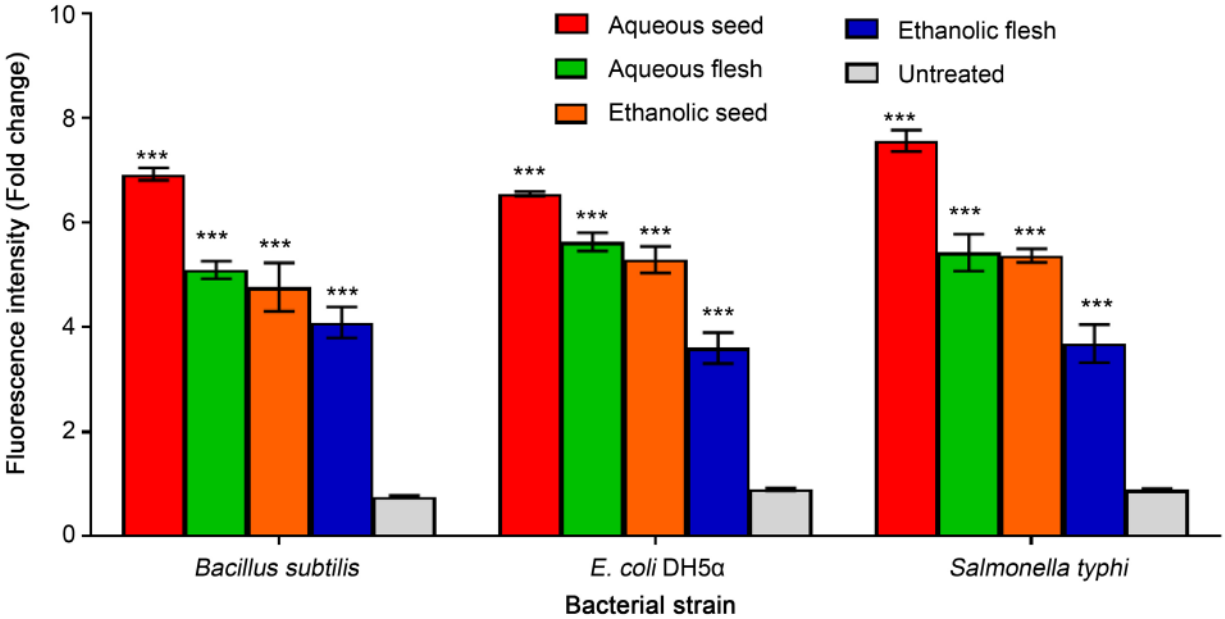

Figure 5. Cell Tox™ Green uptake assay. The fluorescence intensity of bacterial strains (*i.e.*, *B. subtilis*, *E. coli* DH5*α*, and *S. typhi*) treated with of *D. malabarica* seed and flesh extracts was measured at 490 nm using a spectrofluorometer. The data have been presented as an average value of three replications ± SEM (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA (Dunnett test) and significance level was determined (***$p < 0.001$).

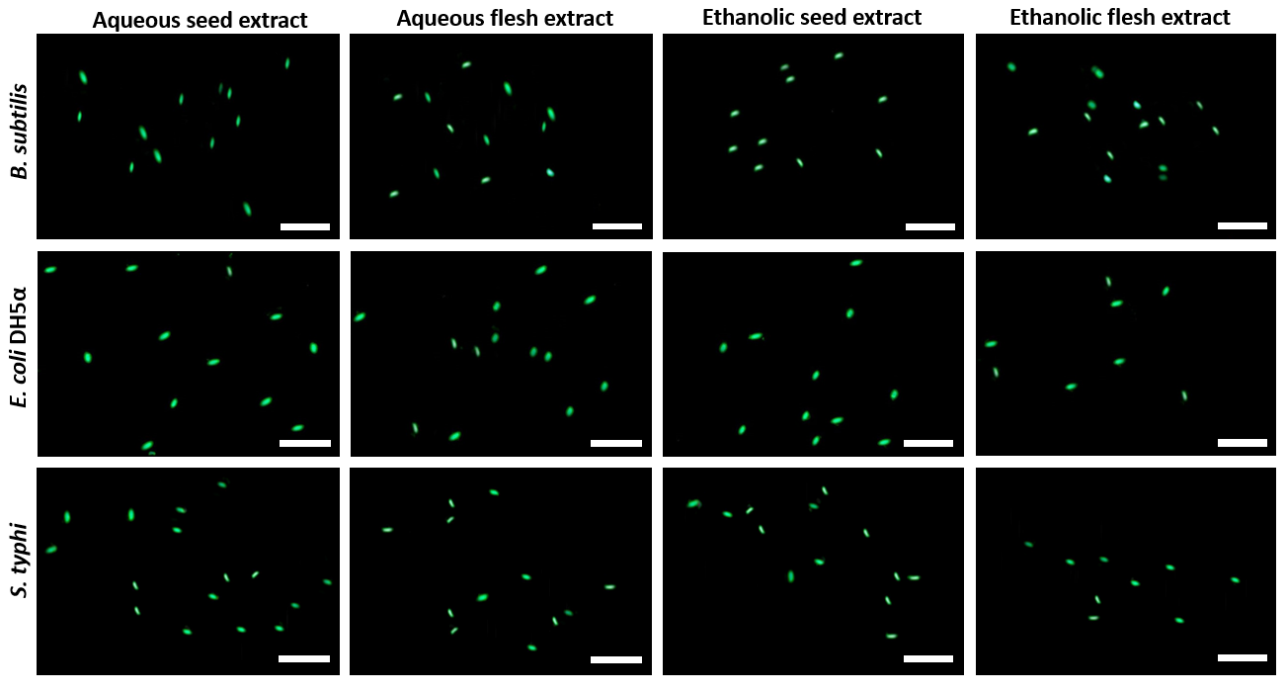

Figure 6. CellTox™ Green assay. *B. subtilis*, *E. coli DH5α*, *and S. typhi* were first treated with aqueous extract of seed, aqueous extract of flesh, ethanolic extract of seed, and ethanolic extract of flesh of *D. malabarica*. The treated bacteria were then incubated with CellTox™ green to stain the cell wall compromised bacterial DNA and green fluorescence was observed under a fluorescence microscope (40×). Scale bar: 20 μm.

