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Research Article

# Phytochemical and Pharmacological Evaluation of Methanolic Extracts of the Leaves of *Nepenthes bicalcarata* Hook. F.

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**ABSTRACT**
Pitcher plants (*Nepenthes* spp.) have a variety of medicinal uses, from simple management of infections to treatment of diabetes. The present study was conducted to evaluate the phytochemical and pharmacological properties of methanolic extracts of the leaves of *Nepenthes bicalcarata*. The screening for various phytochemicals was conducted using a standard procedure. Pharmacological investigation involved the determination of the antioxidant, antimicrobial, toxicity and antidiabetic activities of the extracts. Antioxidant activity determination was carried out using ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazil (DPPH) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assays, and the total phenolic and flavonoid contents were determined using colorimetric methods. Antimicrobial activity was evaluated by the disk-diffusion method and the minimum inhibitory concentration (MIC) was determined by a two-fold agar dilution method. Toxicity was assessed using a brine-shrimp lethality test (BSLT). Antidiabetic activity was evaluated using alloxan (120 mg/kg body weight) induced diabetic Wistar male rats orally treated on alternate days for 8 weeks at an extract dose of 300 mg/kg body weight. The phytochemical screening led to the detection of tannins, alkaloids, flavonoids, anthraquinones, terpenoids, steroids and phlobatannins. The FRAP, DPPH and ABTS antioxidant assays indicated the extracts had a moderate activity of 87.1 ± 2.50 mg Trolox Equivalent/g dry-weight, and $IC_{50}$ values of 12.52 ± 0.49 µg/mL and 16.13 ± 0.33 µg/mL, respectively. The total phenolic and total flavonoid contents were 50.5 ± 0.7 mg Gallic Acid Equivalent/g and 6.63 ± 0.17 mg Quercetin Equivalent (QE)/g, respectively. Antimicrobial tests revealed that the leaf extract was active against selected gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *B. spizizenii*) and a few fungi (*Candida albicans* and *Saccharomyces cerevisiae*), with MIC values ranging from 256 to 1024 µg/mL. An $LC_{50}$ value of 73.3 µg/mL was obtained from the Brine Shrimp Lethality Test. These findings indicate that methanolic extracts of *N. bicalcarata* leaves have various phytopharmacological activities and thus it would be useful to isolate the compounds responsible for these bioactivities in the future.

**Keywords:** *Nepenthes bicalcarata*, phytochemicals, antioxidant, antimicrobial, toxicity, antidiabetic

## INTRODUCTION

Diseases play an important role in a population. Infectious or otherwise, they greatly influence overall population growth. Infectious or communicable diseases are considered to be one of the leading causes of mortality, especially in developing countries. The epidemicity of a disease is often caused by pathogens. Much research has been dedicated to finding solutions to halt the spread of disease-causing organisms, and has led to the discovery of antibiotics such as penicillin, erythromycin and chloramphenicol[1]. However, the rapid evolution of resistant pathogenic infections has become a great concern, because these antibiotics have become less effective[2,3]. Therefore, the demand for new and alternative medicines has exponentially increased in recent years. Non-communicable diseases remain the leading cause of death worldwide, comprising 68% of the total in 2012[4]. In Brunei Darussalam, the three leading causes of death are cancer, cardiovascular diseases and diabetes mellitus[4, 5]. Diabetes is a degenerative metabolic disease where the blood glucose level remains high for a long period because of the insufficient production or utilization of insulin in the body[6]. Type 2 diabetes is a hyperglycemic condition that results from the combination of both insufficient insulin production and utilization and it accounts for the majority of diabetes in Brunei[5]. From 2009 to 2013, 9.14% of the deaths in Brunei were the result of diabetes mellitus[7].

Antioxidants are often associated with a reduction of health risks that contribute to conditions such as diabetes[8,9]. They act as a defense against reactive oxidative species (ROS), compounds responsible for causing damage to cells that give rise to the development of degenerative diseases[9].Plants in general are rich in antioxidants and other phytochemicals. Plant-based traditional medicines are still prevalent because plants are often inexpensive to prepare, are effective and their use for curing common

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ailments results in minimal complications[10]. Thus, plant extracts have become an invaluable source of candidate compounds for the development of new drugs. *Nepenthes* is the only genus in the family Nepenthaceae. In Brunei, plants from this genus are known as '*sumboi-sumboi*' whereas in neighboring countries such as Malaysia, they are commonly called '*periuk kera'*. This genus includes carnivorous plants that utilize nutrients from insects which fall into their pitchers and thus, they are commonly found growing in extremely infertile soils. *Nepenthes* spp. have been used as traditional medicines to cure numerous illnesses. Their medicinal uses include remedies developed from the roots for stomach aches and dysentery, whereas the pitcher has been used for the treatment of diabetes[11,12]. Investigations of the pitcher fluids of *Nepenthes* spp. indicated that the fluids were unsuitable for microbial growth[13]. More specifically, the pitchers of *N. khasiana* showed positive effects against microbial growth[14] and in lowering glucose as well as lipid levels in blood[12]. There are 13 species of *Nepenthes* known in Brunei Darussalam[15]. *N. ampullaria* and *N. gracillis* are the most common species used in traditional medicine, where the liquid of unopened pitchers are administered to regulate the menstrual cycle, ease child birth, relieve asthma, treat eye inflammation and act as a stamina booster[16, 17]. *N. bicalcarata* is an endemic species to Borneo and is recognized by its 'fanged' pitcher. It is a climbing species with large leaves reaching 65 cm in length and 12 cm in width[16,18]. It is found in peat swamp forest and to a lesser degree in heath forest. As its habitat is less accessible than other *Nepenthes* spp., its use as a medicinal plant is not well established and documented. However, as other species of the genus *Nepenthes* have been proven to be of medicinal value, this study was conducted to evaluate *Nepenthes bicalcarata*, for its potential use as a natural source of phytochemicals, antioxidant compounds and other biological agents including those which may show antimicrobial, toxic and/or anti-diabetic activities. Until this work, *Nepenthes bicalcarata* has remained relatively uncharacterized.

