JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4325-4334

Brine Shrimps Toxicity and *In vitro* Antibacterial Potentials of the Crude Methanolic Stem Bark Extract of *Erythrina caffra* thunb. against Bacteria in Infections

Olubunmi A. Wintola, Olufunmiso O. Olajuyigbe and Anthony J. Afolayan*

Medicinal Plants and Economic Development Research Centres,
Department of Botany, University of Fort Hare, Alice, 5700, South Africa.

(Received: 12 May 2014; accepted: 25 June 2014)

The *in vitro* antibacterial and cytotoxicity activities of crude methanolic extract of the stem bark of *Erythrina caffra* Thunb. was investigated. MIC and MBC were used for the antimicrobial assay while the cytotoxic activity of the plants extract was determined using brine shrimp lethality test. In this study, the extract was active against all the Gram-positive and Gram-negative bacteria investigated with *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis* and *Micrococcus luteus* being the most susceptible. The extract exhibited antibacterial activities with inhibition zones being concentration dependent. At the highest concentration (4500 μg/ml), the extract produced inhibition zones ranging between 18 and 22 ± 1.00 mm. While the agar dilution indicated that the bacteria were susceptible at concentrations ranging between ≤100 and ≤ 1000 μg/ml, the macrobroth dilution showed that the MICs of the methanolic extract ranged between 20 μg/ml and 2500 μg/ml and the brine shrimp lethality assay indicated that the LC_{50} and LC_{90} were 40.22 μg/ml and 56.66 μg/ml respectively. Although the extract was effective against the two groups of bacteria, the Gram-negative bacteria were more susceptible to the extract than the Gram-positive bacteria. The ability of this extract to inhibit bacteria at low concentrations is an indication of its broad spectrum antibacterial potential which may be exploited in the management of microbial infections. The study, therefore, suggests that there are phytochemical constituents in the extract which could serve as a source of chemotherapy drugs useful in the treatment of infections and justifies the use of *Erythrina caffra* traditionally for the treatment of gastroenteritis in the rural communities.

Key words: Antibacterial activity, broad spectrum, cytotoxicity, methanolic extract, macro-dilution assay.

Plant-derived medicines have been part of traditional health care in many parts of the world because the antimicrobial properties of their components are well documented[1,2]. These documentations resulted from the systematic screening of these plants in attempts to discover novel effective compounds[3](Tomoko *et al.*, 2002). While many of the identified compounds have been found to be effective in the treatment of infectious diseases and simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials[4], Chemical constituents in plants with great potentials for medicinal use are now being explored by both traditional healers and pharmaceutical drug companies[5-7].

Apart from the significance age long roles played by plants in traditional health-care delivery in most parts of the world, there is increasing interest in plants as sources of agents to fight

* To whom all correspondence should be addressed.
Fax: +27866282295;
E-mail: Aafolayan@ufh.ac.za

microbial diseases[8]. While World Health Organization has recognized medicinal plants as the best sources for obtaining a variety of synthetic drugs, national and international policies are recently encouraging complementary alternative medicines in order to create a balance between modern and traditional medicine. Today, intense studies have been focused on determining whether traditional uses of plants are supported by actual pharmacological effects or merely based on folklore[9,10]. Accordingly, many of the main active ingredients responsible for these antimicrobial activities in plants have been identified and isolated in various studies[11,12].

Erythrina is a genus of flowering plants in the pea faily, Fabaceae. It contains about 130 species which are distributed in tropical and subtropical regions worldwide with approx 100 Erythrina in the warm regions of the world[13]. Nine of these trees are found in southern Africa where the most common species are probably the *Erythrina lysistemon* and *Erythrina caffra* growing up to 30 m in height. *Erythrina caffra* Thunb. is a tree native to southeastern Africa and Mozambique. It is often cultivated and has introduced populations in India and Kenya[14]. Various parts of this plant are commonly used in African folk medicine for stomach pain and microbial infections[15,16]). Although the bark of *E. caffra* is used topically to treat sores, wounds, abscesses and arthritis, ethnobotanical studies[17] indicated that the traditional uses of this plant strongly suggest antibacterial, anti-inflammatory, anti-blood-clotting and analgesic effects. *Erythina* species have many physiologically active alkaloids[18]. Though known to be highly toxic, *Erythrina* alkaloids are attractive synthetic targets resulting from their use in indigenous medicine[19,20]. Prenylated flavonoids, prevalent in the stem and root bark[21,22], however, display a variety of biological activities.

Though different *Erythrina* species is a medicinal plant of pharmacological importance, there is a dearth of information on the antibacterial and cytotoxic activities of crude methanol extract of *Erythrina caffra* Thunb. The ethnobotanical data of this plant prompted an investigation into its antibacterial activity. Hence, the present study was aimed at investigating the antibacterial and brine shrimp toxicity potential of the methanolic stem bark extract of *E. caffra* in order to validate its ethnomedicinal use in the treatment of diarrhea and dysentery infections.