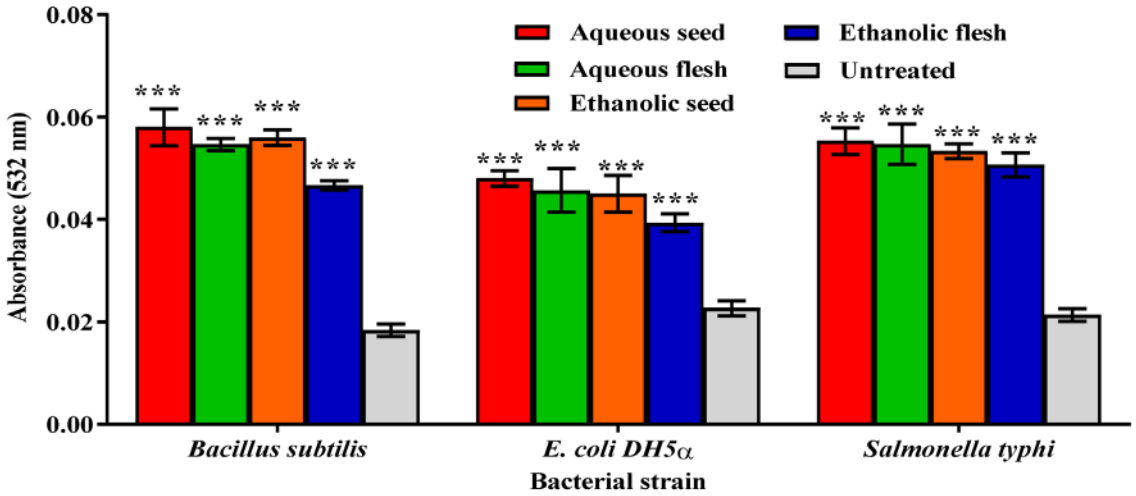

Figure 7. Lipid peroxidation assay of *D. malabarica* seed and flesh extracts against pathogenic (*i.e.*, *S. typhi*) and nonpathogenic (*i.e.*, *B. subtilis* and *E. coli* DH5*α*) bacteria. The cell membrane fatty acid oxidation potential of the plant extracts was measured through malondialdehyde-thiobar-bituric acid (MDA-TBA) adduct assay. The absorbance of MDA-TBA pink adduct was measured at 532 nm (*i.e.*, λmax). The data have been presented as an average value of three replications ± SEM (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA (Dunnett test) and significance level was determined (***$p < 0.001$).

the absorbance, the higher the fatty acid oxidation ability. The aqueous seed extract showed the highest fatty acid oxidation potential against all the tested bacteria and was significantly higher than that of the control (*i.e.*, untreated bacteria) (**Figure 7**). However, these extracts are not effective as fatty acid oxidizers, since *D. malabarica* fruit extracts are rich in natural antioxidants [16]. Previous studies revealed that natural antioxidants might show pro-oxidant effects [39]. Therefore, due to the presence of pro-oxidant influences, *D. malabarica* fruit extracts showed minute amount of fatty acid oxidation potential to bacterial cell membrane fatty acids.

3.2. Antifungal Activity Assay

Antifungal activity of the polar extracts of *D. malabarica* seed and flesh were performed through poison food method against three pathogenic fungus (*i.e.*, *Pestalotiopsis microspore*, *Botrytis cinerea*, and *Fusarium oxysporum*). No fungal growth was observed in PDA medium containing Propiconazole (*i.e.*, positive control) and no inhibition was observed in PDA medium containing 0.9% NaCl (*i.e.*, negative control). The mycelial growth of *P. microspore*, *B. cinerea*, and *F. oxysporum* was 48.66 mm, 59.44 mm, and 15.78 mm, respectively, when grown in PDA medium containing 0.9 % NaCl (**Table 2**). Whereas, in aqueous seed extract, the highest mycelial growth was observed against *P. microspore* (49.16 mm) and in aqueous flesh extract, the highest mycelial growth was obtained against *B. cinerea* (61.33 mm), and *F. oxysporum* (16 mm) (**Table 2**).

Percentage mycelial inhibition (PMI) was calculated to determine the antifungal activity of *D. malabarica* seed and flesh extracts. The higher the value of PMI, the higher the antifungal activity [8]. Aqueous extracts did not show any antifungal activity against *B. cinerea* and *F. oxysporum*. However, the ethanolic seed extract demonstrated the highest antifungal activity (*i.e.*, PMI: 4.55) against *B. cinerea* (**Table 2**). The

Table 2. Mycelial growth and percentage mycelial inhibition (PMI) of *D. malabarica* seed and flesh extracts after 72 hours of incubation.

