Anticonvulsant and behavioral actions of triterpene isolated from *Rubia cordifolia* Linn.

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Effect of a triterpene isolated from the acetone soluble part of petroleum ether extract of *R. cordifolia* was studied on convulsions induced by maximum electro shock (MES), electrical kindling and various chemocconcussants in rats and mice. The effect of triterpene was also investigated on behavior and gamma-aminobutyric acid (GABA) and serotonin (5-HT) content in mouse brain. Triterpene inhibited seizures induced by MES, electrical kindling, pentylenetetrazol (PTZ), and lithium-pilocarpine. However, seizures induced by strychnine were not inhibited. Triterpene reduced locomotion as well as rearing. Pentobarbitone induced sleep was potentiated and amphetamine induced stereotypy was inhibited. The triterpene was found to possess anxiogenic activity. Brain GABA and 5-HT contents were raised by the compound. The study suggests that the triterpene isolated from *R. cordifolia* bear a potential for further study.

*Rubia cordifolia* Linn (Family: Rubiaceae) is a climbing plant growing in north-west Himalaya, and other hilly districts of India. The methanolic extract of roots has cancer activity. Dried roots are used as astringent and diuretic. They are used in folklore medicine for treatment of dropsy, paralysis, jaundice, amenorrhoea, and visceral obstructions. Ethanolic extract of the aerial parts of the plant shows hypoglycemic activity in albino rats. Tripathi and her associates have reported the lipoxygenase activity of ethanolic extract of roots and ethyl acetate fraction of this extract is the most active lipoxygenase inhibitor.

During our preliminary studies, the acetone soluble fraction of petroleum ether extract, which contained a triterpene, was found to possess CNS depressant activity. Therefore, we investigated the effects of the triterpene isolated from the acetone soluble part of the pet.ether extract on chemically induced and maximum electro shock-induced convulsions, and electrically kindled seizures. Its effect on gross behavior, ambulation and rearing, pentobarbitone-induced sleep, and amphetamine-induced stereotyped behavior was also studied.

**Materials and Methods**

**Extraction** — The dried roots and rhizomes of *R. cordifolia* (500 g), collected in March, after identification and authentication by Dr. V.K. Deshmukh, Ex-Prof. of Pharmacognosy, Department of Pharmaceutical Sciences, Nagpur University, Nagpur, were extracted with petroleum ether (60-80°C). The extract was concentrated under vacuum and was then partitioned into acetone soluble and acetone insoluble parts. The yields of pet.ether extract, acetone soluble and insoluble parts were 0.5%, 0.2%, and 0.3% w/w respectively. The acetone soluble part was then separated into four fractions using column chromatography, with neutral alumina as a stationary phase and solvents of varying polarity like benzene, benzene:ethyl acetate (5:5), ethyl acetate and methanol as mobile phase. The benzene:ethyl acetate (5:5) fraction was obtained in sufficient quantity (yield 750 mg). The fraction was thoroughly washed with acetone to obtained white needle shaped crystals, which were triterpene chemically. The crystals of triterpene, (RCT), were suspended in 0.5% (w/v) carboxy-methylcellulose and administered orally.

**Animals** — Albino mice (NIN strain) of either sex weighing 20-22 g and albino rats (NIN strain) of either sex, weighing 150-175 g were housed into groups of 8 in standard laboratory conditions. The experiments were performed during 900-1600 hrs in the laboratory, which was maintained at 23°C ± 1°C.

**Drugs** — The following drugs were used: pentobarbitone sodium, d-amphetamine (Sigma, USA), lithium sulphate (Glenmark, India), pilocarpine (FDC, India), diazepam (Ranbaxy, India). All drug solutions were prepared in distilled water immediately before the experiment and administered in a constant volume (5ml/kg in mice and 1ml/kg in rats).
Acute toxicity — The RCT was administered in doses of 100, 200, 400, and 1000 mg/kg orally and percentage mortality was observed after 24 hr.

Behavioral studies — To investigate the central actions of RCT, (100-200 mg/kg, p.o.), the method described by Irwin was employed. Briefly, animals were observed for 60 min after oral administration of RCT. The procedure involved an initial phase of undisturbed observations and a later manipulative phase during which animals were subjected to the least provoking stimuli. In the initial phase the animal was observed for body position, locomotion, rearing, respiration, tremors, staggering gait, and in the later phase, the effect on grip strength, passivity, righting reflex, pain response and lacrimation was also observed.

