

Phytochemical Screening and *in-Vitro* Antimicrobial Activities of the Leaf Extract of *Acanthospermum hispidum* DC (Asteraceae)

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Abstract

The study into the chemical contents and *in-vitro* antimicrobial activities of the methanolic leaf extract of *Acanthospermum hispidum* were carried out. The extract was evaluated for its antibacterial activity against four Gram positive (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacteria specie* and *Bacillus subtilis*) and four Gram negative bacteria (*Salmonella typhi*, *Klebsiella phumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*). The fungal strains used were *Aspergillus niger*, *Penicillium sp.* and *Candida albicans*. The sensitive microorganisms (*Corynebacteria sp.*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*) had zones of inhibition ranging from 12.20 ± 1.06 mm to 24.00 ± 1.00 mm at 100 mg/ml, while the standard drug (tetracycline 250 mg) had zones of inhibition in the range 20.27 ± 0.64 mm to 27.23 ± 0.68 mm against all the microorganisms tested in this study. The MIC/MBC against the tested organisms ranged from 25 mg/ml to 50 mg/ml and 50 mg/ml to 100 mg/ml respectively. The results obtained from this study revealed that the leaf extract of *Acanthospermum hispidum* possesses antibacterial activity against some pathogenic microorganisms tested. The study confirmed the use of the leaf of *Acanthospermum hispidum* in some parts of Northern Nigeria as a remedy against diarrhoea, dysentery and other related diseases. In view of the reported uses of this plant, the present study investigated the antimicrobial effect of the methanol extract of *A. hispidum* on some Gram positive, Gram negative and some fungal species.

Therefore, this study validates the medicinal use of *Acanthospermum hispidum* traditionally in some parts of Northern Nigeria.

Key words: *Acanthospermum hispidum*, phytochemical screening, antimicrobial, methanolic extract

1. Introduction

Acanthospermum hispidum (DC) (Bristly starbur) from Asteraceae family is found in the tropical and sub-tropical temperate regions of the world. In Nigeria, the plant is common as a weed along the roads and in moist habitats and is reported to have various medicinal values (Smith, 2002). *A. hispidum* can be potential sources of useful drugs (Faleye et al., 2012). *Acanthospermum hispidum* is adapted to a wide range of soil and climatic conditions. It is particularly adapted to light textured soil but also grows well in heavy textured one. It is commonly found in cultivated upland crops, roadsides, pastures, waste area, around corrals, along railroads and cattle trails. Both seed and leaves contain phenolic acids that are allelopathic to other plants (Holm et al., 1997). It is found in a wide range of habitats, commonly on roadsides, disturbed areas and around settlements. It is known to invade pastures and out-compete more desirable native species. It is also a weed of crops and a serious contaminant of wool (Smith, 2002). Sub-inhibitory concentrations of *A. hispidum* (5 mg/ml) enhanced the activity of amoxicillin against *Staph. aureus* and *B. subtilis* but reduced slightly the activity against *Kl. pneumoniae*. Combining ciprofloxacin with the sub-inhibitory concentrations (5 mg/ml) of *A. hispidum* extract modulated the resistance of all the organisms to ciprofloxacin. The resistance modulatory activity of the extracts

on amoxicillin and ciprofloxacin is more pronounced with Gram positive organisms than Gram negative organisms (Adu et al., 2011). Bioactivity-directed purification of the leaf of *A. hispidum* using anti-trichomonal assay yielded subfractions C₆ and C₇ which had activity comparable to metronidazole, the positive control and had better activity than the mother ethyl acetate extract. The study further showed the potential usefulness of *A. hispidum* in treating protozoal infections (Deepa et al., 2004).

The leaves and stems of *Acanthospermum hispidum* were extracted with distilled ethanol using cold extraction and concentrated using a rotary evaporator at 37 °C. The crude extract was partitioned successively using hexane, benzene and methanol. Fractions 19, 20 and 21 purified on Sephadex LH-20 gave a compound elucidated to be 1, 3, 6, 8-tetrahydroxyl-9-anthracene carboxaldehyde, using the state-of-art tools of spectrometry. The results of the antimicrobial test on the isolated compound show activity against *P. mirabilis*, *B. subtilis*, *P. aeruginosa*, *C. albican*, *S. typhi* and *B. cereus* at minimum inhibitory concentration (MIC) value of 100 ppm Olajide et al., (2014). Early workers have reported the use of the stem bark for medicinal purposes (Chakraborty et al., 2012). *Acanthospermum hispidum* plant is important for its medicinal properties. In Nigeria, from information available from the indigenous traditional healers, the crushed herb is used in the form of a paste to treat skin ailments and the leaf juice is taken orally to relieve fevers (Mshana et al., 2000).

