ORIGINAL RESEARCH

THE FLIP SIDE OF HERBAL REMEDIES

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ABSTRACT

Aim: This study was carried out to determine the fate of bacteria exposed to sub lethal concentrations of bioactive extracts of some plants. Methods: Ethanolic extracts of *Tetrapleura tetraptera* (leaves), *Prosopis africana* (bark) and *Buchholzia coriaceae* (seeds) were assayed for phytochemical contents, antibacterial activities and induction of multidrug resistance in bacteria hitherto susceptible to test drugs using standard methods. Results: Results showed that the three plants contain alkaloids, glycosides, saponins, flavonoids and reducing sugars in addition to other metabolites. The extracts showed appreciable inhibitory effects on some species of *Alcaligenes, Bacillus, Enterobacter, Escherichia, Klebsiella, Pseudomonas* and *Salmonella.* The minimum inhibitory concentration (MIC) ranged (mg/ml) from 3.13 – 50.0 for *P. africana;* 3.13 – 6.25 for *B. coriaceae* to 1.25 – 50.0 for *T. tetraptera.* Exposure of drug susceptible bacterial species belonging to the genera *Escherichia, Enterobacter, Klebsiella, Proteus, Pseudomonas* and *Salmonella* to different concentrations of these extracts resulted in emergence of clones resistant to some commonly available antibacterial agents. Conclusion: Plant extracts are capable of dose-related inducement and sustenance of multiple antibiotic resistance among some bacteria. This study has shown that despite its positive contribution, herbal remedies incorporate the hitherto unexplained increase in emergence of multidrug resistance among (enteric) bacteria in our communities.

Key Words: Herbal remedy; Plant extract; Antibacterial effect; Multidrug resistance; Resistance induction.

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INTRODUCTION

Plant medicine is a broad category of medicaments that include drugs used in traditional systems of medicine, folklore and ethno medical products, and drugs discovered from plants that have no hitherto documented therapeutic use. For many decades, herbal therapy formed the foundation for the treatment of many diseases in Africa. Undoubtedly, medicinal plants and drugs derived from them constitute great medical and economically strategic value not only for the African continent but also for other parts of the world (Anon, 1985). Herbal therapy is widely accepted and used as an alternative in the prevention and treatment of physical and mental disorders as well as infectious diseases and antisocial behaviours attributable to spiritual causes. Due to its intrinsic qualities, unique and holistic approaches as well as its accessibility and affordability, it continues to be the best alternative health care available for the majority of the global population, particularly for those in the rural areas of developing countries (Mwambazi, 1996). In developed countries such as the United States of America, many consumers of nutritional supplements have embraced the use of botanicals as a natural approach to their health care (Pamplona-Roger, 2004).

Among the successes achieved by herbal medicine is that many drugs in clinical use today were discovered from the way plants were used in traditional communities. Examples include quinine which was discovered from the way traditional communities in South America especially Peru, Columbia and Bolivia, used plant species of the genus *Cinchona* in managing fevers. Digitoxin is a popular heart tonic and was obtained from *Digitatis purpurea,* a plant that was in use as a heart tonic in traditional communities in Europe. Taxol is a modern day therapy for ovarian cancer obtained from *Taxus brevifolia* which was a traditional medicinal plant in British Columbia (Farnsworth, 1990; Njoroge and Bussmann, 2006).

In addition, herbal therapy is also seen as a panacea to the problems associated with drug resistance among infective agents. Mboya (2003) asserted that with increasing resistance of microorganisms associated with infectious diseases, and increasing environmental pollution which constitutes selective pressure on microorganisms, herbal medicine provides alternative sources for new drugs. Thus emphasis is now being laid on traditional medicine as an alternative to orthodox medicine more than ever before. This is especially true in developing countries like Nigeria. Studies (Okafor *et al.,* 2001, Okafor *et al.,* 2002, Okoli and Iroegbu, 2004, Ofokansi, *et al.,* 2005) have given credence to the folkloric claims on the prophylactic and therapeutic effectiveness of some medicinal plants against infectious agents including multidrug resistant ones. Notable among some of the plants used in folkloric medicine are *Buchholzia coriaceae*, *Prosopis africana* and *Tetrapleura tetraptera*. *B. coriaceae* is otherwise called "Wonderful kola" because of local belief

in its potency as an anti-histamine and anti-inflammatory agent (Enechi and Nwafor, 2001). *P. africana* is a popular source of spice and dietary supplement in many parts of Nigeria. Bark and leaf extracts of *P. africana* are used locally to treat cough, stomach upset and urinary tract infections (Iwu, 1993). In ethno-medicine, *T. tetraptera* products are used to treat ulcer, management of asthma, diabetes, and hypertension (Ojewole and Adewunmi, 2004); as childbirth analgesics (Njoku *et al.,* 1999); and as spices to improve food taste and provide vitamin supplementation (Adewunmi, 2001).

