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Toxicity Assessment of Ethanolic Extracts of *Amaranthus Spinosus* L., in Wistar Rats Using OECD 407 Guidelines

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Abstract

The various parts of *Amaranthus spinosus* (*Amaranthaceae*) extracts have the following activity viz. analgesic, antiinflammatory antidiabetic, antihyperlipidemic, antimalarial and antioxidant properties; antiprotozoal, hepatoprotective, immuno-modulatory and spermatogenic effects Despite its potential therapeutic uses, the toxicity profile of *Amaranthus spinosus* has not been evaluated. This study assessed the sub-acute toxicity effects of whole plants of *Amaranthus spinosus* ethanolic extract (ASE). The sub-acute toxic effect of the ASE (1000 mg/kg) was investigated by administration for 28 consecutive days as per the OECD 407 guideline. The weekly body weights were recorded. The animals were euthanized on the 29th day, and blood samples were obtained for the hematological and biochemical investigations. The kidney and uterus were subjected for histological examinations.

Key Words: OECD 407 guidelines, *Amaranthus spinosus,* biochemical investigations.

Introduction

Amaranthus spinosus is an erect, monoecious herb, up to 100–130 cm tall, much branched, the stems are terete or obtusely angular, glabrous or slightly pubescent, green or variably suffused with purple. (1) The leaves of A. spinosus are alternate and simple without stipules; petiole is approximately as long as the leaf blade; the blade is ovate-lanceolate to rhomboid, acute slightly decurrent at base, obtuse, rounded. A. spinosus flowers are dense clusters, lower ones are axillary, higher ones often collected in an axillary and terminal spike, which is often branched in its lower part; axillary clusters are usually armed with very sharp spines up to 2 cm long. The flowers are unisexual, solitary in the axil of a bract, subtended by 2 bracteoles; bracts and bracteoles scarious, mucronate from a broad base with shorter perianth; male flowers are usually arranged in a terminal spike above the base of the inflorescence, green; tepals 3 to 5, free, subequal, ovate-oblong to oblong-spatulate, up to 2.5 mm long, very convex, membranous, with transparent margins and green or purple median band; male flowers with 5 stamens and long sepals; female flowers with superior, oblong ovary, 1-celled, styles 2–3, ultimately recurved. The fruits are ovoid shaped with a short inflated neck below the style base, circumsessile a little below the middle or indehiscent and the seeds are about 1 mm in diameter, shiny, compressed, black or brownish-black in colour.(2)

A.spinosus plants were reported to contain 7-p-coumaroyl apigenin 4-O- β -D-glucopyranoside, spinoside, xylofuranosyl uracil, β - D-ribofuranosyl adenine, β -sitosterol glucoside, hydroxycinnamates, quercetin and kaempferol glycosides, betalains, betaxanthin,

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betacyanin, amaranthine and isoamaranthine, gomphrenin, betanin, β -sitosterol, stigmasterol, linoleic acid, rutin and β – carotene. (4)

The various parts of Amaranthus spinosus extracts has diverse ethno medicinal uses, (5) a thoughtful attempt has been made here to explore herbal medicines that have a strong traditional or conceptual base and the potential to be useful as antifertility effects in terms of safety and effectiveness. A thoughtful attempt has been made here to explore herbal medicines that have a strong traditional or conceptual in terms of safety and effectiveness. The present study was an attempt to investigate the effects of the whole plants of Amaranthus spinosus ethanolic extracts (ASE) for the preliminary test in repeated dose oral toxicity study in rats using OECD 407 guidelines before the antifertility actions in female rats.

Materials And Methods

The whole plants of Amaranthus spinosus from Kothagiri, Ooty district Tamilnadu and was authenticated by Scientist of Botanical Survey of India, Agricultural University, Coimbatore - 641 003. The plants collected were washed in running water, dried under shade, segregated and pulverized by mechanical grinder and the powder was passed through No 20 sieve. The powdered material was successfully extracted with ethanol by hot continuous percolation method in Soxhlet apparatus for 10 hrs. The residue obtained was then utilized for evaluating sub-acute toxicological assessment by suspending in distilled water in Tween 80 (2%) as suspending agent was given at doses of 1000 mg/ kg.

