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Research Article
LC-ESI MS/MS Profiling, Antioxidant and Anti Epileptic Activity of *Luffa cylindrica* (L.) Roem Extract

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Abstract

Background and Objective: Around 80% people are suffering from central nervous system (CNS) disorder and epilepsy, where most of the patients do not get adequate treatment. Management of epilepsy through synthetic drugs exhibit some side effects and may cause neurological changes. The aim of present study was to examine the *Luffa cylindrica* entire fruit alcoholic extract for polyphenol profiling, antioxidant and anti epileptic activity followed by biochemical and oxidative stress study and report a safe natural source for the management of epilepsy. **Materials and Methods:** The prepared extracts were detected in positive and negative mode of UPLC-MS/MS study followed by antioxidant activity through, 1,1-Diphenyl-2-picryl hydrazyl (DPPH) (227.19±0.84), superoxide dismutase (SOD) (2386.03±0.66) and lipid peroxidation (LPO) (226.14±1.38). The same extract was studied for diazepam induced sleep, pentylenetetrazole induced (PTZ) and maximal electroshock (MES, 70 mA) induced convulsion in wistar rat. Oxidative stress and cholinesterase activity of brain tissue was studied by estimating reduced glutathione and lipid peroxidation, total protein in both convulsive models. The changes at cellular level in rat brain histopathology were examined in microscope. **Results:** The study revealed the presence of total phenol (195.21±3.42 μg mL^{-1}) and flavonoid (641.25±0.24 μg mL^{-1}) and specifically 16 compounds i.e., hyperoside, kaempferol, acacetin, vitexin, leotiolin etc. were identified through LC-MS. The extract (400 mg kg^{-1}) was found potentially active ($p<0.05$) in all the anticonvulsant study but less than the standard (phenytoin and diazepam). Minimal changes were observed in the extract (400 mg kg^{-1}) and standard drug. **Conclusion:** *Luffa cylindrica* is using regularly in daily life as vegetable may be good source of potential antioxidant and anti epileptic activity. The alcoholic entire fruit extract showing the presence of phenolics and flavonoids suppose to be effective for the anti epileptic activity. However, further studies still needed to be carried on the extract for constituent specifically for the reported activity.

Key words: *Luffa cylindrica*, antioxidants, anti epileptic, flavonoids, cholinesterase activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In developing countries, 80% of people suffering from epilepsy, where most of the patients are not acquired adequate treatment[1]. Epilepsy is treated by anticonvulsant drugs, around one-third of population suffers from un-prevented neurological changes induced by epileptic seizures and also exhibit some side effects. Long duration of seizure may cause neurological changes and ultimately ended by neuronal death. It is affecting approximately 70 million people of the world, among whom about 22.5% of patients with consistent access of medical treatment and have drug resistant seizures, thereby making them more prone to comorbid illness[2]. It is known that epileptic attack may cause oxidative stress, free radicals are produced and membrane lipid peroxidation occurs, causing tissue damage. Scientists are interested in using drugs from natural sources that can mask the undesirable effects of synthetic drugs. The drugs from natural sources could serve for effective medication which are more readily accessible and inexpensive and thus would be helpful in improving the status of patient[3].

Luffa cylindrica (LC) family is Cucurbitaceae, cultivated and naturalized plant is widely distributed in the tropical and subtropical region. *Luffa cylindrica* crop grows well in tropical regions up to 1500-1800 m altitude[4]. Many polyphenols are reported such as coumaric acid, diosmetin-7-O-β-D-glucuronide methyl ester, apigenin-7-O-β-D-glucuronide methyl ester, luteolin-7-O-β-D-glucuronide methyl ester to have different pharmacological activity like anti-inflammatory, antitumor, antihyperglycemic, antidiabetic[5] etc.

Liquid chromatography associated with mass spectrometry is a powerful tool in pharmaceutical and plant metabolism analytics and the use of this hyphenated technique is now very common in bioanalytical laboratories[6]. This is a result of recent developments in separation sciences and instrumentations in general and leads to the use of modern Ultra Performance Liquid Chromatography (UPLC) together with the high selectivity and performance of mass spectrometry. The enhanced separation of analytes in faster analysis time, yielding better throughput with superior results, is leading bioanalytical laboratories to shift from traditional High-Performance Liquid Chromatography (HPLC) to UPLC[7]. UPLC method was chosen as it is precise, accurate, reproducible and allows the identification of compounds based on their ESI-MS/MS spectra. The present study aimed to assess the phytochemical constituents present in the extract using UPLC-MS/MS both by positive and negative mode both.

Anti epileptic activity of the LC entire fruit extract in maximal electrical shock-induced (MES) and Pentylenetetrazole induced (PTZ) models was evaluated along with the oxidative stress activity. Acetylcholinesterase, butyrylcholinesterase activity and total protein, reduced glutathione, DPPH, SOD, lipid peroxidation and histopathology of brain tissue were also analyzed.

MATERIALS AND METHODS

Plant material and extract preparation: *Luffa cylindrica* fruit was collected in the market of Varanasi in the month of August-September, 2016. Fresh fruit was thoroughly washed, separated in different part (fruit pulp, skin and entire fruit), sliced into small slice pieces and dried under shade. The dried pieces were coarsely (60#) powdered and stored at room temperature. Successive extraction of pulp, skin and entire fruit dried powdered drug of 100 g was carried out with different organic solvent (hexane, chloroform, ethyl acetate and ethanol) by cold maceration for 7 days at room temperature. The extracts were first filtered through a cotton plug followed by filter paper and were concentrated using vacuum rotavapour (Buchi R-210 Advanced, Switzerland) at 37°C. The dried extracts were kept in freeze (-40°C) until use.

Preliminary phytochemical screening: Preliminary phytochemical screening of *Luffa cylindrica* fruit part (skin, pulp and entire fruit) extracts in hexane, chloroform, ethyl acetate and ethanol was done for the presence of various phytoconstituents[8].

Total phenolics: Total phenolics were estimated based on the Folin-Coicalteu (FC) calorimetric methods proposed by Singleton *et al.*[9] with slight modifications. The FC assay was carried out by pipetting 1 mL of extract into 10 mL of volumetric flask followed by 8 mL water and 0.5 mL of FC reagent addition. The mixture was vortexed for the 30 sec and after 15 min, 1.5 mL of 20% sodium carbonate solution was added. The absorbance of the colored product was measured at 765 nm after 2 h at ambient temperature. A calibration curve was prepared using different concentrations of gallic acid solutions. The level of total phenol (TP) was calculated from the standard calibration curve. Results were expressed in milligram of gallic acid equivalent per gram (mg GAE g^{-1}) of dried ethanolic extract. The total content of tannins was also determined using FC reagent.

Total flavonoid: Total flavonoid content in crude extracts was determined by the aluminium chloride ($AlCl_3$) colorimetric method. About 50 µL of crude extract (1 mg mL^{-1} ethanol) were diluted up to 1 mL with methanol, mixed with 4 mL distilled water and 0.3 mL of 5% $NaNO_2$ solution followed by incubation for 5 min and addition of 0.3 mL of 10% $AlCl_3$ solution. Then, 2 mL of 1 mol L^{-1} NaOH solution was added and the final volume of the mixture was made up to 10 mL with double-distilled water. Mixture was allowed to stand for 15 min and absorbance was taken at 510 nm. The total flavonoid content estimated by calibration curve and the result was expressed as milligram rutin equivalent per gram of dried extract[10].

Antioxidant activity

DPPH free radical scavenging: The free radical scavenging activity of *Luffa cylindrica* entire fruit extract (LC extract) was determined based on the scavenging activity of stable 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical[11]. Different concentration of extracts were added to 3 mL of a 0.004% methanolic solution of DPPH and incubated for 30 min at 37°C and absorbance was recorded at 517 nm. Inhibition percentage of free radical by the sample was calculated using the formula:

$$\text{DPPH free radical scavenging activity (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_t is the absorbance of the sample/standard.

Superoxide radical scavenging: This assay of LC extract was based on the capacity of extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) with little modification[12]. Reaction mixture of each 3 mL contain 0.01 M phosphate buffer (PBS) (pH 7.8), 130 mm methionine, 60 µM riboflavin, 0.5 mM EDTA, 0.75 mM NBT and 1.0 mL test solutions. It was kept in front of the fluorescent light for 6 min and absorbance was noted at 560 nm. The percentage inhibition of superoxide generation was estimated by comparing the absorbance of control with reaction mixture containing the test sample.

