ACUTE TOXICITY AND SAFETY PHARMACOLOGICAL STUDY OF HYDROALCOHOLIC EXTRACT OF *ZANTHOXYLUM ARMATUM* (ZA-A002).

Gurcharan Kour[1*], Pankaj Chibber[2,3], Sukhleen Kaur[2,3], Narinder Kumar[1], Surjeet Singh[2] and Pawan Kumar Tirpathi [1*]

1. School of Life Sciences, Singhania University, Pacheri Bari, Rajasthan-333515-India
2. PK-PD-Toxicology & Formulation Division, CSIR-Indian Institute of Integrative Medicine, Jammu, 180001,J & K-India
3. Academy of Scientific and Innovative Research (AcSIR), CSIR- Indian Institute of Integrative Medicine, Jammu 180001, India

Abstract:

The present study was designed to study the acute toxicity and preclinical safety pharmacological study to evaluate the potential effects of *Zanthoxylum armatum* (ZA-A002), on various organ systems using various rodent models. In the acute toxicity study, 2000 mg/kg dose of the extract was orally administered to mice and rats for the evaluation of toxic effects if any and further observed for 14 days for any mortality, while in case of safety pharmacology, effect of *Zanthoxylum armatum* (ZA-A002) on central nervous, cardiovascular system and gastrointestinal system was studied. No significant adverse effects on body weight changes, hematological and biochemical parameters were observed. Also, ZA-A002 didn't show any adverse effects on cardiovascular system and gastrointestinal system. However, it showed some effect on central nervous system parameters like increased in spontaneous motor activity and significant reduction of the duration of sleeping time and also delayed the onset of sleep. Further, ZA-A002 was shown to act as potent antinociceptive agent as it produced a dose dependent significant increase in the response latency in comparison to the normal control group in case of hot plate model.

Keywords: Safety Pharmacology, Acute Toxicity, Pentobarbitone, *Zanthoxylum*, Sleeping Time, Convulsion

1. Introduction

Herbal medicines are getting significant attention for the widespread acceptability as natural therapeutic agents for various diseases (Pan et al., 2014). According to World Health Organization (WHO), medicinal plants are the best source of different kinds of phytochemicals which could be formulated into variety of drugs (Horhammer and Hansel, 1953, Shakya, 2016) and suggests that that usage of traditional medicines may be as high as eighty per cent of the population in some parts of Asia and Africa, where many people rely upon some form of traditional medicine for their primary healthcare (WHO, 2008). In general, natural

products play a dominant role in the development of novel drug leads for the treatment and prevention of various diseases.

Zanthoxylum armatum DC [syn. *Zanthoxylum alatum* Roxb.] belongs to the family Rutaceae is a perennial shrub or a small tree height up to 6 m with dense glabrous foliage and straight prickles on stem and is an important traditional plant with lots of significant medicinal values growing in the tropical and temperate parts of the world (Neil and Lewis, 1993, Hostettmann, 1987) and is known by many vernaculars such as Tejbal in Hindi, Toothache tree or Indian "Prickly Ash" in English, Tumburi in Sanskrit. In Jammu region, it is commonly referred to as, Timbru (in Dogri) and Timber (in Pahari). In Ayurveda, *Zanthoxylum armatum* is used to pacify vitiated vata, kapha, inflammation, ulcers, stomatitis, gingivitis, anorexia, indigestion, flatulence, diarrhoea and hemorrhoids.

In Indian traditional system of medicine, *Zanthoxylum armatum* is the most important medicinal plant having medicinal properties. In Jammu and Kashmir *Zanthoxylum armatum* is commonly known as Timbru consists of dried seeds of *Zanthoxylum armatum*. Seeds are a well-known ayurvedic medicine (Wealth of India, 1976). Because of their deodorant, disinfectant and antiseptic properties, the fruits are used in dental troubles. Fruits and seeds are employed as aromatic and tonic, in fever, dyspepsia and expelling round worms (Ramchandran and Ali, 1996, Chopra, 2006). The plant has been reported to possess antioxidant (Batool *et al*., 2010, Guo *et al*., 2011, Sati *et al*., 2011, Mehta *et al*., 2012, Karmakar *et al*., 2015), antinociceptive (Guo *et al.*, 2011), anti-inflammatory (Sati *et al*., 2011, Singh and Singh, 2011, Kaur *et al*., 2011, Mehta *et al*., 2011, Mukhija *et al*., 2012, Siddhanadham *et al*., 2017, Nooreen *et al*., 2017, Guo *et al*.,2010), natural pisciside (Ramanujam and Ratha, 2008), antimicrobial (Metha *et al.*, 1981, Parajuli *et al*., 2005, Alum *et al*., 2017), antihelminitic (Metha *et al*., 1981), Hepatoprotective (Dube *et al*., 1990, Verma and Khosa, 2010, Ranawat *et al*., 2010, Sabir *et al.*, 2017, Barua *et al*., 2018), antiproliferative (Kumar and Miller, 1999) and antifungal (Dikshit and Husain, 1984), Antidiabetic (Karki *et al*., 2014, Rynjah *et al*., 2018, Alum *et al*., 2018) and Anticancer activity (Barkatullah *et al*., 2011, Barkatullah *et al*., 2013, Singh *et al*., 2015, Karmarkar *et al*., 2015, Alam *et al*., 2017).

