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Abstract

Aim of the study: The present study was carried out to evaluate the safety of ethanolic extract of celtis timorensis (EECT) by acute and sub-acute toxicity studies.Materials and Methods: Acute toxicity study was conducted in mice by using OECD 425 guidelines whereas sub-acute toxicity study was carried out in rats by using OECD 407 guidelines. In the acute toxicity study, mice were administered a single dose of 2000 mg/kg and 5000 mg/kg orally and then observed individually for the first four hours, then over a period of 24 hours and at least once daily for 14 days. In the subacute toxicity studies, EECT was given orally at doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight daily for 28 days to male and female rats respectively. General behavior, adverse effects and mortality were observed throughout the experimental period. Food intake, water intake, body weight, organ weight, hematological and biochemical parameters, histopathological changes were evaluated.Results: The limit doses of 2000 mg/kg and 5000 mg/kg did not cause any mortality or signs of acute toxicity in the mice tested during the observation period. In sub-acute toxicity tests, the results did not show any treatment related abnormalities in terms of hematological and biochemical parameters.

Index terms— celtis timorensis, acute toxicity study, subacute toxicity study, rodents, biochemical parameters and hematological parameters.

1 Introduction

atural products which included herbs, animals and minerals serve as the lead compounds for the development of new medicines and also for the treatment and prevention of various human ailments. The present accepted modern medicine has gradually developed in the recent years by various efforts done by the researchers. However, traditional medicine still remains as basis in the development of new drugs [1]. In recent years, herbs and herbal medicines have continued to receive interest and attention from the people as these products are safe and free from side effects [2]. The growing number of herbal drug users around the globe and lack of scientific data on the safety profile of herbal products make it necessary to conduct toxicity study of herbal products [3]. Toxicity associated with herbal products has alerted many national and international regulatory authorities to develop and implement various set of guidelines for assessing, monitoring and preventing the toxicity associated with the herbal products. For example, Uppsala monitoring committee (UMC) of the world health organization (WHO) collates and communicates information regarding herbal adverse drug reactions whereas Organization for Economic Cooperation and Development (OECD) sets guidelines for conducting various toxicity studies. Toxicity tests are most widely used to examine specific adverse events or specific endpoints such as cancer, cardiotoxicity and skin/eye irritation. Toxicity testing also helpful in determining the No Observed Adverse Effect Level (NOAEL) dose and is helpful for further clinical trials [4]. Acute, sub-acute and chronic toxicity tests are routine toxicity tests carried out by the pharmaceutical companies in the development of new medicines. In order to assess the toxic nature of a compound, acute oral toxicity is the first step to be carried out [5]. Acute toxicity testing involves the determination of lethal dose, the dose that kills 50% of the tested group of animals, whereas sub-acute and chronic toxicity testing involves the determination of long term effects of the test compound upon

repeated administration. wood or stink wood. The plant has been recommended in ayurveda for the improvement of memory and in the treatment of nervous disorders. The plant extract has been reported for antidepressant, anticonvulsive and nervous disorders. The extract also enhanced learning and memory in humans [6]. It also helps in repairing of neurons which were damaged in specific brain regions. The plant extract also showed a neuroprotective effect against oxidative stress in the hippocampus of rat brain [7]. Traditionally the leaf extract of celtis timorensis is given during dysentery conditions [8]. Despite the various uses over long time periods, no toxicological data is available regarding the safety of repeated exposure to celtis timorensis. As a part of safety evaluation, acute and sub-acute oral dose toxicity studies were carried out to investigate the potential toxicity after single oral dosing of extract in mice and 28day repeated oral dosing of extract in rats.

2 II.

3 Materials and Methods

4 a) Collection and Identification of Plant Materials

Fresh leaves of celtis timorensis were collected from Tirupathi, Andhrapradesh. The plant was identified and authenticated taxonomically by Assistant professor K.Madhava chetty of the Department of Botany, S.V. University, Tirupathi, Andhra Pradesh, India. A voucher specimen of the collected sample was deposited in the herbarium of the institution for future reference.

5 b) Preparation of the Extract

The leaves are shade dried and made into coarse powder and extracted with 70% ethanol by cold maceration method for 72 hours with intermittent shaking. The extract was filtered and concentrated at high vacuum. The extract was stored in the refrigerator till further use.