Despite its positive contributions as outlined above, the adverse effects of plant products and the ultimate fate of the microorganisms treated with these products have not been fully investigated. The possibility of induction and/or selection of drug resistant bacteria from clones of susceptible ones by plant extracts is the subject of this investigation. The above mentioned plants namely *B. coriaceae*, *P. africana* and *T. tetraptera* are employed in this investigation which is aimed at:

(1) Determining the phytoconstituents of the test plants.
(2) Determining the initial susceptibility of selected bacterial isolates to the extracts of the plants.
(3) Determining whether plant products (extracts) are capable of inducing resistance in hitherto drug-susceptible bacterial isolates, and
(4) Determining the percentage of such emergence of resistance in named bacterial species against selected orthodox antibacterial agents.

It is a preliminary look into the consequences of exposing bacteria to sub lethal concentrations of plant extracts.

METHODS

Extraction of plant materials: Extracts from three plants namely: *Buchholzia coriaceae (*seeds), *Prosopis africana* (bark), and *Tetrapleura tetraptera* (leaves) were used in this study. Desired parts of plants were collected from different locations in the Nsukka area of Enugu State, Nigeria and their identity authenticated by the taxonomical section of the Department of Botany, University of Nigeria, Nsukka. Voucher specimens are in the herbarium of the Department of Pharmacognosy, University of Nigeria Nsukka, Nigeria.

The collected parts were sun-dried and then powdered using a corona mill. Each powder (100 g) was divided into two equal parts. A part (50 g) was packed in 500 ml Erlenmayer flasks and soaked with 250 ml absolute ethanol with intermittent shaking for 48 h. Each extract was then filtered through a Whatman number 1 filter paper. The filtrate was evaporated to dryness in a steady air current for about 24 h. The extract was sterilized by exposure to UV rays for 24 h. Sterility was checked by streaking the extract on sterile nutrient agar plates. All extracts were stored at 4^0C until needed.

The second half of the 100 g powder was used to determine the phyto-chemical constituents of the plant parts. The chemical tests were carried out according to the methods described by Harbone (1973) as modified by Trease and Evans (1982). The relative concentration of each constituent was inferred from the intensity of the reaction.

Antimicrobial studies: The cup – plate method described by Collins and Lyne (1979) was employed to determine the activity and the zones of inhibition of the plant extracts against some test bacteria viz. sewage and clinical isolates of species of *Alcaligenes, Bacillus, Enterobacter, Escherichia coli, Klebsiella, Pseudomonas* and *Salmonella.* Dry plant extracts were reconstituted in 5% DMSO (1g in 5 ml of 5% DMSO) and diluted (also with 5% DMSO) to achieve 100, 50, 25, 12.5, 6.5, 3.13 and 1.56 mg/ml concentrations. These were filter sterilized (Millipore Type HC filters). Sterility was confirmed by microscopy and inoculation of test extracts on nutrient agar (NA) plates followed by overnight incubation at 37^0C.

Eighteen hour Mueller Hinton broth cultures of test bacterial isolates were diluted (with sterile 0.85% NaCl solution) to the turbidity (by visual examination) (Baron and Finegold, 1990) of 0.5 McFarland Nephlometer standards. A 1 ml volume of the standardized inoculum of each test bacterium was spread on Mueller Hinon Agar plates and allowed to dry. Six mm – diameter wells were bored in the plates and 100 µl of different concentrations of the extracts introduced into each well. These were allowed to stand for 1 h at room temperature (Okoli and Iroegbu, 2004). After this, the plates were incubated at 37^0C for 24 h. Subsequently, the inhibition zone diameters (IZD) were measured to the nearest mm and minimum inhibitory concentrations (MIC) determined. Experiments were done in duplicate. Wells containing 5% DMSO and ethanol only were separately used as controls. The minimum bactericidal concentration (MBC) of each extract was subsequently determined according to the methods of Okeke *et al.,* (2001) and Okafor *et al.,* (2002). MIC index was also calculated for each test organism.