Several cases of adverse effects of herbal drugs have been reported in developed countries during the last few years, which are allegedly caused by taking herbal products or traditional medicines prescribed by the practitioners of indigenous systems of medicine. These products may be contaminated with excessive banned pesticides, microbial contaminants, heavy metals and chemical toxins which cause various deformities like congenital paralysis, sensori-neural defects, liver and kidney damage etc.

According to literature survey and various documented pharmacological studies on the different parts of *Zanthoxylum armatum* and their active fractions and compounds isolated from it have been carried out all over the world. But no single study on pre-clinical toxicity and safety pharmacological study has been reported on this plant so for. Because of this paucity of information related to drug toxicity and safety

issues, the present study was designed to investigate acute oral toxicity and *in vivo* pharmacological effects of hydroalcoholic extract **of** *Zanthoxylum armatum* (ZA-A002) on various physiological systems of body like cardiovascular, respiratory, gastro-intestinal and central nervous systems in rodents in compliance with the OECD (Fixed Dose Procedure, 420 and ICH, 2001), guidelines respectively.

2. Materials and methods

2.1 Animals

Wistar rats (133-139g), and Swiss mice weighing between 24 and 25g were housed at 22±2^{0}C and 40-60% relative humidity (RH) on a 12:12h light and dark cycles. The room was well ventilated (>10 air changes/h) with 100% fresh air, according to CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines and had free access to standard pelleted diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. The animals were kept in polypropylene cages at room temperature and were acclimatized to laboratory environment for a week prior to start of study. The protocol used in this study was approved by the Animal Ethical Committee of CSIR-Indian Institute of Integrated Medicine, Jammu (67/PCSEA/99).

2.2 Preparation of hydro- alcoholic (1:1) extract of fruits of *Zanthoxylum armatum*

Fruits (seeds) of *Zanthoxylum armatum* were collected from Rajouri and Poonch district of Jammu Province, Jammu and Kashmir State (India) and authenticated by the Botanical Division of CSIR- Indian Institute of Integrative Medicine, Jammu-India.

Dried and powdered fruits of *Zanthoxylum armatum* (500 g) was placed in a separating funnel with ethanol and distilled water mixture (1:1) 2.5 liter and kept for 24 hrs at room tempt. The extract was drained and marc was extracted two times more under similar conditions using ethanol: distilled water (1:1) mixture (2 liter each time). All the three extracts were pooled and centrifuged. Combined extract was concentrated on rota vapour to remove ethanol. Leftover aqueous was freeze dried to get a residue 105gm and coded as ZA-A002.

2.3 Chemicals and reagents

EDTA (Himedia), Phenobarbitone, Pentobarbitone, Paracetamol, Ibuprofen, Atropine sulphate, Diclofenac, Caffeine, Gum Acacia, and Chlorpromazine (all these chemicals purchased from Sigma-Aldrich).

3. Acute toxicity

Acute toxicity study of hydroalcoholic extract of *Zanthoxylum armatum* (ZA-A002) was conducted in accordance with guidelines of Organization for Economic Co-operation and Development (OECD-420, Fixed dose procedure) for testing of chemicals using female Swiss mice and Wistar rats (nulliparous and non pregnant). The animals were fasted overnight (12- 16 hrs) with free access to water. Each animal was administered with a single dose of 2000 mg/kg (Limit Dose) of ZA-A002 by oral route. The animals were

observed for any changes continuously for 30 min, 1, 2 and 4 hours up to 24 hours and thereafter once a day for the next 14 days. The animals were then kept for 14 days to observe daily cage side observations and clinical signs of toxicity (lacrimation, salivation, piloerection), central nervous system effect (tremors, convulsion, drowsiness) skin (fur) and mortality. Body weight changes were recorded weekly, feed and water intake was measured daily, serum biochemistry and hematological parameters were investigated at the end of the study and finally animals were scarified for detailed necropsy in order to detect abnormality in tissue architecture like necrosis, proliferation etc. (if any).

4. Safety pharmacology:

Safety pharmacological study was done according to ICH guideline S7A. The purpose of the safety pharmacological study is to investigate the effects of the test substance on vital functions. In this regard, the cardiovascular, respiratory, gastro-intestinal and central nervous systems are usually considered the vital organ systems which are used in this study.