6 c) Animals

Swiss albino mice (25-30 g) were selected for acute toxicity studies and Wistar albino rats (weighing between 130 gms-200 gms) of both sexes were selected for sub-acute toxicity studies. They had free access to food and water and were maintained under standard laboratory conditions which included 12-hour light-dark cycle and temperature of 28-30 degrees centigrade. Animals are allowed for a one week of acclimatization period prior to the study. The experimental protocol was approved by the IAEC (institutional animal ethical committee) and care of the experimental animals was taken according to the CPCSEA guidelines.

7 d) Acute Toxicity Studies

Acute toxicity studies of ethanolic extract of celtis timorensis (EECT) was carried out in female mice by using Organization for Economic Co-operation and Development (OECD) guideline 425 [9]. Before oral administration of a single dose of the test samples, the mice were deprived of food for 3 h. Doses of 2000 and 5000 mg/kg of the test samples were given using oral gavage to mice of Group I and Group II respectively. All the mice were observed for general behavioral changes; symptoms of toxicity and mortality after treatment for the first four (critical) hours, then over a period of 24 hours, thereafter daily for 14 days. e) Sub-Acute Toxicity Studies Sub-acute toxicity study (28-day repeated oral toxicity study) was carried out according to OECD 407 guidelines [10]. Both sexes of rats (130-200g) were divided into four groups with 10 animals (5 males plus 5 females in each). The group I received 1% CMC vehicle orally at a dose volume of 10 ml/kg body weight and served as a control group whereas group II, group III and group IV received EECT at 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight, p.o. respectively (10ml/kg body weight dissolved in 1% CMC). All the groups of rats were observed twice daily for mortality and morbidity till the completion of the experiment. All the animals were observed for clinical signs and the time of onset, duration of these symptoms, if any were recorded. Body weights of the rats in all groups were recorded once before the start of dosing, once weekly during the treatment period and finally on the day of sacrifice. The amount of food and water intake was recorded on every day and the data were expressed as 7 days cumulative value. At the end of the experiment (on 29th day), blood samples were collected from overnight fasted rats (only water allowed) by retro-orbital bleeding into heparinized and non-heparinized tubes for hematological analysis and biochemical analysis.

8 f) Hematological parameters

The heparinised blood was used for the analysis of hematological parameters such as hemoglobin, red blood cell count, white blood cell count, platelet count were measured using fully automated hematology analyser (PE 6000).

9 g) Biochemical Parameters

The serum was separated from non-heparinized blood and the serum biochemical parameters including total cholesterol, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

(ALP), blood urea nitrogen (BUN), triglycerides, total cholesterol, albumin, bilirubin and total protein were analysed by using semi-automatic biochemical analyser (Star 21plus, India).

10 h) Histopathology

After blood collection on day 29, all the animals are euthanized for gross pathological examinations of all major internal organs. Organs such as liver, kidney, stomach, brain, heart and spleen were collected from all the animals for histopathology. The collected organs were weighed and preserved in 10% neutral buffered formalin, trimmed and a 5? thickness of tissue sections were stained with hematoxylin and eosin for histopathological study.

11 i) Statistical Analysis

Results are expressed as mean ±standard error mean (SEM). Data obtained was analyzed by using one way ANOVA followed by Dunnett's test and $p<0.05$ was considered as statistically significant.

12 III.

13 Results

14 a) Acute Toxicity Studies

In the toxicity study, oral administration of the EECT at 2000 mg/kg and 5000 mg/kg did not produce any deaths and clinical signs of toxicity in mice. As there were no mortality and clinical signs of toxicity in both the tested doses, LD50 value of EECT was found to be greater than 5000 mg/kg.

15 b) Sub-Acute Toxicity Studies

There were no treatment related toxicity signs and mortality observed in both sexes of rats treated at 250mg/kg, 500mg/kg and 1000mg/kg orally during the 4 weeks of treatment. No significant differences in body weight were observed between the initial and final body weight of the rats treated with EECT and control rats (Table 1). A similar absence of toxic effect was observed in the case of food and water consumption (Table 2 and Table 3). There were no significant differences between control and EECT treated groups in organ weight (Table 4). The hematological profile of treated and control group were summarized in Table 5. The results concluded that all hematological parameters such as total red blood cell count, total white blood cell count, platelet count, haemoglobin, hematocrit and differential leukocyte count are with in normal range in both control and treated groups during the experimental period. The data on biochemical parameters in treated and control rats were presented in Table **??**. Sub-acute administration of EECT did not show any significant changes in biochemical parameters such as creatinine, urea, triglycerides, total cholesterol, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin when compared to control groups. There were no statistically significant differences in the hematological parameters and biochemical param-eters measured between control and EECT treated groups. In our study, we performed histopath-ological examinations in control and high dose group-brain, heart, liver, kidney, stomach and spleen and they were revealed no abnormalities. Hence we did not performed histopathology examination of low and medium dose groups. The No-Observed Adverse Effect level (NOAEL) of the extract was estimated to be greater than 1000 mg/kg/day in rats. Hence it can be concluded that EECT is safe for oral administration. Values are expressed as mean±SEM, n=5 females and 5 males. Values are expressed as mean±SEM, n=5 females and 5 males. Values are expressed as mean±SEM, n=5 females and 5 males.