2. Materials and Methods

2.1 Sample Collection and Identification

Fresh samples of the leaves of *Acanthospermum hispidum* were collected from Uvaha village, Gwoza Local Government Area, of Borno State in November, 2012. The plant was identified and authenticated by a plant Taxonomist Prof. S.S. Sanusi, in the Department of Biological Sciences, University of Maiduguri. A voucher specimen No. Chem/09/01 was deposited in the Research Laboratory, Department of Chemistry. The leaves were cleaned, air-dried under shade for seven (7) days, then pulverised to powder and coded “plant material”.

2.2 Extraction of Plant Material

The air-dried powdered plant material (1000 g) was extracted exhaustively with 85% methanol using a Soxhlet apparatus as described by Evans (2002). The methanolic extract obtained was concentrated to dryness at 45 °C on a water bath and coded “CMLE” Crude methanolic leaf extract. About 200 g of the CMLE concentrate were subjected to preliminary phytochemical screening and *in-vitro* antimicrobial susceptibility test while the MIC and MBC determined accordingly.

2.3 Qualitative Phytochemical Screening

The crude methanolic leaf extract “CMLE” of *A. hispidum* was subjected to qualitative phytochemical screening for identification of the various classes of active chemical constituents such as flavonoids, alkaloids, sterols, terpenes, saponins, tannins as methods described by the Harbone (1973), Awe and Sodipo (2001), Evans (2002); Sofowora (2008).

3. Atimicrobial Studies

3.1 Test Microorganisms

A number of microorganisms consisting of both Gram positive and Gram negative (-ve) bacteria were used. The Gram positive (+ve) organisms used were *Streptococcus faecalis*, *Staphylococcus aureus*, *Corynebacterium spp.*, and *Bacillus cereus*, while the Gram negative ones were: *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and some fungi species such as: *Candida albicans*, *Aspergillus niger* and *Penicillium* species were also used. The microorganisms were obtained from the University of Maiduguri Teaching Hospital, Maiduguri, (UMTH) Nigeria.

3.2 In vitro Disc Antibacterial Activity of Methanolic Extract of Leaf

The *in vitro* disc diffusion method was used to test for antibacterial activity of the extract as described by Cheesbrough (2004).

3.3 Preparation of Culture Media

The culture media used in this study were nutrient agar (Biotec Medical Market, UK) for bacteria and *Candida albicans* and sabouraud-2% glucose agar (Merk, Darstadt, Germany) for *Penicillium spp.* and *Aspergillus niger*. The nutrient agar was prepared according to the manufacturer’s specifications (by dissolving 18.5 g powder in 500 ml distilled water) and sterilized at 121 °C for 15 minutes. After autoclaving, the pH was 7.2-7.4 (Bello, 2002). This was poured in 90 mm diameter sterile, disposable plastic petri-dishes to a depth of 4 mm (about 25 ml per plate). Care was taken to pour the plates on a level surface so that depth of the medium would be uniform. The

plates were dried upside down in an incubator at 37 °C with their lids opened and inverted so that water would not condensed back in to the agar. The sabouraud-2%-glucose agar was prepared according to the manufacturer's specification (by dissolving 18.5 g in 400 ml of distilled water) and sterilized at 121 °C for 15 minutes. 1 ml each of the different concentrations of the CMLE (200 mg/ml, 400 mg/ml, 800 mg/ml and 1600 mg/ml) was pipette into eight (8) sterile, disposable petri-dishes i.e. 2 plates for each CMLE concentration 25 ml of the sabouraud-2%-dextrose agar was poured in to the plate, swirled round to mix very well with the CMLE, then allowed to set at low temperature. The other plates were also prepared, but without the CMLE, to act as the control. All the eleven plates were then incubated upside down, with their lides opened at 37 °C in an incubator to dry (Sodipo et al., 2012)

3.4 Preparation of Test Organisms

The microorganisms were propagated and stored on nutrient agar at 4 °C for 24 hours prior to antimicrobial testing.

