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Screening for Bioactive Extracts and Targeted Isolation of Antimicrobial Agents from the Stem Bark of *Adenanthera pavonina L.*

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Abstract

Bioactive secondary metabolites can be isolated from medicinal plants as antimicrobial agents. Based on ethnopharmacological relevance, *Adenanthera pavonina L.* is recognized as a plant with good medicinal values and forms the integral part systems of traditional medicine in Borno state, and other parts of Nigeria. Despite the widespread used, most of the information available to consumers about the plant is not backed by credible scientific data. Only a limited number of studies have probed into the scientific evidence for bioactivity and safety of this medicinal plant. Such studies rarely progress to isolation and evaluation of the active antimicrobial agents. In the present study, four solvent-extract (ethanol, ethyl acetate, chloroform and n-hexane) were subjected to antimicrobial activity test against *E. coli* (E.C), *S. typhi* (S.T), *S. aureus* (S.A), *A. flavus* (A.F), *C. albican* (C.A) and *M. specie* (M.S) and toxicity test against *Artemia salina*. The ethyl acetate extract showed outstanding activity against the microbes (bacteria and fungi) and *Artemia salina*, respectively. The activity and toxicity effects of the ethyl acetate extract suggests the presence of active antimicrobial agents and hence provides a way forward for column chromatographic isolation of the targeted Antimicrobial agents. The targeted compounds isolated (AP-X_{38} and AP-X_{44}) were also subjected to antimicrobial test using three bacterial species and found to be even more active that the solvent extract of ethyl acetate. This study justifies part of ethno medicinal claims on the plant.

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Keywords and phrases: *Adenanthera pavonina*, cytotoxicity, *Artemia salina*, extracts, antimicrobial agents.

Introduction

Adenanthera pavonina is well-known for its medicinal value in folk medicine in Borno state and other parts of Nigeria. It is widely used in the treatment of mastitis and other ailments like: Pile, diarrhoae, ulcer, healing of open wound, skin diseases among others (Abdu and Adamu [1]). The plant is also grown in Asian countries (Malaysia, India, Bangladesh and China) as an ornamental crop and commonly known by English names as coral wood, red sandal wood, red lucky tree, red bead tree, food tree among others (Ezeagu et al. [2]). It is a deciduous tree, about 6-15 m tall, erect and up to 45 cm in diameter depending on the location (Orwa et al. [3] and Chopra et al. [4]).

The stem-bark of the plant is traditionally used for treatment of various disease conditions like gonorrhea, haematuria, ulcers, it is astringent, vulnerary and aphrodisiac in nature (Hussain et al. [5]). The plant is endowed with tremendous potentials in both healthcare and trade, but limited numbers of studies have probed into the scientific evidence for bioactivity and safety of this medicinal plant in Borno state and other parts of Nigeria. Mathew et al. [6] reported that, considerable work has been done to explore the biological activity and medicinal applications of the plant (*Adenanthera pavonina*), still there are available countless possibilities of pharmacological applications which needs to be explored, because Abdu and Adamu [7] reported that, phytochemical screening of stem bark of *Adenanthera pavonina* uncovered the presence of alkaloids, phenols, flavonoids, sterols, tannins, glycosides among others in different extracts. These constituted number of phytochemicals imbedded in *A. pavonina* disclosed its uses for various therapeutic purposes as in the treatment of various health illness and act as hepatoprotective, anti-hyperlipidemic, anti-nociceptive, anti-diarrhoeal, antioxidant, anticancer, antimicrobial, inhibitor of nephrolithiasis and carcinogenesis (Mujahid et al. [8]).

Different parts of *Adenanthera pavonina* (roots, stem-bark, leaves and seeds) have been used for the treatment of different human disorders like chronic rheumatism, diarrhea, dysentery antiseptic paste, pulmonary affections, chronic ophthalmia, and reported to have properties such as antioxidant, antibacterial by Jayasinghe et al. [9]. The plant has been reported to demonstrate anti-inflammatory and analgesic activities (Olajide et al. [10] and Jayakumari et al. [11]), antihypertensive effect (Adedapo et al. [12]), antifungal (Soares et al. [13]), anti-oxidant (Mujahid et al. [14]), anticancer (Chauhan et al. [15], Kumar et al. [16]), hepatoprotective (Mujahid et al. [17]), renal protective (Pandhare et al. [18]), anti-hyperlipidemic (Das et al. [19]) and antibacterial (Hussain et al. [20], Adeyemi et al. [21]). The medicinal properties of the plant are due to

the presence of flavonoids (Rastogi and Mehrotra [22]), glycosides (Yadav et al. [23]), saponins and steroids (Khare [24], Orwa et al. [3]).