16 Discussion

In developing countries, herbal products prepared from medicinal plants have become famous in healthcare and some have been falsely considered as safe as they are obtained from natural sources. Nevertheless, these bioactive compounds from traditional medicinal plants are concluded to be safe without understanding the possible health effects and thus commonly used as self medication [11]. However, there is a lack of data on the toxicological profile and adverse effects of these compounds. Therefore, acute toxicity study is required not only to identify the further range of doses in animal studies but also to explain the probable clinical signs evoked by the test compounds under investigation. It is also an important effective parameter for calculating the therapeutic index of drugs and chemicals [12]. Results obtained from toxicity studies on animals will be critical for positive judgement on the safety of medicinal plants if they are found to have adequate potential for development into pharmacological compounds [13]. As the use of plant based products increases, it is important to screen the toxicological profile of these plants to confirm the safety and efficacy of those natural sources. Hence the present study was undertaken to evaluate the acute and subacute toxicity of ethanolic extract of celtis timorensis (EECT).

Throughout the 14 days of observation period, no morbidity or mortality was observed in the extract treated mice. In the present study, the results showed no adverse events in the dose groups 2000 mg/kg and 5000 mg/kg which indicate that the LD50 was greater than 5000 mg/kg. The sub-acute dose was selected based on the rats LD50 value which kept rats alive, i.e. 1/5, 1/10 and 1/20 of 5000 mg/kg. In the repeated dose 28-day oral toxicity study, there were no deaths and treatment-related signs were observed in all the groups Volume XIV

Issue III Version I of animals. After exposure to a few possible toxic substances, there will be changes in body weight gain and internal organ weights which would reflect toxicity [14]. The body weight changes are markers of adverse effects of drugs and chemicals and if the body weight loss occurred is more than 10% of the initial body weight it will be considered as statistically significant [14,15]. Organ weight also is an important indicator of physiological and pathological status of animals. The relative organ weight is fundamental to confirm whether the organ weight was exposed to the injury or not. The heart, liver, kidney, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicant [16]. There were no significant differences in body weight gain of both control and treated groups. In the present study, organ weights in all the treated groups of both sexes were not significantly different from those of control groups. Hence it can be concluded that EECT is almost non-toxic. It is also important to measure the food intake and water consumption during the study of the safety of a product with medicinal purpose, as proper intake of supplements is necessary to the physiological status of the animal and to the achievement of a better response to the test substance under investigation [17,18]. In this study, the food intake and water consumption also was not affected by the administration of EECT and it did not promote any appetite suppression and had no unfavourable effects. Thus, this indicates there was no interruption in the metabolism of carbohydrate, protein and fat.

Analysis of blood parameters is important in the evaluation of risks associated with test compounds under investigation as the changes in the hematological system have a greater indicative value for human toxicity, when the data are converted from animal studies [19]. In the present study, treatment with EECT for 28 days did not produce any changes in hematological parameters (i.e. hemoglobin, platelet count, white blood cell count, red blood cell count) which indicate that the extract did not affect the blood cellular components or their production. Transaminases such as SGOT and SGPT are well known good of liver function and used as biomarkers to conclude the probable toxicity of drugs and xenobiotics [20]. Normally, destruction to the liver parenchymal cells will result in an increase of both these enzymes in the blood [21]. There were no changes in the ALT and AST levels, which reveal that the extract did not affect the liver function/ or metabolism. Elevated bilirubin levels are an indication of altered liver functions and a small elevation is an important indicator of liver damage in laboratory animals or could be a sign of biliary duct obstruction. In order to assess the synthetic capacity of the liver, determination of plasma proteins like albumin is required and decrease in plasma proteins therefore tend to reflect chronic damage **??**22]. There were no significant differences in the levels of AST, ALT, bilirubin and total protein between the control and treated groups. These indicate that EECT did not cause any damage to the liver. The normal values of kidney parameters such as blood urea nitrogen (BUN) and creatinine suggest that sub-acute administration of EECT did not cause any damage to the kidney. Histopathological studies provide supportive evidence for biochemical and haemat-ological observations. No abnormality was recorded to histopathological examinations of all organs examined. Since there were no signs of toxicity with respect to hematology, biochemistry, organ weight, body weight and histopathological examination noted in all the tested groups, it can be inferred that EECT will not produce any toxicity. Based on the results, the No Observed Adverse Effect Level (NOAEL) of the extract is greater than 1000 mg/kg/day.