MATERIALS AND METHODS

Plant collection and identification

The bark materials of *E. caffra* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University. The bark samples were air-dried at room temperature and pulverized with a milling machine before extraction. The water extract was prepared by soaking 100 g of the pulverized sample in 500 ml of methanol. The mixture was allowed to stand for 72 h on rotary shaker (Stuart Scientific Orbital Shaker, UK) before being filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator (Laborota 4000 - efficient, Heldolph, Germany) at 50°C. The crude extract collected was allowed to dry at room temperature to a constant weight. The dried crude extract was redissolved in methanol before being diluted with sterile distilled water to the required concentrations for the bioassay. The corresponding concentration was expressed as µg of extract per ml of solvent (µg/ml).

The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter. The sterility of the extract after membrane filtration was confirmed by introducing 2 ml of this extract into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. A sterile extract was indicated by absence of turbidity in the broth after the incubation period[23].

Test organisms

Twelve bacteria used in this study included *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Pseudomonas aeruginosa* ATCC 19582, *Proteus vulgaris* CSIR 0030, *Acinetobacter calcoaceuticus anitratus* UP, *Micrococcus luteus*, *Proteus vulgaris* KZN, *Enterobacter faecalis* KZN, *Salmonella typhi* ATCC 13311 and *Escherichia coli* ATCC 25922. These strains were obtained from the Department of Biochemistry and Microbiology,

University of Fort Hare, Alice, South Africa. The organisms were maintained in nutrient broth and nutrient agar (Biolab) while Mueller Hinton II Agar and broth (Biolab) were used for susceptibility, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay.

Determination of zones of inhibition

The susceptibility screening of the test bacteria to the crude methanolic stem bark extract of *Erythrina caffra* and ciprofloxacin, used as control, was carried out in accordance with the methods described by Irobi *et al.*[24] and Akinpelu *et al.*[25]. The inoculum of each test bacterial strain was standardized at 5×10^6 cfu/ml using McFarland Nephe-lometer standard[26]. The antibacterial activity was determined by using the modified Kirby-Bauer diffusion technique[27] involving swabbing Mueller-Hinton agar (MHA) (Oxoids Ltd, Basingstoke, Hampshire, UK) plates with the resultant saline suspension of each adjusted bacterial strain. Wells were bored into the agar media with a sterile 6 mm cork borer. The wells were filled with 100 µl of different concentrations (750, 1500, 3000 and 4500 µg/ml) of the extract and ciprofloxacin (2.5 and 5 µg/ml) without allowing spillage of the extract solution onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract before being incubated at 37°C for 24 h. Wells in Mueller Hinton agar plates containing 5% methanol representing the final methanol concentration in the test plates without the extract served as positive controls. Assays were performed in duplicates. After 24 h incubation period, the diameters of the inhibition zone produced by different concentrations of the extract solutions were measured in millimeters[28] and interpreted using the CLSI zone diameter interpretative standards[29].

Antimicrobial assay by agar dilution methods

To determine the antibacterial activity of the extract by the agar dilution method described by Afolayan and Meyer[30], different concentrations of the extract ranging between 100 and 10000 µg/ml were prepared in molten Mueller Hinton agar maintained in a water bath at 50°C and used for the agar dilution assay. One hundred microlitres (100 µl) of the standardized bacterial cultures were aseptically dispensed and spread evenly on the agar plates. Two Mueller Hinton agar plates containing 5% methanol representing the final methanol concentration in the test plates without the extract served as negative controls. Another two blank plate containing only Mueller Hinton agar served as negative controls. Plates were incubated at 37°C for 24 h. Each test was done in duplicate and any test agar plate lacking visible growth was considered the minimum inhibitory concentration of the extract.

Macrobroth Dilution for Determining Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) defined as the lowest concentration which resulted in maintenance or reduction of inoculums' viability was determined by serial tube dilution technique for the bacterial isolates. Different concentrations (19.5 – 10000) µg/ml of the crude extract was differently prepared by serial dilutions in the Mueller Hinton broth medium. Each tube was then inoculated with 100 µl of each of the adjusted bacterial strain. Two blank Mueller Hinton broth tubes, with and without bacterial inoculation, were used as the growth and sterility controls. The bacteria containing tubes were incubated at 37 °C for 24 h. After the incubation period, the tubes were observed for the MICs by checking the concentration of the first tube in the series (ascending extract concentrations) that showed no visible trace of growth. The first tube in the series with no visible growth after the incubation period was taken as the MIC. The MIC, therefore, was the lowest concentration of the extract that yielded no visible growth after the incubation period[31].

