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

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PEER REVIEW

ABSTRACT

Objective: To evaluate leaves and stem bark extracts of *Bauhinia rufescens* for antimicrobial activities, tyrosinase enzyme inhibition potentials and lethality to brine shrimp larvae (*Artemia salina*).
Methods: The plant materials were extracted based on polarity gradient of petroleum ether, ethyl acetate and methanol. The extracts were investigated for their antimicrobial, antityrosinase and cytotoxicity potentials.
Results: The antimicrobial results 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. While the tyrosinase enzyme inhibition assay of the extracts showed that both the petroleum ether leaves and stem bark extracts exhibited a moderate inhibition (39.60% and 39.89%) towards the action of mushroom tyrosinase enzyme on L−DOPA. In the brine shrimp lethality assay, ethyl acetate and methanol extracts of the leaves of the plant were found toxic to the *Artemia salina* with IC_{50} values of 0.059 mg/mL and 0.389 mg/mL. However, both the petroleum ether and ethyl acetate extracts of the stem bark were nontoxic to the larvae.
Conclusions: The result of the present study suggest the presence of phytochemicals possessing antimicrobial and antityrosinase activity as well as cytotoxic agents.

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 bacteria[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.*

rufescens).

   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, diarrhoae, dysentery, mycosis, pain and inflammatory cases[5,6]. Previous studies have demonstrated their potentials 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. rufecsens* 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
Appearance and percentage yield of leaves and stem bark extracts from *B. rufescens*.

Part	Extracts	Weight (g)	Yield (%)	Appearance
	Petroleum ether	8.57	2.14	Green, Gummy
Leaves	Ethyl acetate	10.88	2.72	Green, Gummy
	Methanol	27.55	6.89	Brown, Gummy
	Petroleum ether	5.82	0.73	Dark brown, Gummy
Stem bark	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), *Kleibsiella pneumonia* (*K. pneumonia*) (ATCC 13883), *Pseudomonas aeroginosa* (*P. aeroginosa*) (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[15]. 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[15]. 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) was 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 LC$_{50}$ 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 percentage yield of the respective extracts were tabulated in Table 1.

The antimicrobial potential of the extracts were investigated against bacterial and fungal strains on the basis of disc diffusion and microdilution assay. The qualitative 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			
													SS		NS	
	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Bacteria																
BS	6.00±0.00	>1 800	7.67±0.25	900	8.33±0.94	900	7.1±0.79	1 800	10.33±1.25	450	8.50±1.08	900	11.30±0.70	56.25	–	–
SA	6.67±0.94	>1 800	8.67±1.25	1 800	9.67±1.25	1 800	8.67±0.70	1 800	8.67±0.17	900	7.67±1.25	1 800	12.30±0.25	14.07	–	–
EF	6.33±0.47	>1 800	7.67±1.70	1 800	7.67±0.47	1 800	6.67±0.94	1 800	8.45±0.30	900	7.67±0.94	1 800	14.00±0.82	28.13	–	–
EC	6.70±0.50	>1 800	7.30±0.90	1 800	8.70±1.09	900	7.00±0.14	1 800	8.70±0.90	900	7.80±0.70	1 800	18.00±0.80	28.13	–	–
PA	6.70±0.90	>1 800	10.00±0.80	450	10.13±1.07	900	12.57±0.42	1 800	10.00±1.60	900	11.00±1.20	450	17.00±0.73	28.13	–	–
KP	6.00±0.04	>1 800	8.67±1.70	1 800	8.00±0.82	1 800	7.00±0.82	1 800	7.67±0.94	1800	7.67±0.47	1 800	11.00±0.82	56.25	–	–
Fungi																
AN	13.30±0.47	225	14.03±0.82	225	11.30±0.47	450	9.67±1.89	900	1 1.70±0.47	450	12.70±0.94	450	–	–	15.00±0.80	14.07
CG	10.33±0.47	450	9.00±0.82	900	10.00±0.82	900	7.33±0.47	1 800	7.33±0.94	1 800	8.67±0.47	900	–	–	18.05±1.01	28.13
SC	8.67±0.47	450	11.70±0.47	450	10.30±0.25	900	8.00±0.82	1 800	9.67±0.94	1 800	9.00±0.82	450	–	–	12.00±0.72	14.07

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. aeroginosa*, 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 pigmentation, 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 LC_{50} of 0.059 mg/mL against *A. salina* compared to positive control potassium dichromate (LC_{50} 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 LC_{50} 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. aeuroginosa* and *B. subtilis* respectively, the same MIC value was recorded with methanol extract of the stem bark against *P. aeroginosa* (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. auereus* and *P. aeuroginosa*[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. aeroginosa*, 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 cereviacea* (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 15-30% at 120 μg[21]. Svetaz *et al.* found that extracts of *B. forficata* were inactive (MIC>1000 μg/mL) against *Candida albicans*, *A. niger* and *Sacchoromyces cereviaceae*[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 LC_{50} 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 (LC_{50}, 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 diarrhoae, 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.

Acknowledgements

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 premises, 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–vitro*.

Related reports

The *in–vitro* 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 IC_{50} 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.

Peer review

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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