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In Vivo Toxicity Study of Ethanolic Extracts of *Evolvulus alsinoides* & *Centella asiatica* in Swiss Albino Mice

Mukesh Kumar Yadav[1*], Santosh Kumar Singh[2], Manish Singh[3], Shashank Shekhar Mishra[4], Anurag Kumar Singh[2], Jyoti Shankar Tripathi[1], Yamini Bhusan Tripathi[5]

[1]Department of Kayachikitsa, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India; [2]Centre of Experimental Medicine & Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India; [3]Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India; [4]Department of Vikriti Vigyan, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India; [5]Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

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Keywords: Centella asiatica; Evolvulus alsinoides L.; Ethanolic extracts; Sub-acute; Toxicity

***Correspondence:** Prof. Jyoti Shankar Tripathi and Mukesh Kumar Yadav. Department of Kayachikitsa, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. E-mail: mukesh.yadav@bhu.ac.in

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Abstract

AIM: We aimed to investigate several parameters after the *in vivo* acute and sub-acute administration of ethanolic extracts from *E. alsinoides* & *C. asiatica.*

METHODS: Malignant Ovarian Germ Cell Tumors for *in vivo* toxicity study guidelines 423 and 407 of Organization for Economic Co-operation and Development (OECD) were followed for acute and sub-acute toxicity assays respectively. For LD50 evaluation, a single dose of ethanolic extracts of Evolvulus alsinoides L. (EEA) and ethanolic extracts of Centella asiatica (ECA) was orally administered to mice at doses of 200, 400, 800, 1600 and 2000 mg/kg. Then the animals were observed for 72 hours. For acute toxicity evaluation, a single dose of both extracts was orally administered to mice at doses of 300, 600, 1200 and 2000 mg/kg and the animals were observed for 14 days. In the sub-acute study, the extracts were orally administered to mice for 28 days at doses of 300, 600, 1200 and 2000 mg/kg. To assess the toxicological effects, animals were closely observed on general behaviour, clinical signs of toxicity, body weight, food and water intake. At the end of the study, it was performed biochemical and hematological evaluations, as well as histopathological analysis from the following organs: brain, heart, liver, and kidney.

RESULTS: The oral administration of E. alsinoides and C. asiatica ethanolic extracts, i.e. EEA 300, EEA 600, EEA 1200, EEA 2000, ECA 300, ECA 600, ECA 1200 & ECA 2000 mg/kg doses showed no moral toxicity effect in LD50, acute and sub-acute toxicity parameters.

CONCLUSION: In this study, we had found that *E. alsinoides* & *C. asiatica* extract at different doses cause no mortality in acute and sub-acute toxicity study. Also, histopathology of kidney, liver, heart, and brain showed no alterations in tissues morphology.

Introduction

Centella asiatica belonging to the family: Apiaceae is native to most of the Asian countries. Being herbaceous, it contains stems which are long, filiform and prostrate with long internodes containing roots, 1-5 leaves per node which are 50-350 cm in radius, uninformed, deeply cordate, long-petioled and oval or orbicular in shape, 3-6 small flowers which are purple to white-green in color and are arranged in umbels arising from the axis of the leaves. *Centella asiatica* grows well in both tropical and sub-tropical countries. The pharmacological activity of *Centella asiatica* is due to several active constituents including kaempferol-3-o-β-d-glucuronide, quercetin-3-o-β-d-glucuronide, castillicetin, apigenin, rutin, luteolin, naringin [1], [2] rosmarinic acid, chlorogenic acid, 3,4-di-o-caffeoyl quinic acid, 1,5-di-o-caffeoyl quinic acid, 3,5-di-o-caffeoyl quinic acid, 4,5-di-o-caffeoyl quinic acid, isochlorogenic acid, asiaticoside, centelloside, madecassoside, brahmoside, brahminoside (saponin glycosides), asiaticentoic acid, centellic acid, madecassic acid, terminolic acid and betulic acid [3].

The plant *Centella asiatica* has been reported

as traditionally used for various ailments including wound healing, bronchitis, asthma, diabetes, allergy, cancer, diuretic, and hypertension and to improve mental ability [4], [5], [6], [7].

