Original Experimental Research 实验论著

Anticonvulsant activity of alcoholic extract of bark of *Pinus roxburghii* Sarg.

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OBJECTIVE: To study the anticonvulsant activity of alcoholic extract of bark of *Pinus roxburghii* Sarg. (AEPR) used in Indian traditional medicine system in treating convulsion.
METHODS: Anticonvulsant activity was evaluated by maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizures in Wistar albino rats. In the MES model, 150 mA current for 0.2 s was given through ear electrodes to induce convulsions in rats. The duration of tonic extension of hind limb was used as the end point, namely, prevention or decrease in the duration of hind limb extension was considered as a protective action. In the PTZ model, the anticonvulsant property of AEPR was assessed by its ability to delay the onset of myoclonic spasm and clonic convulsions produced by intraperitoneal administration of PTZ.
RESULTS: In the MES-induced seizure model, AEPR in doses of 300 and 500 mg/kg body weight reduced all the phases of convulsion significantly ($P<0.01$). Standard drug phenytoin at a dose of 25 mg/kg significantly reduced flexion phase ($P<0.01$) and abolished all phases of convulsion. In the PTZ-induced seizure model, the administration of the extract at doses of 300 and 500 mg/kg 30 min prior to injection of PTZ significantly delayed the onset of clonic seizure ($P<0.01$). AEPR at the dose of 100 mg/kg body weight could not exert any significant protective effect on PTZ-induced convulsions. Standard drug diazepam at a dose of 4 mg/kg showed much delayed onset of clonic seizure.
CONCLUSION: The study suggests that AEPR would be effective against generalized tonic-clonic and partial seizures. Thus AEPR possesses anticonvulsant property against MES- and PTZ-induced seizures in Wistar rats. However, further research is in progress to isolate the compound responsible for its activity.

KEYWORDS: *Pinus*; plant extracts; anticonvulsants; electroshock; pentylenetetrazole; rats

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The Chir pine (*Pinus roxburghii* Sarg., named after William Roxburgh) is a pine native to the Himalaya. The range extends from northern Pakistan across northern India and Nepal to Bhutan[1]. The plant is used in Indian traditional system of medicine to treat a number of disorders, namely, bronchial infection, chronic rheumatism, skin disease, convulsion, ulcers, etc[2]. The chief chemical constituents extracted from resin of plants are α-pinene (18.1%), longifolene (13.8%) and carene (51.8%)[3].

The second most common neurologic disorder after stroke is epilepsy which affects about 1% of the world's population. Epilepsy is a common chronic neurological disorder characterized by seizures[4]. These seizures are transient signs or symptoms of abnormal, excessive or synchronous neuronal activity in the brain[5]. Epilepsy is more likely to occur in young children or people over the age of 65 years; however, it can occur at any time[6,7]. The term seizure refers to a transient alteration of behavior due to the disorder, synchronous and rhythmic firing of population of brain neurons[8]. Researchers are gaining new insight into the traditional medicine in assisting the body to maintain its own self-healing systems while preventing debilitating effects of chronic diseases like epilepsy[9]. The objective of the present study was to evaluate the anticonvulsant activity of alcoholic extract of bark of *Pinus roxburghii* (AEPR) occurring in northern India and also report the active constituent present therein responsible for its anticonvulsant activity.

1 Materials and methods

1.1 Plant materials The stem bark of *P. roxburghii* was collected from the Hilly region of Morni, District Panchkula, Haryana, in the month of December 2008 and was authenticated by Forest Research Institute, Dehradun, Uttrakhand, India where a voucher specimen No. 129 FHH was deposited for future reference.

1.2 Preparation of the extract Shade-dried coarse powdered bark of *P. roxburghii* in a quantity sufficient as per the volume of extractor was packed in thimble (made of filter paper sheet). A sufficient volume of alcohol was added to the reservoir and hot continuous extraction process in a Soxhlet extractor was started. This extraction process was continued for about 48 h or until alcohol coming down the siphoning tube became colorless. The excess of alcohol was distilled under reduced pressure using rotatory vacuum evaporator (Heidolph Laborota 4011, digital). A brown residue was recovered from flask with 12% yield.

1.3 Drugs All chemicals used in the present study were of analytical grade. Pentylenetetrazole (PTZ, Hi-Media, India), diazepam (Oyster lab, India) and phenytoin (Hi-Media, India) were used in this study.

1.4 Animals Wistar rats (150 to 250 g) and Swiss albino mice (20 to 25 g) of either sex brought from the National Institute of Pharmaceutical Education and Research, Mohali were kept in the Animal House of Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra, India. Animals were housed at standard conditions of temperature (22±1) ℃ and a 12/12 h light/dark cycle. They were fed with standard pellet diet (Ashirwad industries, Ropar, Punjab, India) and had free access to water. Permission for conduct of these experiments was obtained from the Institutional Animal Ethics Committee. Before the experiments they were fasted overnight with water *ad libitum*.

