Safety evaluation of enriched fraction from leaves of *Dillenia indica* L. in BALB/c mice

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A B S T R A C T

The enriched fraction derived from *Dillenia indica* L. (*Dilleniaceae*), also known as elephant apple was subjected to acute and sub-acute toxicological study to document its safety issues for use as fumigant. The enriched fractions were orally administered to both sexes of BALB/c mice at doses of 200, 800 and 1600 mg/kg bw for acute toxicity, and 50 and 500 mg/kg bw for 14 days of sub-acute toxicity. Experimental results revealed that there were no signs of adverse toxicity, and mortality, with no significant treatment related effect in the percentage weight gain, daily feed and water intake, and haematological parameters. However, at higher dose in sub-acute toxicity study a patch of mild tubular injuries in kidney of female mice were observed as suggested by histopathological studies and mild abnormalities in levels of serum biochemical parameters. In general, it can be considered that the enriched fraction from *D. indica* leaves on oral feeding does not show any adverse effect on mice of both sexes. Hence, the highest doses 1600 mg/kg bw (acute) and 500 mg/kg bw (sub-acute) can be used as basal dose for the determination of no-observed-adverse-effect level (NOAEL) of enriched fraction from *D. indica* to calculate its safety margin.

1. Introduction

The loss of stored food grains and pulses due to insect infestation is a major problem all over the world, especially in the tropical and sub-tropical regions, where there is already scarcity of food [1,2]. In the earlier period farmers used indigenous traditional knowledges to protect stored product commodities from insect damage. These knowledges include the development of several conventional structures for food grain storage as well as the use of various plant parts or plant extracts as natural insecticides [3]. But with the advent of the chemical era, synthetic insecticides are commonly used as they provide better efficiency and reliability as compared to traditional approaches [4]. However, chemical based synthetic insecticides also possessed several unwanted attributes such as toxicity to humans, livestock and other non-target organisms, ozone depletion, product adulteration, as well as development of resistance [5,6]. These led us to revisit the time before the chemical era to find an alternative approach that could effectively scale down the use of synthetic insecticides. Recently, there is a paradigm shift of interest from chemical based synthetic insecticides to those of bioactive compounds from natural sources like phytochemicals, essential oils, and extracts, since they are rich in insecticidal activity [7,8].

Plant derived botanicals have the potential to effectively control the damage inflicted by stored grain insects without showing any human and environmental hazard [9,10]. However, evaluation of any toxicological effect due to plant derived botanicals intended to be used in human or animal feeds must be assessed before recommendation for clinical use. In view of the recent global trend towards the revival of interest in the traditional system of insecticides, screening of plant derived products for their mammalian safety is of great importance. Only a handful of the several plants derived products have been evaluated for their toxicity and safety viz; Decaleside, [11] azadirachtin [12], linalool, nicotine [13], pyrethrin [14].

*Dillenia indica* L. (*Dilleniaceae*), or elephant apple, is an evergreen perennial middle size tree native to South-Eastern Asia. This plant has...
several important biological activities including insecticidal properties [15,16]. They are known to have antidiabetic, antioxidant activity, anti-inflammatory, as well as anticancer properties [16]. Some literature reported that the spreading of *D. indica* leaves over the stored rice repelled rice weevil, *Sitophilus oryzae* (L.) (Coleoptera) [17]. However, literature survey indicates no reports on the evaluation of toxicity of *D. indica* leaves extracts and enriched fraction on animal model. Therefore, the present study analyses the safety assessment of bioactive enriched fraction isolated from *D. indica* leaves through acute and sub-acute toxicity test on both male and female BALB/c mice.

2. Materials and method

2.1. Plant material and the enriched fraction preparation

Fresh and matured leaves of *D. indica* were collected from Imphal West, Manipur. They were properly washed and semi-dried in shade for 4–5 days. The samples were finely powdered using an electric grinder and packed in air-tight poly bags for further use.

One kilogram of the powdered leaves samples was used for sequential extraction of phytochemicals. Phytochemicals were extracted using different solvents in sequential manner, based on their polarity, in Soxhlet apparatus for 8-9 h. The solvent extracts were filtered and evaporated using rotary vacuum evaporator (Rotavapor R100 (Buchi) Switzerland) under low pressure, at temperature 45 °C. Fumigation bioassay guided separation and isolation of the bioactive enriched fraction was performed using chromatographic techniques such as column chromatography and flash chromatography. In the column chromatography, the extract was eluted with different solvent of varying polarity and at different solvent ratio. Eluted fractions showing the highest fumigant activity were pooled and subjected to Flash chromatography (CombiFlashRF+ Lumen, Teledyne ISCO, USA) with solvent system hexane and ethyl acetate and 0.5% methanol as modifier, for further separation of bioactive compounds (Supplementary Fig. 1). The eluted sub-fractions were again tested for fumigation activity against the test insects. The most active enriched fraction based on corrected mortality was collected and used for further experimental purposes.

