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Original Article

A PILOT STUDY ON THE DETERMINATION OF ANTIOXIDANT POTENTIAL AND LETHAL DOSAGE OF HYDRO ALCOHOLIC FRUIT EXTRACT OF *TERMINALIA CHEBULA*

MARIAPPAN AMUTHA[a], ARUMUGAM GEETHA[a]*, MARIMUTHU AMUTHA[b]

[a]*Department of Biochemistry, Bharathi Women's College, Chennai 600108, India, [a, b] Research and Development Centre, Bharathiar University, Coimbatore 641046, India.
Email: geethav21@yahoo.co.in

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ABSTRACT

Objective: Medicinal plants exhibit therapeutic properties due to their antioxidant potential and the assessment of LD_{50} value is essential to study their biological efficacy against experimentally induced diseases. Hence, the present study is focused on determining the antioxidant potential and the lethal dosage of fruit extract of *Terminalia chebula*, a traditional medicinal plant widely used for various ailments.

Methods: The hydro alcoholic fruit extract of *Terminalia chebula* (HAETC) was quantitatively analyzed for alkaloids and phenolics by HPLC technique and terpenoids by gas chromatography. The antioxidant potential was determined using various models in which free radicals were generated *in vitro*. LD_{50} value was calculated by Karber's method.

Results: Phytochemical analysis of *T. Chebula* revealed the quantity of alkaloids (2.85mg/g), phenols (1.72 mg/g), terpenoids (1.06 mg/g) and flavonoids (0.560 mg/g). Among the flavonoids, quercetin (0.2336 mg/g) was found abundant when compared to rutin (0.017 mg/g) and gallic acid (0.045mg/g). Aqueous fruit extract of *Terminalia chebula* (AFETC) was found to scavenge free radicals effectively. The IC_{50} value of AFETC while scavenging DPPH radicals (37.4µg/ml), superoxides (37.85µg/ml) and nitric oxide (34.37µg/ml) were comparable to that of their corresponding reference compounds ascorbic acid (38.76µg/ml) and rutin (40.57µg/ml and 45.84µg/ml). The acute toxicity profile was assessed and the lethal dosage was found to be 2475mg/Kg body wt.

Conclusions: The study revealed that *T. Chebula* has potent antioxidant capacity which might be accounted for its therapeutic potential. Based on the LD_{50} value of HAETC, 200, 250 and 300 mg/kg body weight may be used as low, mid and high dose respectively to study the therapeutic activity of *T. chebula* in rat models.

Keywords: *Terminalia chebula,* Reactive oxygen species, $LD_{50,}$ scavenging activity, $IC_{50.}$

INTRODUCTION

Recently medicinal plants rich in antioxidant phytochemicals have received growing attention as they are traditionally consumed for various ailments. They are recognized to exhibit disease preventive property by scavenging reactive oxygen species (ROS) and detoxifying potent genotoxic oxidants. The potentially reactive derivatives of oxygen (ROS) include hydroxyl radicals, superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radicals, hypochlorite radical and various lipid peroxides [1]. These free radicals are continuously generated inside the human body as a result of contact with excess of exogenous chemicals and environmental pollutants and / or due to a number of endogenous metabolic processes involving redox enzymes. All these free radicals are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage [2]. Excess production of ROS is implicated in the pathogenesis of atherosclerosis, cancer and inflammatory arthritis [3].

Many plant products have been shown to exhibit medicinal property due to their antioxidant and free radical quenching effects. Active components of plants such as flavonoids, phenols, triterpenes and tannins may be regarded as possible phytochemicals against inflammatory diseases by acting as antioxidants. Antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases in which oxidants or free radicals are involved [4].

Flavonoids and tannins are phenolic compounds which act as primary antioxidants or free radical scavengers [5]. Phenolics or polyphenols have received considerable attention because of their physiological function, including antioxidant, antimutagenic and antitumor activities [6]. Phenolic compounds are widely distributed in plants [7] which have gained much attention due to their antioxidant activities and free radical scavenging abilities [8, 9].