Wistar rats having weight of 180- 220 gm were kept in quarantine for 10 days under standard husbandry conditions (27 ^{0}C, RH 65 ±2 %) for 12 h in dark and light cycle respectively and were given standard food and water ad libitum. All the experiments were performed as per the CPCSEA norms after obtained the approval of the IAEC,C.L.Baid Metha College of Pharmacy, Chennai - 97, Tamil Nadu, (IAEC / II / 02 / CLBMCP / 2013 dated 21.01.2013).(6)

Repeated dose 28-days oral toxicity study:

The study was carried out on adult female young virgin Wistar rats (180-220 g. body weight) between 8 and 12 weeks old, each group containing 6 animals were housed individually in labeled polypropylene cages. Animals were allowed free access to standard pellet diet and tap water ad libitum. They were maintained in controlled laboratory conditions of 12 hrs dark/light cycle, 22±2°C temperatures and 45-60% humidity. After 2 weeks of acclimatization, the animals were divided into four groups of 6 each. Group –I, (control group) rats received tween 80 (2%) 1 ml/ kg/p.o suspension; Group –II, SOE treated rats.

Table- 1: Grouping of rats in repeated dose 28-days oral toxicity study

Sl.No	Groups	Treatment
1.	Group-I (Control)	Rats treated with tween 80 (2%) 1 ml/ kg/p.o suspension for 28 days.
2.	Group-IV (ASE treated)	Rats treated with ASE (1000mg / kg/ p.o.) for 1- 28 days.

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The extract was administered at a fixed time daily for 28 days and observed twice daily for morbidity/mortality and gross behaviour activity. Body weights of the animals were measured weekly. On the 29th day, after an overnight fast, the rats were anaesthetized with ether and blood samples were collected. The haematological, biochemical analysis and hormonal assays were performed. Diagnostic reagents or kits consisting of Cholesterol (Autozyme, Accurex Biomedical, Mumbai), Hormonal kits (Elecsys 2010 Modular Analytics, E 170, Cobase 411), SGOT & SGPT (E- Merck India Ltd. Worli, Mumbai), Total protein (Ecoline-,E- Merck (India) Ltd., Worli, Mumbai) and other kits acid phosphatase, albumin, alkaline phosphatase, ALP, ascorbic acid, cholesterol, creatinine, GGT, glucose, glycogen, LDH, sialic acid, total bilirubin, triglycerides, urea and uric acid were obtained from Agappe Diagnostics, Ernakulam, Kerala, India. The reagents required for investigating the haematological parameters were obtained from Masters Bio-Tech (P) Ltd., Bangalore, India. The organ weights were recorded and necroscopy and histopathological studies were also carried out. (7,8,9)

Results:

Results of Gross behaviour studies of ASE at dose of 1000mg/kg. p.o in rats.

Gross behaviour studies of ASE at dose of 1000mg/kg/oral. in rats													
Sl.no	**Effect on CNS:**	**Time (hrs)**											
		$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	5	6	12	24
Spontaneous motor activity:													
1.	Ataxic gait.	-	-	-	-	-	-	-	-	-	-	-	-
2.	Bizarre behaviour	-	-	-	-	-	-	-	-	-	-	-	-
3.	Chronic convulsions	-	-	-	-	-	-	-	-	-	-	-	-
4.	Convulsions	-	-	-	-	-	-	-	-	-	-	-	-
5.	Grooming behaviour	-	-	-	-	-	-	-	-	-	-	-	-
6.	Lying flat on the back	-	-	-	-	-	-	-	-	-	-	-	-
7.	Lying flat on the belly	-	-	-	-	-	-	-	-	-	-	-	-
8.	Lying flat on the side	-	-	-	-	-	-	-	-	-	-	-	-
9.	Narcosis	-	-	-	-	-	-	-	-	-	-	-	-
10.	Opisthotonus	-	-	-	-	-	-	-	-	-	-	-	-
11.	Restlessness	-	-	-	-	-	-	-	-	-	-	-	-
12.	Rolling and jumping	-	-	-	-	-	-	-	-	-	-	-	-
13.	Sleeping	-	-	-	-	-	-	-	-	-	-	-	-
14.	Straub's phenomenon	-	-	-	-	-	-	-	-	-	-	-	-
15.	Timidity	+	+	+	+	+	+	+	+	+	+	+	+
16.	Tonic convulsions	-	-	-	-	-	-	-	-	-	-	-	-
17.	Tremors	-	-	-	-	-	-	-	-	-	-	-	-
18.	Twitches	-	-	-	-	-	-	-	-	-	-	-	-
19.	Writhing	-	-	-	-	-	-	-	-	-	-	-	-

