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Antimicrobial and Antioxidant Activity of Crude Extracts of *Rauvolfia caffra* var. *caffra* (Apocynaceae) From Tanzania

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Abstract

As part of an effort to search for extracts and compounds with new antimicrobial efficacy to fight against bacterial resistance, the antibacterial activity of *Rauvolfia caffra* var. *caffra* (Sond.), a plant of family Apocynaceae used in Traditional Medicine in Tanzania, was investigated. Ethanol, methanol and water extracts from leaf, stem and root barks were tested against three species of bacteria namely *Escherichia coli* (ATCC 25922) (Gram -ve), *Staphylococcus aureus* (ATCC 25923) (Gram +ve) and *Enterococcus faecalis* (ATCC 51299) (Gram +ve) using Agar-well diffusion assay method and minimum inhibitory concentration on Mueller-Hinton Agar plates. The extracting solvents were removed by vacuo evaporator to obtain gummy-like extracts. This was then dissolved in dimethylsulfoxide (10% DMSO). The DMSO without plant extracts was used as a negative control whereas Gentamicin® as the standard antibiotic was used as a positive control. The Zone of Inhibition (ZOI) measured in mm and Relative Inhibitory Zone Diameter (RIZD) was calculated. Results showed that *R. caffra* exhibited antimicrobial inhibitory activity at a range of 1.25 to 5.0 mg/ml with activity most prominent with methanol extract (ZOI of 28.33 ± 0.33 mm and RIZD of 95% for *S. aureus*; and ZOI of 26.66 ± 0.33 mm for *E. coli* and 19.0 ± 0.57 mm for *E. faecalis* at $P < 0.05$). To characterize further, the alkaloid from the root bark was extracted according to the standard procedure. The antioxidant activity of the alkaloids and ethanolic extracts was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and reducing capacity assays. The results indicated that alkaloid fraction of the root and 80% ethanolic extracts of stem bark exhibited high antioxidant activity. The phytochemical analysis indicated that *R. caffra* is rich in alkaloids, anthraquinones, anthocyanoides, flavonoids, saponins, tannis and reducing sugars. This study provides supportive evidence that methanol and ethanol extracts of *R. caffra* can be used as herbal medicine in control of *E. coli*, *S. aureus* and *E. faecalis*.

Keywords: antimicrobial, *E. faecalis*, *E. coli*, *S. aureus*, *Rauvolfia caffra*, ethnopharmacology, phytochemicals

1. Introduction

The increasing resistance of bacteria and fungi to currently marketed antimicrobial agents is becoming a world-wide medical problem (World Health Organization [WHO], 2004). In recent years many bacteria have developed antimicrobial drug resistance, these include but not limited to *Staphylococcus aureus* and most of the *Enterobacteriaceae*, such as *Klebsiella pneumonia* (WHO, 2004). Statistics indicate that more than 70% of the bacteria causing infections are resistant to at least one of the drugs most commonly used to treat them (WHO, 2004). For underdeveloped countries like Tanzania, HIV/AIDs pandemic, poverty and an upsurge of new and re-emerging infectious diseases, and high cost and side effects of available drugs (Humber, 2002) further aggravate this situation. Antimicrobial resistance has increased both severity of infectious disease and mortality rates from certain infections. This has necessitated studies on potential source of additional effective, safe and cheap antimicrobial alternatives, and plants are one of these sources that have not been exhaustively utilized. Plants have ability to synthesize a wide variety of chemical compounds such as alkaloids, glycoside, saponins, resins, lactose and essential oils (Soraya, 2011). Many of these phytochemicals have beneficial effects on a long term human health and may be used to effectively treat human disease (Lai & Roy, 2004).