Twenty five (25) isolates of *E. coli*, *Klebsiella* spp, *Salmonella* spp, *Enterobacter* spp, *Proteus* spp and *Pseudomonas* spp obtained from clinical and sewage samples were also subjected to sensitivity tests against a panel of ten antibiotics (Optun Nig.) tarivid (10 µg), ciprofloxacin (10 µg), augmentin (30 µg), gentamicin (10 µg), streptomycin (30 µg), ceporex (10 µg), nalidixic acid (30 µg), septrin (30 µg), and ampicillin (30 µg), Standardized broth cultures of isolates were assayed for sensitivity to these antibiotics using the methods of Bauer *et al.,* (1966) on plates. After incubation (24 h at 37^0C) and measurement of inhibition zone diameters, susceptibility ranges were decided following CLSI (NCCLS, 2006). Control plates were incubated without antibiotic discs.

Induction assays: A qualitative analysis (quantitative tests are on going) of the effect (s) of plant extracts on the emergence of drug resistant bacterial clones from hitherto

drug susceptible clones was carried out using 12 drug susceptible isolates each of *E. coli, Klebsiella* spp, *Salmonella* spp, *Enterobacter* spp, *Proteus* spp and 10 of *Pseudomonas* spp. This followed, with some modifications, the methods of Pumbwe *et al.,* (2007) and Eze (2008). Two different concentrations (12.5, and 3.125 mg/ml) of the three plant extracts analysed were selected based on observations and results from a previous study (Eze 2008). Sterile NB preparations containing the above final concentration were distributed (10 ml each) into Bijou bottles. Twenty-four hour standardized NB cultures of selected drug susceptible bacterial isolates were inoculated (40 µL) separately into these NB culture bottles. The contents of each bottle was mixed well and incubated with shaking at 37^0C for 48 h. NB cultures without extracts were also incubated as controls. Following incubation, cultures were assayed for viability by plating 0.1 ml on MacConkey agar (MA) and NA plates and incubating at 37^0C for 24 h. Viable cultures were further subjected to drug susceptibility tests as above. Recovery of drug resistant clone(s) from test clones was recorded and scored as a percentage of the 12 or 10 isolates of each bacterial genus used.

RESULTS

Phyto-chemical analysis showed that saponin, alkaloid, flavonoid, glycoside and reducing sugars were present in the three plants *T. tetraptera, B. coriacea* and *P. africana.* The amounts of these phyto-constituents, however differed between plants. Only *P. africana* contained tannins while proteins were detected in *T. tetraptera* and *B. coriaceae* (Table 1). Leaf extracts of *P. africana* showed activity against all the test bacteria. It had a relatively lower mean MIC against clinical (CS) isolates of *E. coli* (12.5 mg/ml), *Pseudomonas* spp (25 mg/ml) and *Salmonella* spp (12.5 mg/ml) than sewage (SS) isolates of the same organisms against which it had 25, 50 and 25 mg/ml MIC respectively (Table 2).

The antimicrobial activity of *T. tetraptera* against *Enterobacter* spp was high, having mean MIC of 1.25 mg/ml against both SS and CS isolates. It also had a 12.5 mg/ml mean MIC against both CS and SS isolates of *E. coli*. It did not, however, exhibit any activity against SS isolates of *Pseudomonas* and *Bacillus* spp For *B. coriaceae*, higher antibacterial activities were recorded against CS isolates of *Salmonella* and *Klebsiella* spp (mean MIC = 3.125 mg/ml) than SS isolates of species of the same organisms with mean MIC of 6.25 mg/ml (Table 4). Also *B. coriaceae* extracts had a mean minimum bactericidal concentration (MBC) of 25.0 mg/ml against both SS and CS isolates of *E. coli.*

Drug resistance induction assays revealed that three (25%) of the *E. coli* isolates tested developed resistance to streptomycin, augumentin, ampicillin and septrin after exposure to 12.5 mg/ml concentration of *T. tetrapera* for 48 h. *Pseudomonas* spp demonstrated 30% resistance to augumentin while *Salmonella* spp exhibited 16.67% induced resistance to streptomycin, tarivid, augumentin, ampicillin and septrin after the same treatment (Fig. 1). The mean difference in the percentage emergence of resistant clones induced by 3.125 mg/ml *T. tetraptera* was high between *Pseudomonas* spp on one hand and *E. coli, Enterobacter* spp, *Klebsiella Proteus,* and *Salmonella* spp on the other (Fig. 2). A 12.5 mg/ml concentration of *B. coriaceae* induced multiple antibiotic resistance (MAR) in *E. coli* (16.67%), *Enterobacter* spp (16.67%), *Proteus spp* (8.33%) (Fig.3). While 8.33% of *Proteus spp* exposed to 3.125 mg/ml concentration of *B. coriaceae* developed MAR, none of *Klebsiella* spp did. (Fig.4).