1.5 High-performance liquid chromatography analysis Sample of the AEPR was analyzed without any treatment. The high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) consisted of a diode array detector (SPDM10AVP), a solvent delivery module (LC-10ATVP), an online degasser (DGU-14A), an auto-injector (SIL-10ADVP), a flow channel system (FCV-14AH), a system controller (SCL-10AVP), and a reversed-phase HPLC column (RP-18, 250 mm × 4.6 mm, 5 μm particle size, Sigma, USA). The flow rate of the HPLC was 1 mL/min and the mobile phase 0.05% trifluoroacetic acid (TFA) in acetonitrile: 0.05% TFA in water

(gradient) for 70 min. Standards of chlorogenic acid, rutin and quercitin were injected separately (10 μL). Chemical compounds in the samples were identified by comparison of their retention time with the standards. Data analysis was carried out using Class VP V6.12 SP2 software (Shimadzu, Japan).

1.6 Acute toxicity study Toxicity studies were conducted as per internationally accepted protocol drawn under Organistion for Economic Co-operation and Development (OECD) guidelines 425 in Swiss albino mice[10].

1.7 Determination of anticonvulsant activity

1.7.1 Maximum electroshock-induced seizures Male or female Wistar rats with a body weight between 100 and 150 g were used. The animals were divided into five groups ($n=5$). Control group received saline, standard group received phenytoin 25 mg/kg per oral and other groups received a single dose of AEPR (100, 300 and 500 mg/kg per oral), respectively. The test was started 30 min after treating with the extracts, the standard drug and the vehicle. Maximum electroshock (MES, INCO Electroconvulsiometer model# 100-3) of 150 mA current for 0.2 s was given through ear electrodes to induce convulsions in the control and drug-treated animals. MES produced various phases of convulsions, namely, flexion, extensor, and stupor. The duration of tonic extension of hind limb was used as the end point, namely, prevention or decrease in the duration of hind limb extension was considered as a protective action[11].

1.7.2 PTZ-induced seizures Male or female Wistar rats with a body weight between 100 and 150 g were used. The animals were divided into five groups ($n=5$). Control group received saline, standard group received diazepam 4 mg/kg per oral and other groups received a single dose of AEPR (100, 300 and 500 mg/kg per oral), respectively. Thirty minutes after drug administration, 60 mg/kg PTZ was injected subcutaneously. Each animal was placed into an individual polycarbonate cage for observation lasting for 30 min. The anticonvulsant property of AEPR in this model was assessed by its ability to delay the onset of myoclonic spasm and clonic convulsions. Protection against PTZ-induced seizure and the percentage of mortality were measured[12].

1.8 Statistical analysis Data analysis employed Graphpad INSTAT version 2.0 software. All the data were presented as mean±standard error of mean. The data of MES and PTZ tests were analyzed by one-way analysis of variance followed by Dunnett's t-test. $P<0.05$ was considered significant.

2 Results

2.1 HPLC analysis From ultraviolet spectra and retention time of the main peaks, some compound classes contained in the extract have been determined. HPLC revealed the presence of bioflavonoids, quercetin, chlorgenic acid and rutin.

2.2 Acute toxicity study AEPR was found safe at the dose of 5 000 mg/kg according to the OECD guidelines 425.

2.3 Anticonvulsant activity of AEPR on MES-induced seizures In the MES-induced seizure model, AEPR at doses of 100, 300 and 500 mg/kg body weight reduced all the phases of convulsion significantly ($P<0.01$). See Table 1.

Table 1 Effects of AEPR on MES-induced seizures in rats

(Mean±standard error of mean)

Group	n	Duration (s)		
		Flexion	Extensor	Stupor
Control (saline 0.9%, per oral)	5	26.8±3.7	50.2±0.2	208.0±0.2
Standard (phenytoin 25 mg/kg, intraperitoneally)	5	10.0±0.3**	0	0
AEPR 100 mg/kg per oral	5	23.4±0.4**	7.4±1.7**	3.8±0.8**
AEPR 300 mg/kg per oral	5	16.6±2.2**	2.0±0.3**	3.2±1.7**
AEPR 500 mg/kg per oral	5	14.0±2.0**	1.6±0.2**	2.0±0.4**

** $P<0.01$, *vs* control group. AEPR: alcoholic extract of *Pinus roxburghii*; MES: maximum electroshock.

2.4 Anticonvulsant activity of AEPR on PTZ-induced seizures In the PTZ-induced seizure model, the administration of AEPR at doses of 300 and 500 mg/kg 30 min prior to injection of PTZ significantly delayed the onset of clonic seizure ($P<0.01$). AEPR at the dose of 100 mg/kg body weight could not exert any significant protective effect on PTZ-induced seizures. Standard drug diazepam at a dose of 4 mg/kg shows much delayed onset of clonic seizure ($P<0.01$). See Table 2.