Lipid peroxidation: A modified Thiobarbituric Acid-Reactive Species (TBARS) assay was used to measure the lipid peroxide formed using egg yolk homogenate as lipid-rich media[13]. Malondialdehyde (MDA), a secondary product of oxidation of polyunsaturated fatty acid, reacts with two molecules of Thiobarbituric Acid (TBA), yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (250 µL, 10% in distilled water, v/v) and 50 µL of extracts were mixed in test tube and the volume was made to 500 µL by adding distilled water. Further 25 µL freshly prepared $FeSO_4$ (0.07 M) was added to the mixture and incubated for 30 min. Then, 750 µL of 20% acetic acid (pH 3.5), 750 µL of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulfate) and 25 µL of 20% TCA was added, vortexed and heated in a boiling water bath for 60 min. After cooling 3.0 mL of 1-Butanol was added to each test tube and centrifuged at 3000 rpm for 10 min. The absorbance of the upper organic layer was recorded against 3 mL butanol at 532 nm:

$$\text{Concentration} = A \times \frac{V}{E} \times P$$

UPLC-MS/MS based profiling: A Micromass LCT Premier XE (Waters MS Technologies, Manchester, UK) was used for profiling of LC extract. The scanning was performed from 100-1000 m/z. The LC extract was diluted and filtered through a 0.22 mL PVDF syringe filter before being injected into the UPLC-MS/MS system. The capillary and cone voltage were set at 2.4 kV and 35 V, respectively, for positive and negative electrospray modes. The desolvation gas was set at 750 L h^{-1} and temperature on 350°C, the cone gas was set at 30 L h^{-1} and the source temperature was on 110°C. The rate of data acquisition was adjusted to 0.2 sec, with a 0.1 sec interscan delay. Analyses were carried out using lock spray to ensure accuracy and reproducibility. The Software Respect for Phytochemicals (http://spectra.psc.riken.jp/) was used for data analysis.

Pharmacological study

Experimental animals: Adult wistar albino rat (180-200 g) of either sex, were obtained from the Central Animal House, Institute of Medical Sciences, B.H.U. and were randomly distributed into different groups. The rats were kept in groups of six in polypropylene cages at an ambient temperature of 25±1°C and 45-55% relative humidity (RH), with a 12:12 h cycle light and dark. Animals were provided with food pellet and water *ad libitum*. Experiments were carried out between 09:00 and 14:00 h. Animals were acclimatized for at least once 1 week before using for the experiment. Guide for the care and use of laboratory animal guidelines were fully followed[14].

Drugs: The following drugs and chemicals were used and all the reagents and chemicals used were of analytical grade. Diazepam (Ranbaxy, India) (1 mg kg^{-1} i.p.) was used as the standard anxiolytic agent. Phenytoin (Sigma, St. Louis, USA) (25 mg kg^{-1} i.p.) was used as the standard anticonvulsant drug.

Acute and neurotoxicity study: Acute toxicity studies of LC extract was performed as per OECD-423[15] guidelines to determine the safety of doses using female wistar strain albino rats. Rats were divided into six groups and LC extract was orally administered with increasing doses of 100, 500, 1000, 2000 and 4000 mg kg^{-1} b.wt. After treatment, the rats were observed 4 h for any sign of toxicity such as motor tremors, activity, tonic extension, convulsions, loss of righting reflex, muscle spasm, sedation, ataxia, hypnosis, diarrhea, salivation, lacrimation and writhing. Observation was made up to 72 h for any mortality ("OECD Guideline for testing of Chemical Acute Oral Toxicity-Acute Toxic Class Method Introduction[15]). Further, neurotoxicity was assessed using rotarod test. Rats, which remain on the rotating rod, with a speed of 10 rpm for 5 min or more were selected and divided into four groups (n = 6) receiving dose of 100, 200 and 400 mg kg^{-1} (p.o.) and the control. After 60 min of treatment with above dose, average retention time on the rod was recorded. Neurotoxicity was measured as the inability of the animal to maintain balance on the rotating rod for at least 3 min[16].

Diazepam-induced sleep: This test was performed in 4 groups of 6 rats each group, which were treated as, one group received saline and three group received LC extract (100, 200, 400 mg kg^{-1}). Diazepam (1 mg kg^{-1} i.p.) was administered to all the rats 30 min post-drug administration. Each rat was observed for the onset and duration of sleep. The time interval between loss and recovery of righting reflex was used as the index of the hypnotic effect[17].

Pentylenetetrazole-Induced seizures: Rats were randomly divided in 5 groups of 6 rats each group, group 1 was given distilled water and serves as vehicle control group, group 2 was given pentylenetetrazole (PTZ) (60 mg kg^{-1}, i.p.) and standard drug diazepam (1 mg kg^{-1}, i.p.). Group 3-5 was given LC extract of 100, 200 and 400 mg kg^{-1} oral dose by gavage, 30 min before seizure induction with PTZ. Animals were observed for latency in time of seizure and the total time of seizure[18].

Maximal electroshock-induced seizures: The anti epileptic property of the LC extract was measured by its ability to protect against MES induced seizure. The rats were then divided into 5 groups (n = 6). Group 1 received vehicle, group 2 received phenytoin (25 mg kg^{-1}) 60 min prior to the seizure induction as standard drug treatment. Group 3-5 received 100, 200 and 400 mg kg^{-1} b.wt., of LC extract, 60 min before to the seizure induction. The MES (Inco Electroconvulsiometer model No. 100-3) of 50 mA current for 0.2 sec was administered through corneal electrodes to induce seizure in the control and extract treated groups. The severity of effect was measure by the duration of tonic clonic and total seizure time for each animal[19].

Biochemical analysis of isolated brain: Immediately after study animals were sacrificed, the brain was quickly dissected out and cleaned with ice-cold saline (0.9%, w/v) stored at - 40°C. To perform biochemical analyses, 10% (w/v) homogenates prepared with ice-cold 0.1 M phosphate buffer (pH 7.4) followed by centrifugation (10,000 rpm, 15 min). Aliquots of the supernatant was collected and used for biochemical estimation of total protein, Reduced Glutathione (GSH) and Malondialdehyde (MDA) level.

Cholinesterase activity: Ellman *et al.*[20] method was used to assess the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in rat brain. The absorbance was recorded at 412 nm, 30 sec intervals for 3 min using a spectrophotometer. AChE activity was reported as mmoles of acetylthiocholine iodide hydrolyzed min^{-1} mg^{-1} protein. BChE activity was reported as mmoles of butyrylthiocholine iodide hydrolyzed min^{-1} mg^{-1} protein.

Reduced Glutathione (GSH) levels: Ellman *et al.*[20] method with slight modification was used for measurement of concentration of glutathione. The absorbance was recorded at 412 nm in a spectrophotometer with in 15 min. The concentration of reduced glutathione was reported as mg g^{-1} wet brain tissue.

Lipid peroxidation levels: Malondialdehyde (MDA) was used as an indication to assess lipid peroxidation[21]. The absorbance was recorded at 532 nm using spectrophotometer (Thermo Scientific UV1). The concentration of MDA was reported in nmol g^{-1} wet brain tissue.

Total proteins: Biuret method was used for the estimation of the protein amount[22]. Homogenate sample of 10 µL and 3 mL of biuret reagent (Sigma-Aldrich) were placed in a test tube. Mixed properly with inversion and absorbance was taken at 590 nm. Biuret reagent of 3 mL was mixed with 3 mL of NaCl 0.9% and used as blank. To determine the concentration of unknown sample, the weight of protein was plotted against the corresponding absorbance for a standard curve. The concentration of protein was expressed in mg mL^{-1} of protein in brain tissue.

Histology: Rats were sacrificed by decapitation for histology from each group and the brains were rapidly removed, fixed in 4% neutral buffered paraformaldehyde at 4°C and processed for paraffin embedding. Coronal sections, 7 µm thick, were cut serially at the level of the cortex and selected sections were stained with Hematoxylin and Eosin (H and E) for microscopic examination.