Species of *Enterobacter* developed resistance to streptomycin (8.33%), ciporex (8.33%), augumentin (16.67), ampicillin (16.67%) and septrin (8.33%) following incubation in NB containing 12.5 mg/ml of *P. africana* extracts. Thirty percent of *Pseudomonas spp* demonstrated resistance to streptomycin and ampicillin (Fig. 5) Test bacteria exposed to 3.125 mg/ml *P. africana* showed variations in emergence of MAR clones as in the other cases. Only one (10%) isolate of *Pseudomonas* spp developed resistance to ciproflox, nalidixic acid and tarivid (Fig. 6). None of the bacterial clones recovered from the control NB cultures showed any variations from their initial antibiogram.

Table 1: Phyto-constituents of analysed plant materials.

Plant	Partused	Protein	Saponin	Alkaloid	Tannin	Flavonoid	Glycoside	Steroids	Reducing	Terpenoid	Acids
Tetrapleura tetraptera (oshosho)	Leaves	+	++	++	-	+	+	NT	++	NT	+
Buchholzia coriaceace (wonderful kola)	Seed	++	++	++	-	+	++	NT	+	NT	NT
Prosopis africana (okpeye)	Bark	-	+++	++	+	+	++	++	++	++	-

Key: + = Present in trace amount, ++ = Present in large amount, +++ = Present in very large amount, - = Absent, NT = Not tested.

Table 2: Susceptibility of test bacterial isolates to ethanol extracts of *Prosopis africana* (pH = 7.5).

Test organisms	No of clones tested	No of replication	Mean IZD (mm)	Mean MIC (mg/ml)	Mean MBC (mg/ml)	MIC Index
Alcaligenes spp (SS)	3	2	21.25	3.125	25.0	0.13
Bacillus spp (SS)	3	2	13.20	50	> 50.0	< 0.98
Enterobacter spp (SS)	3	2	16.50	6.25	50.0	0.13
Enterobacter spp (CS)	3	2	16.70	6.25	6.25	1.00
Escherichia coli (SS)	3	2	12.80	25	50.0	0.50
Escherichia coli (CS)	3	2	10.50	12.5	50.0	0.25
Klebsiella spp (SS)	3	2	18.34	12.5	> 25.0	< 0.48
Klebsiella spp (CS)	3	2	18.30	12.5	> 25.0	< 0.48
Pseudomonas spp (SS)	3	2	11.51	12.5	> 25.0	< 0.48
Pseudomonas spp (CS)	3	2	10.82	25	> 25.0	< 0.96
Salmoella spp (SS)	3	2	12.50	25	50.0	0.50
Salmoella spp (CS)	3	2	12.50	12.5	50.0	0.50

Key: SS= Sewage Isolate, CS= Clinical Isolate, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, IZD = Inhibition Zone diameter.

Table 3: Susceptibility of test isolates to ethanol extracts of *Tetrapleuta tetraptera* (pH = 6.2).

Test organisms	No of clones tested	No of replications	Mean IZD (mm)	Mean MIC (mg/ml)	Mean MBC (mg/ml)	MIC Index
Alcaligenes spp (SS)	3	2	21.25	12.50	25.0	0.50
Bacillus spp (SS)	3	2	NI	0.00	0.00	NA
Enterobacter spp (SS)	3	2	18.40	1.250	25.0	0.05
Enterobacter spp (CS)	3	2	18.45	1.250	25.0	0.05
Escherichia coli (SS)	3	2	13.10	12.5	50.00	0.25
Escherichia coli (CS)	3	2	13.20	12.5	50.00	0.25
Klebsiella spp (SS)	3	2	16.80	25.0	100.0	0.25
Klebsiella spp (CS)	3	2	16.52	12.50	100.0	0.13
Pseudomonas spp (SS)	3	2	NI	0.00	0.00	NA
Pseudomonas spp (CS)	3	2	12.50	50.00	0.00	0.98
Salmonella spp (SS)	3	2	13.4	25.0	0.00	0.96
Salmonella spp (CS)	3	2	13.50	25.0	0.00	0.97

Key: SS= Sewage Isolate, CS= Clinical Isolate, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, IZD = Inhibition Zone diameter, NI =No inhibition, NA = No activity within the inhibitory concentration range.

