

Research Article

Isolation, Characterization and Evaluation of Anti-Convulsant Activity Of *Rubus Racemosus*

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ABSTRACT

The structure of new compounds Compound I - 1-[20, 21 – dodeacylnanone] – α -1 \rightarrow 6-D-glucotetraose-6'''(P-hydroxy) benzoate. Compound II-9, 10 epoxynonacosane have been isolated from the methanolic aerial extract of the *Rubus racemosus*. The methanolic extract was subjected to anticonvulsant activity by MES induced epilepsy and determination of neurotransmitter concentrations in rat brain after induction of epilepsy.

Keywords: MERR, MEZ, Anticonvulsant, *Rubus racemosus*

INTRODUCTION

Rubus racemosus is a deciduous shrub belongs to the family *Rosaceae*¹. Traditionally, it is used as astringent, abortifacient, muscle relaxant, Anticonvulsant and free radical scavenging agents². Family *Rosaceae* is known as folk medicine for treatment to nervous disorders³. Decoction of the root is useful for relaxed bowel and dysentery⁴. The chief active constituents are flavanoids, phenolic, glycosides and tannins. Literature review revealed no documentation of scientific work on aerial parts of *Rubus racemosus*. An attempt has been made to isolate and characterize the compounds and evaluate the anticonvulsant activity.

MATERIALS AND METHODS

Extraction and isolation

The aerial parts of *Rubus racemosus* were collected from Nilgiri hills authenticated, shade dried for seven days and powdered. Powdered material was extracted with methanol and evaporated under reduced pressure. The methanolic extract was subjected to column chromatography⁵ over silica gel G activated at 100⁰ for one hour. The column was eluted with different organic solvents in increasing order of polarity. The eluate from the column was collected. The fractions were combined and identified by TLC with mobile phase ethylacetate:isopropyl alcohol, ethylalcohol:methanol⁶.

Anti-convulsant activity

Experimental animals

Wistar albino rats weighing 150g-200g were obtained from animal house. They were fed with standard pellet diet and water *adlibitum*. Animals were maintained in a standard animal house. The experiments were designed and conducted according to the ethical guidelines after obtaining the necessary clearance from the committee [Approval No: IAEC/XIII/17/CLBMCP/2007-2008.

Maximal electroshock induced convulsion⁷

Procedure

Seizures are induced to all the groups by using an electroconvulsimeter. Maximal electroshock seizures were elicited by a 60 Hz alternating current of 150 mA intensity for 0.2 sec. A drop of electrolyte solution (0.9% NaCl) with lignocaine was applied to the corneal electrodes prior to application to the rats. This increased the contact and reduced the incidence of fatalities. Different doses of the methanolic extract of *Rubus racemosus* (MERR) were administered for 14 days before induction of seizures. The duration of various phases of epilepsy were observed. The percentage protection was estimated by observing the number of animals showing abolition of Hind Limb Tonic Extension⁶ (HLTE).

Group I Animals treated with 1% SCMC, 1ml/100g

Group II Animals treated with phenytion (25mg/kg) suspended in 1% w/v SCMC

Group III Animals treated with MERR* (200mg/kg) suspended in 1% w/v SCMC

Group IV Animals treated with MERR (400mg/kg) suspended in 1% w/v SCMC

*MERR – Methanolic Extract of *Rubus racemosus*

Determination of the effect of *Rubus racemosus* on neurotransmitter concentrations in rat brain after induction of epilepsy

The various biogenic amines in discrete regions of the rat brain were estimated by spectrofluorimetric method.

Preparation of Tissue Extracts⁸

Dissected frozen rat brains were first cut on a cooled microtome (-20⁰) in to frontal slices (about 1mm thick) at pre determined antero posterior levels. The frontal slices were subsequently placed on the cooled stage (-20⁰) of a punching apparatus where cylindrical tissue samples (usually 1 mm in diameter, same thickness as the slice) were punched out of selected brain areas with a glass tube. The x and y co-ordinates of the center of the area were adjusted is a stereo microscope, the ocular of which contained cross line that were concentric with the

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center of the glass tube. For weight determination the tissue pieces were transferred immediately to pre-cooled microhomogenizers which were closed with glass stored at -25° .

