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Acute Oral Toxicity Studies and Evaluation of Central Analgesic Activity of Various extracts of Leaves of *Rhizophora apiculata*

Annie Mande[1,2], Narender Malothu[1]*, Nagaraju Banadaru[1] and T. E. G. K. Murthy[2]

[1]Department of Pharmaceutical Chemistry, KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur - 522502, Andhra Pradesh, India; narendermalothu@gmail.com

[2]Department of Pharmacology, Bapatla College of Pharmacy, S.N.P. Agraharam, Bapatla, Andhra Pradesh - 522101, India

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Abstract

The present study aimed to determine the acute toxicity and screening of central analgesic activity of various extracts of *Rhizophora apiculata* leaves. The animals (Mice) were divided into control (no drug) and extract-treated groups (n=5), which were treated with diethyl ether, ethyl alcohol, and aqueous extract of *R. apiculata* leaves in various doses for specific regulatory needs. The groups which were given the highest safe doses were observed for 14 days. Then, blood samples were collected from high dose treated live mice through the retro-orbital route and were analysed for haematological, biochemical, and histopathological study. Evaluation of central analgesic activity was carried out by using tail immersion and hot plate methods. No considerable alterations were observed in body weight and organ-to-body weight index with the administration of extracts. An increase in albumin, globulin, total protein content and high-density lipoproteins, white blood cells, mean corpuscular volume, and eosinophils were observed. And a decrease in low-density lipoproteins, very low-density lipoproteins, triglycerides, and red blood cells were observed. Lymphocyte and monocyte levels were also reduced. The results also showed that the ethyl alcohol and aqueous extracts have elevated the time taken to flick response in the tail immersion test, and reduced the number of jumps, paw lick responses in the hot plate method. It was concluded that the diethyl ether and ethanolic extracts were found to be safe, which falls under non-toxic chemicals (LD50>2000 smg/kg) whereas the aqueous extract was found safe up to 550 mg/kg beyond which, it was shown mortality. Ethyl alcohol and aqueous extracts were also proved to have analgesic activity.

*Author for correspondence

1. Introduction

Medicines prepared from natural sources can exert health improvement as well as illness prevention properties in human beings[1]. The use of preparations from natural sources in the prevention of a variety of diseases has been in practice long before the introduction of semi-synthetic and synthetic medicines[2]. Nowadays, herbal preparations can be used in the treatment of serious ailments like ischaemic heart disease and also in the treatment of neurological disorders[3]. Herbal preparations have some advantages over synthetic preparations like low toxicity, cost-effectiveness, and affordability[4]. The natural plants with medicinal properties can be used as drugs only when they are having a low toxicity profile[5]. Acute toxicity studies are mandatory in order to evaluate the safety of herbal medicinal preparations[6]. The importance of acute toxicity studies is to evaluate short term toxic effects of the herbal preparations.

Mangrove ecosystems are unique and are important for human beings in several ways[7]. Mangrove forests have so many beneficial species which are used by the local people of coastal areas for the treatment of health ailments like hepatic disorders, diabetes, cardiac disorders, and neurological disorders[8]. *Rhizophora apiculata* is a mangrove that belongs to the Family *Rhizophoraceae;* Genus Rhizophora & Species *Apiculata*. It is present across the coastal regions of the subtropical and tropical areas of the world including India, Singapore and Pakistan, etc.[9]. This plant is commonly known as ponna or uppu ponna. *R. apiculata* is an Indian mangrove plant that has many medicinal uses like anti-inflammatory, wound healing, anti-diabetic, antioxidant and anti-cancer properties[10]. Although it has medicinal properties there are no studies exploring the acute toxic effects. Therefore, this is the first-ever approach for the determination of acute toxic effects of leaf extracts of this plant.

Long-term use of nonsteroidal anti-inflammatory agents and corticosteroids may result in many adverse effects; thus, it is imperative to find safe and novel drugs[11]. In general, this approach is used to evaluate analgesics among many natural products. A centrally acting analgesic can reduce neuropathic pain by inhibiting the firing of somatosensory neurons involved in pain perception. The best example of centrally acting analgesics is tramadol and opioid analgesics[12]. Considering these ideas the central analgesic effects of plant extracts have been evaluated by thermal methods like the hot plate method and tail immersion test.

2. Materials and Methods

2.1 Plant Identification, Collection and Authentication

The plants were identified and collected in the month of June 2019 from the coastal mangrove forests in Guntur district, Andhra Pradesh, and authenticated by the taxonomist Dr. Raghu Ram, with the voucher specimen (RAR/7-2(872)/ 2019/ANU/BOT) in the Department of Botany, Acharya Nagarjuna University. A fresh specimen with roots, leaves, flowers, and inflorescence was submitted to authenticate the plant. The leaves were separated from the plant and cleaned with a dry cloth and subjected to drying under shade for seven days and then by using a hot air oven at a temperature of 45°C. The dried leaves were made into fine powder.

2.2. Preparation of the Crude Extract

The crude extract was obtained by successive extraction of leaves powder with diethyl ether, ethyl alcohol, and water in the increasing order of their polarity. The diethyl ether extract (DERA) was obtained by the process of maceration for three days whereas the ethyl alcohol (EERA) and aqueous extracts (AERA) were obtained by continuous Soxhlet extraction. The extract was filtered through Whatman filter paper and evaporated to concentrate. The final extract was weighed and stored in a refrigerator.

2.3 Preliminary Phytochemical Screening

The three extracts were subjected to phytochemical screening to know the presence of various constituents[13].

2.4 Approval from Animal Ethics Committee

The present work was conducted after receiving acceptance from the Institutional Animal Ethics Committee of Bapatla College of Pharmacy with Ref. No. IAEC/XIII/10/ BCOP/2019.

2.5. Acute Toxicity Study

2.5.1 Selection of the Animals

Healthy, nulliparous, female non-pregnant albino mice which weigh about 27±5 g at age 9-12 weeks were randomly selected based on the specifications given in OECD guidelines 425 (Up and Down Procedure)[14] and procured from the animal house of Bapatla College of Pharmacy. Mice were weighed and kept under standard conditions for five days. Animals were kept fasting for about 4 h prior to being given doses but had to access water ad libitum.