## MATERIALS AND METHODS

*Chemicals and reagents*

All the chemicals and reagents used in this study were of analytical grade. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ketoconazole, penicillin G, sodium persulfate, quercetin, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Gentamicin (10 µg), erythromycin (15 µg), Mueller-Hinton agar (CM03337), nutrient broth (CM0001), penicillin G (10 units), potato dextrose agar (CM0139), tetracycline (30 µg) and vancomycin (30 µg) were obtained from OXOID Ltd. (Cheshire, UK).

*Test organisms*

*Aspergillus brasiliensis* (ATCC16404), *Bacillus spizizenii* (ATCC6633), *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were obtained from Microbiologics Inc. (St. Cloud, MN). *Bacillus subtilis* (ATCC6633), *Candida albicans (ATCC10231), Saccharomyces cerevisiae (ATCC2601)* and *Staphylococcus aureus* (ATCC25923) were obtained from BioMéreux Inc. (Boston, MA). Brine shrimp eggs (*Artermia salina*) were obtained from Artemia International LLC, Fairview, TX.

*Plant collection and sample preparation*

The leaves of *N. bicalcarata* were collected during December 2012 from Kampong Sungai Mau in the Belait District of Brunei Darussalam. The leaves were cleaned and the midribs were removed. The leaf laminas were cut into smaller pieces and freeze-dried until a constant mass was obtained. They were ground into a fine powder using a micro-grinder with a sieve size of 0.5 mm.

*Solvent Extraction*

Two types of extractions were carried out. For the first extraction, 20 g of ground sample was extracted in 250 mL absolute methanol using a Soxhlet apparatus for 5 h. The extract was evaporated to dryness using a rotary evaporator. The extract was then subjected to phytochemical screening, antioxidant activity tests and other biological assays.

For the second extraction, a methanolic extract was prepared by mixing 0.05 g sample in 10 mL of 80% methanol. The extraction was carried out with constant shaking at 250 rpm at room temperature for 2 h. The mixture was centrifuged at 2496×g for 20 min. The supernatant was collected and subjected to total phenolic content and flavonoid analyses.

*Phytochemical Screening*

The presence of tannins, saponins, terpenoids, steroids and phlobatannins was confirmed according to the standard methods described by Edeoga et al.[19], whereas flavonoids, anthraquinones and cardiac glycosides were measured according to methods published by Ayoola et al.[20] Alkaloids was detected using Dragendorff's method as described by Shanili and Sampathkumar[21].

*Determination of total phenolic content*

The total phenolic content was determined using a method developed by Velioglu et al.[22] Gallic acid in the concentration range of 25 to 500 µg/mL was used as the standard. An aliquot of 0.3 mL of sample extract was mixed with 2.25 mL Folin-Ciocalteu reagent which had been diluted ten-fold with distilled water. The mixture was allowed to stand for 5 min before the addition of 2.25 mL of 0.566 M sodium carbonate. The mixture was left to react at room temperature for a further 90 min and the absorbance was read spectrophotometrically at λ=725 nm. The analysis was replicated six times. The results are expressed as mg of gallic acid equivalent (GE)/g of dry-weight (DW) extract.

*Determination of total flavonoid content*

An assay method described by Ameer et al.[23] was used for the determination of total flavonoid content using 80% methanolic quercetin solution (1 to 50 µg/mL) as the standard. Sample (0.5 mL) extract was mixed with 0.1 mL of 10% aluminum trichloride and 0.1 mL of 1 M potassium acetate. Methanol (80%, 1.5 mL) was then added to the

mixture, followed by 2.8 mL of distilled water. The reaction mixture was incubated at room temperature for 30 min and the absorbance was read at λ=415 nm. The analysis was replicated six times. The results are expressed as mg of quercetin equivalent per gram of dry-weight extract (mg QE/g DW).

*Antioxidant activity*

*DPPH radical scavenging assay*

The DPPH assay was adopted from Rana et al.[24] Methanolic extracts of the leaves of *N. bicalcarata* at different concentrations ranging from 0 to 50 µg/mL were prepared. A methanol solution of 0.1 µM DPPH was added to each extract at a volume ratio of 1:1. The mixture was allowed to react in the dark for 30 min. The absorbance was read at λ=515 nm using a spectrophotometer. Absolute methanol was used as the control and Trolox (concentration ranging from 0 to 10 µg/mL) and BHT (0–20 µg/mL) were used as the standards. The experiment was done in triplicate. The percent inhibition of activity was calculated according to the equation: % inhibition = $[(A_0-A_1/A_0] \times 100$, where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample or standard. The antioxidant activity was expressed as an $IC_{50}$ calculated from the dose-response graph, i.e., a graph of percent inhibition against the concentration of extract.

*ABTS radical scavenging assay*

The ABTS assay was carried out according to the method described by Lobo et al.[25] with minor modifications. A crude extract solution (500 µg/mL) was prepared by dissolving 0.05 g sample powder in 100 mL absolute methanol. The Trolox standard solutions (0–10 µg/mL) and BHT standards (0–20 µg/mL) were also prepared in methanol. The working ABTS solution was made by mixing an equal amount of stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate followed by incubation in the dark for 12 h. The resulting solution (1 mL) was then diluted with distilled water (60 mL) until an absorbance of 0.70 ± 0.01 arbitrary units at λ = 734 nm was obtained using a spectrophotometer. The extract was then allowed to react with the ABTS working solution at a ratio of 1:1 for 30 min and the absorbance was measured at λ = 734 nm. This assay was replicated. The ABTS scavenging capacity of the extract was compared with that of the standard and the percentage inhibition was calculated from the equation: % inhibition = $[(A_{control}-A_{test}/A_{control}] \times 100$, where $A_{control}$ is the absorbance of ABTS in methanol and $A_{test}$ is the absorbance of the extract or standard. The antioxidant activity was expressed as an $IC_{50}$ determined from the graph of percent inhibition versus extract concentration.

*FRAP assay*

The FRAP assay was conducted as described by Thaipong et al.[26]. The test sample (0.02 g) was dissolved in 10 mL absolute methanol. Standard solutions of Trolox at different concentrations (25 to 250 µg/mL) were also prepared. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (prepared by mixing 19.38 g sodium acetate trihydrate and 100 mL glacial acetic acid, pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid and 20 mM iron (III) chloride hexahydrate solution at a ratio of 10:1:1. The reagent was used at 37°C.

