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Phytochemical, Toxicological and Antioxidant Studies of Methanolic and Hyro-methanolic Extracts of *Acoruscalamus*linn

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Abstract—Our present study was aimed to phytochemically screen the methanolic and hydro-methanolic extracts of Acoruscalamus Linn for the evaluation of acute oral toxicity and antioxidant activity (in-vivo and in-vitro). Simple maceration was used to prepare the methanolic and hydro-alcoholic extracts. Standard methods like Mayer's test, Ferric chloride, Shinoda test, Keller-Kiliani test,Million's test, Ferric chloride (1%) test, Salkowski's testetcwere employed to screen the phytochemistry of Acoruscalamaus. Acute toxicity was assessed by monitoring hematology and clinical biochemistry of the test organism (balb mice) Antioxidant activity (in-vivo) was evaluated by employing methods like estimation of SOD activity, estimation of total protein level, and for in-vitro DPPH and ABTS assay were used. Phytochemical analysis revealed the presence of flavones, monoterpenes, xanthone glycosides, sesquiterpenes, lignans, steroids, andphenylpropanoids. Potential toxicity studies revealed that acute administration of methanolic extract (upto1000mg) and hydro-methanolic extract (up to 2500 mg) respectively in Balb mice did not cause any mortality in mice even after 14 days of administration, however, clinical signs like abdominal breathing, piloerection and tremors were observed in first 30 minutes at same dosage. Hematology and biochemical analysis didn't unveil any marked difference in any of the parameter. Antioxidant activity (In-vitro, In-vivo) results revealed concentration dependent antioxidant activity of the extracts.

Keywords*: Acute toxicity; Antioxidant activity; DPPH; ABTS; SOD.*

1. INTRODUCTION

Acoruscalamus(L.) is a perennial, semiaquatic plant seen in the northern temperate and subtropical regions of Asia, North America, and Europe. It is almost six feet tall, with creeping rhizomes. The leaves are long, slender, sword-shaped and simple, arising alternately from the horizontal rhizomes. Flowers are small and fragrant with pale green spadix, fruits are a three-celled fleshy capsule (Nadkarni, 2007). All parts of the plant are known to have volatile oil containingterpenoids, calamine, calamenol, calamenone, eugenol, camphene, pinene, andasaron aldehyde. Acorafuran is a sesquiterpenoid found in calamus oil (Tkachev, 2006). The rhizomes part of the plant is extensively used by the Chinese, Indians and American (Pandy, 2009). Roots and rhizomes are used to overcome various ailments like mental disorders (hysteria, insanity, insomnia, epilepsy, diarrheaand asthma) (Mukherjee, 2007). Having rich phytochemistry and good folk claim our present study focused to evaluate its antioxidant and acute toxicity.

2. MATERIALS AND METHODS

2.1 Collection and air drying of plant material and extraction

*Acoruscalamus*was collected from the Lolab*Valley* of Jammu and Kashmir, India. The plant was authenticated from the Department of Taxonomy, University of Kashmir, Srinagar under voucher specimen No.2436-KASH Herbarium of University of Kashmir, 1/07/2016. A sample specimen was deposited in herbarium for future reference. Extraction was done by simple maceration process. Methanol: water (50:50) % was used as solvent. Concentration was done by using rotatory flash evaporator.

2.2 Tests employed for the detection of various phytoconstituents

Mayer's test,Ferric chloride (5%) test,Shinoda test,Molish's test,Ninhydrin test, Ferric chloride (1%) test were employed to check the phytochemistry of the selected plant.