	Plant extracts	Fungal strains		
		Pestalotiopsis microspora	*Botrytis cinerea*	*Fusarium oxysporum*
Mycelial growth (mm) Mean ± SEM	(-)ve Control (0.9% NaCl)	48.66 ± 0.51	59.44 ± 1.41	15.78 ± 1.11
	Aqueous seed (10 mg)	49.16 ± 0.51	61.33 ± 0.69	15.89 ± 0.68
	Aqueous flesh (10 mg)	48.56 ± 1.06	61.33 ± 1.92	16 ± 0.19
	Ethanolic seed (10 mg)	46.88 ± 1.52	56.73 ± 2.12	15.22 ± 0.91
	Ethanolic flesh (10 mg)	46.79 ± 0.99	57.18 ± 0.4	15.33 ± 0.33
Percentage mycelial inhibition (PMI) (%)	Aqueous seed	No effect	No effect	No effect
	Aqueous flesh	0.23	No effect	No effect
	Ethanolic seed	3.65	4.55	3.52
	Ethanolic flesh	3.84	3.80	2.82

ethanolic flesh extract also showed little antifungal activity against the tested fungi. Previously, it was reported that polar (*i.e.*, methanolic) extract of *D. malabarica* fruit demonstrate antifungal activity at higher concentrations [15]. Therefore, ethanolic extracts of *D. malabarica* could be a natural and effective antifungal agent at higher concentrations.

3.3. Hemocompatibility Assay

Hemolytic potential of different concentrations (*i.e.*, 1 - 4 mg) of *D. malabarica* seed and flesh extracts was performed using human and rat red blood cells (RBCs) to investigate their biocompatibility. All the extracts showed excellent hemocompatibility to human as well as rat RBCs (**Figure 8(a)** and **Figure 8(b)**). At the concentration of 4 mg, the ethanolic seed extract demonstrated the highest hemolytic activity (3.71%) to human RBCs. On the other hand, the aqueous seed extract demonstrated the highest hemolytic activity (4.41%) to rat RBCs. The hemolytic potential of *D. malabarica* fruit extracts was less than the acceptable hemolytic value (*i.e.*, 5%) that is considered as safe for the potential therapeutic applications [23]. The HC_{50} value (the amount required to lyse the 50% of RBCs) for aqueous and ethanolic extracts of *D. malabarica* seed and flesh was ranged from 50 mg to 90 mg which was ~15 times higher than that of the amount used for antibacterial activity assay (**Figure 8(c)**). As the interaction of plant extracts with RBCs provides primary information about their biocompatibility [1], it can be stated that *D. malabarica* seed and flesh extracts are highly biocompatible and could be safe as therapeutics.

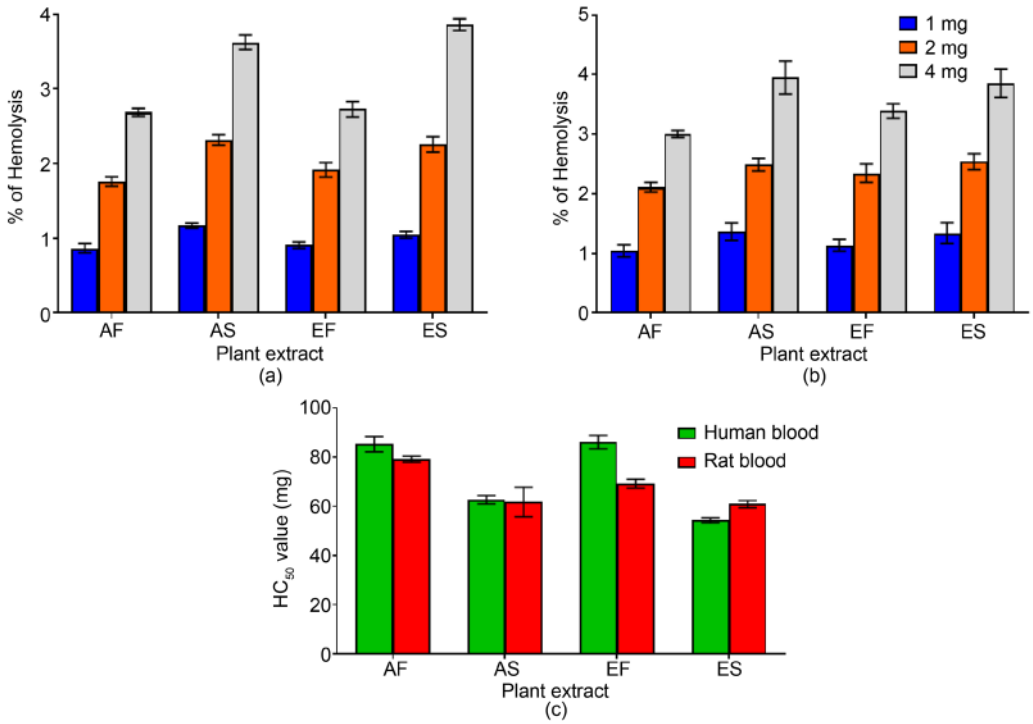

Figure 8. Hemocompatibility of *D. malababarica* seed and flesh extracts to Human (a) and Rat (b) red blood cells (RBCs). Different amounts (*i.e.*, 1 mg, 2 mg, and 4 mg) of *D. malabarica* seed and flesh extracts were taken in the hemocompatibility assay. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, and ES = ethanolic seed extract. (c) The amount of extracts at which 50% hemolysis of RBCs occurred (HC_{50}) was presented and no significant difference was observed between human and rat RBCs. The data have been presented as an average value of different replications ± SEM (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA (Dunnett test) and no significant difference was observed ($p > 0.05$).