Sample extract (0.5 mL) was treated with 2.85 mL FRAP reagent and the mixture was left to react in the dark for 30 min. This assay was carried out in triplicate. The absorbance of samples and standards were read at λ=593 using a spectrophotometer. The result was expressed as mg of Trolox equivalent (TE)/g of dry-weight (DW) extract, which was estimated from the Trolox standard graph.

*Antimicrobial activity*

*Screening for antimicrobial activity using the disk-diffusion method*

The disk-diffusion method of Kirbey-Bauer[27] was used to screen for antimicrobial activity. The leaf extract of *N. bicalcarata* was tested against five different bacteria and three fungi: *E. coli*, *P. aeruginosa*, S. *aureus*, *B. subtilis*, *B. spizizenii*, *S. cerevisiae*, *C. albicans* and *A. brasiliensis*. Standard antibiotic disks, tetracycline (30 µg), gentamicin (10 µg), penicillin G (10 units), erythromycin (15 µg), vancomycin (30 µg), ketoconazole (25 µg) and miconazole (10 µg) were used as positive controls for *E.coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. spizizenii*, *S. cerevisiae*, *C. albicans* and *A. brasiliensis*, respectively.

Five different concentrations of *N. bicalcarata* extract were used in the experiment: 10, 25, 50, 100 and 500 mg/mL. Stock bacterial cultures grown on Mueller-Hinton agar and fungal cultures on potato dextrose agar were first sub-cultured in nutrient broth and potato dextrose broth, respectively, and incubated overnight at 37°C. Sterile Mueller-Hinton and potato dextrose agar plates were then prepared for the determination of inhibition zones for the bacteria and fungi, respectively. Each plate was divided into seven sections and labelled with the five doses of extract, a positive and a negative control (a sterile filter paper disk containing 10 µL of 1% DMSO in absolute methanol). The plate was then inoculated with the overnight microbial culture in broth whose turbidity had been adjusted to 0.5 McFarland standard. Sterile filter paper disks of 6 mm in diameter containing 10 µL *N. bicalcarata* extract of different concentrations (giving final doses of 100, 250, 500, 1000 and 5000 µg) were placed on top of the agar plate, with one disk in each section. The bacterial plates were incubated at 37°C and the fungal plates were incubated at 30°C. The diameters of the zones of inhibition for the bacterial plates were measured after a 24-h incubation. For the fungal plates, the measurements were taken after a 72-h incubation. The experiment was carried out five times for the bacteria and three times for the fungi. The results are expressed as the mm ± standard error of the mean (SEM).

*Determination of minimum inhibitory concentration*

Determination of the minimum inhibitory concentration (MIC) was conducted using a two-fold agar dilution technique, adopting the method described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST)[28]. The MIC was determined only for microorganisms which showed positive results against the plant extract, *S. aureus*, *B. subtilis*, *B. spizizenii*, *S. cerevisiae* and *C. albicans.* Penicillin G, erythromycin and amphotericin B were used as positive controls for *S. aureus*,

*Bacillus* spp. and the fungi respectively whereas absolute methanol containing 1% DMSO (total volume) was used as the negative control. A quality control was also included which consisted of the agar only.

Mueller-Hinton agar was used for bacteria and potato dextrose agar for fungi. Molten agar (19 mL at 50°C) was transferred into a sterile boiling tube containing 1 mL sample or an antimicrobial agent at various concentrations (with final concentrations ranging from 1000 to 0.244 µg/mL) and mixed vigorously using a vortex. The mixed agar (3 mL) was then transferred into a 12-well plate and the agar was allowed to cool and set at room temperature. Each well was then inoculated with 1 µL of the microorganism (having a turbidity equivalent to the 0.5 McFarland standard) using a multi-channel pipette and were left to air-dry. The Mueller-Hinton plates were incubated for 24 h at 37°C and the potato dextrose agar plates were incubated for at least 72 h at 30°C.

The MIC, defined as the minimum concentration of an antimicrobial agent capable of inhibiting visible microbial growth after an overnight incubation, was assessed as the last concentration (arranged in descending order) that was free from visible growth after the incubation period. The determination of the MIC was conducted in triplicate for each concentration.

*Cytotoxicity activity*

*Brine shrimp assay*

The cytotoxicity of the extract was investigated by the brine shrimp lethality bioassay following the method described by Meyer et al.[29] A small aquarium was half-filled with 3.8% brine solution (Ultramarine, Waterlife Research Limited) at pH 8 and partitioned into two sections. One side of the aquarium was covered with aluminum foil and the other side was exposed to a 60-watt lamp. Brine shrimp eggs (*Artemia salina*) were sowed in the dark side of the aquarium and left to hatch for 30 to 36 h. After hatching, brine shrimp nauplii were left to mature for another 24 h. The toxicity test was carried out 52 h after sowing. A stock solution (100 mg/mL) of *N. bicalcarata* leaf extract was prepared in 5% DMSO in absolute methanol. A serial dilution of the stock solution was then carried out to obtain extract from 2 mg/mL to 100 mg/mL. Using a pipette with a wide opening, 1 mL brine solution containing 10 to 15 swimming nauplii was transferred into a 12-well plate. Fresh brine solution (4 mL) was added followed by the addition of 25 µL of sample extract with thorough mixing. As a negative control, 25 µL of 5% DMSO in methanol was added instead of the extract. The plate was loosely covered and left under a light source for 24 h. The number of dead nauplii in each well was counted with the aid of a dissecting microscope. The total number of nauplii in each well was noted and recorded. The assay was carried out in triplicate. Probit analysis was used to determine the lethal concentration at 50% ($LC_{50}$), which is defined as the concentration at which 50% of the nauplii population was killed.

*Antidiabetic activity*

*Experimental animal*

Healthy male Wistar albino rats weighing 200–250 g were obtained from the animal house of the Department of Environmental and Life Sciences of the University. The animals were maintained at 25–30°C and exposed to a 12 h light-dark cycle. They had free access to food (Altromin, Altromin Spezialfutter GmbH & Co. KG, Germany) and water *ad libitum* during the course of the experiment. The experimental procedure was approved by the University Research Ethics Committee (UREC) of Universiti Brunei Darussalam. The animals were fasted but allowed to access water for 12 h prior to the induction of diabetes.

