Assessment of luteolin isolated from Eclipta alba leaves in animal models of epilepsy

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ABSTRACT

Objective: The present study isolates and characterizes luteolin from E. alba and evaluates its antiepileptic potential in chemically induced acute and chronic models in mice.

Materials and methods: The methanol extract (16.85% w/w) of E. alba leaves was subjected to fractionation for isolation of luteolin. In acute pentylentetrazole (PTZ) model, luteolin (5, 10, 20 mg/kg, i.p.) was administered 30 min prior to PTZ injection (100 mg/kg) in Swiss albino mice. Kindling was induced by chronic administration of PTZ (35 mg/kg) on every alternate day (48 days). Luteolin was investigated on the course of kindling development and oxidative stress markers [reduced glutathione (GSH) and malondialdehyde (MDA)] in kindled mice.

Results: Single-dose pretreatment with luteolin (10 and 20 mg/kg, i.p.) was found to be effective in an acute PTZ model (100% protection from mortality) and it did not exhibit any effect on motor coordination at the same doses. PTZ-induced kindling was significantly (p < 0.001) prevented by luteolin (5, 10, 20 mg/kg, i.p.) in a dose-dependent manner. Luteolin restored levels of reduced GSH (p < 0.001) and decreased the level of MDA (p < 0.001), a marker of lipid peroxidation.

Discussion and conclusion: The results of the present study demonstrated that luteolin had an anticonvulsant effect in an acute PTZ model. Luteolin exhibited an inhibitory effect on the course of kindling and associated oxidative stress and hence could be a potential molecule in the treatment of epilepsy.

INTRODUCTION

Epilepsy is a disease characterized by spontaneous recurrence of seizures. It is a chronic neurological disease experienced by millions and a cause of substantial morbidity and mortality. In spite of an expanded array of antiepileptic drugs (AEDs) available in market, many patients either continue to have seizures or suffer from side effects associated with AEDs (Schmidt 2002). Accumulating data supports the hypothesis that oxidative stress might have a major contribution in the development of experimental epilepsy leading to cellular and molecular damage (Ilhan et al. 2005). Thus, there is an unmet need to develop a drug which will be able to overcome the underlying cause and progression of epilepsy rather than being just a mere anticonvulsant. Eclipta alba (Linn) Hassk. (Asteraceae) has been used in Indian traditional systems of medicine and also by traditional healers since ancient times, especially in the southern region of India for the treatment of epilepsy (Reddy et al. 1989). It has been reported that E. alba possessed antiepileptic activity in PTZ and MES models of epilepsy (Shaikh et al. 2012 a,b). A literature survey revealed luteolin, a naturally occurring flavonoid, as one of the major constituents of E. alba. Flavonoids exert antiepileptic activity as they are ligands for benzodiazepine (BDZ) receptors (Medina et al. 1997; Fernandez et al. 2006). Luteolin has been reported to cross the blood–brain barrier, has CNS activity with anxiolytic-like effects and also affinity for the BDZ receptor in vitro (Coleta et al. 2008). Based on the above literature, it was hypothesized that luteolin might exhibit an antiepileptic effect.

This study isolates luteolin, one of the major phytoconstituent of E. alba and evaluates its anticonvulsant and antiepileptogenic activity in animal models of epilepsy. Furthermore, luteolin was evaluated on the course of kindling development and for its antioxidative effects in pentylentetrazole (PTZ) kindled mice.

MATERIALS AND METHODS

Isolation of luteolin

Fresh leaves of E. alba were collected in the month of June–August 2014 from Palghar, Mumbai. The leaves were authenticated by Dr Ganesh Iyer from Botany Department of Ruia College, Mumbai and deposited in the herbarium of ICT department as a voucher specimen number ICT 201015. The dried powder of E. alba leaves (5 kg) was subjected to defatting using petroleum ether and then Soxhlet extracted with 95% methanol (60–80°C) for 18 h. The methanol extract (16.85% w/w) was then precipitated with ice-cold water. Residue and the aqueous extract were separated by centrifugation and both the fractions were analyzed by thin-layer chromatography (TLC) for the presence of luteolin. Aqueous extract was further subjected to fractionation with ethyl acetate in a separating
funnel. The ethyl acetate layer obtained was concentrated to dryness, dissolved in methanol which was then concentrated and evaporated to yield a yellow mass of flavonoid. The above residue was further purified with chloroform to get a crude compound. Repetitive recrystallization of crude compound with methanol was done to yield luteolin.

