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Cytotoxic, antibacterial and analgesic activities of *Rhaphidophora glauca* (Wall.) Schott leaves

Mir Muhammad Nasir Uddin[1*], Raju Dash[2], Mohammad Shah Hafez Kabir[3], Md. Mominur Rahman[3], Mohammad Nazmul Islam[3], Arkajyoti Paul[2]

[1]*Department of Pharmacy, University of Chittagong, Chittagong-4331, Bangladesh*

[2]*Department of Pharmacy, BGC Trust University Bangladesh, Chittagong-4000, Bangladesh*

[3]*Department of Pharmacy, International Islamic University Chittagong, Chittagong-4203, Bangladesh*

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ABSTRACT

Objective: To investigation of cytotoxic, antimicrobial and analgesic activities of different fractions of *Rhaphidophora glauca* (Wall.) Schott.

Methods: Two partially purified aqueous methanolic fractions from ethyl acetate extract (AMF-1) and chloroform extract (AMF-2) obtained from the partitioning were used in study. The cytotoxic effect was determined by brine shrimp lethality bioassay. Antibacterial activity was investigated by disc diffusion and minimal inhibitory concentration methods. Hot plate method and acetic acid test was used for determining analgesic activity.

Results: The LC_{50} values of AMF-1 and AMF-2 were found to be 287.73 and 428.54 μg/mL respectively, where colchicines showed LC_{50} of 11.16 μg/mL. The zone of inhibition of the fractions AMF-1 and AMF-2 was found to be in the range of 8–26 mm in 2000 μg/disc, as compared to reference antibiotics kanamycin (11–28 mm at 30 μg/disc) and ciprofloxacin (20–25 mm at 30 μg/disc) indicating the antibacterial activity. In hot plate test, the highest pain inhibitory activity was found at a dose of 250 mg/kg for AMF-1 which was statistically significant ($P < 0.05$) compared to both positive and negative control at 30 min interval. In acid induced model, both AMF-1 and AMF-2 at a dose of 500 mg/kg showed significant activity compared to positive and negative control.

Conclusions: This study found that *Rhaphidophora glauca* possesses potential cytotoxic, antibacterial and analgesic activity. Further study may be needed to isolate the bioactive compounds responsible for different activities with subsequent mechanistic study.

1. Introduction

Plants have been considered as a rich supply of conventional medication for many years because they produced a wide variety of bioactive molecules, maximum of that have been advanced as pills for the remedy of numerous diseases. In lots of developing countries, conventional medication is one of the number one fitness concern systems[1,2]. Many ancient civilizations like Chinese medicine and Unani medicine have a confirm belief on treatment of plant medicine. Recent reviews indicate that approximately 13000 plant species universally are recognized to be already used as capsules. Any parts of a plant like bark, leaves, flora, roots, end result and seeds can be the prolific originators of the therapeutically active components which might also in the end affect the beneficial medicinal consequences[3]. Large-scale evaluation is a essential primary step for systemic isolation and identity of the lively principles of the nearby flora exploited in conventional medicine with the intention of discovery new medicine.

Cytotoxicity means the adverse effects which result from intervention with the configurations or processes vital for the survival and propagation of cells[4]. Bio-active compounds of

*Corresponding author: Mir Muhammad Nasir Uddin, Assistant Professor, Department of Pharmacy, Faculty of Biological Science, University of Chittagong, Chittagong-4331, Bangladesh.

Tel: +88 031 726311 14, ext. 4486, +88 01712725987

E-mail: nasirmir@cu.ac.bd

All experimental procedures involving animals were conducted in accordance to the guiding principles for research involving animals as recommended by the guidelines for laboratory animal care of the National Institute of Health (National Institute of Health publication No. 85–23, revised 1996) and approved by the Ethical Review Committee, Faculty of Biological Science, University of Chittagong (AERB/FBS/UC/-9991, 2016).

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medicinal plants are the good resource of anticancer research[5].

Nowadays, the increasing bacterial resistance against antibiotics has become a concern[6]. Expanding bacterial resistance is encouraging to analyze the antimicrobial task of herbs against resistant bacteria. A huge range of medicinal plants have been documented as important resource of natural antimicrobial compounds[7,8]. Extract of medicinal plants helps to develop a new effective agent against bacterial resistance[9,10].