*Induction of diabetes and blood glucose level determination*

The animals were given a single intra-peritoneal (I.P.) injection of alloxan (120 mg/kg body wt.) dissolved in 0.9% saline solution. The blood glucose level was determined 72 h after the induction, where the blood was collected from the tail vein of the rats and measured using a Duo-Care glucose meter (Genexel Medical Instrument Inc. Korea). Only rats with a fasting blood glucose level of more than 15 mmol/L were included in the study.

*Evaluation of long-term hypoglycemic effect of the methanolic extract of N. bicalcarata*

Diabetic rats were divided into four groups (alloxan control, vehicle control, normal control and treated group) of six animals each. The alloxan control group was the alloxan-induced group with no further treatment administered throughout the experiment. The vehicle control group was treated the same as the alloxan-induced group but received the vehicle (aqueous solution containing 1% methanol and 1% DMSO) at a dose of 10 mL/kg body weight. The normal control group was the non-alloxan induced group and received no further treatment throughout the experiment. The treated group was the same as the alloxan-induced group and received methanolic extract suspended in the vehicle at a dose of 300 mg/kg body weight. Treatment was done orally every alternate day for 8 weeks. The blood glucose level was recorded every 3 to 4 days (two times a week).

*Intraperitoneal Glucose Tolerance Test (IPGTT) in normal rats*

The method for the glucose tolerance test was adapted from Ali et al.[30] Ten rats were divided into two groups (vehicle control and treated) of five rats (n=5) per group. After at least 12 h of fasting (with free access to water *ad libitum*), treated and vehicle control groups were orally administered with extract suspended in the vehicle (300 mg/kg body wt.) and vehicle (10 mL/kg body wt.), respectively. After 1 h, an intraperitoneal injection of glucose dissolved in distilled water (2 g/kg body wt.) was administered to each rat. The blood glucose level was measured 60 min before oral treatment of the extract and vehicle, 0 min (before the administration of glucose), and 15, 30, 60, 90, 120 and 150 min after glucose administration.

*Statistical analysis*

All the assays and analyses were carried out in triplicate (unless stated) and the results were presented as the mean ± SEM. Statistical and Probit analyses were done using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA), using independent sample *t*-tests and one-way

Table 1. Phytochemicals detected in the methanolic extracts of *N. bicalcarata* leaves

| Phytochemical | Test performed | Result |
|---|---|---|
| Tannin | Ferric chloride test | + |
| Saponin | Olive oil emulsion test | – |
| Alkaloids | Dragendorff's test | + |
| Cardiac glycosides | Keller-Killiani test | – |
| Flavonoids | Aluminum solution test | + |
| Anthraquinones | Borntrager's test | + |
| Terpenoids | Salkowski test | + |

Table 2. Total phenolic and flavonoid content of the extracts of *N. bicalcarata* leaves

| Total phenolic (mg GAE/g DW) | Total flavonoid (mg QE/g DW) |
|---|---|
| 50.5 ± 0.7 | 6.63 ± 0.17 |

Results are expressed as the mean ± SEM of six replicates (n= 6). GAE: Gallic acid equivalent; QE: quercetin equivalent.

Table 3. Antioxidant activity of the extracts of *N. bicalcarata* leaves determined using DPPH, ABTS and FRAP assays.

| | $IC_{50}$ (µg/mL) | | FRAP (mg TE/g DW) |
|---|---|---|---|
| | DPPH | ABTS | |
| *N. bicalcarata* | 12.52 ± 0.49 | 16.13 ± 0.33[b] | 87.1 ± 2.56 |
| Trolox | 6.53 ± 0.15[a] | 6.12 ± 0.03[a] | |
| BHT | 18.34 ± 1.09[a] | 4.87 ± 0.12[a b] | |

ANOVA. The statistical significant level was set at $p \leq 0.05$.

## RESULTS

*Phytochemical screening*

The screening for phytochemicals present in the methanolic extracts of *N. bicalcarata* leaves qualitatively revealed the presence of various secondary metabolites, which included tannins, alkaloids, flavonoids, anthraquinones, terpenoids, steroids and phlobatannins (Table 1).

*Total phenolic and total flavonoid content*

In our study, *N. bicalcarata* leaf extract exhibited low amounts of phenolic and flavonoid compounds (Table 2). The phenolic content was calculated using the standard calibration curve of gallic acid (standard curve equation: $y = 0.006x + 0.032$; $r^2 = 0.998$). The flavonoid content was quantified from the quercetin standard calibration curve (standard curve equation: $y = 0.007x - 0.012$; $r^2 = 0.0998$).

*Antioxidant activity*

Table 4. Diameter of the inhibition zone of the extracts of *N. bicalcarata* leaves tested against selected microorganisms.

| | Mean diameter of the zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram negative | | Gram positive | | | Fungi | | |
| Dose (µg) | *E. coli* | *P. aeruginosa* | *S. aureus* | *B. subtilis* | *B. spizizenii* | *S. cerevisiae* | *C. albicans* | *A. brasiliensis* |
| 100 | 6.00 ± 0.00 | 6.00 ± 0.00 | 19.60 ± 0.51 | 19.40 ± 0.68 | 18.20 ± 0.37 | 10.00 ± 0.00 | 19.00 ± 1.15 | 6.00 ± 0.00 |
| 250 | 6.00 ± 0.00 | 6.00 ± 0.00 | 25.80 ± 0.37 | 22.20 ± 0.58 | 21.20 ± 0.37 | 11.33 ± 0.33 | 23.33 ± 0.67 | 6.00 ± 0.00 |
| 500 | 6.40 ± 0.24 | 6.00 ± 0.00 | 28.60 ± 0.51 | 25.60 ± 0.24 | 22.80 ± 0.73 | 15.00 ± 0.00 | 27.67 ± 0.88 | 6.00 ± 0.00 |
| 1000 | 9.00 ± 0.32 | 6.00 ± 0.00 | 32.00 ± 0.77 | 27.40 ± 0.51 | 26.40 ± 0.40 | 17.67 ± 0.33 | 30.00 ± 0.00 | 6.00 ± 0.00 |
| 5000 | 10.40 ± 0.24 | 7.80 ± 0.37 | 37.60 ± 0.40 | 31.80 ± 0.66 | 27.20 ± 0.20 | ND | ND | ND |
| PC | 23.20 ± 0.51 | 18.00 ± 0.00 | 38.60 ± 0.00 | 29.8 ± 0.37 | 23.40 ± 0.40 | 24.30 ± 0.67 | 26.67 ± 0.67 | 13.33 ± 0.27 |
| NC | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 |