Statistical analysis: All experiments were performed in triplicate and data were expressed as Means±SE. Statistical comparisons were made by one-way ANOVA test followed by one way variance analysis with Dunnet's t test by using Graph pad prism (version 5.0). The $p \leq 0.05$, ≤ 0.01 and ≤ 0.001 were considered highly significant. EC_{50} values were calculated from linear regression analysis.

RESULTS

Extraction and preliminary phytochemical screening: The fruit of *Luffa cylindrica* (skin, pulp and entire fruit) was extracted with different solvent (hexane, chloroform, ethyl acetate and ethanol). The percentage yield of skin extract in hexane, chloroform, ethyl acetate and ethanol was found 3.27, 2.89, 3.92 and 6.01%, respectively. The yield from pulp was 4.25, 3.49, 3.14 and 7.42% in hexane, chloroform, ethyl acetate and ethanol. However, in the entire fruit it was 8.85, 9.51, 10.21 and 15.1% in hexane, chloroform, ethyl acetate and ethanol, which were maximum among all extract.

Maximum yield was found in ethanolic extract of entire fruit among all extracts of LC. The main phytoconstituents found present are flavonoid, phenolic, saponins, amino acid, glycosides and tannin. However ethyl acetate, alcoholic extracts of skin and fruit pulp part, ethyl acetate extract of entire fruit has also some phytoconstituents in good quantity.

Total phenol and flavonoid content: Total phenol content, as determined by Folin-Ciocalteu's method and was measure with gallic acid equivalent found significantly higher in the LC extract (149.28±6.22 µg mL^{-1}). The standard curve ($y = 0.0032x+0.0953$, $R^2 = 0.994$) was prepared with different concentration of gallic acid. Flavonoid content determined by $AlCl_3$ colorimetric method and rutin was used to make a standard curve ($y = 0.0008x+0.1523$, $R^2 = 0.994$). Ethanolic LC extract has significantly higher flavonoid content among all other extracts (988.37±0.05 µg mL^{-1}).

Percentage inhibition of *in vitro* antioxidant studies: The antioxidant with various parameters such as DPPH radical scavenging, SOD and lipid peroxidation of the entire fruit, pulp and skin of the ethyl acetate and ethanolic extract were performed and percentage inhibition was comparatively studied. The percentage antioxidant inhibition activity of ethanolic LC extract was found more as compare to other extracts (Table 1). Correlation analysis was used to make relationships between total phenolics, flavonoid and total antioxidant content measured in LC extracts. There was a very close correlation between the free radical scavenging activity determined by using the DPPH and total polyphenolic compounds (phenolic and flavonoids) of the extract of LC. Linear correlation found between total antioxidant and polyphenolic compounds was higher in the LC extract (Fig. 1).

UPLC-MS/MS of LC extract: LC extract contain possible fragment of phenolics and flavonoids class of compounds. The structure elucidation was done with the spectra obtained by the LC/MS. The spectrum obtained by direct infusion (both in ESI +ve and-ve mode) of entire fruit extracts of LC (Fig. 2, 3).

Table 1: Percentage inhibition of *in vitro* antioxidant activity of LC extracts

Samples	DPPH	SOD	LPO
Entire fruit (ethyl acetate)	233.12±1.11	2387.11±2.57	233.03±2.44
Entire fruit (alcohol)	227.19±0.84	2386.03±0.66	226.14±1.38
Pulp (ethyl acetate)	253.49±0.17	2394.93±2.63	244.13±3.93
Pulp (alcohol)	240.99±0.62	3492.79±1.37	242.24±2.32
Skin (ethyl acetate)	236.33±1.43	3287.31±1.78	240.35±4.11
Skin (alcohol)	232.13±0.73	3392.20±0.59	239.71±0.40
Standard	78.70±0.63 (Ascorbic acid)	330.58±0.58 (Copper sulfate)	76.56±0.03 (Ascorbic acid)

Each values represents Mean±SD

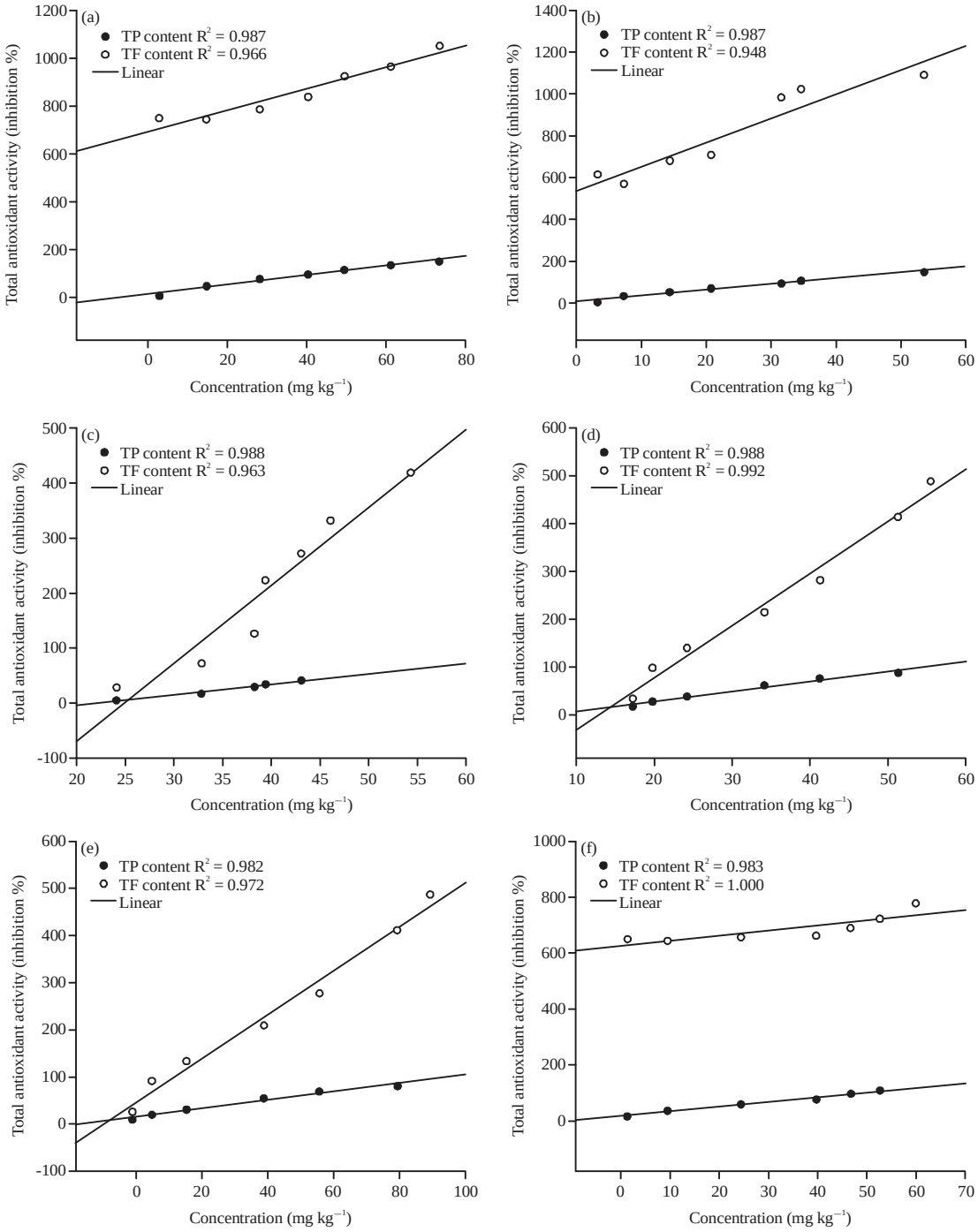


Fig. 1(a-f): Correlation between total antioxidant activity and polyphenolic (TP and TF) contents of LC extracts, (a) Ethyl acetate extract of LC fruit, (b) Alcoholic extract of LC fruit, (c) Ethyl acetate extract of LC fruit pulp, (d) Alcoholic extract of LC fruit pulp, (e) Ethyl acetate extract of LC fruit skin and (f) Alcoholic extract of LC fruit skin

The major peaks in the +ve mode were observed at 313.19, 385.10 while in the -ve mode at 255.17, 283.20, 339.26, 383.20, 385.10 m/z ratio. Identification of the compounds was done with the aid of R_t and (m/z) ratio evaluation of the resulting data by searching against the spectra library (Table 2).

