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Evaluation of Preliminary Toxicity Studies on the Methanolic Leaves Extract of *Tylophora asthmatica* in Experimental Rats

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Abstract: The methanolic leaves extract of *Tylophora asthmatica* was screened for its toxicological and biochemical effects on rats, because of the traditional healers of India uses this plant as an anti-inflammatory and anti-anaphylactic. The extract was safe in the smaller doses needed to produce a therapeutic effect, (LD_{50} = 223.6 mg kg^{-1} body weight) and had significant toxic effect on the liver at extremely high doses leading to death of the animal. In acute toxicity study (72 h), single dose of the methanolic extract of *Tylophora asthmatica* (META) leaves (50, 100, 200, 500 and 1000 mg kg^{-1} body weight) were given to male rats. Smaller doses of META (50, 100 and 200 mg kg^{-1} body weight) produced no signs of poisonous or death in animals while 500 mg kg^{-1} body weight caused death of two animal and 1000 mg kg^{-1} body weight caused death of four animals within 72 h. The degree of protection was also measured by evaluating biochemical indices like serum AST, ALT, ALP, total protein, albumin, globulin and bilirubin. In addition, sub-chronic administration for 15 days showed a significant ($p<0.05$) increase in the serum ALT, ALP and reduction in total protein, albumin and globulin showing that the plant leaves extract i.e. META has hepatoprotective effects after prolonged use. These studies demonstrated that the META is (50-200 mg kg^{-1} body weight) safe and did not cause any detrimental effects *in vivo* under the conditions investigated in this study.

Key words: Toxicity, marker enzymes, *Tylophora asthmatica*, tylophorine, hepatoprotective

INTRODUCTION

Medicinal plants are natural resources yielding valuable phytochemical products which are often used in the treatment of various diseases. Crude extract of these medicinal plants is used in Ayurvedic preparations. Although medicinal plants impart enumerable virtues, their use in the protection of internal body organs has gained impetus in the past decades. Hence, *Tylophora asthmatica,* a wild indigenous plant, belongs to the family Asclepidaceae and is commonly known as Indian ipecac. The powdered leaves, stems and root of *Tylophora asthmatica* contains 0.2-0.3% alkaloids, of these, tylophorine, tylophorinine and tylophorinidine are important alkaloids (Gopalakrishnan *et al*., 1980). Various studies have confirmed the anti-inflammatory activity (Gopalakrishnan *et al*., 1979), direct stimulant of adrenal cortex (Udupa *et al*., 1991), anti-asthmatic (Shivpuri *et al*., 1972) and the treatment of bronchitis, rheumatism and dermatitis (Nadkarni, 1976). The extract from the leaves and stems of *T. asthmatica* possess anti-feedent, insecticidal and anti-tumour properties (Verma *et al*., 1986). So far limited information is available on toxicological and biochemical effects of *T. asthmatica* on experimental rats. So, the present investigation was carried out to obtain data on the safety of META, focusing on the acute toxicity, sub-chronic toxicity and maximum tolerable dose on biochemical parameters.

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MATERIALS AND METHODS

Collection and Processing of Plant Material

The fresh, mature leaves of the plant was collected during the month of February-2006 in the Banks of Cauvery river, Trichy, South India and these were botanically identified and authenticated. Samples of the plant leaves were also preserved for the future reference which was labeled as TAL-12. The leaves were shade dried, powdered and kept in a well-closed container until further use.

Preparation of Plant Extract

The shade dried, powdered leaves (500 g) of the plant were extracted with petroleum ether (60-80°C) using soxhlet apparatus to remove lipids. It was then filtered and filtrate was discarded. The residue was then extracted with methanol using soxhlet apparatus. The extract was completely dried *in vauco*, stored in refrigerator at 4°C and protected from sunlight until the extract administration. The yield of methanolic dried extract was 8.63% (w/w). This was used for both the acute and sub-chronic toxicity.

Animals

Male wistar rats (150-175 g) were housed in under standard animal house conditions of humidity, temperature and provided on rat pellet diet (manufactured by Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. After randomization into various groups, the rats were acclimatized for a period of 2-3 weeks in the new environment before initiation of experiment. The experiment were designed and conducted in accordance with the ethical norms approved by Ministry of Social Justice and Empowerment, Govt. of India and Institutional Animal Ethics Committee Guidelines.