2.5.2 Preparation of Doses

The DERA and EERA extract doses were prepared by using 0.1% carboxymethylcellulose (CMC) as a vehicle, and for AERA distilled water was served as a vehicle.

2.5.3 Grouping of Animals

Six groups with five animals in each group were taken. Three groups served as control groups for three vehicles and the remaining three groups were given DERA, EARA, and AERA in different doses with a washout period of 7 days.

2.5.4 Acute Toxicity Studies Procedure

Acute toxicity studies were conducted based on the OECD guidelines 425 (Up and Down Procedure). The three extracts were administered in doses 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg in preliminary tests. The DERA and EERA extract doses were increased to 3000 mg/kg as they did not show any mortality. A single higher safe dose was given to a single female mouse in each extract-treated group according to body weight. The animals were carefully observed for the first 30 min, and then for 4 h. Food was given after 2 h of dosing. As no mortality was observed in treated mice the remaining four mice were given the same dose under the same conditions. The same procedure was repeated for six groups of animals. Two groups were vehicle-treated which was considered as a control group to whom 1% carboxymethyl cellulose (CMC) was administered. For the third group water was administered in the same volume for the fourth group DERA, for the fifth group EERA and for the sixth group AERA was administered. All the six groups were observed closely for any toxic reactions for the first 6 h which was continued up to a total period of 14 days with regular intervals. Weights of animals were monitored and documented throughout the entire study period. Finally, animals were weighed, physically observed and blood samples were withdrawn by retro-orbital route with inhalational anaesthesia by using diethyl ether as an anaesthetic agent for haematological evaluations. Serum was separated from the blood for biochemical evaluation of various parameters. Organs of the mice were collected by the cervical dislocation method. Weights of each organ were recorded and utilized for histopathological studies[15]. No mortality was found in the group administered with EERA and DERA, but in the group which had given AERA out of five animals, 4 animals died.

2.6 Haematological Analysis

The samples of blood were collected from mice (both extract and vehicle-treated groups) in Ethylene Diamine Tetra Acetic Acid (EDTA) coated chambers and subjected to haematological analysis at Srinivasa Laboratories, Bapatla, Guntur Dt, Andhra Pradesh, for measuring various blood parameters like haemoglobin (Hb), Total Red Blood Cells (RBC), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Packed Cell Volume (PCV), platelet count, White Blood Cells (WBC) Count, Monocytes (M), Neutrophils (N), Eosinophils (E), and Lymphocytes (L) were determined.

2.7 Biochemical Analysis

The serum samples were subjected to biochemical analysis at Srinivasa Laboratories, Bapatla, Andhra Pradesh, to measure various renal parameters like urea, creatinine, hepatic parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase, albumin, globulins, total protein content, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), cholesterol, triglyceride, and high-density lipoprotein (HDL) [16].

2.8 Histopathological Study

The organs like Brain, Heart, Kidney, Liver, Uterus, and Pancreas were collected from sacrificed mice, fixed in 10% formalin and they were subjected to histopathological studies at Andhra Labs, Guntur, Andhra Pradesh[17].

2.9 Analgesic Activity

2.9.1 Hot Plate Method

Eddy's hot plate was used which consists of a plate that was maintained at a specific temperature by adjusting the knob. A cylinder of 17 cm in height and 13 cm in diameter was kept on it. Healthy adult mice of both sexes were selected and grouped into 6 groups with 5 animals in each group. The hot plate was maintained at a temperature of 49°C'±'0.1°C, jumping and paw licking responses were noted before, and after administration of drugs with a cut-off time of 15 secs to avoid injury to the paws of mice. Jumping response and paw licking responses were used to evaluate the pain sensitivity[18].

2.9.2 Tail immersion Test

Adult healthy mice were selected and grouped into 7 groups with 5 animals in each group. All animals have fasted for 12 h and clean drinking water was provided *ad libitum*. One group served as a control and the second group was given standard (Morphine 5 mg/kg). The remaining groups were administered with EERA and AERA extracts at different doses. After that 2-3 cm of the tip of the tail was immersed in hot water maintained at a temperature of 50±1°C and the time taken for the mice to withdraw the tail from the water known as Pain Reaction Time (PRT) was recorded for all the mice. Cut off time was set for 15 secs [18].

2.10 Statistical Analysis

The results were represented as mean ± Standard Error Mean (SEM) and statistical significance between different groups was analysed using one-way ANOVA. Statistical significance was determined by One-way ANOVA plus Tukey HSD to compare variations among groups. Analysed data was represented using tables and graphs.

3. Results

3.1 Phytochemical Studies

The diethyl extract was found tested positive for carbohydrates, flavonoids, and glycosides. The ethyl alcohol extract comprised carbohydrates, flavonoids, tannins, glycosides, alkaloids, and tannins. The aqueous extract was tested positive for carbohydrates, flavonoids, tannins, glycosides, alkaloids, and tannins (Table 1).

Table 1. Phytochemical investigation of *R. apiculata* leaves extracts

S. No	Phytochemical compound	DERA	EERA	AERA
1	Carbohydrates	+	+	+
2	Flavonoids	+	+	+
3	Alkaloids	-	+	+
4	Glycosides	+	+	+
5	Tannins	-	+	+
6	Phenolics	-	-	-
7	Saponins	-	-	-
8	Triterpenoids	-	-	-

"+" -----Present

"- "-----Absent

DERA -----Diethyl ether extract of R. apiculata leaves.

EERA -----Ethyl ether extract of R. apiculata leaves.

AERA -----Aqueous extract of R. apiculata leaves

3.2 Changes in Body Weight

The animal body weights of both control and extract treated groups were increased progressively in the study period as shown in Table 2. No significant change was observed in the body weight of animals during the study period.

3.3 Changes in Behavioural Pattern

Nothing abnormal was observed in the behavioural

Table 2. Effect of various extracts on body weight of mice in acute toxicology study

Groups	1st Day Body weight (gm)	7th Day Body weight (gm)	14th Day Body weight (gm)
Vehicle control	35.11 ± 2.561	36.57 ± 1.231	37.47 ± 1.238
DERA (2000 mg/kg)	36.1±1.651	35.67±1.321	37.18±1.1825
EERA (2000 mg kg)	31.26 ± 2.6076	32.32 ± 2.230	33.12 ± 1.267
AERA (550 mg/kg)	30.07 ± 3.245	31.56 ± 1.432	32.56 ± 1.245

Note: Values are presented as mean ± SEM, (n=5), No significant change was observed in weight of the animal.

