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SENSITIVITY OF EXTENDED-SPECTRUM β - LACTAMASES PRODUCING ENTEROBACTERIACEAE TO *ACALYPHA MACCAFEANA* EXTRACTS

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ABSTRACT
Powdered leaves of Acalypha maccafeana (L.) "Red Acalypha" was extracted with ethanol and fractionated with Petroleum ether, Chloroform and n-butanol using maceration technique. The extract and fractions were tested for antimicrobial activity against confirmed extended spectrum β-lactamases (ESBLs) producing enterobacteriaceae isolates of Escherichia coli, Citrobacter species, Enterobacter species, Klebsiella species, Proteus species and Salmonella species using disc diffusion method and microbroth dilution technique. The extract and fractions were further subjected to screening for phytochemical constituents. Sensitivity test results showed that ethanol extract, petroleum ether and residue fractions of red Acalypha were active against the isolates tested. The results of phytochemical screening indicated the presence of alkaloids, reducing sugars, steroids and tannins in both extract and fractions with flavonoids only present in ethanol extract and residue fractions of the plant.

Keywords: Sensitivity, β-lactamase, Enterobacteriaceae, Acalypha maccafeana

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are plasmid mediated enzymes that confer resistance to bacterial pathogens. The enzymes pose threat to current β-lactam therapies and are of growing concern because at present there exist more than 200 different natural variants worldwide (Duim *et al,* 2006).

ESBL occur predominantly in the family enterobacteriaceae leading to outbreaks of nosocomial infections in intensive care units, burn, oncology and neonatal units (Kohler et al, 1999).

Medicinal plants have long been utilized as a source of therapeutic agents worldwide due to their constituent secondary metabolites (Singh and Bhat, 2003). Some of the well known Nigerian plants such as *Mitracarpus scaber, Acalypha ornate, Acalypha godseffiana, Allium sativum, Aloe barbadensis and Vitex doniana* have been known to exert some inhibitory effects on bacteria and fungi (Akinyanju *et al*, 1986; Benjamin *et al*, 1986; Olonitola *et al*, 1998; Yusha'u and Dabo, 2004).

Acalypha maccafeana is mostly tropical or subtropical, fast growing shrub 4-6 inches long with heart-shaped and pink coloured leaves (Gilman, 1999). It is locally used in the treatment of dermatological and gastrointestinal disorders (Akinde and Odeyemi, 1987) including the devastating typhoid fever.

The common use of β-lactam antibiotics in treatment of bacterial infections coupled with the emergence of ESBLs producing organisms among pathogen calls for the need to search for new antimicrobials especially from natural sources such as plants.

This study was conducted with the objective of determining the phytochemical constituents present in *A. maccafeana* and the sensitivity of ESBLs producing clinical isolates to the plant extracts. It was aimed at finding alternative treatments to infections caused by ESBLs producing organisms.

MATERIALS AND METHODS

Collection and identification of Plant Material

A. maccaffeana (Red Acalypha) was identified by Dr. L. D. Fagwalawa of Biological Sciences Department, Bayero University, Kano and the leaves were handpicked, air dried and ground into powder using mortar and pestle in the laboratory as described by Mukhtar and Tukur (1999).

Extraction

The powdered plant material was subjected to ethanol extraction using the protocol of Fatope and Hamisu (1993) as described below.

Ethanol Extract

Eight batches of fifty grams each of the powdered plant materials were weighed and percolated with 500mls and the ninth batch of twenty grams was weighed and percolated with 200mls of 95% ethanol in separate conical flasks and allowed to stand for two weeks with intermittent shaking. These were filtered and concentrated using Rotavapour machine at 4-10^{o}C. They were combined, air-dried and labeled EE(R).

Petroleum Ether Fraction

Ten grams (10g) of ethanol fraction was macerated with 450mls of Petroleum ether using 50ml for nine macerations. The fractions were evaporated to dryness Red Acalypha while the residue was further subjected to chloroform extraction.

