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Antimicrobial efficacy of fractions from the most active ethanolic column fraction of the stem-bark of *Zizyphusspina christi (Desf)*

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ABSTRACT

The phytochemical study and in vitro antimicrobial studies of further fractionation of the most active column fraction of the ethanolic extract of stem-bark of Zizyphusspina christi Linn were evaluated using Gram positive and Gram negative pathogens as well as Candidaalbican using the disc diffusion method. The powdered most active column fraction (15.8g) was further fractionated using hexane / chloroform methanol as eluting solvents. The resulting portions from column chromatographic separation were combined into five (5) fractions A_1–A_5 on the basis of TLC results. However fraction A_5 was pure compound obtained after crystallization using acetone.Fractions $A_1$to A_4 were again recombined based on polarity where (A_1& A_2) were of lower polaritythan (A_3& A_4). Phytochemical analysis showed moderate presence of terpenoids and cardiac glycoside in (A_1& A_2), while fraction (A_3& A_4) showed copious presence of cardiac glycoside, moderate presence of terpenoids and alkaloids but low flavonoid content. The pure compound showed low presence of terpenoids and cardiac glycosides. Anti-microbial susceptibility studies showed that column fractions (A_1& A_2) and (A_3& A_4) were very active against all organisms tested except Escherichia coli,Streptococcus pyogenes and Pseudomonas aeruginosa while the pure compound was active only on Proteus mirabilis and Bacillus subtilis. The pure compound after spectroscopic studies was confirmed to be betulinic acid. The MIC values of the susceptible organism ranged from 6.25 – 18.75, while the MBC were usually one step higher than the MIC (12.25 to 25). The reference antibiotic showed the same pattern in the MIC and MBC. The fact that the MBC values were only one step away from the MIC implies that the activity of the column fractions were bactericidal. Since betulinic acid, a triperpenoidhas antimicrobial activity; the compound maybe partlyresponsible for the antimicrobial activity of Z. spina-christi. The results have provided scientific validity for the use of this plant in the treatment of bacteria-related infections in herbal medicines.

Keywords: *Zizyphusspina christi* , active column fraction, phytochemicals, stem-bark, *in vitro* and column fractionation

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INTRODUCTION

The plant *Zizyphusspina christi* from the family *Rhamnaceae* is readily distributed in the Sahara and Sahel, from Senegal to the Sudan and Arabia. (Von Maydell, 1990).The plant *Z. spina-christi*, a deciduous tree is found in Borno state and locally called “kurna” in Hausa and Kanuri. The Shuwa Arab of Nigeria, Chad and Cameroon call it “Nabak” (Anthony, 2005). It is a quick growing, strong and long lived deciduous tree. It flowers from October to November and the seed ripens from January to February in Nigeria. The pleasant smelling flowers are hermaphrodite (Levy, 1991).The majority of the rural populations in Arid and Savanna regions use *Zizyphusspina christi* as food and especially in Northern Nigeria, where the fruits have nutritional value (Anthony, 2005).

The aim of this study is to evaluate the antimicrobial activities of fractions from the most active fractionated column fraction of the ethanolic extract of the plant, so as to confirm the folkloric usage in treatment of urinary diseases,

skin and wound infections. Different parts of the plant are used for various medicinal purposes among the local populace of Northern Nigeria. It is used for the treatment of wounds, burns, stomach discomfort and urinary infections (Mohammed *et al*., 2011, 2012). Previous studies suggest that *Zizyphusspina christi* can be very useful in the control of hepatic and nephritic abnormalities (Mohammed *et al*., 2009).

EXPERIMENTAL SECTION

Sample collection and identification of plant

Sample of the stem-bark of *Zizyphusspina christi* Linn was collected from Jiddari Polo of Maiduguri Metropolitan Council Area of Borno State, Nigeria. The plant material was identified and authenticated by a plant taxonomist at the Department of Biological Sciences, University of Maiduguri to be from *Z. spina-christi*. The voucher specimen was deposited and labeled 544c at the herbarium.

