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# Toxicity evaluation of aqueous soluble fraction of *Tamarindus indica* seed coat extract in albino wistar rats

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**Abstract**
The present study was aimed at toxicological evaluation of aqueous soluble fraction of methanolic extract of *Tamarindus indica* seed coat (AMTS) on acute oral exposure in rats and to detect phytoactive constituents present in AMTS. The toxicity study was done as per Organisation for Economic Co-operation Development test guidelines No. 420, Acute Oral Toxicity-Fixed Dose Procedure, while phytochemical screening of AMTS was done by various qualitative tests. The AMTS treated rats survived throughout the study period and did not exhibit any adverse clinical signs at the limit dose level. Besides, necropsy revealed no pathological lesions in AMTS-treated rats. Furthermore, phytochemical screening revealed the presence of alkaloids, phenolic compounds, tannins, flavonoids, saponins and steroids. In summary, acute oral toxicity testing of AMTS revealed no treatment-related detrimental effects up to a dose level of 2000 mg/kg body weight, enabling it to be listed as hazard category 5/unclassified under the Globally Harmonized System.

**Keywords:** *Tamarindus indica* seed coat, acute oral toxicity, OECD guidelines, Globally Harmonized System, Phytochemical screening

## Introduction
Traditional herbal medicines are acquiring great emphasis globally. During the past few decades, clear shift in the public interests towards herbal medicine has been observed, and the acceptance of these herbal remedies is increasing considerably in both developing and the developed countries [1]. Though it is commonly assumed that, they are intrinsically nontoxic, this might not be the reality and toxicological testing is essentially warranted. Hence, sufficient data on chemical and toxicological profiles is imperative for the use of plants that have traditionally been touted as having health benefits [2].
*Tamarindus indica* Linn. is a native herbal medicine with a range of pharmacological effects identified in various parts of the plant [3]. Moreover, *T. indica* seed coat extract is a potential source of polypenolic antioxidant compounds [4] and hence, it was anticipated to have protective effect on acetaminophen induced hepatic and renal damage, the confirmation of which require further investigation. However, prior to efficacy assessment, safety evaluation is essential and this study was aimed at toxicological evaluation of aqueous soluble fraction of methanolic extract of *T. indica* seed coat (AMTS) on acute oral exposure in rats as per OECD guidelines and also to qualitatively detect the phytoactive constituents present in AMTS.

## Materials and methods
### Experimental animals
Five female albino Wistar rats of 6 to 8 week age, weighing 150-200 g body weight were procured from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy and were housed in polypropylene cages with free access to fresh water and fed with laboratory animal feed. The animals were housed under standard conditions of 12 h/12 h light/dark cycle at 25 °C ± 2 °C with 30-70 per cent relative humidity and were acclimatized for a week before experimentation. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/CVASMTY9/18-19).

### Plant Material
The seeds of *T. indica* collected from local commercial suppliers were identified and authenticated at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi.

***Preparation of Aqueous Soluble Fraction of Alcoholic Extract of T. indica Seed Coat***

The seed coat was separated from kernel using mortar and pestle after keeping the Tamarind seeds at hot air oven at a temperature of 50 ºC for 5 h. Aqueous soluble fraction of alcoholic extract of *T. indica* seed coat was prepared as described by Sasidharan *et al* [3], with slight modifications. Weighed quantities of the *T. indica* seed coat powder were initially extracted using Soxhlet apparatus with 95 per cent methanol to prepare the methanolic extract. The extract was then evaporated and concentrated using Rotary vacuum evaporator under reduced pressure and at a temperature of 55ºC. The concentrated extract was air dried at room temperature and stored in refrigerator until use.

The methanolic extract was then refluxed with distilled water (1:10) at 80-90 ºC for 5 h and the water dissolved upper layer of extract was collected and stored in refrigerator. The marc was successively refluxed with fresh distilled water and repeated the procedure thrice and the water soluble fractions so obtained were pooled and filtered with Whatman filter paper grade No. 40 to get clear liquid, which was further concentrated under reduced pressure at a temperature range of 40-50 ˚C using Rotary vacuum evaporator for preparing the aqueous soluble fraction of methanolic extract of *T. indica* seedcoat (AMTS). It was further air dried at room temperature to a powder consistency and stored in airtight containers and kept in refrigerator until use. The percentage yield of methanolic extract and AMTS were also calculated.