Table 4: Susceptibility of test bacterial isolates to ethanol extracts of *Buchholzia coriaceae* (pH = 6.8).

Test organisms	No of clones tested	No of replications	Mean IZD (mm)	Mean MIC (mg/ml)	Mean MBC (mg/ml)	MIC Index
Alcaligenes spp (SS)	3	2	NI	0.00	0.00	NA
Bacillus spp (SS)	3	2	NI	0.00	0.00	NA
Enterobacter spp (SS)	3	2	16.8	6.25	50.0	0.13
Enterobacter spp (CS)	3	2	18.5	6.25	50.0	0.13
Escherichia coli (SS)	3	2	20.6	3.125	25.0	0.13
Escherichia coli (CS)	3	2	21.4	3.125	25.0	0.13
Klebsiella spp (SS)	3	2	14.3	6.25	50.0	0.13
Klebsiella spp (CS)	3	2	14.2	3.125	50.0	0.06
Pseudomonas spp (SS)	3	2	NI	0.00	0.00	NA
Pseudomonas spp (CS)	3	2	NI	0.00	0.00	NA
Salmonella spp (SS)	3	2	12.4	6.25	0.00	0.99
Salmonella spp (CS)	3	2	13.5	3.125	0.0	0.99

Key: SS= Sewage Isolate, CS= Clinical Isolate, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, IZD = Inhibition Zone diameter, NI =No inhibition, NA = No activity within the inhibitory concentration range.

Figure 1: Percentage of clones of drug susceptible bacteria isolates that developed resistance to test drugs after exposure to 12.5 mg/ml *T. tetraptera* extracts for 48 hours.

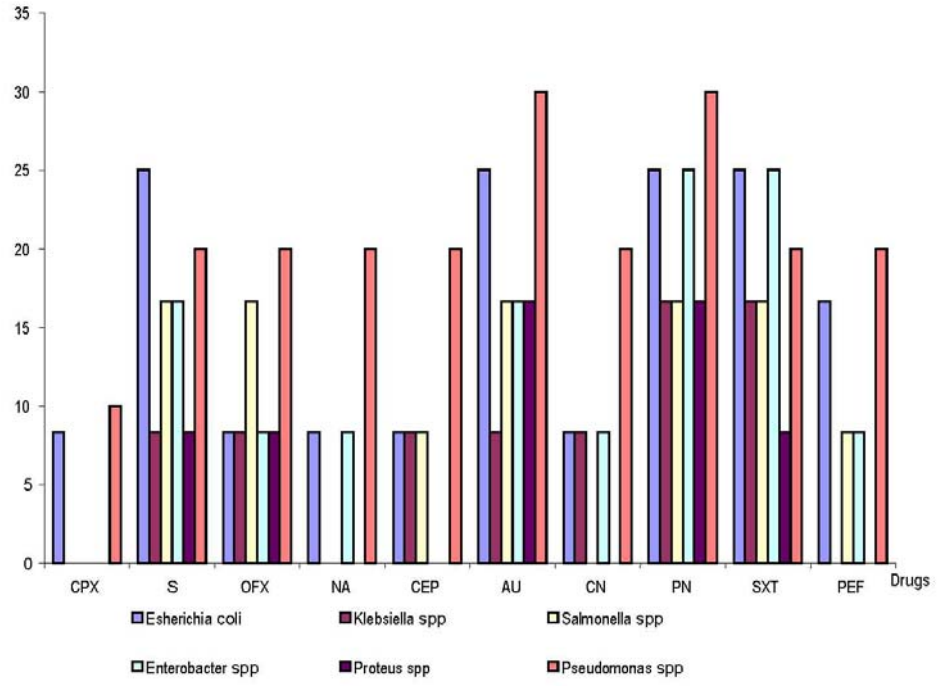


Figure 2: Percentage of clones of drug susceptible bacteria isolates that develop resistance to test drugs after exposure to 3.12mg/ml *T. tetraptera* extracts for 48 hours.

