Antimicrobial, antityrosinase and brine shrimp lethality test of Bauhinia rufescens Lam (Fabaceae)

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1. Introduction

Plants contributed in the development of medicine and pharmaceutical industry due to their diverse bioactive phytochemicals. These chemicals were synthesized as a variety of bioactive compounds in plant tissues as natural product that has broad spectrum of activities against organisms, such as bacteria, fungi, tyrosinase enzyme and brine shrimp larvae. Each of the secondary metabolite has a variable mechanism of action, for example, the toxicity of polyphenols in microorganisms is attributed to enzyme inhibition by oxidation of the compounds[1]. However, the development of resistant strains of pathogenic bacterial[2] and fungi[3] to antibiotics, as well as the involvement of tyrosinase enzyme in melanin production which might be responsible for some of the histopathological features exclusive to skin disorders [4], motivated our investigation for the bioactivity potentials of Bauhinia rufescens (B.
Bauhinia species (Fabaceae) are commonly known as ‘cow’s hoof’, because of the shape of their leaves. They are widely distributed in most tropical countries, including Africa, Asia and South America. *Bauhinia* trees typically reach a height of 6–12 m and their branches spread 3–6 m outwards. The lobed leaves usually are 10–15 cm across. The trees begin flowering in late winter and often continue to flower until early summer. Depending on the species, *Bauhinia* flowers are usually in magenta, pink or white hues with crimson high lights. In traditional medicine, their leaves and stem–bark have been used as a remedy for different kinds of pathologies, particularly diabetes, diarrhoea, dysentery, mycosis, pain and inflammatory cases\(^5,6\). Previous studies have demonstrated their triterpenes, saponins\(^13\) and phenolic compounds\(^14\) in the plant. This work is to evaluate the potentials of leaves and stem bark extracts of *B. rufescens* against pathogenic microbes. *Bauhinia variegata* was found to have significant antimicrobial activity against bacterial strains\(^7\), while *Bauhinia manca* collected from costa rica showed a significant inhibition of phytopathogenic fungi on *Botrytis cinerea, Claviceps viridis, Coprinus cinereus, Rhizoctonia solani* and *Saprolegnia asterophora*\(^6\). Similarly, research carried out on *Bauhinia racemosa*, a plant used in India to treat various pathologies, including infections, confirmed its production of active principles with antimicrobial potential, and it showed a broad–spectrum of antimicrobial activity against a panel of fungi and bacteria due to the presence of phenolic metabolites in the plant\(^8\).

*B. rufescens* has been reported to be used for the treatment of fibrosis, dysentery, jaundice\(^9\), eye diseases, diarrhea\(^10\), mycosis in children\(^11\) and gingivitis\(^12\). Phytochemical screening has revealed the presence of tannin, sterols, triterpenes, saponins\(^13\) and phenolic compounds\(^14\) in the plant. This work is to evaluate the potentials of leaves and stem bark extracts of *B. rufescens* against bacterial strains, fungal strains, tyrosinase enzyme and brine shrimp larvae.

2. Materials and methods

2.1. Plant materials

The leaves and stem barks of *B. rufescens* were collected at Kiru, Kano State, Nigeria in August 2011. A voucher specimen (Acc. 99) was deposited in the herbarium of the Department of Biological Sciences, Bayero University, Kano, Nigeria.

2.2. Preparation of plant extracts

The dried leaves (400 g) and stem barks (800 g) of *B. rufescens* were ground and extracted successively with petroleum ether (4.0 L), ethyl acetate (4.0 L) and methanol (4.0 L) in a soxhlet extractor for 18 h. The samples were concentrated using rotary evaporator to give the respective crude extracts (Table 1). The percentage yield of a crude extract was determined based on the dried weight.

### Table 1

| Part                      | Extracts  | Weight (g) | Yield (%) | Appearance    |
|---------------------------|-----------|------------|-----------|---------------|
| Leaves                    | Petroleum ether | 8.57       | 2.14      | Green, Gummy  |
|                           | Ethyl acetate   | 10.88      | 2.72      | Green, Gummy  |
|                           | Methanol      | 27.55      | 6.89      | Brown, Gummy  |
| Stem bark                 | Petroleum ether | 5.82       | 0.73      | Dark brown, Gummy |
|                           | Ethyl acetate   | 12.52      | 1.56      | Dark brown, Gummy |
|                           | Methanol      | 44.93      | 5.62      | Dark brown, Gummy |