**Phytochemical Screening**

The methanolic extract of *T. indica* seed coat was tested for the presence of various active chemical constituents namely steroids, alkaloids, tannins, phenolic compounds, flavonoids, glycosides, terpenoids and saponins [5, 6].

***Tests for Detection of Steroids***

Salkowski Test: About 5 mg of the extract was mixed with 3 ml of chloroform and then shaken with 3 ml concentrated sulphuric acid. Development of reddish-brown colour indicated the presence of steroids.

Lieberman Burchardt Test: About 5 mg of the extract was mixed with 3 ml of chloroform in a test tube. Then five drops of acetic anhydride and 1 ml of concentrated sulphuric acid were added to it through the sides of the test tube. Development of a reddish ring at the junction of two layers indicated the presence of steroids.

***Tests for Detection of Alkaloids***

About 0.5 g of the extract was mixed with 5 ml of ammonia and then extracted with equal volume of chloroform. To this extract, 5 ml dilute hydrochloric acid was added. The acid layer obtained was used for the following chemical tests for alkaloids.

Mayer's Test: To 1 ml of acid layer, a few drops of Mayer's reagent (1.358 g of mercuric chloride dissolved in 60 ml of water and poured into a solution of 5 g of potassium iodide in 10 ml of water and then made up the volume to 100 ml with distilled water) were added. Development of a creamy white precipitate indicated the presence of alkaloids.

Wagner's Test: A few drops of Wagner's reagent (2 g of iodine and 6 g of potassium iodide dissolved in 100 ml of water) were added to 1 ml of the acid extract. Development of reddish-brown precipitate indicated the presence of alkaloids.

Hager's Test: To 1 ml of the acid extract, a few drops of Hager's reagent (1 g of picric acid dissolved in 100 ml of water) were mixed. Development of yellow precipitate indicated the presence of alkaloids.

***Test for Detection of Phenolic Compounds***

Five milligrams of the extract were dissolved in 1 ml of water and five drops of 10 per cent ferric chloride was added to it. Development of dark blue colour indicated the presence of phenolic compounds**.**

***Test for Detection of Tannins***

Ferric Chloride Test: Two milligrams of the extract were mixed with 3 ml of one per cent ferric chloride solution. Development of a blue, green or brown colour indicates the presence of tannins.

Gelatin Test: About 0.5 g of the extract was mixed with a few drops of one per cent solution of gelatin containing 10 per cent sodium chloride. Development of a white precipitate indicated the presence of tannins.

***Tests for Detection of Flavonoids***

Ferric Chloride Test: To 2 ml of alcoholic solution of the extract (0.5 g extract in 10 ml methanol), a few drops of neutral ferric chloride solution were mixed. Development of green colour indicates the presence of flavonoids.

Lead Acetate Test: To 2 ml of alcoholic solution of the extract (0.5 g extract in 10 ml methanol), a few drops of neutral ten per cent lead acetate was mixed. Development of yellow precipitate indicated the presence of flavonoids.

***Tests for Detection of Glycosides***

Sodium Hydroxide Test: A small amount of the extract (about 5 mg) was mixed with 1 ml water and added 5-6 drops of sodium hydroxide solution (10 per cent). Development of yellow colour indicated the presence of glycosides.

Benedict's Test: The extract (0.5 g) was mixed in 1 ml of water and then 5 ml of Benedict's reagent was added. The mixture was boiled for two min and cooled. Development of brown or red colour indicated the presence of glycosides.

***Tests for Detection of Terpenoids***

About 3 mg of the extract was mixed with 3 ml of chloroform and then it was evaporated to dryness on water bath and then boiled with 3 ml of concentrated sulphuric acid. Development of grey colour indicated the presence of terpenoids.

***Tests for Detection of Saponins***

Foam Test: A small amount of the extract (about 1 g) was shaken with 5 ml of water. Development of the foam that persisted for 10 min indicates the presence of saponins.