4.1. Effect of ZA-A002 on Central Nervous System:

4.2.1 General behavior in mice

Behavior effect of mice with respect to various parameters like body position, locomotion, rearing, respiration, righting reflex and lacrimation in vehicle and ZA-A002 treated animals (125, 250 and 500 mg/kg p.o.) was assessed according to the method of Irwin, 1968. Animals were checked daily for mortality, gross signs of toxicity and abnormal behavior for 14 days post-treatment.

4.2.2. Spontaneous Motor Activity

Female Swiss mice (20-25 g) were employed for the experiment. Animals were divided into six groups (6 mice/group). Group 1, 2 and 3 received hydroalcoholic extract of ZA-A002 (125, 250, 500 mg/kg, p.o.) respectively. Group 4 received the same volume of normal saline and served as control whereas groups 5 and 6 received chlorpromazine (1 mg/kg, i/p.) and caffeine (10 mg/kg, p.o) and served as standard groups respectively. In this method, animals were placed individually in the actophotometer, a polypropylene cage (40.6×20.3×15.2; PAS open-field chamber) in a quiet room with controlled temperature (22 ± 2°C) and allowed to move for a given period of time. When the beam of light falling on the photocell was cut by the moving animal, the motility count was recorded on the display (Scott and Harlan, 1997). Spontaneous Motor Activity (SMA) (counts) was recorded at 0, 30, 60 &120 minutes after administration of test drug as described previously (Rayees *et al.,* 2012). Spontaneous motor activity of each animal was measured and results of test drugs were compared with control and standard.

4.2.3. Anti-convulsive activity

Swiss mice (20–25 g, 6 mice /group) were used for the study. Three groups were administered with ZA-A002 at different doses (125, 250 & 500 mg/kg). Fourth group kept as control and received vehicle only (normal saline) whereas fifth group was administered with standard drug (Phenobarbitone, 30 mg/kg, p.o.). After 1h, animals were given a single electric shock (50Hz frequency, 0.5ms pulse width, 50 mA current for 0.3s) through bi-polar corneal electrodes attached to the ear pinna on both sides to induce convulsion (Yemitan and Adeyemi, 2005). The animals were observed for onset of convulsion up to 30 min after electric shock. Hind limb extension was taken as tonic convulsion. The onset of tonic convulsion and the number of animals convulsing or not convulsing within the observation period were noted. The ability of the testing drug to prevent or delay the onset of the hind limb extension exhibited by the animals was taken as an indication of anticonvulsant activity.

4.2.4. Analgesic activity (Hot plate method)

Analgesic activity was carried out by hot plate method according to the method of Eddy and Leimbach (1953). Mice were screened by placing them on a hot plate maintained at 55 ±1˚C and the reaction time (in seconds) for hind paw licking or jumping was recorded. Only those mice which reacted within 20s and which did not show large variation when tested on four separate occasions, each 15 min apart, were used in this study. The time for hind paw licking or jumping on the heated plate of analgesiometer (Socrel, Ugo Basile, Italy) was taken as the reaction time. The animals were treated with ZA-A002 (125, 250 and 500 mg/kg,p.o.) or with Diclofenac (50 mg/kg, p.o.) 60 minutes prior to the test. The control group received the same volume of vehicle (normal saline). After drug administration (ZA-A002 and Diclofenac), the reaction time was measured at 0, 30, 60, 90 and 120 minutes.

4.2.5. Pentobarbitone induced sleeping time

Swiss mice were divided into six groups (6 mice/group). Group 1 received vehicle (normal saline) and served as control. Groups 2 and 3 received (chlorpromazine 1 mg/kg, i/p.) and (caffeine 10 mg/kg, p.o.) and served as positive controls. Whereas groups 4, 5 and 6 received hydroalcoholic extract of ZA-A002 (125, 250 and 500 mg/kg, p.o.) respectively. 1 h later, each animal received an intra-peritoneal injection of sodium pentobarbitone (50 mg/kg). The onset of sleep and the duration of sleeping time (time during which the righting reflex was lost), of each animal was determined and the mean for each group was calculated (Sonavane *et al.*, 2001 and Owolabi *et al.*, 2008).

4.2.6. Body temperature in normal rats

Male Wistar rats (5 rats /group) with stable rectal temperatures were used to study body temperature. Rectal temperature of animals treated with hydroalcoholic extract of ZA-A002 at different doses (125, 250 and 500 mg/kg) and control group was recorded using a electronic thermometer probe (ISO THERMEX, Colmbus

Instruments, USA) inserted 4.5–5.0 cm in the rectum. Rectal temperatures were recorded at 1 h interval for 3 h after drug administration (Rao *et al*., 2002).

