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Evaluation of bioactive potentials in the crude flavonoid fraction from the leaves of *Butea monosperma* (Lam.)Taub. *in vitro* and *in vivo* in experimental models

Rajni Bala[1], Sakshi Mehta[2*#], Vikas C Roy[2], Ram C Roy[2], Sharda Roy[2] & Jaspreet Singh[2]

[1]Department of Biotechnology, Punjabi University, Patiala, India

[2]Department of Pharmacy, Global College of Pharmacy, Kahanpur, Punjab, India

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Oxidative stress is responsible for the advent of seizure hence, antioxidants are used for the treatment neurodegenerative ailments such as epilepsy. The Sacred tree, *Butea monosperma* (Lam.)Taub. is known to possess several medicinal properties due to the presence of steroids, alkaloids, triterpenoids, polyphenols, flavonoids, tannins and glycosides, particularly in the leaves. In the present study, we evaluated the bioactive potentials in the isolated crude flavonoid fraction (FF) from its leaves. FF was isolated by successive methanol extract method and was evaluated for antioxidant and anticonvulsant activities *in vitro* and *in vivo*. For *in vivo* study, 25 healthy Wistar albino rats were employed in five groups: Gr.I: control; Gr. II: standard group receiving phenytoin sodium; and Gr. III-V: test groups receiving oral doses of FF i.e., 50, 100 and 200 mg/kg, respectively. *In vitro* data showed concentration dependent antioxidant activity (88.86±0.006%), reducing power activity (0.694±0.002%) and nitric oxide scavenging activity (87.98±0.013%). *In vivo*, a significant depression in the phases and percentage incidence of convulsions was observed in the maximal electroshock model in a dose-dependent manner. The pentylene tetrazole-induced seizure model also depicted a significant ($P \leq 0.001$) decrease in the latency of convulsions and an increase in the percentage protection against the convulsions in a dose-dependent manner. Our findings suggest antioxidant and anticonvulsant potential of FF obtained from *Butea monosperma* leaves.

Keywords: Anticonvulsant, Antioxidants, *Dhak*, Epilepsy, Forest flame, Maximal electroshock, Oxidative stress, *Palash*, Pentylene tetrazole, Sacre tree, Seizures

Epilepsy is one of the most common disorders of the brain that affects over 70 million individuals worldwide[1-3]. It is a chronic and progressive neurological disorder, characterized by sudden and transient episodes (seizures) of consciousness disturbance and/or distinctive body movements (convulsions)[4-6]. The underlying pathology of epilepsy involves several mechanistic pathways including inflammation, altered gene expression, and synaptic dysfunction.

Oxidative stress, an emerging mechanism appears to play an important role in the advent of seizure[7-9]. This process is also implicated in the pathogenesis of several diseases such as neuropsychiatric and neurodegenerative disorders. The antioxidants are profoundly used for the treatment of such neuropsychiatric and neurodegenerative ailments such as epilepsy[10-12]. Substances containing polar phytoconstituents namely polyphenols and flavonoids are generally believed to be associated with antioxidant activity[13,14]. The current treatment therapy of epilepsy includes modern antiepileptic drug (AED) which is associated with side effects, chronic toxicity and teratogenic effects. Clinical evidence in the literature reported that approximately 30% of the patients on AED continue to have seizures[15-17]. Thus, more effective and safer agents possessing antioxidant activity should be hunted and/or developed for the treatment of epileptic disorders.

Butea monosperma (Lam.) Taub., commonly called the Sacred tree and popularly known as the "Flame of the Forest or Forest flame" and locally "*palash* or *dhak*", belongs to the family Fabaceae[18]. The different parts of the plant have been used in the folklore medicine since long[18,19]. Also, the medicinal properties of the plant have been validated scientifically such as antifungal, antiviral, antimycobacterial, antihepatotoxic, antiinflammatory, antioxidant, anticonvulsant, etc.[20,21]. Based on our knowledge and literature survey, these properties have

*Correspondence
Phone: +91 9416372927 (Mob.)
E-Mail: samimehta18@gmail.com
#Present add.: School of Basic and Allied Sciences, KR Mangalam University, Sohna, Gurugram, Haryana, India

been studied in the isolated flavonoid fraction from the stem bark and flowers of the plant[22]. However, the bioactive potentials of the flavonoid fraction from the "leaves of *B. monosperma"* have not been studied yet[23]. Therefore, in the present study, we assessed the antioxidant and anticonvulsant properties of the crude flavonoid fraction from the leaves of *B. monosperma* in animal model.

Materials and Methods

Plant material

Mature leaves of *B. monosperma* were collected from Shri Anandpur Sahib, Punjab, India. The leaves were authenticated from Forest Research Institute (FRI), Dehradun (Voucher specimen No.: 172/2011-Bot-15-1) and a specimen was deposited in the institution for future reference.