2.2. Fumigation toxicity test

Fumigant toxicity assays were performed following the methodology from Rajashekar et al. [10]. The assay was performed on the stored grain pests, Rice weevil, *Sitophilus oryzae* (L.) (Coleoptera). One litre volume glass desiccators were adapted to perform as fumigation chambers. In the chambers twenty adults of both sexes of *S. oryzae* were separately released. Filter papers were placed inside the chambers avoiding the direct contact with the insects. Using a Hamilton syringe, leaves extracts of different solvents were infused on the filter paper through rubber septum fitted to the chamber’s lid. Equal volume of pure acetone was also infused on separate chamber for solvent control. After 24 h, the number of dead insects inside the chamber was determined. The percentage of corrected mortality was calculated using the Abbott formula equation (Abbott, 1925). Fumigation assay was performed at every separation step till it reached the most active fraction. Dose response study for the enriched fraction was also perform with concentration ranging from 50 to 400 µg/L against *S. oryzae*.

2.3. Animals

Both sexes of BALB/c mice of 6–8 weeks old and weight range of 15–20 g were taken for the toxicity study from in-house colony of Institute of Life Sciences, Bhubaneswar experimental animal facility. Animals were maintained in compatible groups in individually ventilated cages (Citizen Industries, Ahmedabad, India) with corncob bedding, wood shredding nesting material and fed with standard laboratory rodent diet (VRK Nutritional Solution, India) and water ad libitum. The animal rearing room was well equipped with a heating, ventilation, and air-conditioning (HVAC) system. A constant 22 ± 2°C temperature and 50–70% relative humidity was maintained. The room air changes per hour were 16–20. The 12 h cycle of light and dark were maintained in a noise free environment. The mice were handled and used for experiments in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines after obtaining ethical approval from Institutional Animal Ethics Committee of Institute of Life Sciences, Bhubaneswar (ILS/IAEC-223-AH/APR-21). The mice were free of vital, bacterial and parasitic pathogens such as epizootic diarrhoea of infant mice, mouse hepatitis virus, *Clostridium piliforme*, entremelria virus, lymphocytic choriomeningitis virus, hantaan virus, murine cytomegalovirus, mycoplasma pulmonis, mouse parvovirus, respiratory enteric orphan virus, sendai virus, Theiler’s murine encephalomyelitis virus, Pinworm (*Syphasia obvelata*, *S. muris*), ecto-parasite (*Mycopes musculinis*, *Polyplax spp.*) etc.

2.4. Acute toxicity test

Acute toxicity test was performed orally using female BALB/c mice of 6–8 weeks old. The study was performed in accordance with the OECD (Organization for Economic Co-operation and Development) guideline 423. A total of 16 mice were chosen and randomly divided into 4 groups, each containing 4 mice. Each group, respectively, received a single dose of 1600, 800 and 200 mg/kg body weight of the enriched fraction and whereas the control group was treated with normal drinking water. The mice were kept in fasting condition for 6 h and aqueous enriched fraction of *D. indica* was orally administered to the mice with the use of 18 gauze bent oral gavaging needles at the rate of 10 ml/kg body weight. All the mice received the same feed and water and kept under similar environmental conditions. The animals were observed carefully for the first 4 h and thereafter twice daily (every day at 8.00 am and 4.00 pm) for a 7-day study period. The study parameters were observation for mortality, signs of toxicity and toxicity related behavioural changes. The body weight was measured on 1st day, 4th day, and 7th day, while feed weight and volume of water were recorded on 1st day, 3rd day, 5th day, and 7th day of experiment. The feed consumption, water consumption and percentage body weight change were recorded.

On the 8th day, the mice were weighed and euthanized by overdose of isoflurane inhalation anaesthesia. Collection of blood was done by cardiocentesis for haematological and blood biochemical assay. The vital organs like heart, liver, kidney, and spleen were collected for calculating relative organ weight.

