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Research Article

ANTIMICROBIAL EFFICACIES OF ETHANOLIC EXTRACT AND ACTIVE COLUMN FRACTIONS OF THE STEM-BARK OF *ZIZYPHUS SPINA-CHRISTI* L. (DESF)

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ABSTRACT

The objective of the study is to determine the phytochemical and *In vitro* antimicrobial activity of the extracts of *Zizyphus spina-christi.*

The ethanolic extract was further subjected to column fractionation using hexane/chloroform/methanol as eluting solvents. The fractions obtained were tested for antimicrobial activity against Gram positive, Gram negative bacteria and *Candida albicans*. The result indicated the fractions of ethanolic extract of *Zizyphus spina-christi* containing significant quantity of terpenoids are the most active against the microorganisms tested.

The minimum inhibitory concentration (MIC) values of susceptible organisms ranged from 6.25 to 18.75 while the minimum bactericidal concentration (MBC) was usually one step higher than the MIC (12.5 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 extract is bactericidal. The ethanolic extract and fraction A & C were the most active against the organisms tested. This could be as a result of the presence of some phytochemicals such as terpernoids, flavonoids, alkaloids and cardiac glycosides. The results have provided scientific validity for the use of this plant in the treatment of bacteria-related infections in herbal medicine.

Keywords: *Zizyphus spina christi*, Ethanolic extract, Active column fractions, Phytochemical, stem-bark and *In vitro*

INTRODUCTION

Zizyphus spina-christi L. known as "Kurna" in Kanuri or Hausa, or Jerusalem-thorn or Christ-thorn is a member of the Rhamnaceae family. The majority of the rural population in Northern Nigeria use *Zizyphus spina-christi* extensively for its medicinal and economic importance.

The plant *Zizyphus spina-christi* is readily distributed in the Sahara and Sahel, from Senegal to the Sudan and Arabia where the annual rainfall is about 50 – 300mm or on periodically inundated sites [1]. It grows in dry conditions and are conspicuously thorny, often the thorns in pairs one being straight and other curved. It has numerous small, sometimes minute teeth like leaves [2].

The genus *Zizyphus* has medicinal importance as all parts of the plant are used by the local Arab people to help maintain a healthy life style [3]. *Zizyphus spina-christi* has been reported to have activity against bacterial and fungal pathogens that are normally quite resistant to modern medications [4]. It is used extensively for the treatment of ulcers, wounds, eye diseases, bronchitis febrifuge, diuretic and as anti-inflammatory agent for healing skin diseases such as atopic dermatitis [5,6]. Similarly, 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 [7]. Previous studies suggest that *Zizyphus spina-christi* can be very useful in the control of hepatic and nephrotic abnormalities [8]. The present study was undertaken to provide scientific validity for the folkloric use of *Zizyphus spina-christi* as an antimicrobial agent.

MATERIALS AND METHODS

Sample collection and Identification of plant

Sample of the stem-bark of *Zizyphus spina-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 litres of 95% ethanol. The crude extract obtained after drying, the concentrate was defated 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.

Phytochemical screening of ethanolic extract

The ethanol extract was subjected to phytochemical analysis using standard procedures as described by Sofowara [8]. The various classes of active chemical constituents like tannin, saponins, saponin glycosides, cardiac glycosides, flavonoids, alkaloids, terpenes and steroids etc, were be screened according to the methods of Sofowara [8].

Column Chromatographic Separation

About 105g of the ethanolic extract was subjected to column chromatographic separation using hexane, chloroform and methnol as eluting solvents. These ratios were usually started 100% hexane and then hexane/chloroform ratios to 100% chloroform and then chloroform/methanol to 80/20. Various portions were obtained which were combined into six (6) fractions, fractions A – F base on the similarities of their spots and R_f values on TLC plates. However, only three (3) of the fractions, fractions A, C and F were obtained in sufficient concentration to enable their phytochemical screening and antimicrobial investigation

Antimicrobial Investigation

Test microorganisms

The ethanolic extract was subjected to antimicrobial susceptibility studies against a total of nine microorganisms with five gram negative organisms (*Salmonella typhi, Klebsiella pneumoniae, proteus mirablis, Escherichia coli, pseudomonas aeruginosa*) three gram positive organisms (*staphylococcus aureus, Bacilus subtilis, streptococcus pyogenes*) and one fungal strain (*candida albican*). Standard susceptibility antibiotic disc of ciprofloxacin (5µg/disc); norfloxacin (10µg/disk) erythromycin (10µg/disc) were used to compare extract activities.