Chloroform Fraction
The residue above was further macerated with 850mls of chloroform using 100ml each time for six times and then followed by 50mls aliquot for five times. The fractions were evaporated to dryness and labeled CF(R) while the residue was further subjected to n-Butanol extraction.

n-Butanol Fraction
The residue above was further macerated with 200mls of n-butanol using 50ml each time for four times. The fractions were evaporated to dryness and labeled n-BF(R) while the residue was kept as residue fraction.

Residue Fraction
The residue from n-Butanol extraction above was labeled RF(R). All extracts were kept refrigerated before use.

Phytochemical Screening
The extracts were subjected to phytochemical tests to determine the groups of secondary metabolites present in the plant material.

Test for Alkaloids
To 1.0ml of each extract in two separate test tubes, 2-3 drops of Dragendoff's and Meyer's reagents were separately added. An orange red precipitate (turbidity) with Dragendoff's reagent or white precipitate with Meyer's reagent would denote the presence of alkaloids as reported by Ciulci (1994).

Test for Flavonoids
To two mls of 4mg/ml each of the fractions, a piece of magnesium ribbon were added followed by Conc. HCL drop wise. A colour ranging from orange to red indicates flavones; red to crimson indicates flavonols while crimson to magenta indicates flavonoids (Sofowara, 1993).

Test for Saponins
To 0.5g of the powder in a test tube, 5.0ml of distilled water was added and vigorously shaken. A persistent froth that last for atleast 15minutes would indicate the presence of saponins in accordance with Brain and Turner (1975).

Test for reducing sugars
One ml of each fraction was taken in separate test tubes. These were diluted with 2.0ml of distilled water followed by addition of Fehling's solution (A+B) and the mixtures warmed. Brick-red precipitates at the bottom of the test tubes would denote the presence of reducing sugars in accordance with Brain and Turner (1975).

Test for Steroids
Two ml of the extracts were taken into separate test tubes and evaporated to dryness. The residues were dissolved in acetic anhydride and chloroform was then added. By means of a pipette concentrated sulphuric acid was added by the side of the test tubes. A brown ring at the interface of the two liquids and the appearance of violet colour in the supernatant layer would indicate the presence of steroids as observed by Cuilci (1994).

Test for Tannins
Two ml of the extracts were diluted with distilled water in separate test tubes, 2-3 drops of 5%ferric chloride ($Fecl_3$) solution was added. A green-black or blue-black colouration would indicate the presence of tannins as reported by Ciulci (1994).

Bioassay
Disc Preparation
Sensitivity discs of the extracts were prepared by serial doubling dilution of the extracts in Dimethyl sulfoxide (DMSO) followed by placing sterile Whatman No. 1 filter paper discs of 6mm diameter that take up 0.01ml to make the required disc potency. Disc potencies of 15, 30 and 60µg/disc were prepared.

Inoculum Standardization
Few colonies of confirmed extended spectrum β-lactamase producers were dispensed in sterile normal saline to match the 0.5 McFarland standard for sensitivity tests as described by NCCLS (1999). The McFarland standard was prepared by mixing 0.6ml of 1% (w/v) dihydrate barium chloride solution with 99.4ml of 1% (v/v) sulphuric acid solution.

Preliminary antimicrobial activities
Standardised inocula of the confirmed ESBL producing isolates were swabbed onto the surface of Mueller Hinton Agar in separate petridishes. This was followed by placing the prepared discs of the extracts and standard antibiotic discs onto the surface of inoculated media at intervals. The plates were incubated at 35^oC for 18 hours before observation for and measurement of zones of inhibition formed.