Preparation of plant extract and Phytochemical Screening

The collected leaves of *B. monosperma* were washed, shade dried and milled to a coarse powder using a mechanical grinder. Dried leaf powder (1.4 kg) was extracted in a soxhlet extractor using solvents of increasing polarity from petroleum ether, chloroform, ethyl acetate to methanol. The successive extracts were then concentrated using a rotary vacuum film evaporator at 40±5°C and stored in a refrigerator at 4±1°C till further usage. The prepared extract was aliquoted and was subjected to the preliminary phytochemical screening tests using standard procedures.

Isolation of crude flavonoid fraction

Crude flavonoid fraction (FF) was isolated from successive methanol extract (SME)[23,24] by the following method: SME (50 g) was dissolved in water and fractionated thrice with chloroform, followed by the addition of 10% sodium chloride solution drop wise in order to precipitate tannins. The resultant solution was subjected to centrifugation. The supernatant obtained was partitioned using ethyl acetate and the organic layer obtained was evaporated to yield FF (5 g). The isolated FF was evaluated for the presence of antioxidant and anticonvulsant activities.

In vitro antioxidant assays

Radical scavenging assay (*RSA*)

As per Kaushik *et al*.[25], stock solution (1.0 mg/mL) of standard (ascorbic acid) and FF were prepared in methanol. Working dilutions (20, 40, 60, 80 and 100 µg/mL) were prepared from the stock solution. 1.0 mL of various dilutions of standard/samples were added to 5 mL of prepared 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM). Control solution was prepared in similar manner using 1.0 mL of methanol. The content of the flask was shaken vigorously and allowed to stand in dark for 30 min. The absorbance was measured at 517 nm against a blank (methanol) on a UV-Visible spectrophotometer (Shimadzu UV 1650 PC, Kyoto, Japan).

The RSA was calculated as a percentage (%) of DPPH discolouration by:

$$\% \text{ RSA} = 100 \times (1 - A_{Test} / A_{Control})$$

Reducing power assay

The reducing power assay[25,26] was performed by the following method: Briefly, 1.0 mL of working dilution was mixed with phosphate buffer (2500 µL, pH 7.4) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. TCA (2.5 mL, 10%) was then added to the mixture followed by centrifugation at 3000 rpm for 10 min. Upper layer of the centrifuged solution (2.5 mL) was mixed with distilled water (2.5 mL) and freshly prepared ferric chloride solution (0.5 mL, 0.1%). Absorbance was measured immediately at 700 nm against a blank. Notably, ferric chloride solution was not added to the blank. Distilled water was used as control.

Nitric oxide (*NO*) *assay*

Briefly, 2 mL of sodium nitroprusside (10 mM) in phosphate buffer saline was added in the working dilutions. The mixture was incubated at room temperature (27°C) for 30 min. Thereafter, 0.5 mL of the incubated solution was mixed with 1.0 mL of Griess reagent that led to the formation of pink colored solution. The absorbance was measured at 546 nm. The NO scavenging radical activity was calculated by:

$$100 \times (1 - A_{Test}/A_{Control})$$

In vivo study

Experimental animal models

Twenty-five healthy Wistar Albino rats (150-200 g) of either sex, aged 3 months were procured from Central Animal Facility, NIPER, Punjab, India. The animals were housed in standard isolation cages with paddy husk bedding under environmentally controlled conditions with a 12 h light/dark cycle. The animals were allowed free access to water and standard laboratory chow diet (Hindustan Lever Pvt. Ltd., Mumbai, India). All animals were provided acclimatization period of seven days before experimentation. The protocol for the animal study was

approved by the Institutional Animal Ethics Committee (IAEC), Global College of Pharmacy, Punjab, India. The procedures followed were according to the guidelines of CPCSEA, Government of India[27,28].

Study groups

The animals were employed in five groups (n=5 each): Gr. I served as the control and received 20% tween-20 in normal saline; Gr. II, the standard group receiving phenytoin sodium 25 mg/kg, i.p.; Gr. III-V served as the test groups receiving oral doses of flavonoid fraction (FF) @ 50, 100 and 200 mg/kg, respectively.

Maximal electroshock (*MES*) *induced seizures*

Animals reacting to a pretest before the experiment for tonic hind limb extensions were included in the study. The stimulus for inducing convulsions included electroshock of 150 mA for a duration of 0.2 s through an ear-clip electrode. The animals were given the respective doses 60 min prior to shock exposure. The phases of convulsion, namely tonic Hind Limb Tonic Extensions (HLTE) and clonic were recorded as time taken in seconds. The percent inhibition from convulsions were also determined[29].