2.3. Antimicrobial activity

Microbial strains: *Bacillus subtilis* (*B. subtilis*) (ATCC 6633), *Staphylococcus aureus* (*S. aureus*) (ATCC 29737), *Enterococcus faecalis* (*E. faecalis*) (ATCC 19433), *Escherichia coli* (*E. coli*) (ATCC 10536), *Klebsiella pneumonia* (*K. pneumonia*) (ATCC 13883), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 9027), *Aspergillus niger* (*A. niger*) (ATCC 16888), *Candida glabrata* (*C. glabrata*) (ATCC 2001) and *Saccharomyces cerevisiae* (*S. cerevisiae*) (ATCC 7754) were purchased from Mutiara Scientific, Cheras, Kuala Lumpur, Malaysia. The strains were grown on nutrient agar (Oxoid, Italy) for the bacteria, and sabouraud dextrose agar (SDA) for fungi.

2.3.1. Disc diffusion

Antimicrobial activity of the leaves and stem bark extracts of *B. rufescens* were determined by the agar disc diffusion method\(^5\). The suspension (400 µL) of the test bacteria and fungi which were spread on the nutrient agar and sabouraud dextrose agar respectively. The disc (6 mm diameter) impregnated with 10 µL of the extracts and DMSO (negative control) was placed on the inoculated agar, which was incubated for either 24 h at 37 °C (bacteria) or 48 h at 30 °C (fungi). Streptomycin sulfate (10 µg/mL) and nystatin (100 IU) were used as the positive controls for bacteria and fungi, respectively. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All tests and analyses were carried out in triplicate.

2.3.2. Minimum inhibitory concentration (MIC)

The MIC was determined by the broth micro dilution method using 96–well microplates\(^5\). The inoculate of the microbial strains was prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Each extract (1.8 mg) was dissolved in DMSO (1 mL) to obtain 1 800 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to the well from row B to H. The stock solutions of samples (100 µL) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100 µL) in row B was transferred to each well in order to obtain a twofold serial dilution of
the stock samples (concentration of 1 800, 900, 450, 225, 112.5, 56.5, 28.25 and 14.13 µg/mL). The inoculum (100 µL) was added to each well. The final volume in each well was 200 µL. Streptomycin sulfate for bacteria and nystatin for fungi were used as positive controls. Plates were incubated at 37 °C for 24 h. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well.

2.4. Tyrosinase inhibition activity

Tyrosinase inhibitory activity was determined using the method described by Sirat et al., with slight modifications[16]. Each of the tested extract (0.1 mg/mL, 40 µL) mixed with sodium phosphate buffer (100 mmol/L, 80 µL, pH 6.8) and then L-DOPA solution (2.5 mmol/L, 40 µL) and mushroom tyrosinase enzyme (100 units/mL, 40 µL) were added into a 96–well plate. The test mixture (200 µL) was mixed well and incubated at 37 °C for 10 min. DMSO instead of the compound was used as a control. The absorbance level was obtained with a multiplate reader at 515 nm with reference to 655 nm, and the percentage inhibition of tyrosinase activity was calculated by the following formula:

\[
\text{Inhibition} \% = \left( \frac{\text{Absorbance (blank)} - \text{Absorbance (tyrosinase+sample)}}{\text{Absorbance (blank)}} \right) \times 100
\]

Where, Absorbance (blank) and Absorbance (tyrosinase+sample) were the absorbance values in the presence and absence of inhibitor.

2.5. Brine shrimp lethality test

2.5.1. Hatching shrimp

Brine shrimp eggs, Artemia salina (A. salina) were hatched in artificial seawater prepared by dissolving 19 g of sea salt in 500 mL of distilled water. After 48 h incubation at room temperature (22–29 °C), the larvae was attracted to one side of the vessel with a light source and collected by pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater[17].

2.5.2. Brine shrimp assay

Toxicity of the extract was monitored by the brine shrimp lethality test according to the method of Jo et al., with slight modification[17]. Each of the extract (1 mg/mL) was dissolved in methanol, from which 5 000, 500 and 50 µL of each solution was transferred into vials corresponding to 1.00, 0.10 and 0.01 mg/mL respectively. This was allowed to evaporate to dryness in about 24 h at room temperature. Each dosage was tested in triplicate (9 per test sample). Sea water (4 mL) and 10 larvae were introduced into each vial. The final volume of solution in each vial was adjusted to 5 mL with sea water immediately after adding the shrimps. A negative control was prepared as a drug–free. Survivors were counted after 24 h, and LC50 was determined by probit analysis using SPSS version 16.