Evolvulus alsinoides L. (Family: *Convolvulaceae*) is a small genus composed of about 10–15 species widely distributed in Asian and American countries, with some of its species used medicinally. *Evolvulus alsinoides* is one of the several well-known Ayurvedic crude drugs that have a significant place in the traditional medicinal system of India due to its memory enhancing properties. In *Evolvulus alsinoides* some active chemical constituents present like triacontane, pentatriacontane, evolving, β-sitosterol, two alkaloids betaine and shankpushpin, caffeic acid, 6-methoxy-7-O-b-glucopyranoside coumarin, kaempferol-7-O-b-glucopyranoside, kaempferol-3-O-b-glucopyranoside and kaempferol-3-O-b-glucopyranoside and quercetine- 3-O-b-glucopyra-noside in this species [8].

This plant has some traditional pharmacological activities such as gastro protective [9], antibacterial [10], antiulcer [11], immunomodulatory [12], cytoprotective [13], adaptogenic and antiamnesic [14], anxiolytic [15], diabetes [16], syphilis [17], tonic to brain strength & memory enhancer [18], analgesic and anti-inflammatory activity [19].

We aimed to investigate several parameters after the *in vivo* acute and sub-acute administration of ethanolic extracts from *E. alsinoides* & *C. asiatica.*

Material and Methods

Plant material

Whole plant material of *Centella asiatica* and *Evolvulus alsinoides* were collected from village Ramnapur, Varanasi, Uttar Pradesh, India in October 2015 and authentication was done by Department of Botany, Banaras Hindu University, India and also herbarium of *Evolvulus alsinoides* (voucher specimen no. Convolvul./03/2015) and *Centella asiatica* (voucher specimen no. Apia/02/2015) plants were deposited in the Department of Botany, Banaras Hindu University, India.

Preparation of extracts

The extraction of both plants was done with soxhlet method in ethanolic solvents at 72-82°C for 72 hours. The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources. It is used as a model for the comparison of new extraction alternatives. Generally, a small amount of dry sample is placed in a thimble. The thimble is then placed in distillation flask which contains the solvent of particular interest. After reaching an overflow level, the solution of the thimble-holder is aspirated by a syphon.

Syphon unloads the solution back into the distillation flask. This solution carries extracted solutes into the bulk liquid. The solute has remained in the distillation flask, and solvent passes back to the solid bed of plant. The process repeatedly runs until the extraction is completed. Per cent yield for *Centella Asiatica* and *Evolvulus alsinoides* were 16.7% w/w and 15.3% w/w respectively.

Preparation of extract samples

Ethanolic extracts of *E. alsinoides* (EEA) and *C. asiatica* (ECA) were solubilized in distilled water to obtain solutions of 30, 60, 120 and 200 mg/ml. The doses were evaluated as 300, 600, 1200 and 2000 mg/kg.

Toxicity assays

The safety parameters assessed by conducting the acute and sub-acute toxicity study according to the OECD guidelines [20] 423 and 407 respectively.

Animals

The experimental Swiss albino mice (male and female) 7-8-week-old of 25-30 gm weight were issued by Animal house of Institute of Medical Sciences, Banaras Hindu University Varanasi, Uttar Pradesh. Animals were divided into experimental groups, housed in plastic cages and maintained on a 12-hour light and 12-hour dark cycle. They were given standard food and water ad libitum. The Central Animal Ethical Committee of Banaras Hindu University approved all experimental procedures (CAEC/196).

LD 50 assay

LD50 (Lethal Dose) is the amount of a drug or extracts given at once, which causes the death of 50% population of test animals. This is one way to measure the short-term toxicity of the drug or extract. For LD50 a single dose of ethanolic extracts of both plants *Centella asiatica* and *Evolvulus alsinoides* L. was orally administered to mice at doses of 200, 400, 800, 1600 and 2000 mg/kg. Then the animals were observed for 72 hours.

Acute toxicity assay

The animals were divided into nine experimental groups of 6 animals each (3 male and 3 female). Group 1 received 10 µl/g of distilled water and served as control.

Groups 2 to 5 treated with ethanolic extract of *E. alsinoides* (EEA) at the doses of 300, 600, 1200 and 2000 mg/kg.

Groups 6 to 9 were treated with ethanolic extract of *C. asiatica* (ECA) at doses of 300, 600, 1200 and 2000 mg/kg respectively.

All treatments were administered once by oral gavage. Animals were closely observed for 4 hours following administration and once a day for 14 days on general behaviour, clinical signs of toxicity, mortality, food and water intake. Body weight was measured before and after administration on days 4, 7, 10 and 14. At the end of the experiment, animals were anaesthetized with ketamine (20 mg/kg i.p.). After the anaesthesia has reached depth, the cardiac puncture was performed to collect blood for biochemical and haematological evaluations.