Convolusions — Mice (n = 6/8) were pretreated with either RCT (25-200 mg/kg p.o.) or vehicle or diazepam (2mg/kg i.p.), 30 min before the administration of either pentyleneetrazol (80 mg/kg s.c.), strychnine (1mg/kg i.p.), or an electric shock (42mA for 0.2 sec using corneal electrodes). The animals were placed in isolated cages and observed for the presence or absence of seizures. The onset of convulsions and the incidence of seizures were recorded in case of chemoconvulsants, whereas the duration of tonic hind leg extension and incidence of convulsions were observed in case of MES induced convulsions.

Lithium-pilocarpine-induced seizures — The rats (n = 8) received lithium sulphate (3 meq/kg i.p.) 24 hr before pilocarpine (30 mg/kg i.p.). The animals received vehicle or RCT (100-200 mg/kg) orally 30 min before pilocarpine. The severity of convulsions was assessed for 90 min using the following scoring system suggested by Patel et al.

No response = 0, fictive scratching = 1, tremors = 2, head nodding = 3, forelimb clonus = 4, rearing, falling and clonus = 5.

Electrical kindled seizures — Rats (n=6) received electric shock daily, (70 mA, for 0.1sec, twice a day, 3 hr apart using Techno Electroconvulsiometer) till all the animals exhibited full blown seizures. All animals received equal number of shocks (11 shocks), although some exhibited complete development of kindling with less number of shocks. The vehicle, diazepam (2mg/kg i.p.) or RCT (100 mg/kg p.o.) was administered 30 min before the electric shock and the severity of seizures was recorded as score 1 = facial movements, 2 = head nodding, 3 = forelimb clonus, 4 = rearing with forelimb clonus, 5 = clonic seizures. The scores representing the intensity of pilocarpine induced behavior in lithium pretreated rats were noted every 10 min for 90 min and the latency to first forelimb clonus was noted.

Acute safety test — The inverted screen procedure was used to study the acute safety of RCT, as described by Coughenour and his associates. The apparatus consisted of a 12.6cm square platform of 0.6cm wire mesh supported by metal bars and mounted on a steel rod. Mice were pretrained by placing them on the platform and gently rotating it upside down and observing the animals for their ability to climb on the platform within 1 min. The animals failing the task were not used for the test on the next day. For the test, animals (n = 6) were administered vehicle or RCT (100 mg/kg) orally or diazepam (1mg/kg i.p.) and were placed on the platform and the rod rotated through an arc of 180°. Mice unable to climb to an upright position within 1 min were considered to be neurally impaired. Six animals were used in each group.

Activity on rotating rod — Mice were previously trained to remain on the rod (2.54 cm diameter) rotating at a speed of 20 rev/min for a period of 5 min. On the next day the animals were randomly divided into groups of 5 each. The RCT (100 or 200 mg/kg p.o.) or diazepam (1mg/kg i.p.) or vehicle (5 ml/kg, p.o.) was administered 30 min before the test and the time required to fall off the rotating rod was noted for each animal.

Locomotion and rearing — The effect of RCT was observed on locomotion and rearing using open field test. The open field test apparatus (24x24x15") made up of plywood and the floor divided in 16 squares of same size. Mice (n = 5) were treated with vehicle or RCT (100 mg/kg) orally or diazepam (1 mg/kg i.p.) and 30 min later the animals were placed (individually) gently in one corner of the apparatus and the number of squares traversed and rearings made in 5 min were noted.

Pentobarbitone-induced sleep — Female mice in groups of 5 each were treated with vehicle or RCT (100/200 mg/kg) orally 30 min before pentobarbitone (40 mg/kg i.p.) and the duration of loss of righting reflex was observed in each group. The righting reflex was considered to be lost when the animal placed on its back, failed to regain its normal posture within 10 sec.

Amphetamine antagonism — Mice were divided in groups of five each. Mice were pretreated with vehicle or RCT (200 mg/kg) p.o. or chlorpromazine (10 mg/kg i.p.) and 30 min later, d-amphetamine
(1 mg/kg i.p.)$^{15}$ was administered. The animals were observed for the onset of tremors and biting.

**Effect on haloperidol-induced catalepsy**—Catalepsy was measured using “Bar test”$^{16}$. In brief, mice (n = 5) were treated with vehicle or RCT (100 mg/kg) p.o. 30 min before haloperidol (1 mg/kg i.p.) and their forepaws were placed on a bar (1 cm dia. placed 2.5 cm above the table) and the duration of catalepsy was measured at 5, 15, 30, 60 and 90 min intervals.