Toxicity study: In acute toxicity study, LC extract in alcohol did not show any mortality in rats even at higher dose up to 4000 mg kg^{-1}, no gross behavioral changes were found. Behavioral activity was done at 100, 200, 400 mg kg^{-1} and vehicle treated dose in rotarod test showing no symptom

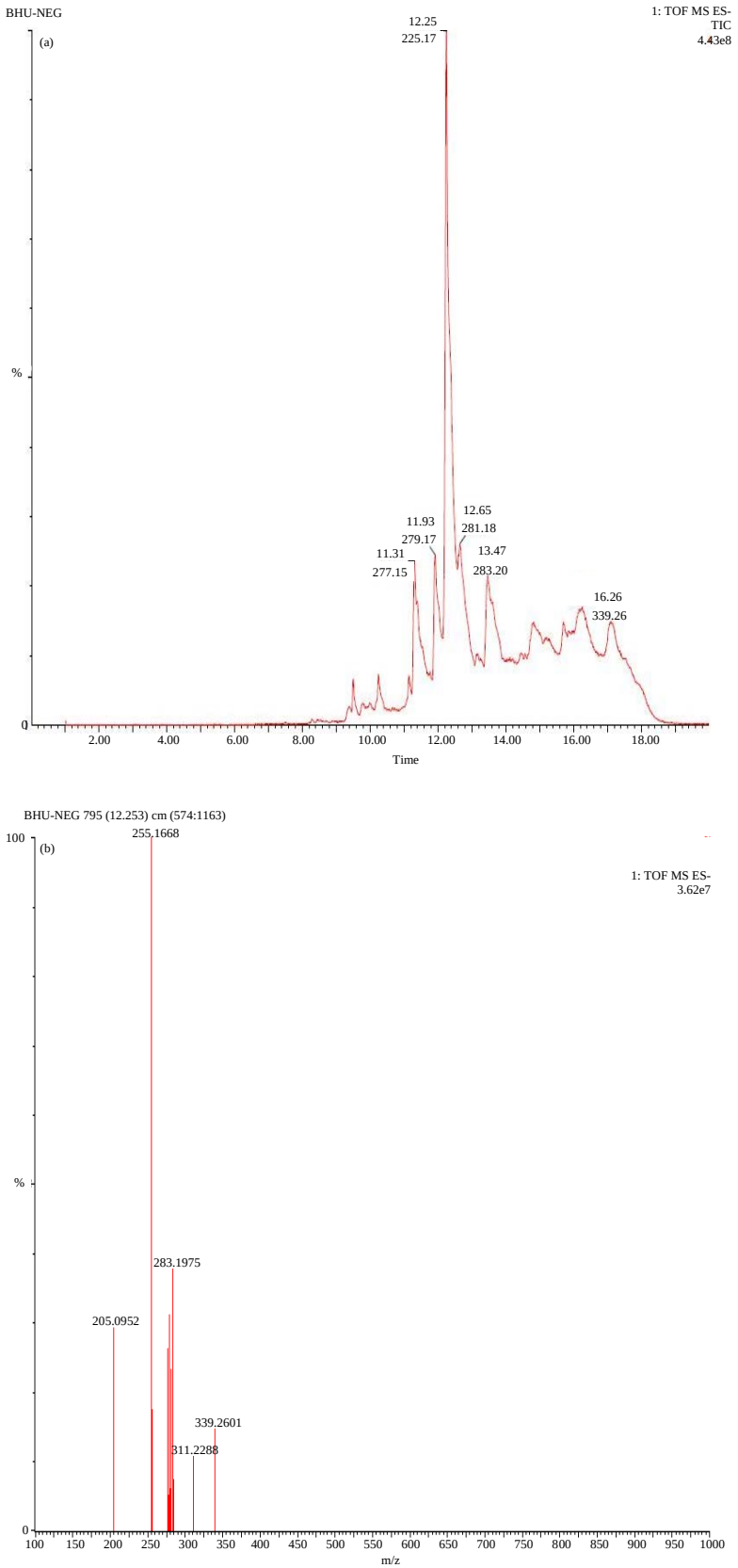


Fig. 2(a-b): (a) Chromatogram (negative mode) and (b) MS/MS spectra (negative mode) of LC LC fruit extract fruit extract

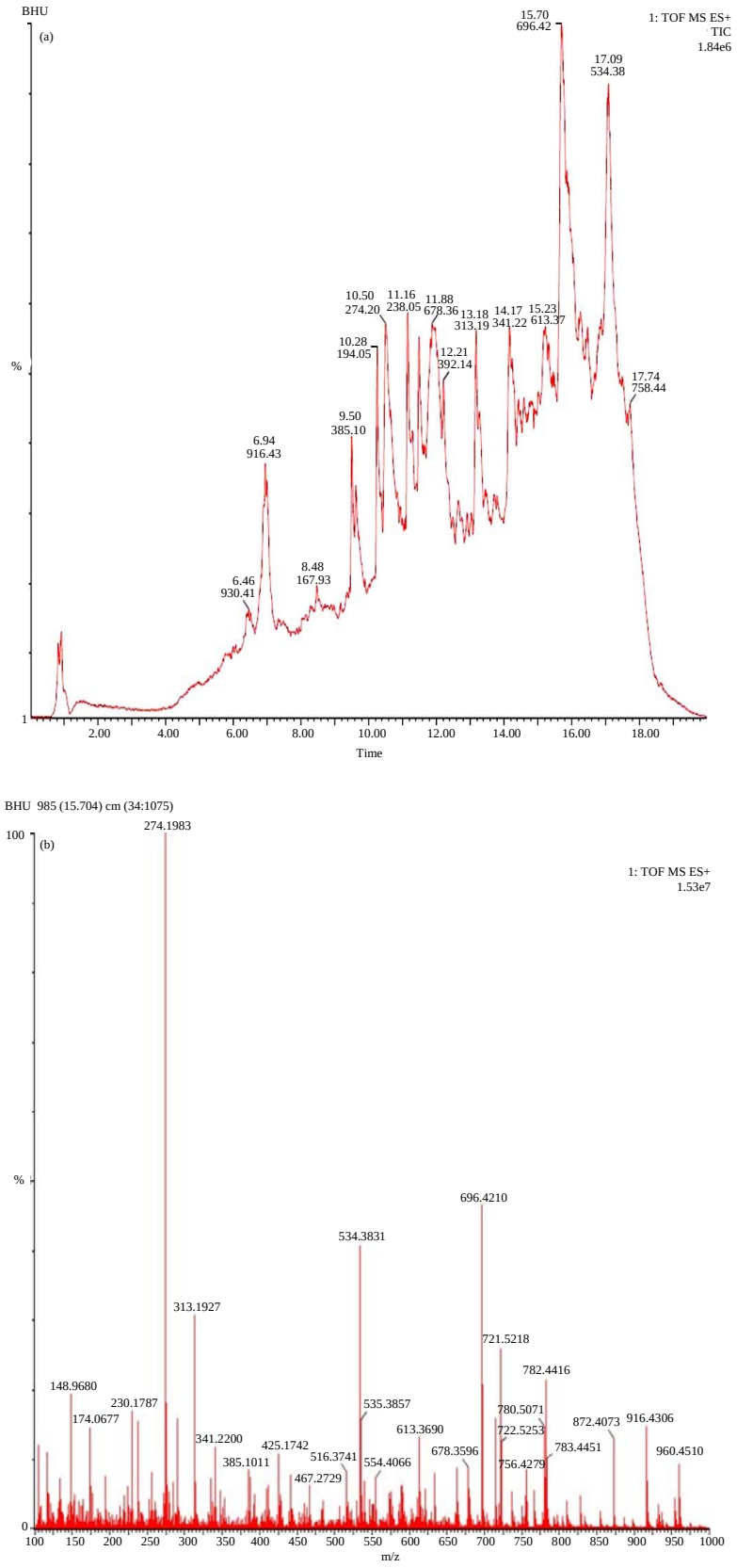


Fig. 3(a-b): (a) Chromatogram (positive mode) of LC and (b) MS/MS (positive mode) spectra of LC fruit extract fruit extract

Table 2: Structure of tentatively identified molecules through UPLC-MS spectra of LC extract R_t