3.4. Inhibition of H_2O_2 Mediated Hemolysis

D. malabarica fruit extracts are rich in polyphenolics, flavonoids and vitamin C and are an excellent source of natural antioxidants [16]. Hence, the extracts prevent the generation of reactive oxygen species (ROS) (*i.e.*, superoxide, singlet oxygen, and H_2O_2 etc.) that cause the oxidation of cellular protein, lipid and DNA. **Figure 9** showed the ability of the *D. malabarica* seed and flesh extracts to prevent H_2O_2 induced hemolysis. Furthermore, the extracts can inhibit H_2O_2 induced hemolysis in a dose dependent manner (**Figure 9(a)**). The IC_{50} values of aqueous seed, aqueous flesh, ethanolic seed, and ethanolic flesh extracts were 2.58 mg, 3.27 mg, 2.67 mg, and 2.95 mg, respectively, and the IC_{50} value of ascorbic acid was 1.77 mg (**Figure 9(b)**). The IC_{50} value of aqueous seed, aqueous flesh, ethanolic seed and ethanolic flesh was significant with respect to that of ascorbic acid (**Figure 9(b)**). The lowest IC_{50} value was obtained for aqueous seed extract (*i.e.*, 2.58 mg) and is the most efficient free radical scavenger. Therefore, it can be said that *D. malabarica* fruit extracts are not only hemocompatible but also they can protect blood cells from degradation induced by reactive oxygen species (ROS).

3.5. *In Vivo* Cytotoxicity Assay

The aqueous extracts of *D. malabarica* seed and flesh were administered orally to rats for 14 days to investigate any toxic effects on animal tissues and organs. During this time, the rats were weighed every day and checked properly for any physiological changes (*i.e.*, skin color, salivation, respiration rate and so on) and compared with that of untreated rats (*i.e.*, control). The body weights increased gradually (**Figure 10(a)**), no physiological changes observed and all the experimental rats were alive (**Table 3**). After 14 days, the rats were sacrificed and the organs of the treated (*i.e.*, administered with aqueous extracts of *D. malabarica* seed and flesh) and untreated (*i.e.*, administered with PBS only) rats were weighed. No significant changes was observed in terms of the weight of organs of the treated rats when compared to that of untreated rats (**Table 4** and **Figures 10(b)-(d)**).

To determine the toxic effects of the aqueous extracts of *D. malabarica* seed and flesh on liver and kidney, the level of serum ALT, AST, γ-GT enzymes (*i.e.*, liver function biomarker) and creatinine (*i.e.*, kidney function biomarker) of both the control and experimental rats were measured. Previous studies

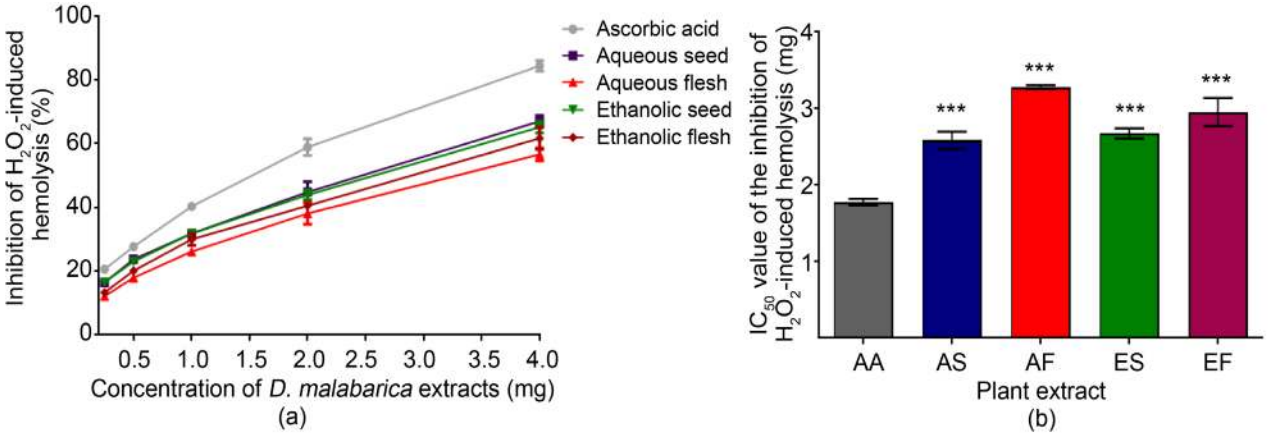

Figure 9. Percentage inhibition of H_2O_2 induced hemolysis by the plant extracts (a) and their IC_{50} values (b). Different amounts (*i.e.*, 0.25 mg, 0.5 mg, 1 mg, 2 mg, and 4mg) of control (*i.e.*, ascorbic acid) and *D. malabarica* seed and flesh extracts were taken in this assay. Here, AA = ascorbic acid, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, and ES = ethanolic seed extract. The data have been presented an average value of different replications ± SEM (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA (Dunnett test) and significance level was determined (*$p < 0.001$).**