Extraction

The tissue (1.5 – 5 mg) was homogenized in 0.1ml HCl-Butanol (0.85ml of 37% HCl in 1liter of butanol for spectroscopy) for 1 minute in a glass homogenizer made from a small centrifuge tube (vol. 1.5ml). The total volume was considered to give 0.105ml, taking account of the tissue volume (1mg = 0.001ml). The sample was then centrifuged for 10 min at 2000 rpm. An aliquot of the supernatant phase (0.08ml) was added to an Eppendroff reagent tube (vol.1.5ml) containing 0.2ml heptane (for spectroscopy) and 0.025ml HCl 0.1M. After 10 min of vigorous shaking, the tube was centrifuged under the same condition as above in order to separate the two phases and the overlaying organic phase was discarded. The aqueous phase (0.02ml) was then taken either for a 5-HT or NA and DA assay. All steps were carried out at 0°C .

Nor-Adrenaline and Dopamine assay⁸

The assay represents miniaturization of trihydroxyindole method. To 0.02ml of HCl phase, 0.005ml 0.4M HCl and 0.01ml EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.01ml iodine solution (0.1M in ethanol) for oxidation. The reaction was stopped after two minutes by the addition of 0.01ml sodium thiosulphate in 5M sodium hydroxide (0.5g Na_2SO_3 in 2ml H_2O + 18ml 5M NaOH). Acetic acid (0.01ml, 10M) was added 1.5 minutes later. The solution was then heated to 100°C for 6 minutes. When the sample again reached room temperature, excitation and emission spectra were read (330 and 375 nm for Dopamine and 395 – 485 nm for Nor-adrenaline) in a spectrofluorimeter compared the tissue values (fluorescence of tissue extract minus fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard minus fluorescence of internal reagent blank). Tissue blanks for the assay were prepared by adding the reagents of the oxidation step in reversed order (sodium thiosulphate before iodine). Internal reagent standards were obtained by adding 0.005ml distilled water and 0.1 ml HCl Butanol to 20 ng of dopamine and Nor-adrenaline standard. For the internal reagent blank 0.005ml of water was added to 0.1ml Hcl butanol.

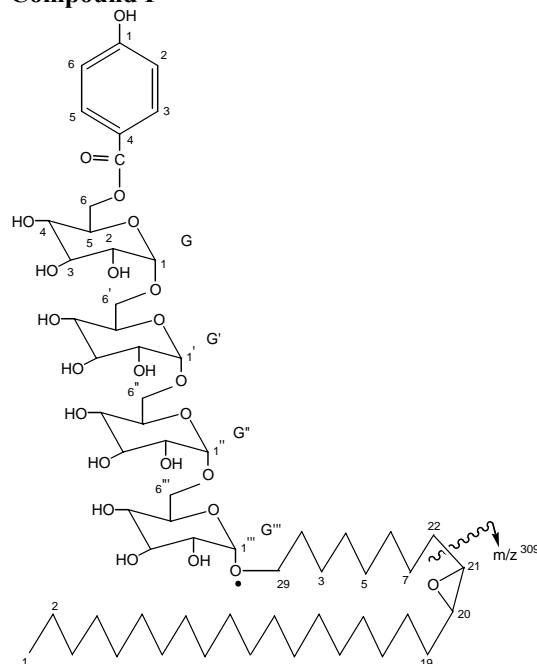
Serotonin Assay⁹

In order to obtain in a good fluorescence yield with reduced volume for 5-HT determination, the o-

phthaldialdehyde (OPT) method was employed. From the OPT reagent (20mg% in conc. HCl) 0.025ml were added to 0.08ml of the HCl extract. The fluorophore was developed by heating to 100°C for 10min. For the serotonin tissue blank 0.025 of HCl without OPT was added. After the samples reached equilibrium with the ambient temperature, excitation and emission spectra or intensity reading at 360 and 470nm were recorded.

RESULTS

Compound I



It was obtained as an amorphous and yellow in colour. The IR spectrum of the isolated compound I from *Rubus racemosus* shows characteristic absorption bands at 3411cm^{-1} for $-\text{OH}$ group and 1651cm^{-1} for α, β - unsaturated carbonyl group.