4.3. Effect of ZA-A002 on the digestive system:

4.3.1. Charcoal propulsion in mice

The effect of hydroalcoholic extract of ZA-A002 on small intestinal transit was studied in overnight fasted Swiss mice which were divided in different groups. Group I received vehicle (normal saline) and served as control. Group II served as positive control and received atropine (3 mg/kg, i/p.). Groups III, IV and V were administered with hydroalcoholic extract of ZA-A002 (125, 250 and 500 mg/kg), orally before 45 min. after the administration of 1 ml of marker (charcoal). The animals were sacrificed after 30 min, and the distance traveled by charcoal meal through the pylorus was measured and expressed as percentage of the total length of the intestine from the pylorus to caecum (Ali and Bashir (1993) according to the formula:

Charcoal meal transit = Distance covered by the charcoal powder / Total length of small intestine × 100

4.3.2. Billiary secretion

The billiary secretion test was conducted in male Wistar rats according to the method of Shetler, *et al*., (1993). Rats (6 animal/group) were anesthetized with pentobarbital (50 mg/kg body weight, i.p). A catheter was placed in the jugular vein for administration of test drug (12.5, 25 and 50 mg/ml i/v.). The abdominal cavity was opened by making a midline incision and the common bile duct was cannulated. Billiary secretion was collected by gravity for 2 hours.

4.4. Effects of ZA-A002 on the cardiovascular system:

4.4.1. Blood pressure and heart rate in conscious, normotensive rats (Telemetry method)

Male Wistar rats (250–300 g) were anesthetized using a combination of xylazine hydrochloride, 5 mg/kg (Indian Immunologicals Ltd., India) and ketamine hydrochloride, 40 mg/kg, i/p. (Themis Medicare Ltd., India). A radio transmitter (TL11M2-C50PXT, Data Sciences, St. Paul, MN, USA) was implanted in the peritoneal cavity of the animal by performing surgery (Mirza *et al*., 2007). Individual rats were placed in a polypropylene rat cage on top of a receiver (RLA 2000, Data Science) for measurement of systolic and diastolic blood pressure and heart rate (Brokway *et al.*, 1991; Guiol et al., 1992). Experiments were performed after 15 days of surgery. The data were interpreted by the Dataquest program A.R.T. version 2.2 Gold Version. The data were recorded up to 1 h post drug treatment.

5. Statistical analysis

The data obtained were calculated and expressed in percent frequency and the significance at different dose levels was tested using primer software through Student-Newman-Keuls test with values showing $p<0.05$ as the criterion of significance.

6. Results

6.1. Acute toxicity

The acute toxicity study was performed according to OECD guideline 420. No treatment-related mortality was observed at 2000 mg/kg, and throughout the 14-day observation period, there were no clinical signs of behavior and toxicity like lacrimation, salivation, piloerection, tremors, convulsion, drowsiness and mortality in any of the treated animal as compared to control animals. No abnormal changes in feed and water consumption, body weight, respiration rate, or heart rate attributable to the treatment were noted (Feed and water data not shown). There are no significant changes in biochemical and hematological parameters when compare to control group (Table 1-6).

6. 2. Safety pharmacology

6.2.1. Effect of ZA-A002 on the Central nervous System

6.2.1.1. General behavior

Animals administered with different doses of test material, ZA-A002 showed normal behavior during the 14-day examined post-treatment. There were no signs of toxicity and abnormality in behavior observed during this 14-day period.

6.2.1.2. Spontaneous Motor Activity

Spontaneous ambulatory activity (indicated by the distance traveled) gradually decreased over the 120 min recording period as the animals became acclimatized to the new environment. ZA-A002 at all doses (125, 250 and 500 mg/kg, p.o.) has exhibited significantly increase in locomotor activity when compared with that of control animals which received normal saline only and standard drug chlorpromazine (1 mg/kg, i/p.).
The caffeine, a known CNS stimulant at a dose of (10 mg/kg, p.o.) showed statiscally significant increase in locomotor activity whereas chlorpromazine, a CNS depressant at a dose of (1 mg/kg, i/p.) reduced locomotor activity in mice when compared with control animals (Table 7).

6.2.3. Anti-convulsive activity in mice

In the maximal electroshock seizure (MES) test, the control group exhibited 100% hind limb tonic extensions (HLTE) seizure. Whereas phenobarbitone (30 mg/kg, p.o.) provided 100 % protection against MES seizure. Treatment of hydroalcoholic extract of ZA-A002 (125, 250 and 500 mg/kg, p.o.), did not show any protection against tonic extensions (HLTE) seizure (Table 8).