Rauvolfia caffra var. *caffra* is a plant species belonging to family Apocynaceae. It is commonly known as “quinine tree” and is widely used in Sub-sahara Africa by natives as a medicine (Nkunya, 1992). In Tanzania, *R. caffra* is widely distributed in riverine *Brachystegia* woodlands, lowlands, in dry montane rainforests and in swamps (Food and Agricultural Organization [FAO], 1986). It is commonly found in highlands of Arusha, Kilimanjaro, Morogoro, Mbeya and Tanga regions in Tanzania. It is known as **Mwembemwitu, or mkufi** (Kiswahili), **Msesewe** (Kichagga), **Olchapukalyan** or **Oljabokaryan** (Maasai) (Njau, 2001; Mbuya, Msanga, Ruffo, Birnie, & Tengnäs, 1994). Ethnobotanical information indicates that *R. caffra* has been used widely for treatment of various diseases. Decoctions derived from stem bark are taken as an astringent, purgative or emetic to treat fever, swellings, abscesses, hepatitis and pneumonia (Tshikalange, Meyer, & Hussein, 2005). The pounded stem bark is applied against measles skin lesions or itching rashes (Schmelzer & Gumb-Fakin, 2008). A stem bark is chewed to cure cough and toothaches and also for treatment of venereal diseases (Njau, 2001). Root decoction is taken to treat fever and swollen legs (Tshikalange et al., 2005). The bitter bark is strongly purgative and said to produce severe abdominal pains; nevertheless the Pondo of South Africa use the bark for abdominal disorders (Bryant, 1995). The Vendas people of South Africa regard the plant as insecticidal and use the powdered bark to kill maggots in wounds. Bryant (1995) reported that Zulus use *R. caffra* bark along with other plants in a decoction for scrofula and also employ the powdered bark as an application to skin rashes caused by measles, urticaria and other forms of rashes. Chagga and Meru of North Eastern Tanzania use stem bark, which yields an astringent latex and mix with banana and millet to increase the potency of local brew commonly known as (**pombe ya mbege**) (Njau, 2001). Furthermore, *Rauvolfia* species are commonly used in the treatment of malaria, diabetes, and both parasitic and microbial infections (Amole, Onabanjo, & Agbaje, 1998; Campbell, Mortensen, Mølgaard, 2006). Although different species of *Rauvolfia* grow in Tanzania, *R. caffra* var. *caffra* is the most used species in North Eastern Tanzania in the treatment of various diseases. It is used in the treatment of coughs, gastrointestinal disturbances, skin infections, hypertension, diarrhea, dysentery, scabies, worm infections and malaria (Pesewu, Cutler, & Humber, 2008; Oyediji, 2007). The ethnomedicinal uses make it one of the most important medicinal plants used in the suppression of skin diseases and opportunistic infections in HIV/ AIDS patients in Tanzania (McMillen, 2004). *R. caffra* var. *caffra* (Apocynaceae) are rich in indole alkaloids most of which have been isolated and identified (Malik & Siddiqui, 1979; Nasser & Court, 1984). These alkaloids have various pharmacological properties including antimalarial, antitumor and antidiabetes efficacy (Katic, Kusan, Prosek, & Bano, 1980; Dewick, 2002). Furthermore, the extracts and alkaloids derived thereof may have high antimicrobial activity which is due to inhibition of some redox pathways and other biochemical processes in the bacterium cell (Mazza, Fukumoto, Delaquis, Girard & Ewert, 1999). As part of our efforts to search for extracts and compounds with high antibacterial efficacy, the stem bark, root barks and leaf extracts of *R. caffra* were screened for antibacterial activity. The most effective and active extracts was screened for antioxidant activity and evaluated further for pharmacological effects for its use in ethnomedicines.

Although studies on *Rauvolfia caffra* have been done elsewhere little information exist about the Tanzania species. In this study, the antimicrobial efficacy of *R. caffra* var. *caffra* was examined using three species of bacteria namely *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*; The antioxidant properties and phytochemical potential of the plant was also examined. The results will be useful and may contribute in the development of pharmaceutical industry.

2. Materials and Methods

2.1 Collection of Plant Materials

Plant material of *R. caffra* var. *caffra* (Figure 1) was collected from a coffee plantation at Njari village, in Uru North, Moshi, Tanzania, near a Catholic church (grid 3°16' 60'' S and 37°22'0''E) about 15 km north of Moshi Town in February, 2013. The village where *R. caffra* plant parts were collected has typical volcanic soils and receives biannual rainfall with short rain in October to December and long rains in March to June. The plant was authenticated by Mr. Emmanuel Mboya, a Botanist from National Herbarium of Tanzania (NHT) from Tropical Pesticides Research Institute (TPRI)-Arusha. A voucher specimen was kept at the herbarium with reference no. EN 2981. Leaves stem barks and root barks were collected, washed with tap water to remove soil debris followed with distilled water. They were then allowed to dry under shade at The Nelson Mandela African Institute of Science and Technology for 2 weeks. The plant material was pounded to fine powder using motor and pestle, packed and sealed in cellophane papers and transported by DHL to University of Saskatchewan-Canada, College of Pharmacy and Nutrition laboratories for analysis. All research ethical issues were adhered to, including material transfer agreement, sanitary and phytosanitary, plant export permit and biosafety regulation for plant material handling.



Figure 1. *Rauvolfia caffra* as found in the field at Uru North-Moshi

Source: field photo.

2.2 Extraction of the Plant

Powdered (100 g) *R. caffra* plant parts were weighed into three different conical flasks. To each of these flasks, 500 ml of one of the following solvents was added (95 % methanol, ethanol, or distilled water) and then stirred by means of magnetic stirrer for 30 minutes. The mixtures were macerated under sonicator (Bransonic Ultrasonic 5510 OR-DTH, Alberta, Canada). Sonication was carried in water bath, at room temperature (25°C) for 30 minutes. These mixtures contained in the three conical flasks were thereafter transferred to a shaker. The shaker was set at 60 rpm for 48 hours.

2.3 Filtration and Evaporation of the Sample

The extracts were filtered using Whatman® no.1 filter paper in Büchi funnel under vacuum pump. To evaporate, the samples were poured in a 250 ml round bottomed flask (Pyrex® USA). Concentration by evaporation was achieved using vacuum evaporation (Büchi Vacuum V-850 Switzerland). The bath temperature was set at 45°C. Evaporation was run until a gummy like material was formed. The concentrate extracts were stored in a refrigerator at 4°C until used.

2.4 Phytochemical Analysis

Powdered (20 g) *R. caffra* plant parts namely root barks; stem barks and leaf were weighed into separate conical flasks. To each of these flasks, 250 ml of one of the following solvents was added (95% methanol, ethanol or distilled water) and boiled. Thereafter, the solution was filtered in Whatman® no.1 filter paper in Büchi funnel using vacuum pump. The resulting plant filtrates were used for phytochemical screening for secondary metabolites. The different phytochemicals such as alkaloids, flavonoids, saponins, phenolics, anthraquinones, anthocyanosides and reducing sugars were tested using established methods.