**Acute Oral Toxicity Evaluation of AMTS**

Acute oral toxicity test of AMTS was performed as per the Organisation for Economic Co-operation Development (OECD) test guidelines No. 420, Acute Oral Toxicity-Fixed Dose Procedure [7]. Accordingly, AMTS was solubilized in distilled water and administered orally in a sequential manner in one animal at 2000 mg (limit test of sighting study) followed by four animals at 2000 mg/kg in the main study.

All the animals were observed for mortality and any clinical signs for the first 10 min, 30 min, 1 h, 2 h and 4 h after dosing and thereafter twice daily for mortality and once a day for clinical signs for next 14 days. The clinical signs observed during the first 4 h of the drug administration were gross behavioural changes like hyperactivity, grooming, convulsions, sedation and loss of righting reflex along with

changes in respiration, salivation, urination and defecation, if any. Body weights of animals were recorded individually before dosing and at weekly intervals thereafter.

After the observation period of 14 days, the animals were euthanized and subjected to detailed gross pathology examination. As the present study was intended for the evaluation of AMTS for the protective effect against acetaminophen/ paracetamol induced hepato and nephrotoxicity, histopathological examination of liver and kidney were also conducted at the end of the experiment to ensure the safety of the test substance [8].

## Results

The extractive yield of methanolic extract of *T. indica* from the powdered Tamarind seed coat was found to be 38.51 % w/w, whereas the yield of aqueous fraction from the methanolic extract of Tamarind was 33.33 % w/w.

### Phytochemical screening of AMTS

The result of the phytochemical screening of AMTS is summarized in Table 1. Phytochemical screening revealed that AMTS contains a mixture of active chemical constituents such as steroids, alkaloids, phenolic compounds, tannins, flavonoids, terpenoids and saponins.

### Acute oral toxicity evaluation of AMTS

The female Wistar albino rats treated with aqueous fraction of methanolic extract of *T. indica* seed coat did not show any mortality throughout the study period of 14 days in both the sighting, and main studies of acute oral toxicity evaluation, conducted as per OECD Test Guideline No. 420. At the limit dose of 2000 mg/kg body weight, rats did not show any adverse signs of intoxication immediately following dosing as well as during the 14 days of observation period, except for the hypoactivity observed in two animals. The overall weight gain in all the treated rats was found to be normal at the end of 14 days. On necropsy, no major gross pathological changes were observed in any of the treated rats (Tables 2 and 3).

The histopathological examination of the liver and kidney from the AMTS treated rats showed normal structure and architecture without any pathological changes, indicating the safety potential of AMTS after the acute oral administration (Figure 2). Thus, aqueous fraction of methanolic extract of *T. indica* seed coat was found to be safe up to the tested limit dose level of 2000 mg/kg body weight.

## Discussion

*Tamarindus indica* L., or Tamarind, a worldwide proverbial herbal medicine to humankind is reported to contain a multitude of phytochemicals and consequently different parts of the plant have been documented for antioxidant, anti-inflammatory, antimutagenic, antihyperlipidemic, antimicrobial, carminative, and expectorant activities [9]. Various studies have detailed the polyphenolics profile of Tamarind pericarp in which proanthocyanidins in various forms, such as apigenin, catechin, procyanidins, epicatechin etc. were reported as the major constituents [10, 11]. Phenolic compounds are predominantly renowned for their antioxidant and anti-inflammatory properties and hence, have been demonstrated to have significant protective and therapeutic effects in a variety of liver diseases as evidenced by both *in vivo* and *in vitro* studies [12]. Hence, a study was planned to evaluate hepato- and nephro protective effects of a herbal preparation containing *T. indica* seedcoat on acetaminophen/paracetamol toxicity for which a prior safety evaluation was mandatory and this study thus focused on preliminary phytochemical and acute toxicity evaluation of aqueous soluble fraction of methanolic extract of *T. indica* seedcoat (AMTS).