Determination of Minimum Bactericidal Concentrations (MBC)

The MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette and a 100 µl aliquot was removed. Each aliquot was placed on a sterile extract-free nutrient agar plate in a single streak down the center of the plate in accordance with the method of Shanholtzer *et al.*,[32]. The samples were allowed to be absorbed into the agar until the plate surface appeared dry (after 30 min). The aliquot was then spread over the plate by making a lawn of the bacterial culture with a sterile cotton swab. The MBC determining lawned plates were incubated

for 24 h at 37°C. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as the MBC values for this crude extract[33]. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation. In order to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic, MIC_{index} (MBC/MIC) ratios were calculated against each test strain. MIC_{index} ratios greater than 1 was considered bacteriostatic. MIC_{index} ratios less than or equal to 1were considered bactericidal. If the ratio was greater than or equal to 16.0, the extract was considered ineffective.

Brine shrimp lethality test

The brine shrimp lethality test using the larvae of brine shrimp nauplii, *Artemia salina* L was carried out using the standard procedure[34,35]. For the cytotoxicity assay different concentrations (0.9765 - 125 µg/ml) of the extract were prepared from the stock solution of the extract by serial tube dilution technique in different vials. Ten nauplii were transferred into each vial using Pasteur pipettes and were not given food because hatched brine shrimp can survive for up to 48 h without food[36] as they still feed on their yolk-sac[37]. The control vials were prepared using 5% methanol only and the experiment was replicated three times. After 24 h of incubation, larvae were considered dead if they did not exhibit any observable movement during several seconds of observation. The numbers of survivors in each vial were counted and percentages of mortality were calculated for each concentration. The extract was regarded as being mildly toxic if LC_{50} >30<100 µg/ml and non-toxic if its LC_{50} is greater than 100 µg/ml in the brine shrimp lethality assay[38]. The mean mortality percentage and lethal concentrations (LC_{50}) and (LC_{90}) were determined using statistical analysis and the graph of Logarithm of concentration against percent lethality[39].

RESULTS

The initial screening of the antibacterial activities of the extract was assayed *in vitro* by the agar dilution and diffusion methods. The two assay methods which are complementary showed that the bacteria exhibited varied susceptibility to the extract at the different concentrations used. The crude methanolic bark extract of *E. caffra* inhibited all the tested Gram-negative and Gram-positive bacteria producing concentration dependent inhibition zones with varied sizes (Table 1). The degree of the antibacterial activity of the extract on the test organisms was indicated by the varied minimum inhibitory and bactericidal

Table 1. Inhibition zone diameters indicating activity of the crude methanolic bark extract of Erythrina caffra Thunb

	Average Inhibition Zone Diameters (± 1.0 mm) from agar diffusion assay					
	Ciprofloxacin			Methanolic crude extract		
	2.5	5	750	150	3000	4500
	µg/ml					
Micrococcus luteus	22	24	13	16	18	21
Proteus vulgaris KZN	20	23	10	14	16	19
Enterococcus faecalis KZN	16	18	14	17	20	21
Staphylococcus aureus OK_{2b}	17	20	12	14	17	19
Proteus vulgaris CSIR 0030	21	24	11	15	17	20
Bacillus cereus ATCC 10702	19	21	11	15	18	20
Escherichia coli ATCC 25922	19	22	13	15	17	29
Salmonella typhi ATCC 13311	18	20	14	17	19	21
Bacillus pumilis ATCC 14884	18	20	13	17	20	22
Acinetobacter calcoaceuticus UP	16	19	14	17	18	21
Staphylococcus aureus ATCC 6538	19	21	12	16	18	20
Pseudomonas aeruginosa ATCC 19582	20	22	10	12	15	18

concentrations (Table 2). In agar diffusion assay, 100 µl of 4500 µg/ml produced inhibition zones ranging between (18 - 22) ± 1.00 mm and that of 750 µg/ml produced inhibition zones ranging (10 - 14) ± 1.00 mm. The agar dilution indicated that the bacteria were susceptible to the extract at concentrations ranging between ≤100 and ≤ 1000 µg/ml.

From the macrobroth dilution, the MICs of the methanolic extract ranged between 20 µg/ml and 2500 µg/ml while the MBCs ranged between 20 and 2500 µg/ml. *Micrococcus luteus*, *Pseudomonas aeruginosa* (ATCC 19582), *Enterococcus faecalis* KZN and *Proteus vulgaris* CSIR 0030 had their MICs to be less than 100 µg/ml. *Acinetobacter calcoaceuticus* UP, *Bacillus pumilus* ATCC 14884, Escherichia coli ATCC 25922, *Proteus vulgaris* KZN, *Staphylococcus aureus* OK_{2b}, *Salmonella typhi* ATCC 13311 and *Staphylococcus aureus* ATCC 6538 had their MICs less than or equaled to 625 µg/ml while *Bacillus cereus* 10702 had a higher MIC value greater than 1000 µg/ml. The MIC values of the extract were higher than those of ciprofloxacin used as a control. While the crude extract was considered bactericidal because more bacteria had MIC_{index} values equal to 1 and MIC_{index} greater than 1 was considered as bacteriostatic, the inhibition zones obtained at the lowest concentration, however, affirm the MIC values of the extract obtained in the macrobroth dilution assay and indicated its significant medicinal potential. The MBC values being similar