Study of Acute Toxicity

Acute toxicity (LD_{50}) was determined in rats according to Lorke's method (1983) to the oral dose levels ranges from 50, 100, 200, 500, 1000 mg kg^{-1} body weight. The clinical signs such as weakness or aggressiveness, food refusal, loss of weight, diarrhoea, discharge from eyes and ears, noisy breathing and the number of deaths in each group of rats (within 72 h) were monitored carefully. Thereafter, the animals were sacrificed by cervical dislocation and serum was separated to determine some of marker enzymes and other biochemical indices. The LD_{50} is the geometric mean of the product of the dose A that produced 100% clinical signs in one group and dose B that produced 0% mortality in another group. If there was any inconsistency, then probit log was applied. Therefore, the acute toxicity was determined as $LD_{50} = \sqrt{(A \times B)} = \sqrt{(1000 \times 50)} = 223.6$ mg kg^{-1} body weight.

Study of Sub-chronic Toxicity

Four groups of 6 wistar rats, each were given orally saline-control; 100, 200 and 300 mg kg^{-1} body weight of the META (experimental groups), respectively for 15 days based on the above calculated LD_{50} value. The animals were observed for all external symptoms of toxicity and mortality. The animals were anesthetized and sacrificed 24 h after the last treatment by cervical dislocation and serum was separated for marker enzymes and other biochemical examinations.

Relative Organ Weight

Different organs namely the liver, kidney, spleen and heart were surgically dissected out and weighed in grams (absolute organ weight). The relative organ weight of each animal was then calculated as follows;

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}} \times 100$$

Assessment of Liver Functions

Serum was separated by centrifugation and aspartate transaminase (AST) and alanine transaminase (ALT) were estimated according to King's (1965a) method. The activity of serum alkaline phosphatase was estimated by the method of King (1965b). Bilurubin concentration was calculated by the method of Malloy and Evelyn (1937). Total protein was determined by Lowry *et al*. (1951). Lastly, the albumin and globulin values were estimated using Bromocresol green (BCG) method (Doumas and Biggs, 1972).

Statistical Analysis

All the grouped data were statistically evaluated with SPSS/10.0 software. The results were expressed as mean±SEM for six animals in each group. Differences between control and experimental groups were assessed by hypothesis testing methods included one-way analysis of variance followed by Least Significant Difference (LSD) test. $p<0.05$ was considered to indicate statistical significance.

RESULTS

The effect of META on the body weight of rats during sub-chronic administration for 15 days was showed in Table 1. The mean body weight loss was significantly ($p<0.05$) increased in the group treated with 300 mg kg $^{-1}$ body weight as compared to control group.

There was no significant ($p<0.05$) changes in relative organ weight of liver, kidney, spleen and heart of rats treated with META up to 200 mg kg^{-1} body weight in relation to control groups. However, 500 and 1000 mg kg^{-1} body weight of META produced a significant ($p<0.05$) decrease in the relative organ weight of liver, kidney, spleen and heart (Table 2). Similar changes in the relative organ weight of rats treated with META for 15 days were showed in the Table 3. Also, a significant ($p<0.05$) decrease in the relative organ weight of liver, kidney and spleen of rats treated with 300 mg kg^{-1} body weight as compared to control rats (Table 3).

Table 1: Effects of META on the body weight of rats treated with sub-chronic oral administration for 15 days

Doses (mg kg^{-1})	Body weight at day 1	Body weight at day 15	Body weight loss (days 1-15)
	(g)		
Control	150.9±3.65	147.1±3.72	3.8±0.187
100	151.5±3.35	148.0±3.26	3.5±0.833
200	155.2±4.26	151.2±4.35	4.0±0.563
300	153.8±5.11	146.5±5.21	7.3±0.219*

Values are Mean±SEM; *: $p<0.05$ vs control

Table 2: Relative weight of selected organs 72 h after administration of a single (oral acute) dose of META

Doses (mg kg^{-1})	Relative organ weight			
	Liver	Kidney	Spleen	Heart
Control	4.01±0.10	0.66±0.01	0.56±0.01	0.39±0.01
50	4.09±0.11	0.66±0.02	0.53±0.01	0.40±0.03
100	3.84±0.12	0.69±0.01	0.53±0.02	0.39±0.02
200	3.96±0.04	0.65±0.05	0.57±0.03	0.41±0.03
500	3.05±0.07*	0.58±0.21*	0.45±0.01*	0.37±0.01
1000	2.85±0.02*	0.51±0.09*	0.39±0.02*	0.30±0.01*

Values are Mean±SEM; *: $p<0.05$ vs control

Table 3: Relative weight of selected organs after 15 days of sub-chronic oral administration of META

Doses (mg kg^{-1})	Relative organ weight			
	Liver	Kidney	Spleen	Heart
Control	4.44±0.10	0.63±0.01	0.54±0.01	0.41±0.01
100	4.41±0.04	0.64±0.01	0.56±0.01	0.39±0.01
200	4.47±0.02	0.62±0.02	0.55±0.02	0.39±0.01
300	3.65±0.02*	0.52±0.01*	0.43±0.01*	0.40±0.02