3.5 Preparation of Agar Medium

Nutrient agar (25 g) was dissolved in one litre (1L) of distilled water in a sterilized conical flask and heated to dissolve by stirring. The prepared medium was tightly corked with aluminium foil and sterilized in an autoclave at 121 °C at 15 mm Hg for 15 minutes. The conical flask was put on a water bath at 55 °C to cool for 20 minutes. 10 ml of peptone water was put into a sterile inoculating bottle and sterilized in an autoclave at 121°C at 15 mm Hg for 15 minutes. Normal saline was prepared by the same process for 30 minutes. The nutrient agar was poured into sterilized petri dishes and allowed to set and dry in an incubator at room temperature (Sodipo et al., 2010).

3.6 Preparation of Discs Containing Graded Concentrations of the CMLE of the Leaves of *A. hispidum* and Tetracycline Discs

Whatman filter paper No.1 was punched into circular discs (each 6 mm indiameter), with the aid of an office punch. The discs were then put in a glass petri-dish and sterilized in a hot air oven at 60 °C for 30 minutes. 1 ml of each of the different concentrations of the extract were put in sterile glass plates and thirteen (13) sterile discs were put in their using sterile forceps to soak the extract, then they were allowed to dry. The discs were checked to be sure that they were not sticking together (Lamikanra, 1999). These CMLE discs were used for the antibacterial tests and that of *Candida albicans*. One capsule tetracycline 250 gm powder was dissolved in 1 ml distilled water. Sterile discs were then put inside it so as to be soaked with the tetracycline and then left to dry. This gave tetracycline discs of 250 mg/ml which is equivalent to 2.5×10^5 µg/ml. This concentration of tetracycline disc was prepared because the pilot study revealed that the commercially available tetracycline disc, 50 mg/ml is too low to be effective on both the bacterial and fungal species under test (Sodipo et al., 2012).

3.7 Disc Diffusion Antibacterial Selectivity Test

A stock solution was prepared with sterile distilled water at a concentration of 1 mg/ml. Tetracycline was used as a standard drug. Solutions of different concentrations (200, 400, 800, 1600, 3200, 6400 mg /ml) of the methanolic leaf extract and tetracycline (250 mg / ml) were prepared. Filter papers of 7 mm in diameter were cut and sterilized for one hour at 160 °C. The paper discs were placed on the prepared solutions to allow for absorption of the solution. The standard discs were allowed to dry in the oven and then placed on the dried surface of the culture plates already inoculated with bacteria (Agar Plates) and left at 37°C for incubation. The zones of inhibition were measured after 24 hours (Soodipo, et al., 2010) and recorded if it was greater than 10mm (Vlietinek et al., 1995).

3.8 Disc Diffusion Antifungal Selectivity Test

The antibiotic disc tetracycline (2.5×10^5 µg/disc) were placed on the already prepared sabouraud-2%-dextrose agar containing graded concentrations of the CMLE (8 in all) and the control (2 pates). The *Penicillium spp.* and the *Aspergillus niger* were then removed from their pure culture with a pair of sterile forceps and placed on the plates so that the organisms could spread on the antibiotic disc and the extract in the plates. The plates were incubated at 25-30 °C and examined every 2-3 days and kept for four weeks before being considered negative for the fungi (Bello, 2002).

3.9 Minimum Inhibitory Concentration (MIC)

MIC can be defined as the lowest concentration where no visible turbidity is observed in the test tube. MIC is a technique employed to know at what concentration the extract can inhibit the microbial activity. MIC was determined using the broth dilution technique Vollekova et al. (2001). The minimum inhibitory concentration was determined from micro-organisms that where sensitive to the extract under study (leaf extract). Equal volume of nutrient broth was dispensed in to tubes (bijou bottles) were known concentrations of the extract diluted at concentration ranging from lowest to highest i.e 12.5 mg/ml to 200 mg/ml were prepared. Also 0.2 ml suspension

of microbial isolates (*Pseudomonas aeruginosa* and *Corynebacteria* species) were inoculated to various concentrations.

3.10 Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed (Reuben et al., 2009). MBC was determined by using the broth dilution technique described by Usman et al. (2007) by assaying the test tubes resulting from MIC determinations. A loop of the content of each test tube was inoculated by streaking on a solidified nutrients agar plate incubating at 37 °C for 18 hours and observed for bacterial growth. The lowest concentration of the subculture with no growth was considered the minimum bactericidal concentration.

4. Results

The result of the qualitative phytochemical analysis of methanolic leaf extract is shown in Table 1. The result showed the presence of flavonoids, cardiac glycosides, alkaloids, steroids, terpenes, saponins, carbohydrates and tannins, while anthraquinones and Phlobatanins were absent.

