

## Original Research Article

# Anticonvulsant, Antimicrobial and Cytotoxic Activities of *Berberis calliobotrys* Aitch ex Koehne (Berberidaceae)

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## Abstract

**Purpose:** To evaluate the anticonvulsant, antimicrobial and cytotoxic activities of *Berberis calliobotrys*.

**Methods:** The powdered plant material (10 kg) was extracted thrice with methanol (3 × 12 L) by dipping for seven days. The methanol extract was concentrated to dryness under reduced pressure, and then successively fractionated with solvents of different polarity, including n-hexane, chloroform, ethyl acetate and n-butanol. The anticonvulsant effect of the extract and fractions (at oral doses 500 and 1000 mg/kg) was studied against picrotoxin-, pentylenetetrazole (PTZ)- and strychnine-induced seizures in Swiss albino mice of either sex divided into 12 groups (n = 6). Diazepam was used as standard drug. Antimicrobial activity of the extract against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* as well as against *Candida albicans*, *Penicillium notatum* was conducted by disc diffusion method and minimum inhibitory concentration (MIC). Cytotoxicity of the extract/fractions was analyzed by haemolytic method while the phenolic compounds present in the ethyl acetate fraction of the plant were determined by high performance liquid chromatography (HPLC).

**Results:** The extract and its ethyl acetate and n-butanol fractions showed maximum response against drug-induced convulsions and provided 100 % protection to animals at both doses. They also showed zones of inhibition of 27.00 ± 2.51, and 22.00 ± 2.51 mm against all bacterial and fungal strains, respectively, especially *Staphylococcus aureus*. The methanol extract and ethyl acetate fraction also showed high MIC against all bacterial and fungal strains. Cytotoxicity data from hemolytic assay indicate that the extract/fractions are safe. The highest amount of phenolic found was chlorogenic acid (84.44 ± 0.06 ppm).

**Conclusion:** The plant is thus a potential source of new lead compounds for the development of new clinically effective anticonvulsant and antimicrobial compounds.

**Keywords:** *Berberis calliobotrys*, Anticonvulsant, Antimicrobial, Haemolytic, Phenolics, Chlorogenic acid

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## INTRODUCTION

*Berberis calliobotrys* Aitch. ex Koehne (Berberidaceae), commonly known as Choweng, is an important medicinal plant mostly found in

North Temperate regions, tropical mountains and South America [1].

This plant is used in various systems of medicines for treating different types of ailments. The fruits of the plant are crushed and boiled in

water and used for fever. A paste is prepared from the bark of the rhizome by mixing with equal weight of black pepper and butter and is applied locally for backache. Decoction of bark is used as a gargle for pharyngitis. This plant is also used in some liver infections and intestinal colitis [2].

An aporphine – benzyloquinoline alkaloid, khyberine has been reported in *Berberis calliobotrys* (*B. calliobotrys*) in about one part per million. A complete scheme for the biogenesis of khyberine was presented which described that it was formed by the condensation of two coclaurine units [3,4].

Western medicines became the main system of health care, but different communities still continue to benefit from the folk knowledge of the use of plants as medicines. Unfortunately, most of this knowledge base has never been documented and thus threatened to disappear with the rapid demographic changes. The extensive folklore uses of *B. calliobotrys* for the treatment of different diseases have led to ensure its pharmacological activities. In this paper, the anticonvulsant, antimicrobial and *in vitro* cytotoxic effects of this plant species were evaluated which might be utilized to standardize the plant for therapeutic purposes. The plant was also estimated for the presence of phenolic constituents by HPLC which might be responsible for these pharmacological activities.