Pentylene tetrazole (*PTZ*) *induced seizures*

The respective doses of FF standard and phenytoin were administered 60 min prior to the administration of PTZ. Seizures were induced in the test animals using single dose administration of PTZ (80 mg/kg, i.p.). The animals were placed individually in polypropylene cages (50×50×40 cm) and observed for the next 30 min. The latency of the convulsions was noted and the % protection from the same was calculated.

Statistical analysis

The data obtained have been expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 5.0 software. The *P* values for *in vivo* studies were calculated using one way analysis of variance (ANOVA, 95% confidence interval) followed by Duncan's multiple range test. In all cases, values of $P \leq 0.05$ were considered as statistical significant.

Results

Preliminary phytochemical screening of the prepared extracts

The prepared extract from the leaves of *Butea monosperma* indicated the presence of steroids, triterpenoids, alkaloids, tannins, flavonoids, glycosides and polyphenols. The SME revealed the presence of flavonoids in the preliminary phytochemical screening studies.

***In vitro* antioxidant assays**

As mentioned earlier, the FF dilutions were assessed for antioxidant potential using RSA, reducing power assay and NO scavenging assay. The antioxidant assays showed augmentation in the percentage of scavenging and absorbance values in a dose-dependent manner. The highest concentration of FF (100 µg/mL) exhibited the maximum percentage of scavenging of 88.86±0.006% using RSA and 87.98±0.013% by NO scavenging activity. Lastly, the reducing activity of 0.694±0.002 was observed with 100 µg/mL of FF. The IC_{50} values for the RSA and NO scavenging assay were found to be 50±0.001 µg/mL and 38.2±0.002 µg/mL, respectively. The results of antioxidant assays with different dilutions of FF are presented in Table 1.

Effect of different doses of FF on the MES model

The anticonvulsant activity was assessed in animal models by MES. Compared to control group, a significant reduction in both the phases of convulsions (HLTE and clonic) was observed in Group III, IV and V animals which received different concentrations of FF. Among these FF groups (Group III-V), the reduction in clonus and tonic convulsions was found with increasing dose. The maximum reduction in convulsion activity was observed in Gr. V rats. Similar reduction was obtained for percentage incidence of convulsion in FF group animals. As expected for a positive control, phenytoin showed no clonus or tonic convulsions at all (Table 2).

Table 1 — *In vitro* antioxidant assays with different dilutions of flavonoid fraction

Conc. (µg/mL)	DPPH assay (% scavenging)	Reducing power assay (Absorbance)	NO assay (% scavenging)
20	38.16±0.009	0.512±0.004	42.45±0.001
40	41.98±0.008	0.596±0.006	54.85±0.003
60	60.63±0.018	0.626±0.035	65.10±0.03
80	70.71±0.011	0.644±0.009	74.04±0.008
100	88.86±0.006	0.694±0.002	87.98±0.013

[DPPH, 2,2-Diphenyl-1-picrylhydrazyl; NO, Nitric oxide. Values are expressed as mean ± SEM (n=3)]

Table 2 — Effect of different doses of FF on the maximal electroshock-induced seizure model

Treatment group	Dose (mg/kg)	Tonic (HLTE)	Clonus	% Incidence of convulsions
Control	10 mL/kg	12.80±2.03	3.20±0.89	100
Standard	25	0	0	0
FF	50	10.70±1.06	3.16±0.71	77.69
	100	7.6±2.63**	3.11±0.82	54.26
	200	7.0±0.57**	2.47±0.57*	46.51

[FF, Flavonoid fraction; HLTE, Hind limb tonic extensions. *$P \leq 0.05$, **$P \leq 0.001$ *vs*. vehicle treated group]

Table 3 — Effect of the various doses of FF on the PTZ-induced seizure model

Treatment Group	Dose (mg/kg)	Latency of convulsions	% Protection
Control	10 mL/kg	13.74±0.17	0
Standard	25	0	100
FF	50	6.95±0.29	23.36
	100	22.66±0.18**	64.91
	200	25.11±0.18**	86.90

[FF, Flavonoid fraction. Values are expressed as mean ± SEM (n=5 each). Data was analyzed by ANOVA followed by Duncan's multiple range test.** $P \leq 0.001$ *vs*. vehicle treated group]

Effect of different FF doses on the PTZ-induced convulsion model

As compared to the vehicle treated group, different doses of FF delayed the onset of convulsions in a dose-dependent manner. However, the data was found to be non-significant with 50 mg/kg of FF dose ($P > 0.05$) and significant with 100 and 200 mg/kg of FF doses ($P < 0.001$). Moreover, a subsequent increase in the % protection from convulsions was also observed with FF in a dose dependent manner. The standard drug, phenytoin did not allow the onset of convulsion in the animals thus, offering a 100% protection from seizures in the animals of the standard group (Table 3).