Pain is an undesired and emotional experience due to the injury of tissue[11]. Drugs that are used currently for the managing of pain are either steroidal like corticosteroids or non steroidal like aspirin. All of those drugs possess more or less toxic effect like renal failure, kidney failure, allergic reaction, *etc*.[12,13]. The extract of plant which contains effective analgesic compound helps to discover a new drug without any toxic effects[14].

Rhaphidophora glauca (Wall.) Schott (Araceae) (*R. glauca*) grows in Alutila and Khagrachori, Bangladesh. Literature confirmed that it also occurs in Himalaya, Nepal, Moulvibazar (Sylhet, Bangladesh) *etc*. *R. glauca* enlisted as "new plant species and file of Bangladesh", which is published with the aid of the Bangladesh National Herbarium, Ministry of Surroundings and Forest, Authorities of Bangladesh. The conventional plant has as a substitute small leaves (30 cm long or less) with between two and five pinnae and has been used as a traditional medicine in Nepal, India, Myanmar, Thailand and Vietnam. To our best knowledge, the pharmacological properties of this plant have not been explored yet[15].

In this study, we aimed to investigate the cytotoxic, antimicrobial and analgesic activities of two partially purified fractions of *R. glauca*.

2. Materials and methods

2.1. Plant material collection

Leaves of *R. glauca* were collected from Alutila, Khagrachari and Chittagong, Bangladesh in September, 2014. It was authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh. A specimen of the plant has been preserved in the National Herbarium with the Accession No. 30145.

2.2. Extract preparation and partial purification

Extraction of the air dried ground powder (300 g) previously defatted with *n*-hexane was successively carried out with chloroform and ethyl acetate. The extracts were evaporated to dryness in vacuum at 50 °C under reduced pressure. The crude chloroform and ethyl acetate extracts were partitioned using aqueous methanol (90% methanol + 10% water) and *n*-hexane to get the aqueous methanolic fractions from ethyl acetate extract (AMF-1) and from chloroform extract (AMF-2). *n*-Hexane was used to ensure complete removal of residual fatty and oily substances to produce partially purified fractions.

2.3. Cytotoxicity screening

The fractions AMF-1 and AMF-2 were investigated for cytotoxic activity by brine shrimp lethality bioassay method[16]. The eggs of brine shrimp were collected from Katabon market, Dhaka. For hatching the eggs, sea water prepared by dissolving 38 g NaCl in 1 L of distilled water was kept in a small tank, which was divided into two parts by a net. The eggs of the shrimp were placed into one part of the dividing tank. The other side of the tank containing a light source attracted the hatched shrimp through the perforation into it. The shrimps were hatched for 2 days and considered to be matured as nauplii. In this bioassay, the test compounds dissolved in distilled water were applied at a concentration of 25, 50, 100, 200, 400, 800 μg/mL. However, not more than 1 mL of distilled water was added to the nauplii in each vial. For each concentration, one vial containing the same volume of distilled water plus sea water was used as a negative control and colchicine was used as a positive control for its cytotoxic activity. After 24 h, the vials were observed for mortality or changes in behavior if any.

2.4. Antibacterial activity screening

Test organisms were collected from Medinova Diagnostic Center, Dhaka and University of Dhaka. The antibacterial activity was determined by disc diffusion technique[17]. Bacterial strains were grown on a nutrient agar at 37 °C for 24 h. The suspension was used to inoculate 90 mm diameter Petri dishes. Wells (diameter 6 mm) were punched into the agar and filled with 2 000 μg/disc extracts. Standard kanamycin disc (30 μg/disc) and ciprofloxacin disc (30 μg/disc) were used as positive control and wells filled with distilled water were used as negative control. The plates were incubated at 37 °C for 24 h. Antibacterial activities were evaluated by measuring the diameter of the zone of inhibition.