The phytochemical screening of AMTS revealed the presence of steroids, alkaloids, phenolic compounds, tannins flavonoids, terpenoids and saponins, which was in accordance to the previous report in which the presence of phenolics, alkaloids, tannins, terpenes and saponins have been documented in aqueous extract, methanolic extract and its hexane, chloroform, butanol and water fractions of *T. indica* seed coat [13]. Scientific reports are available stating that methanolic extracts of raw and dry heated seed coat samples of *T. indica* contained higher level of phenolic acids like flavonoids and high molecular tannins. It has been specified that these extracts presented high free radical scavenging activities on superoxide, DPPH and hydroxyl free radicals indicating their promising antioxidant capacity [14].

Similarly, sterols and triterpenoids derived from plants and foods are also known to exhibit antioxidant, metabolic-regulating, immunomodulatory, and anti-inflammatory properties. Due to the existence of the steroid nucleus and structural similarity to cholesterol and bile acids, it is currently claimed to have a hepatoprotective impact and seem to be a viable alternative for the prevention and treatment of chronic metabolic-related liver diseases, cirrhosis, and hepatocellular carcinoma [15]. Saponins are also essential secondary metabolites synthesised by a variety of plant species, possessing a range of medicinal properties including antimicrobial, antitumor, anti-insect, hepatoprotective, haemolytic, and anti-inflammatory effects [16].

Owing to the presence of active phytochemicals, majorly polyphenols as tannins along with steroids, alkaloids, flavonoids, terpenoids and saponins in the extract, it can be suggested that AMTS might be a good candidate for the preclinical evaluation for the protective effect against acetaminophen induced hepatic and renal damage. However, the evidence on safety aspect of different parts of T. indica is scarce despite the existence of numerous empirical proofs of its excellent pharmacological impact.

The acute oral toxicity assessment of AMTS in this study was performed as per OECD test guidelines No. 420 (Acute Oral Toxicity-Fixed Dose Procedure, OECD, 2001)[7] to determine the preliminary safety specifics. A single oral administration of AMTS at a dosage of 2000 mg/kg body weight in both sighting and main studies showed neither any mortality and adverse clinical signs nor any significant changes in body weight. Moreover, the gross and histopathological examination of liver and kidney showed normal appearance and architecture, indicating the test substance's promising preliminary safety profile and thereby was able to be labelled as Category 5/unclassified in the hazard category according to globally harmonised system.

Thus, the findings of acute oral toxicity help to classify the test substance using the global harmonised system (GHS) for chemical classification [7, 17]. The GHS is a classification and labelling scheme for chemicals that aims to standardise and harmonise classification and labelling. The harmonised classification scheme defines and categorises chemical product hazards, as well as provide health and safety information on labels and safety data sheets. Substances can be classified into one of five toxicity categories based on 'acute toxicity results', according to the GHS. Substances in GHS category 5 have a low acute toxicity but can pose a risk to vulnerable populations in some circumstances. The oral or

dermal LD50 of these substances is expected to be in the range of 2000-5000 mg/kg bodyweight [17].

According to GHS, there are three categories of hazard groups based on 'target organ toxicity' after a single exposure. When there is toxicity of a particular target organ linked to a single exposure of a drug, it can be denoted as 'classified' and are further allotted to one of the three categories based on the specifications, whereas, if a drug is labelled as 'unclassified', it means that the toxicity found in particular target organs was deemed unimportant or irrelevant to humans. However, the drug will be 'classified' and 'categorised' if subsequent human injury data reveals a particular target organ toxic impact [17]. Hence, based on the findings of the present study, AMTS can be labelled as category 5/unclassified in the hazard category according to globally harmonised system.