## EXPERIMENTAL

### Collection and preparation of plant material

*B. calliobotrys* was collected from the hilly areas of Quetta, Balochistan, Pakistan. The specimen was further identified and authenticated by taxonomists, Prof Dr Rasool Bakhsh Tareen, Department of Botany, University of Baluchistan, Quetta and Prof Dr. Zaheer Ahmad Khan, Department of Botany, GC University, Lahore, Pakistan. Voucher specimen number 1912 was deposited in the Sultan Ayoub Herbarium, GC University Lahore for further reference. Stems and branches of plant were spread on laboratory tables and dried under the shade for seven days at room temperature. The plant was pulverized prior to use by using an electric mill. The powdered plant material (10 kg) was extracted thrice with methanol (3 × 12 L) by dipping for seven days. The methanol extract was concentrated to dryness under reduced pressure using a rotary evaporator (Heidolph, model Laborata 4000, Schwabach, Germany). The methanol extract was further fractioned using a

successive solvent extraction method with different polarity based solvents such as n-hexane, chloroform, ethyl acetate and n-butanol. After fractionation samples were concentrated to dryness under reduced pressure and stored in a refrigerator at 4 °C, until used for analysis [5].

### Preparation of test microorganisms

The pathogenic bacterial strains, namely, *Bacillus subtilis* (ATCC 6633) *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 12228) and fungal strains namely *Candida albicans* (ATCC 18804) and *Penicillium notatum* (ATCC 11709) were obtained from Musaji Adam and Sons, Rawalpindi, Pakistan and used for antibacterial and antifungal activity respectively. All the microbial strains were transferred to nutrient agar slants, then shifted to nutrient broth and stored at 4 °C until used.

### Animals

Healthy lab bred adult male/female Swiss albino mice (22-30 gm body weight) were used for experimental purposes. The animals were housed in metabolic animal cages and maintained at controlled room temperature of 25 ± 10 °C. All the animals had free access to food and water *ad libitum*. Treatment of experimental animals was performed according to protocols approved by the animal ethical committee of University College of Pharmacy, University of the Punjab, Lahore, Pakistan (vide reference no. 2009). The protocols, ethics and regulations governing the care and use of experimental animals in "Principles of laboratory animal care" were followed [6].

### Phytochemical screening

Different chemical tests were conducted, using reported methods, to check out the presence of various phytoconstituents from methanol extract and its fractions [7-9].

### Evaluation of anticonvulsant activity

The anticonvulsant effect of plant crude extract and its various fractions was performed according to method with minor modifications as described by Swinyard *et al* [10]. Swiss albino mice of either sex were randomly divided into 12 groups of 6 mice each: (1) negative control group with normal saline and Tween (10 mL/kg); (2) positive control group with diazepam (4 mg/kg); extract-treated groups (500 and 1000 mg/kg, as groups 3 and 4, respectively); n-butanol fraction-treated groups (500 and 1000 mg/kg, as groups 5 and 6 respectively); ethyl acetate fraction-

treated groups (500 and 1000 mg/kg, as groups 7 and 8 respectively); chloroform fraction-treated groups (500 and 1000 mg/kg, as groups 9 and 10, respectively); and n-hexane fraction-treated groups (500 and 1000 mg/kg, as groups 11 and 12, respectively). All the animals were fasted for 24 h before the start of the experiment, but were granted free access to water. Treatment was oral, 30 min prior to administration of convulsion-inducing drugs, i.e., PTZ (80 mg/kg), strychnine (2.5 mg/kg) and picrotoxin (10 mg/kg) intraperitoneally. The animals were placed individually in plastic boxes and observed immediately for a period of 30 min and after 24 h. The onset time of hind limb tonic extensions (HLTEs), duration of tonic convulsions and percent against mortality protection (mortality protection) were recorded.

#### **Determination of antimicrobial activity**

The antimicrobial assay for plant extract and fractions against different bacterial and fungal strains was conducted by disc diffusion method and minimum inhibitory concentration (MIC) [11].