**Characterization of compound**

The isolated luteolin was subjected to various analytical tests like TLC, UV spectroscopy, IR spectroscopy, MS spectroscopy, NMR spectroscopy, and percent purity determination by HPLC for luteolin confirmation. TLC was performed on silica gel 60 F_{254} precoated plates using ethyl acetate: toluene: formic acid: methanol (3:3:0.8:0.2) as the mobile phase and NP-PEG was used as the spraying agent (Shaikh et al. 2012b). The absorption maxima ($\lambda_{max}$) of the isolated luteolin were recorded on Shimadzu UV/Vis spectrophotometer. The column was maintained at ambient temperature (25 ± 1°C). Each sample was run for 10 min. Methanol was used for the preparation of sample solution.

**Antiepileptic studies**

**Animals**

Swiss albino mice in the weight range of 18–25 g were procured from Haffkine Biopharmaceuticals (Mumbai, India) and were maintained at ambient temperature of 25 ± 1°C, relative humidity of 50 ± 10% and 12:12 h light:dark cycles. The animals had free access to standard pellet diet and water ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Chemical Technology, Mumbai and experiments performed as per the norms laid by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (ICT/IAEC/2011/P78).

**Drugs**

Pentylentetrazole (PTZ, Sigma-Aldrich Co. St. Louis, MO), diazepam (Valium®, Abbott Healthcare Pvt. Ltd., Himachal Pradesh, India) and luteolin Standard from Chromadex, Bangalore, India were used in the present study. All the standard chemicals used in the study were of analytical grade. The isolated luteolin powder was suspended in 0.5% sodium carboxymethyl cellulose (Na CMC) whereas all the other solutions were prepared in saline for in vivo studies.

**Assessment of rotarod activity**

Rotarod test was performed 30 min after the drug treatment as an index of neurotoxic side effect (motor in coordination) before the convulsions were induced by PTZ. Only those mice which were capable to remain on rotating rod at 12 rpm for at least 150 s were included in the study. The animals were placed on the rotarod 30 min after intraperitoneal administration of the drug (luteolin/diazepam) and the latency to fall from the rotarod in a period of 5 min at 12 rpm was recorded (Coleta et al. 2008).

**Pentylentetrazole (PTZ)-induced convulsions**

Mice were divided into five groups each containing six animals wherein Group I: 0.5% CMC (Vehicle control); Group II: diazepam (2 mg/kg, i.p.) treated; Group III–V: luteolin (5, 10, 20 mg/kg respectively, i.p.) treated. All the treatments were done intraperitoneally 30 min prior to the PTZ injection. Seizures were induced by a single-dose administration of PTZ (100 mg/kg, i.p.) to the mice which were subsequently placed in individual plexiglass cage and observed initially for 30 min. The latency to first myoclonic jerk, onset of clonic seizure, onset of hind limb extension (HLE), time taken for death and percentage of animal protected from death were noted (Mahendran et al. 2011).

**PTZ-induced kindling test**

In this test, mice were divided into six groups each containing ten animals and all the treatments were done by intraperitoneal route. Group I: Normal control (Biochemical parameter estimation); Group II: PTZ control (Vehicle + PTZ); Group III: diazepam 2 mg/kg + PTZ (diazepam 2 mg/kg); Group IV: luteolin 5 mg/kg + PTZ (luteolin 5 mg/kg); Group V: luteolin 10 mg/kg + PTZ (luteolin 10 mg/kg); Group VI: luteolin 20 mg/kg + PTZ (luteolin 20 mg/kg). Kindling was induced by a sub convulsive dose of PTZ (35 mg/kg, i.p.) and were treated on every alternate days till kindling was attained. After each PTZ injection animals were observed for 30 min and behavioural seizure activity was rated according to the scale adapted from Ilhan et al. (2005) with some modifications as follows: 0 (No response); 1 (Myoclonic jerk); 2 (Clonic jerk without loss of righting reflex); 3 (Clonic seizure with loss of righting reflex); 4 (Clonic seizure without loss of righting reflex); 5 (Tonic seizure); 6 (Death).