Table 1. Phytochemical analysis of the leaf extract of *Acanthospermum hispidum*

S/No	Plant Constituents	Test	Result	Observation
1.	Tannins	Ferric Chloride Test	+	Deep blue black
2.	Alkaloids	General test: Dragendorff's Test	+	Orange-red
		Mayer's Test	-	No ppt formed
3.	Saponins	Froth Test	+	Foam formed
4.	Cardiac Glycosides	(i) Keller-Killiani's Test	+	Greenish colour
		(ii) Liebermann-Burchard Test	-	Bluish green
		(iii) Salkowski Test	+	Reddish brown
5.	Steroids	Liebermann-Burchard Test	+	Bluish green
6.	Flavonoids	(i) Ferric Chloride Test	+	Bluish green
		(ii) Lead Ethanoate Test	+	Buff color ppt
		(iii) (Shinada's Test)	+	Light pink colour
		(iv) (Sodium Hydroxide Test)	-	Yellow colour
7.	Phlobatanins	Hydrochloric Acid Test	-	No color change
8.	Anthroquinones	(i) Free Anthroquinone Test	-	No colour formed
		(ii) Combined Anthroquinone Test	-	No colour formed
9.	Carbohydrates	(i) General test (Molish's Test)	+	Purple colour
		(ii) Monosaccharides (Barfoed's Test)	+	Brick red ppt
		(iii) Free Reducing Sugar	+	Deep brick red
		(iv) Combined Reducing Sugar	+	Deep brick red
		(v) Ketoses (Resorcinol or Selivanoff's Test)	+	Deep rose colour
10.	Terpenenoids	General Test	+	Pink colour

Key: + = present; - = absent (not detected).

In vitro disc diffusion method was used to study the antimicrobial activity of methanol leaf extract. The result of the antimicrobial activity on some bacterial pathogens shows that the extract at various concentrations inhibited the growth of *Staphylococcus aureus*, *Corynebacteria* sp., *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were presented in Table 2, but there was no activity against *Streptococcus pyogenes*, *E. coli*, *Salmonella typhi*, *Candida albican*, *Aspergillus niger* and *Pencillium* species. Among the sensitive microorganisms, *Corynebacteria* specie recorded the largest zone of inhibition (24.00 ± 1.00 mm) and *Staphylococcus aureus* recorded the least zone of inhibition (12.20 ± 1.06 mm). While other microorganisms such

as *Bacillus cereus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* had 13.00 ± 1.00 mm, 13.33 ± 1.15 mm and 18.23 ± 1.08 mm zone of inhibition respectively at 1000 mg/ml concentration. The standard drug (tetracycline 250 mg) inhibited the growth of the microorganisms tested in this study. The result of the minimum inhibitory concentration and minimum bactericidal concentration (/MIC/MBC) assay are as presented in Table 3 and 4 respectively. They revealed the concentration of the extract which could inhibit the growth of the bacterial species under test (bacteriostatic / bactericidal concentrations) with the values ranges from 25 to 50 mg/ml and 50 mg/ml to 100 mg/ml respectively.

Table 2. In vitro disc antimicrobial susceptibility test of the methanolic leaf extract of *A. hispidum*

Extract/drug conc. mg/ml	Micro organisms/Diameter of zones of inhibition (mm)										
	<i>Staph. aureus</i>	<i>Strep. Pyogene</i>	<i>Con. bacterium specie</i>	<i>Bacillus Subtilis</i>	<i>Salmo nella typhi</i>	<i>Klebsiella Pnumoniae</i>	<i>Pseudo monas aeruginosa</i>	<i>E. coli</i>	<i>Asper gillus niger</i>	<i>Penici llium specie</i>	<i>Con. Albican</i>
200	7.00±1.00	R	14.50±0.50	7.07±1.07	R	7.93±0.12	9.67±1.53	R	R	R	R
400	8.66±0.58	R	18.33±0.58	9.00±0.00	R	8.53±0.50	14.33±0.58	R	R	R	R
600	10.00±1.00	R	21.33±0.58	9.67±1.53	R	10.67±0.58	14.00±1.00	R	R	R	R
800	11.00±0.00	R	23.67±1.53	11.67±0.58	R	12.00±0.00	16.00±1.00	R	R	R	R
1000	12.20±1.06	R	24.00±1.00	13.00±1.00	R	13.33±1.15	18.23±1.08	R	R	R	R
*250 mg (TTC)	23.33±0.58	23.00±1.00	27.23±0.68	22.17±0.77	22.00±1.00	20.27±0.64	23.00±1.00	27.23±0.68	20.70±1.54	21.00±1.00	20.07±1.05

Key: R = Resistant (i.e not sensitive), TTC = Tetracycline, * = standard drug ($2.5 \times 10^5 \mu\text{g}/\text{disc}$), Data are presented as means±S.D and n = 3.