V.

17 Conclusion

Treatment with single oral doses of 2000 mg/kg and 5000 mg/kg did not result in any toxic signs or mortality in the acute toxicity studies. Daily oral administration of ethanolic extract of celtis timorensis for a period of 28 days did not cause mortality, changes in body weight and body weight gain. Also, no significant changes in hematological, biochemical and histopathological alterations were observed at the end of the duration of the experiment. Hence, the no-observed adverse-effect level of the extract was found to be exceed 1000 mg/kg/day p.o. Overall, it can be concluded that the ethanolic extract was well tolerated in daily dose at 1000 mg/kg for a period of 28 days. [1]

Figure 1: N

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Study'

Figure 2: Table 1 :

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Treatment group	Sex	Gms First week	Second week	Third week	Fourth week
Control (1% CMC)	Males (n=5)	44.72±1.30	39.11±2.81	42.58±2.2	43.25±1.46
	Females(n=5)	40.15±1.50	40.55±1.75	39.17±2.42	41.45±1.68
250 mg/kg	Males (n=5)	40.87±1.67	36.12±2.24	38.50±1.84	44.85±2.03

[Note: B© 2014 Global Journals Inc. (US)]

Figure 3: Table 2 :

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Treatment group	Sex	(ml) First week	Second week	Third week	Fourth week
Control (1% CMC)	Males (n=5)	55.85±3.33	46.71±2.86	49.71±2.69	58.42±2.67
	Females(n=5)	45.57±2.71	44.92±2.32	48.21±2.21	48.42±3.82
250 mg/kg	Males (n=5)	49.28±2.74	43.28±1.86	57.00±3.65	53.28±2.56
	Females(n=5)	48.00±2.13	46.42±2.09	49.28±3.62	46.85±2.26
500 mg/kg	Males (n=5)	52.57±3.19	56.14±3.54	55.57±3.06	51.57±1.51
	Females(n=5)	44.71±2.37	51.85±2.29	47.85±3.32	52.01±0.95
1000 mg/kg	Males (n=5)	49.85±2.08	55.57±1.41	50.57±3.18	51.57±3.48
	Females(n=5)	48.64±1.78	50.71±2.84	48.21±2.14	47.14±2.36

Figure 4: Table 3 :

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Study

Figure 5: Table 4 :

5

		Toxicity Study			
Hematological Parameter	Sex	Control	Treatment group 250 mg/kg	500 mg/kg	1000 mg/kg
Hemoglobin (g/dl)	Males (n=5)	14.74±0.50	14.30±0.75	14.34±0.58	13.72±0.67
	Females(n=5)	15.68±0.59	16.24±0.30	15.76±0.79	15.94±0.42
RBC count (x10 6 /?l)	Males (n=5)	4.92±0.23	4.68±0.25	4.72±0.19	4.84±0.34
	Females(n=5)	5.52±0.22	6.26±0.17	6.44±0.26	6.06±0.41
WBC count (x10 3 /?l)	Males (n=5)	8.84±0.70	9.56±0.61	9.90±0.41	8.46±0.60
	Females(n=5)	10.48±0.48	9.12±0.60	9.36±0.57	9.10±0.38
Platelet count (x10 3 /?l)	Males (n=5)	806.32±38.21	813.99±41.88	797.31±53.46	806.25±36.90
	Females(n=5)	834.31±39.97	859.00±42.11	817.15±38.96	830.45±40.24
Hematocrit (%)	Males(n=5)	47.42±2.40	47.94±2.36	46.36±1.83	45.46±2.11
	Females(n=5)	42.06±1.05	42.70±1.22	41.84±0.81	42.22±0.75

Figure 6: Table 5 :

Values are expressed as mean±SEM, n=5 females and 5 males. Values are expressed as mean±SEM, n=5 females and 5 males.

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