Terminalia chebula Retz commonly known as chebulic myrobalan, is a popular medicinal plant in the Ayurvedic system of medicine. It belongs to the genus Terminalia and family Combretaceae. *T. chebula* has tannins (32%), fructose, aminoacids, succinic acid, β–sitosterol, anthroquinone and sennosine [10]. The chief constituents of tannin are chebulagic acid, chebulic acid, corilagin and gallic acid [11]. Traditionally, *T.chebula* has been used to treat kidney stones and urinary disorders, nervous disorders, colic pain, chronic cough, sore throat, asthma etc. There are a number of formulations like Chooranam (powder), Ennai (Medicated Oil), Ilagam (Confectionary) and Maathirai (Pills) in which *T.chebula* is a main ingredient. *T.chebula* has been reported to exhibit anticancer, antidiabetic, antimutagenic, antibacterial, gastroprotective, hepatoprotective and cardioprotective [12-14] activities. *T.chebula* is also reported to possess strong anti-inflammatory activity [15]. Since *T.chebula* has a wide application in treating human diseases, a scientific validation is essential to evaluate the safe dose to be recommended to avoid side effects. The aqueous and hydro alcoholic fruit extracts of *T.chebula* were used for the study.

MATERIALS AND METHODS

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl(DPPH) (Sigma Chemicals, USA), Ascorbate (HiMedia Laboratory Pvt.Ltd, Mumbai, India), Gallic acid, nitroblue tetrazolium (NBT) and phenazine methosulphate (PMS) (Sigma Aldrich, India, Bangalore) were used for the study. All other chemicals and solvents used for the analysis were of analytical grade. Absorbance was measured with Shimadzu 1601 UV- visible spectrophotometer for the antioxidant studies.

Sample collection

The dried fruits of *T.Chebula* were purchased from three different places at Chennai. The fruits were authenticated by Dr. P. Jayaraman,

Director, Plant Anatomy Research Center, Chennai (Voucher. No. PARC/2013/2102). The fruits were coarsely grounded into homogeneous powder using domestic grinder and extracted with water and 70% ethanol.

Preparation of HAETC

100 gm of *T. chebula* powder was soaked in 250 ml of 70% ethanol (v/v) for 3-5 days with intermediate shaking and filtered using What man filter paper No 42 (125 nm). The filtrate obtained was evaporated to dryness using rotary evaporator. The concentrate was lyophilized and used for phytochemical screening and LD_{50} value determination.

Preparation of AFETC

The AFETC was prepared by macerating 10g of finely powdered *T. chebula* in 100 ml of distilled water at 60°C for 2 hours and the resulting mixture was filtered. The filtrate was evaporated to dryness to one third of the original volume and stored at 40°C. The filtrate was lyophilized and the dried material obtained was collected, stored at 4ºC and used for the assessment of antioxidant property *in vitro*.

HPLC analysis

The amount of phytochemicals in HAETC was determined by HPLC analysis (LCGC AGLIENT). For flavonoids determination, the stationary phase was octadecyl silyl silica gel and the mobile phase was a linear gradient with methanol, water and phosphoric acid (100:100:1). The flow rate was adjusted 1.5 ml per min with the injection volume of 20µL. Rutin, quercetin, gallic acid, galangin and thymoquinone were used as standards. The UV spectra were monitored at 270 nm. For alkaloids estimation, the mobile phase used was monobasic potassium phosphate in distilled water – acetonitrile. The flow rate of sample was 1.8ml/min with the injection volume of 10µL.

Gas chromatography (GC) analysis of terpenoids

The terpenoids level was quantified in GC system using capillary column coated with macrogol 20000 R and nitrogen as carrier gas. The flame ionisation detector was set at the flow rate of 0.4 ml/min for 1 µL sample and anethole was used as standard.

DPPH radical scavenging activity

The DPPH radical scavenging assay is based on the ability of DPPH, a stable free radical, to get decolorized in the presence of antioxidants. To 300 µL of methanolic solution of DPPH (100 µM), various concentrations of the extract (20-100 µg /ml) in water were added. It was then incubated at 37 °C for 30 min in dark and the absorbance was measured at 490nm against the corresponding blank solution. The percentage scavenging of DPPH free radicals by the test material was calculated by comparing with that of standard ascorbic acid.