2.5. Sub-acute toxicity test

Sub-acute toxicity study was conducted orally as per the OECD guideline 407 by using 15 mice each of both sexes of 6–8 weeks old. The mice of each sex were randomly grouped into 3 groups, each group having 5 mice. The first two groups of each sex, respectively, received a dose of 50, 500 mg/kg body weight of the enriched fraction. While the 3rd control group was treated with normal drinking water.

The aqueous enriched fraction of *D. indica* was orally administered to the mice with the use of 18 gauze bent oral gavaging needles at the rate of 10 ml/kg body weight. All the mice were gavaged orally every day for 14 days and received the same feed and water and kept under similar environmental conditions. The different concentration of enriched fraction was prepared daily just before administration. The animals were observed carefully for the first 4 h and thereafter twice daily (every day at 8.00 am and 4.00 pm) for a 14 -day study period. The study parameters were observation for mortality, signs of toxicity and toxicity related behavioural changes. The body weight was measured on 1st day, 7th day, and 14th day, while feed weight and volume of water were recorded on 1st day, 4th day, 7th day, and 14th day of experiment. The feed consumption, water consumption and percentage body weight exchange were recorded. On the 15thday, the mice were
weighed and euthanized by overdose of isoflurane inhalation anaesthesia. Collection of blood was done by cardiocentesis for haematological and blood biochemical assay. The organs like heart, liver, kidney, uterus for female and testes for male were collected for gross and histopathological analysis and organ weight.

2.6. Haematological parameters

Blood samples were collected in tubes containing EDTA for the haematological analysis. The analysis was performed using Exigo haematology analyzer. Blood parameters studied were White blood cell (WBC), Lymphocyte (LYM), Monocyte (MONO), Granulocyte (GRAN), Haemoglobin (HGB), Haematocrit (HCT), Red blood cell (RBC), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RDW), and Mean platelet volume (MPV).

2.7. Biochemical assay

Biochemical analysis of the serum was performed using a semi-automated biochemistry analyzer (Merilyzer, CliniQuant). The solidified blood samples were centrifuged at 3000 rpm in 4 °C for 10 min to extract sera. Serum biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine (CRE), were measured using the kits provided by the CliniQuant.

2.8. Organ weight and histopathological analysis

The vital organs, namely, liver, kidney, heart, uterus, and testis were carefully excised and weighed on an analytical balance. For sub-acute toxicity test uterus (female) and testis (male) was collected instead of spleen. The relative organ weight (ROW) of each animal was calculated using the formula; Relative Organ weight (%) = (organ weight/body weight) X 100 [18]. The collected organs were preserved in a fixation medium of 10% buffered formalin for histopathological study. The organ paraffin section of 3–4 µm thickness was prepared and stained with haematoxylin and eosin. The organ section was then visualised under the microscope (Leica microscope, Leica DM500).

2.9. Statistical analysis

All the data were expressed as mean ± standard error (SEM). Analysis of one-way ANOVA performed using GraphPad prism 8.4.0 and statistical significance was determined using Holm-Sidak method. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1. Fumigation assay

Fumigant bioassay experiment result reveal that ethyl acetate extract among all other solvents shows highest fumigant activities against S. oryzae (Fig. 1A). Dose response study of the bioactive enriched fraction isolated from the leaves of D. indica showed toxicity against S. oryzae with LC50 value of 101.88 µg/L of air after 24 h exposure (Fig. 1B).

3.2. Acute toxicity test

The acute toxicity effect of the bioactive enriched fraction isolated from the leaves of D. indica was determined as per the OECD guidelines 423. The enriched fraction at a dose of 1600 mg/kg body weight showed no toxic symptoms or mortality on the treated animals after oral administration and observed for 7 days. The general behaviour of the enriched fraction treated animals and control animals did not display any treatment related changes (Table 1).

External appearances of the animals such as changes in skin and changes in eyes were also normal. There was also no sign of respiratory depression, salivation, and diarrhoea on the treated animals. No significant treatment related changes in the body weight of the treated animals (200, 800, and 1600 mg/kg bw) could be seen as compared to control animals (P > 0.05). The feed intake and water consumption were also normal in all the enriched fraction treated animals when compared to control animals (Table 2).

Haematological study also revealed that there were no significant changes in the blood parameters of the treated animals (Supplementary Table 1).