Adenanthera pavonina L. belongs to Fabaceae family. The scientific name is derived from a combination of two Greek words *aden*, "a gland," and *anthera*, "anther" (Khan and Khanum [25]). It is commonly known by English names as coral wood, red sandal wood, red lucky tree, red bead tree, red bread tree, food tree and also known by vernacular names Raktakambal, Manjadi, Anikundumani, Lopa (Hindi), Raktakambal (Bengali), among other (Chopra et al. [4], Binggeli et al. [26], Orwa et al. [3], Mathew et al. [6]).

Adenanthera pavonina L. is a plant naturally grown in the southern part of Borno, Nigeria and is well known to Babur/Bura people of the state by a local name "Ghrini". It is a medium-sized to large deciduous tree about 8-14 m tall and 40-45 cm thick, depending on location. Generally, the plant is erect with a dark brown to greyish bark and a soft-pale brown inner bark. It bears leaves, flowers and seeds. The leaves are bi-pinnate in nature with 8-21 leaflets on short stalks, which turns yellow with age (Abdu and Adamu [1]).

Materials and Methods

Sample Collection and Authentication

Fresh stem bark sample along with leaves and seeds of the plant were collected from Kwaya Bura village, Hawul Local Government, Borno State, Nigeria. The plant was identified at the Department of Plant Biology, Bayero University, Kano, Nigeria. A text report from Herbarium unit of the department confirmed the authenticity of the plant material as *Adenanthera pavonina L.* with accession number 0493.

Figure 1 shows pictures of leaves and seeds of *Adenanthera pavonina* that helps in the identification and authentication of the plant.

Figure 1. Picture of leaves and seeds of *A. pavonina.*

Sample Preparation

Fresh stem-bark of *Adenanthera pavonina L.* was washed with distilled water, cut into smaller pieces and air dried under shade at ambient temperature for about three (3) weeks. It was then pulverized mechanically to form a coarse powder. The powdered sample was stored in an air tight container in cool and dry place away from any form of contaminant and was later subjected to extraction using different solvents (ethanol, ethyl acetate, chloroform and n-hexane).

Figure 2 is a representation of *A. pavonina* stem bark sample (A: unwashed-fresh stem bark, B: washed-fresh stem bark, C: sliced-fresh stem bark and D: powdered-stem bark).

A **B** **C** **D**

Figure 2. Pictures showing fresh and dried grounded stem bark of *A. pavonina*.

Solvents Extraction

Each of the powdered sample (200 g) were introduced into Four (4) different amber bottles labeled (W, X, Y and Z) and percolated with 1.0 L of ethanol (W), ethyl acetate (X), chloroform (Y) and n-hexane (Z) with shaking at regular intervals for one week. Their extracts were separated from the debris by filtration. The filtrates were concentrated using a rotary vapour (R110 at 40^{o}C) and then coded as AP-W, AP-X, AP-Y and AP-Z. They were weighed, kept in a cool and dry place away from any form of contaminant (Sarkar et al. [27]).

***In vitro* Antimicrobial Activity**

Test Organisms

Standard clinical bacterial and fungal isolates obtained from Microbiology Department, Bayero University, Kano, were used in this assay. These bacterial isolates were gram negative and positive including *E.coli*, *S.typhi* and *S.aureus* respectively and fungal isolates including: *A.Flavus*, *C.albican* and *M. specie.*

Preparation of Stock Solutions and Working Concentrations

The stock solutions of the extracts were prepared by dissolving each extract (60.0 mg) in 1.0 mL DMSO separately to produce 60.0 mg/mL. From each stock solution, solutions of lower concentrations (30.0 and 15.0 mg/mL) were obtained using serial double dilution (SDD).

Antimicrobial Screening (Agar Well Diffusion Method)

Antimicrobial (antibacterial and antifungal) activity of the extracts and isolated compounds were screened using agar well diffusion method. In this method, nutrient agar (NA) and potatoes dextrose agar (PDA) plates were swabbed with eight-hours-old broth culture of bacteria and fungi using sterile cotton swab respectively. On each sensitivity disc, four wells (6 mm each) were made using a sterile Cork borer and three different concentrations (60.0, 30.0 and 15.0 mg/mL) of ethanol extract (AP-W), ethyl acetate extract (AP-X), chloroform extract (AP-Y) and n-hexane extract (AP-Z) were impregnated (100 µL) in to each well using a sterile micropipette to investigate the dose dependent activity of the extracts on both standard bacterial and fungal isolates in use. At the same time Gentamicin, 125 mg/mL and Ketoconazole, 100 mg/mL were used as standard. The sensitivity plates were incubated at 37°C for about 24 hours. The diameter of zones of inhibition around each well was noted and the values were measured for the eventual antimicrobial activity (Azoro [28], Renilda et al. [29], Balouiri et al. [30]).