2.4.1 Alkaloid Test

The presence of alkaloids in extracts was tested by using Wagner reagent prepared by dissolving 2 g of iodine and 6 g of potassium iodide in 100 ml of water as previously described by (Mamta & Jyoti, 2012). Two milliliters of Wagner reagents was added to 2 ml of extracts. The formation of reddish brown precipitate indicates the presence of alkaloids.

2.4.2 Test for Steroids

Test for steroids was done according to the method described by (Mohammad, Khulood, Salim, & Zawan, 2013) with modification. The plant extracts (1 mg) was taken in a test tube and dissolved with chloroform (10 ml) then added equal volume of concentrated H₂SO₄ to the test tube by sides. The upper layer in the test tube appears red and Sulphuric acid layer showed yellow with green fluorescence, which indicates the presence of steroids.

2.4.3 Flavonoids Test

A stock solution (2 ml) was taken in a test tube and 2-3 drops of dilute NaOH were added as per (Mohammad et al., 2013). An intense yellow colour appeared in the test tube. This solution becomes colourless when few drops of dilute H₂SO₄ are added confirming the presence of flavonoids.

2.4.4 Test for Saponins

Two grams (2 g) of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered as previously described by (Doherty, Olaniran, & Kanife, 2010). To the filtered sample (10 ml), about (5 ml distilled water) was added, shaken vigorously and observed for a stable persistent frothing for 25 minutes.

2.4.5 Test for Tannins

Test for tannins was done according to (Doherty et al., 2010) with some modification. Dried powdered sample (0.5 g) was boiled in water (20 ml) in a test tube and then filtered. One milliliters of 0.1% ferric chloride (0.01 Mol/dm^3) was added to 2 ml of each extract sample. Brownish green colourations indicate the presence of tannins.

2.4.6 Anthraquinones

Approximately 1 ml of the plant extract to be tested was shaken with 10 ml of benzene and then filtered as previously described (Doughari, Ndakidemi, Human, & Benade, 2012; Doherty et al., 2010). Five millilitres of the 10 % ammonia solution was then added to the filtrate and shaken. Appearance of a pink, red or violet color in the ammoniacal (lower) phase is an indication of presence of free anthraquinones.

2.4.7 Anthocyanosides

Test for anthocyanosides was done according to method previous descibed (Mamta & Jyoti, 2012). One millilitre of the plant filtrate was mixed with 5 ml of dilute HCl; a pale pink color indicates positive test.

2.4.8 Reducing Sugars

One millilitre of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown color with Fehling B and a green color with Fehling A indicate the presence of reducing sugars as previously described (Doughari et al., 2012).

2.5 Chemicals

Gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] trichloroacetic acid and ferric chloride were purchased from Sigma Aldrich (Alberta, Canada). All solvents were of HPLC grade and purchased from (Alberta, Canada). Middlebrook 7H9 broth base was obtained from HIMEDIA. Gentamicin® was purchased from Sigma (UK), 96 wells microtitre plates supplied by KAS Medics.

2.6 Test organisms

The testing microorganisms were obtained from the Western College of Veterinary Medicine, Department of Microbiology, University of Saskatchewan-Canada. These bacterial samples were pure and imported from the American Type Culture Collection (ATCC) as indicated in Table 1.

Table 1. Microorganisms used for activity tests on *R. caffra*

Name of bacteria	Stain type	ATCC number
<i>Escherichia coli</i>	Gram-	ATCC 25922
<i>Enterococcus faecalis</i>	Gram +	ATCC 51299
<i>Staphylococcus aureus</i>	Gram +	ATCC 25923

Key: Gram+ = Gram positive bacteria; Gram- = Gram negative bacteria.

2.7 Inoculum Preparation

Eighteen-hour broth culture of the test organism was suspended into sterile Mueller Hinton broth (MHB). It was standardized according to National Committee for Clinical Laboratory Standards (NCCLS, 2002) by gradually adding 9% normal saline to compare its turbidity to McFarland standard of 0.5, which is approximately 1.0×10^8 CFU/mL

2.8 Susceptibility Tests

Overnight broth culture was diluted to approximately 0.1 ml using McFarland scale (0.5 McFarland which is about 1×10^8 CFU/ml). The molten sterile Mueller-Hinton agar (20ml) was poured into sterile Petri plates and allowed to settle. The sterile Mueller-Hinton agar plates were swabbed (sterile cotton swabs) with the 18 hour old-broth culture of respective bacteria by careful striking and rotating the plates about 360 degrees. Wells (6 mm in

diameter and about 4 cm apart) were made in each of these plates using sterile borer no. 4. The extracts of the *R. caffra* plant were tested as follows: - An extract (0.2 g) was weighted and dissolved in 10 ml of dimethyl sulphoxide (DMSO) to obtain a concentration of 20 mg/ml. Extractions of 10 mg/ml of methanol, ethanol and water extracts of *R. caffra* stem bark, root bark and leaves for the same solvents were tested in turn. 100 µl of the different concentrations (20 mg/ml - 2.5mg/ml) of the extract was added to fill the bore holes. The negative control was prepared by putting 100 µl of (10 % DMSO) in one of the bored holes. Gentamicin® 10 µg discs were placed in each of the testing Petri plates as positive controls. One hour of pre-diffusion time was allowed, after which the plates were incubated at 37 °C for 18 to 24 hours. The diameters of zone of inhibition were then measured in millimeters. The above method was carried out in triplicate and the mean of the triplicate results were taken.