Table 2 Effect of AEPR on PTZ-induced seizures in rats

(Mean±standard error of mean)

Group	n	Onset of convulsion (s)
Control (saline 0.9%, per oral)	5	3.4±1.1
Standard (diazepam 4 mg/kg, intraperitoneally)	5	234.0±0.5**
AEPR 100 mg/kg per oral	5	38.0±1.0
AEPR 300 mg/kg per oral	5	115.0±1.0**
AEPR 500 mg/kg per oral	5	227.0±0.5**

** $P<0.01$, *vs* control group. AEPR: alcoholic extract of *Pinus roxburghii*; PTZ: pentylenetetrazole.

3 Discussion

The MES test is the most frequently used test as an animal model for identification of anticonvulsant activity of drugs for the generalized tonic-clonic seizures "grand mal"[13,14]. This model is based on observation of the stimulation by repeated electrical pulses[15]. The MES model is used to identify compounds which prevent seizure spread, corresponding to generalized tonic-clonic seizures in humans[16,17]. Moreover, MES-induced tonic extension can be prevented either by drugs that inhibit voltage-dependent Na^+ channels, such as phenytoin, valproate, felbamate and lamotrigine or by drugs that block glutamatergic excitation mediated by the *N*-methyl-D-aspartate (NMDA) receptor, such as felbamate[18]. Currently used anticonvulsant drugs such as phenytoin and carbamazepines which are effective in treatment of generalized tonic-clonic and partial seizures have been found to show strong anticonvulsant action in MES test[19,20].

PTZ-induced seizure is analogous to petitmal seizure and human generalized seizure[13]. Compounds effective against this experimentally induced seizure models are effective against petitmal type of epilepsy[21]. Drugs that are effective against petitmal seizures reduce T-type calcium currents and these types of seizures can also be prevented by drugs that enhance γ-aminobutyric acid (GABA) or benzodiazepine receptor which mediated neurotransmission such as benzodiazepines and phenobarbitone[20,22]. Studies have shown that activation of receptor are also involved in the initiation and generalization of PTZ-induced seizures[23,24]. Drugs that block glutametargic excitation mediated by NMDA receptors, such as felbamate, have anticonvulsant property against PTZ-induced seizures[20].

The HPLC analysis showed the presence of phytochemicals such as quercetin[25] and rutin which were reported to have anticonvulsant activity; it is likely that flavonoidal compounds, present in this plant may be involved in its anticonvulsant activity. Hence, AEPR is able to modulate the function of GABA or glutamate receptors[26].

Therefore, it can be concluded that the anticonvulsant activity exhibited by the AEPR is due to the blocking of the seizures spread by inhibiting Na^+ channels and glutamatergic excitation through NMDA receptor. The study also suggests that AEPR would be effective against generalized tonic-clonic and partial seizures. Several drugs are thought to inhibit seizures by regulating GABA-mediated synaptic inhibition through an action at distinct sites of the synapse.

Thus AEPR possesses anticonvulsant property against MES- and PTZ-induced seizures in Wistar rats. However, further research is in progress to isolate the compound responsible for its activity.

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5 Competing interests

The authors declare that they have no competing interests.

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西藏长叶松树皮乙醇提取物的抗惊厥作用

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目的：研究西藏长叶松树皮乙醇提取物(alcoholic extract of bark of *Pinus roxburghii* Sarg., AEPR)的抗惊厥作用。

方法：用最大电休克和戊四唑诱发白化 Wistar 大鼠癫痫。最大电休克模型经由大鼠耳电极给予 150 mA 电流刺激 0.2 s 诱发癫痫，以后肢强直性伸展持续时间的变化作为结局指标衡量 AEPR 的抗惊厥作用，即后肢强直性伸展持续时间减少或停止。戊四唑模型大鼠经腹膜内注射戊四唑诱发肌阵挛性发作和阵发性抽搐，以痉挛发作的延迟衡量 AEPR 的抗惊厥作用。

结果：在最大电休克惊厥模型中，AEPR 剂量分别为 300 和 500 mg/kg 体质量，均显著减轻所有阶段的大鼠惊厥发作($P<0.01$)；标准对照药物苯妥英钠组用量 25 mg/kg，能显著减轻屈曲阶段的发作($P<0.01$)，并抑制所有阶段的惊厥发作。戊四唑惊厥模型大鼠于注射戊四唑前 30 min 分别给予 AEPR 300 和 500 mg/kg，均能显著延迟阵挛性发作($P<0.01$)。100 mg/kg 体质量的 AEPR 在戊四唑诱发的癫痫模型中没有显著的抗惊厥作用；标准对照药物地西泮 4 mg/kg 能大幅度延迟阵挛性发作。

结论：本研究提示 AEPR 能有效抑制普遍强直性肌阵挛和部分癫痫发作。因此，AEPR 对最大电休克及戊四唑诱发的大鼠癫痫有抗惊厥作用。然而需要进一步的研究确认是 AEPR 中的何种成分对这种抗惊厥作用起主导作用。

关键词：松属；植物提取物；抗惊厥药；电休克；戊四唑；大鼠