Superoxide anion scavenging activity

The method of Nishkimi *et al* [16] was used to measure the superoxide anion scavenging activity of the extract. Briefly, 1 ml of NBT (156 µM in 100 mM phosphate buffer, pH 7.4), 1 ml of reduced nicotinamide adenine dinucleotide (NADH) in 100mM phosphate buffer, pH 7.4 were added to 0.1 ml of the test extract with concentration ranging from 20-100 µg. The reaction was started by adding 100 µL of PMS (60 µM in 100mM phosphate buffer, pH 7.4) and the reaction mixture was incubated at 25 °C for 5 min. The decrease in absorbance was measured at 560nm against water blank. Rutin was used as the positive control.

Nitric oxide scavenging activity

The method of Sreejayan and Rao [17] was used to measure the nitric oxide scavenging activity of the AFETC. Various concentrations of the test material and standard rutin (20-100 µg) were dissolved in phosphate buffer (0.025 M, pH 7.4) and incubated with sodium nitroprusside (5 µM) in standard phosphate buffer at 25 °C for 5 hrs. After incubation, 0.5 ml of the reaction mixture was added with 0.5 ml of Griess reagent (equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% napthyl ethylene diamine dihydochloride in water). The absorbance of the chromophore formed was read at 540nm. The scavenging activity was compared with that of rutin at similar concentration.

Reducing power

The reducing power of the plant extract was determined by the method of Oyaizu et al [18] Varying concentrations of the extracts (20-100 µg) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml of 1% potassium ferricyanide and incubated at 50 °C for 20 min. 1.5 ml of 10% trichloroacetic acid was then added to the reaction mixture and the contents were centrifuged at 3000 rpm for 10 min. 0.5 ml of the supernatant was collected and mixed with 1 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Ascorbic acid was used as the standard. Increase in absorbance at 700nm indicates increased reducing power.

Acute Toxicity profile of HAETC in Albino rats

Acute toxicity test was performed according to the World Health Organization (WHO) guideline (WHO 2000) and the organization of Economic Co-operation and Development (OECD) guideline 420 for testing of chemicals (OECD 2001).

Animals and husbandry

Male albino Wistar rats (175-200 g) were housed four per polycarbonate cage under controlled conditions (20-25°C/RH 40-45%) in a 12: 12 h L:D cycle and fed standard pellet diet containing required amount of minerals and vitamins obtained from M/S: Provimi Animal Nutrition India Pvt Ltd., Bangalore, during 7 day acclimatization period. Diet and water were provided *ad libitum*. Different groups of rats were administered 100, 200, 500, 1000, 2000 & 2500 mg/ Kg b.wt of HAETC in two equally divided doses during a period of 8 hrs. The drug was dissolved in 2 to 3 ml of distilled water (freshly prepared) and administered by gastric intubation. The animals were observed for next 72 hrs for behavioral parameters like alertness, grooming, aggressiveness, touch response, tremor, sleep, convulsion, muscle spasm, analgesia, lacrimation, diarrhea, salivation and number of deaths (mortality). The mortality rate observed was subjected to Karber formula for the determination of LD_{50} value.

LD_{50} = Least lethal dose in a group - $\sum$ (axb) / N, Where N is the number of animals in each group, a- dose difference and b- mean mortality. The animals were sacrificed after 72 hr; blood samples collected were immediately used for serum separation and stored at 40°C until analysis. Enzyme assays were performed within 2 hrs of sample collection. The whole blood collected was subjected to hemoglobin estimation and cell counting. In serum samples aspartate transaminase, alanine transaminase, alkaline phosphatase, urea, creatinine and protein were assayed by using standard kits and semi autoanalyzer.

Histopathological Studies

For histopathology examination, liver, brain, kidney, intestine and heart were excised and rinsed with ice-cold saline (0.9% sodium chloride) to remove blood and adhering tissues debris. The tissues were then fixed in 10% formalin for 24 h. The fixative was removed by washing through running tap water and after dehydration through a graded concentration of alcohol. The tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut into 5 µM thickness and stained with hematoxylin and eosin and observed under light microscope for details.

Statistical analysis

The data represent mean ± standard deviation (SD) and the results were analyzed statistically by student's t test. Changes were considered statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$.