Fig. 1. Fumigant toxicity of A). Different solvent extracts from D. indica against S. oryzae at 50 mg/L, B). Dose response of bioactive enriched fraction eluted from the leaves of D. indica against S. oryzae.
Table 1
General appearance and behavioural observations of acute toxicity study for control and treated groups.

| Observation       | Control | 200 mg/Kg bw | 800 mg/Kg bw | 1600 mg/Kg bw |
|-------------------|---------|--------------|--------------|---------------|
| Body weight       | Normal  | No abnormal change | No abnormal change | No abnormal change |
| External appearance | Mortality | Alive | Alive | Alive | Alive |
| Sign of toxicity | Changes in skin | No change | No change | No change | No change |
|                   | Changes in eyes | No change | No change | No change | No change |
|                   | Changes in mucus membrane | No change | No change | No change | No change |
| Behavioural changes | Respiratory depression | Not observed | Not observed | Not observed | Not observed |
|                   | Salivation | No effect | No effect | No effect | No effect |
|                   | Diarrhoea | Normal | No effect | No effect | No effect |
|                   | Sleep | Normal | No effect | No effect | No effect |
|                   | Coma | Not observed | Not observed | Not observed | Not observed |
|                   | Lethargy | Normal | No effect | No effect | No effect |
|                   | Urination | Normal | No effect | No effect | No effect |
|                   | Drowsiness | Not present | Not present | Not present | Not present |
| Food and water intake | Normal | No effect | No effect | No effect | No effect |

**Table 2**
Effect of the enriched fraction on body weight, food intake and water intake on BALB/c mice.

| Parameters                      | Sub-acute toxicity | Acute toxicity |
|---------------------------------|--------------------|----------------|
|                                 | Control            | 200 mg/Kg bw   | 800 mg/Kg bw   | 1600 mg/Kg bw |
| Female                          |                    |                |                |               |
| Initial weight                  | 18.62 ± 0.35       | 17.74 ± 0.18   | 18.76 ± 0.33   |                |
| Day 4 (g)                       | – – – – – – – – – – |                |                |               |
| Day 7 (g)                       | 18.64 ± 0.38       | 17.9 ± 0.13    | 19.02 ± 0.23   |                |
| Day 14 (g)                      | 19.94 ± 0.39       | 18.52 ± 0.17   | 19.76 ± 0.31   |                |
| Food intake (g/day/mice)        | 3.21 ± 0.06        | 2.84 ± 0.09    | 2.79 ± 0.13    |                |
| Water intake (ml/day/mice)      | 3.58 ± 0.11        | 3.15 ± 0.10    | 3.23 ± 0.11    |                |
| Male                            |                    |                |                |               |
| Initial weight                  | 21.76 ± 0.46       | 20.28 ± 0.21   | 21.66 ± 0.44   |                |
| Day 4 (g)                       | – – – – – – – – – – |                |                |               |
| Day 7 (g)                       | 22.38 ± 0.48       | 20.82 ± 0.28   | 22.89 ± 0.35   |                |
| Day 14 (g)                      | 23.98 ± 0.41       | 22.38 ± 0.19   | 23.06 ± 0.47   |                |
| Food intake (g/day/mice)        | 3.43 ± 0.12        | 3.40 ± 0.10    | 3.29 ± 0.13    |                |
| Water intake (ml/day/mice)      | 4.32 ± 0.15        | 4.30 ± 0.19    | 3.97 ± 0.12    |                |

Values are expressed as mean ± SEM, p > 0.05 when compared to normal control group.

### 3.3. **Sub-acute toxicity test**

#### 3.3.1. **Feed consumption, water intake and body weight**

The sub-acute toxicity studies did not show any significant abnormalities in the body weight of the treated animals of both sexes. There was no sign of toxicity as well as any significant changes in the external appearance of the treated animals. In the case of treated male, for both the doses of 50 mg/kg and 500 mg/kg enriched fraction, the feed consumption and water intake were normal and were in accordance with their respective body weight. While in females, feed consumption was lower in case of treated animals (2.84 and 2.79 g/day/mice for 50 and 500 mg/kg bw respectively) as compared to the control group (3.21 g/day/mice) (Table 2). No significant changes on the water intake of the treated female mice were observed. Percentage gain in body weight in untreated control male was 9.2% while that of treated mice was 9.38% and 6.07% for 50 and 500 mg/kg respectively. In the case of females, the percentage weight gains were 5.06% (Control), 3.7% (50 mg/kg) and 4.2% (500 mg/kg). Statistically, the changes in body weight of the treated mice were insignificant as compared to the control group (P > 0.05) (Table 2).

#### 3.3.2. **Haematological parameters**

Various blood parameters were analysed of which only the major parameters were listed in the table. In both acute and sub-acute studies blood parameters such as WBC, LYM, MONO, GRAN, HGB, HCT, RBC, MCV, MCH, MCHC, RDW, and MPV from the treated mice did not differ significantly from those of the control group (P > 0.05) (Table 3).