#### **Disc diffusion**

Filter paper discs prepared from Whatman no. 1 having a diameter of 6 millimetres were loaded with 2 mg of dried extract per disc. Discs were dried and sterilized by using autoclave at 121 °C and 15 pound/sq. inch pressure for 15 min [12]. Thereafter, 0.1 mL of culture ( $3 \times 10^8$  cells/ml) according to McFarland standard was spread over Muller Hinton agar medium for antibacterial assay and Sabouraud dextrose liquid medium for antifungal assay. Filter paper discs were placed over the surface of the media with flamed forceps pressed gently to ensure contact. The plates were placed for 2 h in the refrigerator to ensure pre diffusion of the extract into the surface of the agar. The plates were incubated at 37 °C for 24 h and at 28 °C for 48 h against bacterial and fungal strains respectively. Commercial antibiotic ciprofloxacin and antifungal fluconazole were used as positive control. After the incubation period, antimicrobial activity was determined by measuring the zone of inhibition (mm), i.e., the diameter of the paper disc plus the diameter of the zone [11,12].

#### **Minimum inhibitory concentration (MIC)**

Broth micro-dilution method was used for the determination of MIC with some modifications of the procedure as adopted by Sarker *et al* [13]. For this purpose, sterilized 96-microtitre well plates were used. A stock solution of methanol extract and all the fractions was prepared up to

20 mg/mL. From the stock solution, a volume of 50 µL of extract and all fractions was added into the first well of the plate. A volume of 50 µL of nutrient broth was added in the first well of the plate. After mixing, 50 µL of mixture from the first well was transferred to the next well and mixed with 50 µL of broth. Similarly, this serial dilution procedure was repeated up to the last well and then 50 µL was discarded. The tip of micropipette was discarded after single use. Then, 10 µL of resazurin indicator solution (270 mg in 40 ml sterilized distilled water) was added to each well. Finally, 10 µL of inoculum (microorganism suspension) was added to all wells except the last well, which was taken as negative control (extract and medium). Positive control for bacterial growth was also included without extract and medium. The plates were incubated at 37 °C for 24 h for bacterial and at 28 °C for 48 h for fungal strains. After incubation, the change in colour was observed and well numbers were noted. A colour change from purple to pink was considered as positive. The lowest concentration at which colour change occurred was taken as MIC value.

#### **Evaluation of *in vitro* cytotoxic activity**

The cytotoxicity of the plant crude extract and its subsequent fractions was analyzed by the haemolytic method as described by Sharma and Aslam with minor modifications [14,15]. A solution of plant extract and each fraction was prepared at a concentration of 1 mg/ml in DMSO (dimethyl sulfoxide) solution. A volume of 3 mL of heparinized human blood (freshly obtained) was poured into 15 mL polystyrene tube and centrifuged for 5 min. The supernatant obtained was poured off and RBCs were washed with 5 mL of chilled (4 °C) sterile isotonic phosphate-buffered saline (PBS) solution thrice and its pH is adjusted to 7.4. The cells were then counted on a haemocytometer. The erythrocyte count was maintained to  $7.068 \times 10^8$  cells/mL for each test. An aliquot of 20 µL of plant extract and fractions was taken in 2.0 mL eppendorff's tubes aseptically and 180 µL of diluted RBC suspension was added to them. 0.1 % Triton X-100 was used as positive control and PBS as negative control. The samples were incubated at 37 °C for 35 min. After incubation the tubes were immediately placed on ice for 5 min and then centrifuged for 5 min. 100 µL of supernatant obtained after centrifugation was diluted with 900 µL of chilled PBS solution in eppendorff's tubes. The tubes were then maintained on water ice. After this, 200 µL mixture from each tube was transferred in 96 well microtitre plates. Three replicates were taken, including one positive and one negative. Absorbance was measured at 576

nm using BioTek,  $\mu$  Quant<sup>TM</sup> instrument (BioTeck, Winooski, VT, USA). The % lysis of RBCs was calculated as in Eq 1.

$$\text{RBC lysis (\%)} = (\text{As}/\text{At})100 \dots\dots\dots (1)$$

where As and At are the absorbance of sample and triton X-100, respectively

**HPLC analysis**

Phenolic compounds present in the ethyl acetate fraction of the plant were estimated by HPLC using a procedure with some modification as mentioned by Sultana [16].

The sample for HPLC analysis was prepared by mixing small amounts of plant ethyl acetate fraction in 5 ml of distilled water. Thereafter, 12 mL of methanol was added to it. After shaking 6 mL of distilled water was added and left for 5 min. Finally 10 mL of 15 M HCl was added to it and kept in oven at 90 °C for 2 h. The solution was filtered using syringe filter and analyzed for the presence of phenolics.