**Assessment of analgesic activity**—Elevated plus maze was used as described earlier.$^{17}$ In brief, mice (n = 5) were placed individually in the centre of elevated plus maze (35×6 cm, open arm and 35 × 6 x 15 cm, closed arm, elevated to the height of 25 cm) facing an enclosed arm. The time spent by the mice in open and closed arm was noted. The mice were treated with vehicle or RCT (100 mg/kg) p.o. or diazepam (1 mg/kg i.p.) 30 min before the test.

**Assessment of analgesic activity**—The analgesic activity was studied using the hot plate analgesiometer$^{18}$. Mice (n = 5) were treated with vehicle or RCT (100 mg/kg) p.o. or pentazocine (10 mg/kg i.p.) and they were placed individually on the hot plate maintained at $55^\circ\pm 1^\circ$C. The time required to lick paws was noted.

**Estimation of gamma-aminobutyric acid and serotonin in brain**—Whole brain contents of GABA and 5-HT were measured 30 min after vehicle or RCT (100 mg/kg) p.o. by the method of Klingman and Mennel$^{19}$ and Curzon and Green$^{20}$ respectively.

**Phytochemical study**—Chemical tests were carried out to determine the chemical nature of RCT. Its melting point was determined using conventional method. This was followed by UV, IR, Mass, PMR and $^{13}$C NMR spectra and elemental analysis.

**Statistical analysis**—The data obtained were analyzed using one way analysis of variance (ANOVA) followed by Dunnett’s test or Student’s $t$ test. Mann-Whitney U test was used for non-parametric data and the complete effect or lack of effect was analyzed using Fisher exact test.

**Results**

**Acute toxicity**—The approximate LD$_{50}$ of the triterpene, RCT, was found to be 400 mg/kg orally. The signs preceding death usually included prostration, ataxia and convulsions. Convulsions were observed before death. They were not observed in the surviving animals.

**Behavioral studies**—The RCT had no effect on body position and respiration, grip strength, righting reflex, and passivity at the doses used (100 /200 mg/kg, p.o.), however locomotion, rearing, and reaction to painful stimuli decreased noticeably. Tremors, staggering gait, and lacrimation were not observed.

**Convulsions**—The RCT dose dependently inhibited the incidence of convulsions and significantly delayed the onset of spasm and clonus in the PTZ treated mice. However, it failed to inhibit strychnine-induced seizures (Tables 1 and 2).

In the MES test, the duration of tonic extensor phase reduced dose dependently, RCT at the dose of 200 mg/kg, p.o. prevented the tonic extensor phase completely. Diazepam (2 mg/kg, i.p.) afforded complete protection to mice (Table 3). The severity of electrically kindled seizures was reduced and latency to attain forelimb clonus in lithium pilocarpine treated rats was delayed significantly ($P<0.05$, Fig 1 and 2).**

**Acute safety study**—All the mice treated with RCT (100-200 mg/kg, p.o.) could climb the screen within 1 min. However, animals treated with diazepam (1 mg/kg, i.p.) failed to climb the screen within 1 min.

**Activity on rotating rod**—Mice treated with vehicle could remain on the rotating rod for 300 sec and the mice treated with RCT (100-200 mg/kg, p.o.) remained on the rotating rod for 264.4±30.7 and 248.4±34.9 sec respectively. This difference missed the statistical significance. Animals treated with

Table 1—Effect of a triterpene (RCT) isolated from pet ether of *Rubia cordifolia* on pentylenetetrazol induced seizures in mice.

<table>
<thead>
<tr>
<th>Treatment (Dose:mg/kg)</th>
<th>Latency to spasm in sec</th>
<th>Latency to convulsions in sec</th>
<th>Incidence of seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (25)</td>
<td>58.0±5.4</td>
<td>108.4±7.5</td>
<td>8/8</td>
</tr>
<tr>
<td>RCT (50)</td>
<td>68.7±5.9</td>
<td>139.5±8.9</td>
<td>6/8</td>
</tr>
<tr>
<td>(100)</td>
<td>104.7±8.2**</td>
<td>164.6±12.4</td>
<td>5/8</td>
</tr>
<tr>
<td>Diazepam (1)</td>
<td>135.7±7.2**</td>
<td>189.3±11.4**</td>
<td>4/8</td>
</tr>
<tr>
<td>(200)</td>
<td>--</td>
<td>--</td>
<td>0/8</td>
</tr>
</tbody>
</table>

Pentylenetetrazol was administered s.c. in a dose of 80 mg/kg. $P$ values: *$<0.05$, **$<0.01$, ***$<0.001$, ****$<0.0001$ (Student’s $t$ test).