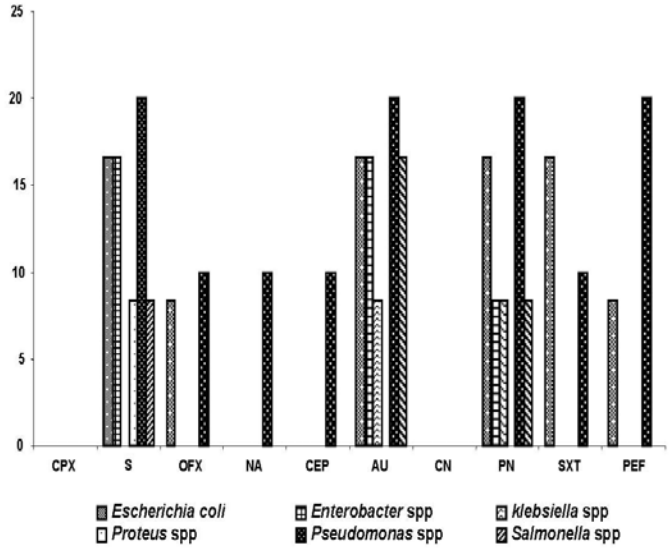


Figure 3: Percentage of clones of drug susceptible isolates that develop resistance to test drugs after exposure to 12.5 mg/ml *B. coriaceae* extracts for 48 hours.

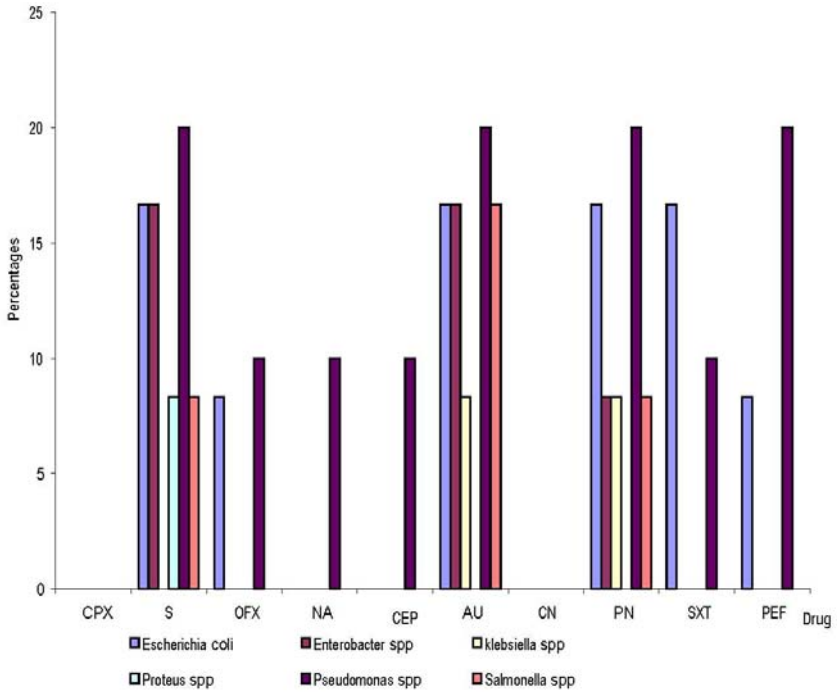


Figure 4: Percentage of clones of drug susceptible bacteria isolates that developed resistance to test drugs after exposure to 3.12 mg/ml *B. coriaceae* extracts for 48 hours.