Values are Mean±SEM; *: $p<0.05$ vs control

Table 4: Effects of META on the serum AST, ALT and ALP levels of rats 72 h after administration of a single (oral acute) dose

Doses (mg kg^{-1})	AST	ALT	ALP
	---------- (IU L^{-1}) ----------		
Control	25.4±0.94	15.25±0.94	106.17±3.35
50	23.9±1.04	21.10±1.21*	100.80±3.14
100	26.3±2.17	15.84±0.56	104.12±3.08
200	24.6±1.20	16.79±1.08	99.45±5.08
500	20.3±1.78*	20.64±0.38*	98.69±2.59
1000	19.7±0.85*	21.93±0.24*	128.57±3.48*

AST = Aspartate transaminase; ALT = Alanine transaminase; ALP = Alkaline phosphatase; Values are Mean±SEM; *: p<0.05 vs control

Table 5: Effects of META on the serum AST, ALT and ALP levels of rats during sub-chronic (oral) administration for 15 days

Doses (mg kg^{-1})	AST	ALT	ALP
	---------- (IU L^{-1}) ----------		
Control	29.30±2.10	16.41±1.16	106.06±3.27
100	28.67±1.24	14.90±1.60	99.59±2.31
200	29.89±1.50	18.15±1.09	109.04±2.84
300	28.91±2.07	24.80±1.26*	116.18±1.66*

AST = Aspartate transaminase; ALT = Alanine transaminase; ALP = Alkaline phosphatase; Values are Mean±SEM; *: p<0.05 vs control

Table 6: Effects of META on the total protein, albumin, globulin, A/G ratio and serum bilirubin of rats 72 h after administration of a single (oral acute) dose

Doses (mg kg^{-1})	Total protein	Albumin	Globulin	Bilirubin	A/G ratio
	---------- (mg dL^{-1}) ----------				
Control	7.9±0.18	3.3±0.04	4.6±0.11	0.33±0.06	0.72±0.01
50	7.7±0.24	3.3±0.14	4.4±0.12	0.31±0.01	0.75±0.01
100	7.8±0.22	3.4±0.16	4.4±0.24	0.35±0.02	0.77±0.03
200	8.0±0.25	3.5±0.08	4.5±0.13	0.34±0.01	0.79±0.02
500	6.5±0.19*	2.6±0.12*	3.9±0.07*	0.41±0.03*	0.65±0.01*
1000	6.1±0.23*	2.4±0.09*	3.7±0.05*	0.44±0.01*	0.63±0.02*

Values are Mean±SEM; *: p<0.05 vs control

Table 7: Effects of META on the total protein, albumin, globulin, A/G ratio and serum bilirubin of rats during sub-chronic administration (oral) for 15 days

Doses (mg kg^{-1})	Total protein	Albumin	Globulin	Bilirubin	A/G ratio
	---------- (mg dL^{-1}) ----------				
Control	7.6±0.13	3.3±0.01	4.3±0.04	0.33±0.03	0.74±0.02
100	8.1±0.27	3.5±0.01	4.6±0.13	0.32±0.02	0.76±0.01
200	7.4±0.34	3.1±0.03	4.4±0.18	0.34±0.01	0.73±0.04
300	6.3±0.19*	2.4±0.01*	3.6±0.07*	0.33±0.02	0.65±0.03*

Values are Mean±SEM; *: p<0.05 vs control

There was a significant (p<0.05) decrease in AST and significant (p<0.05) increase in ALT activity after 72 h of treatment, in the group administered 500 and 100 mg kg^{-1} body weight of the META. Also ALP activity showed a significant (p<0.05) decrease in the group administered 100 mg kg^{-1} body weight of META. In addition, a remarkable (p<0.05) increase in the ALT activity in group treated with 500 mg kg^{-1} body weight as compared to control group (Table 4).

Similar results also found in the administration of META to the rats for 15 days (sub chronic toxicity). There was a significant (p<0.05) elevation of ALT and ALP levels in the group of rats treated with 300 mg kg^{-1} body weight as compared to control group. No significant (p<0.05) changes was observed in AST activities after 15 days treatment with META (Table 5).

The results also depict a significant (p<0.05) decrease in total protein, albumin, globulin and A/G ratio and a significant (p<0.05) increase in total bilirubin with group of rats treated with 500 and 100 mg kg^{-1} body weight (Table 6) in relation to control. Similarly, the group treated with 300 mg kg^{-1} body weight for 15 days showed the similar changes. However, there was no significant change in the total bilirubin level in groups treated with META for 15 days (Table 7).