Table 2—Effect of a triterpene (RCT) isolated from pet ether of *Rubia cordifolia* on strychnine induced seizures in mice.

<table>
<thead>
<tr>
<th>Treatment (Dose:mg/kg)</th>
<th>Latency to spasm in sec</th>
<th>Latency to convulsions in sec</th>
<th>Incidence of seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (100)</td>
<td>124.6±9.7</td>
<td>168.2±7.8</td>
<td>*8/8</td>
</tr>
<tr>
<td>RCT (200)</td>
<td>132.9±6.1</td>
<td>154.3±6.2</td>
<td>8/8</td>
</tr>
</tbody>
</table>

Strychnine was administered in a dose of 1.2 mg/kg i.p.
diazepam (1 mg/kg i.p.) could remain on the rotating rod for 46.4±5.4 sec only \( (P<0.001) \).

**Locomotion and rearing**—Locomotion was significantly reduced by RCT (100 mg/kg p.o.). Whereas rearing was completely suppressed. RCT was more effective than diazepam (1 mg/kg i.p.) in suppressing locomotion and rearing (Table 4).

**Pentobarbitone-induced sleep**—The pretreatment with RCT (100 and 200 mg/kg) orally prolonged sleeping time from 69.6±5.4 min to 98.4±3.7 and 110.4±13.4 min respectively \( (P<0.05) \).

**Amphetamine-induced stereotypy**—Prior treatment with RCT (200 mg/kg p.o.) delayed the onset of tremors from 36.4±5.8 sec to 119.7±8.9 sec and the onset of biting from 79.6±8.4 sec to 320.6±31.5 sec \( (P<0.05) \).

**Effect on haloperidol-induced catalepsy**—The triterpene RCT, as such was devoid of cataleptic activity but potentiated and abbreviated the haloperidol-induced catalepsy significantly (Fig. 3).

**Assessment of anxiolytic activity**—The animals treated with RCT remained for most of the time in the closed arm, whereas animals treated with diazepam spent more time in the open arm \( (P<0.05, \text{Table 5}) \).

**Assessment of analgesic activity**—Animals treated with RCT exhibited increase in reaction time when placed on hot surface \( (P<0.05) \). The activity was comparable with that of pentazocine, 10mg/kg i.p. (Table 6).

**Estimation of brain GABA and 5-HT**—The RCT in a dose of 100 mg/kg p.o. increased the brain content of GABA from 19.6±0.3 to 33.59±2.1 \( \mu \text{g/g} \) and brain content of 5-HT was raised from 492.0±43.1 to 1180.8±23.4 ng/g. The increase in

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**Table 3**—Effect of a triterpene (RCT) isolated from pet ether extract of *Rubia cordifolia* on maximum electroshock-induced seizures in mice

<table>
<thead>
<tr>
<th>Treatment (Dose: mg/kg)</th>
<th>Duration of tonic hindlimb extension in sec</th>
<th>Incidence of convulsions</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>14.9±5.4</td>
<td>8/8</td>
<td>100</td>
</tr>
<tr>
<td>RCT (25)</td>
<td>10.4±3.2</td>
<td>6/8</td>
<td>75</td>
</tr>
<tr>
<td>(50)</td>
<td>7.8±2.5*</td>
<td>5/8</td>
<td>50</td>
</tr>
<tr>
<td>(100)</td>
<td>5.4±2.2*</td>
<td>4/8***</td>
<td>25</td>
</tr>
<tr>
<td>(150)</td>
<td>3.2±0.2*</td>
<td>2/8***</td>
<td>00</td>
</tr>
<tr>
<td>(200)</td>
<td>—</td>
<td>0/8***</td>
<td>00</td>
</tr>
<tr>
<td>Diazepam (2)</td>
<td>—</td>
<td>0/8***</td>
<td>00</td>
</tr>
</tbody>
</table>

*\( P<0.05, \) Student's t test.
**\( P<0.05, \) Fisher exact test.

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**Fig. 1**—Effect of RCT on severity of electrically induced kindling seizures in rats. \( *P<0.05 \) (Student's t test).

**Fig. 2**—Effect of RCT on severity of lithium-pilocarpine induced seizures. \( *P<0.05 \) (Student's t test)
brain contents of both the neurotransmitters was significant at \( P < 0.01 \).

**Phytochemical study of RCT** — The Liebermann-Burchard test indicated triterpene nature of the compound. Its melting point was 110°C. UV spectrum (\( \lambda \) max) at 217,250, 255 and 316 nm. IR (KBr) spectra-3450, 3370, 2900, 2800, 1660, 1640, 1480, 1320, 1060, 1010, and 780 cm\(^{-1}\). Mass spectra indicated molecular ion peak at 478. Other peaks were observed at 430, 426, 412, 396, 379, 271, 202, 148.