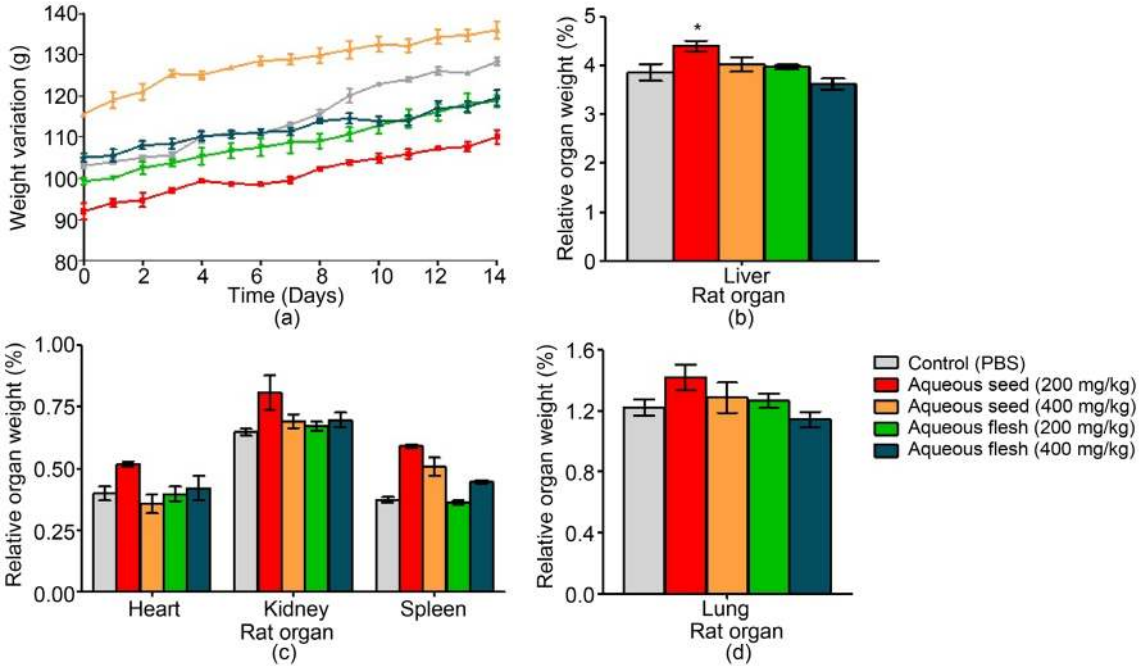

Figure 10. Body weight (a), relative organ weight of liver (b), relative organ weight of heart, kidney, and spleen (c) and relative organ weight of lung (d) of rats after feeding the aqueous extract of *D. malabarica* seed and flesh for 14 days. The values presented are mean ± SEM of multiple samples (3 animals per group). Data were analyzed by GraphPad Prism 5.0 (GraphPad software) using one way ANOVA test. Dunnett test was used for post hoc comparison and significance level was determined (*$p < 0.05$).

Table 3. General appearance and behavioral observations for control and treated groups fed with aqueous extracts of *D. malabarica* seed and flesh.

Parameters	Control	Treatment			
	Only PBS	Aqueous seed (200 mg/kg)	Aqueous seed (400 mg/kg)	Aqueous flesh (200 mg/kg)	Aqueous flesh (400 mg/kg)
Skin and fur	No effect	No effect	No effect	No effect	No effect
Eyes	Normal	Normal	Normal	Normal	Normal
Mucus membrane	Normal	Normal	Normal	Normal	Normal
Behavioral patterns	Normal	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal	Normal
Respiration	Normal	Normal	Normal	Normal	Normal
Urination (color)	Normal	Normal	Normal	Normal	Normal
Feces (color)	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not observed	Not observed	Not observed	Not observed	Not observed
Coma	Not observed	Not observed	Not observed	Not observed	Not observed
Mortality	Alive	Alive	Alive	Alive	Alive

Table 4. Effects of oral administration of aqueous extracts of *D. malabarica* seed and flesh on the average weight (g) of rat organs.

Organ	Average organ weight (g)				
	Treatment				
	Control (PBS)	Aqueous seed (200 mg/kg)	Aqueous seed (400 mg/kg)	Aqueous flesh (200 mg/kg)	Aqueous flesh (400 mg/kg)
Heart	0.51 ± 0.03	0.57 ± 0.01	0.49 ± 0.04	0.47 ± 0.03	0.50 ± 0.05
Kidney	0.83 ± 0.01	0.88 ± 0.06	0.94 ± 0.03	0.80 ± 0.02	0.83 ± 0.03
Liver	4.95 ± 0.17	5.24 ± 0.07	5.46 ± 0.15	4.71 ± 0.05	4.32 ± 0.11
Spleen	0.48 ± 0.01	0.65 ± 0.006	0.69 ± 0.04	0.42 ± 0.009	0.53 ± 0.007
Lung	1.56 ± 0.05	1.56 ± 0.08	1.74 ± 0.11	1.49 ± 0.04	1.36 ± 0.05
Average body weight	128.33 ± 0.72	110 ± 1.41	136 ± 1.69	118.67 ± 1.18	119.5 ± 1.54