2.5. Determination of minimum inhibitory concentration (MIC)

MIC was determined by the spectrophotometric assay[18]. The dilute solutions of AMF-1 and AMF-2 were incubated with test culture to give a concentration of 500, 750, 1 000, 1 250 and 1 500 μg/mL. About 2 mL of each dilution in three replicates per dilution was applied to the wells. Control wells received 2 mL of the culture inoculated nutrient or tryptic soy broth. Optical density (OD) was determined in a spectrophotometer at 620 nm prior to incubation (T_0). After 18 h of incubation, wells were again read in a spectrophotometer at 620 nm (T_{18}). MIC values were evaluated in three distinct values, *viz*. MIC_0 (the highest bioactive compound concentration which results in no retardation of biomass growth),

MIC_{50} (the bioactive compound concentration which results in 50% retardation of biomass growth) and MIC_{100} (the lowest bioactive compound concentration which results in 100% retardation of biomass growth). Percent inhibition of bacterial growth was determined by the following formula:

Inhibition (%) = [1 – (OD test well/ OD of control well)] × 100

The concentration ranges of AMF-1 and AMF-2 used for microbes were 500, 750, 1 000, 1 250 and 1 500 µg/mL, as this is shown to be the concentration range where an inhibitory effect could be observed.

2.6. Analgesic activity testing

2.6.1. Experimental animals

Swiss-albino mice, aged 6–7 weeks old, with average weights of 25–30 g, were used in this study. They were acclimatized in standard environmental condition [temperature (23 ± 2) ˚C, humidity 55%] for 1 week in the animal house of the Department of Pharmacy, North South University for adaptation prior to the experiment. The mice were provided with standard laboratory food and maintained at natural day-night cycle. The design and the performance of this study have been approved by the Ethical Review Committee, Faculty of Biological Science, University of Chittagong through the submission of the research protocol before the study and got ethical permission which was given as AERB/FBS/UC/-9991, 2016.

2.6.2. Samples and doses

The fractions AMF-1 and AMF-2 were administered at doses of 250 and 500 mg/kg *p.o.* in the form of suspension prepared in water. The doses were selected based on the human dose mentioned in the Ayurvedic literature.

2.6.3. Hot plate method

The mice were divided into six groups with five mice in each group. Group I received vehicle (water 10 mL/kg body weight); Group II received ketorolac as a positive control at 2.5 mg/kg body weight while Groups III–VI received 250 and 500 mg/kg body weight (*p.o.*) of AMF-1 and AMF-2, respectively. The mice were placed on Eddy's hot plate kept at a temperature of (55.0 ± 0.5) ˚C. A cut-off period of 20 s was observed to avoid damage to the paw[19]. Reaction time was recorded when mice licked their fore or hind paws or jumped prior to at 0, 30, 60 and 120 min after oral administration of the samples[20]. After reading the mean reaction time, percent (%) inhibition of pain by each sample was calculated according to the following formula:

Inhibition (%) = [(Post-drug latency – Pre-drug latency)/(Cut-off time – Pre-drug latency)] × 100

2.6.4. Acetic acid-induced writhing test

Acetic acid-induced writhing test was performed to evaluate the peripheral analgesic activity of AMF-1 and AMF-2 in chemical-induced pain. The mice were treated with standard drug or AMF-1/2 and then the writhing was induced by injecting 0.6% acetic acid after 15 and 30 min, respectively, at the dose of 10 mL/kg body weight. Five minutes after the injection of acetic acid, the mice were observed and the number of writhing was counted for 30 min[21]. The contractions of the abdomen, elongation of the body, twisting of the trunk and/or pelvis ending with the extension of the limbs were considered as complete writhing.

Inhibition (%) = [(Test – Control)/Control] × 100

2.7. Statistical analysis

Statistical analysis was carried out with One-way ANOVA using the statistical software SPSS (version 19.0, IBM Corporation, Somers, NY, USA) for cytotoxicity and antimicrobial screening. The results obtained were compared with the negative control group. Values were expressed as mean ± SEM and $P < 0.05$ was considered to be statistically significant. Tukey multiple comparison test with $P < 0.05$ was taken as significant (GraphPad Prism 5.0) for analgesic activity.