Elemental analysis: % C=78.91, % H=11.03, %O=10.06.\(^1\)C NMR (200 Hz, CDCl\(_3\)): Peaks were observed at 130.82, 128.8, 121.62, 77.31, 77.20, 77.00, 76.6, 71.71, 56.7, 56.08, 55.2, 45.09, 42.03, 37.27, 36.1, 34.0, 31.87, 29.62, 29.26, 29.08, 27.93, 27.70, 26.24, 24.74, 23.11, 22.61, 19.74, 19.34, 19.05, 14.01, 11.94, 11.83 (Total 32 carbon atoms). PMR (300MHz): 0.69, 0.86, 1.0, 1.29, 1.5, 1.54, 1.66, 1.82, 1.89, 2.0, 2.4, 3.54, 4.9, 5.05, 5.15, 5.2, 5.35. From the elemental analysis and other spectral data, the empirical formula was determined as C\(_{32}\)H\(_{49}\)O\(_3\). The compound has not been reported in the literature.

**Discussion**

The results of behavioral assessment indicate that the triterpene RCT, isolated from the acetone soluble part of petroleum ether extract of *R.cordifolia* produced a depressant effect on the central nervous system. The depression which occurred did not resemble the sedative effect of benzodiazepines, as the animals were still responsive and did not show prominent muscle relaxation. The compound inhibited MES and PTZ-induced convulsions but not strychnine-induced convulsions. These observations indicate that the anticonvulsant effect of the triterpene is possibly mediated by chloride channels of GABA/benzodiazepine receptor complex but not by chloride channel of glycine receptors \(^2\).

The triterpene, RCT, inhibited electrical kindling as well as lithium-pilocarpine-induced seizures. Lithium alone does not have general proconvulsant effect in rats\(^2\). However, rats pretreated with lithium have limbic seizures, following subconvulsive doses of picrotoxin\(^2\). The combined treatment with lithium and picrotoxin results in decrease in cortical inositol and accumulation of inositol monophosphate that are

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Latency to lick paws in sec (mean±SE) at n=5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4.0±0.31</td>
<td>4.2±0.6</td>
<td>3.9±0.6</td>
<td>4.2±0.7</td>
<td></td>
</tr>
<tr>
<td>RCT (100)</td>
<td>12.3±3.2</td>
<td>14.4±2.4</td>
<td>15.7±4.1</td>
<td>23.2±2.1</td>
<td></td>
</tr>
<tr>
<td>Penta (10)</td>
<td>10.2±0.4</td>
<td>12.3±2.7</td>
<td>10.1±2.3</td>
<td>8.3±1.4</td>
<td></td>
</tr>
</tbody>
</table>

Penta= pentazocine.

All differences were significant at \( P<0.05 \) (Student's \( t \) test) when compared with vehicle treated group.

**Fig. 3** — Effect of RCT on haloperidol-induced catalepsy. 
\( *P<0.05 \) (Student's \( t \) test). It is the logarithm to the base \( n \).
about 10 times greater than the effects obtained with either drug alone. These seizures are prevented by diazepam. It is reported that GABA level in the brain decreases in both the electrical kindling and lithium-pilocarpine induced seizures. The RCT increased both GABA and 5-HT concentration in the brain. The 5-HT, like GABA is an endogenous anticonvulsant and elevation of 5-HT is responsible for the anxiogenic effect observed in the elevated plus maze paradigm. In the elevated plus maze test, reduction in the time spent in the open arm provides a measure of fear induced inhibition of exploratory activity which is increased by anxiogenic agents.

A reciprocal relationship has been observed in the concentration of 5-HT and dopamine (DA). The potentiation of haloperidol induced catalepsy is indicative of decrease in the DA transmission in the substantia nigra. This observation has also been reflected in the amphetamine antagonism as observed from the delay in onset of seizures. It is reported that GABA level in the brain decreases in both the electrical kindling and lithium-pilocarpine induced seizures. The RCT increased both GABA and 5-HT concentration in the brain. The 5-HT, like GABA is an endogenous anticonvulsant and elevation of 5-HT is responsible for the anticonvulsant, anxiogenic, and other behavioral actions of the RCT suggestive of central depressant action of the RCT.

Thus functional alterations of GABA and 5-HT systems may be responsible for anticonvulsant, anxiogenic, and other behavioral actions of the RCT isolated from Rubia cordifolia.

References