RESULTS

Phytochemical analysis

HPLC analysis revealed that one gram of the dry fruit contain phenolics (1.72mg), alkaloids (2.85mg), flavonoids (0.56mg), rutin

(0.017mg), gallic acid (0.045mg), galangin (0.049mg), thymoquinone (0.0012 mg) and quercetin (0.2336 mg). GC analysis showed the level of terpenoids as 1.06 mg/g. The major flavonoid detected was quercetin (0.2336 mg/g) (Table 1; Figure 1 (A, B)).

Table 1: **Quantity of phytochemicals in HAETC**

Phytochemicals	Quantity (mg/g of dry fruit)
Alkaloids	2.85
Total phenols	1.72
Terpenoids	1.06
Flavonoids	0.56
Rutin	0.017
Gallic acid	0.045
Galangin	0.049
Thymoquinone	0.0012
Quercetin	0.2336

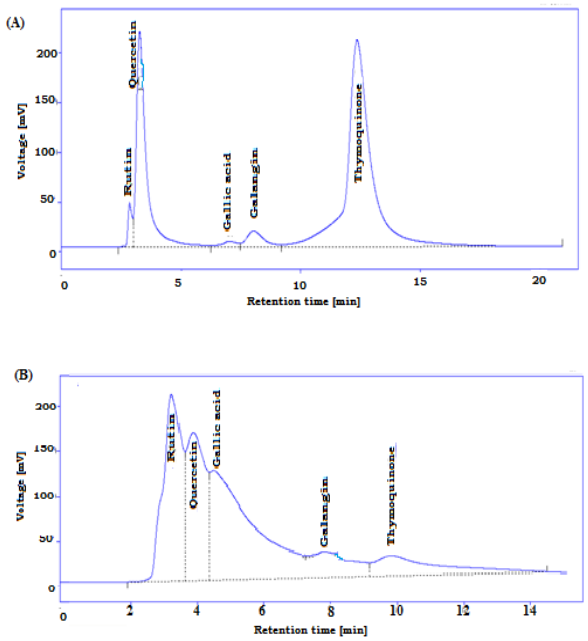

Fig. 1: HPLC chromatogram of HAETC. (A) HPLC fingerprint of mixed standard (Rutin, quercetin, gallic acid, galangin and thymoquinone) (B) HPLC fingerprint of HAETC.

Free radical scavenging activity

Figure 2 illustrates the concentration dependent free radical scavenging activity of AFETC .The IC_{50} value of AFETC (37.40 µg /ml)) was found to be nearer to that of standard ascorbic acid (38.76 µg /ml).

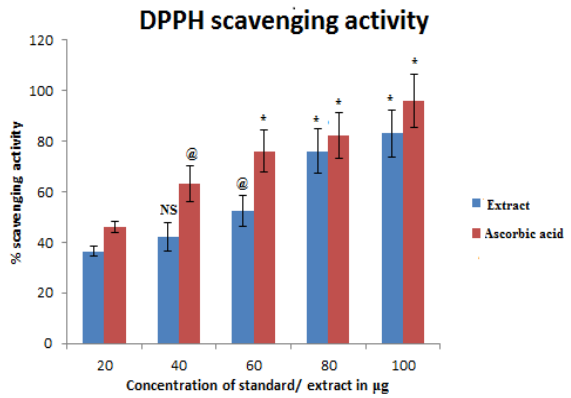

Fig. 2: DPPH scavenging activity of aqueous fruit extract of *Terminalia chebula* and standard ascorbic acid.

Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

Superoxide radical scavenging activity of the extract is shown in Figure 3. The IC_{50} value of aqueous extract of *T.chebula* and rutin was 37.85 µg /ml and 40.57 µg /ml respectively. The results show that AFETC is found to exhibit a potent superoxide scavenging activity which is an essential criteria for medicinal plants used for various therapeutic purposes.

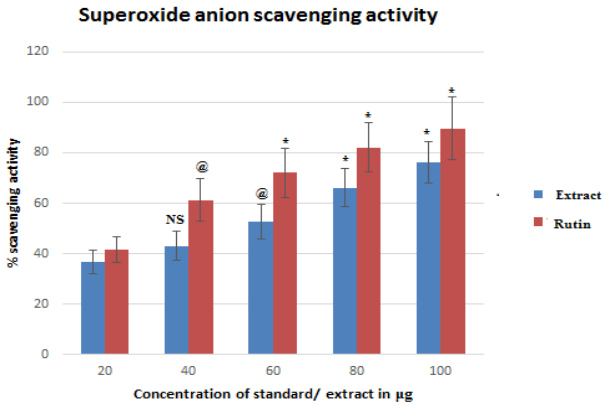

Fig. 3: Superoxide anion scavenging activity of aqueous fruit extract of *Terminalia chebula* and standard rutin.

Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are

expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

The nitric oxide scavenging activity is shown in figure 4. The IC_{50} value of the extract and standard rutin were 34.37 µg /ml and 45.84 µg /ml respectively. The data indicates that AFETC showed a stronger potency than standard rutin in scavenging nitric oxide.

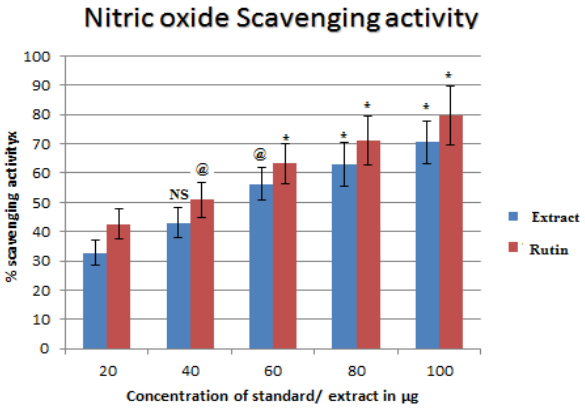

Fig. 4: Nitric oxide scavenging activity of aqueous fruit extract of *Terminalia chebula* and standard rutin

Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

The figure 5 shows the dose response curve for the reducing power of AFETC and standard ascorbic acid. In the reducing power assay, the presence of antioxidants in the sample would have resulted in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700nm. Increasing absorbance at 700nm indicated increase in the reductive ability. The results indicate that AFETC possess a good reducing potential.

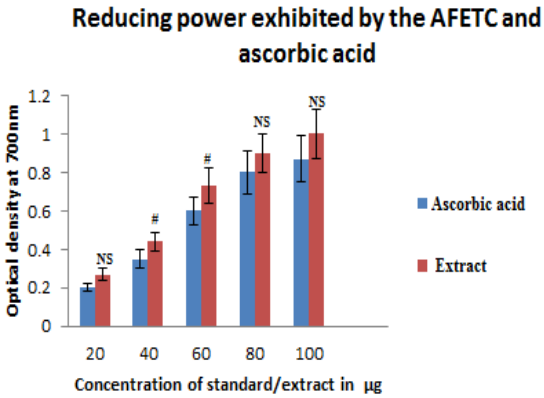

Fig. 5: Reducing power of aqueous fruit extract of *Terminalia chebula* and standard ascorbic acid.

Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

Acute toxicity study

We found that the animals did not show any abnormal symptoms up to the dose of 2000 mg/ kg b.wt of HAETC. There were no changes in the normal behavioral pattern and no signs and symptoms of toxicity were observed. The animals did not show any signs of tremor, convulsion, salivation, diarrhea, lethargy, sleep, coma, dyspepsia, nasal bleeding etc., up to the dose of 2000mg/kg b.wt of HAETC .The mortality was zero at this dosage. Mild alterations were observed in the animals received 2500 mg/ kg b.wt of HAETC. The LD_{50} value of HAETC was found to be 2475mg according to the method of Karber (Table 2).

Table 2: Determination of LD_{50} value of HAETC by arithmetic method of Karber

Group	Dose(mg/Kg b.wt)	No of animals dead	
1	0	0	
2	100	0	
3	200	0	
4	500	0	
5	1000	0	
6	2000	0	
7	2500	1	
Group	Dose difference(a)	Mean mortality(b)	Probit (axb)
8	(2500-2000) 500	0.5	250

Sum of the product = 250, LD_{50} = Least lethal dose in a group – Σ (axb)/N

LD_{50} = 2500-250/10, = 2500-25, = 2475 mg/ kg b. wt

Table 3: Activity of serum marker enzymes, hematological parameters and level of serum metabolites in rats administered with HAETC(2000 mg/kg b. wt)