The $^1\text{H-NMR}$ spectrum revealed three distinct patterns of proton resonances. The first is typical for esterified p-hydroxy benzoic acid and contained two signals at $\delta 6.85$ (d, 2H) and $\delta 7.11$ (d, 2H). The second pattern is characteristic for sugar protons and contained four anomeric resonances at $\delta 5.3$, $\delta 5.2$, $\delta 4.91$ and $\delta 4.92$. The chemical shift values of these doublet signals indicated esterification of the anomeric hydroxyl group and four sugar units were present. Other sugar protons resonate at chemical shift values between $\delta 3.04$ to $\delta 3.84$. The third pattern of H-NMR signals is indicative of a long chain epoxide and contained signals at $\delta 0.84$ (t, 3H, CH_3), a

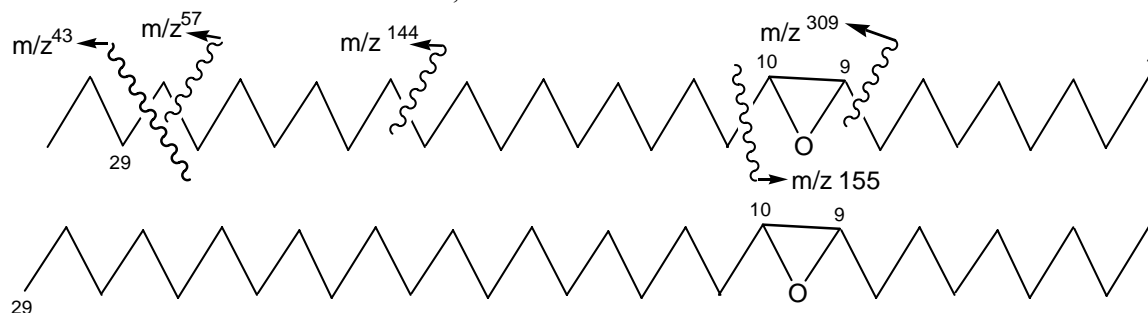


Table 1: TLC of isolated compounds

Compound	Solvent System	Detecting agent	Rf
Compound I	Ethyl acetate :	UV	0.62
Compound II	Isopropanol : Ethyl alcohol : Methyl alcohol	radiation	0.59

strong singlet at δ 1.22 for long chain methylene protons, two multiplets at δ 2.83 and δ 2.69 for epoxide hydrogens and a signal at δ 1.57 for the α – methylene protons to the epoxide ring. The above datas were strongly supported by the ^{13}C -NMR spectral datas. It exhibited a signal at δ 174.99 for ester carbonyl group and at 156.46 (C-1), 143.36 (C-4), 114 (C-2, C-6) and 127.89 (C-3, C-5) for aromatic ring carbons. The downfield signal at δ 156.46 is due to the substitution with a –OH group in that position. The spectrum also indicated the presence of four anomeric carbon signals at δ 102.44, δ 98.49, δ 97.35 and δ 92.68. The other carbon signals of the sugar moiety appeared between δ 63.38 to δ 82.35. Further the ^{13}C -NMR spectral data exhibited the signals at δ 14.39 (CH_3), δ 61.69 and δ 63.38 (epoxide ring carbons), 26.00 and 27.00 for α – carbons to the epoxide ring. The presence of fragment ions at m/z 309 and m/z 153 arising from fragmentations adjacent to the oxygen bearing carbons suggest that the epoxide was located on C-20 and C-21 of the chain. Considering the above data into consideration the following structure is proposed for the compound I (1-[20,21 – dodeacylnanone] – α -1 \rightarrow 6-D-glucotetraose-6 (P-hydroxy) benzoate).

Compound II

Compound II is yellow in colour and hygroscopic in nature. The molecular formulae of the isolated

compound II is found to be $\text{C}_{29}\text{H}_{58}\text{O}$ from the E1 mass spectrum $[\text{MH}]^+$ m/z 423. The ^1H and ^{13}C NMR spectra indicated that Compound II was a linear hydrocarbon with an epoxide ring (δ_{H} , 2.90; δ_{C} , 58.19). The 200MHz ^1H -NMR data further showed two very close methyl resonances at δ 0.84 and δ 0.88. The methylene protons α to the epoxide ring resonated at δ 1.47 and gave the chemical shifts of the corresponding carbons at δ_{C} 27.42 and 28.65. The strong singlet at δ_{H} 2.25 and δ_{C} 30.52 corresponds to the long chain methylene protons and carbons respectively. The epoxide position in the aliphatic chain was determined using mass spectrometry. The presence of two fragments at m/z 155(10%) and m/z 309 (5%) arising from fragmentations adjacent to the oxygen bearing carbons, suggested that the epoxide was located on C-9 and C-10 of the chain.¹⁰The compound II may be 9,10 epoxynonacosane. The TLC for isolated compounds are tabulated in Table 1

Anticonvulsant activity**Effects of MERR on MES induced Epilepsy**

Phenytoin treated animals have shown 100% protection against MES induced seizures where as MERR 200mg/kg and 400 mg/kg have shown 54.35% and 67.31% protection respectively against MES induced seizures. The MERR at both doses and standard treated rats did not show any significant change in duration of tonic flexion and clonic convulsions. MERR 200 mg/kg and 400 mg/kg had shown a significant decrease in the duration of tonic extensor phase and comparable $p < 0.001$ with the standard. Results are shown in Table 2.