The diameters of the inhibition zone are presented as mean (mm) of five replicates (bacteria) and three replicates (fungi) ± SEM. A 6-mm inhibition zone represents the diameter of the disk thus no inhibition. ND: not determined; PC: positive control; NC: negative control

The scavenging activity of the methanolic leaf extract of *N. bicalcarata* determined using DPPH (Fig. 1A) and ABTS

(Fig. 1B) assays increased with an increase in the concentration of the extract. Both Trolox and BHT were used as the standards in each case. This investigation also revealed that the leaf extract was more active than the standard BHT in the DPPH assay, while it was the least active compared with the two standards using the ABTS assay as indicated by its higher $IC_{50}$ value (Table 3). The results are expressed as the mean ± SEM of three independent experiments (carried out in triplicate). TE: Trolox equivalent. [a] Significant difference from *N. bicalcarata* extract within the assay and [b] significant difference between assays. $p < 0.05$, based on ANOVA test followed by Tukey.

*Antimicrobial activity*

The leaf extract of *N. bicalcarata* was found to be active against gram-positive bacteria (*S. aureus, B. subtilis* and *B. spizizenii*) and non-filamentous fungi (*S. cerevisiae* and *C. albicans*). It was observed that the zone of inhibition increased with concentration (Table 4). The zone of inhibition of *N. bicalcarata* was comparable to that of the positive control at a dose of 5000 µg, 1000 µg, 500 µg and 500 µg for *S. aureus, B. subtilis, B. spizizenii* and *C. albicans*, respectively. The leaf extract of *N. bicalcarata* exhibited much higher MIC values than the positive controls with the highest MIC being found for *S. cerevisiae* as shown in Table 5. *Cytotoxicity activity*

The brine shrimp lethality toxicity at 50% ($LC_{50}$) determined through probit analysis was estimated to be 73.3 µg/mL (Fig. 2).

*Antidiabetic activity*

The diabetic rats treated for 8 weeks with *N. bicalcarata* leaf extract showed a decreasing trend in their blood glucose levels (Fig. 3A). As shown in Fig. 3B, the blood glucose level for the treated rats was significantly reduced from 23.52 ± 2.94 to 12.10 ± 6.59 mmol/mL ($p < 0.05$), but was still significantly higher ($p < 0.05$) than the normal control group (5.6 ± 0.34 mmol/mL). The normal rats that were treated with *N. bicalcarata* extract did not show a fast response to the glucose challenge and their blood glucose level started to significantly decrease only 60 min after glucose administration in the IPGTT (Fig. 4). The blood glucose level remained also significantly higher than the vehicle control until 150 min after the glucose load.

Table 5. Minimum inhibitory concentration of the extracts of *N. bicalcarata* leaves toward selected microbial strains

| | Gram positive bacteria MIC (µg/mL) | | | Fungi MIC (µg/mL) | |
|---|---|---|---|---|---|
| | *S. aureus* | *B. subtilis* | *B. spizizenii* | *S. cerevisiae* | *C. albicans* |
| *N. bicalcarata* | 256 | 256 | 256 | 1024 | 256 |
| Antibiotic | 0.0625 | 1 | 1 | NA | NA |
| Antifungal agent | NA | NA | NA | 1 | 1 |

Results are represented as the mean concentration in µg/mL of triplicate measurements. NA: not applicable.

## DISCUSSION

We evaluated a number of biological activities of a methanolic leaf extract of *N. bicalcarata*, although this particular species has no record of any medicinal use, unlike other species of the same genus. The methanolic extract of *N. bicalcarata* was found to be positive for almost all the phytochemicals tested. The majority of these phytochemicals are known to have antimicrobial, antidiarrheal and anthelmintic effects involving different mechanisms[31]. This may support the claim of the use of *Nepenthes* species for the treatment of various ailments of which many are caused by pathogens. Among the phytochemicals detected in the extract are alkaloids, a group of poisonous chemicals known to have different physiological effects[32]. Many successful drugs have been derived from alkaloids, such as morphine and codeine[33]. The presence of flavonoids in the extract might be responsible for the antioxidant properties of the extract. Flavones, which are classified as flavonoids, are proven to have antidiabetic activities[34] which might warrant the use of *Nepenthes* as a treatment for diabetes in India[12]. The presence of anthraquinones, terpenoids and phlobatannins are important as they exhibit antibacterial and antifungal properties[31]. Steroids are a group of phytochemicals which consist of compounds known to have effects on body hormones[35]. Thus, the traditional use of *Nepenthes* for menstrual cycle regulation may be brought about by the action of steroids present in the plants.

Although phytochemical screening of *N. bicalcarata* leaf extract supports the observation that the plant has antimicrobial properties, it was only true against gram-positive bacteria and yeast fungi. This finding is in agreement with the study conducted by Buch et al.[13] on the pitcher fluid of *N. alata, N. fusca, N. gracilis, N. mirabilis, N. superba, N. thorelii* and *N. ventricosa*. An *in vitro* experiment showed that pitcher fluid did not affect the bacterial growth of *E. coli* and *P. syringae* (both are gram-negative bacteria) but did inhibit the growth of *S. cerevisiae*. However, another study conducted on *N. gracilis* showed the leaf extract was active against *A. brasiliensis*[36] contrary to the negative result reported herein. Apart from the fact that they are both different species, the difference in the findings could be a result of the types of solvent used for the extraction. We used methanol as the extracting solvent, whereas the previous study used sequential extraction with several solvents, starting from non-polar hexane to water. Additionally, when sequential extraction is used, the yield of compounds should be higher, compared with our single solvent extraction and this could lead to the differences observed in the bioactivities of the extract. Environmental factors such as localities, climates, soils and collection times may also contribute to the differences in the chemical constituents of a plant[37]. Phytochemicals are secondary plant metabolites produced in response to environmental