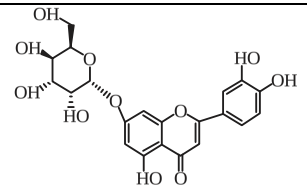
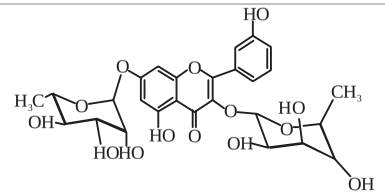
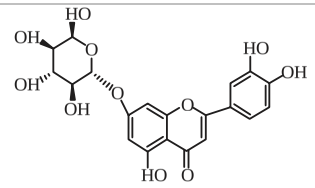
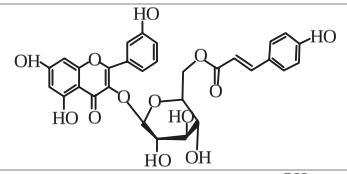
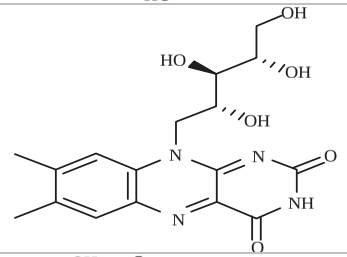
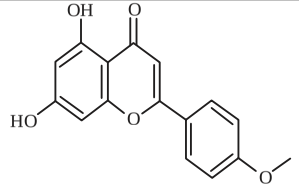
Peak	R_t	Polarity	Tentative identification	Chemical formulae	Exact mass	Chemical structure	Class	m/z value
255.17	12.25	Negative	Hyperoside quercetin-3-beta-O-galactoside	$C_{21}H_{20}O_{12}$	464.38		Flavonoid Flavonol	463.087
		Negative	Kaempferol-3,7-O-bis-alpha-L-rhamnoside	$C_{27}H_{30}O_{14}$	578.53		Flavonoid Flavonol	577.155
		Negative	Quercitrin, quercetin-3-L-rhamnoside	$C_{21}H_{20}O_{11}$	448.38		Flavonoid Flavonol	447.092
		Negative	Tiliroside, Kaempferol-3-glucoside-6-p-coumaroyl	$C_{30}H_{26}O_{13}$	594.52		Flavonoid Flavonol	593.129
		Negative	Riboflavin	$C_{17}H_{20}N_4O_6$	376.36		Vitamin	375.130
283.20	13.47	Negative	Acacetin, 5,7-Dihydroxy-2-(4-methoxyphenyl) chromen-4-one	$C_{16}H_{12}O_5$	284.06		Flavonoid Flavonol	283.250

Table 2: Continue

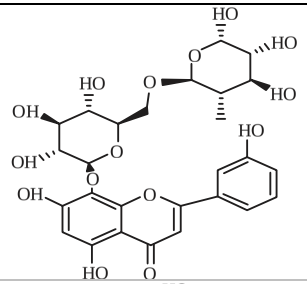
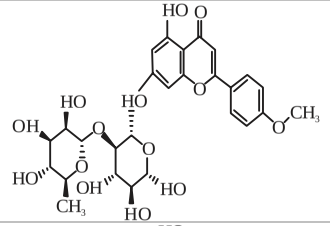
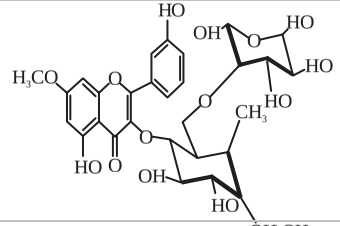
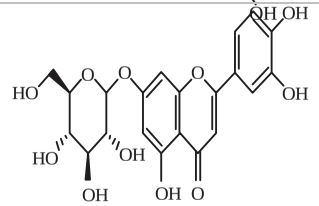
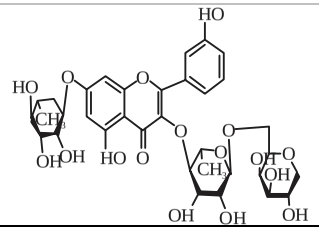
Peak	R_t	Polarity	Tentative identification	Chemical formulae	Exact mass	Chemical structure	Class	m/z value
		Negative	Datiscin, datiscetin-3-O-rutinoside	$C_{27}H_{30}O_{15}$	594.158		Flavonoid Flavonol	593.510
	Negative		Fortunellin, Acacetin-7-O-neohesperidoside	$C_{28}H_{32}O_{14}$	592.56		Flavonoid Flavone	591.171
	Negative		Linarin, acacetin-7-O-rutinoside	$C_{28}H_{32}O_{14}$	592.55		Flavonoid Flavone	591.171
	Negative		Luteolin, luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$	448.1		Flavonoid Flavonol	447.410
		Negative	Robinin, kaempferol-3-O-robinoside-7-O-rhamnoside	$C_{33}H_{40}O_{19}$	740.67		Flavonoid Flavonol	739.208

Table 2: Continue

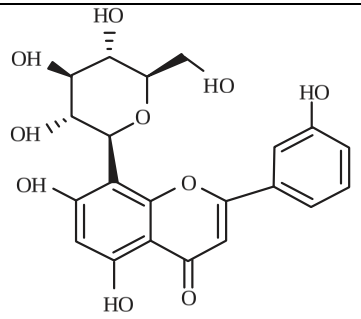
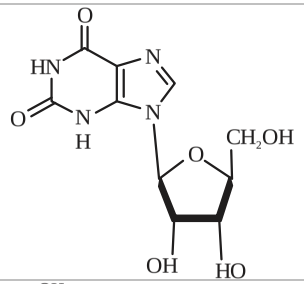
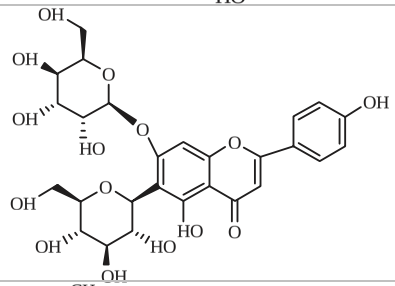
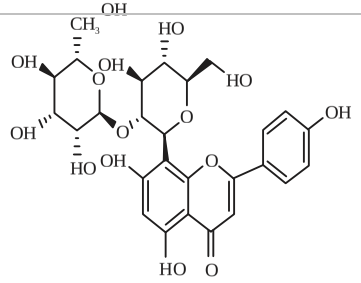
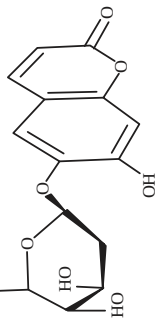
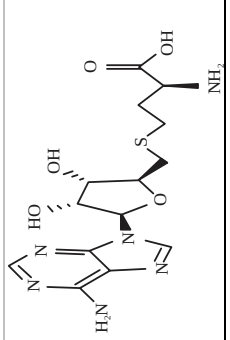
Peak	R_t	Polarity	Tentative identification	Chemical formulae	Exact mass	Chemical structure	Class	m/z value
		Negative	Vitexin, apigenin glycoside	$C_{21}H_{20}O_{10}$	432.38		Flavonoid Flavonol	431.097
383.20	13.47	Negative	Xanthosine, 9-beta-D-ribofuranosylxanthine	$C_{10}H_{12}N_4O_6$	219.23		Nucleoside	283.067
313.19	13.18	Positive	Saponarin, apigenin glycoside	$C_{27}H_{30}O_{15}$	594.15		Flavonoid Flavonol	595.470
		Positive	Vitexin-2''-O-rhamnoside	$C_{27}H_{30}O_{14}$	578.53		Flavonoid Flavone	579.171

Table 2: Continue

Peak	R_t	Polarity	Tentative identification	Chemical formulae	Exact mass	Chemical structure	Class	m/z value
339.26	16.26	Negative	Esculin sesquihydrate, 6,7-Dihydroxycoumarin-6-glucoside	$C_{15}H_{16}O_9$	340.28		Suger phosphate	339.071
385.10	9.50	Positive	S-Adenosyl-L-homocysteine	$C_{14}H_{20}N_6O_5S$	384.41		Amino acid	385.129