3. Results

3.1. Cytotoxic activity

The cytotoxic effects of AMF-1 and AMF-2 are given in Table 1. LC_{50} values of the samples were determined by plotting the percent mortality of shrimp against log concentration of the sample. Both the fractions showed cytotoxic activity against brine shrimp and the LC_{50} values of AMF-1 and AMF-2 were found to be 287.73 and 428.54 µg/mL.

Table 1
Cytotoxic activity of AMF-1 and AMF-2 against *Artemia salina*.

Test sample	Concentration of sample (µg/mL)	LC_{50} (µg/mL)
AMF-1	800:400:200:100:50:25	287.73
AMF-2	800:400:200:100:50:25	428.54
Colchicine	800:400:200:100:50:25	11.16

3.2. Antibacterial activity

The antibacterial activity of fractions, AMF-1 and AMF-2 were tested against 16 pathogenic bacteria and exhibited a significant antibacterial activity against both Gram-positive and Gram-negative bacteria at the concentration of 2 000 µg/disc, which is shown in Table 2. The inhibitory activities showed by the test samples were compared with standard broad spectrum antibiotic kanamycin (30 µg/disc) and ciprofloxacin (30 µg/disc). The zone of inhibition produced by AMF-1 against Gram-positive bacteria were found to be 13–26 mm and against Gram-negative bacteria were found to be 8–14 mm. AMF-2 produced zone of inhibition against Gram-positive bacteria in the range of 8–20 mm and against Gram-negative bacteria in range of 7–11 mm. On the

other hand, kanamycin showed a zone of inhibition against Gram-positive bacteria in the range of 11–28 mm and against Gram-negative bacteria in the range of 13–27 mm. Ciprofloxacin showed a zone of inhibition against Gram-positive bacteria in the range of 23–25 mm and against Gram-negative bacteria were found to be 20–25 mm.

MIC values of the test organisms were determined by plotting the percent inhibition of growth *vs*. log concentration of the fractions. From the plotted results MIC values of AMF-1 for each organism were determined, which is shown in Table 3. All bacterial species demonstrated a near linear relationship between fractions concentration and percent inhibition. For example, *Bacillus cereus* in both fractions (AMF-1 and AMF-2) demonstrated a near-linear relationship between fractions concentration and percent inhibition, with the regression analysis (SPSS 19.0) giving an *R*-value of 0.964 and 0.930, respectively.

Table 2

Results of antibacterial activity testing of AMF-1 and AMF-2.

Bacterial strain	Zone of inhibition (mm)			
	AMF-1	AMF-2	Kanamycin	Ciprofloxacin
Gram positive strain				
Bacillus cereus	20	18	25	23
Bacillus subtilis	15	12	18	24
Staphylococcus aureus	15	18	22	25
Streptococcus pyogens	13	8	11	Nd
Enterococcus faecalis	26	20	18	Nd
Streptococcus agalactiae	20	10	15	Nd
Sarcina lutea	18	20	28	25
Gram-negative strain				
Salmonella typhi	13	9	25	23
Salmonella paratyphi	10	–	19	20
Pseudomonas aeruginosa	9	10	27	25
Escherichia coli	11	9	25	23
Klebsiella	8	7	13	Nd
Shigella dysenteriae	14	11	25	23

–: No zone of inhibition; Nd: Not determined.

3.3. Analgesic activity

In this study, we have demonstrated the effect of both AMF-1 and AMF-2 fractions (250 and 500 mg/kg) on hot plate test and the result is shown in Table 4. Both fractions (250 and 500 mg/kg) showed significant ($P < 0.05$) increase in mean reaction time in a dose-dependent manner when compared with negative control.

The fraction AMF-1 at 250 mg/kg produced a statistically significant ($P < 0.05$) increase in the pain threshold, after 30 min of drug administration as compared to control. On the other hand, at 500 mg/kg dose, the mean reaction time also increased significantly compared to positive control. The effect or activity was rather low but enough for treatment or blocking the pain. This activity was comparable to that of ketorolac (2.5 mg/kg). The analgesic activity of fraction AMF-1 was increased until the 60 min. At 60 min, fraction AMF-1 at both dose (250 and 500 mg/kg) produced a statistically significant increase ($P < 0.05$) in the pain threshold after 30 min of drug administration as compared to the negative control. The increase of mean reaction time at 60 min, at dose of 250 mg/kg, was similar to ketorolac (2.5 mg/kg). However, at 90 and 120 min, fraction AMF-1, at dose 500 mg/kg produced a statistically significant increase ($P < 0.05$) in the pain threshold after 30 min of the drug taken as compared to the negative control.