Antimicrobial Susceptibility Testing

The ethanolic extract and column fractions A, C and F 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 *candida albican*. The extracts 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 where as 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 [9]. At the end of inhibition 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 extract that can inhibit the microbial activity. MIC was determined using the broth dilution technique as described by Baker and Silverton [10]. The minimum inhibitory concentration was evaluated from microorganisms that were sensitive to the extracts under study (stem-bark).

Equal volumes of nutrient broth (0.5ml) were dispensed into sterile test-tubes where known concentrations of the extracts 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 (*Salmonella typhi*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus* pyogenes, *Candida albican*) 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 [10], 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 inhibition exhibited by the extract, 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

About nine hundred (900) gram of the plant stem-bark was soxhlet extracted with ethanol and was defatted with petroleum ether and about 74.5g of reddish brown-gummy flakes of the ethanol extract was obtained with 8.28% w/w yield. Also 105g of the ethanol extract was subjected to column fractionation and fractions A, C and F were obtained. The values obtained were about 9.3 g light brown-gummy mass of fraction A with 8.86% w/w yield. About 6.6g orange powder of fraction C with 6.29% w/w yield and fraction F had about 6g light orange – glassy gummy mass with 5.71% w/w yield.

Phytochemical analysis for the ethanolic extract, column fraction A, C and F were carried out. For the ethanolic extract, copious presence of carbohydrate, tannins, flavonoids, terpenoids and saponin glycosides were obtained. Moderate presence of soluble starch, cardiac glycoside and alkaloids was obtained. While free and combined anthraquinones were absent. The analysis for fraction A give moderate presence of terpenoids and cardiac glycosides while flavonoids and alkaloids were in low concentration. Fraction C gave moderate presence of terpenoids and cardiac glycosides, while fraction F had moderate presence of cardiac glycoside and low presence of terpenoids (Table 1).

Table 1: Phytochemical constituents of ethanolic extract and the column fractions from the stem-bark of *Z. spina-christi* Linn

S. No.	Chemical constituents	Ethanol extract	A	C	F
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 (+ + +) = Copious concentration

Many secondary metabolites have therapeutic potential for a number of diseases and are useful as medicines. Some of them could serve as "lead compounds" for the synthesis of more effective drugs [11].

Research has shown that the potential use of plant extract for treatment was due to the phytochemicals present in the extract, for example tannins have astringent properties which are important in wound healing. They act by precipitating proteins and making nutritional protein unavailable to them and thereby protecting underlying tissues [12]. Some terpenes have effects on microorganisms, for instance plants oils containing terpenes such as cinnamon oil have in vivo inhibited multiple species of bacteria, with broad spectrum activity against pseudomonas aeruginosa [13]. Saponin is naturally occurring glycosides that has the common characteristics of foam formation in aqueous solution, bitter taste and ability to haemolyse red blood cells, highly toxic to cold blooded animals and their toxicity being related to their activities in lowering surface tension [14]. Previous studies have shown that the species of *zizyphus* are very rich in saponins. Saponins are known to have expectorant properties and these have been made use of in treatment of upper respiratory tract inflammation [15].

The mechanism of anti-carcinogenic properties of saponins include anti oxidant effects, direct and selected cytotoxicity of cancer cells, immune-modulation, acid and neutral sterol metabolism and regulation of cell proliferation [16]. Saponins are believed to prevent colon cancer. Normally bile acids pour into the stomach to help absorb fat food. Some bacteria in the large intestine turn the bile into a substance that in highly carcinogenic. Research suggests that saponins stop the toxic materials from forming [17].