2.3 Determination of antioxidant activity.

2.3.1*In Vivo* Antioxidant Studies

(i) Estimation of Brain superoxide dismutase (SOD) activity

20mg of the brain was homogenized in 2ml of0.01mM phosphate buffer. The homogenates were centrifuged at 10,000 rpm at 4 ºC in a cooling centrifuge for 20 minutes. 100µl of this supernatant was added to 3 ml of HCl buffer, pH

8.5 followed by 25µl of pyrogallol and then mixed thoroughly. The change in absorbance at 420 nm was recorded at 1-minute interval for 3 minutes.

$$\frac{\text{Unitof SOD}}{\text{ml of Sample}} = \frac{(A - B) \times 100}{A \times 50}$$

Where, **A** = Change in absorbance per minute of Standard,

B = Change in absorbance per minute of test Sample

(ii) Estimation of total protein levels

Protein estimation was done for determining the levels and activity of numerous antioxidant enzymes in the brain. Standard concentrations of protein (250, 500, 750 & 1500µg/mL, samples extracts methanolic (25, 50,100) & hydro-methanolic (100,200,400), standards (imipramine &fluoxetine) and control group were prepared respectively. 100 µL of each of the above were taken to separate tubes and 1ml coomassie blue was added to each tube. the spectrophotometer was adjusted to a wavelength of 595 nmand using the blank tube which contains no protein. After 2 minutes the absorbance of standard and samples at 595nm was read.

Total protein (g/dl) =Y=mx + c

Where Y=0.0365x + 0.497

Thus X= Y-0.497/0.0365

From the available value of Y we can calculate value of X (Protein concentration).

2.3.2 *In Vitro* Antioxidant Screening

(i) Di Phenyl PicrylHydrazyl (DPPH) radical scavenging activity

The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517nm in ELISA. Ascorbic acid (1mg/ml DMSO) was used as reference. Dissolved 1mg of DPPH in 25ml of ethanol and 1mg of ascorbic acid in 1ml of distilled water. Different volumes (10 - 200µl) of plant extracts were made with 1ml DMSO and 20µl DPPH (0.1mM) solution was added (Table.1). The reaction mixture was incubated in dark condition at room temperature for 40 min using 96 plate well. After 40 min, the absorbance of the mixture was read at 517nm in ELISA. The % radical scavenging activity of the plant extracts was calculated using the following formula, $\boldsymbol{DPPH\ Scavenging\ activity}$ (% = $\frac{[(Abs.control - Abs.sample)]}{(Abs.control)]} \times 100$

Where Abs.control is the absorbance of DPPH + methanol; and

Abs. sample is the absorbance of DPPH radical + sample (ie.extract or standard).

2.3.4 ABTS assay (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic)

ABTS substrate and different volumes (10 -100µl) of plant extracts, using ascorbic acid as standard were prepared, total volume up to 1 ml (Table.2). The reaction mixture was incubated in dark condition at room temperature for 40 min using 96 plate well. After 40 min, the absorbance of the mixture was read at 51 nm in ELISA.

2.4 Acute toxicity

2.4.1 Preparation of Dosing Solution

Methanolic extract (MEAC) (200, 500, 700 and 1000 mg) was dissolved in 10 ml of olive oil each to obtained concentration of 20, 50, 70 and 100 mg/ml, respectively, whereas hydro-alcoholic (HAAC) (200,500, 1,000, 2,000 and 2,500 mg) was triturated with 200mg of gum acacia dissolved in 10 ml of distilled water each to obtained concentration of 20, 50,100,200 and 250 mg/ml, respectively and were administered orally at the dose volume of 10 ml/kg body weight to achieve the dose level of 10 mg/kg body weight.

2.4.2 Acute Toxicity study.

Female Balb mice, 7-8 weeks old (25-29 g) were obtained from animal house of IIIM CSIR lab Jammu. Animals were kept under controlled environmental conditions (22±0.5°C, relative humidity 65-67%, 6 am to 6 pm alternate light-dark cycles, food, and water *ad libitum*) in poly-propylene cages covered stainless steel grid and an autoclaved clean rice husk breeding. The animal protocol was approved by the Institutional Animal Ethical Committee (IAEC) protocol number R-290 as per provisions of Committee for the purpose of control and supervision of experimental animals (CPCSEA). MEAC was suspended in 1ml of olive oil on the day of the experiment while as HAAC was triturated with 200mg of gum acacia in 1ml of distilled water on the day of the experiment Dose progression slope 2 of OECD 425 (Haye, 1989) was followed throughout the study with the starting dose of higher limit 2000 mg/kg body weight to 5000 mg/kg body weight. The mice were observed for signs of toxicity and mortality at 0.5, 1, 2, 3, 4 and 6 hours post dosing. Subsequently, the MEAC dose was decreased from 1000 to 700mg/kg body weight whereas HAAC dose was decreased to 2000mg/kg.