2.9 Minimum Inhibitory Concentrations (M.I.Cs) and Minimum Bactericidal Concentrations (M.B.Cs)

Graded concentrations of the extracts ranging from 20 mg/ml to 0.625 mg/ml were used. Extract solution (concentration 20 mg/ml) was serially diluted two fold in Mueller-Hinton broth (MHB) to give decreasing concentration of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml. An aliquot (0.1 ml) of overnight broth culture of test microorganism (concentration 1.5×10^8 CFU/ml) in a sterile normal saline was introduced into each extract dilution. The mixture in test tubes were incubated at 37°C for 24 hrs and observed for turbidity (signifying growth) or absence of it (signifying inhibition). Gentamicin®, a standard antibacterial drug was used as a positive control and sterile normal saline without extract nor drug was used as a negative control. The minimum inhibitory concentration was the lowest concentration of extract solution that inhibited microbial growth.

2.10 Percentage Relative Inhibitory Zone Diameter for *E. coli*, *S. aureus* and *E. faecalis* for Different Extracts

Antibacterial activity was determined by measuring the inhibition zone diameter (mm) against each test organism. The antimicrobial activity expressed as percentage relative inhibition zone diameter (RIZD) was calculated according to (Rojas, Veronica, Saul, & John, 2006) as follows:

$$\%RIZD = \left(\frac{IZD \text{ sample} - IZD \text{ negative control}}{IZD \text{ standard antibiotic}} \right) \times 100 \quad (1)$$

RIZD is the percentage of relative inhibition zone diameter and IZD is the inhibition zone diameter (mm).

2.11 Total Alkaloid Extraction

A total alkaloid extraction from *R. caffra* root was done according to (Rujjanawate, Kanjanapothi, & Pathong, 2003) with some modification. Approximately 15.58 g of the extracts was dissolved in 350 ml distilled water in a 500 ml flask. The flask and its contents were shaken in the water bath set at 40°C to further dissolve the extracts and obtain a homogenous solution. Thereafter the mixture was acidified by adding 10 ml of 5% sulphuric acid in water. Subsequently the acid solution was repeatedly washed with 100 ml Chloroform to remove neutral substances. The aqueous acidic solution was then made basic with 15 ml of 20% Ammonium hydroxide and extracted again with Chloroform until the aqueous layer was free of alkaloids. The combined total chloroform extracts were evaporated in vacuo evaporator (Büchi vacuum V-850, Switzerland) to yield 2.91 g of alkaloid as shiny brown powder. The crude alkaloids were spotted on thin layer chromatographic plates and developed using dichloromethane / methanol (10: 1). In order to confirm the presence of alkaloids, the TLC plates were sprayed with Dragendorff reagent to give three major orange spots and some other minor compounds.

2.12 Determination of DPPH Radical Scavenging Activity

In order to determine the DPPH radical scavenging activity of *Rauvolfia caffra* stem bark extracts and alkaloid extracts from the root the method described by (Liyana-Pathirana & Shahid, 2005; Cuendet, Hostettmann, & Potterat, 1997) was employed with some modifications. A volume of 0.5 ml of 0.12mM DPPH solution in methanol was separately mixed with 2 ml of 0.01, 0.025, 0.05 and 0.075 mg/ml of the extracts/ alkaloids in methanol and vortexed thoroughly. The absorbance of the mixture at ambient temperature (25°C) was recorded for 30 minutes at 10 minute intervals. Gallic acid (GA) was used as reference antioxidant compound. The absorbance of remaining DPPH radical was read at 519 nm using a Jenway 6505 UV/Vis spectrophotometer (Cole-Parmer, Canada). The analysis of each assay solution was replicated thrice. The scavenging of DPPH radical was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \quad (2)$$

Where A control is the absorbance of DPPH radical in methanol, A sample is the absorbance of DPPH radical + sample extract or / standard.

2.13 Reducing Capacity

For reducing capacity determination, the method of Oyaizu (1986) was adapted with some modification. Stem bark extracts at 0.01, 0.025, 0.05 and 0.075 mg/ml were mixed with 2.5 ml of 0.02 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was then incubated at 50°C for 20 min. Aliquots (2.5ml) of 10 % trichloroacetic acid were added to the mixture, which was then centrifuged for 10 minutes at 1000 rpm. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % $FeCl_3$, and absorbance was measured at 700 nm in a Jenway 6505 UV / Vis spectrophotometer (Cole-Parmer, Canada). The same procedure was done using alkaloid extract of the root part of *R. caffra*. Gallic acid was used as a standard antioxidant compound. The analysis of each assay was done in triplicates.

2.14 Statistical Analysis

The statistical analysis was performed according to (Steel, Torrie, & Dickey, 1980) using the one way analysis of variance (ANOVA) with the computations being performed with STATISTICA software program. The Fisher's Least Significance Difference (L.S.D) was used to compare treatment means at $P=0.05$ level of significance. Results are expressed as mean \pm standard error (Mean \pm S.E).