Table 2. Minimum inhibitory and bactericidal concentrations (MIC/MBC) of the methanolic bark extract of *Erythrina caffra* Thunb

Ciprofloxacin	Methanolic crude extract				
	Agar dilution		Macrobroth dilution		
	MIC	MIC	MIC (mg/ml) µg/ml	MBC (mg/ml)	MIC_{index}
Micrococcus luteus	0.156	≤ 100	78	78	1.0
Proteus vulgaris KZN	0.313	≤ 1000	625	625	1.0
Enterococcus faecalis KZN	0.156	≤ 100	39	78	2.0
Staphylococcus aureus OK_{2b}	0.313	≤ 1000	625	625	1.0
Proteus vulgaris CSIR 0030	0.156	≤ 100	39	39	1.0
Bacillus cereus ATCC 10702	0.078	≤ 2500	1250	2500	2.0
Escherichia coli ATCC 25922	0.020	≤ 1000	625	1250	2.0
Salmonella typhi ATCC 13311	0.020	≤ 1000	625	1250	2.0
Bacillus pumilus ATCC 14884	0.0391	≤ 1000	625	625	1.0
Acinetobacter calcoaceuticus UP	0.313	≤ 1000	625	625	1.0
Staphylococcus aureus ATCC 6538	0.313	≤ 1000	625	625	1.0
Pseudomonas aeruginosa ATCC 19582	0.078	≤ 100	20	20	1.0

Table 3. Cytotoxicity effects of methanolic stem bark extract of *Erythrina caffra* on brine shrimps (*Artemia salina*) larvae

		No. of Dead Shrimps/test					
Conc. (µg/ml)	Total No.	Test 1	Test 2	Test 3	Av. Mortality	% Mortality	Log. of Conc.
3.91	10	0	0	0	0	0	0.5922
7.8	10	1	1	2	1.33	13.3	0.8921
15.6	10	2	3	2	2.33	23.3	1.1931
31.3	10	4	3	4	4.33	43.3	1.6365
62.5	10	7	6	7	6.67	66.7	1.7959
125	10	10	10	10	10	100	2.0969

LC_{50} = 40.22 µg/mlLC90 = 56.66 µg/ml

or two-fold higher than the MIC values indicated that the extract would be more bactericidal than being more bacteriostatic at very lower concentrations.

The cytotoxic activity of the plants extract was determined using brine shrimp lethality test. Based on the percentage mortality, the LC_{50} and LC_{90} of the extract was determined using probit scale[40]. Methanol extract of *E. caffra* showed different mortality rate of brine shrimp which increased proportionally with the increasing concentration of the extract (Fig. 1). The LC_{50} and LC_{90} were 40.22 µg/ml and 56.66 µg/ml respectively as shown in Table 3.

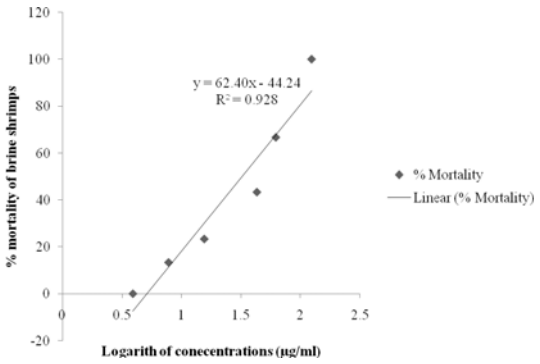

Fig. 1. Graph of logarithm of concentrations versus percentage mortality of brine shrimps interacted with crude methanolic extract of the bark of *E. caffra*

DISCUSSION

Plants are the natural reservoir of many antimicrobial and anticancer agents. They produce some chemical constituents which are naturally toxic to bacteria and fungi[41]. Contrary to the general reports of many workers who had indicated that Gram-positive bacteria are more susceptible to plant extracts than Gram-negative bacteria[42-45] the Gram-negative bacteria were more susceptible to the methanolic extract of *E. caffra* than the Gram-positive strains. The activity of the extract, however, agreed with earlier reports indicating that plant extract show good inhibitory effect against Gram negative bacteria with low MIC and MBC[46,47]. Since Van Den Berghe and Vlietinck[48] reported that an antibacterial effect obtained from $^1/_2$ dilution only is less promising for further investigation in liquid screening methods, the antibacterial activity of higher plants obtained from $^1/_2$, $^1/_8$ and $^1/_{32}$ dilutions is worthy of further investi-gation. Considering the degree of antibacterial activities and the MIC_{index} values obtained from this study, the broad spectrum antibacterial potential of the extract and its ability to inhibit the growth and proliferation of infectious and opportunistic pathogens in both healthy and immunocompromised patients in a disease state may not be over-emphasized. Although the activities of the extracts may be bacteriostatic at lower concentrations, MIC_{index} indicated bactericidal effect at higher concentrations. These correlate with some previous studies documenting the inhibitory effects of methanol extracts of some other plants[49,50].