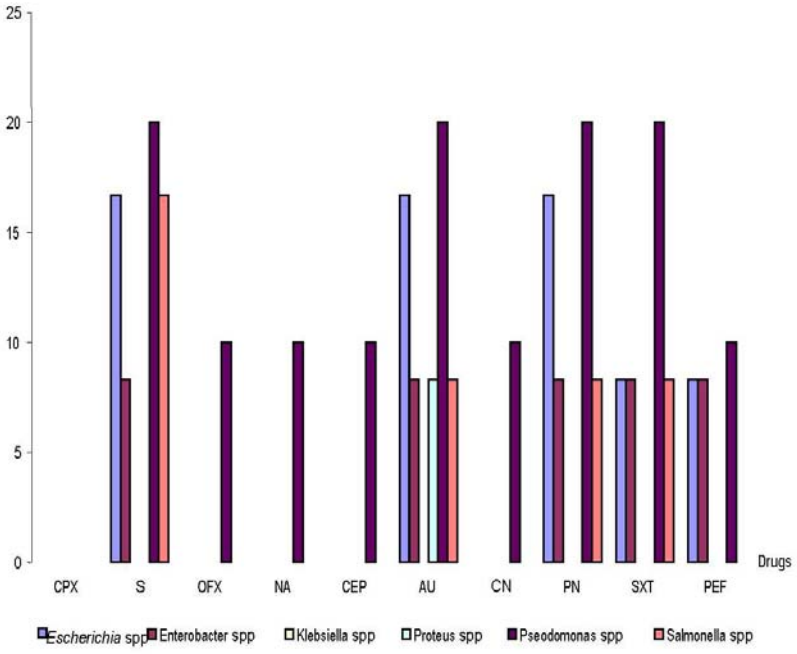


Figure 5: Percentage of clones of drug susceptible bacteria isolates that developed resistance to test antimicrobial agents after exposure to12.5 mg/ml *P. africana* extracts for 48 hours.

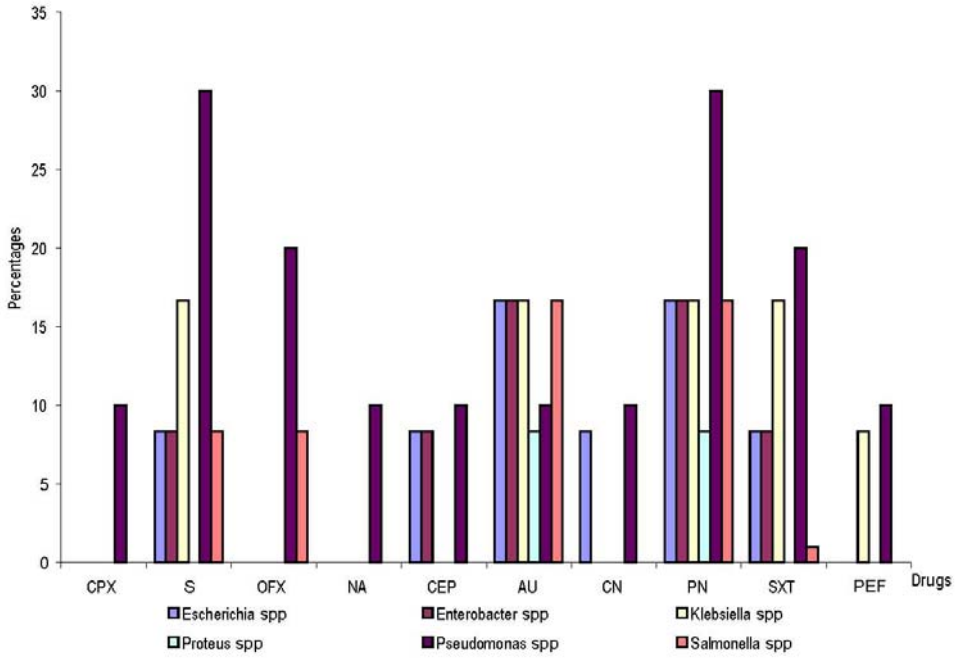
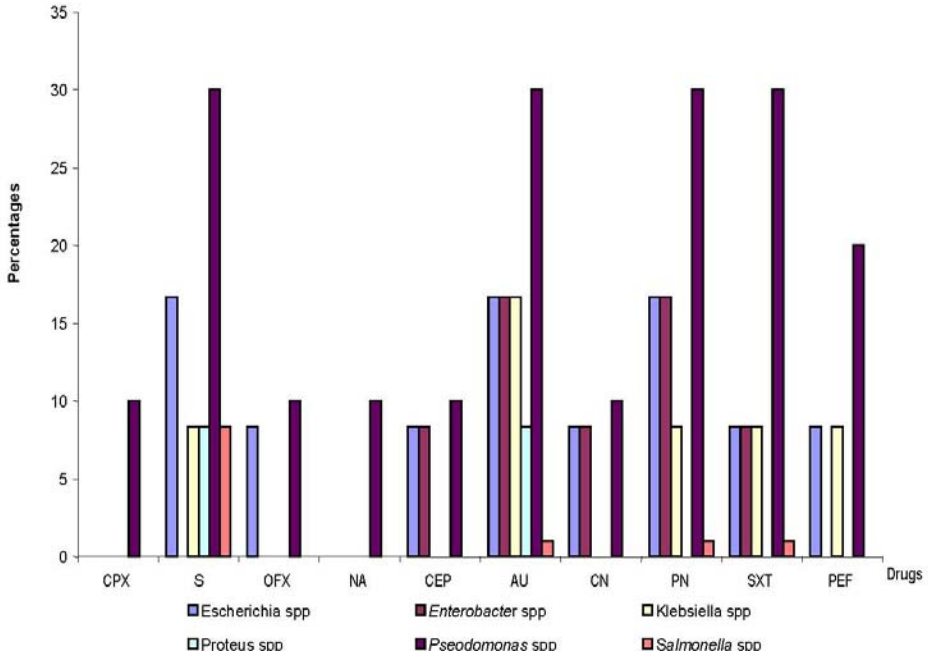