Sub-acute toxicity assay

The animals were divided into nine experimental groups of 6 animals each (3 male and 3 female). Group 1 received 10 µl/g of distilled water and served as control.

Groups 2 to 5 treated with ethanolic extract of *E. alsinoides* (EEA) at the doses of 300, 600, 1200 and 2000 mg/kg.

Groups 6 to 9 were treated with ethanolic extract of *C. asiatica* (ECA) at doses of 300, 600, 1200 and 2000 mg/kg respectively.

All treatments were administered once by oral gavage daily 7 days each week for 28 days. Animals were closely observed for 28 days on general behaviour, clinical signs of toxicity, mortality, food and water intake. Body weight was measured before and after administration on days 7, 14, 21 and 28. At the end of the experiment, animals were anaesthetised with ketamine (20 mg/kg i.p.). After the anaesthesia has reached depth, the cardiac puncture was performed to collect blood for biochemical and haematological evaluations.

Haematological analysis

The haematological evaluation was performed in all surviving animals at the end of the experiment. The complete blood count was performed using an automated haematology analyser. Haematological evaluations included haemoglobin concentration (HGB), red blood cell count (RBC), platelet count (PLT), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and white blood cell count (WBC).

Blood serum biochemistry analysis

The biochemical evaluation was performed in all surviving animals at the end of the experiment. The collected blood was transferred to tubes without anticoagulant and allowed to stand for 60 min at room temperature and centrifuged at 4000 rpm for 10 min. The serum from each blood sample was recovered and stored in cryogenic tubes at -80°C deep freezer. Urea, Creatinine, Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase were evaluated.

Histopathology

The organs were collected from all surviving animals, washed with saline solution 0.9% (w/v), weight and fixed in 40% formaldehyde solution. Then organs were processed for paraffin embedding. Five µm thick sections were prepared and stained with hematoxylin and eosin (H&E). The tissues were analysed in an optical microscope for their general structure, signs of inflammation, degenerative changes and necrosis evidence. The images were captured with the microscope Motic B1 series, scanned through micro camera Moticam 480 using the Motic Images Plus 2.0ML Application Suite software.

Statistical analysis

The relative haematological and biochemical data were expressed as mean ± standard error of the mean (SEM). Data were submitted to analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparison tests. The results were expressed as mean ± SEM. The software GraphPad Prism 6.0 (GraphPad Software, USA) was used for statistical analysis. $P < 0.05$ were considered statistically significant.

Results

Acute toxicity & Sub-acute toxicity

General signs and mortality

No deaths were recorded within 72 hours in LD50 assay after administration of the extracts. No signs of toxicity were observed in animal groups after the treatment with EEA 300, EEA 600, EEA 1200, EEA 2000, ECA 300, ECA 600, ECA 1200 and ECA 2000 mg/kg.

Body weight, relative organ weight, food and water intake

Animal treated with EEA and ECA at the three evaluated doses showed weight gain throughout the entire experiment duration. The increase was the same in treated and control group animals, and the treatment did not affect relative organs weights, food, and water intake (Table 1).

Table: 1 List of different parameters and general signs assessed during toxicity study

Parameters	Dose (mg/kg)							
	EEA 300	EEA 600	EEA 1200	EEA 2000	ECA 300	ECA 600	ECA 1200	ECA 2000
Body weight	N	N	N	N	N	N	N	N
Feed Intake	N	N	N	N	N	N	N	N
Water Intake	N	N	N	N	N	N	N	N
Fur Condition	N	N	N	N	N	N	N	N
Nails Colour	N	N	N	N	N	N	N	N
Eye Colour	N	N	N	N	N	N	N	N
Convulsion	N	N	N	N	N	N	N	N
Locomotion	N	N	N	N	N	N	N	N
Dysponea	N	N	N	N	N	N	N	N
Sedation	N	N	N	N	N	N	N	N
Aggressive Behavior	N	N	N	N	N	N	N	N

(Normal = N, Abnormal = Ab).

Table: 2 Hematological parameters of Swiss mice treated for 28 days with different doses (300, 600, 1200 and 2000 mg/kg) of ethanolic extracts of *E. alsinoides* (EEA) and *C. asiatica* (ECA) n = 6 swiss albino mice.