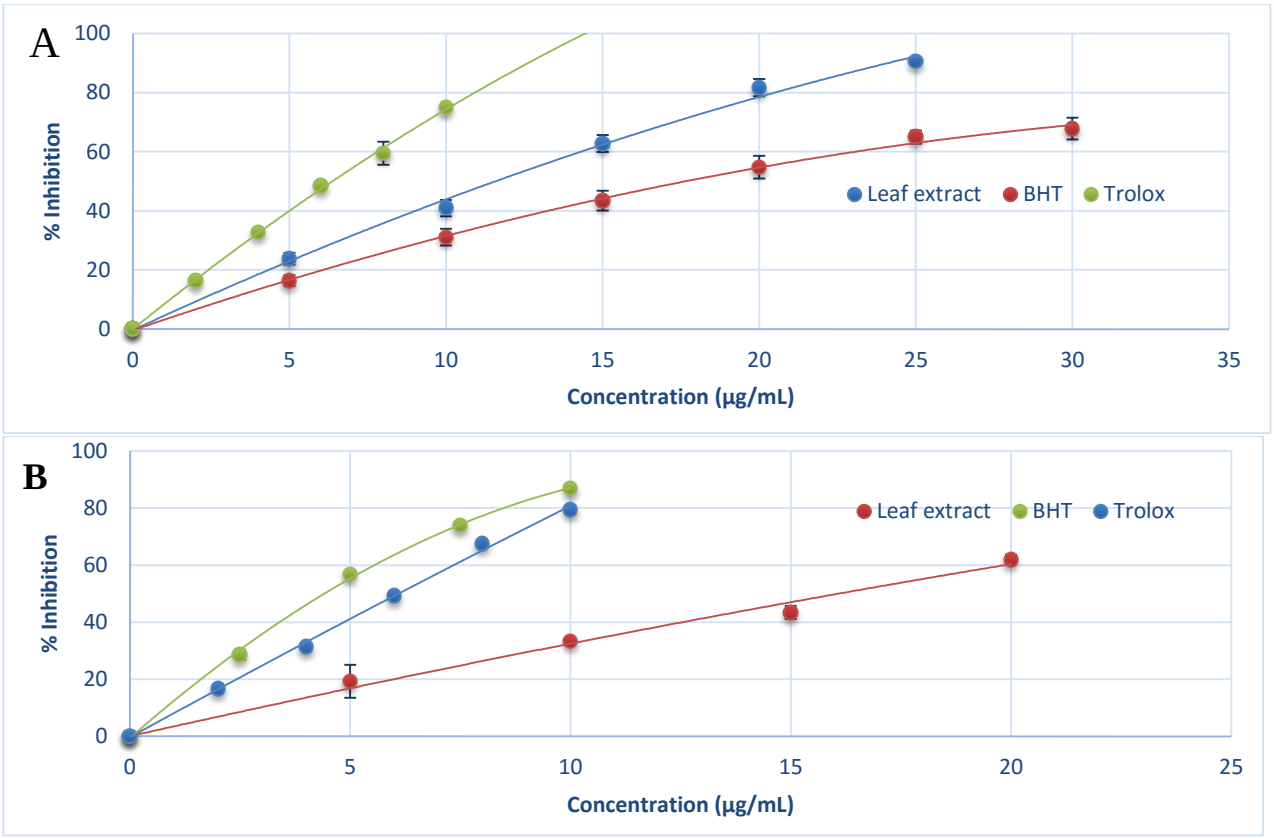


Figure 1. Scavenging activity of the methanolic extract of leaves of *N. bicalcarata* and the standards Trolox and BHT using A. DPPH assay, B. ABTS assay. Results represent mean values of three independent experiments (carried out in triplicate) at different concentrations and the bars represent the standard errors.

stimuli[38,39]. As a protective mechanism against herbivory or diseases, plants produce metabolites that are toxic to predators or parasites. This was observed in the study mentioned above[13], where although the pitcher fluid was found to be inactive against gram-negative bacteria, introduction of these bacteria into the pitcher fluid *in vivo* resulted in inhibition of the bacterial growth. This result indicated that plants can be stimulated to produce bioactive compounds necessary for their survival.

The MICs were determined for the microorganisms that were susceptible to the *N. bicalcarata* extract. A good antimicrobial agent is indicated by a low MIC value. The inhibitory action of *N. bicalcarata* leaf extract appeared to be lower compared with that of the positive controls (Table 5). This is probably because the potency of an extract is often masked as the bioactive compound(s) are present in lower concentrations compared with isolated and purified antimicrobial agent. Comparing the zones of inhibition of the bacteria and fungi tested at a dose of 1000 µg (Table 4), the inhibition zone of *S. cerevisiae* was found to be the lowest, which is in agreement with its MIC value being the highest. The MIC values for the remaining microorganisms are the same although they have different inhibition zones. However, it is also important to note that the MICs obtained from the agar two-fold dilution method may not reflect the actual values. The actual MIC may fall between two concentrations, one being the last showing visible growth and the other being the first that inhibits the growth[40].

We also investigated the antioxidant properties of *N. bicalcarata* leaf extract. Antioxidants are compounds capable of protecting the cells from harmful, damaging reactive oxygen species, thus lowering the risk of degenerative diseases such as cancer and diabetes. Although the amounts of phenolic and flavonoid compounds in the extract were low, the leaf extract exhibited high antioxidant activities. Assessment of the antioxidant activities was carried out using the FRAP, DPPH and ABTS assays. Each assay relies on a different mechanism to test for antioxidant activity. FRAP is an assay that evaluates the ability of a sample to reduce a ferric (III)-TPTZ complex to ferrous (II)-TPTZ *in vitro*[41, 42]. Although it is similar to DPPH and ABTS in terms of inhibiting the reactive species, the FRAP assay demonstrates the ability of antioxidants to prevent cellular oxidative damage in the body by becoming the oxidized substrate to the oxidizing species[43]. The leaf extract of *N. bicalcarata* exhibited a reducing power of 87.1 ± 2.56 mg TE/g DW, which is much higher than the reducing power of other plants reported using the same method[41, 44–45]. Phenolic compounds are thought to be responsible for antioxidant activity and several studies have shown a strong positive correlation between the phenolic and flavonoid content of a plant and its antioxidant activity [42, 46]. For the present study, although no correlation study was conducted, it would likely be negative as the extract had a low phenolic content but high antioxidant activity, suggesting that the antioxidant reducing power of *N.*