On one hand, the fraction AMF-2 at dose 250 mg/kg increased mean reaction time as compared to control group, which was statistically significant. On the other hand, at dose 500 mg/kg, it was not statistically significant ($P < 0.05$) after 30 min of drug administration as compared with the negative control. This activity is lower than positive control (ketorolac 2.5 mg/kg), but it is enough for inhibition of pain. At 60 min, both doses increased the mean reaction time, which was statistically significant ($P < 0.05$) as compared to control group. At 90 and 120 min, fraction AMF-2 at both dose (250 and 500 mg/kg) produced a statistically significant increase ($P < 0.05$) in the pain threshold after 30 min of drug administration as compared to the control.

The AMF-1 at 250 and 500 mg/kg dose showed 50.50% and 16.92% inhibition of pain at 30 min respectively. However, both doses showed an increase in inhibition as compared to ketorolac (37.68%). At 60 min, both doses showed 57.88% and 66.17% inhibition, respectively, which was also higher than ketorolac (56.08% inhibition). However, at 60 min AMF-1 showed the higher percent of inhibition at 500 mg/kg dose. The AMF-2 at the dose of 250 and 500 mg/kg showed 38.27% and 16.16% inhibition of pain

Table 3

The concentration of AMF-1 and AMF-2 required in growth medium to inhibit the growth of bacteria in spectrophotometric assay tested over an 18 h incubation period.

Bacterial strain	MIC_0 (μg/mL)		MIC_{50} (μg/mL)		MIC_{100} (μg/mL)		*R* value	
	AMF-1	AMF-2	AMF-1	AMF-2	AMF-1	AMF-2	AMF-1	AMF-2
Bacillus cereus	22.10	–	630.95	478.63	630.95	1 109.17	0.964*	0.930**
Bacillus subtilis	478.73	501.18	868.96	1 023.20	868.96	1 603.24	0.983*	0.978**
Staphylococcus aureus	–	–	467.73	524.80	467.73	1 348.90	0.918*	0.978**
Sarcina lutea	–	–	489.77	467.73	489.77	1 380.38	0.913*	0.994**
Salmonella typhi	104.11	104.71	758.57	860.99	758.57	1 603.24	0.993*	0.990**
Salmonella paratyphi	478.63	–	870.66	–	870.66	–	0.995*	–
Pseudomonas aeruginosa	104.11	104.11	776.24	758.57	776.24	1 610.64	0.994*	0.983**
Escherichia coli	22.13	446.68	630.95	860.99	630.95	1 603.95	0.966*	0.996**
Shigella dysenteriae	22.18	21.87	707.94	724.43	707.94	1 348.96	0.984*	0.986**
Shigella boydii	100.00	–	794.32	524.80	794.32	860.99	0.998*	0.902**

*: Correlation is significant at $P < 0.05$ level; **: Correlation is significant at $P < 0.01$ level.

Table 4
Antinociceptive effect of *R. glauca* extracts and ketorolac in hot plate test.