The analysis of phenolics was performed by using HPLC (model LC-10AT, Shimadzu, Japan) equipped with two LC-10 AS pumps, SCL-10A system control unit, Rheodyne injector, CTO-10A column oven, SPD-10AV, UV-VIS detector and data acquisition class CSW32 software. The 20  $\mu$ L volume of the filtered sample was injected into an analytical column (Shim-Pak CLC-ODS, 25 cm  $\times$  4.6 mm, 5  $\mu$ m). The mobile phase used was gradient; (a) water: Acetic acid 94: 6, (b) Acetonitrile 100 %. The detection was carried out at 280 nm. The identification was made by

external standard methods by comparing with the known standard used in the same conditions.

**Statistical analysis**

The results are presented as mean  $\pm$  SEM. Statistical significance between the groups was analyzed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.  $P < 0.05$  was considered as significant.

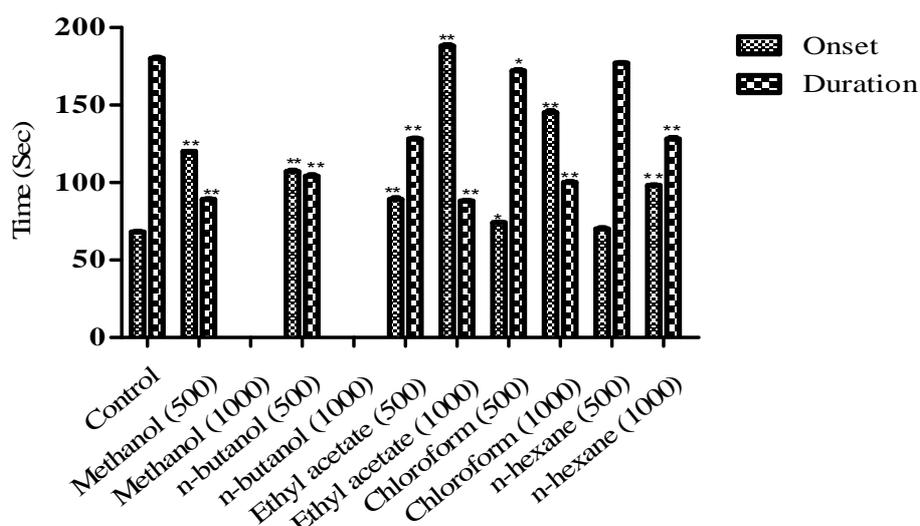
**RESULTS**

**Phytochemical profile**

The qualitative phytochemical analysis showed the occurrence of different chemical constituents like alkaloids, glycosides, tannins, saponins, terpenoids, fats, phenols and flavonoids in methanol extract and its various fractions.

**Anticonvulsant activity**

The results of PTZ induced convulsions are depicted in Figure 1. The methanol extract and n-butanol fraction of *B. calliobotrys* showed maximum protection (100 %) to the animals against PTZ-induced convulsions at both doses. However, at the dose of 1000 mg/kg they provided result compared with the standard drug diazepam. The n-hexane fraction at a dose of 500 mg/kg did not produce any significant delay in the onset of convulsions and also duration of convulsions however, some protection was provided to animals against death.



