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

Mir Muhammad Nasir Uddin¹*, Raju Dash², Mohammad Shah Hafez Kabir³, Md. Mominur Rahman¹, Mohammad Nazmul Islam¹, Arkajyoti Paul²

¹Department of Pharmacy, University of Chittagong, Chittagong-4331, Bangladesh
²Department of Pharmacy, BGC Trust University Bangladesh, Chittagong-4000, Bangladesh
³Department of Pharmacy, International Islamic University Chittagong, Chittagong-4203, Bangladesh

ARTICLE INFO

Article history:
Received 18 Jul 2016
Received in revised form 5 Aug 2016
Accepted 28 Aug 2016
Available online 12 Sep 2016

Keywords:
Rhaphidophora glauca
Cytotoxicity
Antimicrobial
Analgesic
Kanamycin

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 LC50 values of AMF-1 and AMF-2 were found to be 287.73 and 428.54 µg/mL respectively, where colchicines showed LC50 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 2 000 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 an 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
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].

*Rhipidophora 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 methanic 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 2000 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 (T0). After 18 h of incubation, wells were again read in a spectrophotometer at 620 nm (T18). MIC values were evaluated in three distinct values, viz. MIC0 (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:

\[
\text{Inhibition (\%)} = \left[ 1 - \left( \frac{\text{OD test well}}{\text{OD of control well}} \right) \right] \times 100
\]

The concentration ranges of AMF-1 and AMF-2 used for microbes were 500, 750, 1000, 1250 and 1500 \(\mu\text{g/mL}\), 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 \(\mu\text{g/kg body weight}\) 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 (%) = \left[ \frac{(\text{Post-drug latency} – \text{Pre-drug latency})}{(\text{Cut-off time} – \text{Pre-drug latency})} \right] \times 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.

\[
\text{Inhibition (\%)} = \left[ \frac{(\text{Test} – \text{Control})}{\text{Control}} \right] \times 100
\]

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 \(\mu\text{g/mL}\).

| Test sample  | Concentration of sample (\(\mu\text{g/mL}\)) | LC$_{50}$ (\(\mu\text{g/mL}\)) |
|--------------|------------------------------------------|-------------------------------|
| 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 \(\mu\text{g/disc}\), 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 | 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 pyogenes* | 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<sub>50</sub> (µg/mL) | MIC<sub>90</sub> (µg/mL) | MIC<sub>100</sub> (µg/mL) | R value |
|------------------|---------------------------|--------------------------|--------------------------|---------|
| AMF-1            | AMF-2                     | AMF-1                    | AMF-2                    |         |
| *Bacillus cereus* | 22.10                     | –                        | 630.95                   | 1109.17 | 0.964<sup>*</sup> 0.930<sup>**</sup> |
| *Bacillus subtilis* | 478.73                   | 501.18                   | 868.96 1023.20           | 868.96 1603.24 | 0.983<sup>**</sup> 0.978<sup>**</sup> |
| *Staphylococcus aureus* | –                       | –                        | 467.73 524.80           | 467.73 1348.90 | 0.918<sup>**</sup> 0.978<sup>**</sup> |
| *Sarcina lutea* | –                         | 489.77                   | 467.73 524.80           | 489.77 1380.38 | 0.913<sup>**</sup> 0.994<sup>**</sup> |
| *Salmonella typhi* | 104.11                    | 104.71                   | 758.57 860.99           | 758.57 1603.24 | 0.993<sup>**</sup> 0.990<sup>**</sup> |
| *Salmonella paratyphi* | 478.63                   | –                        | 870.66                   | –       870.66 | 0.995<sup>**</sup> – |
| *Pseudomonas aeruginosa* | 104.11                   | 104.11                   | 776.24 758.57           | 776.24 1610.64 | 0.994<sup>**</sup> 0.983<sup>**</sup> |
| *Escherichia coli* | 22.13                     | 446.68                   | 630.95 860.99           | 630.95 1603.95 | 0.966<sup>**</sup> 0.996<sup>**</sup> |
| *Shigella dysenteriae* | 22.18                     | 21.87                    | 707.94 724.43           | 707.94 1348.96 | 0.984<sup>**</sup> 0.986<sup>**</sup> |
| *Shigella boydii* | 100.00                     | –                        | 794.32 524.80           | 794.32 860.99 | 0.998<sup>**</sup> 0.902<sup>**</sup> |

<sup>*</sup>: Correlation is significant at P < 0.05 level; <sup>**</sup>: Correlation is significant at P < 0.01 level.
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 the dose of 250 and 500 mg/kg 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).