3. Results

3.1 The Determination of Diameter of Zone of Inhibition (mm) using Agar wells Assay

The inhibitory effects of different parts of *R. caffra* namely stem and roots barks as well as leaf using different extracting solvents against the three pathogens *E. coli*, *S. aureus* and *E. faecalis* were determined. The results of zone of inhibition (ZOI) against tested pathogens are presented in Table 2. A general trend shows that methanolic root bark extracts (RBMe) was more effective against all the three tested pathogens with values of 28.33 ± 0.33 mm, 26.66 ± 0.33 mm and 19.0 ± 0.57 mm against *S. aureus*, *E. coli* and *E. faecalis*, respectively. This was followed by stem bark ethanolic extracts (SBEt). Root bark (RBWa) and stem bark (SBWa) aqueous extracts demonstrated moderate effectiveness against the tested pathogens. The lowest value was given by leaf aqueous extracts with value of 8.3 ± 0.67 mm, 6.67 ± 0.33 mm and zero against *S. aureus*, *E. coli* and *E. faecalis*, respectively. However, Gentamicin®, the standard antibiotic demonstrated the highest zone of inhibition diameter with values ranging from zero to 30.0 ± 0.57 mm (*E. coli*), 29.6 ± 0.33 mm (*S. aureus*) and 27.0 ± 1.2 (*E. faecalis*) at ($P \leq 0.05$) level of significance. The higher ZOI values for methanol and ethanol extracts may be explained in terms of solvent polarity. It is likely that *R. caffra* contains active ingredients that are more soluble in polar solvent and has been the reason for higher activity.

Table 2. Activity of plant extracts indicating zone of inhibition (mm) on selected bacterial species

Zone of inhibition in (mm) for different bacteria species			
Extract/ drug	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>
SBEt	$20.66 \pm 0.33e$	$18.66 \pm 0.33e$	$11.0 \pm 0.57e$
RBEt	$22.66 \pm 0.33d$	$20.66 \pm 0.66d$	$12.66 \pm 0.88d$
SBMe	$24.66 \pm 0.33c$	$24.33 \pm 0.33c$	$16.67 \pm 0.33c$
RBMe	$26.66 \pm 0.33b$	$28.33 \pm 0.33b$	$19.0 \pm 0.57b$
SBWa	$17.66 \pm 0.33g$	$14.33 \pm 0.33g$	$7.0 \pm 0.33g$
RBWa	$19.33 \pm 0.33f$	$17.0 \pm 0.0f$	$9.0 \pm 0.0f$
LEt	$11.33 \pm 0.33i$	$13.0 \pm 0.0h$	$0 \pm 0.0h$
LMe	$13.33 \pm 0.33h$	$15.0 \pm 0.0g$	$0 \pm 0.0h$
LWa	$6.67 \pm 0.33j$	$8.3 \pm 0.67i$	$0 \pm 0.0h$
DMSO	$0 \pm 0.0k$	$0 \pm 0.0j$	$0 \pm 0.0h$
Gentam	$30.0 \pm 0.57a$	$29.6 \pm 0.33a$	$27.0 \pm 1.2a$
One way ANOVA F –statistics value			
Extract/drug	66.55***	57.08***	30.04**

Values presented are Mean \pm SE; **, *** significant at $P \leq 0.01$, $P \leq 0.001$ respectively, ns = not significant; SE = standard error; Means followed by dissimilar letter(s) in a column are significantly different from each other at $P=0.05$ according to Fischer Least Significance Difference (LSD); **Key:** SBEt-Stem Bark Ethanolic extract; RBEt-Root Bark Ethanolic extract; SBMe-Stem Bark Methanolic extract; RBMe-Root Bark Methanolic extract; SBWa-Stem Bark Water extract; RBWa-Root Bark Water extract; LEt-Leaf Ethanolic extract; LMe- Leaf Methanolic extract; LWa- Leaf Water extract; DMSO- Dimethylsulfoxide; Gentam-Gentamicin, standard antibiotic.

3.2. Percentage RIZD for *E. coli*, *S. aureus* and *E. faecalis* for Different Extracts

The results of percentage relative inhibition zone diameter (RIZD) against the tested pathogens are presented in Table 3. The general trend revealed that methanolic root barks extracts of *R. caffra* (RBMe) gave higher percentage relative inhibition zone diameter with values ranging from zero to $96.0 \pm 1.2\%$, $89.0 \pm 2.0\%$ and $69.0 \pm 0.88\%$ against *S. aureus*, *E. coli* and *E. faecalis*, respectively. The ethanolic root barks (RBet) and stem bark (SBet) had RIZD values ranging between 60-70%. Lowest values were given by aqueous extracts of leaf parts. However, it was observed further that the ethanolic, methanolic and aqueous extracts of leaf part of *R. caffra* had zero effect against *E. faecalis*. This indicates that *E. faecalis* has higher resistance against these extractions and / or drugs.