Also, consistent with the earlier reports of Kubmarawa *et al.*[51], the antibacterial activities of the crude extract were lower than that of the ciprofloxacin used as control. This significant difference could be due to the extract being crude and contained other constituents that do not possess antibacterial property or inhibitory to the antibacterial agents in the extracts. This may also be attributed to the presence of very little quantity of bioactive compounds in the extract or large molecular weight bioactive compounds difficult to diffuse across the exposed lipid domains of the outer membrane. In any case, the total activity of the extract is very high[52] and the antibacterial activities demonstrated that there are bioactive compounds inhibitory to the microorganisms in the extract at lower concentrations. Thus, the antibacterial activity of the extract may be due to the presence of flavonoids, phenols, tannins and triterpenoids earlier implicated in pharmacological activities of medicinal plants[53,54]. It may, also, be due to the fact that triterpenoids in d plant weaken the membranous tissue resulting in dissolution of microbial cell walls[55] while its tannins form irreversible complexes with prolene rich protein to inhibit cell wall synthesis[56].

Although studies on the toxicological effects of plant extracts are, at times, disregarded, some reports have indicated antimicrobial screening together with other pharmacological investigations including toxicity[57,58]. Solis *et al.*,[59] and Mackeen *et al.*,[60] showed that there is a good relationship between brine shrimp lethality and pharmacological activities of terrestrial plant extracts as it has been

used to predict the presence of bioactive compounds in the extract[34] and detect general toxicity[61]. While a compound or an extract with an LC_{50} value greater than 100 µg/ml was considered inactive[62] and *in vitro* antimicrobial activity could provide the needed preliminary observations necessary to select medicinal plants with potentially useful properties for the development of novel antibiotic prototypes[63], the cytotoxicity test in this study showed that the methanolic extract of this plant was toxic to brine shrimp larvae giving LC_{50} and LC_{90} values below 100 µg/ml. Consequently, based on the LC_{50} and LC_{90} values and the degree of antibacterial activities of the extract, the extract was considered highly active and possibly emphasized the medicinal importance of the plant. Consequently, the antibacterial and cytotoxicity of this extract showing that this plant would be effective in the treatment of microbial infections at lower concentrations may account for the rationale behind the use of the plant by traditional medical practitioners for the effective treatment of their patients over a reasonable period of time.

CONCLUSIONS

In conclusion, the good antibacterial and cytotoxic activities of the extract suggest that the plant has potential to yield bactericidal and possibly cytotoxic compounds. This study, therefore, suggests that there are phytochemical constituents in the extract which could serve as a source of chemotherapy drugs useful in the treatment of infections initiated by the selected bacteria and justifies the use of this plant traditionally for the treatment of gastroenteritis in rural communities. Further studies to investigate the bioactive compounds responsible for the observed biological effects are ongoing in our laboratory.

ACKNOWLEDGEMENTS

The authors are grateful to the National Research Foundation (NRF) and Govan Mbeki Research Development Centre (GMRDC) of South Africa for supporting this research.