Table 3. Observations of behaviour changes of mice in vehicle treated and DERA treated groups

Observations of vehicle control and leaf extract treated groups												
Parameter	30 mins		4		24		48		7 days		14 days	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Fur & Skin	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Respiration	N	↑	N	↑	N	N	N	N	N	N	N	N
Urination (colour)	N	N	N	N	N	N	N	N	N	N	N	N
Faeces (consistency)	N	N	N	N	N	N	N	N	N	N	N	N
Samatomotor activity behaviour	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N
Mucus Membrane	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion & Tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Itching	NF	P	NF	P	NF	P	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note: CG: Control Group (Vehicle Treated); TG: Treated Group (DERA Treated)

N: Normal; NF: Not Found; ↑: Increased rate; P: Present

pattern of the test animals. The colour and texture of the skin, mucus membrane, and fur were normal. Salivation was normal. A little rise in respiratory rate was observed in all extract-treated groups for the first 4 h after the administration of the extracts. The frequency and colour of urination were normal. Somatomotor activity was normal in the control and test groups. There was no drowsiness and sleepiness observed in both groups. The itching was noticed in both groups for the first 48 h. Behavioural observations were summarized in Tables 3-5.

3.4 Biochemical Analysis

Serum urea level was enhanced in all extract-treated groups Table 6. significant change was observed in serum urea and creatinine levels in EERA and AERA treated groups at $p<0.1$ The level of Alkaline phosphatases (Significant at $p<0.1$) is reduced in all extract-treated

Table 4. Observations of behaviour changes of mice in vehicle treated & EERA treated Groups

Parameter	Observations of vehicle control and leaf extract treated groups											
	30 mins		4		24		48		7 days		14 days	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Fur & Skin	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Respiration	N	↑	N	↑	N	N	N	N	N	N	N	N
Urination (colour)	N	N	N	N	N	N	N	N	N	N	N	N
Faeces (consistency)	N	N	N	N	N	N	N	N	N	N	N	N
Samatomotor activity behaviour	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N
Mucus membrane	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion & tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Itching	NF	P	NF	P	NF	P	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note: CG: Control Group (Vehicle Treated); TG: Treated Group (EERA Treated) group;
N: Normal; NF: Not Found; ↑: Increased rate; P: Present

Table 5. Observations of behavioural changes in vehicle and AERA treated group

Parameter	Observations of vehicle control & *R. apiculata* leaf extract treated groups											
	30 mins		4		24		48		7 days		14 days	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Fur & skin	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Respiration	N	↑	N	↑	N	N	N	N	N	N	N	N
Urination (colour)	N	N	N	N	N	N	N	N	N	N	N	N
Faeces (consistency)	N	N	N	N	N	N	N	N	N	N	N	N
Samatomotor activity behaviour	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N
Mucus membrane	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion & tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Itching	NF	P	NF	P	NF	P	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note: CG: Control Group (Vehicle Treated); TG: Treated Group (AERA Treated) group;
N: Normal; NF: Not Found; ↑: Increased rate; P: Present

Table 6. Effect of the extract and vehicle treatment on renal function

Parameters	Vehicle control group (CMC 1 % gel)	DERA treated group (2000 mg/ kg)	Vehicle control group (CMC 1% gel)	EERA treated groups (2000 mg/ kg)	Vehicle control group (Water)	AERA Treated group (550 mg/ kg)
Creatinine (Serum)	0.7 ± 0.026	0.6 ± 0.164	0.8 ± 0.017	0.7 ± 0.023*	0.8 ± 0.0145	0.7 ± 0.041*
Urea (Serum)	17 ± 0.216	18 ± 0.101	18 ± 0.231	24 ± 0.231*	19 ± 0.325	22 ± 0.236*

Note: Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change in specified parameter at $p<0.01$ when compared with the control group.

Table 7. Effect of the extract and vehicle treated groups on Liver function test in mice

Parameters	Vehicle control group (CMC 1%gel)	DERA treated group (2000 mg/kg)	Vehicle control group (CMC 1% gel)	EERA treated group (2000 mg/kg)	Vehicle control group (Water)	AERA treated group (550mg/kg)
SGPT (IU/ml)	159 ± 0.120	158 ± 0.10	160 ± 3.180	150 ± 5.774*	155 ± 2.023	146 ± 3.224*
SGOT (IU/ml)	210 ± 0.45	211 ± 0.27	220 ± 4.041	210 ± 3.464*	212 ± 4.041	200 ± 2.050*
Alkaline Phosphatase (IU/ml)	165 ± 0.32	167 ± 0.38*	164 ± 2.887	122 ± 1.732*	152 ± 1.807	120 ±01.501*
Total Bilirubin (mg/dl)	0.7 ± 0.68	0.7 ± 0.21	0.9 ± 0.012	0.9 ± 0.035	0.8 ± 0.010	0.8 ± 0.020
Direct Bilirubin (mg/dl)	0.1 ± 0.168	0.1 ± 0.18	0.1 ± 0.020	0.2 ± 0.012*	0.1 ± 0.035	0.2± 0.025*
Indirect Bilirubin (mg/dl)	0.6 ± 0.452	0.5 ± 0.2	0.6 ± 0.021	0.7 ± 0.011*	0.5 ± 0.021	0.8 ± 0.023*
Albumins (mg/dl)	4.0 ± 0.89	4.5 ± 0.8	4.5 ± 0.089	5.0 ± 0.231*	3.5 ± 0.079	5.5 ± 0.632*
Globulins (mg/dl)	3.3 ± 0.183	2.5 ± 0.136*	2.3 ± 0.173	3.0 ±0.012*	1.3 ± 0.142	3.45±0.015*
A/G Ratio (mg/dl)	1.21 ± 0.024	1.8 ± 0.13*	2.3 ± 0.035	1.66 ± 0.115*	1.6 ± 0.022	1.54± 0.1245
Total proteins (mg/dl)	6.5 ± 1.112	7.0 ± 1.20	6.8 ± 0.112	8.0 ± 0.241*	5.6 ± 0.112	8.95 ± 0.241*

Note: Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change in specified parameter at $p<0.01$ when compared with the control group.

groups when compared with control group Table 7. The levels of albumin and globulin levels were increased in the test group when compared to the control group. No remarkable changes were noticed in total bilirubin, direct as well as indirect bilirubin levels. Both the extract-treated groups have lower levels of cholesterol, LDL, VLDL, and triglycerides. Both the extracts EERA and AERA have raised the levels of HDL (Table 8) but DERA has no effect on the level HDL. With reference to EERA and AERA treated groups significant change was observed in the parameters like SGOT, SGPT, Alkaline phosphatases, Albumins, Bilirubin, Globulins, and total proteins at $p<0.1$ when compared with the vehicle-treated groups.