Disscussion

The sacred tree, *Butea monosperma* has been in use in the traditional and ayurvedic system of medicine since ages due to its neuroprotective properties[23]. The main constituents of the plant including flavonoids and polyphenols have antioxidant potential[22,23]. Hence, flavonoids and polyphenols play an important role in the prevention and treatment of several CNS-related disease and other disorders. Epilepsy is one such neuropsychiatric disorder and oxidative stress is considered as one of its underlying mechanisms[10,11]. Thus, the present study was designed to evaluate the bioactive potentials in the isolated flavonoid fraction from the leaves of the *B. monosperma*.

Firstly, the preliminary phytochemical analysis of the prepared extracts revealed the presence of flavonoids in SME. Accordingly, the extract was used for the isolation of the crude flavonoid fraction (FF). The isolated FF was then evaluated *in vitro* for antioxidant potentials and *in vivo* for anticonvulsant properties using rodent models of MES- and PTZ-induced seizures.

Free radicals are produced in the human body as a metabolic by-product and the natural levels of antioxidants protect body from these free radicals. An additional burden of free radicals leads to oxidative stress and thereby cause related disorders. In order to check the antioxidant activity of FF, different dilutions (20-100 µg/mL) were evaluated using *in vitro* assays such as RSA, reducing power assay and NO scavenging assay. RSA/DPPH assay involves conversion of 2,2'-diphenyl-1-picrylhydrazyl (purple solution) to 2,2'-diphenyl-1-picrylhydrazyine (yellow solution) accompanied with decreased absorbance in the presence of antioxidants[31,32]. Our results showed that the antioxidant potential of FF intensifies in a concentration-dependent manner. Further, reducing power assay evaluates the reductive abilities of the test substance that involves initially yellow colored solution (potassium ferricyanide + ferric chloride) changes to different shades of bluish-green (potassium ferrocyanide + ferrous chloride) accompanied with increased absorbance[33,34]. The FF exhibited a dose-dependent reductive ability with maximum activity observed at 100 µg/mL concentration. Lastly, nitric oxide is a pleiotropic mediator which acts in various physiological and pathophysiological processes in the body[35]. Sodium nitroprusside in an aqueous medium generates NO which further interacts with oxygen to produce nitrite ions[36]. The NO formed plays an important role in the activation of N-methyl-D-aspartate (NMDA) receptors, responsible for convulsions mediated via increased Ca++ concentration in brain[37]. The NO scavenging ability of FF was also found to be increased in a concentration-dependent manner. Several studies exist in the literature that show similar results of anti-oxidant activities in parts of *B. monosperma*. For instance, Jamkhande *et al*.[38] reported *in vitro* antioxidant activity of *B. monosperma* flower fraction using RSA assay, reducing power assay and NO scavenging assay, which is in concordance to our results. However, in our study, the antioxidant potential was assessed in different dilutions of crude flavonoid fraction, extracted from leaves of the plant.

Further, the results of *in vitro* data were confirmed *in vivo* using rat models of MES- and PTZ-induced seizures and evaluated for anticonvulsant property. The NMDA and gamma aminobutyric acid (GABA) play a significant role in the pathogenesis/treatment of convulsions[39]. The MES model is believed to induce convulsions in animals by the stimulation of NMDA receptors which produce tonic and clonic convulsions characterized by HLTE. In the present study, a

significant reduction in convulsion phases and % incidence of convulsions was observed in a dose-dependent manner. In a study, Kasture *et al*.[40] assessed anticonvulsive activity of *Albizzia lebbeck*, *Hbiscus rosa* and *B. monosperma* in experimental models and reported significant decrease in convulsion phases with all the three plants. The results were in agreement to our data. The standard drug phenytoin protected the animals completely convulsions as compared to the vehicle treated group animals.

In case of PTZ-induced convulsions, the drugs which reduce T-type Ca++ currents or enhance GABA type A receptor-mediated inhibition, prevent seizures. In the present study, the FF from the leaves of *B. monosperma* significantly decreased the onset of convulsions in a dose-dependent manner and offered a maximum protection of 86.9% against the induced convulsions in PTZ model. *B. monosperma* has anticonvulsant properties that significantly reduces convulsion in MES and PTZ models[41]. As far as the mechanism of action of FF is concerned it may be acting by either blocking the NMDA receptors or by increasing the concentration of GABA in the brain.

Conclusion

The above study on the antioxidant and anticonvulsant properties of the crude flavonoid fraction (FF) from the leaves of the sacred tree, *Butea monosperma* has demonstrated its high potential. The administered doses of FF showed a significant ($P \leq 0.05$-0.001) depression in the latency in various phases and % incidence of convulsions in MES and PTZ models in a dose-dependent manner. The underlying mechanism responsible for these activities needs further investigation.

Conflict of interest

Authors declare no competing interests.

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