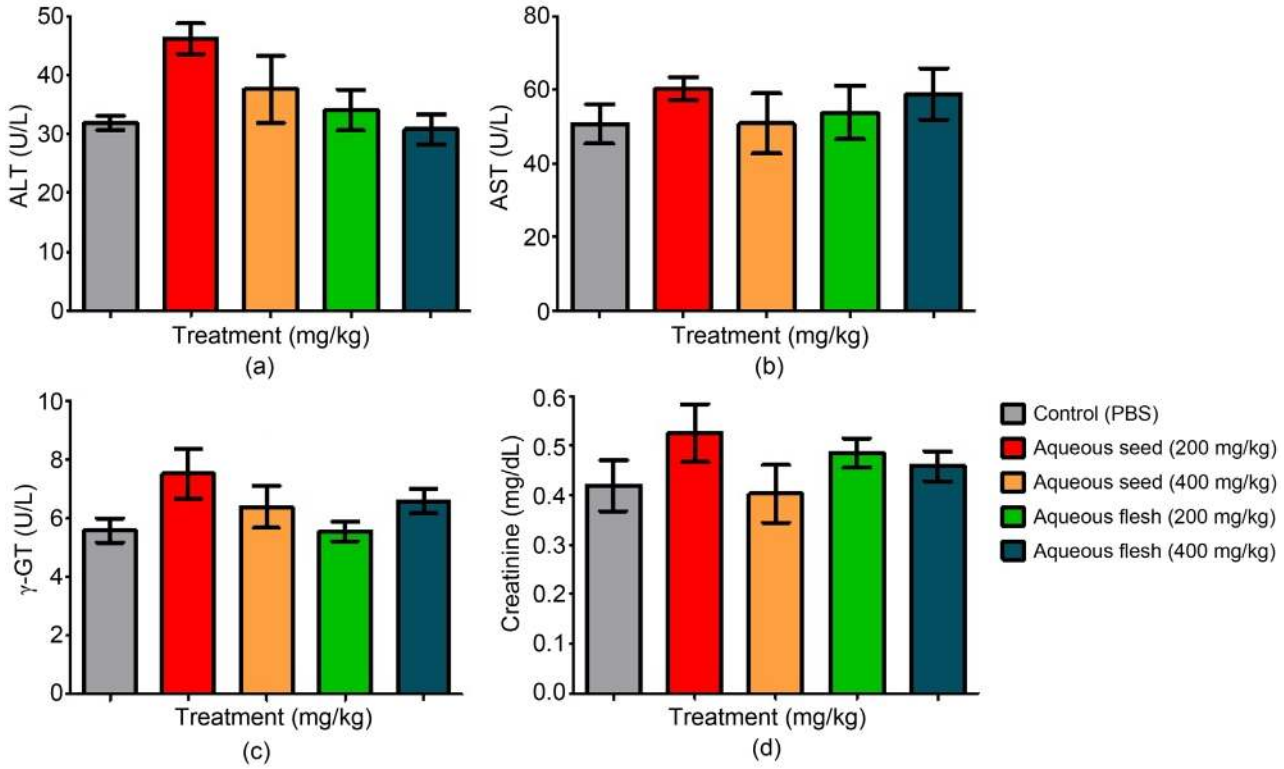

Figure 11. *In vivo* cytotoxicity assay. The effect of aqueous extracts of *D. malabarica* seed and flesh on liver function was investigated by measuring the levels of serum ALT (a), AST (b) and γ-GT (c). One the other hand, the effects of aqueous extracts of *D. malabarica* seed and flesh on kidney functions was investigated by measuring the level of serum creatinine (d). The values presented are mean ± SE of multiple samples (3 animals per group). Data were analyzed by GraphPad Prism 5.0 (GraphPad software) using one way ANOVA test. Dunnett test was used for posthoc comparison. No significant difference was observed ($p > 0.05$).

revealed that the level of ALT, AST, and γ-GT enzymes increased whenever there is any damage in liver due to the decrease of ATP (a high-energy phosphate compound) or any impairment of the bile flow [23, 40, 41]. On the other hand, the increased level of creatinine indicates the inability of kidney to filter the waste product from blood [23, 42]. In this study, no significant differences was found in the level of serum ALT, AST, γ-GT enzymes and creatinine between experimental and control rats (**Figures 11(a)-(d)**). This indicates that *D. malabarica* seed and flesh extracts have no significant toxicity to liver as well as kidney and aqueous extracts of *D. malabarica* seed and flesh are safe for therapeutic applications.

4. CONCLUSION

The antimicrobial activity of *D. malabarica* fruit extracts was investigated in terms of their antibacterial activity as well as antifungal activity. The bactericidal effect of *D. malabarica* extracts was the highest after 2 h of incubation against all the bacterial strains in disk diffusion assay. On the other hand, the ethanolic extract showed slight antifungal activity against the pathogenic fungi. They showed excellent hemocompatibility and did not show any significant toxicity to rat liver and kidneys even at a high dose.

Therefore, it can be concluded that the aqueous and ethanolic extracts of *D. malabarica* fruit have moderate antibacterial propensity and excellent biocompatibility.

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CONFLICTS OF INTEREST

The authors have no conflict of interests.

REFERENCES

1. Kumar, G., Karthik, L. and Rao, K.V.B. (2011) Hemolytic Activity of Indian Medicinal Plants towards Human Erythrocytes: An *in Vitro* Study. *Elixir Applied Botany*, **40**, 5534-5537. https://www.elixirpublishers.com/articles/1350647393_40%20(2011)%205534-5537.pdf
2. Karimi, A., Majlesi, M. and Rafieian-Kopaei, M. (2015) Herbal versus Synthetic Drugs; Beliefs and Facts. *Journal of Nephropharmacology*, **4**, 27. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5297475
3. Mahady, G.B. (2005) Medicinal Plants for the Prevention and Treatment of Bacterial Infections. *Current Pharmaceutical Design*, **11**, 2405-2427. https://doi.org/10.2174/1381612054367481
4. Wadud, A., Prasad, P.V., Rao, M.M. and Narayana, A. (2007) Evolution of Drug: A Historical Perspective. *Bulletin of the Indian Institute of History of Medicine* (*Hyderabad*), **37**, 69-80. http://ccras.nic.in/sites/default/files/viewpdf/jimh/BIIHM_2007/69%20to%2080.pdf
5. Li, X., *et al.* (2014) Functional Gold Nanoparticles as Potent Antimicrobial Agents against Multi-Drug-Resistant Bacteria. *ACS Nano*, **8**, 10682-10686. https://doi.org/10.1021/nn5042625
6. Boxall, A.B. (2004) The Environmental Side Effects of Medication. *EMBO Reports*, **5**, 1110-1116. https://doi.org/10.1038/sj.embor.7400307
7. Polash, S.A., Hossain, M.M., Saha, T. and Sarker, S.R. (2021) Biogenic Silver Nanoparticles: A Potent Therapeutic Agent. In: *Emerging Trends in Nanomedicine*, Springer, Berlin, 81-127. https://doi.org/10.1007/978-981-15-9920-0_4
8. Mohana, D.C. and Raveesha, K.A. (2007) Anti-Fungal Evaluation of Some Plant Extracts against Some Plant