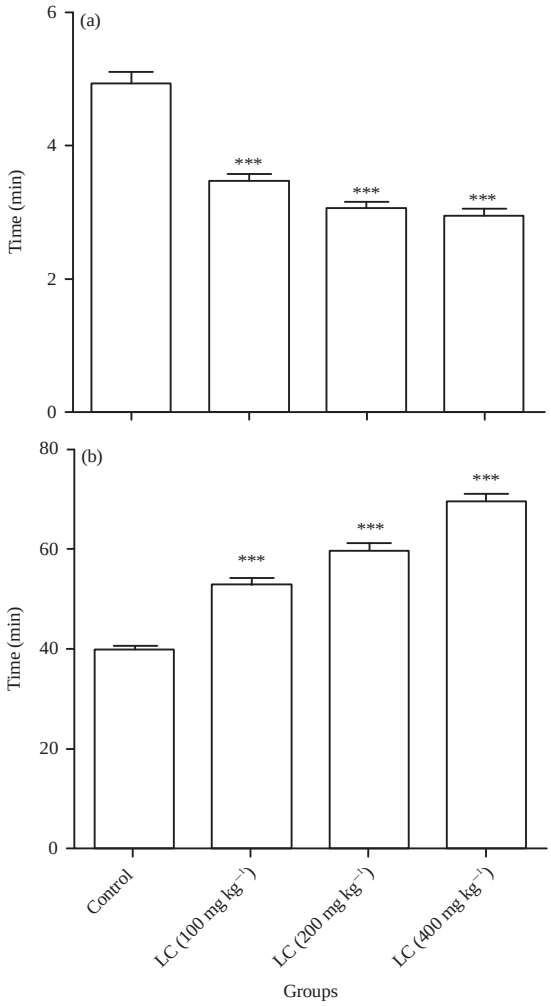


Fig. 4(a-b): Effect of LC extract on diazepam induced sleep, (a) Onset of sleep and (b) Duration of sleep in rats
Data presented as Mean±SEM, n = 6. ***p<0.001 as compare with the control group (one way ANOVA followed by Dunnett's multi-comparison test)

of impaired motor co-ordination. Each rat was capable of performing test, i.e., the mean time spent on rotarod apparatus was 180 sec. Thus, the LC extract was found to have no acute and neurotoxicity (Table 3).

Behavioral studies

Diazepam-induced sleeping time: The LC extract at 400 mg kg^{-1} dose was found to have more sedative property as compare to other doses (100-200 mg kg^{-1}). The time recorded after 30 min of diazepam induction (onset of sleep) has less at 400 mg kg^{-1} dose level in comparison with other doses and found significant (*p<0.05). At the same dose it also prolonged the sleeping time more significantly (*p<0.05) as comparison with other doses (Fig. 4).

Table 3: Acute toxicity and neurotoxicity screening of alcoholic entire fruit LC extract

Treatments	Acute toxicity test Locomotor activity count/5 min	Neurotoxicity screening Retention time (sec)
Vehicle	349.00±4.59	325.12±1.62
LC extract (100 mg kg^{-1})	330.12±4.57	320.21±1.06
LC extract (200 mg kg^{-1})	336.89±3.31	321.38±1.96
LC extract (400 mg kg^{-1})	340.20±4.91	322.54±2.16

Each values represents Mean±SD

Table 4: *In vivo* studies of alcoholic extract LC on PTZ-induced seizures on rat brain

Groups	AChE activity	BChE activity	GSH content	MDA level	Total protein
Control (saline)	0.19±0.005	0.092±0.005	0.20±0.01	0.12±0.03	31.79±5.5
PTZ (negative control)	0.08±0.003***	0.034±0.003***	0.07±0.01***	0.35±0.03***	11.15±0.4***
Standard (diazepam)	0.18±0.006$^{\$\$\$}$	0.089±0.001$^{\$\$\$}$	0.23±0.01$^{\$\$\$}$	0.18±0.07$^{\$\$}$	26.53±1.9$^{\$\$\$}$
LC extract (100 mg kg^{-1})	0.11±0.002***$^{\$\$\$\#\#\#}$	0.043±0.003******$^{\#\#\#}$	0.12±0.01***$^{\$\#\#\#}$	0.27±0.05*	17.22±0.1**$^{\$\#\#}$
LC extract (200 mg kg^{-1})	0.12±0.004***$^{\$\$\$\#\#\#}$	0.049±0.002***$^{\$\$\#\#\#}$	0.15±0.02*$^{\$\$\$\#\#}$	0.24±0.01	20.17±2.6*$^{\$\$\$\#}$
LC extract (400 mg kg^{-1})	0.15±0.007***$^{\$\$\$\#\#}$	0.057±0.005***$^{\$\$\$\#\#\#}$	0.18±0.01$^{\$\$\$\#}$	0.21±0.01	22.04±0.3$^{\$\$\$}$

Data presented as Mean±SEM, n = 6. ***p<0.001, **p<0.01, *p<0.05 as compare with the control group, $^{\$\$\$}$p<0.001, $^{\$\$}$p<0.01, $^{\$}$p<0.05 as compare with the negative control and $^{\#\#\#}$p<0.001, $^{\#\#}$p<0.01, $^{\#}$p<0.05 as compared with standard (one-way ANOVA followed by Dunnett's multi-comparison test)

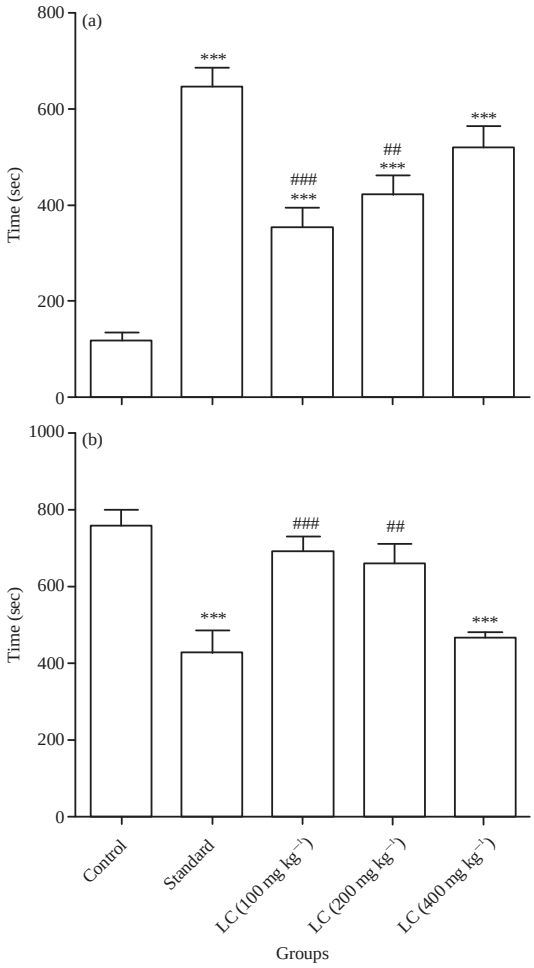


Fig. 5(a-b): Effect of LC extract on PTZ induced seizure, (a) Latency of seizure and (b) Total seizure time in rats

Data presented as Mean±SEM, n = 6. ***p<0.001 as compare with the control group and $^{\#\#\#}$p<0.001, $^{\#\#}$p<0.01, as compared with standard (one way ANOVA followed by Dunnett's multi-comparison test)

Anticonvulsant effect

PTZ induced model: In PTZ induced model, LC extract 400 mg kg^{-1} prolonged the latency time and decrease the total time of seizure more significantly (*p<0.05) as compared to other dose (100 and 200 mg kg^{-1} b.wt.) treated group and found significant compared to control group (Fig. 5). However, LC extract at 400 mg kg^{-1} (*p<0.05) showed the less effect compare to standard drug (diazepam).

MES induced model: MES induction in rat, LC extract 400 mg kg^{-1} (*p<0.05) decreased the tonic clonic and total seizure time among all other dose (100-200 mg kg^{-1} b.wt.) treated group and found significant with control group. Though it was indicated increase in time, when it was compared with standard drug (phenytoin) treated group (Fig. 6).

Biochemical parameter of rat brain: For estimation of biochemical nature from brain tissue, standard drug (phenytoin) and LC extract 400 mg kg^{-1} showing more significant effect from other dose (100-200 mg kg^{-1} b.wt.) treated group. Enzymatic (acetylcholinesterase and butyrylcholinesterase) activity in PTZ model was found less but comparable to the standard at 400 mg kg^{-1} dose level ($^{*\#\$}$p<0.05). The GSH and total protein content was also affected in a dose dependent manner ($^{*\#\$}$p<0.05). The MDA content was not effected with dose of 400 mg kg^{-1}, though the 100 mg kg^{-1} was found little bit effective (*p<0.05) (Table 4).