Figure 6: Percentage of clones of drug susceptible bacteria isolates that developed resistance to antimicrobial agents after exposure to 3.12 mg/ml *P. africana* extracts for 48 hours.



DISCUSSION

As part of the general response to the public health problems associated with increasing drug resistance among bacteria, attention is more and more focused on ethnomedicine and dietotherapy. This work evaluated by *in vitro* analysis the antibacterial activity of extracts of *Prosopis africana, Tetrapleura tetraptera* and *Buchholzia coriaceae*. Phyto-chemical analysis of the extracts revealed, among others, the presence of alkaloids, saponins, flavonoids and terpenoids in the plants. These secondary metabolites have been previously shown to have antibacterial activities (Levin *et al.,* 1979; Okafor *et al;* 2001; Okafor *et al;* 2002; Okoli and Iroegbu, 2004; Esimone *et al;* 2005). Results obtained portray these plants as having appreciable activities against the test organisms. For instance, the low MIC (1.25 mg/ml) of *T. tetraptera* extracts against both CS and SS isolates of *Enterobacter* spp is remarkable. Also *B. coriaceae* demonstrated relatively low MIC (3.125 mg/ml) against CS isolates of *Salmonella* and *Klebsiella* spp. These data recommend the plants for further analysis in the multidimensional search for a solution to the apparently intractable problem of multidrug resistance of bacteria. The crude extracts have shown potential as veritable sources of resistance modifying agents.

A hitherto unexplored side to the much acclaimed bioactive effect of medicinal plants or their products in medicine is their possible role in the emergence and sustenance of drug resistance in bacteria. This study took a maiden look into the consequences of exposing bacteria especially enteric bacteria to sub-lethal concentrations of some plant extracts. This study was conducted against the background knowledge that herbal remedies are often administered in unspecific and non standardized doses. Results indicate that these (test) plants containing biologically active secondary metabolites such as tannins, saponins, alkaloids, flavonoids and terpenoids are capable of inducing and/ or selecting (by yet undefined mechanisms) bacterial clones resistant to antibacterial agents from populations initially susceptible to the drugs. The induced MAR is such that it has generic effects, manifesting as cross – resistance to ciproflox (a quinolone), nalidixic acid (an older quinolone), streptomycin (an aminoglycoside), ceporex (a cephalosporin), augumentin (an amoxicillin / clavulanic acid), ampicillin (a penicillin) and septrin (a sulphonamide / trimethoprim). It does appear that plant extracts with more potency as shown by *T. tetraptera* also have a higher likelihood of inducing MAR especially at low concentrations. These results show that the MAR induction is dose–dependent and it is recommended (on this basis) that herbal remedies should be dose-regulated and monitored.

The data obtained further suggest that bacteria belonging to a genus known to have intrinsic resistance abilities are more easily induced than those with less of such capabilities. Members of the genus *Pseudomonas* showed higher MAR acquisition than other test bacteria. Members of the genus *Proteus* appeared to be least prone to MAR induction.

The potential impact of this demonstrable emergence of drug resistant bacteria as a result of exposure to (sub lethal concentrations) plant metabolites is high, considering the re-emerging trust being bestowed on ethno medicine and herbal remedies. This is even more worrisome in view of the fact that traditional healing is not dose–defined and is still the first point of healthcare

for many people in sub–Saharan Africa and other resource poor areas (TDR News, 2007). On the other hand the findings of this study may explain, in part, the resurgence of multidrug resistant bacteria in our communities. Despite its positive contribution, therefore, herbal remedies may also incorporate some detrimental public health effects. We therefore call for caution in the recommendation and usage of herbal remedies. We also recommend that this area becomes a general focus of research using these and other plants as well as other genera of bacteria and fungi, in order to further evaluate our results. Part of these studies are already on-going in our laboratory.

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