3. Results

The soxhlet extraction of leaves and stem barks of B. rufescens were employed in order to extract substances of low and medium volatility as well as thermally stable constituents based on polarity gradient of petroleum ether, ethyl acetate and methanol. The appearance and quantitative evaluations were investigated on some selected microorganisms, namely, three Gram-positive bacteria: B. subtilis, S. aureus, E. faecalis, three Gram-negative bacteria: E. coli, P. aeroginosa, K. pneumonia, and three fungi: A. niger, C. glabrata, S. cerevisiae on the basis of clinical pathogens.

Table 2

Antimicrobial activity of B. rufescens extracts.

| Organisms | Petroleum ether of leaf exact | Ethyl acetate of leaf exact | Methanol of leaf exact | Petroleum ether of stem bark extract | Ethyl acetate of stem bark extract | Methanol of stem bark extract | Positive control |
|-----------|-----------------------------|---------------------------|------------------------|-------------------------------------|----------------------------------|------------------------------|----------------|
| Bacteria  |                             |                           |                        |                                     |                                  |                              |                |
| BB        | 6.00±0.00                   | >1800                     | 7.67±0.25              | 900                                 | 8.33±0.94                        | 900                           | 7.1±0.79        | 1800            |
| SA        | 6.67±0.79                   | >1800                     | 8.67±1.25              | 1800                                | 9.67±1.25                        | 1800                          | 8.67±0.70       | 1800            |
| EF        | 6.33±0.47                   | >1800                     | 7.67±1.70              | 1800                                | 7.67±0.47                        | 1800                          | 8.45±0.30       | 1800            |
| EC        | 6.70±0.50                   | >1800                     | 7.30±0.90              | 1800                                | 8.70±0.10                        | 900                           | 7.80±0.70       | 1800            |
| PA        | 6.70±0.90                   | >1800                     | 10.0±0.50              | 450                                 | 10.1±0.07                        | 900                           | 10.0±0.07       | 1800            |
| KP        | 6.00±0.04                   | >1800                     | 8.67±1.70              | 1800                                | 8.00±0.82                        | 1800                          | 7.67±0.47       | 1800            |
| Fungi     |                             |                           |                        |                                     |                                  |                              |                |
| AN        | 13.30±0.47                  | 225                       | 14.0±1.42              | 225                                 | 11.30±0.47                       | 450                           | 11.70±0.47      | 450             |
| CG        | 10.33±0.79                  | 450                       | 9.00±0.82              | 900                                 | 7.33±0.47                        | 900                           | 8.67±0.70       | 900             |
| SC        | 8.67±0.47                   | 450                       | 11.70±0.47             | 450                                 | 10.30±0.25                       | 900                           | 9.00±0.82       | 450             |

Data represent mean±standard deviation of three independent experiments; BS=B. subtilis; SA=S. aureus; EF=E. faecalis; EC=E. coli; PA=P. aeroginosa; KP=K. pneumonia; AN=A. niger; CG=C. glabrata; SC=S. cerevisiae; DD=disc diffusion method (including the diameter of disc 6 mm); MIC=minimum inhibitory concentration (µg/mL); SS=streptomycin sulfate.
The range of inhibition of the bacterial growth summarized in Table 2, varied from 6.00–10.13 mm and 6.67–12.57 mm for the leaves and stem bark extracts of *B. rufescens*, respectively. The petroleum ether extract of the leaves was not active against all the tested bacteria, whereas a maximum inhibition was observed against *P. aeruginosa*, in a similar extract of the stem bark, moderate activity was observed. A quantitative determination of the extracts against the selected bacterial strains recorded a MIC values in the range of 450–1 800 µg/mL. The efficacy of ethyl acetate and methanol extracts from the leaves of the plant were found to be weakly active against *S. aureus*, *E. faecalis* and *K. pneumonia* inhibiting their growth in the range of 7.67–9.67 mm diameter and a minimum concentration of 1 800 µg/mL quantitatively, while the *B. subtilis* growth was inhibited at MIC value of 900 µg/mL. Furthermore, ethyl acetate extract of stem bark was found to be more active than methanol extract against all the tested bacteria with exception of *K. pneumonia*.

Tyrosinase is an enzyme in melanin biosynthesis which is involved in determining the colour of mammalian skin and hair. The pigment melanin in human skin is a major defense mechanism against the ultraviolet light of the sun. The production of abnormal pigmentations, such as melasma, freckles, age spots, liver spots and other forms of melanin hyperpigmentation are dermatological disorders associated with the melanin biosynthesis[18]. Therefore, in search of tyrosinase inhibitors from plants, the leaves and stem bark extracts of *B. rufescens* were screened against mushroom tyrosinase enzyme for their potential inhibition properties. The inhibitory effect of the leaves and stem bark extracts of *B. rufescens* were found in the range of 16.85–39.60% and 28.47–39.89% respectively.