Contents	Control	Test
Aspartate transaminase(IU/L)	22.6 ± 3.1	21.9 ± 3.2 NS
Alanine transaminase (IU/L)	21.0 ± 2.6	19.9 ± 2.1 NS
Alkaline phosphatase (IU/L)	109.5 ± 12.6	113.6 ± 12.9 NS
RBC count(million/cu.mm)	4.2 ± 0.52	4.6 ± 0.51 NS
WBC count(cells/cu.mm)	12900 ± 300	12200 ± 150 NS
Lymphocyte (%)	68.3 ±6.2	70.1± 8.5 NS
Eosinophil (%)	2.1 ±0.2	2.3 ±0.3 NS
Platelet count (lakhs/cumm)	4.2 ± 0.56	4.1± 0.56 NS
Hemoglobin (gm %)	12.7 ± 1.7	13.2 ± 1.6 NS
Glucose (mg/100 ml)	83.5 ± 9.5	84.6 ± 9.2 NS
Protein (g/100 ml)	5.4± 0.61	5.6 ± 0.7 NS
Urea (mg/100 ml)	20.6 ± 3.5	22.5 ± 3.1 NS
Creatinine (mg/100 ml)	0.8 ± 0.07	0.7 ± 0.06 NS

Values are expressed as mean ± S.D for n=6. Statistically significant values are expressed as NS- non significant when compared to that of control rats.

Table 3 show the levels of serum marker enzymes aspartate transaminase, alanine transaminase and alkaline phosphatase, hematological parameters and level of serum metabolites in control and test rats administered with HAETC. There were no alterations in the serum enzymes level, hematological parameters and serum metabolites when compared to those of control rats.

Histological observations in liver, heart, kidney, intestine and brain revealed that HAETC treatment did not produce any significant alterations in tissue architecture when compared with those of normal rats (Figure 6A-E).The histopathological score in each group received HAETC was zero and this shows the non toxic nature of the test drug on major organs.

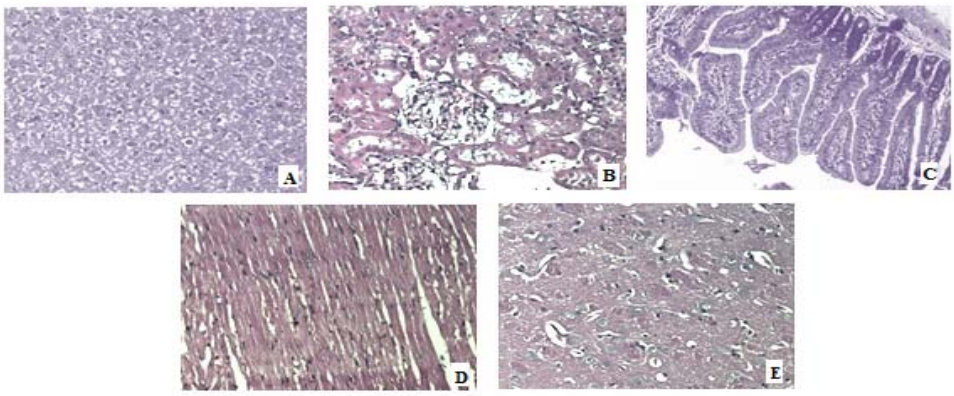

Fig. 6 (A-E): Effect of HAETC (2000mg/kg b.wt.) on liver, kidney, intestine, heart and brain tissues.

Histopathological changes

The photomicrograph of (A) liver showing normal tissue architecture with hepatic cell nuclei (B) kidney showing normal tissue architecture with prominent normal glomeruli (C) intestine showing normal structure and villi membrane (D) heart showing normal striated cardiac muscle cells without any necrosis or edema (E) brain showing normal glial cells without any pathological deposits (H & E, 100x).

DISCUSSION

The wide spread use of *T. chebula* as a traditional medicine demands a preliminary study on the determination of phytochemicals and the evaluation of safer dose to be used for further study. All the phytochemicals present in *T.chebula* possess various medicinal properties. Phenolic compounds are a class of antioxidants acting as free radical terminators [19]. The flavonoids are promising metal chelators which prevent free radical formation in cells [20, 21]. Terpenoids have potent anti- inflammatory property [22].