REFERENCES

1. Sibanda, T., Okoh, A.I. In vitro evaluation of the interactions between acetone extracts of Garcinia kola seeds and some antibiotics. *Afr. J. Biotechnol.* 2008; **7**: 1672-1678.
2. Chomnawang, M.T., Surassmo, S., Wongsariya, K., Bunyapraphatsara, N. Antibacterial activity of Thai medicinal plants against methicillin-resistant *Staphylococcus aureus. Fitoterapia*, 2009; **80**: 102-104.
3. Tomoko, N., Takashi, A., Hiromu, T., Yuka, I., Hiroko, M., Munekazu, I., Totshiyuki, T., Tetsuro, I., Fujio, A., Iriya, I., Tsutomu, N., Kazuhito, W. Antibacte-rial activity of extracts prepared from tropical and subtropical plants on methicillin-resistant *Staphylo-coccus aureus*. *J. Health Sci*., 2002; **48**: 273-276.
4. Rajeshwar, Y., Gupta, M., Mazumder, U.K. *In vitro* lipid peroxidation and antimicrobial activity of *Mucuna pruriens* seeds. *Iran J. Pharmacol. Ther.*, 2005; **4**: 32-35.
5. Coopoosamy, R.M., Magwa, M.L. Antibacterial activity of aloe, emodin and aloin A isolated from *Aloe excelsa*. *Afr. J. Biotechnol.*, 2006; **5**: 1092-1094.
6. Keymanesh, K., Hamedi, J., Moradi, S., Mohammadipanah, F., Sardari, S. Antibacterial, antifungal and toxicity of rare Iranian plants. *Int. J. Pharmacol.*, 2009; **5**: 81-85.
7. Hasani-Ranjbar, S., Vahidi, H., Taslimi, S., Karimi, N., Larijani, B., Abdollahi, M. A systematic review on the efficacy of herbal medicines in the management of human drug-induced hyperprolactinemia; Potential sources for the development of novel drugs. *Int. J. Pharmacol.*, 2010; **6**: 691-695.
8. Chariandy, C.M., Seaforth, C.E., Phelps, R.H., Pollard, G.V., Khambay, B.P. Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. *J. Ethnopharmacol* 1999; **3**: 265-270.
9. Builders, M.I., Wannang, N.N., Ajoku, G.A., Builders, P.F., Orisadipe, A., Aguiyi, J.C. Evaluation of the antimalarial potential of *Vernonia ambigua* Kotschy and Peyr (Asteraceae). *Int. J. Pharmacol.*, 2011; **7**: 238-247.
10. Yusufoglu, H.S., Alqasoumi, S.I. Anti-inflammatory and wound healing activities of herbal gel containing an antioxidant *Tamarix aphylla* leaf extract. *Int. J. Pharmacol.*, 2011; **7**: 829-835.
11. Carson, C.F., Riley, T.V. Antimicrobial activity of the major components of the essential oil of

Melaleuca alternifolia. *J. Appl. Bacteriol.* 1995; **76**: 264-9.
12. Okemy-Andissa, N., Ouamba, J.M., Koudou, J., Diatewa, M., Gbeassor, M., Abena, A.A. Comparative study of analgesic activities of Tetra® and an association of three plants: *Ageratum conyzoides, Combopogon citratus* and *Lippia multiflora*. *Int. J. Pharmacol.,* 2006; **2**: 42-44.
13. Bever, B.O. Medicinal Plants in Tropical West Africa. Cambridge University Press, New York, USA., ISBN-13: 9780521268158, 1986; pp. 100.
14. Gillett, J.B., Polhill, B.A., Verdcourt, B. Leguminosae, Papilionoideae, Part 1, Part 2. In: *Flora of Tropical East Africa*, Milne-Redhead, E. and R.M. Polhill (Eds.). Crown Agents for Overseas Governments and Administrations, London, UK., 1971, 1-502, 503-1109
15. Mitscher, A.L., Drake, S., Gollapudi, S.R., Simon, K., Okwute, S.R. A modern look at folkloric Use of anti-infective agents. *J. Nat. Prod.*, 1987; **50**: 1025-1040.
16. Burkill, H.M. The Useful Plants of West Tropical Africa. 1995; 3, Families J-L. Royal Botanic Gardens, Kew.