	Haematological parameters							
Groups	HGB (g/dL)	RBC (10^6 /µL)	PLT (10^3/µL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	WBC (10^3/µL)
Control	12.3 ± 0.28	6.30 ± 0.30	901.2 ± 17.2	41.2 ± 1.09	57.0 ± 0.99	19.5 ± 0.50	32.1 ± 1.01	5.94 ± 0.20
EEA 300	13.0 ± 0.46	6.90 ± 0.17	771.3 ± 39.8	39.6 ± 1.07	54.0 ± 0.36	17.2 ± 0.72	31.3 ± 1.65	5.85 ± 0.55
EEA 600	12.7 ± 0.28	6.24 ± 0.24	936.4 ± 41.0	41.7 ± 1.06	57.4 ± 1.56	20.4 ± 1.06	33.7 ± 1.61	5.89 ± 0.42
EEA 1200	12.93 ± 0.73	6.46 ± 0.44	928.8 ± 23.8	43.66 ± 1.45	57.1 ± 0.96	19.26 ± 0.76	32.56 ± 0.63	5.48 ± 0.63
EEA 2000	13.33 ± 0.63	7.06 ± 0.69	795.9 ± 26.1	43.16 ± 2.00	57.20 ± 1.83	20.33 ± 1.80	31.86 ± 1.68	5.51 ± 0.39
ECA 300	13.63 ± 0.52	7.22 ± 0.31	816.1 ± 10.2	41.52 ± 1.29	55.09 ± 1.09	18.75 ± 1.10	30.77 ± 1.21	5.46 ± 0.29
ECA 600	12.6 ± 0.3	6.66 ± 0.24	795.7 ± 9.1	39.2 ± 0.46	53.43 ± 0.66	16.86 ± 0.17	28.36 ± 1.53	4.89 ± 0.16
ECA 1200	13.5 ± 0.4	7.25 ± 0.5	862.5 ±14.7	45.5 ± 1.9	55.8 ± 0.8	18.8 ± 0.7	32.9 ± 0.7	5.27 ± 0.5
ECA 2000	13.6 ± 0.57	7.46 ± 0.78	784.3 ± 25.5	46.2 ± 1.12	57.5 ± 1.47	20.8 ± 1.28	33.5 ± 0.40	5.79 ± 0.21

One-way ANOVA followed by Dunett's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: Hemoglobin concentration (HGB), Red blood cell count (RBC), platelet count (PLT), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell count (WBC).

Haematological parameters

Treatment with EEA and ECA at all doses did not produce any changes on animal haematological parameters.

Biochemical parameter

Treatment with EEA and ECA at all doses did not produce any statistically significant changes on Urea, Creatinine, SGOT, SGPT and alkaline phosphatase (Table 3).

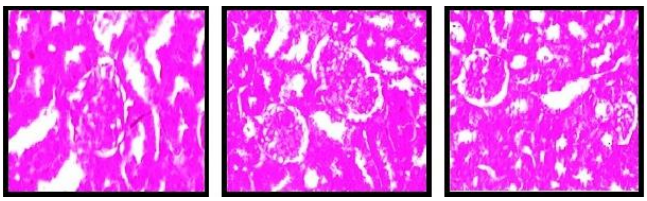
Table 3: Blood serum biochemical parameters of Swiss albino mice treated with dosage (300, 600, 1200 & 2000 mg/kg) of ethanolic extracts of *E. alsinoides* & *C. asiatica.* N = 6 swiss albino mice.

Groups	Urea Normal value = 25-30 mg/dl	Creatinine Normal value = 0.2-0.9 mg/dl	SGOT Normal value = 54-298 mg/dl	SGPT Normal value = 17-77 mg/dl	Alkaline phosphatase Normal value = 35-96 mg/dl
Normal	26.80 ± 1.12	0.3 ± 0.06	57.92 ± 1.62	58.18 ± 3.22	39.41 ± 3.22
EEA 300	26.54 ± 0.82	0.4 ± 0.08	58.59 ± 4.19	52.69 ± 1.11	55.45 ± 4.76
EEA 600	27.45 ± 0.62	0.6 ± 0.1	55.19 ± 2.06	62.67 ± 6.26	46.51 ± 5.73
EEA 1200	27.68 ± 0.68	0.4 ± 0.08	55.26 ± 1.30	59.10 ± 2.8	48.87 ± 2.05
EEA 2000	27.88 ± 0.82	0.4 ± 0.12	61.82 ± 4.28	51.45 ± 8.93	54.38 ± 3.33
ECA 300	27.38 ± 1.02	0.5 ± 0.13	55.08 ± 0.57	63.82 ± 5.79	51.54 ± 4.08
EAC 600	26.33 ± 0.68	0.4 ± 0.12	56.24 ± 1.24	52.80 ± 1.77	55.55 ± 5.08
ECA 1200	27.36 ± 1.80	0.5 ± 0.05	58.24 ± 2.00	59.36 ± 3.56	41.32 ± 3.69
ECA 2000	26.24 ± 0.83	0.2 ± 0.03	57.94 ± 4.51	57.49 ± 2.53	50.82 ± 2.08