### 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 LC₅₀ 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

### 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 | 7.67 ± 0.51 | 6.07 ± 0.35 | 5.70 ± 0.28 |
| Positive control   | 7.66 ± 0.66  | 12.31 ± 0.60 | 14.58 ± 0.62 | 12.83 ± 0.41 | 10.29 ± 0.34 |
| AMF-1 250 (mg/kg)  | 6.99 ± 0.97  | 13.56 ± 1.07 (50.50%) | 14.52 ± 0.56 (57.88%) | 12.84 ± 0.40 (44.97%) | 9.30 ± 0.32 (17.76%) |
| AMF-1 500 (mg/kg)  | 5.28 ± 1.21  | 7.77 ± 0.24 (16.92%) | 15.02 ± 0.55 (66.17%) | 10.86 ± 0.39 (37.91%) | 8.36 ± 0.27 (20.92%) |
| AMF-2 250 (mg/kg)  | 6.96 ± 0.33  | 11.95 ± 0.45 (38.27%) | 13.20 ± 0.43 (47.85%) | 10.52 ± 0.43 (27.30%) | 8.37 ± 0.32 (10.81%) |
| AMF-2 500 (mg/kg)  | 7.56 ± 0.48  | 9.57 ± 0.38 (16.16%) | 14.12 ± 0.41 (32.73%) | 12.21 ± 0.45 (37.38%) | 10.35 ± 0.32 (22.43%) |

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.

### 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    | 57.84%                    |
| Positive control  | 23.82 ± 0.86    | 54.35%                    |
| AMF-1 250 (mg/kg)| 25.91 ± 0.47    | 54.35%                    |
| AMF-1 500 (mg/kg)| 17.13 ± 0.53    | 67.25%                    |
| AMF-2 250 (mg/kg)| 27.92 ± 0.68    | 51.29%                    |
| AMF-2 500 (mg/kg)| 19.69 ± 0.47    | 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.
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 tempers 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[55]. 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.

**Acknowledgments**

The authors are greatly indebted to the managing committee of the Department of Pharmacy, University of Chittagong, Bangladesh, for providing all the laboratory amenities and support with a research grant to accomplish the study (Grant No. 182-Pharm-P&D-7-32/39).

**References**

[1] Farnsworth NR. Ethnopharmacology and future drug development: the North American experience. *J Ethnopharmacol* 1993; **38**(2-3): 145-52.
[2] Houghton PJ. The role of plants in traditional medicine and current therapy. *J Altern Complement Med* 1995; **1**(2): 131-43.
[3] Patanjayak P, Behera P, Das D, Panda SK. *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: an overview. *Pharmacogn Rev* 2010; **4**(7): 95-105.
[4] Ekwall B. Screening of toxic compounds in mammalian cell cultures. *Ann N Y Acad Sci* 1983; **407**: 64-77.
[5] Amin AR, Kucuk O, Khuri FR, Shin DM. Perspectives for cancer prevention with natural compounds. *J Clin Oncol* 2009; **27**(16): 2712-25.
[6] Naik SK, Mohanty S, Padhi A, Pati R, Sonawane A. Evaluation of antibacterial and cytotoxic activity of *Artemisia nilagirica* and *Murraya koenigii* leaf extracts against mycobacteria and macrophages. *BMC Complement Altern Med* 2014; **14**: 87.
[7] Rios JL, Recio MC. Medicinal plants and antimicrobial activity. *J Ethnopharmacol* 2005; **100**(1-2): 80-4.
[8] Kokoska L, Polesny Z, Rada V, Nepovim A, Vanek T. Screening of some Siberian medicinal plants for antimicrobial activity. *J Ethnopharmacol* 2002; **82**(1): 51-3.
[9] Khan R, Islam B, Akram M, Shakil S, Ahmad AA, Ali SM, et al. Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. *Molecules* 2009; **14**(2): 586-97.
[10] Adeniyi BA, Groves MJ, Gangadharam PR. *In vitro* antimycobacterial activities of three species of *Cola* plant extracts (Sterculiaceae). *Phytother Res* 2004; **18**(5): 414-8.
[11] Loeser JD, Treede RD. The Kyoto protocol of IASP Basic Pain Terminology. *Pain* 2008; **137**(3): 473-7.
[12] Kabir MG, Rahman MM, Ahmed NU, Fakruddin M, Islam S, Mazumdar RM. Antioxidant, antimicrobial, toxicity and analgesic properties of ethanol extract of *Solena amplexicaulis* root. *Biol Res* 2014; **47**(1): 36.
[13] Thomas MC. Diuretics, ACE inhibitors and NSAIDs--the triple whammy. *Med J Aust* 2000; **172**(4): 184-5.
[14] Dewan SMR, Amin MN, Adnan T, Uddin SM, Shahid-Ud-Daula AF, Sarwar G, et al. Investigation of analgesic potential and *in vitro* antioxidant activity of two plants of Asteraceae family growing in Bangladesh. *J Pharm Res* 2013; **6**(6): 599-603.
[15] Boyce P. Plate 377. *Rhaphidophora glauca*. *Curtis's Bot Mag* 1999; **16**(4): 273-80.
[16] Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE,
McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 1982; 45(5): 31-4.