Table 3. Calculated percentage RIZD for *E. coli*, *S. aureus* and *E. faecalis* for different extracts

Extract/ drug	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>
SBet	$69.0 \pm 1.5d$	$63.0 \pm 1.73d$	$40.0 \pm 3.0d$
RBet	$75.0 \pm 1.8c$	$69.0 \pm 1.76c$	$46.0 \pm 1.85c$
SBMe	$82.0 \pm 1.8b$	$82.0 \pm 1.0b$	$60.0 \pm 2.40b$
RBMe	$89.0 \pm 2.0a$	$96.0 \pm 1.2a$	$69.0 \pm 0.88a$
SBWa	$59.0 \pm 0.57f$	$48.0 \pm 0.88f$	$27.0 \pm 2.33f$
RBWa	$64.0 \pm 0.88e$	$57.0 \pm 0.66e$	$33.0 \pm 1.45e$
LEt	$39.0 \pm 1.2h$	$43.0 \pm 0.66g$	$0.0 \pm 0.0g$
LMe	$44.0 \pm 0.66g$	$50.0 \pm 0.66f$	$0.0 \pm 0.0g$
LWa	$22.0 \pm 0.66i$	$28.0 \pm 2.0h$	$0.0 \pm 0.0g$
DMSO	$0.0 \pm 0.0j$	$0.0 \pm 0.0i$	$0.0 \pm 0.0g$
One way ANOVA F-statistics value			
Extract/drug	64.9***	69.1***	28.9**

Values presented are Mean \pm SE; **, *** significant at $P \leq 0.01$, $P \leq 0.001$ respectively; ns = not significant; SE = standard error; Means followed by dissimilar letter(s) in a column are significantly different from each other at $P = 0.05$ according to Fischer Least Significance Difference (LSD). **Key:** SBet-Stem Bark Ethanolic extract; RBet-Root Bark Ethanolic extract; SBMe-Stem Bark Methanolic extract; RBMe-Root Bark Methanolic extract; SBWa-Stem Bark Water extract; RBWa-Root Bark Water extract; LEt- Leaf Ethanolic extract; LMe- Leaf Methanolic extract; LWa- Leaf Water extract; DMSO- Dimethylsulfoxide; Gentam-Gentamicin, standard antibiotic.

3.3 Qualitative Phytochemicals Analysis of *R. caffra*

The phytochemical screening of *R. caffra* for secondary metabolites indicated the presence of the following major compounds namely alkaloids, anthracene, flavonoids, glycoside, reducing sugars, saponins and tannis.

Table 4: Results of qualitative phytochemicals analysis of *R. caffra*

Phytochemical	SBet	RBet	SBMe	RBMe	SBWa	RBWa	LEt	LMe	LWa
Alkaloid	+	+	+	+	+	+	+	+	+
Anthracene	—	—	+	+	+	+	—	—	—
Flavonoids	+	+	+	+	+	+	+	+	+
Glycoside	+	+	+	+	+	+	+	+	+
Saponin	+	+	+	+	+	+	+	+	+
Reducing sugars	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+

Key: SBet-Stem Bark Ethanolic extract; RBet-Root Bark Ethanolic extract; SBMe-Stem Bark Methanolic extract; RBMe-Root Bark Methanolic extract; SBWa-Stem Bark Water extract; RBWa-Root Bark Water extract; LEt- Leaf Ethanolic extract; LMe-Leaf Methanolic extract; LWa-Leaf Water extract.

3.4 Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) for different extracts of *R. caffra* are presented in Table 5. The results showed that root extracts generally had more promising activity when compared with the leaf extracts at a given concentration. The best extracts that demonstrated inhibitory effects at lower concentrations include both methanol and ethanol root extracts with a value of 1.25 mg/ml against *E. coli* and *S. aureus*. The ethanol and methanol leaf extracts followed with the MIC value of 2.50 mg/ml against *E. coli* and *S. aureus*. In contrast the minimum inhibitory concentration that had effect against *E. faecalis* had a value of 5.00 mg/ml for both root and leaves methanol and ethanol extracts. However, *E. faecalis* showed resistance against extracts of leaves and using ethanol as well as methanol.

Table 5. Minimum inhibitory concentration of *R. caffra* extracts with antibacterial activity

Bacteria spp	Minimum inhibitory concentration of <i>R. caffra</i> extracts mg/ml									
	SBEt	RBET	SBMe	RBMe	SBWa	RBWa	LEt	LMe	ALk	Gent
<i>E.coli</i>	1.25	1.25	1.25	1.25	2.50	2.50	5.0	5.0	1.25	1.25
<i>S.aureus</i>	1.25	1.25	1.25	1.25	2.50	2.50	5.0	5.0	0.625	1.25
<i>E. faecalis</i>	5.00	5.00	5.00	5.00	5.00	5.00	N/A	N/A	2.50	5.00

Key: SBEt-Stem Bark Ethanolic extract; RBET-Root Bark Ethanolic extract; SBMe-Stem Bark Methanolic extract; RBMe-Root Bark Methanolic extract; SBWa-Stem Bark Water extract; RBWa-Root Bark Water extract; LEt-Leaf Ethanolic extract; LMe-Leaf Methanolic extract; ALk-Alkaloid; DMSO- Dimethylsulfoxide; Gentam-Gentamicin, standard antibiotic; N/A-no activity observed.

3.5 DPPH Radical Scavenging Activity

The free radical scavenging activity of 80% ethanolic aqueous extract of the stem bark (SBEt) and alkaloids from the root of *Rauvolfia caffra* was determined from a reduction of absorbencies of DPPH radical at 519 nm. In this assay, both 80% ethanolic stem bark aqueous and alkaloid extracts exhibited higher antioxidant activity than gallic acid, a standard and natural antioxidant compound (Figure 2). The order of activity was alkaloids > 80% ethanolic (SBEt) aqueous > Gallic acid. At 0.03 mg /ml the alkaloid extract scavenged 98 ± 0.88 % of the DPPH radical while 80% ethanolic aqueous extract had 86 ± 0.88 % and gallic acid had 82 ± 1.2 % (Figure 2). The observed trend was such that, the activity of each test sample increased with concentration and time.