#### 3.3.3. **Biochemical parameters**

Serum biochemistry results of three parameters that are Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Creatinine (CRE) are summarised in the Fig. 2. It was observed that in both the treatment of 50 mg/kg bw and 500 mg/Kg BW the ALT value was slightly increased with p < 0.05 (Fig. 2). No significant changes in AST values in the treatment of 50 mg/kg bw when compared to the normal group, but significant changes could be observed in case of higher dose of 500 mg/kg bw (p < 0.05) (Fig. 2). While the creatinine level in treated male BALB/c mice was found to be lower than that of the control group. In case of female treated mice the drop in the level of creatinine was much higher (p < 0.05) with 22% and 32% for lower and higher dose respectively (Fig. 2).

#### 3.3.4. **Organ weight**

The macroscopic analysis of the vital organs of both control and treated animals shows no adverse changes in the weight, shape, and texture. The organs did not show any external treatment related symptoms. Relative organ weight (ROW) of both acute and sub-acute toxicity study was calculated and summarised in the Table 4. ROW recorded in the entire treated group did not show any significant difference (p > 0.05) as compared to that of the control group.

#### 3.3.5. **Histopathological analysis**

Histological sections of liver, kidney, heart, uterus, and testis of both male and female BALB/c mice in the control and treated group with doses of 50 and 500 mg/kg bw were analyzed. The microscopic observation reveal that the tissue sections did not show any lesions in the vital organs and were comparable with the histopathological profile of the control groups. Both sexes of mice orally administered with enriched fractions did not show variations indicative of toxicity on vital organs. In both lower (Fig. 3B1, B2) and higher dose (Fig. 3C1, C2) treatment, liver...
showed normal histo-architecture and was comparable to the control (Fig. 3A1, A2) and no sign of necrosis were observed in both male and female mice. In case of kidney both the glomeruli, tubules and interstitium were all normal in lower dose treatment (Fig. 4B1, B2) and control group (Fig. 4A1, A2). However, in higher dose administration, a patch of mild tubular injuries (Fig. 4C1) in female mice were observed. Some mild glomerular injury and interstitial edema were observed in renal tubular epithelial cells of male mice (Fig. 4C2). No significant visual difference was observed between the heart (no damage on myocardium cells), uterus (no rupture in three layers; endometrium, myometrium and perimetrium) and testis (no damage in seminiferous tubules) of treated and control group of mice (Supplementary Fig. 2).

4. Discussions

In the Global market, trends towards biopesticides are progressively focused on natural products especially of plant origin. As these plants extracts or fractions consist of mixture of complex chemical compounds, the safety evaluation of the plant derived products becomes necessary to ensure human safety. Several plants derived insecticides including botanical formulations have not been thoroughly evaluated for their toxicity on experimental animals or man. Recently, some of the plant derived products have been investigated for safety and these are having insecticidal properties [19–22]. Leaves and fruits of D. indica are traditionally used as insect repellents and have insecticidal properties. However, there are no studies on mammalian safety evaluation of products derived from D. indica as insecticides. Our previous study has shown that the enriched bioactive fraction extracted from the leaves of D. indica (Supplementary Fig. 1) has biofumigant properties against S. oryzae, T. castaneum and R. dominica. The current study aims to evaluate the acute and sub-acute toxicity of the enriched fraction on BALB/c mice to understand the safety and adverse effect of the fraction on humans.

Our experimental results indicated that enriched fraction of D. indica is not toxic to the laboratory BALB/c mice on both acute and sub-acute toxicity study. In acute study, tests showed that no sign of respiratory depression, salivation, diarrhoea, no significant changes in the body weight, feed intake and water consumption were also normal in all the enriched fraction treated animals when compared to control animals and haematological study also revealed that there were no significant changes in the blood parameters of the treated animals. Hence the enriched fraction was not significantly toxic up to 1600 mg/kg bw in acute toxicity study [21,23].