**Evaluation of malondialdehyde and reduced glutathione levels**

Kindled mice were sacrificed at the end of the study, brain was quickly removed and rinsed with 0.9% saline. The brain tissues were homogenized using ice-cold 0.1 M phosphate buffer saline (pH 7.4). The homogenate were centrifuged at 4°C (3000 rpm; R-248M of CPR-223 24 plus Instrument, Remi, India) for 15 min. The aliquots obtained were used for the estimation of glutathione (GSH) and malondialdehyde (MDA) using methods described in Ellman (1959) and Ohkawa et al. (1979) respectively.

**Statistical analysis**

The data are expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered to be statistically significant when *p < 0.05, **p < 0.01, ***p < 0.001 when compared with PTZ control. All statistical analysis were performed using GraphPad Prism software version 5.

**Results**

**Characterization of compound**

The isolated compound exhibited melting point in the range 250–252°C. The R$_f$ value of isolated compound (0.72) matched...
with the standard luteolin. The UV spectrum of luteolin in methanol was obtained at 268 and 345 nm. The IR spectrum exhibited peaks at 3420 cm\(^{-1}\) due to the OH groups and a strong band at 2919 cm\(^{-1}\) (aliphatic C–H stretching) indicating the presence of –CH\(_2\)– and –C–H in addition to peaks at 1618, 1508, and 1454 cm\(^{-1}\) which indicated the presence of an aromatic ring system. The Mass spectrum showed a molecular ion peak of 287 (M\(^+\)) The \(^1\)H NMR (500 MHz) showed the presence of hydroxy groups at \(\delta\) 13.04 (1 H, S), and signals at \(\delta\) 6.25 (1 H, d), 6.53 (1 H, d), 6.55 (1 H, s), 7.00 (1 H, d), 7.46 (1 H, d), 7.48 (1 H, d). The data obtained from chemical and spectral studies of the isolated compound was found to match the specification mentioned in the literature for luteolin. The percentage purity (%) of the isolated compound was found to be 94.90%.

Antiepileptic study

Rotarod model

Pretreatment with luteolin at doses 5, 10, and 20 mg/kg did not exhibit any effect on motor coordination as determined by rotarod test. However, the diazepam-treated group showed significant decrease (\(p < 0.001\)) in the onset of time required to fall from rotarod apparatus as compared to the control (Table 1).

PTZ-induced seizure test

The results from the study revealed that administration of luteolin at a dose of 10 or 20 mg/kg delayed the onset of myoclonic jerks (\(p < 0.001\)), onset of clonic seizures (\(p < 0.01\)) and onset of HLE (\(p < 0.05\) and \(p < 0.01\)) when compared with PTZ control animals. Luteolin (5 mg/kg) did not exhibit any significant effect in PTZ-induced seizure test. Luteolin at the dose of 10 or 20 mg/kg showed complete protection (100%) against PTZ-induced mortality comparable to diazepam. Luteolin exhibited its antiepileptic activity in the PTZ-induced seizure model in a dose-dependent manner (Table 2).

PTZ-induced kindling test

Repeated administration of sub-convulsive dose of PTZ (35 mg/kg) on every second day in control group (for 48 days, 24 injections) resulted in increasing convulsive activity leading to generalized clonic-tonic seizure score of 5. Administration of luteolin at a dose of 5, 10, and 20 mg/kg significantly decreased the seizure score on 4th injection. As the study continued, luteolin at all the doses very efficiently protected the animals from PTZ-induced seizures and decreased the seizure score significantly (\(p < 0.001\)) on day 8, 12, 16, and 20. On the last day of treatment i.e., 24th injection, luteolin at a dose of 10 and 20 mg/kg (\(p < 0.001\)) was found to be more effective in decreasing seizure score as compared to the dose of 5 mg/kg (\(p < 0.01\)). Luteolin at a dose of 10 mg/kg exhibited similar effect as diazepam in suppressing PTZ-induced kindling (Figure 1).