Table 8. Effect of extracts and vehicle treated groups on Lipid profile of mice

Parameters	Vehicle control group (CMC 1%gel)	DERA treated group (2000 mg/kg)	Vehicle control group (CMC 1%gel)	EERA treated group (2000 mg/kg)	Vehicle control group (Water)	AERA treated group (550 mg/kg)
Cholesterol	128 ± 0.56	125 ± 0.121*	135 ± 1.69	128 ±0.962*	132 ± 2.103	126 ±0.861*
Triglycerides	115 ± 0.20	110 ± 0.35*	120 ± 0.721	110 ± 0.120*	118 ± 0.721	100 ± 0.111*
HDL	20 ± 0.192	22 ± 0.6*	20 ± 0.693	25 ± 0.134*	18 ± 0.452	28 ± 0.134*
LDL	88 ± 1.267	85 ± 1.09*	90 ± 1.386	81 ± 0.262*	93± 1.214	78 ± 0.202*
VLDL	23 ± 1.067	22 ± 0.67	24± 0.689	22 ± 0.120*	22 ± 0.5345	17 ± 0.140*
Cholesterol/ HDL ratio	6.4 ± 0.167	5.0 ± 0.32*	6.7 ± 0.115	5.12 ± 0.085*	7.3 ± 0.14	4.5 ± 0.085*

Note: Parameters in mg/dl; Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change in specified parameter at $p<0.01$ when compared with the control group.

3.5 Haematological Analysis

The effect of three extracts on the hematological profile was represented in Table 9. No considerable changes were observed in levels of Hb, MCHC, neutrophils, and platelets, with the administration of extracts. There was a reduction in levels of total RBCs, lymphocytes, and monocytes in extract-treated groups. There was elevated levels of MCV, WBC, and eosinophils. EERA and AERA treated groups have shown significant change in various parameters at $p<0.1$ when compared with the vehicle-treated group.

Table 9. Effect of extracts and vehicle treated groups on complete blood profile of mice

Parameters	Vehicle control group (CMC 1% gel)	DERA treated group (2000 mg/kg)	Vehicle control group (CMC 1% gel)	EERA treated group (2000 mg/kg)	Vehicle control group (Water)	AERA Treated group (550 mg/kg)
HB (gm/dl)	10 ± 0.37	8.7 ± 0.21*	11.8 ± 0.075	10 ± 0.121*	10.6 ± 0.015	9 ± 0.623*
Total RBC (M/cumm)	9.64 ± 0.92	6.8 ± 0.45*	7.45 ± 0.173	4.0 ± 0.058*	8.45 ± 0.162	5.0 ± 0.023*
MCV (Cumu)	44.2 ± 0.55	62 ± 0.48*	45.2 ± 0.035	75 ± 0.577*	43.2 ± 0.025	68 ± 0.261*
MCHC (%)	35 ± 1.20	34 ± 0.68	35 ± 0.154	33 ± 1.155	38 ± 0.131	34 ± 1.210*
Platelet Count (Lakhs/ cumm)	1.7 ± 0.125	1.8 ± 1.22	1.8 ± 0.126	1.9 ± 1.193	1.6 ± 0.021	1.7 ± 0.0623*

WBC count (cells/cumm)	5400 ± 5.29	7200 ±6.89*	5600 ± 4.619	8400 ± 8.660*	5200 ± 4.321	8200 ± 7.660*
Neutrophile (%)	9 ± 0.37	9 ± 0.2	10 ± 0.48	10 ± 0.208	11 ± 0.23	11 ± 0.102
Lymphocytes (%)	81 ± 1.12	75 ± 2.6*	80 ± 2.209	68 ± 0.052*	82 ± 0.201	65± 0.045*
Monocytes (%)	2 ± 0.104	1 ± 0.65*	3 ± 0.144	1 ± 0.745*	4 ± 0.213	2 ± 0.132*
Eosinophils (%)	2 ± 0.173	6 ± 0.97*	1 ± 0.125	8 ± 0.98*	2 ± 0.121	9 ± 0.76*
MCHC (uugms)	14 ± 0.678	15 ± 0.018*	16 ± 0.577	25 ± 0.017*	15 ± 0.567	23 ± 0.024*

Note: Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change in specified parameter at $p<0.01$ when compared with the control group.

3.6 Histopathology

No mortality was observed in the group which had taken EERA and DERA, but four animals out of five animals died in the group which had taken an AERA of *R. apiculata* extract. Further, the main test was conducted with maximum safe dose, i.e., for EERA and DERA it was 2000 mg/kg, and for aqueous extract of *R. apiculata* extract, it was 550 mg/kg.

Lesions were not observed in any of the samples of vital organs which includes the brain, heart, kidney, liver, and pancreas. There is no evidence of necrosis or ischemic areas in the brain (Figure 1). There was no evidence of inflammatory infiltrates or necrosis in the kidney (Figure 3). The heart sample also showed normal histological features (Figure 2). Mild interphase and peritoneal hepatitis were reported in liver (Figure 4), extramedullary haematopoiesis was observed in spleen and pancreas (Figure 6) was histologically normal. The uterus of extract-treated groups was also found to be normal (Figure 5). There was no significant variation