The sub-acute toxicity studies did not show any significant abnormalities in the behaviour of treated animals of both sexes. There was no sign of toxicity as well as any significant changes in the external appearance of the treated animals. In the case of the treated male, for both doses the feed consumption and water intake were normal. While in females, feed consumption was lower in case of treated animals as compared to the control group. Generally, any abnormal changes in the rate of feed and water intake are associated with toxic effects of chemicals and drugs. However, prolonged administrations of the chemicals or drugs at higher dose sometimes induce stress to the animals, thereby reducing the food intake [24,25]. The organs were removed and weighed at the end of the 14-days subacute period. Insignificant changes in all vital organs between control and treated groups for both sexes. Furthermore, haematological, serum biochemical and histopathological parameters are necessary to determine the toxicity of enriched fraction from D. indica. Toxic effects of the any substance are reflected on blood parameters and serum diagnostic enzyme markers as they are very sensitive to xenobiotics, and as a result haematological and serum biochemistry studies are generally used for assessing physiological and pathological status in humans and animals [26,27]. Haematological profile was unaltered as all the blood parameters studied were well within the normal range.

In addition, the evaluation of serum diagnostic enzymes are associated with liver necrosis, architecture, hepatitis, renal function, glomerular swelling, and liver toxicity [28,29]. In the present work, the levels of ALT of the 50 and 500 mg/kg bw treatment of both sex groups were slightly elevated (6–7%) and were statistically significant (p < 0.05; p > 0.001), while the levels of AST on lower dose treatment showed non-significant change (p > 0.05) of 0.1% elevation as compared to the control group. For higher dose treatment significant increase in the level of AST was observed (6% elevation) (p < 0.05; p > 0.001), suggesting

### Table 3

Effect of the oral administration of enriched fraction on haematological parameters in the sub-acute toxicity studies.

| Parameters                           | Unit         | Male: Normal group | Male: 50 mg/kg bw enriched fraction | Male: 500 mg/kg bw enriched fraction |
|--------------------------------------|--------------|--------------------|-------------------------------------|-------------------------------------|
| White blood cell (WBC)               | 10^9/l       | 7.46 ± 0.15       | 6.29 ± 0.5                          | 6.2 ± 0.69                          |
| Lymphocyte (LYM)                     | %            | 71.84 ± 3.2       | 75.25 ± 2.04                        | 73.2 ± 2.13                         |
| Monocyte (MONO)                      | %            | 5.76 ± 0.35       | 5.67 ± 0.35                         | 5.97 ± 0.59                         |
| Granulocyte (GRAN)                   | 10^9/l       | 1.68 ± 0.44       | 1.18 ± 0.22                         | 1.25 ± 0.22                         |
| Granulocyte (GRAN)                   | %            | 22.4 ± 3.01       | 19 ± 1.96                           | 20.8 ± 1.86                         |
| Haemoglobin (HGB)                    | g/dl         | 18.32 ± 0.67      | 18.18 ± 0.34                        | 16.42 ± 1.8                         |
| Haematocrit (HCT)                    | %            | 18.32 ± 0.67      | 17.5 ± 1.69                         | 41.25 ± 4.5                         |
| Red blood cell (RBC)                 | 10^12/l      | 9.99 ± 0.38       | 9.93 ± 0.26                         | 8.97 ± 0.94                         |
| Mean corpuscular volume (MCV)        | fl           | 45.58 ± 0.35      | 47.78 ± 0.54                        | 46 ± 0.23                           |
| Mean corpuscular haemoglobin (MCH)   | Pg           | 18.28 ± 0.11      | 18.32 ± 0.17                        | 18.22 ± 0.16                        |
| Mean corpuscular haemoglobin concentration (MCHC) | g/dl | 40.16 ± 0.42 | 38.42 ± 0.73                        | 39.6 ± 0.32                         |
| Red cell distribution width (RDW)    | %            | 19.86 ± 0.13      | 18.63 ± 0.52                        | 18.58 ± 0.33                        |
| Mean platelet volume (MPV)           | fl           | 5.24 ± 0.11       | 5.17 ± 0.18                         | 5.4 ± 0.09                          |
| Male: Normal group                   | %            | 19.86 ± 0.13      | 18.63 ± 0.52                        | 18.58 ± 0.33                        |
| White blood cell (WBC)               | 10^9/l       | 4.06 ± 0.27       | 5.9 ± 0.43                          | 6.3 ± 0.19                          |
| Lymphocyte (LYM)                     | %            | 77.98 ± 1.05      | 81.28 ± 1.39                        | 74.8 ± 4.22                         |
| Monocyte (MONO)                      | %            | 6.4 ± 0.02        | 5.14 ± 0.3                         | 6.24 ± 0.77                         |
| Granulocyte (GRAN)                   | 10^9/l       | 0.58 ± 0.01       | 0.78 ± 0.12                         | 0.88 ± 0.13                         |
| Granulocyte (GRAN)                   | %            | 15.62 ± 0.09      | 13.5 ± 1.34                        | 14.9 ± 1.98                         |
| Haemoglobin (HGB)                    | g/dl         | 18.06 ± 0.78      | 18.92 ± 0.9                         | 18.12 ± 0.39                        |
| Haematocrit (HCT)                    | %            | 46.8 ± 2.37       | 47.2 ± 2.6                         | 46.42 ± 1.45                        |
| Red blood cell (RBC)                 | 10^12/l      | 9.89 ± 0.46       | 10.03 ± 0.48                        | 9.82 ± 0.26                         |
| Mean corpuscular volume (MCV)        | fl           | 47.26 ± 0.18      | 46.96 ± 0.40                        | 47.2 ± 0.25                         |
| Mean corpuscular haemoglobin (MCH)   | Pg           | 18.4 ± 0.09       | 18.42 ± 0.06                        | 18.42 ± 0.17                        |
| Mean corpuscular haemoglobin concentration (MCHC) | g/dl | 38.94 ± 0.2 | 39.14 ± 0.24                        | 39.06 ± 0.49                        |
| Red cell distribution width (RDW)    | %            | 17.02 ± 0.42      | 17.48 ± 0.23                        | 17.54 ± 0.32                        |
| Mean platelet volume (MPV)           | fl           | 5 ± 0.15          | 5.16 ± 0.22                         | 4.96 ± 0.17                         |