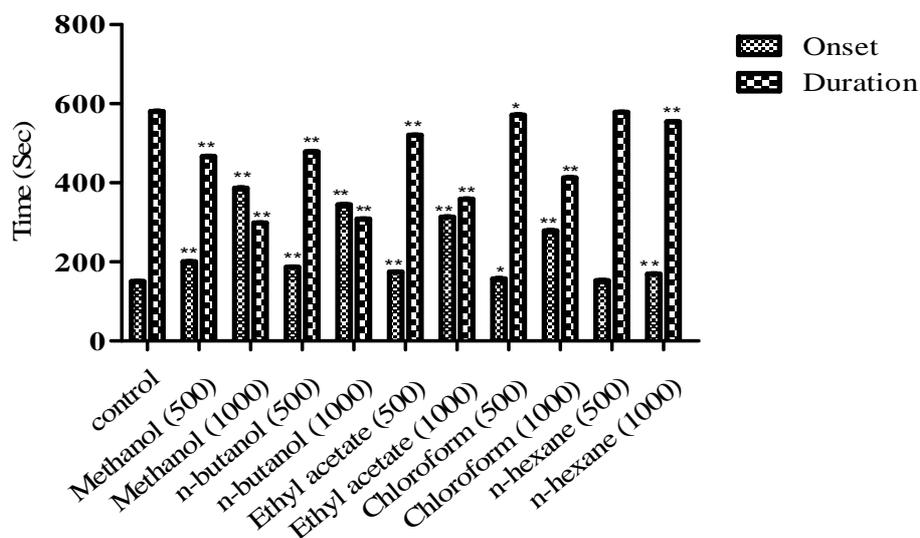
**Figure 1:** Anticonvulsant effect of *B. calliobotrys* extract and fractions against PTZ induced convulsions. Values are expressed as Mean  $\pm$  SEM (n = 6); \* $p < 0.05$ , \*\* $p < 0.01$  when compared with control

The results of strychnine induced convulsions are shown in Figure 2. All the fractions and methanol extract of the plant significantly delayed the onset time and reduces the duration of convulsions except n-hexane fraction at the dose of 500 mg/kg. However, chloroform and n-hexane fraction provides no protection to animals against mortality.

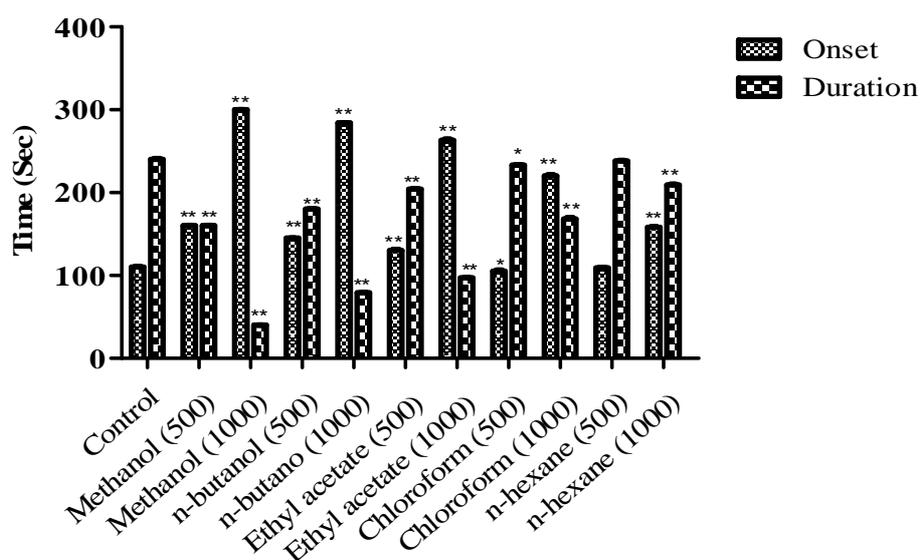
The results of picrotoxin induced convulsions are given in Figure 3. All the fractions and methanol extract produced highly significant anticonvulsant effects against picrotoxin induced convulsions except n-hexane fraction.

### Antimicrobial activity

All the fractions and methanol extract of *B. calliobotrys* were evaluated for their antibacterial activity by disc diffusion method against *P. aeruginosa*, *S. aureus* and *B. subtilis* and their results were compared against standard antibacterial agent ciprofloxacin. An Inhibition zone of 14 mm was considered as significant. The methanol extract of the plant, ethyl acetate and n-butanol fractions showed maximum zone of inhibition against all bacterial strains especially *S. aureus*.