Extraction of Plant Materials

The stem-bark of *Z. spina-christi* was cleaned, air-dried in the laboratory, for number days and pulverized into a coarse powder using a mortar and pestle. The coarse powder was weighed and stored at room temperature in a plastic container. Nine hundred grams (900g) of *Z. spina-christi* air-dried powder was placed in a thimble. The thimble and its contents were introduced into a soxhlet extractor which was connected to a condenser. The powder was extracted for eight (8) hours with 2 L of 95% ethanol. The crude extract obtained after drying, the concentrate, was defatted with petroleum ether and concentrated to dryness in vacuum at 40°C.The extract concentrate was weight and labeled and stored at room temperature for further analysis.

Column Chromatographic Separation

The powdered most active column fraction (15.8g) was further fractionated using hexane / chloroform / methanol as eluting solvents. These ratios were usually started with 100% hexane and then hexane / chloroform ratios to 100% chloroform and then chloroform / methanol to 80 / 20. The resulting portions from column chromatographic separation were combined into five (5) fractions $A_1$to A_5 on the basis of TLC results. However fraction A_5 was pure compound obtained after crystallization using acetone. Fraction $A_1$to A_4 were again recombined based on polarity where (A_1& A_2) were of lower polaritythan (A_3& A_4). (A_1& A_2) weighed 4.5g while (A_3& A_4) weighed 2.3g, and the pure compound from A_5 weighed 30mg.(A_1& A_2) was a brown gummy mass while (A_3& A_4) was yellowish brown-gummy solid mass and pure compound was amorphous white powder.

Phytochemical screening of most active column fraction

Being a bioassay guided study the most active column fractionsA & C were subjected to further column fractionation.The obtained fractions combined into(A_1& A_2) and (A_3& A_4) which were then subjected to phytochemical analysis using standard procedures. The various classes of active chemical constituents like tannins, saponins, saponin glycosides, cardiac glycoside, flavonoids, alkaloids, terpenes and steroids etc were screened according to the methods of (Trease and Evans 2002; Sofowora, 2008).

Antimicrobial studies

Test microorganisms

The column fractions (A_1& A_2), (A_3& A_4) and pure compound of *Z. spina-christi* were subjected to antimicrobial susceptibility studies against a total of nine microorganisms with five gram negative organisms (*Salmonella typhi, Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa*) three gram positive organisms (*Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes*) and one fungal strain (*Candidaalbican*). Standard susceptibility antibiotic disc of ciprofloxacin (5µg/disc); norfloxacin (10µg/disk) erythromycin (10µg/disc) were used to compare extract activities.

Antimicrobial Susceptibility Testing

The column fractions, (A_1& A_2), (A_3& A_4) and pure compound resulting from the most active column fraction of the ethanolic extract of*Z. spina-christi* were subjected to preliminary antimicrobial evaluation on five (5) Gram negative and three (3) Gram positive organisms and one (1) fungal strain *Candidaalbican*. The column fractions were made in four stock concentrations of 6mg/ml, 4mg/ml, 2mg/ml and 1mg/ml; prepared by dissolving 60mg, 40mg, 20mg and 10mg respectively in 10ml each of sterilized distilled water.

The disc diffusion method was used where a 6mm diameter disc was used. Agar plates for the different organisms were prepared and allowed to dry.One milliliter of an overnight broth culture of each test microorganism was dispensed on the nutrient agar plate and spread evenly using a sterile glass rod. The disc was allowed to soak the ethanolic extract at various concentrations, 6mg/ml, 4mg/ml, 2mg/ml and 1mg/ml. After thirty minutes the various concentrations of the disc containing the extract were applied on the surface of the culture plates before incubation

at 37^{o}C for 24hrs. Sensitive organisms showed a zone of inhibition whereas resistant organisms showed growth right to the edge of the disc. Zone of inhibition gave relative activity of the microbial agent against each test organism. Susceptibility test were carried out according to standard methods (Monica, 2000). At the end of incubation period inhibition zone were recorded in millimeter as the diameter of growth free zones around the 6mm disc using a transparent meter rule.

Determination of minimum inhibitory concentration (MIC)

MIC is a technique employed to determine the concentration of the column fractions (A_1& A_2), (A_3& A_4) and pure compound that can inhibit the microbial activity. MIC was determined using the broth dilution technique as described by (Baker and Silverton, 2007). The minimum inhibitory concentration was evaluated from microorganisms that were sensitive to the column fractions under study (stem-bark).