One-way AVOVA followed by Dunett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

Histopathological analysis

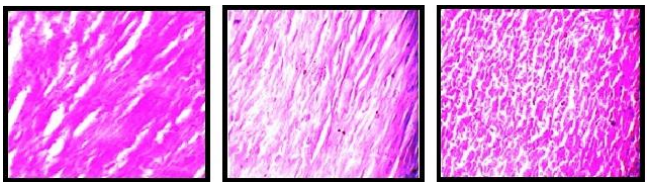
The oral administration of ECA and EEA did not produce significant dose-dependent Histopathological alterations. At the four evaluated doses, it was not observed any tissue damage on the kidney, heart, liver and brain of mice.



(a) Normal (b) EEA (c) ECA

Figure 1: Histopathological slides of kidney organ shown no changes in Swiss albino mice treated with (b) EEA & (c) ECA extracts

Microscopic histological slides from different organs of Swiss albino mice treated with ethanolic extracts of *E. alsinoides* & *C. asiatica,* i.e. EEA & ECA respectively are shown in Figures 1 to 4.



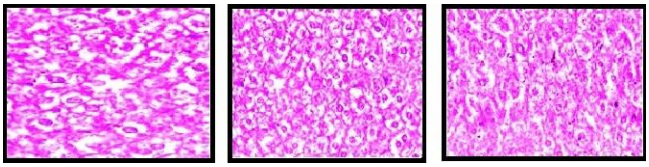
(a) Normal (b) EEA (c) ECA

Figure 2: Histopathological slides of heart organ shown no changes in Swiss albino mice treated with (b) EEA & (c) ECA extracts

Discussion

To assess preliminary toxicity study animal models are widely used because the early

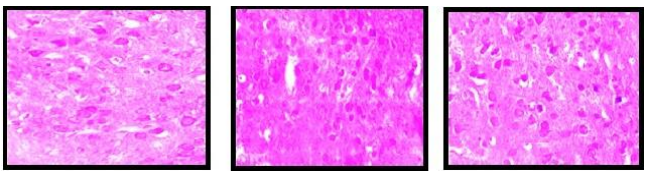
identification of side effects is usually predictive of the toxicity in humans and can save time, resources and efforts [21]. In this study, several parameters evaluated after the *in vivo* acute and sub-acute administration of ethanolic extracts from *E. alsinoides* & *C. asiatica* were investigated. In toxicological evaluation mortality is an important criterion [22] and there was no mortality seen in both acute and sub-acute evaluation of extracts. For LD50 no death was recorded in 72 hours of administration of extracts.



(a) Normal (b) EEA (c) ECA

Figure 3: Histopathological slides of LIVER organ shown no changes in Swiss albino mice treated with (b) EEA & (c) ECA extracts

In acute toxicity, no death was recorded in 14 days extracts administration and in sub-acute toxicity study also no death recorded for 28 days extract administration. Clinical signs of toxicity were observed after the acute administration and during the sub-acute evaluation for all extract dosage. Liver damage is usually assessed by the determination of SGOT, SGPT, and alkaline phosphatase.



(a) Normal (b) EEA (c) ECA

Figure 4: Histopathological slides of brain organ shown no changes in Swiss albino mice treated with (b) EEA & (c) ECA extracts

It was not observed any significant alterations in serum levels of these three markers of liver function after acute and sub-acute administration of extracts and histopathological analysis did not show liver damage. Renal function was evaluated by serum levels of urea, creatinine and by histological analysis.

The histopathological evaluation did not reveal alterations in this organ of any treated groups of sub-acute toxicity. Also, no tissue alterations found in the heart and brain of animals treated with 28 days *E. alsinoides* & *C. asiatica* extracts.

In this study, we had found that *E. alsinoides* & *C. asiatica* extracts at different doses cause no mortality in acute and sub-acute toxicity study. In addition, histopathology of kidney, liver, heart, and brain showed no alterations in tissues morphology.

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