Table 1: Procedure for DPPH

	CONTR	**10µg/ml**	**25µg/ml**	**50µg/ml**	**100µg/ml**	**150µg/ml**	**200µg/ml**	**25µg/ml**	**50µg/ml**
Methanol	1ml	990µl	975µl	950µl	900µl	850µl	800µl	975µl	950 µl
Sample	0	10µl	25µl	50µl	100µl	150µl	200µl	0	0
A.A	0	0	0	0	0	0	0	25µl	50µl

DPPH	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µ
Total	1020 µl	1020 µl	1020 µl	1020 µl	1020 µl	1020 µl	1020 µl	1020 µl	1020µl

Table 2: Procedure for ABTS

S. NO	CONTROL	10µg/ml	25µg/ml	50µg/ml	100µg/ml	25µg/ml	50µg/ml
ABTS	1ml	990µl	975µl	950µl	900µl	975µl	950µl
SAMPLE	0	10µl	25µl	50µl	100µl	0	0
A.A	0	0	0	0	0	25µl	50µ
Total	1ml	1ml	1ml	1ml	1ml	1ml	1ml

body weight for morbidity and mortality and initial signs after dosing were recorded and animals were kept under observation for a period of 14 days following 100g feed and 100ml water daily. The clinical signs were recorded once a day. Individual animal body weights were recorded prior to dosing on days 0, 7 and 14. At the end of the 14 days observation period, the mice were kept on fasting whole night and next day blood samples were collected for hematological and biochemical studies.

2.4.3 Hematology

Total red blood cells (RBC), leukocyte (WBC), platelet count, clotting time, erythrocyte indices: -mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and differential leucocyte count, reticulocyte count of blood sample were recorded.

2.4.4 Clinical biochemistry

Glucose, total cholesterol, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, cholesterol, triglycerides, uric acid, total protein, total bilirubin, albumin, globulin, albumin: globulin ratio (A/G ratio), total creatinine, urea, alkaline phosphate were determined using auto analyser.

2.5 Statistical analysis

Results were expressed as Mean ± SEM of six animals (n=6). The difference between experimental groups was compared by one- Way Analysis Of Variance (ANOVA) using the software Graph Pad Instat.

3. RESULTS

The extracts MEAC & HAAC were subjected to phytochemical screening which revealed the presence of flavonoids, tannins, saponins, in high concentration. Methanolic extract shows a higher concentration of flavonoids, phenols, terpenoids, while Hydro-alcoholic extract showed a higher concentration of tannins and alkaloids (Table 3).

3.1 *In vivo* antioxidant study

3.1.1 Super Oxide Dismutase (SOD) levels

From Table.4 we see that at the dose of 400mg/kg b.w/day, of HAAC Group VI showed a highly significant increase in SOD levels (15.44 ± 0.96 U/mg protein × 10^{-5}), whereas, at the dose of 100mg/kg b.w/day of MEAC when administered to Group IX showed a very highly significant increase in SOD level (17.88±1.80 U/mg protein × 10^{-5}) compared to control group which receive the only vehicle for 14 days.

3.1.2 Protein levels

The result of protein estimation as depicted in Table.5 shows that, the dose of 400mg/kg b.w/day, of HAAC extract administered to Group VI for 14 days showed a significant increase in Protein levels (23.51±2.06mg/ml while as the dose of 100mg/kg b.w/day, of (MEAC) extract when administered to Group IX showed a very highly significant increase in Protein level (38.28±3.31mg/ml).