**Figure 2:** Anticonvulsant effect of *B. calliobotrys* extract and fractions against strychnine induced convulsions. Values are expressed as Mean  $\pm$  SEM (n = 6); \*p < 0.05, \*\*p < 0.01 when compared with control



**Figure 3:** Anticonvulsant effect of *B. calliobotrys* extract and fractions against picrotoxin induced convulsions. Values are expressed as Mean  $\pm$  SEM (n = 6); \*p < 0.05, \*\*p < 0.01 when compared with control

Chloroform and n-hexane fractions, however, did not show any promising results (Table 1).

All the fractions of the plant showed antifungal effects when compared with the standard antifungal fluconazole, however the results were not significant with n-hexane and chloroform fractions. The ethyl acetate, n-butanol fractions and methanolic extract significantly produced antifungal effects; however the results were not as promising as antibacterial effects (Table 1).

MIC value was also determined against bacterial and fungal strains. The methanol extract and ethyl acetate fraction showed highly significant MIC against all bacterial and fungal strains. However, n-hexane fraction did not show any significant results in the MIC assay (Table 1).

### **In vitro cytotoxic effect**

To evaluate the cytotoxicity of the selected medicinal plant extract and its various organic fractions, the hemolytic activity method was used against human red blood cells (RBCs). The highest hemolytic effect was shown by methanol extract (8.96 %) followed by the n - butanol fraction (6.32 %), ethyl acetate (4.80 %), and n-hexane fraction (3.05 %). Chloroform fraction showed the least hemolytic effect. However, the data obtained is in a safe range and the plant extracts and fractions may be safe for human use.

### **HPLC data**

Ethyl acetate fraction of *B. calliobotrys* showed significant results for anticonvulsant and antimicrobial activities and so it was subjected to HPLC analysis to ascertain its phenolic content. The chromatogram is shown in Fig 4.

The phenolics (ppm) in the ethyl acetate fraction was quercetin ( $4.45 \pm 0.02$ ), gallic acid ( $2.83 \pm 0.04$ ), caffeic acid ( $3.26 \pm 0.03$ ), vanillic acid ( $40.23 \pm 0.03$ ), chlorogenic acid ( $84.44 \pm 0.06$ ), P-coumeric acid ( $13.21 \pm 0.04$ ) and trans-4-hydroxyl, 3-methoxy cinnamic acid ( $3.11 \pm 0.02$ ).

## **DISCUSSION**

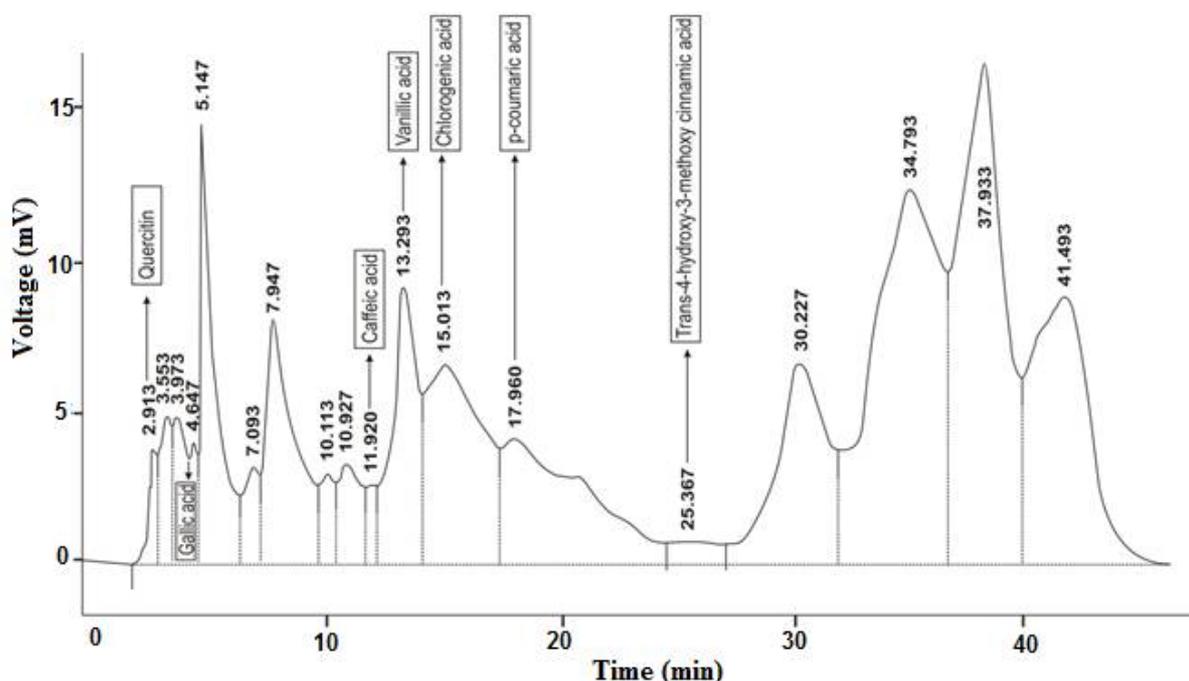
Herbal drugs have gained massive importance during the last few decades and there has been an ever increasing demand for these drugs due to their various therapeutic benefits. In the current study pharmacological activities, cytotoxicity and HPLC profile of *B. calliobotrys* were evaluated.