Pathogenic Field and Storage Fungi. *Journal of Agricultural Technology*, **4**, 119-137. http://www.ijat-aatsea.com/pdf/june_v4_n1_08/ijat2008_11_raveesha.pdf

9. Rahman, M.H. and Uddin, M.J. (2017) BLAST: A Threat to Cereal Crops in Bangladesh. *Barisal University Journal Part* 1, **4**, 237-260. http://bu.ac.bd/uploads/BUJ1V4I2/2.%20Hasinur%20Rahman.pdf

10. Harris, C.A., Renfrew, M.J. and Woolridge, M.W. (2001) Assessing the Risks of Pesticide Residues to Consumers: Recent and Future Developments. *Food Additives & Contaminants*, **18**, 1124-1129. https://doi.org/10.1080/02652030110050122

11. Saleem, U., *et al.* (2017) Acute oral Toxicity Evaluation of Aqueous Ethanolic Extract of *Saccharum munja* Roxb. Roots in Albino Mice as per OECD 425 TG. *Toxicology Reports*, **4**, 580-585. https://doi.org/10.1016/j.toxrep.2017.10.005

12. Koley, H., Howlader, D.R. and Bhaumik, U. (2019) Assessment of Antimicrobial Activity of Different Phytochemicals against Enteric Diseases in Different Animal Models. In: *New Look to Phytomedicine*, Academic Press, Cambridge, 563-580. https://doi.org/10.1016/B978-0-12-814619-4.00022-7

13. Gratiana, E., Tenggara, N. and Jøker, D. (2004) *Diospyros malabarica* (Desr.) Kostel. *Seed Leaflet*, (105). https://curis.ku.dk/ws/files/20546962/105_diospyros_int.pdf

14. Ravikumar, A., Vengalrao, P., Shobhana, K. and Kishore, S. (2014) An Overview on *Diospyros malabarica*. *IJNTPS*, **4**, 93-96. http://www.ijntps.com/sites/default/files/IntJNTPharmSci-4-5-93.pdf

15. Kaushik, V., Saini, V., Pandurangan, A., Khosa, R.L. and Parcha, V. (2012) A Review of Phytochemical and Biological Studies of *Diospyros malabarica*. *International Journal of Pharmaceutical Sciences Letters*, **2**, 167-169. https://www.researchgate.net/profile/Dr_Pandurangan/publication/263657168_A_review_of_Phytochemical_and_biological_studies_of_Diospyros_malabarica/links/55f39ba308ae1d980394a4b6.pdf

16. Shubhra, R.D., *et al.* (2019) Investigation of the Phytoconstituents and Antioxidant Activity of *Diospyros malabarica* Fruit Extracts. *Advances in Bioscience and Biotechnology*, **10**, 431-454. https://doi.org/10.4236/abb.2019.1012031

17. Mondal, R. (2017) Investigation of the Phytoconstituents and Bioactivityof Various Parts of Wild Type and Cultivated *Phyllanthus emblica* L. *Advances in Bioscience and Biotechnology*, **8**, 211-227. http://www.scirp.org/journal/PaperInformation.aspx?paperID=77426 https://doi.org/10.4236/abb.2017.87016

18. Polash, S.A., Saha, T., Hossain, M.S. and Sarker, S.R. (2017) Investigation of thePhytochemicals, Antioxidant, and Antimicrobial Activity of the *Andrographis paniculata* Leaf and Stem Extracts. *Advances in Bioscience and Biotechnology*, **8**, 149-162. https://file.scirp.org/pdf/ABB_2017052315481908.pdf https://doi.org/10.4236/abb.2017.85012

19. Polash, S.A., Saha, T., Hossain, M.S. and Sarker, S.R. (2017) Phytochemical Contents, Antioxidant and Antibacterial Activity of the Ethanolic Extracts of Centellaasiatica (L.) Urb. Leaf and Stem. *Jahangirnagar University Journal of Biological Sciences*, **6**, 51-57. https://doi.org/10.3329/jujbs.v6i1.33731

20. Azwanida, N.N. (2015) A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Medicinal and Aromatic Plants*, **4**, 196.