Micro broth Dilution Test for Minimum Inhibitory Concentration (MIC)
Different concentrations of the plant extracts (62.5, 125, 250, 500, 1000, 2000 µg/ml) were prepared by serial doubling dilution and incorporated into test-tubes of Mueller-Hinton broth (Scharlau Microbiology, Belgium). Standardized inocula were introduced and the tubes incubated at 37^0C for 24 hours. Tube containing broth without plant extracts were inoculated and incubated along side to serve as positive control. Un-inoculated tube containing broth and plant extracts were incubated along side to serve as negative control. The tubes were observed after incubation to determine the minimum inhibitory concentration (MIC) as the lowest concentration that shows no evidence of growth (turbidity).

Minimum Bactericidal Concentration (MBC)
Mueller-Hinton agar plates were inoculated with sample from each of the tubes that showed no turbidity and the plates were incubated at 37^0C for 24 hours to determine the minimum bactericidal concentration (MBC) as the dilution from which there occurs no growth.

RESULTS

The plant *Acalypha maccafeana* used in this investigation yielded extracts amounting to 16.79% of the initial powdered plant material when subjected to ethanol extraction. The product was gummy in texture and slightly greenish brown in appearance (Table 1).

The plant species was found to contain some secondary metabolites including; alkaloids, flavonoids, reducing sugars, steroids and tannins (Table 2).

Ethanolic extract of red Acalypha was active on *E. coli, Klebsiella* and *Salmonella species* at 30 µg/disc and Proteus at 60µg/disc potencies. It has no activity on *Citrobacter* and *Enterobacter species* at all concentrations tested.

The sensitivity test of Petroleum ether fraction of the plant on the isolates showed that Acalypha fraction was active on *E. coli* and *Klebsiella specie* at all concentrations tested but inactive against *Citrobacter* and *Enterobacter species*. The fraction was active against proteus at 30µg/disc but only at 60µg/disc potency against *Salmonella specie*.

Chloroform fraction of the plant extract was active against *E. coli* at 30µg/disc while active against *Salmonella specie* only at 60µg/disc but inactive against *Citrobacter, Enterobacter, Klebsiella* and *Proteus species* at all concentrations tested.

Sensitivity test of the isolates to n-butanol fraction showed that *Klebsiella specie* was sensitive at all concentrations tested while *Proteus specie* was sensitive to the fraction at 30µg/disc concentration. Moreover, *E. coli and Salmonella specie* were sensitive to red Acalypha only at 30µg/disc concentration. *Citrobacter* and *Enterobacter species* were insensitive to the fraction.

The residue fraction of the plant was active against *Klebsiella specie* but inactive against *Citrobacter* and *Enterobacter species* at all concentrations tested.

Minimum Inhibitory Concentration (MIC) of the plant against *Citrobacter specie* was 1000µg/ml and 2000µg/ml against all other isolates tested. The Minimum Bactericidal Concentration (MBC) of ethanolic extract of the plant was 2000µg/ml on *Citrobacter specie* and the MBC against *E. coli, Enterobacter, Klebsiella, Proteus* and *Salmonella species* was greater than 2000µg/ml.

Petroleum ether fraction was active against *Proteus specie* at all concentrations tested and active against *Citrobacter, Enterobacter* and *Salmonella species* at concentrations of 2000µg/ml (MIC). The fraction was active against *E. coli* and *Klebsiella specie* at 250 and 500µg/ml concentrations (MIC) with an MBC greater than 2000µg/ml (Table 4).

Red Acalypha fraction exhibited MIC of 1000µg/ml against *E. coli* and 2000µg/ml against *Citrobacter, Enterobacter* and *Salmonella species* but greater against *Klebsiella* and *Proteus species*. The plant fraction had MBC greater than 2000µg/ml.

DISCUSSION

The high yield of ethanolic extracts in the plant may be due to the stronger extraction capacity of ethanol as indicated by Tschehe (1971).

Flavonoids were only present in ethanol extract and residue fraction which may be due to the fact that all the solvents used in fractionation were not able to dissolve an appreciable amount of the metabolite to be detected by phytochemical screening procedure employed. Also, tannins were not detected in the n-butanol fraction of Acalypha extract. Some of these metabolites particularly the flavonoids were reported to be responsible for antimicrobial activity associated with some ethnomedicinal plants (Singh and Bhat, 2003).