[17] Rios JL, Recio MC, Villar A. Screening methods for natural products with antimicrobial activity: a review of the literature. J Ethnopharmacol 1988; 23(2-3): 127-49.

[18] Patton T, Barrett J, Brennan J, Moran N. Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. J Microbiol Methods 2006; 64(1): 84-95.

[19] Franzotti EM, Santos CV, Rodrigues HM, Mourao RH, Andrade MR, Antoniolli AR. Anti-inflammatory, analgesic activity and acute toxicity of Sida cordifolia L. (Malva-branca). J Ethnopharmacol 2000; 72(1-2): 273-7.

[20] Toma W, Gracioso JS, Hiruma-Lima CA, Andrade FD, Vilegas W, Souza Brito AR. Evaluation of the analogic and antiedematogenic activities of Quassa amara bark extract. J Ethnopharmacol 2003; 85(1): 19-23.

[21] Sulaiman MR, Tengku Mohamad TA, Shaik Mossadeq WM, Moin S, Yusof M, Mokhtar AF, et al. Antinociceptive activity of the essential oil of Zingiber zerumbet. Planta Med 2010; 76(2): 107-12.

[22] Zaraini Z, Yoga Latha, Suryani S, Sasidhuran S. Anticandida albicans activity of crude extract of the plant, winged beans leaf. Plant Kuala Lumpur 2007; 80: 653-7.

[23] Rahman MA, Imran TB, Islam S. Antioxidative, antimicrobial and cytotoxic effects of the phenolics of Leea indica leaf extract. Saudi J Biol Sci 2013; 20(3): 213-25.

[24] Gabbainn SN, Bergh Ø, Dixon B, Donachie L, Carson J, Coyne R, et al. The precision and robustness of published protocols for disc diffusion assays of antimicrobial agent susceptibility: an inter-laboratory study. Aquaculture 2004; 240(1-4): 1-18.

[25] Lozano-Chiu M, Nelson PW, Paetznick VL, Rex JH. Disk diffusion method for determining susceptibilities of Candida spp. to MK-0991. J Clin Microbiol 1999; 37(5): 1625-7.

[26] Hewitt W, Vincent S. Microbiological assay: an overview. In: Hewitt W, editor. Theory and application of microbiological assay. Cambridge: Academic Press; 1989, p. 1-8.

[27] Archer MH, Dillon VM, Campbell-Platt G, Owens JD. Effect of diacetyl on growth rate of Salmonella typhimurium determined from detection times measured in a microwell plate photometer. Food Control 1996; 7(2): 63-7.

[28] Casey JT, O’Cleirigh C, Walsh PK, O’Shea DG. Development of a robust microtiter plate-based assay method for assessment of bioactivity. J Microbiol Methods 2004; 58(3): 327-34.