Table 3. The minimum inhibitory concentration (MIC) values of some organisms to the methanolic extract of *A. hispidum*

Bacteria	Extract Concentrations (mg/ml)				
	12.5	25	50	100	200
<i>Conynebacterium</i> species	+	β	-	-	-
<i>Pseudomonas aeruginosa</i>	+	+	β	-	-

Key: - = No growth (inhibition of bacterial growth); + = There was growth (Resistant); β = Least concentration showing no turbidity (MIC).

Table 4. The Minimum Bactericidal Concentration (MBC) of some organism to the methanolic extract of *A. hispidum*

Bacteria	Extract Concentration (mg/ml)				
	12.5	25	50	100	200
<i>Conynebacterium</i> species	+	+	+	-	-
<i>Pseudomonas aeruginosa</i>	+	+	+	β	+

Key: - = No growth; + = There was growth; β = Minimum bactericidal concentration (MBC).

5. Discussion

Previous research in to the phytochemistry of the leaves of *Acanthospermum hispidum* revealed the presence of flavonoids, cardiac glycosides, alkaloids, terpenes, saponins, carbohydrates and tannins, while steroids, amino acid and Phlobatanins were absent (Harekrishna et al., 2010). These secondary metabolites are responsible for most physiological and chemotherapeutic effects exhibited by plant extractives both in *vitro* and *vivo* (Usman, 2012). The extract under study had shown the presence of tannins and flavonoids; these phytochemicals had been known to inhibit the growth of microorganisms (Havagiray et al., 2004; Ogundaini, 2005; Usman et al.,

2007; Usman, 2012).

Alkaloids in the extract under study produce analgesic, anti-inflammatory and adaptogenic effects which help to develop resistance against diseases and endurance against stress (Gani, 1990; Gupta, 1994; Aska, 2008).

Carbohydrates in this extract occupy an important position in metabolism so the method of their detection is useful in phytochemistry. Carbohydrates have no therapeutic actions but they possibly increase the effectiveness of the biological active principles in the plant, thus most therapeutic principles isolated from plants occur in combination with sugar as glycosides (Iwu, 1984; Vollekowa et al., 2001).

In vitro disc diffusion method was used to study the antimicrobial activity of methanol leaf extract. The microorganisms tested in the study are widely distributed in air, water, faeces and can contaminate our food, thus producing enterotoxin which leads to some infections of the gastrointestinal tract including diarrhoea (Lucas and Gilles, 2003). The result of the antimicrobial activity on some bacterial pathogens shows that the extract at various concentrations inhibited the growth of *Staphylococcus aureus*, *Corynebacteria sp.*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were presented in Table 2, but there was no activity against *Streptococcus pyogenes*, *E. coli*, *Salmonella typhi*, *Candida albicans*, *Aspergillus niger* and *Penicillium* species. The result obtained showed that methanolic leaf extract of *Acanthospermum hispidum* exhibited inhibitory activities against some microorganisms tested at varying degrees of concentration as demonstrated by the diameters of the zones of inhibitions. These result where in conformity with those earlier reported by Harekrishna et al. (2010) and Chakraborty et al. (2012). The ability of the extract to inhibit the growth of these organisms *in vitro* may be due to the presence of some active chemical constituents found in the extract (Ogundaini, 2005).

The MIC/MBC data obtained from the evaluation of CMLE against the tested organisms ranges from 25 mg/ml to 50 mg/ml and 50 mg/ml to 100 mg/ml respectively, shown in Table 3 and 4. There is a need to consider the use of this leaf extract that have shown some measures of antimicrobial activities, judging by the antimicrobial activity, low Minimum Inhibitory Concentration (MIC) and low Minimum Bactericidal Concentration (MBC) on tested microorganisms.

6. Conclusion

In conclusion, the extract was found to be effective against the tested pathogenic microorganisms at varying concentrations. Therefore, this study validates the use of the leaf of *Acanthospermum hispidum* traditionally in some parts of Northern Nigeria.

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