Serotonin

A significant $p < 0.001$ decrease in brain Serotonin levels was observed in forebrain of epileptic control animals.

Table 2: Effect of MERR on MES induced epilepsy

Groups	% Protection	Flexion	Extensor	Clonus	Stupor	Recovery
I. Control (CMC)	0	6.87 \pm 0.41	13.89 \pm 0.81	9.89 \pm 0.52	16 \pm 0.32	190.82
II. PHT	100	5.27 \pm 0.38	0	9 \pm 0.32**	12.17 \pm 0.58***	109.34
III. MERR 200	51.69	6.18 \pm 0.35 ^{ns}	6.28 \pm 0.61***	10 \pm 0.43 ^{ns}	15.98 \pm 0.47 ^{ns}	147.35
IV. MERR 400	61	5.87 \pm 0.37***	5.07 \pm 0.37***	8.78 \pm 0.55 ^{ns}	14.68 \pm 0.57 ^{ns}	122.81

Values are expressed as mean \pm SEM of six observations. Comparison between Group I Vs Group II, Group II Vs Group III & Group IV. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns-non significant. Values expressed in seconds.

Table 3: The effect of MERR on Neurotransmitter levels in MES induced epilepsy

Groups	Drug used	Serotonin (ng/mg)	Nor adrenaline (ng/mg)	Dopamine (ng/mg)
I	Control	181.33 \pm 1.84	718.3 \pm 2.50	813.3 \pm 0.84
II	MES	77.83 \pm 1.34a***	440.16 \pm 1.02a***	478.16 \pm 1.29a***
III	PHT	98.16 \pm 0.9b**	597.66 \pm 1.90b***	810 \pm 1.12b***
IV	MERR 200	84.5 \pm 1.73b***	490.83 \pm 3.17b**	626.66 \pm 3.45b***
V	MERR 400	88.83 \pm 1.02b***	520.3 \pm 2.52b***	710.3 \pm 2.71b***

Values are expressed as mean \pm SEM of six observations. Comparison between Group I Vs Group II, Group II Vs Group III Group IV & Group V. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns-non significant. Units = ng/mg of wet tissue.

MERR 200mg/kg, 400 mg/kg and PHT treated rats have shown a significant ($p < 0.001$) increase in Serotonin levels in forebrain.

Noradrenaline

A significant $p < 0.001$ decrease is observed in forebrain in epileptic control animals. MERR 200mg/kg, 400 mg/kg and PHT treated animals showed a significant $p < 0.01$ & $p < 0.001$ increase in Noradrenaline levels in forebrain of MERR treated animals.

Dopamine

A significant $p < 0.001$ decrease in the dopamine levels is observed in forebrain in epileptic control animals and a significant $p < 0.001$ increase is observed in forebrain on MERR 200mg/kg, 400 mg/kg and PHT treated animals showed a significant $p < 0.001$ increase forebrain. Results are shown in Table 3

DISCUSSION

The compounds isolated from methanolic extract of *Rubus racemosus* was found to be **Compound I - 1-[20, 21 - dodeacylnanone] - α -1 \rightarrow 6-D-glucotetraose-6'''' (P-hydroxy) benzoate. Compound II-9, 10 epoxynonacosane**. The R_f values of the isolated compounds were found to be 0.62, 0.59. In epilepsy, normal pattern of neuronal activity becomes disturbed briefly when the nerves in the brain "Fire" spontaneously causing strange sensations, emotions, behaviours and often times seizures with muscle spasms as well as loss of consciousness. It has been reported to increase the brain levels of Dopamine and Noradrenaline which causes an inhibition of seizure activity. MES induced epilepsy was altering the levels of monoamines like noradrenaline, serotonin, dopamine. It is found that treatment with MERR on rats significantly reduces in tonic hind limb extensor stage in MES induced epilepsy and markedly protects epilepsy induced by MES by increasing levels of monoamines. From the observations of the studies performed it could be predicted that the

MERR at both 200 mg/kg and 400 mg/kg exhibited significant anti-epileptic activity.

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