[29] Kuda T, Shimizu K, Yano T. Comparison of rapid and simple colorimetric microplate assays as an index of bacterial count. Food Control 2004; 15(6): 421-5.

[30] Turcotte C, Lacroix C, Kheadr E, Grignon L, Fliss I. A rapid turbidimetric microplate bioassay for accurate quantification of lactic acid bacteria bacteriocins. Int J Food Microbiol 2004; 90(3): 283-93.

[31] Swenson JM, Hill BC, Thornsberry C. Problems with the disk diffusion test for detection of vancomycin resistance in enterococci. J Clin Microbiol 1989; 27(9): 2140-2.

[32] Piliouras P, Ulett GC, Ashhurst-Smith C, Hirst RG, Norton RE. A comparison of antibiotic susceptibility testing methods for cotrimoxazole with Burkholderia pseudomallei. Int J Antimicrob Agents 2002; 19(5): 427-9.

[33] Parente E, Brienza C, Moleas M, Ricciardi A. A comparison of methods for the measurement of bacteriocin activity. J Microbiol Methods 1995; 22(1): 95-108.

[34] Muhammad N, Saed M, Khan H. Antipyretic, analgesic and anti-inflammatory activity of Viola betonicifolia whole plant. BMC Complement Altern Med 2012; 12: 59.

[35] Elisabeths E, Amador TA, Albuquerque RR, Nunes DS, Carvalho ADO C. Analgesic activity of Psychotria colorata (Willd. ex R. & S.) Muell. Arg. alkaloids. J Ethnopharmacol 1995; 48(2): 77-83.

[36] Pal S, Sen T, Chaudhuri AK. Neuropsychopharmacological profile of the methanolic fraction of Bryophyllum pinnatum leaf extract. J Pharm Pharmacol 1999; 51(3): 313-8.

[37] Khan MA, Islam MT. Analgesic and cytotoxic activity of Acorus calamus L., Kigelia pinnata L., Mangifera indica L. and Tabernaemontana divaricata L. J Pharm Bioallied Sci 2012; 4(2): 149-54.

[38] Urban MK. COX-2 specific inhibitors offer improved advantages over traditional NSAIDs. Orthopedics 2000; 23(7 Suppl): S761-4.

[39] Adeyemi OO, Yemitan OK, Afolabi L. Inhibition of chemically induced inflammation and pain by orally and topically administered leaf extract of Manihot esculenta Crantz in rodents. J Ethnopharmacol 2008; 119(1): 6-11.

[40] Zhang L, Hu JJ, Lin JW, Fang WS, Du GH. Anti-inflammatory and analgesic effects of ethanol and aqueous extracts of Pterocephalus hookeri (C.B. Clarke) Hoeck. J Ethnopharmacol 2009; 123(3): 510-4.

[41] Lu TC, Ko YZ, Huang HW, Hung YC, Lin YC, Peng WH. Analgesic and anti-inflammatory activities of aqueous extract from Glycine tomentella root in mice. J Ethnopharmacol 2007; 113(1): 142-8.

[42] Duarte ID, Nakamura M, Ferreira SH. Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz J Med Biol Res 1988; 21(2): 341-3.

[43] Mbiancha M, Teronno RB, Nguelefack TB, Tapondjou AL, Watcho J, Kamel G, et al. Analgesic and anti-inflammatory properties of extracts from the bulbs of Dioscorea bulbifera L. var sativa (Dioscoreaceae) in mice and rats. Planta Med 2008; 74: PA316.

[44] Ferreira SH, Vane JR. New aspects of the mode of action of nonsteroid anti-inflammatory drugs. Annu Rev Pharmacol 1974; 14(1): 57-73.

[45] Berkenkopf JW, Weichman BM. Production of prostacyclin in mice following intraperitoneal injection of acetic acid, phenylbenzoquione and zynosan: its role in the writhing response. Prostaglandins 1988; 36(5): 693-709.

[46] Soumaya KJ, Dhekra M, Fadwa C, Zied G, Ilef L, Kamel G, et al. Pharmacological, antioxidant, genotoxic studies and modulation of rat splenocyte functions by Cyperus rotundus extracts. BMC Complement Altern Med 2013; 13: 28.