3.2 In *vitro* antioxidant study by (DPPH, and ABTS assay) of hydro-alcoholic extract.

3.2.1 Di phenylpicryl hydrazyl (DPPH) radical scavenging activity.

With the increase in concentration, the Radical Scavenging Activity increased as depicted in Table.6**.** At the concentration of 100,150,200 µg/ml**,** the (HAAC) of the rhizome parts of*AcorusCalamus* was found to produce a greater %age of inhibition 66%, 71%, 84%, respectively indicating a greater antioxidant effect of a Hydro-alcoholic*e*xtract of the rhizome part of *Acoruscalamus*.

3.2.2 ABTS assay (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic.

With increase in concentration, the radical scavenging activity increased as depicted in Table.7**,** has shown that at the concentration of100µg/ml, extract of the (HAAC) rhizome parts *of Acoruscalamus* was found to produce a greater % inhibition 69%**,** indicating a greater antioxidant effect than the 50µg/ml, extract which shows 37.6% inhibition of Hydro-alcoholic extract of the rhizome part of *Acoruscalamus*

3.3 Acute oral toxicity of Methanolic (MEAC) &Hydro-alcoholic (HAAC) extract of *Acoruscalamus* Linn.

3.3.1 Acute Toxicity Study

The results of the acute toxicity study of MEAC& HAAC are presented in Table.8. No mortality or change in body weight was observed in mice at a dose level of the 500 mg/kg, and 700 mg/kg body weight of MEAC extract and dose levels of 1000 mg/kg, 2000 mg/kg body weight of HAAC extract of *Acoruscalamus*Linn. Some clinical signs such as tremors, pilo erection,and abdominal breathing were observed immediately after the oral dose No significant changes were observed on day 14.

3.3.2 Clinical signs:-

No clinical signs like tremors, convulsions, piloerection, aggression, lethargy, abdominal breathing, gait, and licking were observed throughout the study period of 14 days (Table.8)

3.3.3Hematological parameters:-

The values for the hematological parameters in treated and control mice are shown in Table.9. On acute oral administration of MEAC & HAAC (for 14 days) did not exhibit any significant changes in any of hematological parameters. All values remained within physiological range throughout the treatment period

3.3.4 Biochemical parameters

The values for biochemical parameters in treated and control mice of MEAC & HAAC are presented in Table.10. Acute oral administration of HAAC & MEAC (for 14 days) did not show any significant changes in any of the biochemical parameters. Although the values remained within physiological range during the treatment period.

Table 3: PRELIMINARY PHYTOCHEMICAL RESULTS

S. No.	PHYTOCONSTITUENTS	MEAC	HEAC
1	Alkaloids	++	+++
2	Saponins	+	++
3	Tannins	+	++
4	Flavonoids	+++	++
6	Terpenoids	++	+
8	Proteins	+	+
9	Phenolics	++	+
10	Amino acids	+	+

MEAC: Hydro-alcholic extract of Acoruscalamus**HAAC:**Methanolic extract of Acoruscalamus. (-) = Absent; (+) = Slight coloration; (++) = Deep coloration; (+++) = Very deep coloration(showing concentration of the constituents in the extract

Table 4: Effect of Methanolic and Hydro-alcoholic extracts of the Rhizome parts of Acoruscalamus on Superoxide Dismutase (SOD) levels.

	Group (i)	Group (ii)	Group (iii)	Group (iv)	Group (v)	Group (vi)	Group (vii)	Group (viii)	Group (ix)
Mice	**Control**	**Imipramine (STD - i)**	**Fluoxetine (STD-ii)**	**HAAC-100**	**HAAC-200**	**HAAC-400**	**MEAC -25**	**MEAC -50**	**MEAC-100**
1	9.25	20.86	18.44	10.33	11.73	15.44	9.35	15.21	22.14
2	10.18	16.33	17.12	10.98	13.01	12.2	12.54	18.04	17.88
3	9.38	17.65	15.24	13.33	12.48	14.81	8.25	16.22	24.77
4	4.21	18	12.45	9.04	12.61	16.4	11.56	14.55	15.97
5	11.68	23.41	22.57	14.58	9.45	18.07	11.57	19.5	15.55
	Control	**Imipramine**	**Fluoxetine**	**HAAC. 100**	**HAAC. 200**	**HAAC. 400**	**MEAC 25**	**MEAC 50**	**MEAC100**
MEAN	9.38	18	17.12	10.98	12.48	15.44	11.56	16.22	17.88
STDEV	2.815484	2.852481	3.767576	2.258909	1.422526	2.163176	1.781609	2.043803	4.035687
SEM	1.259123	1.275668	1.684911	1.010215	0.636173	0.967402	0.79676	0.914016	1.804814
MEAN± SEM	9.38± 1.25	18±1.27	17.12±1.68	10.98±1.01	12.48±0.63	15.44±0.96	11.56±0.76	16.22±0.91	17.88± 1.80