Estimation of malondialdehyde levels

Repeated treatment with PTZ-induced oxidative stress as indicated by a significant rise in the whole brain MDA levels in the PTZ control group as compared to vehicle treated normal control group (\(p < 0.001\)). Luteolin at a dose of 5, 10, 20 mg/kg (\(p < 0.001\)) along with diazepam at a dose of 2 mg/kg (\(p < 0.05\)) showed significant reductions in the levels of MDA as compared to PTZ control group (Figure 2).

Estimation of reduced glutathione levels

Pretreatment of luteolin at a dose of 10 and 20 mg/kg (\(p < 0.001\)) restored the depleted GSH levels as compared to PTZ control group. The effect was observed to be dose-dependent with ceiling effect at 20 mg/kg better than diazepam (Figure 3).

Discussion

The isolation of luteolin was successfully carried out which was confirmed by the data obtained from TLC and other spectral studies. Luteolin was observed to be devoid of neurotoxic side effects like motor in co-ordination whereas diazepam (2 mg/kg) showed marked impairment of motor co-ordination. The results from the above study revealed that administration of luteolin at a dose of 10 mg/kg and 20 mg/kg delayed the onset of myoclonic

### Table 1. Effect of luteolin on motor coordination in rotarod model.

| Treatment       | Latency to fall from rotarod |
|-----------------|-----------------------------|
| Control (CMC)   | 299 ± 0.91                  |
| Diazepam 2 mg/kg| 84.33 ± 19.87***            |
| Luteolin 5 mg/kg| 300 ± 0.00                  |
| Luteolin 10 mg/kg| 293.33 ± 2.43              |
| Luteolin 20 mg/kg| 297.5 ± 2.28               |

The data are expressed as mean±SEM (n = 6) and were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. Differences were considered to be statistically significant when ***\(p < 0.001\) compared to normal control.

### Table 2. Effect of luteolin in PTZ-induced seizure test.

| Treatments   | Onset of myoclonic jerks | Onset of clonic seizures | Onset of HLE | Onset of death | % Protection from death |
|--------------|--------------------------|--------------------------|--------------|---------------|------------------------|
| PTZ Control  | 50.14 ± 2.71             | 57.17 ± 1.13             | 122.34 ± 9.20| 223.17 ± 22.46| 0                      |
| Diazepam 2 mg/kg| 612.11 ± 11.42***        | 0 ± 0.00                 | 0 ± 0.00     | 0 ± 0.00      | 100                    |
| Luteolin 5 mg/kg| 69.17 ± 3.86             | 79.67 ± 5.65             | 112.5 ± 17.44| 543.25 ± 24.15***| 66.66                |
| Luteolin 10 mg/kg| 169.49 ± 16.30***       | 214.10 ± 34.76***       | 238.25 ± 18.67| 0 ± 0.00      | 100                    |
| Luteolin 20 mg/kg| 267.51 ± 10.84***        | 536.12 ± 34.34***        | 672.67 ± 57.16***| 0 ± 0.00      | 100                    |

The data are expressed as mean±SEM (n = 6) and were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. Differences were considered to be statistically significant when *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) compared to PTZ control.
jerks, onset of clonic seizures and onset of HLE along with complete protection (100%) against PTZ-induced mortality comparable to diazepam in a dose-dependent manner. Generally, compounds with antiepileptic activity in petit mal epilepsy are effective in PTZ-induced seizure model. The above fact supports the notion that luteolin might be active against petit mal seizures. The data obtained is in contrast to the previous reports by Shaikh et al. (2013) in exhibiting anticonvulsant effects in acute PTZ model probably due to differences in the dose used for screening. In the present study, 10 and 20 mg/kg showed anticonvulsant effect in PTZ-induced convulsion whereas in the above mentioned literature a low dose (3 mg/kg) of luteolin was used for screening.