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Effect on reflexes:													
20.	Corneal reflexes	-	-	-	-	-	-	-	-	-	-	-	-
21.	Pain following stimulation	-	-	-	-	-	-	-	-	-	-	-	-
22.	Pinna reflex	+	+	+	+	+	+	+	+	+	+	+	+
Effect on autonomic nervous system:													
23.	Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-
24.	Defecation	-	-	-	-	-	-	-	-	-	-	-	-
25.	Eyelids(closure/exophthalamus)	-	-	-	-	-	-	-	-	-	-	-	-
26.	Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-
27.	Piloerection	-	-	-	-	-	-	-	-	-	-	-	-
28.	Pupil diameter (constriction/dilatation)	-	-	-	-	-	-	-	-	-	-	-	-
29.	Salivation	-	-	-	-	-	-	-	-	-	-	-	-
30.	Secretion of sweat	-	-	-	-	-	-	-	-	-	-	-	-
31.	Urination	-	-	-	-	-	-	-	-	-	-	-	-
Effect after manipulations:													
32.	Auditory stimulus response	+	+	+	+	+	+	+	+	+	+	+	+
33.	Catalepsy in induced position	-	-	-	-	-	-	-	-	-	-	-	-
34.	Escape after touch	+	+	+	+	+	+	+	+	+	+	+	+
35.	Paralysis of fore paws	-	-	-	-	-	-	-	-	-	-	-	-
36.	Paralysis of hind limbs	-	-	-	-	-	-	-	-	-	-	-	-
37.	Writhing reflex	-	-	-	-	-	-	-	-	-	-	-	-

(-): Normal or non response, (+): positive response

Table-3 : Results of the haematological parameters of ASE (1000 mg/kg/oral) inrats

Test	**Control**	**SOE (1000 mg/kg/oral)**
WBC (thous/mcl)	8.46±1.87	9.08±2.25
RBC (mill/mcl)	8.64±2.58	8.98±1.84
Hb (g/dl)	15.94±3.46	15.68±2.4
MCV (fl)	58.64± 3.24	57.36 ±2.5
MCH (pg)	21.22±2.25	22.39±1.89
MCHC (%)	42.54±1.24	42.30±2.5
HT (%)	40.12±2.09	41.64±2.54
Lymphocytes (%)	44±2.9	44±2.84
Monocytes (%)	3±0.1.22	4±0.87
Heterophils (%)	45±2.60	42±3.31

Values are mean ± SEM; n=6 in each group, SOEwas compared with control group showed non- significant results.

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Table 4: Results of the biochemical parameters of ASE (1000 mg/kg/oral) in rats.

BIOCHEMICAL PARAMETERS	Control	SOE (1000 mg/kg/oral)
Albumin (g/dl)	3.7±0.5	4.12±0.8
Alkaline Phosphates (U/l)	86.24±2.4	67.14±1.4*
Creatinine (mg/dl)	0.6±0.05	0.8±0.13
Cholesterol (mg/dl)	72.04±0.14	144.64±2.41
Glucose (mg/dl)	90.24±3.47	75.47±2.14*
Gamma Glutamyl Transferase (GGT) (U/l)	43.74±1.33	43.24±2.14
Lactate Dehydrogenate (LDH) (U/l)	136.45±5.8	141.44±2.14
Glutamic Oxaloacetic Transaminase (SGOT) (U/l)	68.24±4.2	69.44±2.54
Glutamic Pyruvic Transaminase (SGPT) (U/l)	26.14±1.74	28.64±2.4
Urea (mg/dl)	21.54±1.13	22.36±1.17
Uric acid (mg/dl)	5.24±0.44	6.67±1.14
Total bilirubin (mg/dl)	0.62±0.05	0.61±0.03
Total Protein (g/dl)	6.24±0.98	6.28±0.98
Triglycerides (mg/dl)	134.72±4.4	144.58±4.6

Values are mean ± SEM; n=6 in each group **ASE** were compared with control group albumin, creatinine, GGT, LDH, SGOT, SGPT, urea, uric acid, total bilirubin and total protein values were not altered significantly ALP, glucose values were significantly less and cholesterol level was significantly high.(*= P<0.05 significant).

Table-5: Results of serum hormonal parameters of ASE (1000 mg/kg/oral) treated rats.