6.2.4. Analgesic activity (Hot Plate Method) in mice

Analgesic screening was done experimentally by the hot-plate method. Hydroalcoholic extract of ZA-A002 orally at the doses of (125, 250 and 500 mg/kg, p.o.) significantly protected the mice against thermally induced pain stimulus in mice when compared with control. The activity potential (increase in reaction time) increases in a dose-dependent manner along with time. Diclofenac (50 mg/kg, p.o.) treated animals also showed a significant increase in the reaction time (Table 9).

6.2.5. Pentobarbitone induced sleeping time in mice

In the pentobarbitone induced sleeping time in mice, the delay in onset was significantly different from the control at all doses of the extract (250 and 500 mg/kg, p.o.), respectively.

The delay of the onset of sleep produced and the reduction in duration of the sleep time at higher dose of ZA-A002 (500 mg/kg, p.o.) was significantly ($p < 0.01$) lower than produced by caffeine (10 mg/kg, p.o.), a known CNS stimulant indicating that ZA-A002 at high dose (500 mg/kg, p.o.) showed better stimulant effect (Table 10).

6.2.6 Body temperature:

ZA-A002 treated group of animals did not show any significant changes in rectal temperature of rats over the four h time course of the experiment as compared with control group (Table 11).

6.3. Effect of ZA-A002 on the Gastrointestinal System

6.3.1. Charcoal propulsion in mice

The peristaltic distance traveled by the activated charcoal during the 1h test period was 88.73 ± 4.61 of whole gastrointestinal length in the vehicle control group. In comparison with vehicle control group, ZA-A002 treatment (125, 250 and 500 mg/kg, p.o.), did not produce any significant changes in the gastrointestinal transit distance travelled by activated charcoal at any doses (Table 12). However, the positive control, atropine sulphate (3 mg/ kg, i/p.) treated group showed a significant suppressed gastrointestinal motility (gastrointestinal transit distance = 58.31 ± 2.17 ($p<0.01$)

6.3.2. Billiary secretion in rats

ZA-A002 treated (12.5, 25 and 50 mg/ml, i/v.) groups of animals did not show any significant change in the volume of bile collected for 2 hrs (Table 13).

6.4. Effect of ZA-A002 on the Cardiovascular System

6.4.1. Effect on Blood pressure and heart rate in conscious rats

Treatment with ZA-A002 (125, 250 and 500 mg/kg, p.o.), did not alter the systolic, diastolic blood pressure and heart rate as compared to the rats treated with vehicle (normal control) during the 1 h post drug treatment (Table 14).

Table 1: Mean Weekly Body Weight Changes of Female Wistar rats in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Mean Body Weight Changes in Rats			
Group	0 Day	1st Week	2nd Week
Control	133.4± 3.71	145.24±3.0	154.2±3.6
ZA-A002 (2000 mg/kg, p.o)	139.21 ±2.1	152.6±2.7	157.22±4.6

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 2: Mean Hematological Parameters of Female Wistar Rats in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Parameters	Control	ZA-A002 (2000 mg/kg, p.o)
WBC (10^3/uL)	12.11±1.2	14.9±2.3
RBC (10^6/uL)	7.15±0.2	7.25±0.3
HCB (g/dL)	13.16 ± 0.2	12.9 ± 0.4
HCT (%)	38.56 ± 0.6	37.56 ± 1.1
MCV (fL)	54.04 ± 1.1	51.04 ± 2.3
MCH (pg)	18.42 ± 0.4	17.50 ± 0.5
MCHC (g/dL)	34.16 ± 0.2	34.36 ± 0.7
PLT (10^3/uL)	1001.4 ± 68.5	1022.2 ± 71.7
NEUT (%)	15.2 ± 1.6	16.4 ± 3.3
LYMPH (%)	77.1 ± 2.4	74.94 ± 3.3
MONO (%)	5.02 ± 0.7	5.28 ± 0.4
EO (%)	2.48 ± 1.2	3.24 ± 0.36
BASO (%)	0.2 ± 0.05	0.14± 0.2

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 2: Mean Biochemical Parameters of Female Wistar Rats in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Parameters	Control	ZA-A002 (2000 mg/kg, p.o)
GLU (mg/dL)	64.6±3.3	48.36±1.5
ALP (IU/L)	236.3±47.8	247.05±21.4
SGOT (IU/L)	134.8 ± 31.7	151.65 ± 14.2
SGPT (IU/L)	44.2 ± 2.8	45.96 ± 1.3
UA (mg/dL)	2.27.1 ± 0.2	1.6 ± 0.1
TRI (mg/dL)	213.6 ± 14.7	193.2 ± 9.6
CRE (mg/dL))	0.86 ± 0.05	0.73 ± 0.03
TP (g/dL)	8.03 ± 1.7	7.65 ± 0.7
CHOL (mg/dl)	73.17±7.147	79.95±12.62