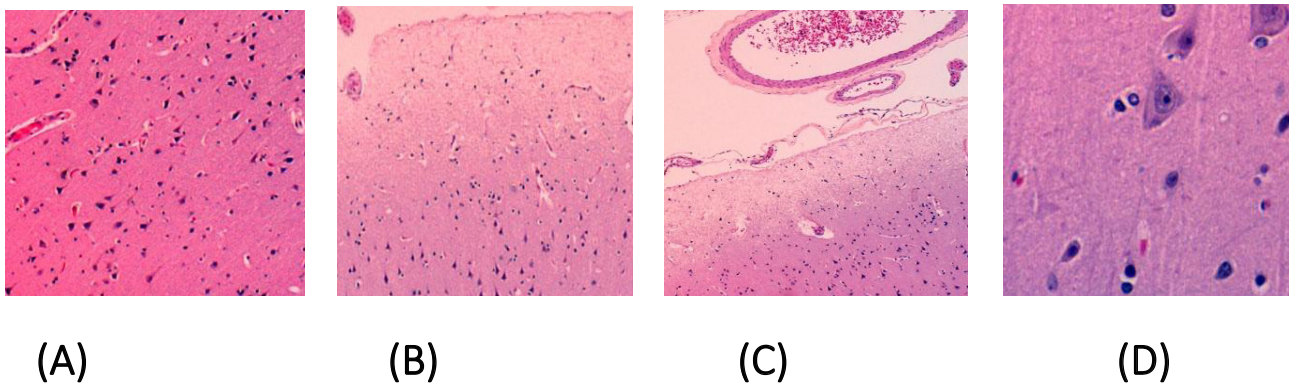


Figure 1. Effect of extracts on histology of brain. **(A)** Normal brain histology of mice; **(B)** Brain histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** Brain histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** Brain histology after administration of AERA (550 mg/kg); No abnormality was recorded.

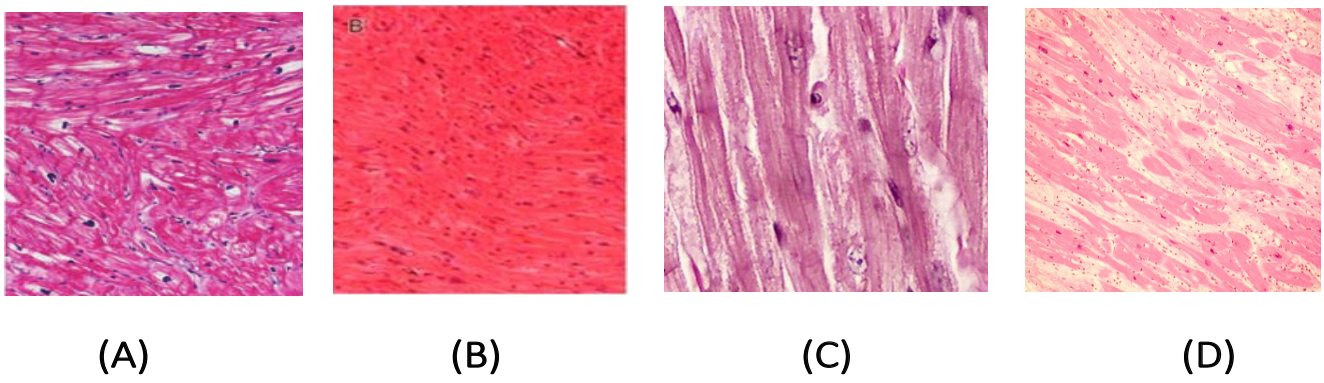


Figure 2. Effect of extracts on histology of Heart. **(A)** Normal Heart histology of mice; **(B)** Heart histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** Heart histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** Heart histology after administration of AERA (550 mg/kg); No abnormality was recorded.

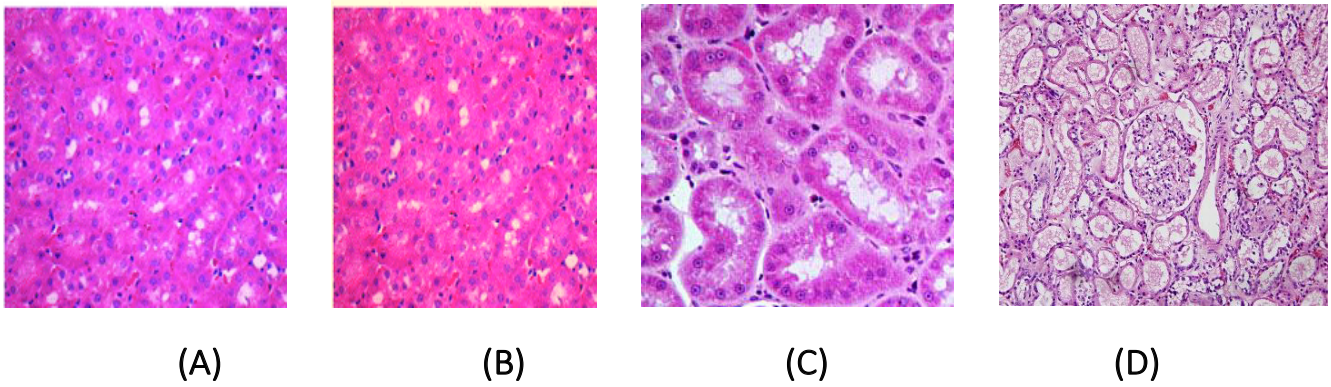


Figure 3. Effect of extracts on histology of Kidney. **(A)** Normal Kidney histology of mice; **(B)** Kidney histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** kidney histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** kidney histology after administration of AERA (550 mg/kg); No abnormality was recorded.

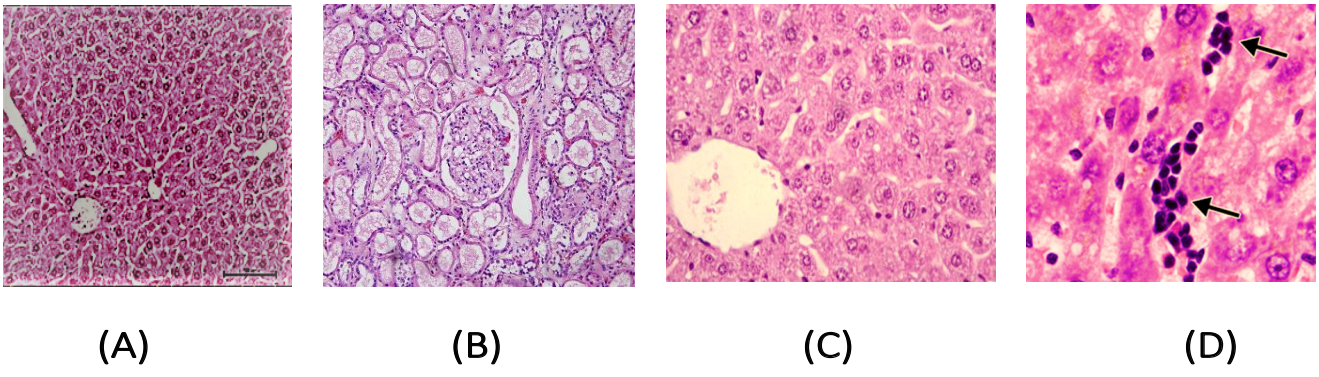


Figure 4. Effect of extracts on histology of Liver. **(A)** Normal liver histology of mice; **(B)** Liver histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** Liver histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** Liver histology after administration of AERA (550 mg/kg); Extramedullary haematopoiesis was observed.