DISCUSSION

The present study showed some interesting biological activities of the plant extract of *Tylophora asthmatica*. Few phytochemical - UV absorption, TLC and HPLC - studies have been conducted on this plant extract (data not shown). Known alkaloids, anthocyanins and flavanoids have been used as reference substances for phytochemical examinations. These examinations rendered possible us to confirm the presence of the phenanthroindolizidine alkaloids-tylophorine and tylophoridine and those biological activities of the plant extract.

The toxicological investigation of the META studied has shown that a dose of 200 mg kg^{-1} body weight can be tolerated. The toxic effects were observed from 500 mg kg^{-1} body weight. The LD_{50} of 223.6 mg kg^{-1} body weight calculated from the acute administration of the META showed that the plant extract is moderately toxic, which is due to the presence of the cytotoxic alkaloids-tylophorine, tylophorinine and tylophoridine in the META (Wei *et al*., 2006).

The significant decrease ($p<0.05$) in body weight could be due to decrease in food intake which probably resulted from the decrease in food efficiency ratio. However, the same extract when given in the increased concentration (500 and 1000 mg kg^{-1} body weight) caused irritation, restlessness, anorexia, hyperexcitability, dyspenia, salivation, diarrohea, nasal discharge and death. Finally, 500 mg kg^{-1} body weight caused death of two animals and 1000 mg kg^{-1} body weight caused death of four animals within 72 h.

The significant changes in the relative organ weight of liver, kidney, spleen and heart could be as a result of the above said phenanthroindolizidine alkaloids induced cytotoxic injury by the META to the cells and tissues of these organs and therefore explain the possible cytotoxicity of these alkaloids at higher doses (Wei *et al*., 2006). And also the decrease in the weight of these organs at higher concentrations of META is due to the disintegration of cytoplasmic materials and necrotic changes as observed in the findings. These changes are further associated with the decrease in the protein contents which is clearly observed in the present experiment.

Proteins are considered as important constituents for the formation of tissues and various enzymes. Higher concentrations of META are known to be degrading the proteins and also to form new types. It is demonstrated that tylophora alkaloids irreversibly inhibit the protein synthesis at the degradation stages of the translation (Wei *et al*., 2006) and then found to induce cell apoptosis (Ganguly and Khar, 2002).

The extent of liver injury is associated by the increased serum levels of AST and ALT. These injuries could be acute or chronic, reversible or irreversible (Mandal *et al*., 2000; Bhakta *et al*., 2001; Janbaz *et al*., 2002). Of the two enzymes, ALT is thought to be more specific for hepatic injury because it is present mainly in the cytosol of the liver cells and in low concentration elsewhere. AST has cytosolic (20%) and mitochondrial forms (80%) (Mayne, 1996). Therefore, AST appears in higher concentrations in number of tissues (liver, kidney, heart and pancreas) and is released slowly in comparison to ALT. But since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocelluar damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Al-Mamary *et al*., 2002). Thus, administration of META shown significant ($p<0.05$) decrease in total protein, AST and increase in ALT levels.

The activity of ALT may therefore indicate more hepatic injury in the administration of META at higher doses leading to decrease in the liver weight and toxicity and chronic administration decreased the AST, total protein and could therefore serve to protect the liver. Moreover, the normal level of Bilurubin at acute and sub-chronic administration may indicate the normal breakdown of RBC and support the fact that META can serve as a liver protective agent at lower doses.

Globulins are major group of proteins in the body directed specifically to fight against infections. The decrease observed in acute and sub-chronic administration of levels of globulin and albumin could imply that the administration of the META does not reduces the power of the subjects to fight against infections and inflammation of essential organs, such as liver.

ALP is an enzyme that transports metabolites across the cell membrane. Liver and bone diseases are the most common causes of pathological elevation of ALP levels, although ALP may originate from other tissues (Mayne, 1996). Hence, the significant increase in ALP levels shows that the possible cholestasis occurred at higher doses.

Based on the AST, ALT, ALP, total protein, bilurubin, albumin and globulin levels in the serum of rats, META (at lower doses) has been suggested to possess hepatoprotective activity.

CONCLUSIONS

The toxicity effects of META was due to its phenanthroindolizidine alkaloids-tylophorine, tylophorinine and tylophoridine-at higher concentrations. This study also shows that acute administration of META could be more dangerous than chronic administration at higher concentrations, which could be mainly due to hepatocytes toxicity. However, chronic administration of the extract had hepatoprotective potential at lower doses. The hepatoprotective potential of META and also its moderate toxic effects on essential organs in acute and sub-chronic form may indicate that the plant extract should be used in lower concentrations with great caution and be treated as a drug.

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