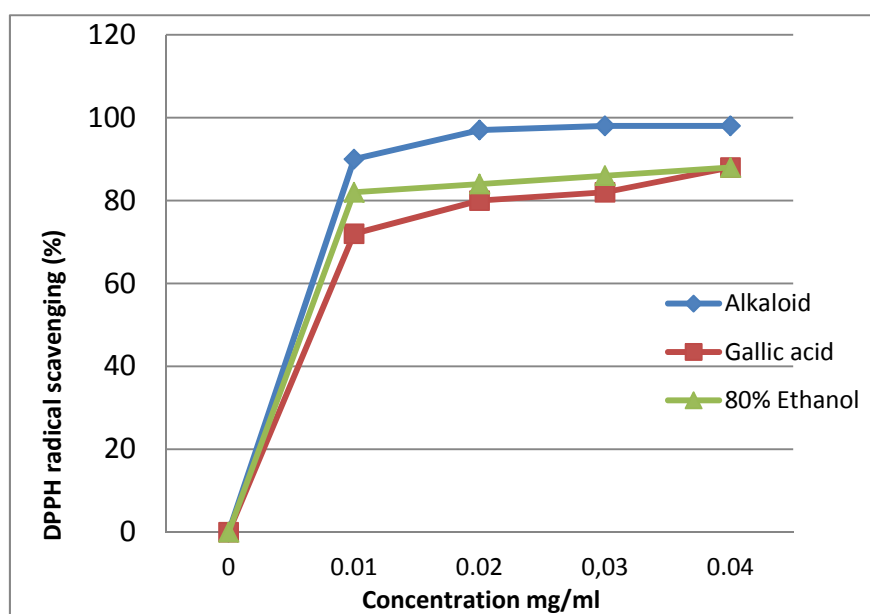


Figure 2. The DPPH radical scavenging activity of ethanolic aqueous and alkaloid extract from the stem bark and root bark of *R. caffra* compared with gallic acid after 60 minutes of reaction. Each value is expressed as mean \pm S.E (n=3)

3.6 Reducing Capacity

The antioxidant activity of the stem bark of *R. caffra* was further manifested through their reducing power as shown in (Figure 3). In this assay, the $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ transformation was established as reducing capacity. Again alkaloid had superior reducing power than the other assayed samples, followed by 80% ethanolic aqueous (SBET) extract and then gallic acid. At 0.03 mg/ml the absorbancies of alkaloids, 80% ethanolic aqueous extract and gallic acid (at 700nm) were 0.75 ± 0.057 , 0.72 ± 0.057 and 0.65 ± 0.09 , respectively. However, at 0.075 mg /ml the absorbencies of all samples were of the same order (Figure 3). This trend shows that reducing capacity increased with increasing concentration of the sample. This implies that the strength of donation of electrons is directly related to the concentration of extracts.

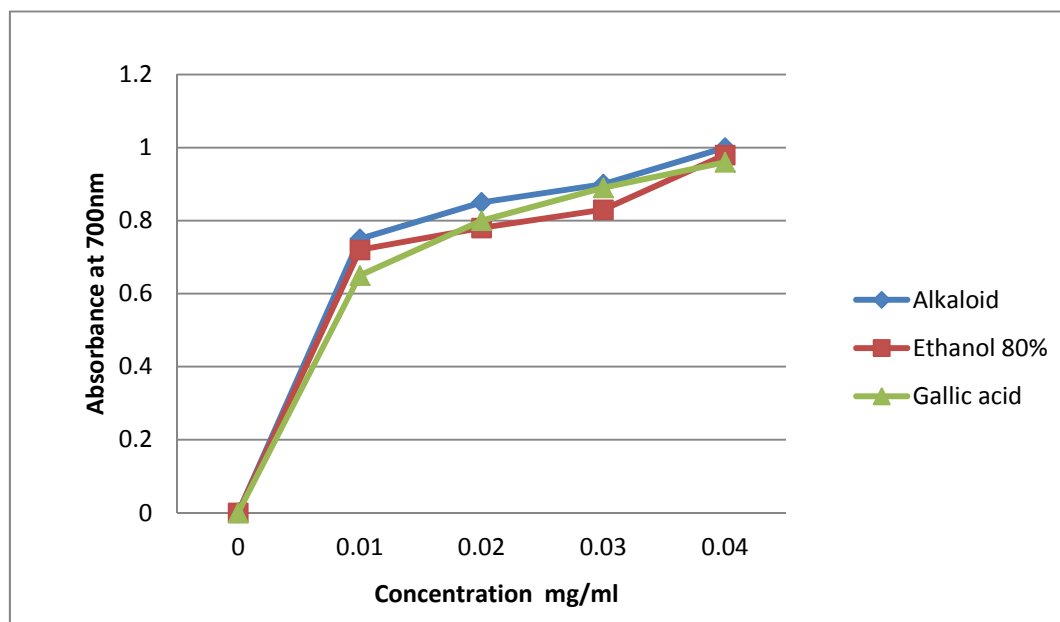


Figure 3. Reducing capacity of different amounts of ethanolic aqueous of stem bark and alkaloid extract from the root bark of *R. caffra* compared with gallic acid (a standard antioxidant compound) using spectrophotometric detection of $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ transformation. Each value is expressed as mean \pm S.E (n=3)