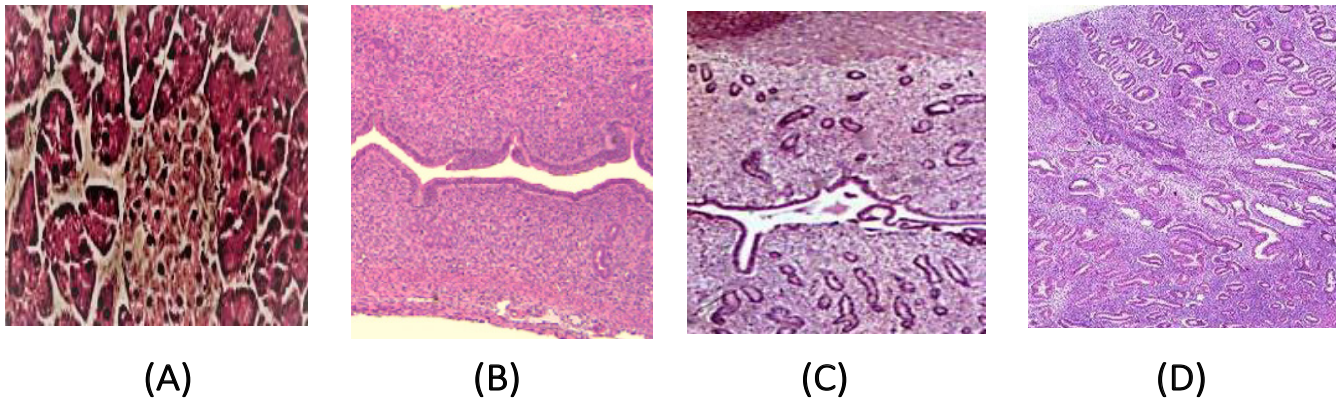


Figure 5. Effect of extracts on histology of Uterus. **(A)** Normal uterus histology of mice; **(B)** uterus histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** uterus histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** Uterus histology after administration of AERA (550 mg/kg); No abnormality was recorded.

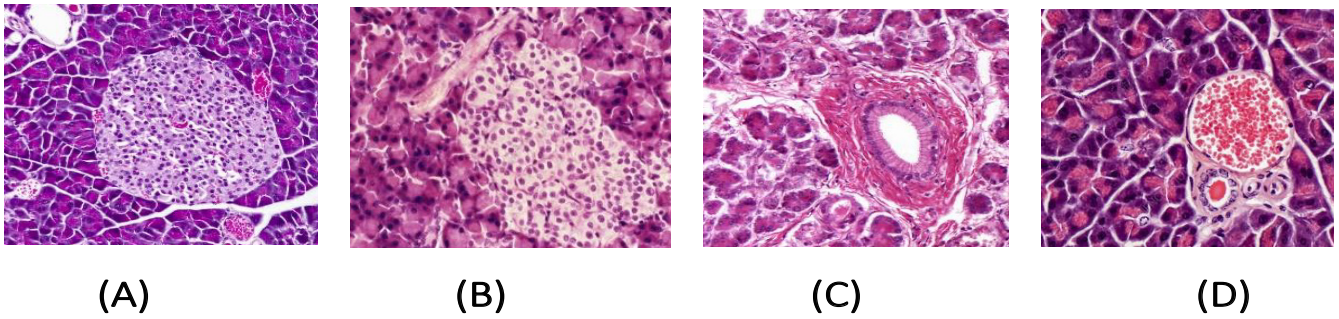


Figure 6. Effect of extracts on histology of Pancreas. **(A)** Normal Pancreas histology of mice; **(B)** Pancreas histology after administration of DERA (2000 mg/kg); No Abnormality was found **(C)** Pancreas histology after administration of EERA (2000 mg/kg); No Abnormality was found **(D)** Pancreas histology after administration of AERA (550 mg/kg); No abnormality was recorded.

Table 10. Effect of organ to body weight indices on mice of control and extract treated groups

Organs	Vehicle control group (CMC 1% gel)	DERA treated Group (2000 mg/ kg)	Vehicle Control Group (CMC 1% gel)	EERA Treated Group (2000 mg/ kg)	Vehicle Control Group (Water)	AERA Treated Group (550 mg/ kg)
Heart	0.615 ±0.09	0.610 ± 0.08	0.814 ± 0.021	0.716 ± 0.029*	0.716 ± 0.011	0.612 ± 0.030*
Kidney	1.465 ± 0.010	1.456 ± 0.020	1.495 ± 0.040	1.572 ± 0.030	1.594 ± 0.020	1.623 ± 0.023
Liver	5.654 ± 0.120	5.641 ± 0.110	6.748 ± 0.296	7.125 ± 0.256	5.732 ± 0.134	6.786 ± 0.321*

Note: Organ to Body Weight index = (Organ weight * 100)/ body weight.

Values are presented as mean ± SEM, (N=5) the values with * mark shown significant change in specified parameter at $p<0.01$ when compared with the control group.

observed in either body weights or body weight indices among the group's (Table 10).

3.7 Analgesic Activity

3.7.1 Hot Plate Method

The results of the hot plate method were shown in Table 11. The EERA treated group in high dose showed that there was a significant decrease in reaction time to jumping & paw licking response when compared to other groups (Figure 7).