Treatment	Pretreatment	30 min	60 min	90 min	120 min
Control	4.58 ± 0.89	6.93 ± 0.57 (15.24%)	7.67 ± 0.51 (20.04%)	6.07 ± 0.35 (9.66%)	5.70 ± 0.28 (7.26%)
Positive control	7.66 ± 0.66	12.31 ± 0.60 (37.68%)	14.58 ± 0.62 (56.08%)	12.83 ± 0.41 (41.90%)	10.29 ± 0.34 (21.31%)
AMF-1 250 (mg/kg)	6.99 ± 0.97	13.56 ± 1.07[a] (50.50%)	14.52 ± 0.50[a] (57.88%)	12.84 ± 0.40[a] (44.97%)	9.30 ± 0.32[a] (17.76%)
AMF-1 500 (mg/kg)	5.28 ± 1.21	7.77 ± 0.24[b] (16.92%)	15.02 ± 0.55[a] (66.17%)	10.86 ± 0.39[a] (37.91%)	8.36 ± 0.27[a] (20.92%)
AMF-2 250 (mg/kg)	6.96 ± 0.33	11.95 ± 0.45[a] (38.27%)	13.20 ± 0.43[a] (47.85%)	10.52 ± 0.43[a] (27.30%)	8.37 ± 0.32[a] (10.81%)
AMF-2 500 (mg/kg)	7.56 ± 0.48	9.57 ± 0.38 (16.16%)	14.12 ± 0.41[a] (52.73%)	12.21 ± 0.45[a] (37.38%)	10.35 ± 0.32[a] (22.43%)

Values are expressed as mean ± SEM, n = 5. Values in parentheses shows the percent inhibition (%) of pain by each sample. [a]: $P < 0.001$ significantly different in comparison with control; [b]: $P<0.001$ significantly different in comparison with positive control. The data were analyzed by ANOVA followed by Tukey test.

at 30 min, respectively. However, both doses showed an increase in percent inhibition as compared to ketorolac (Table 4). At 60 min, both doses showed 47.85% and 52.73% inhibition, respectively, which was also lower than ketorolac (56.08% inhibition). Respectively the AMF-1 at 250 and 500 mg/kg dose showed 44.97% and 37.91% inhibition of pain at 90 min, which was also higher than ketorolac (41.90% inhibition). On the other hand, at 90 min the AMF-2 at the dose of 250 and 500 mg/kg showed 27.30% and 37.38% inhibition of pain. At 120 min, the AMF-1 at 250 and 500 mg/kg dose showed 17.76% and 20.92% inhibition of pain and the AMF-2 at 250 and 500 mg/kg dose showed 10.81% and 22.43% inhibition of pain , where only AMF-2 at 500 mg/kg dose was higher than ketorolac (21.31% inhibition).

3.4. Acetic acid-induced writhing test

In acetic acid-induced test, we have established the effect of both fractions (250 and 500 mg/kg) in Table 5. AMF-1 (250 and 500 mg/kg dose) and AMF-2 (250 and 500 mg/kg dose) were statistically significant after drug administration as compared to negative control. The AMF-1 at 250 and 500 mg/kg dose showed 54.35% and 67.25% inhibition of pain respectively. Only 500 mg/kg doses showed an increase in inhibition as compared to ketorolac (57.84%). However, The AMF-2 at 250 and 500 mg/kg dose showed 51.29% and 65.38% inhibition of pain, respectively. Besides, 500 mg/kg dose also showed an increase in inhibition as compared to ketorolac (57.84%). In comparison to positive control, significant activity was observed in both AMF-1 and AMF-2 at dose of 500 mg/kg (Table 5).

Table 5
Antinociceptive effect of *R. glauca* extracts and ketorolac in acetic acid test.

Group	Mean of writhes	Inhibition of writhing (%)
Control	55.55 ± 0.66	
Positive control	23.82 ± 0.86	57.84%
AFM-1 250 (mg/kg)	25.91 ± 0.47[a]	54.35%
AFM-1 500 (mg/kg)	17.13 ± 0.53[a,b]	67.25%
AFM-2 250 (mg/kg)	27.92 ± 0.68[a]	51.29%
AFM-2 500 (mg/kg)	19.69 ± 0.47[a,b]	65.38%

Values are expressed as mean ± SEM, n = 5. [a]: $P<0.001$ significantly different in comparison with control; [b]: $P<0.001$ significantly different in comparison with positive control. The data were analyzed by ANOVA followed by Tukey test.

4. Discussion

Phytomedicines contributed to the management of tropical and other diseases from ancient time. And also many present pharmaceutical drugs are discovered from plant sources. Our attempt is to identify whether *R. glauca* leaves have any biological effect or not, which is determined by *in vitro* and *in vivo* methods. From the results of cytotoxic test, it is cleared that both the fractions AMF-1 and AMF-2 exhibited cytotoxic effect, though their LC_{50} is low. These findings indicated the presence of bioactive compounds, which are responsible for cytotoxicity.