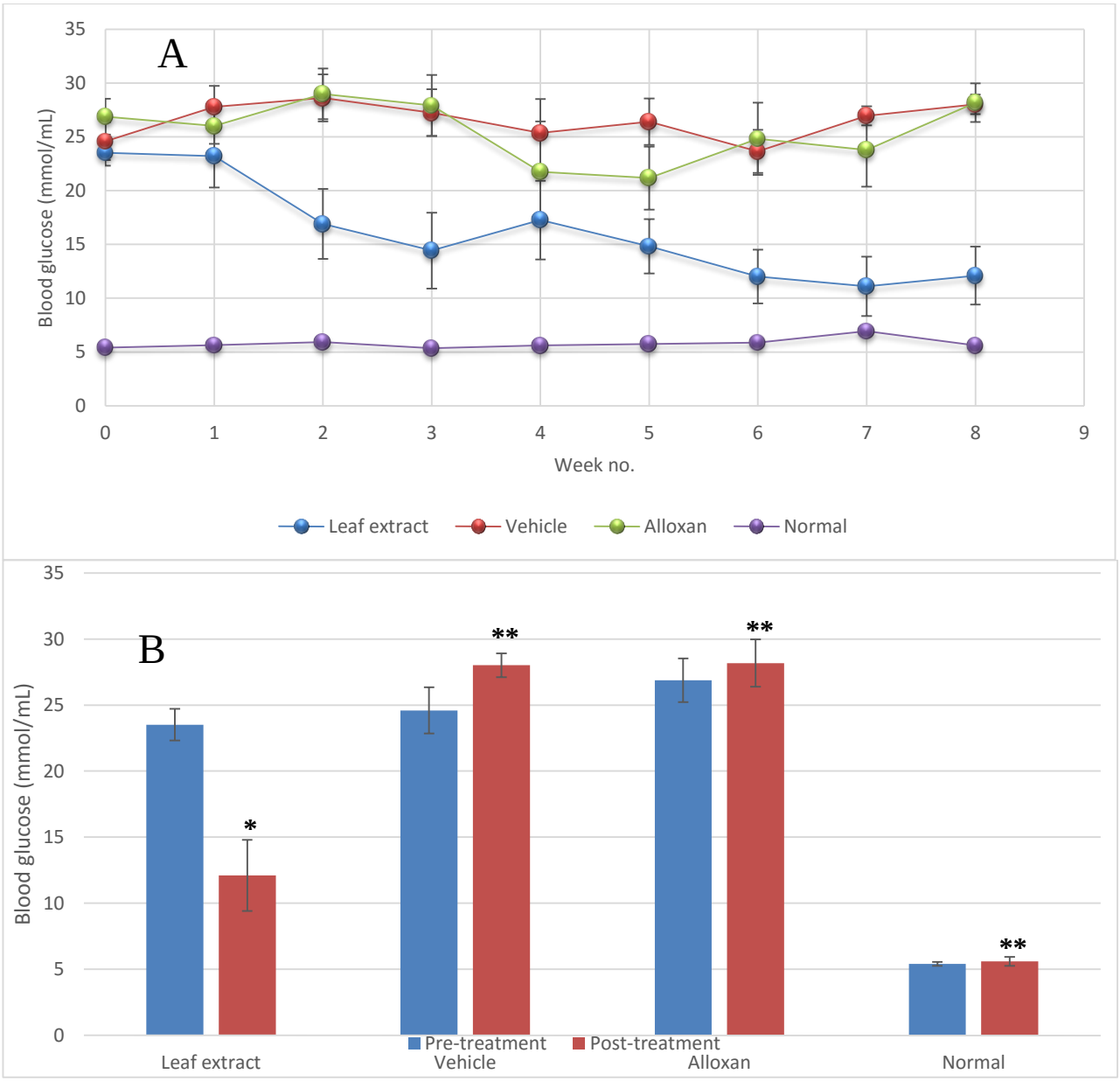


Figure 3. Blood glucose level of rats over 8 weeks of treatment (A), and before and after 8 weeks of treatment (B). Values are displayed as the means with the associated standard error bars. * Significant difference between pre- and post-treatment. ** Post-treatment glucose level significantly different between different media and that of leaf extract. $p < 0.05$, ANOVA test followed by Games-Howell.

*bicalcarata* leaves might not be a result of the action of phenolic compounds.

Antioxidant scavenging activities were also determined using DPPH and ABTS assays. Both methods use the same principle, where the antioxidants, which are also known to be radical scavengers, react with oxidative radicals formed as a result of cellular metabolic reactions. In both methods, stable free radicals of ABTS and DPPH were used. As shown in Fig. 1, the best scavengers for DPPH were Trolox > leaf extract > BHT and for ABTS, the scavengers were in the order BHT > Trolox > leaf extract. The results were also expressed as $IC_{50}$s (Table 3), which is defined as the concentration of a sample that can inhibit the action of free radicals by 50%, thus lower the $IC_{50}$ value, the greater the antioxidant activity of the sample[47]. The leaf extract of *N. bicalcarata* exhibited an $IC_{50}$ of 12.52 ± 0.50 µg/mL using the DPPH assay, which is approximately twice the $IC_{50}$ value of Trolox, but is significantly lower than the value obtained using the ABTS method. This may be because the compounds in the leaf extract scavenge DPPH better than ABTS radicals. The standard compound BHT is better suited to the ABTS assay, whereas Trolox has no preference for either substrate, exhibiting similar $IC_{50}$ values in both assays.

The leaf extract of *N. bicalcarata* has higher antioxidant activity than other carnivorous plants such as *Drosera indica* which is known to have anticancer properties[48]. Based on this observation, it can be concluded that *N. bicalcarata* may also have anticancer potential. Traditionally, there are no reports of *Nepenthes* species