3.8.2 Tail Immersion Method

The results of the hot plate method were shown in Table 12. The EERA in high dose increased the time of

Table 11. Results of hot plate method

Group	Latency time		
	0 Min	30 Min	60 Min
Control	4.39 ± 0.37	3.22 ± 0.19	3.69 ± 0.20
Standard	3.81 ±0.69	7.44 ± 0.53	8.57 ± 0.35
EERA 150 mg/kg	3.47 ± 0.17	4.38 ± 0.29	4.75 ± 0.27
EERA 300 mg/kg	3.72 ± 0.16	4.34 ± 0.15	4.68 ± 0.19*
EERA 600 mg/kg	3.88 ± 0.16	6.61 ± 0.20	10.30 ± 0.32*
AERA 100 mg/kg	3.47 ± 0.17	3.92 ± 0.13	4.12 ± 0.31
AERA 200 mg/kg	3.55 ± 0.21	4.78 ± 0.16	4.92 ± 0.29*
AERA 500 mg/kg	3.41 ± 0.29	6.01 ± 0.42	9.61 ± 0.37*

Note: Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change at * p< 0.001, value is significant.

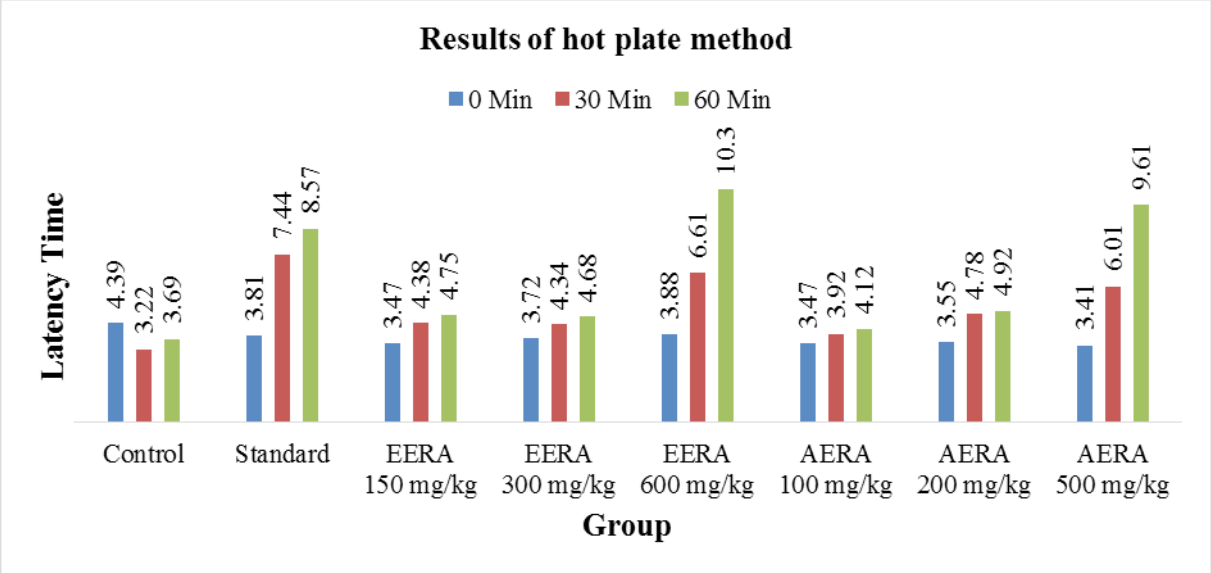


Figure 7. Results of hot plate method showing latency time of paw licking, jumping responses between control, standard and extract treated groups. The values are represented as mean. ± SEM, (N=5).

Table 12. Results of tail immersion method

Group	Latency time		
	0 Min	30 Min	60 Min
Control	2.38 ± 0.20	2.34 ± 0.24	2.27 ± 0.27
Standard	2.10 ± 0.18	4.55 ± 0.38	6.03 ± 0.24
EERA 150 mg/kg	2.50 ± 0.16	2.81 ± 0.14	2.87 ± 0.15
EERA 300 mg/kg	2.55 ± 0.18	4.24 ± 0.17	5.05 ± 0.31*
EERA 600 mg/kg	2.36 ± 0.16	4.27 ± 0.36	5.71 ± 0.17*
AERA 100 mg/kg	2.43 ± 0.18	2.73 ± 0.24	2.88 ± 0.24
AERA 200 mg/kg	2.47 ± 0.16	3.88 ± 0.22	4.61 ± 0.23*
AERA 500 mg/kg	2.53 ± 0.15	4.03 ± 0.26	4.75 ± 0.17*

Note: Values are presented as mean ± SEM, (n=5) the values with * mark shown significant change at * $p < 0.001$, value is significant

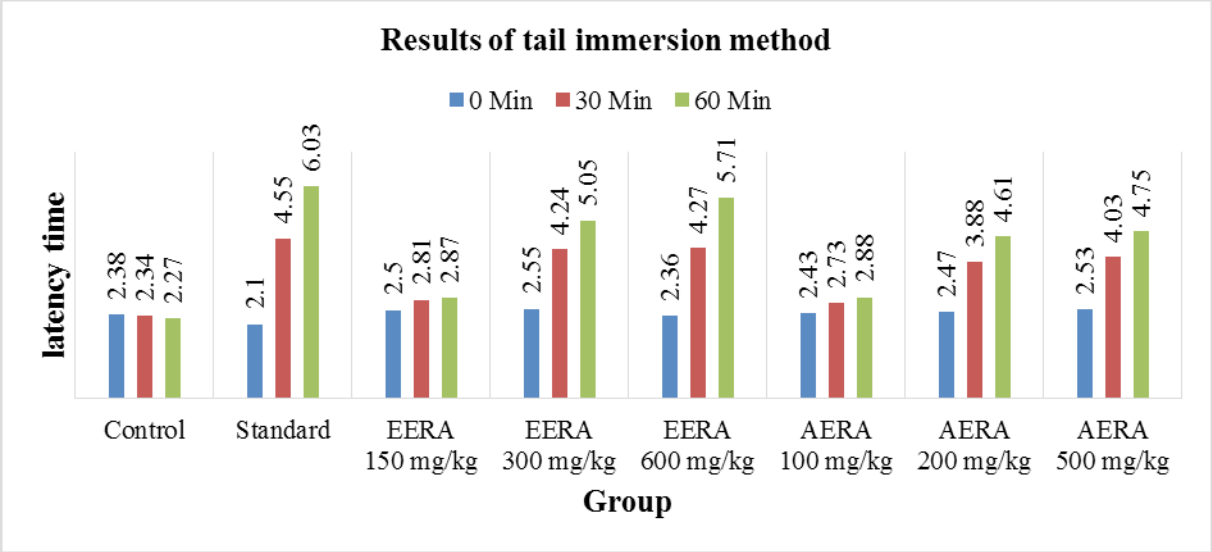


Figure 8. Results of tail immersion method showing latency time of tail withdrawal between control, standard and extract treated groups. The values are represented as mean ± SEM, (N=5).

withdrawal from the hot water bath when compared to the remaining groups (Figure 8).