Flavonoids are naturally occurring phenolic compounds in plants. The disease fighting potential of flavonoids stems from their ability to reduce inflammation, prevent the release of histamine (which causes allergic symptoms such as congestion), fight free radicals, boost immunity, strengthen blood vessels and increase blood flow [18]. They are also potent antioxidants; some are even more powerful than vitamin C or E in preventing cell damage caused by unstable oxygen molecules [18]. Alkaloids are also a class of chemical compounds which have varied medicinal properties, eg alkaloids like reserpine, deserpidine and rescinamine are used in psychiatric cases and as anti-hypertensive [8].

Table 2: Susceptibility pattern of column fractions, ethanolic and pure compounds at 6mg 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	10.75±0.84	R	8.15±0.18	12.61±0.85	12.65±0.78	9.05±0.46	R	12.17±1.10	7.49±0.11
2.	C	9.19±1.06	R	9.54±1.04	10.75±0.81	10.73±1.41	8.52±0.94	R	11.43±0.84	R
3.	F	R	R	7.61±1.21	9.20±0.54	10.15±0.81	R	R	10.32±1.13	R
4	Ethanolic Extract	15.23±0.45	R	14.61±1.24	16.49±1.06	16.51±0.11	14.12±0.79	R	16.63±0.94	13.45±1.02
5.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.37±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.13±1.17
6.	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
7.	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, 2mg and 1mg, (Tables 3-5 the activities gradually became less pronounced but showing similar trend as mentioned for the higher 6mg activities.

Table 3: Susceptibility pattern of column fractions, ethanolic and pure compound at 4mg 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	10.19±0.55	R	7.18±0.45	10.52±2.11	10.31±0.83	8.54±1.28	R	10.31±1.23	R
2.	C	7.58±0.84	R	8.92±1.15	9.71±0.91	9.42±1.08	7.31±0.76	R	10.59±1.51	R
3.	F	R	R	R	8.28±1.49	9.57±0.94	R	R	9.64±0.16	R
4.	Ethanolic extract	12.52±0.78	R	13.37±63	10.18±0.74	11.04±1.32	R	R	12.24±1.09	R
5.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.37±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.13±1.17
6.	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
7.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

Table 4: Susceptibility pattern of column fractions, ethanolic and pure compound at 2mg 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	8	R	R	8.65±0.73	9.54±23	R	R	9.19±0.74	R
2.	C	R	R	R	8.34±1.46	8.06±1.97	R	R	7.68±1.16	R
3.	F	R	R	R	7.11±0.89	8.44±1.21	R	R	8.31±1.33	R
4.	Ethanolic extract	R	R	R	6.76±1.53	7.06±0.19	R	R	8.27±0.74	R
5.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.37±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.13±1.17
6.	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
7.	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 1mg 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	R	R	R	7.33±0.17	8.14±0.54	R	R	8.29±0.88	R
2.	C	R	R	R	7.07±0.81	7.42±0.35	R	R	7.08±1.45	R
3.	F	R	R	R	R	7.03±1.02	R	R	6.96±2.32	R
4.	Ethanolic extract	R	R	R	R	6.78±0.94	R	R	7.37±1.11	R
5.	Ciprofloxacin	27.20±2.45	30.91±1.33	20.37±3.54	22.09±0.78	31.54±1.87	25.32±1.74	22.67±0.73	26.43±1.05	10.13±1.17
6.	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
7.	Norfloxacin	16.16±1.76	18.41±1.78	17.81±2.51	R	10.15±3.11	R	R	R	R

The result of the diameters of inhibition zones of the ethanolic extract and column fractions A, C and F as well as the standard antibiotics were presented in tables 2 – 5. Diameters of inhibition zones exhibited by the ethanolic extract and column fractions A, C and F at 6mg/ disc is presented in table 2.