4. Discussion

The study showed that the aqueous and methanolic extract of the stem bark, root and leaf of *R. caffra* exhibit antibacterial activities against tested bacterial species. However, the methanolic extracts of the root and stem barks showed more antibacterial activity against gram negative bacteria (*E. coli*) than the gram positive bacteria. The ethanolic aqueous and alkaloid extracts of the roots of *R. caffra*, exhibited moderate to high antimicrobial activity against the tested bacterial strains. The alkaloid extracts of the roots of *R. caffra* were more active than all test samples with MIC values ranging from 0.625 mg /ml to 1.25 mg/ml and 5.00 mg /ml against *S. aureus*, *E. coli*, and *E. faecalis*, respectively. The presence of the chemical constituents, tannin, alkaloid, glycoside, saponin, flavonoids, reducing sugars in the stem and root barks of *Rauvolfia caffra* has added to the claim that plants possess chemical substances in their various parts. These classes of chemical compounds in the plant extracts are known to show curative effects against several pathogens. The mechanism of inhibitory action of these phytochemicals on microorganisms may be due to the impairments of variety of enzymes systems, including those involved in energy production, interference with the integrity of cell membrane and structural component synthesis (Okwu & Morah, 2007; Ali & Dixit, 2012). From previous studies about thirty two alkaloids have been reported by many workers to exhibit antimicrobial activities (Nasser & Court, 1984; Elisabetsky & Costa-Campos, 2006). This is a good indication that these extract have a mechanism of overcoming the barriers of the gram-negative cell wall. *Escherichia coli* was found to be more susceptible to the extract than the other organisms, this might suggest that the plant may contain some antidiarrhoeal properties. *E. faecalis* showed little susceptibility and only to the aqueous extract of the stem bark and the ethanolic extract of the leaf. This organism has been reported to be resistant to antimicrobial substances (Livermore, Winstanley, & Shannon, 2001).

Stem bark and the root extracts demonstrated no significant differences in antibacterial activities with root and leaf extracts at $P > 0.05$ but significant difference was observed in the antibacterial activities of the stem bark and leaf extract against *E. coli* at $P < 0.05$. Generally, the root bark extract was observed to be more potential than the stem bark and the leaf extracts. The stem bark of *R. caffra*, from previous reports, has more medicinal uses as compared with roots and leaf (Tshikalange et al., 2005; Schmelzer & Fakin, 2008). The ethanolic extract showed more antibacterial activities than the aqueous extracts. The plant may contain active ingredients that are more soluble in polar solvent and these were responsible for the activity. The higher ZOI values for methanol and ethanol extracts may be explained in terms of solvent polarity. It is likely that *R. caffra* may contain active ingredients that are more soluble in polar solvent and has been the reason for higher activity. Cowan (1999), reported that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily be solubilized in organic solvents. This can justify the traditional use of methanol in extracting plants components in the control of pathogens (Pandit & Langfield, 2004). The activity of ethanolic extract of stem bark of this plant can compete favorably with the activity of gentamicin in this study. The antibacterial activities of the aqueous and ethanolic extracts of the stem bark, root of *R. caffra* shown in this study may justify the traditional use of the plant in the treatment of bacterial induced ailments. However, there has been no report of use synergistically the extract of the stem bark, root and leaf of *Rauvolfia caffra* in the treatment of diarrhea or related diseases and this can be an important aspect of the plant. Gentamicin has been reported to be active against many strains of Gram positive and Gram negative strains.

The alkaloid extracts demonstrated higher free radical scavenging activity than gallic acid and ethanolic aqueous extracts of the stem of *R. caffra*. Furthermore, they also exhibited good electron donating ability, which implies that alkaloids may be inhibiting some redox pathways in the bacterial cell thereby slowing their growth or causing death of microbes (Hamilton, Finlay, Stewart, & Bonner, 2009). This pharmacological property adds value to the potential antimicrobial efficacy of the alkaloids from the roots of *R. caffra*. The electron donating ability of the alkaloid is very important in the inhibition of the bacterial cell growth as the bacterial cell utilizes NADPH dependent reductase enzymes to maintain an intracellular reduced environment in the cells (Hamilton et al., 2009). The DPPH free radical scavenging activity displayed by *R. caffra* methanolic extract compare closely to the currently known scavenging bioorganic molecules and could indicate a potential source of natural anti-oxidants that can be formulated into commercial products. Naturally antioxidants have a fundamental physiological role in the human body by reducing tissue damaging free radical (Tapiero, Tew, Nguyen, & Mathe, 2002; Demo et al., 1998). The provision of these possibly free radical quenching agents from the medicinal plants can be envisaged to contribute towards the antioxidant preventive measures; when consumed, they can improve the digestive system, can function on reduction of coronary heart diseases, and some types of cancer and inflammations (Jayasri et al., 2009).

5. Conclusion

The present investigation indicates that *R. caffra* contains potential antimicrobial bioactive compounds that may be of great use for the development by pharmaceutical industries as a therapy against bacterial related diseases. The ethanol, methanol and aqueous extracts of *R. caffra* possess significant inhibitory effects against tested pathogens namely *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*. The results of study supports the Indigenous knowledge on the use of the plant as medicine along with the development of new antimicrobial drug from both roots and stems parts of this plant.

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