17. Olajuyigbe, O.O., Afolayan, A.J. Ethnobotanical survey of medicinal plants used in the treatment of gastrointestinal disorders in the Eastern Cape Province, South Africa. *J. Med. Plants Res.* 2012; **6**(18): 3415-3424.
18. Tsuda, Y., Sano, T. Erythrina and Related Alkaloids. In *The Alkaloids*, Cordell, G. (Ed.). Vol. 48. Academic Press, Orlando, FL., USA., 1996; pp: 249-335.
19. Chawla, A.S., Kapoor, V.K. *Erythrina Alkaloids.* In: The Alkaloids: Chemical and Biological Perspectives, Pelletier, S.W. (Ed.)., Pergamon Press, New York, USA., 1995; **9**: 86-153.
20. Tanaka, H., Tanaka, T., Etoh, H., Goto, S., Terada, Y. Two new erythrinan alkaloids from Erythrina x bidwillii. *Heterocycles*, 1999; **51**: 2759-2764.
21. McKee, T.C., Bokesch, H.R., McCormick, L.J., Rashid, A., Spielvogel, D., Gustafson, K.R., Alavanja, M.M., Cardellina, J.H., Boyd, M.R. Isolation and characterization of new anti-HIV and cytotoxic leads from plants, marine and microbial organisms. *J. Nat. Prod.*, 1997; **60**: 431-438.
22. El-Masry, S., Amer, M.E., Abdel-Kader, M.S., Zaatout, H.H. Prenylated flavonoids of *Erythrina lysistemon* growing in Egypt. *Phytochem*. 2002; **60**: 783-787.
23. Ronald, M.A. Micro-Organisms in our World. Mosby Year Book Inc., St. Louis, MO., USA., 1995; pp 765.
24. Irobi, O.N., Young, M., Anderson, W.A. Antimicrobial activity of Annato (*Bixa orella*) extract. *Int. J. Pharmacog.*, 1994; **34**: 87-90.
25. Akinpelu, D.A., Aiyegoro, O.A., Okoh, A.I. *In vitro* antibacterial and phytochemical properties of crude extract of stem bark of *Afzelia Africana* (Smith). *Afr. J. Biotechnol.* 2008; **7**: 3665-3670.
26. NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard, M7-A3. NCCLS, Villanova, PA,. 1993.
27. Cheesbrough, M. *Medical Laboratory Manual for Tropical Countries*, ELBS ed; Tropical health technology publications and Butterworth–Heinemann Ltd: Cambridge, UK, 2002; **2**: 2–392.
28. CLSI Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. Pennsylvania, USA, part M2-A9. M100-S17, 2007.
29. CLSI, Performance standards for Antimicrobial Susceptibility Testing Eighteenth informational supplement, 2008.
30. Afolayan, A.J., Meyer, J.J.M. The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoot of *Helichrysum aureonitens*. *J. Ethnopharmacol.*, 1996; **57** (3): 177-181.
31. Sung, W.S., Jung, H.J., Lee, I.S., Kim, H.S., Lee, D.G. Antimicrobial effect of furaneol against human pathogenic bacteria and fungi. *J. Microbiol. Biotechnol.*, 2006; **16**: 349-354.
32. Shanholtzer, C.J., Peterson, L.R., Mohn, M.L., Moody, J.A., Gerding, D.N. MBCs for *Staphylococcus aureus* as determined by macrodilution and microdilution techniques. *Antimicrob. Agents Chemother.*, 1984; **26**(2): 214–219.
33. Irkin, R., Korukluoglu, M. Control of *Aspergillus niger* with garlic, onion and leek extracts. *Afr. J. Biotechnol.*, 2007; **6**: 384–387.
34. Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L. Brine Shrimp: A convenient general bioassay for active plant constituents. *Plant med.*, 1982; **45**: 31-34
35. Mclaughlin, J.L., Rogers, L.L. The use of biological assays to evaluate the botanicals. *Drug Inf. J.*, 1998; **32**: 513-24.
36. Lewis, G.E. Testing the toxicity of extracts of Southern African plants using brine shrimp (*Artemia salina*). *S. Afr. J. Sci.*, 1995; **91**: 382-384.
37. Pelka, M., Danzl, C., Distler, W., Petschelt, A. A new screening test for toxicity testing of dental materials. *J. Dent.*, 2000; 28**:** 341-345.