Each value represents the mean. N=5, *Percent inhibition expressed as mean± SEM Experimental group .p<0.0001, considered extremely significant.

Table 5: Effects of Methanolic (MEAC) and Hydro-alcoholic (HAAC) extracts of the Rhizome parts of Acoruscalamuson Protein.

S.No	Group (i)	Group (ii)	Group (iii)	Group (iv)	Group (v)	Group (vi)	Group (vii)	Group (viii)	Group (ix)
Mice	**Control**	**Imipramine**	**Fluoxetine**	**HAAC-100**	**HAAC-200**	**HAAC-400**	**MEAC-25**	**MEAC-50**	**MEAC-100**
1	12.36	14.06	29.12	12.31	14.03	20.16	17.12	14.08	38.28
2	15.2	20.85	37.45	19.5	18.3	17.87	22.84	21.09	39.25
3	12	18.52	33.94	16.24	16.2	29.35	20.02	16.23	22.15
4	14.23	17.83	24.12	20.16	20.17	26.4	20.15	17.25	40.02
5	12.1	18.01	32.27	19.38	19.84	23.51	19.64	16.11	34.49
	Control	**Imipramine(DOSE)**	**Fluoxetine**	**HAAC-100**	**HAAC-200**	**HAAC-400**	**MEAC-25**	**MEAC-50**	**MEAC-100**
Mean	12.36	18.01	32.27	19.38	18.3	23.51	20.02	16.23	38.28
Stdev	1.450352	2.441256	5.049896	3.283751	2.586459	4.622669	2.031128	2.583141	7.403929
Sem	0.648617	1.091763	2.258382	1.468538	1.15667	2.06732	0.908348	1.155216	3.311138

mean ±sem	12.36±0.64	18±1.09	32.27±2.25	19.38±1.46	18.3±1.15	23.51±2.06	20.02±0.90	16.31±1.15	38.28±3.31
Each value represents the mean. N=5, *Percent inhibition expressed as Mean± SEM Experimental group .p<0.0001, considered extremely significant									

TABLE 6: Effect of Hydro-alcoholic extracts of Acoruscalamus on DPPH Radical Scavenging Activity (200µl/well).

S. No	Control	Std(1)	Std(2)	Test(1)	Test(2)	Test (3)	Test(3)	Test (4)	Test (5)
	control	25aa	50aa	10µM	25µM	50µM	100µM	150µM	200 µM
A	0.319039	0.27116	0.22134	0.286429	0.27216	0.23677	0.10986	0.09871	0.05078
B	0.331409	0.28132	0.23434	0.289599	0.273299	0.24817	0.09768	0.07454	0.05339
C	0.31308	0.27432	0.24316	0.29114	0.271919	0.24271	0.11786	0.09768	0.04823
Average	**0.321176**	0.2756	0.23294	**0.289056**	**0.272459**	**0.24255**	**0.108467**	**0.09031**	**0.0508**
%	0	14.19035	27.47	10.0007	15.17	24.5	66.22	71.88	84.18
Each value presented as mean of triplet treatments, LSD least significantly different at p ≤0.01 according to Duncan'smultiple range test.									