Equal volumes of nutrient broth (0.5ml) were dispensed into sterile test-tubes where known concentrations of the column fractions (A_1& A_2), (A_3& A_4) and pure compound ranging from highest to lowest, (25mg/ml, 18.75mg/ml, 12.5mg/ml, 6.25mg/ml,) were prepared. Also 0.5ml suspension of the microbial isolates (*Salmonellatyphi*, *Klebsiellapneumoniae*, *Proteusmirabilis*, *Escherichiacoli*,*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcuspyogenes*,*Candidaalbican*) were inoculated into the above four concentrations respectively for susceptible organisms. They were incubated at 37^{o}C for 24hours and observed with the naked eye for turbidity. Turbidity showed growth while clear test tubes showed inhibition.

Determination of Minimum Bactericidal Concentration (MBC)

MBC was determined by using the broth dilution technique described by Baker and Silverton (2007) by assaying the test-tubes resulting from MIC determinations. A loopful of the content of the test tubes were inoculated by streaking on a solidified nutrient agar plate, incubating at 37^{o}C for 18–24 hrs and observed for bacterial growth. The lowest concentrations of the subculture with no growth were considered the minimum bactericidal concentration.

Statistics

The result of zone of inhibition exhibited by the column fraction, pure compound and fractions and standard antibiotics against each organism were presented as Mean ± Standard Deviation utilizing statistical package for social sciences version 16, 2007 software for computers.

RESULTS AND DISCUSSION

The powdered most active column fraction (15.8g) was further fractionated using hexane / chloroform / methanol as eluting solvents. The resulting portions from column chlromatographic separation were combined into five (5) fractions($A_1$to A_5) on the basis of TLC results. However fraction A_5 was pure compound obtained after crystallization using acetone.Fraction ($A_1$to A_4) were again recombined based on polarity where (A_1& A_2) were of lower polaritythan (A_3& A_4). (A& A_2) weighed 4.5g while (A_3& A_4) weighed 2.3g, the pure compound from A_5 weighed 30mg.(A_1& A_2) was a brown gummy mass while (A_3& A_4) was yellowish brown -gummy solid mass and pure compound was amorphous white powder. The colour, form, percentage yield of column fractions and the pure compound from ethanolic extract of *Z. spina-christi* are presented in Table 1.

Table 1: The Colour, form, percentage yield of column fractions and the pure compound from ethanolic extract of stem-bark of *Z. spina-christi*

S/No	Fractions	Weight (g)	Colour	Texture	% yield
1	A_1& A_2	4.5	Brown	Gummy -mass	28.5
2	A_3& A_4	2.3	Yellowish brown	Gummy solid mass	14.56
3.	Pure compound	0.75	Amorphous White powder	Amorphous Powder	4.75

Phytochemical analysis was carried out on (A_1& A_2) which showed moderate presence of terpenoids and cardiac glycoside, while faction (A_3& A_4) showed copious presence of cardiac glycoside, moderate presence of terpenoids and alkaloids while flavonoids showed low presence. The pure compound showed low presence of terpenoids and cardiac glycosides. The result for phytochemical constituents of column fraction and the pure compound are presented in table 2.

Table 2: Phytochemical constituents of ethanol extract, the column fractions and the pure compound from the stem-bark of *Z. spina-christi*Linn

S/No	Chemical constituents	(A_1&A_2)	(A_3 & A_4)	Pure compound
1	Carbohydrate	-	-	-
2	Soluble starch	-	-	-
3	Tannins	-	-	-
4	Flavonoid	-	+	-
5	Anthraquinone	-	-	-
6	Terpernoid	+ +	+ +	+
7	Cardiac glycoside	+ +	+ + +	+
8	Saponin glycoside	-	-	-
9	Alkaloids	-	+ +	-

(-) = Absent; (+) = low concentration, (+ +) = Moderate concentration; and (+ + +) = High concentration.

Research has shown that potential use of plant extract for treatment was due to the phytochemicals present in the extract. For example plant synthesizes secondary metabolites which serve as plant defense mechanisms against predation by microorganisms, insects and herbivous.Flavonoids are alsosynthesized by plants in response to microbial infection and consequently have been found in vitro to be effective antimicrobial agents against a wide array of microorganisms (Dixon *et al*, 1983). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*, 1996).

Terpenenes or terpenoids are active against bacteria, fungi, and viruses (Himejima*et al*, 1992). The triterpenoidbetulinic acid is one of the terpenoids which has been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Fugioka and Kashiwada, 1994).