The result showed that activities against Gram negative and Gram positive organism ranged from 7 – 31 and 8 – 26mm zone of inhibition respectively. The antimicrobial susceptibility test of the extract demonstrated activity to varying extents against organisms tested by the disc diffusion test. Fractions A, C and ethanolic extract were the most active against organism testing. Only *E. coli* and streptococcus pyogenes did not show any susceptibility to them, while *candida* was weakly sensitive to fraction A but resistant to fraction C. At lower amounts of 4mg, 2mg and 1mg tables (3 – 5). The activities gradually became less pronounced by showing similar trend as mentioned for the higher 6mg activities. At 4mg / disc the result showed that the zone of inhibition as measure of activities against Gram negative and gram positive organisms ranged from 7 – 10 and 7 – 10mm in both cases while at 2mg and 1ml / disc the results showed that activities against Gram negative and Gram positive organisms ranged from 7 – 9 and 8 – 9mm. Also at 4mg *Salemonella typyi, Klebsiella Pneumoniae, proteus mirabilis and bacillus subtilis* were sensitive, while pseudomonas aeruginosa and *staphylococcus* aureus were weakly sensitive and remaining organisms were all resistant. For fraction C only bacillus subtilis was sensitive with three (3) organisms resistant (*E. coli, streptococcus pyogenes and candida albican*) while the remaining organisms were weakly sensitive. For fraction F, three (3) organisms (klebsiella, proteus and bacillus subtilis were weakly sensitive while the other organisms were all resistant. At 2mg and 1mg most of the organisms were resistant while the remaining few microorganisms were weakly sensitive for which the results for susceptibility tests values ≥ 10mm zone of inhibition which according to Zwadyk [19], that only diameter zones of inhibition ≥ 10mm exhibited by plant extracts were considered active.

Table 6: Minimum inhibitory and minimum bactericidal concentrations of the column fractions, ethanol extract 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	12.5* 18.75**			18.75* 25**	12.5* 18.75**			12.5* 18.75**	
2.	C				12.5* 18.75**	18.75* 25**			12.5* 18.75**	
3.	F					12.5* 18.75**			12.5* 18.75**	
4.	Ethanolic Extract	18.75* 25**		18.75* 25**	18.75* 25**	12.5* 18.75**	18.75* 25**		12.5* 18.75**	12.5* 18.75**
5.	Ciprofloxacin5ug	1ug* 1ug**	1* 1**	1* 2**	1* 1**	1* 1**	1* 1**	1* 2**	1* 1**	2* 3**
6.	Erythromycin10ug						7* 8**	7* 7**	5* 6**	
7.	Norfloxacin 10ug	6* 7**	5* 5**	5* 6**		7* 8**				

Table 6 shows the minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the ethanolic extract and column fraction A, C and F. The results 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 antibiotics showed the same pattern in the MIC and MBC. The result showed that the ethanolic extract was bactericidal and bacteriostatic on seven microorganisms including *Candida* out of nine organisms with only *Escherichia coli* and *Streptococcus pyogenes* being resistant. It also showed that fraction A, was bacteristatic and bactericidal on four (4) microorganisms, *Salmonella typhi, Klebsiella pneumoniae, Proteus mirabilis and Bacillus subtilis*. Fraction C was bacteristatic and bactericidal on three (3) microorganisms, *Klebsiella pneumoniae, Proteus mirabilis, Bacillus subtilis* while fraction F was bacteristatic and bactericidal on two (2) microorganisms, *Proteus mirabilis* and *Bacillus subtilis*.

The reference antibiotic, ciprofloxacin was bacteristatic and bactericidal on all organisms including *Candida*. However, erythromycin was bacteristatic and bactericidal on only the three (3) Gram positive microorganisms under study. Likewise norfloxacin was bacteristatic and bactericidal on four (4) out of the five (5) Gram negative organisms studied. The fact the MBC values were one (1) step away from the MIC implies that activity of the extract is bactericidal.

From the antimicrobial susceptibility studies, the ethanolic extract and fraction A and C were the most active against the organisms tested. This could be as a result of some phytochemicals such as terpenoids, flavonoids, tannins, saponins, cardiac glycosides and alkaloids for the ethanolic extract. While for fractions A, C and F could be as a result of terpenoids, flavonoids, alkaloids and cardiac glycosides [20, 21]. The ethanolic extract at 6mg performed better than erythromycin (10µg) as well as indicated similar or better effect on *Candida albican* than the broth spectrum ciprofloxacin (5µg). Therefore the results have provided scientific validity for the use of the plant in the treatment of bacteria-related infections like urinary tract diseases, intestinal infections and skin infections in some parts of Northern Nigeria.

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