Table 7: Effect of Hydro-alcoholic extracts Acoruscalamus on ABTS Radical Scavenging Activity

S.No	Control	Test(1)	Test(2)	Test(3)	Test(4)	Std(1)	Std(2)
	Cont	10µM	25µM	50µM	100µM	25 A.A	50A.A
A	0.328839	0.26792	0.273219	0.23582	0.11852	0.29377	0.21479
B	0.42729	0.332749	0.253489	0.2371	0.11654	0.293369	0.21064
C	0.38338	0.31181		0.2372	0.11868	0.287459	0.21755
Average	0.379836	0.304159	0.263354	0.236706	0.117913	0.291533	0.214327
%age	0	20	30	37.6	69	23	43.5
Each value presented as mean of triplet treatments, LSD least significantly different at p ≤0.01 according to Duncan'smultiple range test							

Table. 8: Clinical signs of toxicity observed during acute oral toxicity study of Methanolic(MEAC) &hydro alcoholic (HAAC) extract of Acoruscalamusin female mice

METHANOLIC (MEAC)			HYDRO – ALCOHOLIC (HAAC)		
Dose (mg/kg)	Latency	Symptoms	Dose (mg/kg)	Latency	Symptoms
175		None			
250		None	200		None
500		Pilierection	500		None
700		Tremar, piloerection, abdominal breathing	1000		Abdominal breathing, Piloerection
750		Tremar, piloerection, abdominal breathing	2000		Abdominal breathing,Piloerection

Table 9: Effect of Methanolic (MEAC) & hydro-alcoholic (HAAC) extract ofAcoruscalamuson hematological parameters in Acute toxicity study for 14 days

Methanolic (MEAC)			Hydro-alcoholic (HAAC)		
Parameters	**Control**	**Treated**	**Parameters**	**Control**	**Treated**
WBC(10^3/µL)	6.74±1.41	6.29±1.55	WBC(10^3/µL)	6.74±1.41	5.16±0.55
RBC(10^6/µL)	9.90±0.21	10.43±0.18	RBC(10^6/µL)	9.90±0.21	10.83±0.25
HGB(g/dL)	13.96±0.27	14.5±0.22	HGB(g/dL)	13.96±0.27	14.48±0.19
HCT(%)	45.14±1.06	47.8±0.60	HCT(%)	45.14±1.06	45.52±0.91
MCV(fL)	45.66±1.29	45.88±0.75	MCV(fL)	45.66±1.29	42.04±0.80
MCH(pg)	14.14±0.32	13.92±0.28	MCH(pg)	14.14±0.32	13.38±0.23
MCHC((g/dL)	30.94±0.17	30.34±0.25	MCHC((g/dL)	30.94±0.17	31.82±0.30
PLT(10^3/µL)	1443.40±181.23	1628.2±59.83	PLT(10^3/µL)	1443.40±181.23	1689.6±41.21
NEUT(%)	28.44±4.97	23.52±4.2	NEUT(%)	28.44±4.97	26.38±2.86
LYMPH(%)	68.68±4.80	63.1±3.06	LYMPH(%)	68.68±4.80	69.3±2.94
MONO(%)	1.08±0.21	0.98±0.34	MONO(%)	1.08±0.21	1±0.12
EO(%)	1.78±0.68	1.34±0.51	EO(%)	1.78±0.68	3.26±0.22
BASO(%)	0.02±0.02	0.04±0.02	BASO(%)	0.02±0.02	0.06±0.04

Table 10: Effect of Methanolic(MEAC) & hydro-alcoholic (HAAC) extract of Acoruscalamuson biochemistry parameters in Acute toxicity study for 14 days.