Crude from leaves extract contains bioactive compounds[22]. For studying antibacterial study, suitable bacterial strains were chosen as they are vital pathogen and quickly develop antibiotic resistance[23]. There are many methods available to determine antibacterial activity, like agar diffusion, disc diffusion, broth diffusion, or other variants. Among all these methods, disc diffusion method is the most preferable, particularly in the area of antibacterial effect testing of plant extract[24-26]. And recently, there has been an enhanced use of microtiter plate assays[27-30]. Disc diffusion method cannot be reliable in certain state, due to the subjectivity connected with visual determinations as well as time, sample and also cost implications[31,32]. On the other hand, the bacterial growth inhibition can be easily identified by single tube optical density measurement[33] and this technique is less time consuming and cheaper. The results, in our study, indicate that *R. glauca* possessed significant antibacterial effect against tested bacteria. This activity may be indicative of the presence of active compounds, which have antibacterial-like activity. Therefore, this plant extract should be analyzed further, because it might contain a number of unknown compounds that are effective against pathogens.

Thermal nociception models like hot plate and tail immersion tests were used for measuring central analgesic activity[34]. The central analgesic activity of *R. glauca* was studied using the hot plate method. The hot plate method is assumed to be selective to check compounds acting through the opioid receptor. The aqueous methanolic fractions AMF-1 and AMF-2 increase in mean reaction time as compared to negative control, which indicates that it may act via centrally mediated analgesic mechanism[35,36]. This type of pain stimulus results in release of free arachidonic acid from

tissue phospholipids[37]. In this method, the plant extract exhibited analgesic effect.

The squirming of mice, which is peripheral analgesic effect may be interceded throughout the inhibition of cyclooxygenases[38-40] and this acetic acid-induced squirming may be highly sensitive and useful test for analgesic drug development, specially peripherally acting analgesics. Acetic acid tempts pain by liberating endogenous substances (bradykinin, serotonin, histamine)[41] as well as some pain mediators like arachidonic acid via cyclooxygenase and prostaglandin biosynthesis[34,42] which in turn stimulate the pain nerve endings. The pain paradigm is used for the treatment of peripheral analgesic activity because of its compassion and reaction to the compounds at a dose which is not effective in alternative methods[43]. In our observation, aqueous methanolic fractions (AMF-1 and AMF-2) significantly ($P < 0.001$) reduced the abdominal constriction response which is induced by the acetic acid in a dose-dependent manner (Table 5). This result might be explained by the presence of active analgesic compounds in the aqueous methanolic fractions. Besides, this test is also useful for the assessment of mild analgesic non-steroidal, anti-inflammatory compounds[44,45]. This suggests a peripherally induced mechanism of the analgesic action of aqueous methanolic fractions of the *R. glauca*[45]. As a result, one possible mechanism of the analgesic activity by *R. glauca* might be attributable to the obstruction of the effect and might be due to the release of endogenous substances that excite pain nerve endings by the pharmacologically active compounds of *R. glauca* extract. From a mechanistic point of view, the lack of specificity in acetic acid-induced squirming check test suggests the association of various nociceptive mechanisms in the decrease of muscular constriction[46].

Narcotic analgesics hinder each peripheral and central mechanism of pain whereas non-steroidal anti-inflammatory drugs inhibit only peripheral pain[35]. The fractions inhibited both mechanisms of pain, suggesting that the plant extract may act as a narcotic analgesic.

The cytotoxic and analgesic activities of various fractions of *R. glauca* leaves found in this study proved that this plant has potential medicinal effect. The results as well indicate that leaves of *R. glauca* possessed significant activity against bacteria cultures. This activity may be indicative of the presence of metabolic toxins. The effects of the extract emphasize the identification of active compounds present in the fractions with the help of more appropriate animal models, cell lines and enzyme inhibition studies to find out its unknown efficacy, which can be a potential source of chemically interesting and biologically important drug candidates.

Conflict of interest statement

We declare that we have no conflict of interest.

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