Diterpenoid alkaloids commonly isolated from the plants of the *Ranunculaceae*, or buttercup family are commonly found to have antmicrobial properties (Rahman and Choudhary, 1995). Solamargine, a glycoalkaloid from the berries of *Solanumkhasianum* and other alkaloids may be useful against HIV infection as well as intestinal infections associated with AIDS (McMahon *et al*, 1995). Alkaloids also have been reported to have microbiological effects (Ghoshal*et al*, 1996).

The activities of column fractions (A_1& A_2), (A_3& A_4) and the pure compound were found to be concentration-dependent against the Gram positive and Gram negative species; particularly most active column fractions were more active than the individual column fraction which could be indicative of the presence of broad spectrum antimicrobial compounds in the most column active fraction notably terpenes, flavonoid, cardiac glycosides, and alkaloids. While the column fractions (A_1& A_2) showed the presence of terpenoids, cardiac glycoside and column fractions (A_3& A_4) showed presence of terpenoids, cardiac glycosides, flavonoids and alkaloids, while the pure compound showed the presence of terpenoids and cardiac glycoside.

The result of the diameters of inhibition zones of column fractions (A_1& A_2), (A_3& A_4) and pure compound as well as the standard antibiotics were presented in tables 3 – 6. Diameters of inhibition zones at 6mg/ml / disc is presented in table 3.while 4mg/ml to 1mg/ml were presented in table 4 to 6.

The result showed that activities against Gram negative and Gram positive organisms range from 8 – 14 and 8 – 10mm respectively. Further fractionation of the most active column fraction (7 – 16 and 8- 16mm) demonstrated a good range of sensitivity against the test organism, but only on 50% of the organism. Fraction (A_3& A_4) was the most active against the organism tested. Only *E. coli*, *Streptococcus pyogenes*and*Pseudomonas aeruginosa* did not show any susceptibility to it.While*Candida*was weakly sensitive to fractions (A_3& A_4) but resistant to fractions (A_1& A_2) and the pure compound. The fact that the column fractions are more active than the pure compound at 6mg/ml concentrations may be explained due to synergistic effect of the constituents of the fractions or more concentrations above 6mg/ml of the pure compound may be required for better effect. At lower amounts of concentration of 4mg/ml, 2mg/ml and 1mg/ml tables 4 – 6. The activities were fair at 4mg/ml for the column fractions when compared to the 6mg/ml level of concentration. However, at 2mg/ml and 1mg/ml concentrations virtually all the organisms were resistant to the column fractions and the pure compound. The result revealed that by the dilution test the MIC values of susceptible organisms ranged from 6.25mg/ml to 18.75mg/ml, while MBC were usually one step higher than the MIC (12.5 to 25mg/ml). The reference antibiotic showed the same pattern in the MIC and MBC. The result for bacteriostatic and bacteriocidal effect (Table 7).

Table 3: Susceptibility pattern of column fractions, ethanolic and pure compound at 6mg/ml concentration

		Organism/Diameters Of Inhibition Zone (mm)								
S/No.	**Column Fractions**	***Salmonella typli***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Bacillus subtilis***	***Candida albican***
1.	$A_{1\,\&}A_2$	8.15±0.18	R	R	10.75±0.84	12.61±0.85	8.15±0.18	R	10.15±0.81	R
2.	$A_{3\,\&}A_4$	10.75±0.84	R	R	10.15±0.81	14.61±1.24	9.19±1.06	R	10.75±0.84	7.49±0.11
3.	Pure Compound	R	R	R	R	9.19±1.06	R	R	9.54±104	R
4.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.73±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.75±0.84
5.	Erythromycin	7.33±0.15	R	R	R	11.23±2.46	11.52±0.95	12.11±0.89	14.91±0.86	R
6.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

At lower amounts of 4mg/ml, 2mg/ml and 1mg/ml, the activities gradually became less pronounced but showing similar trend as mentioned for the higher 6mg activities.