**Fig 1:** *Tamarindus indica* (a) Tamarind pod with the leaves (b) Tamarind seeds (c) Tamarind seed coat

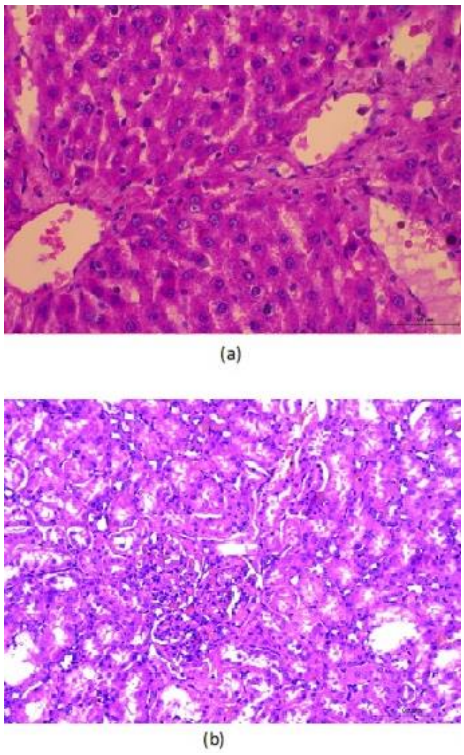


**Fig 2:** Histology of sections of liver and kidney from AMTS treated group after acute oral toxicity test (H &E) (a) Liver (400X) showing normal structure and architecture (b) Kidney (200X) showing normal structure and architecture; AMTS: Aqueous fraction of methanolic extract of *T. indica* seed coat

**Table 1:** Phytochemical screening of aqueous soluble fraction of methanolic extract of *T. indica* seed coat.

| Active principle- Test | AMTS |
|---|---|
| ***Alkaloids*** | |
| Mayer's test | + |
| Wagner's test | + |
| Hager's test | + |
| ***Steroids*** | |
| Salkowski's test | + |
| Lieberman Burchardt Test | + |
| ***Phenolic compounds*** detection test | + |
| ***Tannins*** | |
| Ferric chloride test | + |
| Gelatin test | + |
| ***Flavonoids*** | |
| Lead Acetate test | + |
| Ferric chloride test | + |
| ***Glycosides*** | |
| Sodium hydroxide test | -- |
| Benedict's test | -- |
| ***Terpenoid*** detection test | + |
| ***Saponins*** | |
| Foam test | + |

Symbols '+' and '--'indicated 'presence' and 'Not detected' respectively
AMTS: Aqueous soluble fraction of methanolic extract of *T. indica* seed coat

**Table 2:** Clinical signs, mortality and gross pathology findings observed in female Wistar rats treated with AMTS

| Study | Dose (mg/kg) | Mortality (death/ total) | Cage side observations | | Gross pathology findings |
|---|---|---|---|---|---|
| | | | Signs | Period (onset-recovery) | |
| Sighting (n=1) | 2000 | 0/1 | Nil | - | NAD |
| Main (n=4) | 2000 | 0/1 | Hypoactive | 50 min - 3 h post- dosing | NAD |
| | 2000 | 0/1 | Nil | - | NAD |
| | 2000 | 0/1 | Hypoactive | 40 min - 2 h post- dosing | NAD |
| | 2000 | 0/1 | Nil | - | NAD |

n = number of animals; NAD = No abnormality detected
AMTS: aqueous soluble fraction of methanolic extract of *T. indica* seed coat

**Table 3:** Effect of AMTS on body weight and body weight gain in female Wistar rats

| Study | Dose (mg/kg) | Body weight (g) | | | Body weight gain (%) | | |
|---|---|---|---|---|---|---|---|
| | | Day 0 | Day 7 | Day 14 | Days 0-7 | Days 7-14 | Days 0-14 |
| Sighting (n=1) | 2000 | 149 | 165 | 187 | 14.73 | 7.12 | 26.86 |
| Main (n=4) | 2000 | 153 | 185 | 196 | 21.55 | 8.05 | 29.42 |
| | 2000 | 156 | 192 | 207 | 18.75 | 7.24 | 24.64 |
| | 2000 | 149 | 182 | 194 | 18.13 | 6.19 | 23.19 |
| | 2000 | 154 | 189 | 201 | 18.51 | 6.34 | 23.38 |

n = number of animals
AMTS: aqueous soluble fraction of methanolic extract of *T. indica* seed coat

## Conclusion

To conclude, the aqueous soluble fraction of methanolic extract of *T. indica* seed coat (AMTS) did not exhibit any signs of evident toxicity on acute oral administration up to a dose level of 2000 mg/kg body weight, enabling it to be listed as hazard category 5/unclassified under the Globally Harmonized System, indicating a promising preliminary safety profile. Moreover, it contain many pharmacologically active constituents especially phenolic compounds, steroids, terpenoids etc. Therefore, AMTS can be positively considered as a potential candidate for therapeutic activity evaluation.

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