38. Moshi. M.J., Innocent, E., Magadula, J.J., Otieno, D.F., Weisheit, A., Mbabazi, P.K., Nondo, R.S.O. Brine Shrimp of Some Plants used as Traditional Medicine in Kagera Region, North West Tanzania. *Tanzania J. Health. Res.* 2010; **12**(1): 63–67.
39. Gupta, M.P., Monge A., Karitas, G., Lopez De Cerain, A., Solis, P.N., Leon, E., De Trujilo, M., Surez, O., Wilson F., Montenegro G., Noriega, Y., Santana, A.I., Correa, M., Sanchez, C. Screening of Panamanian medicinal Plants for brine shrimp toxicity, crown gall tumor inhibition, cytotoxicity and DNA interaction. *Int. J. Pharmacol.,* 1996; **34**: 123-127.
40. Miller, L.C., Tainter, M.L. Estimation of EC_{50} and its error by means of log-probit graph paper. *Proc. Soc. Exp. Biol. Med.* 1944; **57***:* 261-269.
41. Basile, A., Giordano, S., López-Sáez, J.A., Cobianchi, R.C. Antibacterial activity of pure flavonoids isolated from mosses. *Phytochem.*, 1999; **52**: 1479-1482.
42. Madamombe, I.T., Afolayan, A.J. Evaluation of antimicrobial activity of extracts from South African *Usnea barbata*. *Pharm. Biol.*, 2003; **41**: 199-202.
43. Erasto, P., Adebola, P.O., Grierson, D.S., Afolayan, A.J. An Ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province, South Africa. *Afr. J. Biotechnol.*, 2005; **4**: 1458-1460.
44. Afolayan, A.J., Ashafa, A.O.T. Chemical composition and antimicrobial activity of the essential oil from *Chrysocoma ciliate* L. leaves. *J. Med. Plants Res.*, 2009; **3**: 390-394.
45. Renukadevi, K.P., Sultana, S.S. Determination of antibacterial, antioxidant and cytotoxicity effect of Indigofera tinctoria on lung cancer cell line NCI-h69. Int. J. Pharmacol., 2011; 7:356-362. *Int. J. Pharmacol.*, 2011; **7**: 356- 362.
46. Sittiwet, C., Puangpronpitag, D. Antibacterial Activity of *Phellinus gilvus* aqueous extract. *Int. J. Pharmacol.*, 2008; **4**: 500-502.
47. Olajuyigbe, O.O., Afolayan, A.J. *In vitro* antibacterial activities of the methanol extract of *Ziziphus mucronata* Willd. subsp. mucronata Willd. *J. Med. Plants Res.* 2011a; **5**: 3791-3795.
48. Van Den Berghe, D.A., Vlietinck, A.J. Screen-ing Methods for Antibacterial and Antiviral Agents from Higher Plants. In: Methods in Plant Biochemis-try: Assays for Bioactivity, Dey, P.M., J.B. Harbone and K. Hostettman (Eds.). 1991; **6:** 47-69.Academic Press, London, UK.
49. Adiguzel, A., Ozer, H., Kilic, H., Cetin, B. Screening of antimicrobial activity of essential oil and methanol extract of *Satureja hortensis* on foodborne bacteria and fungi. *Czech. J. Food Sci.,* 2006; **25**: 81-89.
50. Kursat, M., Erecevit, P. The antimicrobial activities of methanolic extracts of some *Lamiaceae* members collected from Turkey. *Turk. J. Sci. Technol.,* 2009; **4**: 81-85.
51. Kubmarawa, D., Ajoku G., Okorie, A.D. Antimicrobial spectrum of hexane extract of *Commiphora kerstingii* (Brseraceae). *Technol. Dev.,* 2002; **8**: 29-32.
52. Eloff, J.N. Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. *Phytomed.*, 2004; **11**: 370-371.
53. Momtaz, S., Abdollahi, M. An Update on Pharmacology of *Satureja Species:* From antioxidant, antimicrobial, antidiabetes and anti-hyperlipidemic to reproductive stimulation. *Int. J. Pharmacol.*, 2010; **6**: 346-353.
54. Olajuyigbe, O.O., Afolayan, A.J. Phytochemical assessment and antioxidant activities of alcoholic and aqueous extracts of *Acacia mearnsii* De Wild. *Int. J. Pharmacol.,* 2011b; **7**(8): 856-861.
55. Hernandez, N.E., Tereschuk, M.L., Abdala, L.R. Antimicrobial activity of flavonoids in medicinal plants from Tafí del Valle (Tucumán, Argentina). *J. Ethnopharmacol.,* 2000; **73**(1-2): 317-22.
56. Mamtha, B., Kavitha, K., Srinivasan, K.K., Shivananda, P.G. An *in vitro* study of the effect of *Centella asiatica* [Indian pennywort] on enteric pathogens. *Ind. J. Pharmacol.*, 2004; **36**: 1-41.
57. Kamatou, G.P.P., Viljoen, A.M., Figueiredo, A.C., Tilney, P.M., Van Zyl, R.L., Barroso, J.G., Pedro, L.G., Van Vuuren, S.F. Trichomes, essential oil composition and biological activities of *Salvia albicaulis* Benth. and *S. dolomitica* Codd, two species from the Cape region of South Africa. *S. Afr. J. Bot.,* 2007; **73**: 102–108.
58. Viljoen AM, Moolla A., VanVuuren, S.F., Van Zyl, R.L., Baser, K.H.C., Demirci, B., Özek, T., Trinder-Smith, T.H. The biological activity and essential oil composition of 17 *Agathosma* (Rutaceae) species. *J. Essential Oil Res.*, 2006; **18**: 2–16.
59. Solís, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P., Phillipson, J.D. A microwell cytotoxicity assay using *Artemia salina*. *Plant Med.*, 1993; **59**: 250-252
60. Mackeen, M.M., Ali, A.M., Lajis, N.H., Kawazu K., Hassan, Z., Amran, M., Habsah, M., Mooi, L.Y., Mohamed, S.M. Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. Ex T. Anders. *J. Ethnopharmacol.,* 2000; **72**: 395-402.
61. Persoone, G., Wells, P.G. Artemia in aquatic toxicology: a review. In: Artemia Research and

its Applications. Morphology, Genetics, Strain characterization Toxicology (Edited by: Sorgeloos P) Belgium, Universita Press 1987; 259-275.

62. Nondo, R.S.O., Mbwambo, Z.H., Kidukuli, A.W., Innocent, E.M., Mihale, M.J., Erasto, P., Moshi, M.J. Larvicidal, antimicrobial and brine shrimp activities of extracts from *Cissampelos mucronata* and *Tephrosia villosa* from coast region, Tanzania. *BMC Compl. and Alt. Med.*, 2011; **11**: 33.

63. Koduru, S., Grierson, D.S., Afolayan. A.J. Antimicrobial activity of *Solanum aculeastrum*. *Pharm. Biol.*, 2006; **44**(4): 283-286.