The results show that HAETC contains rich amount of these bioactive compounds which might be accounted for the antioxidant and anti-inflammatory properties. Free radicals are constantly generated in living cells and cause extensive damage in biomolecules leading to various diseases. The effective antioxidants from plant products are the only alternatives to synthetic antioxidants in counteracting the onset of free radical mediated diseases [23]. The antioxidant property showed that AFETC has the power to scavenge nitric oxide and superoxide anions in different *in vitro* models studied. The proton radical scavenging action has been known as an important mechanism of antioxidation. DPPH radical is a stable organic free radical which has been used for evaluating the free radical scavenging potential of natural antioxidants. In the present study, concentration of AFETC necessary to decrease the initial concentration of DPPH by 50% (IC_{50}) under experimental condition was calculated. A lower value of IC_{50} indicates higher antioxidant activity. The DPPH scavenging activity can also be attributed to the nature of phenolics contributing to their electron transfer/ hydrogen donating ability [24]. The data obtained revealed that the AFETC can reduce free radical formation and hence can alleviate the risk of free radical mediated human diseases. Nitric oxide (NO), an important chemical messenger formed by the enzyme nitric oxide synthase (NOS) in endothelial cells, macrophages, platelets, neurons, etc., is involved in many physiological processes. Excess NO brings about cytotoxic effects as observed in various disorders like AIDS, cancer, Alzheimer's disease and leads to the production of nitrite and peroxynitrite anions, which are potent free radicals [25]. AFETC acts a scavenger of nitric oxide and might be competed with oxygen, leading to reduced production of nitrite ions. This might be due to the antioxidant principles of the extract which might have prevented the formation of No.

Superoxide anion is harmful reactive oxygen species as it damages the cellular components in living cells [26]. Measurement of superoxide anion scavenging activity of the test drug and standard rutin were done based on the reduction of NBT. Superoxide radicals were generated by a non-enzymatic system of phenazine methosulfate and reduced nicotinamide adenine dinucleotide. These radicals reduce nitro blue tetrazolium (NBT) into a purple coloured formazan. The results show that AFETC is found to exhibit a significant superoxide scavenging activity and the effect was comparable to that of rutin.

Reducing Power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant compounds [27]. The AFETC exhibited a good reducing power that was comparable to that of ascorbic acid. It is clear that the extract has reducing potential and could serve as electron donor to terminate the radical chain reaction.

Acute toxicity study

Acute toxicity profiling is the first step in the preclinical assessment and evaluation of the toxic characteristics of a substance to be evaluated for therapeutic action. It can provide data on health hazards that are likely to arise from a short term exposure. The experimental rats treated with different doses of HAETC for a short period of time (72 hrs) were examined for physical discomfort in the form of tremor, convulsion, salivation, diarrhea, lethargy, sleep, coma, dyspea, nasal bleeding, etc. The experimental rats received up to 2000mg/kg b.wt of HAETC did not show any abnormal signs and symptoms during 72 hrs which shows the non toxic nature of the drug. The mortality rate observed was subjected to Karber's formula for the determination of LD_{50} value which was found to be 2475 mg/kg b.wt. The animals which received the test drug of 2500 mg/kg b.wt showed only mild variations and very low mortality. Study of hematological status is one of the important aspects for the diagnosis of a large number of diseases. Alterations in blood parameters may be due to changes in cellular integrity, membrane permeability and exposure to toxic metabolic intermediates. The hematological parameters did not show any significant alterations in RBC, WBC, neutrophil count and eosinophil count and hemoglobin concentration when compared to those of normal rats. The serum

biochemistry was also not altered in drug treated animals showing the safety aspects of T.*chebula*. The serum level of the liver marker enzymes were also not altered in drug treated animals when compared to that of control. The histopathological observation revealed that all the organs tested showed the normal tissue architecture without significant alterations such as necrosis, accumulation of lipid and inflammation in HAETC treated rats.

CONCLUSION

It can be concluded that the hydro alcoholic fruit extract of *T.chebula* is nontoxic and is safe for consumption up to 2000 mg/kg b.wt. Based on the LD_{50} value calculated, 200, 250 and 300 mg/kg b.wt would be chosen as low, mid and high dose for the evaluation of biological activity. The free radical scavenging activity of the test drug may probably down regulate pathological events such as inflammation by preventing the formation and harmful action of toxic oxygen free radicals.

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