Among many experimental models used for induction of convulsions, pentylenetetrazole (PTZ) has been most frequently employed for preliminary screening of anticonvulsant effect. PTZ produces petit mal type of epilepsy and convulsions in experimental animals by counteracting the action of inhibitory neurotransmitter GABA and thus producing hyper excitation and excessive neuronal firing, which leads to the development of epilepsy [17]. PTZ also produces convulsions by destabilizing neuronal membranes [18,19]. The results of the present study have clearly shown potent anticonvulsant activity of plant methanolic extract and its n-butanol and ethyl acetate fractions. The tested fractions have been found to exert anticonvulsant effect due to their potent ability to increase the time of onset of convulsions and to decrease the time of duration of convulsions caused by administration of PTZ (Figure 1). The strong anticonvulsant action of *B. calliobotrys* against PTZ-induced convulsions might be due to their action as a GABA agonist [20].

**Table 1:** Antimicrobial activity of methanol extract and fractions of *B. calliobotrys*

Test microorganism	Diameter of inhibition zone (mm)						
	<i>Methanol</i>	<i>n-butanol</i>	<i>Ethyl-acetate</i>	<i>Chloroform</i>	<i>n-hexane</i>	<i>Cipro-floxacin</i>	<i>Fluconazole</i>
<i>P. aeruginosa</i>	20±3	20±1.52	16±1.52	12±2.08	11±2	36±20	
<i>S. aureus</i>	27±2.51	22±2.51	22±2.51	18±2	15±2	35±10	
<i>B. subtilis</i>	18±1.52	16±1	13±1.52	11±1.52	9±1.52	30±2.51	
<i>P. notatum</i>	13±1.52	11±1.52	13±2	4±2.12	3±1.52		19±1.52
<i>C. albicans</i>	16±1.52	10±2.08	13±2.08	6±1.52	3±1.52		20±1.52
	<b>Minimum Inhibitory Concentration (mg/mL)</b>						
<i>P. aeruginosa</i>	2.06	2.52	2.32	2.52	2.80	0.07	
<i>S. aureus</i>	2.05	N.A	2.42	2.35	N.A	0.02	
<i>B. subtilis</i>	2.08	2.72	2.50	2.57	2.75	0.06	
<i>P. notatum</i>	2.12	N.A	2.18	N.A	2.92		0.10
<i>C. albicans</i>	2.50	2.90	2.30	N.A	N.A		0.01

Values are expressed as Mean  $\pm$  SEM (n = 3); \*p < 0.05, when compared with control; N.A= no activity



**Figure 4:** Chromatogram showing the phenolic profile of ethyl acetate fraction of *B. calliobotrys*

In addition, strychnine, another very potent convulsant has been shown to act on the spinal cord and produce an inhibitory effect on the inhibitory neurotransmitter glycine, which produces hyper excitability in CNS and ultimately convulsions [21]. Strychnine counteracts the action of excitatory neurotransmitters like AMPA and NMDA and it also produces inhibitory effects on calcium-induced excitotoxicity in neurons. These factors are pro-epileptogenic and precipitate seizures. So, the suppression of the inhibitory neurotransmitter, glycine leads to tonic-clonic seizures in experimental animals [22]. In our study, methanolic extract, n-butanol and ethyl acetate fractions showed anticonvulsant activity against strychnine-induced convulsions in a dose-dependent manner. However, the effect was relatively more potent against PTZ-induced convulsions (Figure 2). The anticonvulsant effect of plant against strychnine-induced convulsions has indicated their ability to interfere with glycine transmission in the spinal cord.