BIT (mg/dl)	0.35±0.03	0.17±0.02

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 4: Mean Weekly Body Weight Changes of Female Swiss Mice in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Mean Body Weight Changes in Mice			
Group	0 Day	1st Week	2nd Week
Control	24.92 ± 0.2	26.88 ± 0.4	29.17 ± 0.3
ZA-A002 (2000 mg/kg, p.o)	25.32 ± 0.5	26.78 ± 1.0	28.39 ± 0.9

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 5: Mean Hematological Parameters of Female Swiss Mice in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Parameters	Control	ZA-A002 (2000 mg/kg, p.o)
WBC (10^3/uL)	7.895 ± 1.2	8.36 ± 1.02
RBC (10^6/uL)	10.07 ± 0.24	9.29 ± 0.17
HCB (g/dL)	14.23 ± 0.24	13.24 ± 0.18
HCT (%)	44.43 ± 0.47	42.64 ± 0.9
MCV (fL)	44.15 ± 0.77	45.88 ± 0.76
MCH (pg)	14.13 ± 0.2	14.24 ± 0.2
MCHC (g/dL)	32.03 ± 0.37	31.08 ± 0.32
PLT (10^3/uL)	985.5 ± 101.3	1050.4 ± 126.1
NEUT (%)	13.83 ± 1.12	15.68 ± 2.9
LYMPH (%)	77.87 ± 2.26	77.34 ± 3.66
MONO (%)	3.3 ± 0.87	3.72 ± 0.85
EO (%)	4.87 ± 0.88	3.14 ± 0.58
BASO (%)	0.13 ± 0.05	0.12 ± 0.4

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 6: Mean Biochemical Parameters of Female Swiss Mice in Acute Toxicity of Hydroalcoholic extract of ZA-A002

Parameters	Control	ZA-A002 (2000 mg/kg, p.o)
GLU (mg/dL)	118.97 ± 9.7	144.84 ± 4.1
ALP (IU/L)	105.03 ± 10.7	92.76 ± 4.3
SGOT (IU/L)	72.5 ± 12.8	84.85 ± 15.2
SGPT (IU/L)	28.7 ± 8.4	31.82 ± 4.5
UA (mg/dL)	0.56 ± 0.04	0.658 ± 0.05
TRI (mg/dL)	69.63 ± 4.8	68.5 ± 7.8
CRE (mg/dL))	0.54 ± 0.01	0.5 ± 0.8
TP (g/dL)	6.41 ± 0.2	6.6 ± 0.36
CHOL (mg/dl)	131.20±5.37	134.65±6.32
BIT (mg/dl)	0.11±0.01	0.14±0.01

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test

Table 7: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on spontaneous motor activity

Treatment	Activity Counts			
	0 min	30 min	60 min	120 min
Control	41.93±4.57	38.15±4.91	42.43±4.41	39.90±2.22
Chlorpromazine (1 mg/kg, i/p.)	53.47±5.21	25.67±6.33	14.77±1.0**	18.53±2.56
Caffeine 10 mg/kg	47.56±3.62	62.66±2.55**	56.33±3.56	40.12±3.21
ZA-A002 125 mg/kg	46.20±4.23	50.32±3.62*	48.18±4.24	42.18±5.32
ZA-A002 250 mg/kg	44.00±2.33	56.18±5.21*	51.23±3.55	41.20±4.56
ZA-A002 500 mg/kg	41.63±3.22	60.09±1.52**	54.33±5.26	44.58±4.21

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.
Statistically significant differences compared to the vehicle group (*p<0.05, **p<0.01)

Table 8: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on MES-induced convulsions in mice

Group	Dose (mg/kg, p.o)	Duration of convulsion (s)	Protection	% of Protection
Control	Vehicle	21.6 ± 1.63	1/5	20
Phenobarbitone	30	10.2 ± 1.39	5/5	100**
ZA-A002	500	22.6 ± 1.43	1/5	20
	250	19.2 ± 1.14	1/5	20
	125	22.1 ± 2.21	1/5	20

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.
Statistically significant differences compared to the vehicle group (**p<0.001)

Table 9: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on analgesia by hot plate in mice

Traetment	Reaction Time (sec)				
	0 min	30 min	60 min	90 min	120 min
Control	4.40± 0.19	4.0±0.17	4.26± 0.15	4.40±0.11	4.5± 0.05
Diclofenac (50 mg/kg, p.o)	4.44±0.34	6.28±0.05	7.75±0.29	7.75±0.55**	5.46±0.28
ZA-A002 125 mg/kg, p.o.)	4.45±0.17	5.17±0.24*	5.09±0.04	4.95±0.14	4.28±0.14
ZA-A002 250 mg/kg, p.o.)	4.45±0.33	5.10±0.07	5.80±0.29+	5.40±0.31	4.83±0.06
ZA-A002 500 mg/kg, p.o.)	4.19±0.36	5.76±0.28	7.01±0.12**	6.12±0.09	4.98±0.03