Same pattern was obtained with MES induced model, LC extract at 400 mg kg^{-1} dose level showing more effect as compared to the other doses but less to the standard ($^{*\#\$}$p<0.05) (Table 5).

Table 5: *In vivo* studies of alcoholic extract LC on MES-induced seizures on rat brain

Groups	AChE activity	BChE activity	GSH content	MDA level	Total protein
Control (saline)	0.19±0.005	0.092±0.005	0.20±0.01	0.12±0.03	31.79±5.5
MES (negative control)	0.05±0.008***	0.040±0.001***	0.08±0.01***	0.58±0.07**	14.51±5.5***
Standard (phenytoin)	0.17±0.002$$$	0.086±0.002$$$	0.24±0.01$$$	0.20±0.17$	29.81±5.1$$$
LC extract (100 mg kg^{-1})	0.11±0.005***$$$###	0.054±0.003***$###	0.12±0.01***###	0.45±0.06*	21.65±1.8*##
LC extract (200 mg kg^{-1})	0.12±0.004***$$$###	0.063±0.003***$$$##	0.15±0.02*$$###	0.37±0.02	23.58±2.1$#
LC extract (400 mg kg^{-1})	0.14±0.007***$$$###	0.075±0.007 **$$$	0.19±0.01$$$##	0.34±0.06	26.12±2.5$$$

Data presented as Mean±SEM, n = 6. ***p<0.001, **p<0.01, *p<0.05 as compare with the control group, $$$p<0.001, $$p<0.01, $p<0.05 as compare with the negative control and ###p<0.001, ##p<0.01, #p<0.05 as compared with standard (one-way ANOVA followed by Dunnett's multi-comparison test)

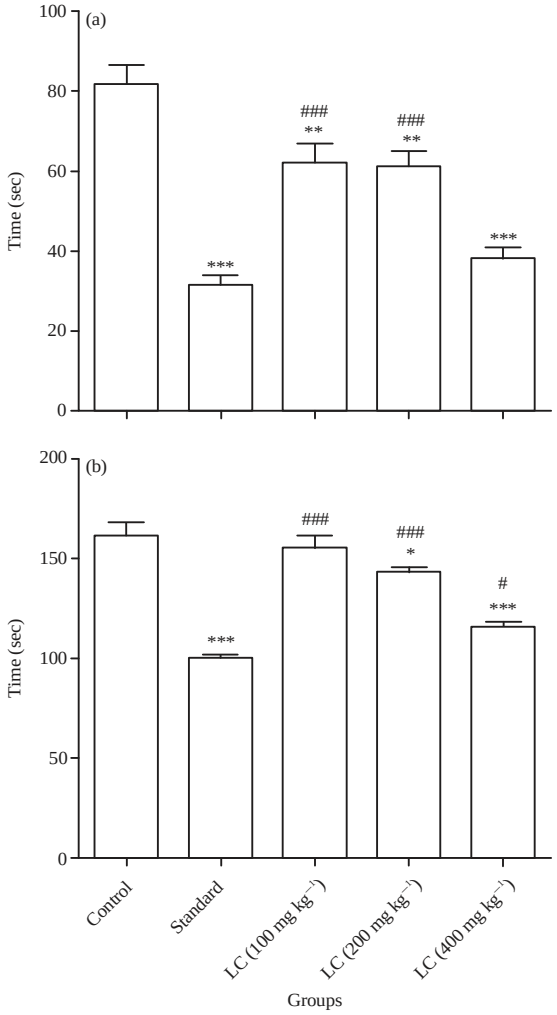


Fig. 6(a-b): Effect of LC extract on MES induced seizure, (a) Tonic clonic seizure time and (b) Total seizure time in rats

Data presented as Mean±SEM, n = 6. ***p<0.001, **p<0.01, *p<0.05 as compare with the control group and ###p<0.001, #p<0.05 as compared with standard (one way ANOVA followed by Dunnett's multi-comparison test)

Histopathological examination: Rat brain was examined and slides were compared with control, negative control (PTZ and MES), standard drug (phenytoin and diazepam) and LC extract (400 mg kg^{-1}) treated group. LC extract (400 mg kg^{-1}) showing less cellular damage as compared to other doses (Fig. 7, 8).

DISCUSSION

The present study showed that the presence of phenol, flavonoid, glycosides carbohydrates, saponin, terpenoids and tannin etc. in the extract. The extract also exhibited antioxidant and anticonvulsant activity which may be due to the presence of bioactive constituents in the extract. The phytochemicals, present in the plant and the food products are generally nontoxic and contains many medicinal properties[23].

Phytoconstituents identified in LC extract was performed by UPLC-(TOF)-ESI-MS/MS with their retention times, m/z ratio, detected mass, molecular formula and MS/MS fragment ions. The different class of constituents were identified but those belonging to class polyphenols and flavonoids are reported in the treatment and management of some serious or life style related neurodegenerative disorders, diabetes, cardiovascular, epilepsy and cancer[24]. These constituents belong to natural antioxidants that were present in fruits and vegetables are responsible for inhibiting or preventing the deleterious consequences of oxidative stress because they contain free radical scavengers, such as polyphenols and flavonoids class of compounds[25]. Apigenin, vitexin, luteolin, quercetin, linarin identified in the LC extract has already been reported for the CNS disorders and protective effect against epileptic seizure[26]. Due to hydrogen donating ability, these constituents show DPPH scavenging activity. The reduction capability of DPPH radicals were determined by the decrease in its absorbance at 517 nm induced by antioxidants. Many antioxidants that react quickly with peroxyl radicals may react slowly or may be inert to DPPH[27].

Superoxide radical is precursor of reactive oxygen species, very harmful for cellular component. The conversion of superoxide and hydrogen peroxide in to more reactive

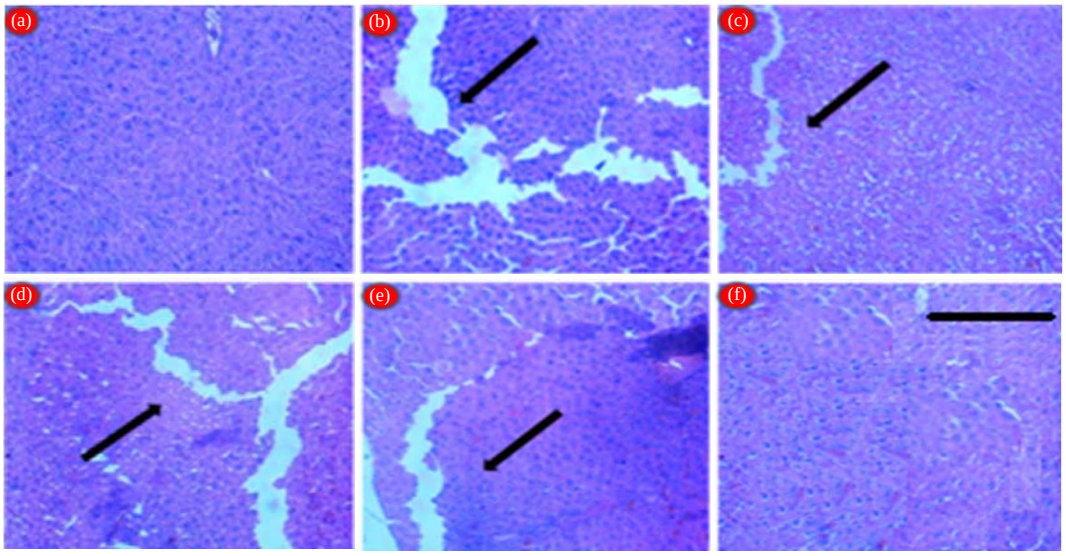


Fig. 7(a-f): Histology of hippocampal region of rat brain (H and E, 100X), black arrow denoted the cellular level changes in tissue (a) Control group, (b) MES treated group (negative control), (c) Phenytoin (25 mg kg^{-1}) treated, (d) LC extract 100 mg kg^{-1} treated, (e) LC extract 200 mg kg^{-1} treated and (f) LC extract 400 mg kg^{-1} treated