Furthermore, picrotoxin, a non-competitive antagonist at GABA receptors, has been reported to block the GABA-activated chloride ionophore [23]. Methanolic extract, n-butanol and ethyl acetate fraction have also demonstrated anticonvulsant activity against picrotoxin induced convulsions (Figure 3). This also indicated that the plant extract and its various fractions exert their anticonvulsant effect due to modification of function of GABA receptor-mediated chloride channels. The results of different studies have

provided evidence that some medicinal plants do possess antimicrobial activities and may act as a potential source of new antibacterial agents even against some antibiotic-resistant strains [24]. In the present study conducted by using the disc diffusion method, it has been observed that the extract and fractions of plant possessed significant antibacterial activity against certain gram negative (*P. aeruginosa*) and gram positive (*S. aureus*, *B. subtilis*) pathogens, i.e. bacteria. The results of this study were in agreement with the earlier studies [25-27].

Antimicrobial activity of the methanolic extract and fractions of *B. calliobotrys* have also exposed noticeable difference in sensitivity against pathogenic fungi *P. notatum* and *C. albicans*. The variation in the effectiveness of the extracts against different microorganisms would be due to differences in the chemical composition of the extracts. In the disc diffusion technique lessening in microbial growth is determined on the basis of zone of inhibition around the disc. An inhibition zone of 14 mm or more has been reckoned to be a significant antimicrobial activity [28]. In our study high antimicrobial activity, i.e. (inhibition zone of 14 mm) was produced by some fractions of the plant.

The minimum inhibitory concentration (MIC) results showed that methanol extract and ethyl acetate fraction had highly significant MIC against all bacterial and fungal strains. The least

MIC value exhibited that less concentration was required to inhibit the selected bacterial and fungal strains. The higher MIC represented that the tested samples had less antimicrobial activity.

The differences in anticonvulsant and antimicrobial activities of plant extract and its various fractions were possibly due to different concentration of their constituents. The phytochemicals are produced in many plant species and exhibit pharmacological and biological activities. Antioxidant and antimicrobial activities were reported in various plants due to the presence of tannins, alkaloids, steroids, saponins and phenolic compounds [28,29]. Terpenoids are found in various plant parts having many therapeutic uses like antimicrobials and also used in many pharmaceutical preparations [30]. The analyzed phytoconstituents in this study might be responsible for their pharmacological actions. Ethyl acetate fraction exhibited very promising pharmacological effects, i.e. Anticonvulsant and antimicrobial. So, the phenolic constituents present in this fraction were estimated by HPLC which might be responsible for these actions. The earlier reports showed that the phenolic compounds present in various plant extracts play a vital role for their antimicrobial and other activities [31].

The mechanical stability of the red blood cell membrane is essential to reduce the cytotoxic effects of different compounds. The least in vitro cytotoxicity of different plant extracts is of great importance for their safe use in different diseases other than cancer [32]. In the present research all the samples tested for *in vitro* hemolytic activity were found in a safe range i.e. below 10.0 %, so it could be expected that the methanol extract and its various organic fractions have a minor cytotoxicity [33]. The plant extracts or fractions having minor cytotoxicity might be used as herbal medicines [34].

## CONCLUSION

The findings of this study indicate that the crude methanol extract and its organic fractions of *B. calliobotrys* possess strong anticonvulsant and antimicrobial effect. The plant is thus a potential source of new lead compounds for the development of new clinically effective anticonvulsant and antimicrobial compounds.

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