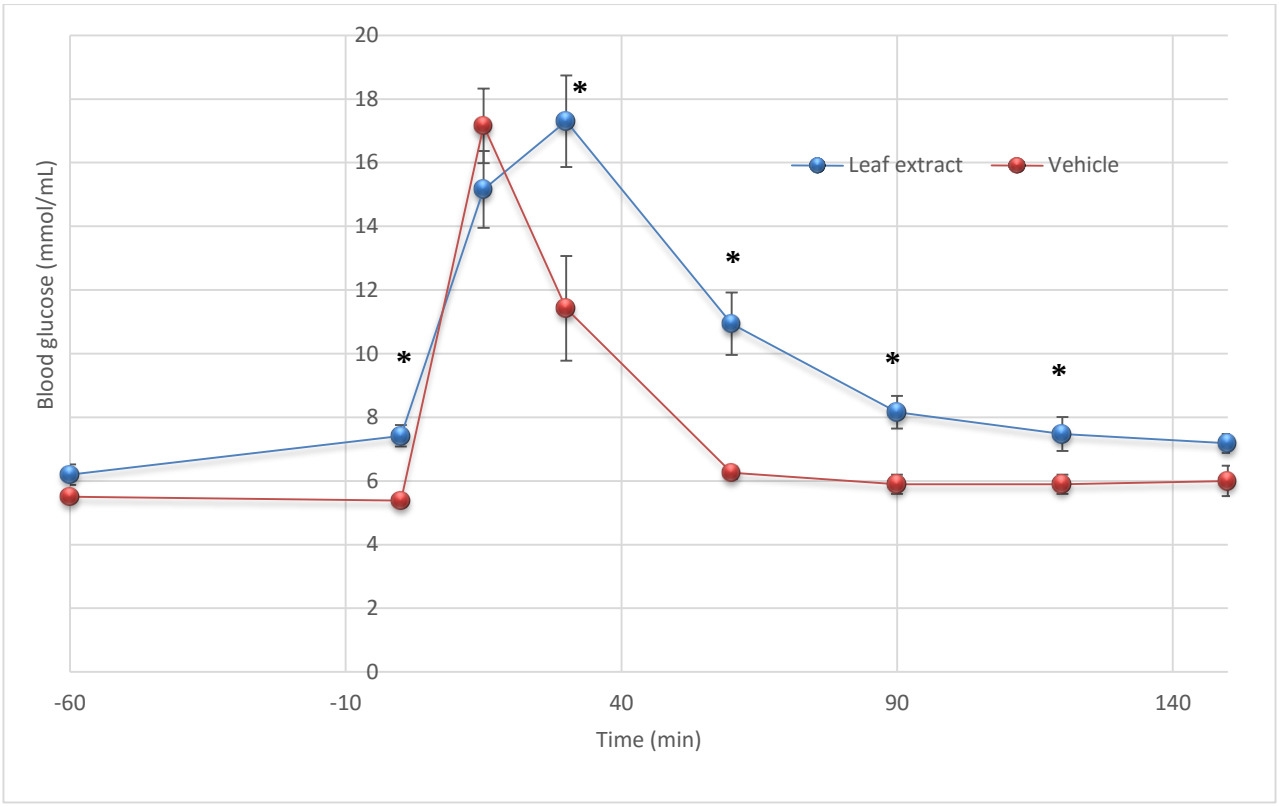


Figure 4. Effect of *N. bicalcarata* leaf extract on the blood glucose level before and after intra-peritoneal administration of glucose (2 g/kg body wt.) in normal rats. Values are expressed as the mean ± SEM (shown as error bars). * Significant difference between the leaf extract and vehicle control. $p < 0.05$, independent sample *t*-test.

being used as treatment for cancers. Carnivorous plants are known to have naphtoquinones[36, 48–51], a group of substances with significant pharmacological properties including anti-cancer activities related to their strong cytotoxic effects[50]. Plumbagin, which is a natural naphtoquinone, was found in *N. gracilis*[49] and *N. mirabilis*[51], supporting the potential use of *Nepenthes* for cancer treatment. In this study, the toxicity activity of *N. bicalcarata* was investigated using the BSLT method. The results from the brine shrimp lethality assay revealed that the leaf extract has a $LC_{50}$ value of 73.3 µg/mL, which according to Meyer et al.[29], may be considered as mildly toxic (highly toxic is indicated by a $LC_{50}$ < 30 µg/mL). Despite the less than remarkable toxicity of the extract, further investigation into the biologically active compounds present is worthwhile as it is expected that fractionating the extract could contribute to the quantification of stronger activity.

The assessment of the hypoglycemic effect of *N. bicalcarata* extract is presented in Fig. 3A and 3B. Alloxan is a chemical that specifically destroys β-cells in the pancreas[52]; hence, reducing its ability to produce sufficient insulin which leads to hyperglycemia. In our study, the hyperglycemia in the model rats was achieved by administration of alloxan. A dose of 300 mg/kg body weight was used and maintained throughout the experiment and no mortality or toxicity was observed within 72 h after the first day of treatment. The blood glucose level of the treated rats was significantly reduced after 8 weeks, while both alloxan and vehicle controls remained high. As we did not have an antidiabetic drug to use as a positive control, we included another group consisting of non-alloxan induced normal rats to act as the reference group. Although treatment of diabetic rats using the *N. bicalcarata* leaf extract was positive, the blood glucose level after the treatment period was still higher than normal ($p<0.05$). Extending the treatment duration or increasing the dose may improve the activity of the extract. The mechanism involved in the lowering of the glucose level in the blood is still unknown. One hypothesis is that an active compound present in the extract may increase the glycolysis process by increasing hexokinase activity as well as enhancing glucose absorption into cells by improving GLUT4 translocation[53]. Alternatively, the extract may be able to help regenerate the damaged pancreatic β-cells, allowing recovery of insulin in the rats. However, further histological study needs to be done to confirm these hypotheses.

An IPGTT was carried out to evaluate the ability of a body to process the sudden increase in glucose level. In this experiment, normal rats pretreated with *N. bicalcarata* leaf extract did not produce the expected result. Their response to the glucose challenge was found to be even slower than that in the rats treated with the vehicle alone (Fig. 4). It is also interesting to note that within 1 h after the administration of the extract and before the glucose intraperitoneal injection, the blood glucose level had increased slightly. As the insulin levels during the IPGTT were not monitored, we could not postulate whether or not insulin production was compromised in the rats treated

with *N. bicalcarata* leaf extract. Consequently, additional studies should be undertaken to investigate the mechanisms pertaining to the observed findings.

## CONCLUSION

In conclusion, the leaf extract of *N. bicalcarata* shows various pharmacological activities, including toxicity and anti-diabetic activity. The leaf extract also exhibited selective inhibition against gram-positive bacteria and yeast fungi. The high antioxidant activities and toxic properties of the extract suggest that *N. bicalcarata* leaves could be a potential treatment for cancer. Based on these findings, it is worthwhile to test the extract further using human cell lines as well as isolating and identifying the bioactive compound(s) present in the extract to confirm their activities.

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