Values are expressed as mean ± SEM. p > 0.05 when compared to normal control group.
that high dose of enriched fraction may possess mild toxicity to the liver, it may be profound hepatic dysfunction. However, increase levels of hepatic enzymes (transaminase enzyme, SGPT and SGOT) cannot be directly link to the toxicity of chemicals as any injury to the liver other than the injury due to the enriched fraction could have also elevated the serum levels of hepatic enzymes [30,31]. In case of kidney showed normal glomeruli with mild necrosis of tubular cells in both sexes, at higher dose mild glomerular injury, and interstitial edema observed in renal tubular epithelial cells of male mice. Earlier reports revealed nephroprotective and hepatoprotective effects of D. indica extracts [15, 32], indicating the need for further study on specific mechanisms of toxic action of enriched fraction from D. indica.

5. Conclusions

The finding of the present study reveals that enriched fraction from D. indica did not cause adverse toxicity and mortality in the experimental BALB/c mice at highest dose of 1600 mg/kg bw and 500 mg/kg bw in acute and sub-acute toxicity tests respectively. Biochemical and histopathological studies on vital organs suggest mild toxicity on liver and kidney at higher dose. This encourages further in-depth studies of the toxicity on longer days exposure and higher dose of the enriched fraction. Further, phytochemical identification of D. indica enriched fractions would provide more information regarding its target site and mode of action.

Ethics approval and consent to participate

The use of animal experimental protocols was approved by the Institutional Animal Ethics Committee of Institute of Life Sciences, Bhubaneswar (ILS/IAEC-223-AH/APR-21).
Fig. 3. Photomicrograph of the liver section of BALB/c mice administered with distilled water at the rate of 1 ml/100 g body weight and enriched fraction for 14 days. A1: Only distilled water treated liver of female mice, B1: 50 mg/kg body weight enriched fraction treated liver of female mice, C1: 500 mg/kg body weight enriched fraction treated liver of female mice. A2: Only distilled water treated liver of male mice, B2: 50 mg/kg body weight enriched fraction treated liver of male mice, C2: 500 mg/kg body weight enriched fraction treated liver of male mice.

Fig. 4. Photomicrograph of the kidney section of BALB/c mice administered with distilled water at the rate of 1 ml/100 g body weight and enriched fraction for 14 days. A1: Only distilled water treated kidney of female mice, B1: 50 mg/kg body weight enriched fraction treated kidney of female mice, C1: 500 mg/kg body weight enriched fraction treated kidney of female mice. A2: Only distilled water treated kidney of male mice, B2: 50 mg/kg body weight enriched fraction treated kidney of male mice, C2: 500 mg/kg body weight enriched fraction treated kidney of male mice.

CRediT authorship contribution statement

Kabrambam D Singh: Methodology, Investigation, Writing – original draft. Sarita Jena: Conceptualization, Investigation, Data curation, review and editing. Biswajit Patra: Investigation. Thiyam B Devi: Investigation. Saurabh Chawla: Investigation and review. Rupjyoti Bharali: Review, editing, Supervision. Ajay Parida: Review, Validation. Pulok K Mukherjee: Investigation and review. Yallappa Rajashekar: Methodology, Conceptualization, Writing – review & editing, Supervision.