Methanolic (MEAC)			Hydro-alcoholic (HAAC)		
Parameters	**Control**	**Treated**	**Parameters**	**Control**	**Treated**
Alkaline phosphate U/L	70.2±18.7	94.00±28.40	Alkaline phosphate/L	70.2±18.7	66.8±23.27
bilirubin total mg/dl	0.09±0.00	0.20±0.01	bilirubin total mg/dl	0.09±0.00	0.06±0.02
Creatinine mg/dl	0.29±0.01	0.23±0.01	Creatinine mg/dl	0.29±0.01	0.22±0.02

Uric acid mg/dl	2.7±1.3	3.80±0.42	Glucose mg/dl	99.0±15.6	95.3±14.9
Total protein mg/dl	8.17±0.34	6.57±0.52	Triglycerides mg/dl	73.2±9.4	64.3±8.40
Glucose mg/dl	99.0±15.6	102.20±10.17	Cholesterol mg/dl	136±39.9	118±8.82
Triglycerides mg/dl	73.2±9.4	90.76±15.45	Uric acid mg/dl	2.7±1.3	1.94±0.40
Cholesterol mg/dl	136±39.9	117.40±4.82	Total protein mg/dl	8.17±0.34	6.91±0.30
AST/GOT U/L	35.22±5.24	34.1±8.46	AST/GOT U/L	35.8±7.66	34.28±5.45
ALT/GPTU/L	42.1±3.10	55.40±9.10	ALT/GPTU/L	42.1±3.10	39.4±3.03
Urea mg/dl	47.52±2.05	40.12±14.06	Urea mg/dl	47.52±2.05	47.56±4.32

4. DISCUSSION

The extracts (MEAC & HAAC) were subjected to phytochemical screening which revealed the presence of flavonoids, tannins, saponins, carbohydrates, steroids in high concentration.

MEAC extract showed a higher concentration of flavonoids, phenols, terpenoids, and steroids while HAAC extract showed a higher concentration of saponinstannins and alkaloids. Besides glycosides and amino acids were found in smaller concentration as evident from the colour intensity. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2. This property may explain the mechanisms of antioxidative action of *Acoruscalamus*.

The current study affirms the *in-vivo* antioxidant potential of crude extracts of rhizomes of *Acoruscalamus*, with results comparable to those of the standard compounds such as Imipramine (18±1. 27

U/mg protein × 10^{-5}) and Fluoxetine(17.12±1.78± U/mg protein × 10^{-5}) and control group (9.38±1.25 U/mg protein × 10^{-5}). Both MEAC & HAAC groups show a dose-dependent increase in SOD levels. The antioxidant potential of this plant provides justification for its use in folklore medicine for treatment of depression because SOD levels are decreased in depressed patients as compared to healthy controls. Here the antioxidant activity of *Acoruscalam us* has shown dose-dependent increase in SOD levels which is mostly decreased during depression. Both (MEAC) and (HAAC) extracts of the rhizome parts of *Acoruscalamus* showed dose-dependent increase in Protein levels (By Bradford method) in the brain homogenates of mice administered with extracts for 14-days when compared to the Control Group. A very highly significant decrease in the protein levels of Control Group I (12.36±0.64mg/ml) administered with vehicle, when compared to the Group II (18±1.09mg/ml) which received 15mg/kg/day of Imipramine and Group III (32.27±2.25mg/ml) which received 20mg/Kg/day of Fluoxetine. While the values of both extracts MEAC &HAAC ranges in between control group and standard group.

The DPPH radical scavenging activity shown by Hydro-alcoholic extract (HAAC) of the rhizome part of *Acoruscalamus* for DPPH radical scavenging activity was found to be concentration dependent. With the increase in concentration, the radical Scavenging activity increased as depicted in Table.6. Ascorbic acid was taken as a standard. At the concentration of 10, 25, 50,100,150,200 µg/ml, the (HAAC) of the rhizome parts *of Acoruscalamus* was found to produce a greater percentage of inhibition of 10%, 15%, 24%, 66% ,71%,84%, respectively indicating a greater antioxidant effect of Hydro-alcoholicextract of the rhizome part of *Acoruscalamus* .

The ABTS radical scavenging activity shown by HAAC was found to be concentration dependent. With the increase in concentration, the radical scavenging activity increased as depicted in Table.7, taking ascorbic acid as a standard. At the concentration of 10, 25, 50,100, µg/ml, of HAAC extracts were found to produce a greater % inhibition of 20 %, 30%, 37.6%, 69%, respectively indicating a greater antioxidant effect of a Hydro-alcoholic extract of the rhizome part of *Acoruscalamus.*The*Acoruscalamus*extract showed a remarkable increased and decreased levels of certain parameters due to the exposure to noise-stress which ultimately proves their antioxidant activity (Devi *et al.*, 2009). *Acoruscalamus*has been found to render the protection against γ-radiation-induced oxidative stress (Sandeep *et al.*, 2010).