The leaves and stem bark extracts of *B. rufescens* were also tested against the brine shrimps larvae at three different concentrations (0.01, 0.10 and 1.00 mg/mL), and their mortality were analyzed after 24 h for the determination of 50% lethal concentration. The toxicity effect of the leaves and stem bark extracts of *B. rufescens* were found in the range of 0.059–119.1 mg/mL, and 0.278–230.3 mg/mL respectively. The ethyl acetate extract from the leaves of the plant showed significant lethality with LC₅₀ of 0.059 mg/mL against *A. salina* compared to positive control potassium dichromate (LC₅₀ 0.004 mg/mL). Similarly, methanol extracts from leaves (0.389 mg/mL) and stem bark (0.278 mg/mL) were also found toxic against the larvae with LC₅₀ value less than 1 mg/mL. However, other extracts were nontoxic to the brine shrimp.

4. Discussion

In the present study, methanol extract of leaves and stem barks were found to have a wide range of percentage yield in comparison to their respective petroleum ether and ethyl acetate extracts (Table 1). This might be associated with the high content of polar constituents in the matrices of the plant materials. All the stem bark extracts were gummy and dark brown in colour, whereas the leaves extract were found greenish in colour with the exception of the methanol extracts.

The antimicrobial activity indicated higher activity index (Table 2) in the ethyl acetate extract of leaves (450, 900 µg/mL) and stem bark (900, 450 µg/mL) against *P. aeruginosa* and *B. subtilis* respectively, the same MIC value was recorded with methanol extract of the stem bark against *P. aeruginosa* (450 µg/mL). This efficacy of extracts of *B. rufescens* against the selected bacterial strains indicated the presence of bioactive compounds at relative concentration in the respective extracts. This is consistent with a significant antibacterial activity of ethyl acetate (1.56, 1.56 mg/mL), n–butanol (3.13, 1.56 mg/mL) and methanol (6.25, 6.25 mg/mL) extracts from the stem bark of *B. rufescens* against *S. aureus* and *P. aeruginosa*[19]. In addition, the n–hexane and methanol extracts of *Bauhinia racemosa* and *Bacillus variegata* were investigated against *Bacillus cereus*, *E. coli*, *S. aureus* and *P. aeruginosa*, in which methanol extract demonstrated higher activity (<100 mg/mL) compared to n–hexane extract. On the other hand, *B. forficata* and *B. microstachya*, proved inactive (1 000 mg/mL) when tested against *E. coli* and *S. aureus*[5].

Evaluation of the antifungal properties of the leaves and stem bark extracts, tabulated in Table 2, shows inhibition of fungal growth in the range of 8.67 to 14.03 mm and 7.33 to 12.70 mm, respectively. The MIC value of petroleum ether and ethyl acetate extracts from the leaves of *B. rufescens* against *A. niger* was observed at 225 µg/mL, while other extracts inhibited its growth at 450 µg/mL with exception of petroleum ether of stem bark (900 µg/mL). A moderate activity (450–1800 µg/mL) was observed for the extracts against *C. glabrata* and *Saccharomyces cerevisiae* (ATCC 7754). The leaves extracts recorded higher activity against tested fungal strains compared to the stem bark extracts. In a study carried out by Maillard et al. dichloromethane extract from the root of *B. rufescens* exhibited antifungal activity (<100 µg/mL) against plant phytopathogenic fungus *Cladosporium cucumarium*[20]. Furthermore a lectin from *B. monandra* inhibited the growth of *Fusarium* species in the range of 15–50% at 120 µg/mL[21]. Svetaz et al. found that extracts of *B. forficata* were inactive (MIC>1000 µg/mL) against *Candida albicans*, *A. niger* and *Saccharomyces cerevisiae*[22].

In the tyrosinase inhibitory activity, leaves and stem bark extracts inhibit the growth of tyrosinase enzyme in the range of 16.85–39.89%. The petroleum ether extracts (leaves 39.60% and stem bark 39.89%) were moderately inhibited tyrosinase enzyme as compared to the standard kojic acid (85%). It was also observed that ethyl acetate (leaves 16.85% and stem bark 28.81%) and methanol (leaves 25.83% and stem bark 28.47%) extracts exhibited a weak activity towards tyrosinase enzyme. The moderate activity exhibited...
by the B. rufescens is in accordance with the inhibitory activity (33.8%) demonstrated by the ethyl acetate extract from the leaves of Tibouchina semidecandra[16]. This is the first report of tyrosinase inhibitory activity of B. rufescens, and the inhibitory activity demonstrated by the plant could be associated to its secondary metabolite present in the tested extracts.