Declaration of Conflict of Interest

The authors declare that they have no conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] D. Prusky, Reduction of the incidence of postharvest quality losses, and future prospects, Food Secur. 3 (2011) 463–474, https://doi.org/10.1016/j.s122751-011-0147-y.

[2] Y. Rajashekar, L.J.M. Rao, T. Shivanandappa, Decaleside: a new class of natural insecticide targeting tarsal gustatory sites, Naturwissenschaften 99 (2012) 843–852, https://doi.org/10.1007/s00114-012-0966-5.

[3] A.J. Mobolade, N. Bunindro, D. Sahoo, Y. Rajashekar, Traditional methods of food grains preservation and storage in Nigeria and India, Ann. Agric. Sci. 64 (2) (2019) 196–205, https://doi.org/10.1016/j.asan.2019.12.005.

[4] M.B. Isman, Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world, Annu. Rev. Entomol. 51 (2006) 45–66, https://doi.org/10.1146/annurev.ento.51.110104.151146.

[5] L.J. Graham-Bryce, Crop protection: a consideration of the effectiveness and disadvantages of current methods and of the scope for improvement, Philos. Trans. R. Soc. B 281 (1977) 163–179, https://doi.org/10.1098/rstb.1977.0131.

[6] F. Horn, P. Horn, J. Sullivan, Current practice in fresh fruit fumigation with phosphine in Chile, in: Proceedings of the Annual Research Conference on Methyl Bromide Alternatives and Emissions Reductions, (2005) 31.

[7] C. Regnault-Roger, C. Vincent, J.T. Arman, Essential oils in insect control: low-risk products in a high-stakes world, Annu. Rev. Entomol. 57 (2012) 405–424, https://doi.org/10.1146/annurev-ento-120710-100554.

[8] A. Saroj, O.V. Oriyomi, A.K. Nayak, S.Z. Haider, S.Z. Phytochemicals of plant-derived essential oils: a novel green approach against pests. Natural Remedies for Pest, Disease and Weed Control, Academic Press, 2000, pp. 65–79, https://doi.org/10.1016/S0961-9525(00)80006-5.

[9] M.B. Isman, Botanical insecticides for: richer, for poorer, Pest Manag. Sci. 64 (2008) 8–11, https://doi.org/10.1002/ps.1470.

[10] Y. Rajashekar, N. Tonsing, T. Shanthibala, J.R. Manjunath, 2, 3-Dimethylmalonic anhydride (3, 4-Dimethyl-2, 5-furandione): a plant derived insecticidal molecule from Colocasia esculenta var. esculenta (L.) Schott, Sci. Rep. 6 (2016) 20546, https://doi.org/10.1038/srep20546.

[11] Y. Rajashekar, T. Shivanandappa, Mammalian safety of Decaleside II in the laboratory mouse, Toxicol. Rep. 1 (2014) 969–972, https://doi.org/10.1016/j.j.torep.2014.01.001.

[12] R.B. Raizada, M.K. Srivastava, R.A. Kaushal, R.P. Singh, Azadirachtin, a neem biosticide: subchronic toxicity assessment in rats, Food Chem. Toxicol. 39 (5) (2001) 477–483, https://doi.org/10.1016/S0278-6915(00)00153-8.

[13] M. Tomizawa, D.L. Lee, J.E. Casida, Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors, J. Agric. Food Chem. 48 (12) (2000) 6016–6024, https://doi.org/10.1021/jf000873r.

[14] R.D. Verschoyle, J.M. Barnes, Toxicity of natural and synthetic pyrethrins to rats, Pestic. Biochem. Physiol. 2 (3) (1972) 308–311, https://doi.org/10.1016/0048-3575(72)90034-X.

[15] K.H. Reddy, V. Tharanath, K.B.N. Reddy, P.V.G.K. Sharma, O.V.S. Reddy, Studies on hepato protective effect of hexane extract of Dillenia indica against CCl4 induced toxicity and its safety evaluation in wistar albino rats, Res. J. Pharm. Biol. Chem. Sci. 1 (3) (2010) 441–450.

[16] C.C. Banar, N. Yasmin, L. Buragohain, A review update on Dillenia indica, its morphology, phytochemistry and pharmacological activity with reference to its anticancer activity, MOJ Bioequiv. Bioavailabil. 5 (5) (2018) 244–254.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.j.torep.2022.05.007.