21. Saha, T., *et al.* (2021) Nanodiamond-Chitosan Functionalized Hernia Mesh for Biocompatibility and Antimicrobial Activity. *Journal of Biomedical Materials Research Part A*. https://doi.org/10.1002/jbm.a.37237

22. Mizielińska, M., Salachna, P., Ordon, M. and Łopusiewicz, Ł. (2017) Antimicrobial Activity of Water and Acetone Extracts of Some *Eucomis taxa*. *Asian Pacific Journal of Tropical Medicine*, **10**, 892-895. https://doi.org/10.1016/j.apjtm.2017.08.018

23. Hossain, M.M., *et al.* (2019) Investigation of the Antibacterial Activity and *in Vivo* Cytotoxicity of Biogenic Sil-

ver Nanoparticles as Potent Therapeutics. *Frontiers in Bioengineering and Biotechnology*, **7**, 239. https://doi.org/10.3389/fbioe.2019.00239

24. Niloy, M.S., *et al.* (2020) Synthesis of Biogenic Silver Nanoparticles Using *Caesalpinia digyna* and Investigation of Their Antimicrobial Activity and *in Vivo* Biocompatibility. *ACS Applied BioMaterials*, **3**, 7722-7733. https://doi.org/10.1021/acsabm.0c00926
25. Ranjan Sarker, S., *et al.* (2019) Functionalization of Elongated Tetrahexahedral Au Nanoparticles and Their Antimicrobial Activity Assay. *ACS Applied Materials & Interfaces*, **11**, 13450-13459. https://doi.org/10.1021/acsami.9b02279
26. Pareja, E., *et al.* (2017) Inhibition of Erythrocyte Hemolysis Induced by H_2O_2 with *Mangifera indica* L. Extract. *VII Latin American Congress on Biomedical Engineering CLAIB* 2016, Bucaramanga, 26-28 October 2016, 457-460. https://link.springer.com/chapter/10.1007/978-981-10-4086-3_115 https://doi.org/10.1007/978-981-10-4086-3_115
27. OECD (2008) Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris.
28. Schumann, G. and Klauke, R.J. (2003) New IFCC Reference Procedures for the Determination of Catalytic Activity Concentrations of Five Enzymes in Serum: Preliminary Upper Reference Limits Obtained in Hospitalized Subjects. *Clinica Chimica Acta*, **327**, 69-79. https://doi.org/10.1016/S0009-8981(02)00341-8
29. Bergmeyer, H., Scheibe, P. and Wahlefeld, A. (1978) Optimization of Methods for Aspartate Aminotransferase and Alanine Aminotransferase. *Clinical Chemistry*, **24**, 58-73. https://doi.org/10.1093/clinchem/24.1.58
30. Szasz, G. (1969) A Kinetic Photometric Method for Serum γ-Glutamyl transpeptidase. *Clinical Chemistry*, **15**, 124-136. https://doi.org/10.1093/clinchem/15.2.124
31. Bowers, L.D. and Wong, E.T. (1980) Kinetic Serum Creatinine Assays. II. A Critical Evaluation and Review. *Clinical Chemistry*, **26**, 555-561. https://doi.org/10.1093/clinchem/26.5.555
32. Fabiny, D.L. and Ertingshausen, G. (1971) Automated Reaction-Rate Method for Determination of Serum Creatinine with the CentrifiChem. *Clinical Chemistry*, **17**, 696-700. https://doi.org/10.1093/clinchem/17.8.696
33. Adeyemi, O.S. and Adewumi, I. (2014) Biochemical Evaluation of Silver Nanoparticles in Wistar Rats. *International Scholarly Research Notices*, **2014**, Article ID: 196091. https://doi.org/10.1155/2014/196091
34. Mazid, M., Khan, T.A. and Mohammad, F. (2011) Role of Secondary Metabolites in Defense Mechanisms of Plants. *Biology and Medicine*, **3**, 232-249. https://www.longdom.org/open-access/role-of-secondary-metabolites-in-defense-mechanisms-of-plants-0974-8369-3-128.pdf
35. Das, K., Tiwari, R.K.S. and Shrivastava, D.K. (2010) Techniques for Evaluation of Medicinal Plant Products as Antimicrobial Agent: Current Methods and Future Trends. *Journal of Medicinal Plants Research*, **4**, 104-111. https://academicjournals.org/journal/JMPR/article-full-text-pdf/7B1A27415011
36. Wallace, R.J. (2004) Antimicrobial Properties of Plant Secondary Metabolites. *Proceedings of the Nutrition Society*, **63**, 621-629. https://doi.org/10.1079/PNS2004393
37. Tran, S.L., Puhar, A., Ngo-Camus, M. and Ramarao, N. (2011) Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of *Bacillus cereus*. *PLoS ONE*, **6**, e22876. https://doi.org/10.1371/journal.pone.0022876
38. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High throughput Screening Assays. *Journal of Biomolecular Screening*, **4**, 67-73. https://doi.org/10.1177/108705719900400206
39. Özen, B.Ö. and Soyer, A. (2018) Effect of Plant Extracts on Lipid and Protein Oxidation of Mackerel (*Scomber*

scombrus) Mince during Frozen Storage. *Journal of Food Science and Technology*, **55**, 120-127. https://doi.org/10.1007/s13197-017-2847-6

40. Gupta, R.C. and Goad, J.T. (2000) Role of High-Energy Phosphates and Their Metabolites in Protection of Carbofuran-Induced Biochemical Changes in Diaphragm Muscle by Memantine. *Archives of Toxicology*, **74**, 13-20. https://doi.org/10.1007/s002040050646
41. Ramaiah, S.K. (2007) A Toxicologist Guide to the Diagnostic Interpretation of Hepatic Biochemical Parameters. *Food and Chemical Toxicology*, **45**, 1551-1557. https://doi.org/10.1016/j.fct.2007.06.007
42. Kaur, B., Khera, A. and Sandhir, R. (2012) Attenuation of Cellular Antioxidant Defense Mechanisms in Kidney of Rats Intoxicated with Carbofuran. *Journal of Biochemical and Molecular Toxicology*, **26**, 393-398. https://doi.org/10.1002/jbt.21433