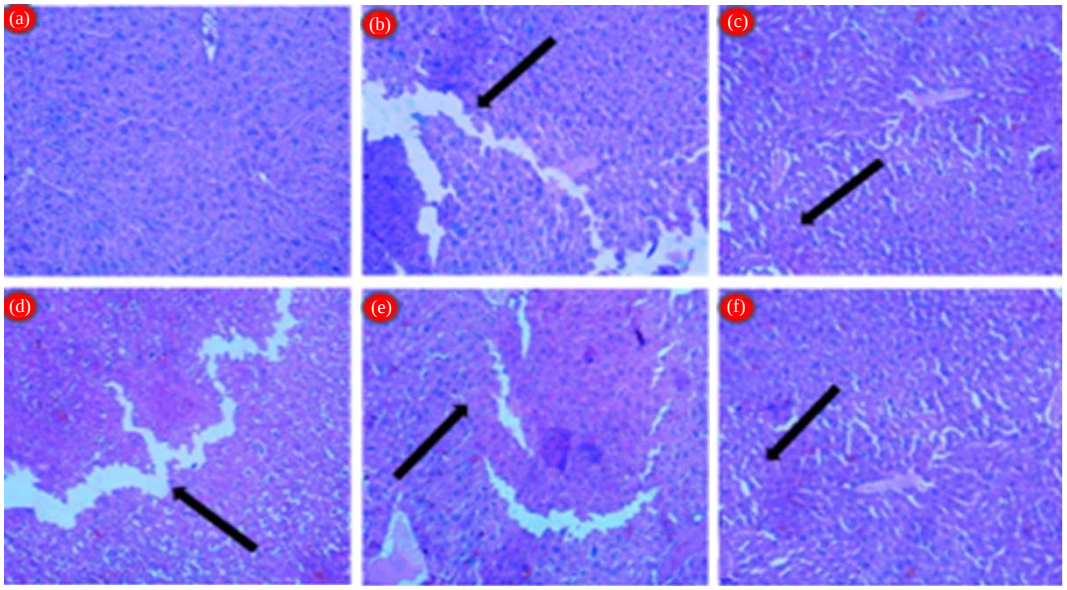


Fig. 8(a-f): Histology of hippocampal region of rat brain (H and E, 100X), black arrow denoted the cellular level changes in tissue (a) Control group, (b) PTZ treated group (negative control), (c) Diazepam (1 mg kg^{-1}) treated, (d) LC extract 100 mg kg^{-1} treated, (e) LC extract 200 mg kg^{-1} treated and (f) LC extract 400 mg kg^{-1} treated

species, hydroxyl radical is unfavorable effect due to superoxides. The LC extract was found to be an efficient scavenger of superoxide radical generated in riboflavin-NBT-light system *in vitro*[28].

Formation of lipid peroxides through an oxidative chain reaction of one lipid molecule after another and become oxidized to the possible extent, it is called lipid peroxidation. After substrate depletion, chain reaction is terminated. Egg yolk used as substrate, indicated that the reaction of LC extract is non-enzymatic oxidation[29]. These salient properties of polyphenols help to reduce hall marks of neurodegeneration, that is, oxidative damage and inflammation[30].

To assess the acute and neurotoxicity, alcoholic LC extracts was selected as it has positive test for the presence of polyphenols and flavonoids as constituents and has high extractive value during phytochemical screening and good *in vitro* antioxidant activity. The same extract as not showing any toxicity was used for antiepileptic study.

Results of the study suggested that LC extract at 400 mg kg^{-1} showing to prolong the duration of sleep, lessen time taken to sleep compared to all other doses, this may be due to the presence of different kind of constituents. Alcoholic extract of entire fruit of LC has anticonvulsant activity at dose of 400 mg kg^{-1} as it also delayed the onset of convulsion, duration of convulsion and no mortality was found in the rat against pentylenetetrazole-induced (PTZ). The protection of rats against PTZ-induced seizures by the standard anticonvulsant drugs, phenobarbitone and diazepam is expected, since various authors have shown that they exert their anticonvulsant activities by enhancing GABA mediated inhibition[31]. Binding of GABA to the site opens chloride channel, resulting in a hyperpolarized cell membrane that blocks further excitation of the cell[15]. The standard anti epileptic drug diazepam (1 mg kg^{-1}) antagonized the seizures induced by pentylenetetrazole. PTZ would be exerting convulsing effect by inhibiting the activity of GABA.

The maximal electroshock induced convulsion in animals is called grandmal type of epilepsy. The tonic extensor phase is selectively inhibits by the drugs effective in generalized tonic clonic seizure. The most outstanding action of phenytoin showed inhibition of tonic extensor phase of MES seizure, many drugs that increase the brain content of Gama Amino Butyric Acid (GABA) have exhibited anticonvulsant activity against seizures induced by MES. There are numerous molecular mechanisms through which drugs can block seizure spread and or elevate seizure threshold[19].

Decrease in AChE activity in brain, 30 min after a PTZ induced seizures and 2 min after convulsion induced electrically have also been reported by Bhosle[32]. Current results show decrease in AChE and BuChE activity following PTZ and MES induced seizure. LC extract (400 mg kg^{-1}) pre-treatment brings AChE activity in whole brain to the levels that are comparable to vehicle control, with no significant changes in BuChE activity. Thus, the results of the present study showed anticonvulsant activity of LC extract (400 mg kg^{-1}) against PTZ and MES-induced seizures and also its protective effect against seizure induced oxidative stress. The role of cholinergic neurotransmission, acetylcholinesterase (AChE) is well known in epilepsy[33].

Indeed, attempts to correlate the anticonvulsant profiles of anti epileptic drugs with specific mechanisms of action revealed certain notable trends. Certain drugs such as phenytoin, carbamazepine, lamotrigine, valproate and felbamate, have an effective role to block the tonic extension by inhibiting the voltage dependent Na^+ channels[32].

Therefore, the effect of LC extract and standard drug (diazepam and phenytoin) treated group on oxidative stress in PTZ and MES induced seizures were also evaluated. In both model pretreated with standard, LC extract at 100, 200 and 400 mg kg^{-1} showed significant decrease in MDA content in rat brain compared with the negative control group. Glutathione is an endogenous antioxidant form within the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals[34]. The decreased level of reduced glutathione in negative control was observed in the present study indicating that there was an increased generation of free radicals and that the reduced glutathione was depleted during process of combating oxidative stress. Standard treated (diazepam and phenytoin), LC extract 100, 200 and 400 mg kg^{-1} dose showed significant increases the GSH level in rat brain. The decreases in MDA and increase in the glutathione level in LC extract+PTZ and LC extract+MES rats indicates that LC extract exerted good antioxidant effect. Among LC extracts the dose of 400 mg kg^{-1} exerted more significant effect in rat brain due to the presence of polyphenols and flavonoids class of compounds.

Histology of the rat brain tissue show more damage in the PTZ and MES induced rat and in the treated group less change occur in standard treated group and LC extract at 400 mg kg^{-1} dose level. These less changes confirmed the anti-convulsive effect of LC extract at 400 mg kg^{-1} in both induced model. On the basis of qualitative and quantitative phytochemicals, alcoholic extract of entire fruit *Luffa cylindrica* indicated the presence of phenolic and flavonoids. Presence of phenol and flavonoid revealed the strong antioxidant and anticonvulsive property of this fruit. It is also reported that many flavonoids and polyphenols present in the *Luffa* extract could act as benzodiazepine like molecules in the central nervous system and modulate GABA-generated chloride currents in animal models of anxiety, sedation and convulsion[35]. The results show that the drug may play an important role in management of epilepsy through a food drug which is very commonly used in society. The constituents identified may be treated as a lead compound of the epileptic disorder.

CONCLUSION

The alcoholic entire fruit extract of *Luffa cylindrica* demonstrated the presence of phenolics and flavonoids responsible for potential antioxidant and anticonvulsant

activity with less toxicity in the experimental animals. However, further studies still needed to be carried on the extract for understanding of exact mechanism.

SIGNIFICANCE STATEMENT

This study provides a good natural source for the effective management of epilepsy by reducing oxidative stress. The tentative phytoconstituents detected through UPLC study will provide many lead compounds which will be useful in many CNS disorders since it is naturally safe for use due the lack of neurotoxicity.

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