PTZ, a GABA<sub>A</sub> receptor antagonist depicts a model of absence seizure in humans and interacts with neurotransmission of GABA. It has been very well documented that AEDs that delay clonic convulsions induced by PTZ act by elevating the seizure threshold (Khan & Mukhram 2011). The observations of the present study indicate that luteolin may act as anticonvulsant by enhancing the activation of GABA<sub>A</sub> receptors, thus facilitating the GABA-mediated opening of chloride channels and by increasing the seizure threshold. This data advocates the use of luteolin in the management of convulsions without producing side effects.

Kindling is characterized by repeated administration of a subconvulsive electrical or chemical stimulus resulting in progressive intensification of seizure activity which culminates in generalized seizures (Sancheti et al. 2014). A major problem associated with available AEDs is their lack of efficacy in preventing the progression of epilepsy (i.e., antiepileptogenic). Since spontaneity and recurrence of seizures are the basic features of human epilepsy, chronic models like kindling have advantage over acute models (Ali et al. 2005). Kindling is one of the leading models for identification and quantification of
epileptogenesis. It has been suggested that the hyper-excitability of a kindled animal is due to the enhanced function of glutamatergic synapses using N-methyl-D-aspartate (NMDA) receptors and due to the increase in NMDA receptors in the cortex of kindled animals (De Oliveira et al. 2008). The main activities of AEDs in synapses include GABAergic inhibitory neurotransmission enhancement and a decrease in glutamatergic excitatory neurotransmission directly or via inhibition of voltage-dependent sodium and calcium channels pathways (Szyndler et al. 2006).

The results of the above study revealed that luteolin suppressed PTZ-kindled seizures in a dose-dependent manner and thus could be a potential candidate in inhibiting the seizure genesis caused by excitotoxic agents.

Oxidative stress in the central nervous system (CNS) has been shown to be produced in various rodent models of experimental epilepsy like PTZ kindling model. One of the causes for seizure generation can be attributed to a homeostatic imbalance of oxidant and antioxidant mechanisms (Shin et al. 2011). Experimental seizures are known to be associated with a massive release of reactive oxygen species (ROS) (Obay et al. 2008). Marked alteration in the metabolism of membrane phospholipids results in the production of free fatty acids (FFAs), lipid peroxides and free radicals (Ilhan et al. 2005). Free radicals have been implicated in the development of seizures. Therefore, free radical involvement in pathological conditions has generally been inferred from the measurement of indirect markers of oxidative stress, which include the changes in the levels of lipid peroxidation and GSH (Obay et al. 2008). The present study supports this hypothesis that PTZ-induced seizure activity coincides with an enhanced oxidative stress in brain tissue as indicated by increased MDA (end product of free radical formation) and significant decrease in reduced GSH (endogenous antioxidant) levels (Khan & Mukhram 2011).

Luteolin at the dose of 10 and 20 mg/kg lowered the levels of MDA in the brain thus attenuating lipid peroxidation. The decreased levels of reduced GSH in vehicle-treated PTZ group indicated that there was an increased generation of free radicals and the reduced GSH was depleted during the process of combating oxidative stress. Further, the decreased levels of GSH were significantly restored by luteolin at the dose of 10 and 20 mg/kg. The present study demonstrated that luteolin significantly attenuated oxidative stress induced by PTZ kindling due to its free radical scavenging activity.

Conclusions

It can be concluded that luteolin exhibited an anticonvulsant effect as well as prevented the progression of PTZ-induced kindling and ameliorated the oxidative stress induced by kindling, supporting the notion that it exhibits antiepileptogenic activity. Further mechanistic studies would be beneficial for establishing luteolin as a potentially effective antiepileptic compound.

Acknowledgements

The authors would like to thank UGC-SAP for funding the research.

Disclosure statement

The authors report no declarations of interest.

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