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.
Statistically significant differences compared to the vehicle group (*p<0.05, **p<0.001)

Table 10: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on pentobarbitone induced sleep-induction and sleep duration in mice

Groups	Treatment	Onset of sleep (min)	Duration of sleep (min)
Control	Vehicle	2.55 ± 1.14	79.90 ± 2.65
Chlorpromazine	1 mg/kg, i. p.)	3.4 ± 0.93	202.23 ± 3.67**
Caffeine	10 mg/kg, p.o.)	2.25 ± 1.12	53.83 ± 1.30*
ZA-A002	125 mg/kg, p.o.)	3.8 ± 1.23	76.0 ± 4.78
	250 mg/kg, p.o.)	4.6 ± 1.08	63.4 ± 2.0
	500 mg/kg, p.o.)	5.8 ± 1.62	52.73 ± 3.85**

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.
Statistically significant differences compared to the vehicle group (*p<0.05, **p<0.01)

Table 11: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on bodt temperature for 4 h

Time intervals (hr)	Control	ZA-A002		
		125 mg/kg, p.o	250 mg/kg, p.o	500 mg/kg, p.o
	Rectal temperature (ºC)			
0h	37.28 ± 0.11	36.26 ± 0.07	36.35 ± 0.09	36.32 ± 0.09
1	37.20 ± 0.12	36.26 ± 0.10	37.36 ± 0.05	36.96 ± 0.10
2	37.32 ± 0.10	35.64 ± 0.12	36.54 ± 0.09	35.50 ± 0.09
3	36.20 ± 0.11	36.72 ± 0.07	36.62 ± 0.06	36.10 ± 0.10
4	35.64 ± 0.12	36.68 ± 0.13	3556 ± 0.01	37.02 ± 0.19

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.

Table 12: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on charcoal propulsion (gastrointestinal motility), in mice

Groups	Treatment	% age travelled by charcoal meal
Control	Vehicle	88.73 ± 4.61
Atropine sulphate	3 mg/kg (*i.p.*)	58.31 ± 2.17**
ZA-A002	125 mg/kg, p.o.	87.88±3.13
	250 mg/kg, p.o.	87.03 ±2.41
	500 mg/kg, p.o.	90.02 ± 4.22

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.
Statistically significant differences compared to the vehicle group (**p<0.01)

Table 13: Effect of hydroalcoholic extract, *Zanthozylym armatum* (ZA-A002) on Billiary secretion in rats

Groups	Treatment (i/v.)	Bile collected (uL) (Mean ± SEM)	
		Before drug treatment	After drug treatment
Control	Vehicle	1.41 ± 0.13	1.35 ± 0.04
	12.5 (mg/ml)	1.40 ± 0.05	1.41 ± 0.11

ZA-A002	25 (mg/ml)	1.29 ± 0.14	1.28 ± 0.11
	50 (mg/ml)	1.35 ± 0.05	1.34 ± 0.06

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.

Table 14: Effect of ZA-A002 on Blood pressure and Heart rate in conscious normotensive rats

Treatment groups	Parameters	0 min.	15 mins	30 mins	1 hr
ZA-A002 (125 mg/kg, p. o.)	Systolic blood pressure (mmHg)	125.1 ± 2.5	128.7 ± 3.5	128.3 ± 3.38	129.6 ± 3.24
	Diastolic blood pressure (mmHg)	53.54 ± 5.1	62.54 ± 4.9	64.31 ± 6.01	64.68 ± 6.8
	Heart rate (beats/min)	318 .1 ± 40.1	370 .1 ± 67.5	323.5 ± 81.51	306 .1 ± 33.6
ZA-A002 (250 mg/kg, p. o.)	Systolic Blood pressure (mmHg)	127.5 ± 4.4	124.4 ± 3.7	123.3 ± 5.3	124.1 ± 6.4
	Diastolic Blood pressure (mmHg)	75.47 ± 8.0	68.64 ± 5.7	73.74 ± 10.3	72.22 ± 9.0
	Heart rate (beats/min)	278.0 ± 22.7	244.9 ± 23.9	245.1 ± 24.6	266 .1 ± 26.6
ZA-A002 (500 mg/kg, p. o.)	Systolic Blood pressure (mmHg)	131.1 ± 8.2	133.5 ± 9.1	129.2 ± 9.1	126.3 ± 7.9
	diastolic Blood pressure (mmHg)	71.19 ± 4.76	72.34 ± 6.71	70.34 ± 6.99	74.78 ± 7.22
	Heart rate (beats/min)	273.5 ± 30.83	296.9 ± 39.63	213.8 ± 28.24	237.5± 11.35

Values are Mean ± SEM (N=5. Group), Student-Newman-Keuls test.