Table 4: Susceptibility pattern of column fractions, ethanolic and pure compound at 4mg/ml concentration

		Organism/Diameters Of Inhibition Zone (mm)								
S/No.	**Column Fractions**	***Salmonella typli***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Bacillus subtilis***	***Candida albican***
1.	$A_{1\,\&}A_2$	7.58±0.84	R	R	9.42±1.08	10.31±1.23	7.33±0.15	R	7.31±0.76	R
2.	$A_{3\,\&}A_4$	9.71±0.91	R	R	9.57±0.94	12.52±0.78	8.15±0.18	R	9.64±0.16	R
3.	Pure compound	R	R	R	R	7.18±0.45	R	R	9.42±1.08	R
4.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.73±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.75±0.84
5.	Erythromycin	7.33±0.15	R	R	R	11.23±2.46	11.52±0.95	12.11±0.89	14.91±0.86	R
6.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

Table 5: Susceptibility pattern of column fractions, ethanolic and pure compound at 2mg/ml concentration

		Organism/Diameters Of Inhibition Zone (mm)								
S/No.	**Column Fractions**	***Salmonella typli***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Bacillus subtilis***	***Candida albican***
1.	$A_{1\,\&}A_2$	R	R	R	R	R	R	R	R	R
2.	$A_{3\,\&}A_4$	R	R	R	R	7.68±1.16	R	R	7.06±0.19	R
3.	Pure compd	R	R	R	R	R	R	R	7.11±0.89	R
4.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.73±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.75±0.84
5.	Erythromycin	7.33±0.15	R	R	R	11.23±2.46	11.52±0.95	12.11±0.89	14.91±0.86	R
6.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

Table 6: Susceptibility pattern of column fractions, ethanolic and pure compound at 1mg/ml concentration

		Organism/Diameters Of Inhibition Zone (mm)								
S/No.	**Column Fractions**	***Salmonella typli***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Bacillus subtilis***	***Candida albican***
1.	$A_{1\,\&}A_2$	R	R	R	R	R	R	R	R	R
2.	$A_{3\,\&}A_4$	R	R	R	R	R	R	R	R	R
3.	Pure compd	R	R	R	R	R	R	R	R	R
4.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.73±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.75±0.84
5.	Erythromycin	7.33±0.15	R	R	R	11.23±2.46	11.52±0.95	12.11±0.89	14.91±0.86	R
6.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

Table 7: Minimum inhibitory and minimum bactericidal concentrations of the column fractions, ethanol extract, pure compound of the stem bark of *Z. Spina-Christi*and standard control drugs

		Organisms' MIC and MBC								
S/No.	**Column Fractions**	***Salmonella typli***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Bacillus subtilis***	***Candida alblican***
1	$A_{1\,\&}A_2$				18.75* 25**	6.25* 12.5**			18.75* 25**	
2	$A_{3\,\&}A_4$	12.5* 18.75**			12.5* 18.75**	12.5* 18.75**			12.5* 18.75**	
3	Pure Compd.									
4	Ciprofloxacin5ug	1ug* 1ug**	1* 1**	1* 2**	1* 1**	1* 1**	1* 1**	1* 2**	1* 1**	2* 3**
5	Erythromycin10ug						7* 8**	7* 7**	5* 6**	
6	Norfloxacin 10ug	6* 7**	5* 5**	5* 6**		7* 8**				

Key: MIC; MBC**; EE = Ethanolic extract; PC = Pure compound*

Showed that fraction (A_3& A_4) was bacteriostatic and bactericidal on four (4) microorganisms, *Salmonella typhi, Klebsiella pneumoniae, Proteus mirabilis and Bacillus subtilis*. Fraction (A_1& A_2) was bacteriostatic and bactericidal on three (3) microorganisms, *Klebsiellapneumoniae,Proteusmirabilis* and *Bacillussubtilis* while *Proteus mirablis* and *Bacillussubtilis* were the only organisms that were sensitive to the pure compound but was neither bacteriostatic nor bactericidal on them. This was because the sensitivity was weak as it was not equal to or greater than (≥) 10mm zone of inhibition which according to Zwadyk(1972), that only diameter zone of inhibition (≥) 10mm exhibited by plant extracts were considered active.

CONCLUSION

From the antimicrobial susceptibility studies, fractions (A_1& A_2), (A_3& A_4) and the pure compound (P_1) known as betulinic acid were found to have antimicrobial efficacies on some of the test microorganisms. This could be as a result of presence of some phytochemicals such as terpenoids, flavonoids, alkaloids and cardiac glycosides. The result shows betulinic acid has antimicrobial activity even though its effect is not as pronounced as that of the standard antibacterial.

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