Generally, the extent of activity exhibited by the ethanol extract and residue fractions was greater than that of other fractions which may be related to the presence of flavonoids in these fractions (Singh and Bhat, 2003) inaddition to alkaloids and tannins that are well documented for antimicrobial activity (Tschehe, 1971).

The variation in the sensitivity of the bacterial species tested as the extract and fractions may be as a result of the differences in the type of ESBLs harboured by these organisms since there exist more than 200 different phenotypes identified worldwide (Bradford, 2001; Marchandin *et al*., 1999) and different ESBLs vary in their resistance to different antibiotic substances.

Conclusion

Acalypha maccafeana (red Acalypha) extracts and fractions were having inhibitory effect on extended spectrum β-lactamase producing enterobacterial isolates tested in this work.

Table 1: Some physical parameters of Red Acalypha

Physical parameters	Red Acalypha
Weight extracted	420g
Weight of extract	70.5g
Colour	Greenish brown
Texture	Gummy

Table 2: Some phytochemical agents in extracts Acalypha

	Phytochemical Tests				
Fractions	**Alkaloids**	**Flavonoids**	**Reducing sugars**	**Steroids**	**Tannins**
EF (R)	+	+	+	+	+
PEF (R)	+	-	+	+	+
CF (R)	+	-	+	+	+
nBF (R)	+	-	+	+	+
R (R)	+	+	+	+	+

Key:
PEF – Petroleum Ether Fraction, CF – Chloroform Fraction, n-BF – n-Butanol Fraction, R – Residue, (R) – Red Acalypha, + - Present, - - Absent

Table 3: Sensitivity of Extended Spectrum β-Lactamases Producers (mm) to Red Acalypha Extracts using Disc Diffusion Method

	EE (µg/disc)			PEF (µg/disc)			CF (µg/disc)			BF (µg/disc)			RF (µg/disc)		
Isolate	**15**	**30**	**60**	**15**	**30**	**60**	**15**	**30**	**60**	**15**	**30**	**60**	**15**	**30**	**60**
Citrobacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter sp	0	9	10	0	0	0	0	0	0	0	0	0	0	0	0
E. coli	0	8	9	10	14	16	0	8	8	0	0	9	8	8	8
Klebsiella sp.	0	10	11	10	13	13	0	0	0	8	8	13	8	10	12
Proteus sp.	0	0	9	0	0	8	8	0	0	0	8	8	0	0	8
Salmonella sp.	0	8	8	0	0	12	0	0	8	0	0	12	0	0	13

Key:
EE – Ethanol Extracts, PEF – Petroleum Ether Fraction, CF – Chloroform Fraction, BF – Butanol Fraction, RF – Residue Fraction

Table 4: Sensitivity of Extended Spectrum β-Lactamases Producers to Red Acalypha Extracts using Microbroth Dilution Technique

	EE (µg/ml)		PEF (µg/ml)		CF(µg/ml)		BF(µg/ml)		RF(µg/ml)	
Isolates	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Citrobacter spp.	1000	**	2000	**	2000	**	**	**	**	**
Enterobacter spp.	2000	**	2000	**	2000	**	**	**	**	**
E. coli	2000	**	250	**	1000	**	2000	**	**	**
Klebsiella spp.	2000	**	500	**	**	**	**	**	**	**
Proteus spp.	2000	**	**	**	**	**	**	**	**	**
Salmonella spp.	2000	**	2000	**	2000	**	2000	**	**	**

Key:
MIC – Minimum Inhibitory Concentration, EE – Ethanol Extracts, PEF – Petroleum Ether Fraction,
MBC – Minimum Bactericidal Concentration, CF – Chloroform Fraction, BF – Butanol Fraction, RF – Residue Fraction
** - MIC or MBC above 2000 µg/ml

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