4. Discussion

Medicinal plants are a boon to mankind because most of the global population relies on drugs derived from natural sources even to cure serious diseases like severe acute respiratory syndrome (SARS), Acquired Immune Deficiency Syndrome (AIDS) infections till today based on the results of one of the surveys conducted by World Health Organization (WHO)[19]. Most of the medicines

which are now in use owe their origin to natural resources[20]. Even though natural plants have medicinal properties they can also have harmful effects when their safety level is exceeded. Hence it is mandatory to determine the safe dose for any natural plant extract[21]. Based on the results of phytochemical investigation all three extracts are tested positive for pharmacologically useful chemical constituents like alkaloids, flavonoids and glycosides[22]. *R. apiculata* leaves have more valuable pharmacological effects[23] like antioxidant activity[24] and antitumor activities[25] based on the previous literature but a toxic profile has been lacking. Therefore, the present study was planned to evaluate the acute toxic profile of diethyl ether, ethanol and aqueous extracts of *R. apiculata* leave *invivo* model by following OECD guidelines 425[26]. In this study, it was scientifically proven that lethal dose data collected from mice will correlate more with the toxic profile in human beings (Uzma Saleem *et al*).

No considerable changes were observed in any of the behavioural parameters of the extract-treated group animals and there was no mortality observed in the animals treated with DERA and EERA. Based on these results it was concluded that the LD50 value of DERA and EERA would be more than 2000 mg/kg[27]. The toxic signs of the extracts were exposed in the behavioural pattern, on histology of vital organs like heart, liver, kidney, pancreas, etc., in biochemical parameters and through hematological parameters also[16]. No mortality was found in EERA and DERA extract-treated groups even at high doses 2000 mg/kg but mortality was observed with AERA hence its dose was reduced to 550 mg/kg and again subjected to the same study with another group of animals[14]. Based on a globally harmonized classification system of chemicals on the basis of their LD50 values. The EARA and DERA extracts can be placed in group 5 (LD50 > 2000 mg/kg), which falls in the lower toxicity class[28].

In this study period, the behavioural pattern of all the animals was observed normally in all animals except itching was observed in the first 48 h of the study but the respiratory rate and somatomotor activities were normal. During the 14 days study period, water and food intake were normal with no significant body weight changes. This suggests that the general metabolism of carbohydrates, proteins and lipids was normal as these are major nutrients that play a vital role in the entire physiology of the body[29], no lesion or necrosis was found. This indicates the extract does not show any cellular toxicity. There were no considerable changes in the histopathology of organs, which may indicate the drug has no toxic effects on vital organs[30]. Mild interphase and peritoneal hepatitis were reported which may indicate the present drug has shown mild toxic effects on the liver. Extramedullary haematopoiesis was observed in the spleen which could be due to age-dependent causes or due to toxicity of the extract[31].

In this study, the health condition of the animals was also assessed by other hematological and biochemical parameters. The serum creatinine levels were normal whereas a little increase in the levels of serum urea levels may indicate some dysfunction in the kidney which may be due to the effect of extracts[32,33]. The level of Alkaline phosphatases (ALP) can be taken as the key indicator for the obstruction of the biliary tract[34]. Total protein, albumin, and globulin levels were enhanced in the extract-treated group when compared to the control group. In general, liver, kidney injury, or some blood disorders may elevate the levels of proteins[35]. Both the EERA and EARA treated groups have lowered cholesterol, triglycerides, LDL, VLDL levels, and a rise in the levels of HDL may indicate this plant has an antihyperlipidemic effect[36]. No considerable changes were observed in the levels of Hb, MCHC, neutrophils, and platelets when compared with the control group.

Haematological parameters which are very sensitive biomarkers of the physiological changes in the body, there were reductions in levels of total RBCs, lymphocytes, and monocytes, when compared with the normal control group, suggesting it has no potential effect against microorganisms and also it has no tendency to reduce the cellular inflammatory process[37].

Traditionally this plant was used to relieve pain by local people so it was a small approach to evaluate its analgesic activity. Both EERA and AERA have shown significant ($p<0.0001$, $p<0.0001$, $p<0.0005$, $p<0.0001$) analgesic activity in medium and high doses respectively. The plants which were proved to reduce inflammation and pain perception may have beneficial effects in improving neuronal cognition activity. Hence further studies are required to evaluate the cognition improving activity by this plant extract.

5. Conclusion

As per the toxicological investigations of *R. apiculata* leaves extracts, i.e., DERA and EERA were found to be safe which suggests the presence of nontoxic substances (LD50 > 2000 mg/kg). On the other hand, the AERA was proved to be toxic at a dose of 2000 mg/kg while it was found safe at 550 mg/kg. All three extracts were not completely devoid of toxic effects as they have shown some toxic signs on the liver kidney and pancreas based on the histopathological, biochemical, and haematological studies. Present plant extracts can be used in a limited dose if their pharmacological effects are proved. However, the results suggest that it should be further evaluated for sub chronic and chronic toxicity profiles in order to ensure safety. The central analgesic activity results explored its significant analgesic activity as per methods employed. The phytochemical results indicated the presence of alkaloids and flavonoids perhaps due to the presence of these constituents the plant possess its analgesic activity[38,39].

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7. Conflict Interest

None

8. Funding

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9. Author Contributions

Mrs. Annie M has contributed her efforts in collecting and hypothesizing the investigative and experimentation work. Dr. Narender M guided the project and reviewed the interpreted data. Dr Nagaraju Banadaru and Dr. T E G K Murthy were helped in the process of analysing the data.

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