Sl. No	Groups	Oestradiol (Pg/ml)	ProgeSOErone (Pg/ml)	Tri iodothyronine (µU/ml)	Thyroxin (µU/ml)	Thyroid stimulating hormone (µU/ml)
1	Group-I (Control)	69.37±2.15	13.13±0.94	0.33 ± 0.11	1.28 ± 0.11	67.09 ± 1.12
2	Group-IV (SRE treated)	22.17±2.98**	06.94±0.87*	0.32 ± 0.08	1.64 ± 0.09	63.46 ± 1.34

Values are mean ± SEM; n=6 in each group SOE were compared with control group. Triiodothyronine, thyroxin, thyroid stimulating hormone values were not altered significantly while oestradiol, progeSOErone values were significantly less.(**= P<0.01) moderately significant,(*= P<0.05 significant).

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Table-6: Results of tissue weight analysis of ASE (1000 mg/kg/oral) treated rats.

Sl.No	Groups	Brain (g)	Heart (g)	Kidney(g)	Liver(g)	Uterus(g)
1.	Group-I (Control)	1.612±0.09	0.457±0.04	0.987±0.02	3.07±0.09	0.535±0.01
2.	Group-IV (SOE treated)	1.647±0.58	0.494±0.03	0.968±0.07	3.04±0.08	0.347±0.14*

Values are mean ± SEM; n=6 in each group SOEwere compared with control group brain, heart, kidney, liver values were not altered significantly uterus values was significantly less (*= P<0.05 significant).

Table-7: Results of macroscopic analysis of ASE (1000 mg/kg/oral) treated rats.

Sl.No	Groups	Brain (g)	Heart (g)	Kidney(g)	Liver(g)	Uterus(g)
1.	**Group-I (Control)**	N	N	N	N	N
2.	**Group-IV (SOE treated)**	N	N	N	N	AbN

N= Normal; Ab N = abnormal

Results of Sub-Acute Toxicological Study:

Repeated dose 28-days oral toxicity study was performed in adult female young virgin Wistar rats and the gross behavioural studies were observed after administering ASE at a dose of 1000mg/kg/oral and the spontaneous motor activity, effect on reflexes, effect on autonomic nervous system and effect after manipulations parameters were observed visually. The effects of drugs on the central and peripheral nervous systems can be easily recognized in normal animals. Several neurological and neuropsychological tests are described which can be used as first screen for behaviour abnormalities in mice or rats. Some of the plant extract contains potent drugs may act on ANS and CNS results in an increase or decrease activity in motor nerves which may alter the behavioural changes.(10)

Repeated dose 28-days oral toxicity study of the test compound ASE at 1000 mg/kg/oral does not altered the normal behaviour such as ataxic gait, bizarre behaviour, grooming behaviour, lying flat on the back, lying flat on the belly, lying flat on the side, narcosis, restlessness, sleeping, straub's phenomenon, timidity and also absence of chronic convulsions, opisthotonus, rolling and jumping, tonic convulsions, tremors, twitches and writhing. The results were compared with control animals and found to be normal (Table: 2). Further corneal reflexes, pain following stimulation and pinna reflex were also normal. Effect on autonomic nervous system such as cyanosis, defecation, eyelids (closure/exophthalamus), lacrimation, piloerection, pupil diameter (constriction /dilatation), salivation, secretion of sweat and urination were normal when compared to control group animals. Effect after manipulations such as auditory stimulus response, escape after touch, writhing reflex, paralysis of hind & fore paws and catalepsy in induced position were not observed. All the above parameters proved that the repeated dose 28-days oral toxicity study in rats after

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administering ASE at a dose of 1000mg/kg/oral respectively were safe and did not alter the normal behaviour of the treated rats.(10)

The hematological parameters such as WBC count, RBC count, haemoglobin, MCV, MCH, MCHC, HT, lymphocytes, monocytes and heterophils the results were not altered significantly when compared to control group animals (Table:3). (10) Serum biochemical parameters such as albumin, creatinine, GGT, LDH, SGOT, SGPT, urea, uric acid, total bilirubin and total protein were not altered significantly when compared to control group animals. (11) The level of ALP, glucose were less and cholesterol level was very high and the results were found statistically significant when compared to control group animals (Table:4).The hormonal levels of oestrogen, progesterone were altered significantly and the T3, T4 and TSH levels were not altered. The histopathological results supported the above findings (Table: 5).