The doses starting at 200, 500, 1000 mg/kg and limit dose 2,000 mg/kg were selected for acute toxicity study, in balb mice as per OECD 425. No mortality was observed in the animal at all selected dose levels. Some signs of toxicity were observed on the administration of doses above 2000mg/kg body weight, but they were all reversible in a maximum period of 24 h after the administration of the extract. The LD_{50} value was found to be1000mg/kg and more than 2,000 mg/kg body weight for HAAC whereas for MEAC it was found to be 700mg/kg body weight. No mortality or clinical signs were observed with the above mentioned dose levels. However, it is very important as it indicates the capability of the reaction of the organism that received the drug. No statistical reduction in feed/water consumption was shown from Ist day to 14th day in comparison to control, and no change in body weight was seen weekly during 14 days respectively. Body weight changes if any indicator of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight. After 14 days of treatment, there were no significant changes in the hematological parameters between control and treated groups

The results indicate that the *Acoruscalamus*extract was neither toxic to the circulating red cells, white blood cells and platelets nor interfered with their production. Hematopoiesis and leucopoiesis were also not affected even though the hematopoietic system is one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in humans and animals. Therefore, it plausible to assume that the extracts are not hemotoxic.

All biochemical parameters of treated and control were in range for 14 days indicating no severe liver or kidney damage. Significant changes in enzymes like ALP, AST, and ALTrepresent liver impairment since these are important indices of liver toxicity. Serum cholesterol and proteins are mainly regulated via synthesis in the liver and increase in serum concentrations of constituents suggest liver toxicity.

Kidney toxicity has also been reported after use of phytotherapeutic products what makes essential its evaluation. In that case, creatinine and urea determinations are critical as these substances are markers of kidney function. The studies carried out suggest that at 700 mg/kg dose of MEAC & 2000 mg/kg dose of HAAC the extract's seems to be safe no acute toxicity was seen after 14 days. Thus, considering that Hydro-alcoholic *Acoruscalamus* (HAAC) & Methanolic *Acoruscalamu* (MEAC)extract to be used for an undetermined time, further studies in non-rodents must be performed to prove its safety.

5. CONCLUSION

The Methanolic (MEAC) and hydro-alcoholic (HAAC) extract of the rhizome part of *Acoruscalamus* significantly reduced the oxidative stress in a dose-dependent manner. Again, the effect was more prominent at the dose of 400 mg/kg, (HAAC) than the doses of 200 and 100mg/kg b.w/day, evidenced by improved/increasing the activities of antioxidant enzymes, (Superoxide Dismutase (SOD)), in the brains of balb mice and again methanolic extract (MEAC) showed highly significant effect as compared to hydro-alcoholic extract.

Both Methanolic (MEAC) and Hydro-alcoholic (HAAC) extract of the rhizome part of *Acoruscalamus* showed dose-dependent increase in the Protein levels (Dose of 400 mg/kg b.w/day HAAC showed more increase than the dose of 200,100 mg/kg b.w), again methanolic extract showed more prominent increase as compared to hydro-alcoholic extract.

The Hydro-alcoholic (HAAC) extract of the rhizome part of Acoruscalamus showed concentration-dependent increase in the antioxidant Activity (DPPH, ABTS Assay), The concentration of 200µg/ml showed very highly significant decrease in the mean % inhibition as compared to the concentration of 10, 25, 50,100,150 µg/ml).

LD_{50} of Methanolic (MEAC) and hydro-alcoholic (HAAC) extract of rhizome part of *Acoruscalamus*was seen at above > 700 mg/kg b.w for methanolic (MEAC) and above > 2000 mg/kg b.w for hydro-alcoholic (HAAC.)

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