6.5. Discussion

In the acute toxicity study, oral treatment with ZA-A002 was well tolerated. A dose of 2000 mg/kg, p.o. administered to female mice and rats did not cause any signs of toxicity, changes in behavior, or any other physiological activities and mortality. Also there are no significant changes in biochemical and hematological parameters. Treatment also did not produce any significant changes in body weight, food and water intake and was found to be safe in acute toxicity study up to a dose of 2000 mg/kg p.o. in both female Swiss mice and Wistar rats. As the overall weight gain was found to be normal in treated animals as compared to control animals, the ZA-A002 is labeled unclassified (category 5) in the hazard category according to Globally Harmonized System of classification of chemicals of Organization of Economic Cooperation and Development (OECD) and considered relatively safe (Kennedy *et al*., 1986)

The pre-clinical safety pharmacological study was designed to evaluate the potential side effects of ZA-A002 on a number of physiological systems (parameters) in rodents. The ZA-A002 (125, 250 and 500 mg/kg, p.o.) did not produce any significant effect on central nervous system (general behavior, MES induced convulsions and body temperature), aside from the dose dependent significant increase in spontaneous motor activity. Spontaneous motor activity attributes the effects on central nervous system. Increased motor activity indicates the excitability of the central nervous system whereas decrease in motor

activity is related to depression and sedative effect of central nervous system (Yadav *et al*., 2008). This increasing activity indicate that extract possesses CNS stimulant properties which probably act via competitive antagonism at adenosine receptors leading to increase in nor-epinephrine secretion and enhanced neural activity in numerous brain areas as like that of in case of caffeine (Palaksha *et al*., 2018).

In pentobarbitone induced sleep time assay ZA-A002 at all doses (125, 250 and 500 mg/kg, p.o.), caused significant reduction of the duration of sleeping time and also delayed the onset of sleep when compared to that of vehicle control group. In comparison with caffeine (10 mg/kg, p.o.), the effect of ZA-A002 at higher dose (500 mg.kg, p.o.) was also significantly ($p<0.05$) better than caffeine. The results further showed that ZA-A002 in all doses (125, 250 and 500 mg/kg, p.o.) did not produce sedation without causing any side effects whereas codeine, which is the most widely used naturally occurring narcotic drug causing serious side effects like sedation (Kim *et al*., 2002). This stimulant property is probably act via competitive antagonism at adenosine receptors leading to increase in nor-epinephrine secretion and enhanced neural activity in numerous brain areas (Owolabi et al., 2008).

Further, ZA-A002 was shown to act as potent antinociceptive agent as the hydroalcoholic extract of ZA-A002 produced a dose dependent significant ($p<0.01$) increase in the response latency in comparison to the normal control group in case of hot plate model. This may in part be mediated by the peripheral acting nociception system rather than the centrally acting opioid receptors because the compounds which act via opioid receptor activation show CNS depression whereas, ZA-A002 is CNS stimulant, hence may be acting through peripheral or some other mechanism for the antinociception.

With respect to gastrointestinal system, hydroalcoholic extract of ZA-A002 did not affect gastrointestinal motility determined by the charcoal propulsion test as compared to Atropine sulphate (3 mg/kg, i/p.) which significantly decreased the intestinal motility. It is very interesting that Atropine which is a human medication to treat certain types of nerve agent and pesticide poisonings as well as some types of slow heart rate and to decrease saliva production during surgery causes severe constipation as side effect. The results of the present study revealed that the extract of ZA-A002 did not alter the intestinal motility and also have potent antinociceptive activity without causing any side affect on the intestine. The extract also did not alter the bile secretion.

Further, ZA-A002 at all doses neither show any significant changes in rectal temperature of rats compared with control group nor alter the systolic, diastolic blood pressure and heart rate as compared to the rats treated with vehicle (normal control) during the 2 h test period

7. Conclusion

In the acute toxicity study, oral treatment with ZA-A002 at a dose of 2000 mg/kg, p.o. administered to female mice and rats did not cause any signs of toxicity, changes in behavior, or any other physiological activities and mortality. Also there are no significant changes in biochemical and hematological parameters

and labeled as category 5 in the hazard category according to G H S of OECD. The safety pharmacological profile of *Zanthoxylum armatum* (ZA-A002) was generated as per ICH S7A guidelines. *Zanthoxylum armatum* was found to increase the motor activity thus indicate the CNS stimulant properties of the extract. ZA-A002 at all doses caused significant reduction of the duration of sleeping time and also delayed the onset of sleep when compared to that of vehicle control group. Further, ZA-A002 was shown to act as potent antinociceptive and produced a dose dependent significant increase in the response latency in comparison to the normal control group.

ZA-A002 at all doses did not show any effects on general behavior, seizure, intestinal motility, bile secretion, body temperature, blood pressure and heart rate.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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