The histopathological slides of kidney 1000 mg/kg/oral treated rat (Fig-1-2) showed normal cellular architecture showed normal cellular architecture of kidney pattern of glomeruli and tubular cells, conforms the safety of ASE when compared to normal group cells of kidney (Fig-7.30). The histopathological slides of uterus 1000 mg/kg/oral treated rat (Fig-3-4) showed normal uterus cells with prominent epithelium lining, conforms the safety of ASE when compared to normal group cells of uterus.

Histopathological Results Of Ase (1000 Mg/Kg/Oral) Treated Rats
Photomicrograph of sections (H&E staining, magnificationX10)

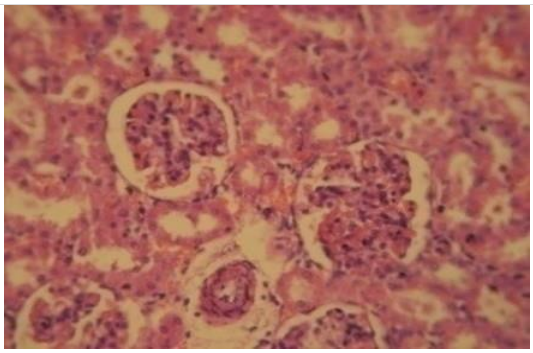

Fig: 1 Tween 80 (2%) 1 ml/ kg/p.o treated kidney cells of rats.

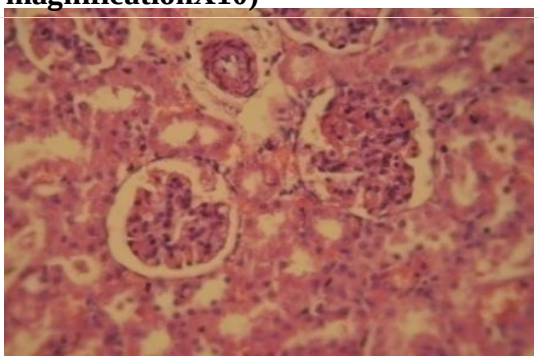

Fig:2 ASE treated kidney cells of rats

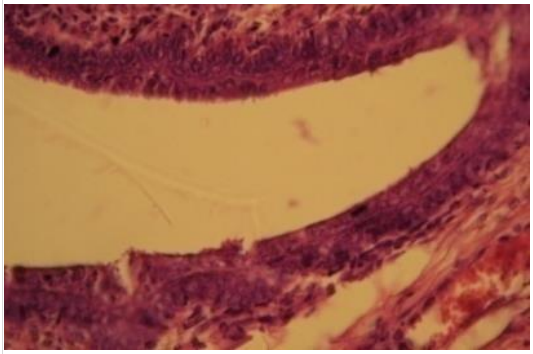

Fig: 3 Tween 80 (2%) 1 ml/ kg/p.o treated utures of rats.

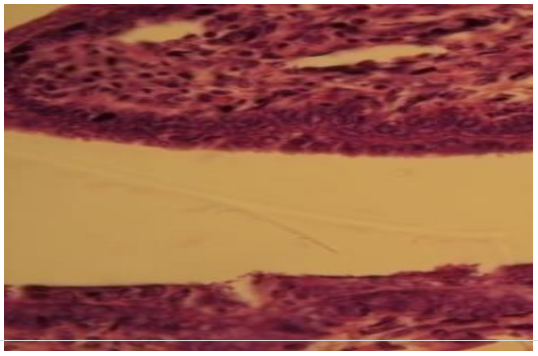

Fig: 4 ASE treated utures of rats.

Conclusion:

In summary, repeated oral doses of ethanolic extract of Solanum torvum L., in wistar rats to rats for 28 days resulted in devoid of abnormalities in the hematological parameters, gross behavior studies, macroscopic analysis, serum hormonal parameters, biochemical parameters. Further, the assessment of histopathology of hepato, and cardiac are devoid of toxicity in nature at the level of ethanolic extract of Solanum torvum L at 1000 mg/kg per day. The study provides scientific evidence for the future studies in Solanum torvum L.

Conflicts of interest: None declared.

Ethical approval: IAEC, C.L.Baid Metha College of Pharmacy, Chennai - 97, Tamil Nadu, (IAEC / II / 02 / CLBMCP / 2013 dated 21.01.2013).

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