The brine shrimp bioassay is a simple and low cost technique for predicting the toxicity of the plant extract in order to consider the safety of the therapeutic agents as well as correlation with other biological models[23]. Plant extract resulting in LC₅₀ less than 1 mg/mL are considered toxic to the larvae[24]. The present study showed that ethyl acetate extract (leaves) and methanol extracts (leaves and stem bark) of B. rufescens were toxic (0.059–0.389 mg/mL) to brine shrimp larvae, while the other extracts were found to be nontoxic (119.1–230.3 mg/mL) to the larvae. Jo et al., found that the methanol extract of Etlingera elatior was nontoxic (LC₅₀ 2.52 mg/mL) to the brine shrimp[17]. Thus, the toxicity of a plant extract could be associated to the type and concentration of its constituents.

In conclusion, the extraction of leaves and stem bark from B. rufescens showed that methanol gave the highest percentage yield of extracts in both leaves and stem bark of the plant. It was also observed that the percentage yield of leaves extracts were higher than the corresponding stem bark extracts. The result of antimicrobial activity revealed that, the extracts from the leaves and stem bark of B. rufescens exhibited a moderate activity towards the tested bacterial and fungal strains, and the potency of the extracts towards fungi was stronger than that of bacterial strains. However, tyrosinase inhibitory activity of the extracts indicated moderate activity of the extracts. While the brine shrimp lethality test showed the potent toxicity of the extracts towards brine shrimp larvae, especially the ethyl acetate of leaves extract, methanol of leaves and stem bark extracts were found toxic to the larvae.

Therefore, antimicrobial, antityrosinase and brine shrimp lethality test activities of leaves and stem bark extracts from B. rufescens found in this study may explain some of the traditional medicinal uses of the plant which includes treatment of diarrhoea, dysentery, mycosis, inflammation etc[25]. These could be of particular interest for the isolation of bioactive constituents as important drugs candidates from B. rufescens.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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**Comments**

**Background**

The acceptance of traditional medicinal plants as safe remedies by WHO increase the value of medicinal plants and their bio–active principles with therapeutic potential. B. rufescens commonly known as ‘cow’s hoof’ is a traditional African medicinal plant used to treat diabetes, diarrhea, mycosis, pain and inflammation. On this premise, this study was programmed to screen the antimicrobial, cytotoxic and antityrosinase activities of B. rufescens.

**Research frontiers**

In the present study, the extracts of B. rufescens leaves and bark were evaluated for antimicrobial, cytotoxic and tyrosinase inhibitory activities. Antimicrobial activity was determined by disc diffusion and MIC method using streptomycin sulfate for bacteria and nystatin for fungi as positive controls. Cytotoxic activity was measured through brine shrimp lethality bioassay. Further, tyrosinase inhibitory property was evaluated in vivito.

**Related reports**

The in vivito antimicrobial study indicated that the extracts inhibited the microbial growth in the range of 6.00–12.57 mm (450–1 800 µg/mL) for bacterial strains and 7.33–14.03 mm (225–1 800 µg/mL) for fungal strains. Brine shrimp lethality bioassay revealed the positive cytotoxic activity of ethyl acetate and methanol plant extracts against A. salina with IC₅₀ values of 0.059 mg/mL and 0.389 mg/mL respectively. Moreover, there are reported articles on antimicrobial activity of stem bark of the plant [Hassan HS, et al., Preliminary phytochemical and antimicrobial screening of the stem bark extracts of B. rufescens lam using some selected pathogens. Bajopas. 2(2) 2009].

**Innovations and breakthroughs**

Tyrosinase inhibitory activity of the plant suggests it can be potential herbal drug to treat dermatological disorders associated with hyperpigmentation.

**Applications**

From the literature survey, it has been found that B. rufescens is safe to humans. This scientific study supports and suggests the use of this plant as an adjuvant along with commonly used antimicrobial, cytotoxic and anti–melanin drug.
This is a well planned research work executed by authors where antimicrobial, cytotoxic and tyrosinase inhibitory activities of the extracts of *B. rufescens* were established. The antimicrobial and cytotoxic potentiality of the plant extracts were accessed through disc diffusion assay and brine shrimp lethality bioassay respectively. Further, antityrosinase potentially reveals its therapeutic activity against dermatological disorders. Further work on this plant will be stimulated if the paper is accepted for publication.

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