Lethality Assay using *Artemia Cyst* (Brine Shrimp)

Hatching of *Artemia Cyst*

Artemia Cyst (Brine shrimps) collected from Professor O.A Adoum, Department of Pure and Industrial Chemistry, Bayero University, Kano, was used as the test organism. Natural sea water (100 mL) was taken in a beaker (500 mL) and *Artemia Cyst* (6.4 g) was added. The *Cyst* started hatching after 48 hrs and was allowed to stand for about 72 hrs for complete hatching and the shrimps were matured as nauplii in the presence of light. This process is carried out under cross ventilation in order to ensure constant supply of oxygen and the nauplii were taken for this assay (Saha et al. [31]).

Preparation of Stock Solutions and Working Concentrations

The ethanol extract (20 mg) and ethyl acetate extract (20 mg) were separately dissolved in methanol (2 mL) to obtain 20,000 µg per 2 mL stock solutions. From each stock solution of the extract, series of solutions of lower concentrations (1000, 100, and

10 µg/mL) were obtained by two-fold serial dilution and were used for the assay (McLaughlin et al. [32], Otang et al. [33]).

Potassium dichromate as a cytotoxic agent was used as a positive control. It was prepared by dissolving 20 mg in Seawater (2 mL) and then serially diluted to solutions of different concentrations (1000, 100 and 10µg/mL). Seawater was used as a negative control (Unuofin et al. [34]).

The standard solutions of the ethanol extract (AP-W), ethyl acetate extract (AP-X) and potassium dichromate (positive control) were impregnated into dry and clean glass tubes in three (3) replicates of each concentration and the glass-tubes were allowed to stand for 24 hours away from any form of contaminant for the diluting solvent to evaporate. After evaporation, few drops of DMSO were introduced in to each test-tubes and Sea water (2.5 mL) was filled into each to make it ready for the application of brine shrimps nauplii (Otang et al. [33], Asaduzzaman et al. [35]).

Lethality Assay Procedure

With the help of Pasteur pipette, ten (10) living and healthy brine shrimp nauplii along with Seawater were introduced into each test-tube containing the test solution of the sample and the control solutions up to the pre-marked volume (5 mL) on the glass tube. For convenient application, a magnifying glass was used for counting the nauplii and also to avoid taking *Artemia Cyst* in to the test-tube (Saha et al. [31]). After 24 hours incubation, the same magnifying glass was used for convenient counting of the survived shrimp nauplii in each test-tube and the results obtained were recorded. From these results, the percentage mortality of shrimp nauplii was calculated for each concentration using the equation three (3) below (Unuofin et al. [34]).

$$\%\text{Mortality} = \frac{\text{Total nauplii} - \text{Living nauplii}}{\text{Total nauplii}} \times \frac{100}{1} \rightarrow \quad (1)$$

Column and Thin Layer Chromatography

The Ethyl acetate extract (AP-X) of stem bark of *Adenanthera pavonina L.* was subjected to silica gel chromatographic isolation. About 5.0 g of this extract (AP-X) was mixed with silica gel (10 g) and 10 mL of solvent (ethyl acetate) in a glass beaker (50 mL). The mixture was stirred thoroughly to facilitate mixing and drying. The sample was then loaded and then 10 g silica gel was added as a protective layer to the sample in the glass colunm. The content within the chromatographic column was eluted with ratios of different solvent mixtures (n-hexane-ethylacetate, followed by ethylacetate-methanol)

based on polarity gradient. Fractions (APX_1-APX_{61}) were collected in labeled bottles based on colour and quantity at different time intervals and were monitored using thin layer chromatographic analysis. Fraction that appeared as a single spot on TLC plates were selected and their R_f values were calculated using the following expression.

$$R_f = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent front}} \rightarrow (2)$$

Results and Discussion

Extraction

Table 1 presents the physical properties of four different solvent-extracts of stem bark of *Adenanthera pavonina*. It is seen from this table that, ethanol extract (AP-W) and ethyl acetate extract (AP-X) gave good yield, chloroform extract (AP-Y) gave small yield and n-hexane extract (AP-Z) presented the least yield. This finding entails that, the stem bark of *A. pavonina* contained much polar compounds than non-polar. This is because, the four different solvent-extracts presented were obtained from same mass (200 g) of plant sample using same volume (1.0 L) of different solvent of varying polarity gradient.

Table 1. Physical properties of the four extracts.

Extract	Colour	Texture	Weight (g)	%Yield
AP-W	Reddish brown	crystal	34.20	17.10
AP-X	Reddish brown	crystal	19.30	9.65
AP-Y	Whitish-blue	Gummy	3.20	1.60
AP-Z	Orange	Oily	1.80	0.90

In vitro Antimicrobial Assay

In vitro antimicrobial activity of the four solvent-extracts (AP-W, AP-X, AP-Y and AP-Z) of stem bark of *A. pavonina* uncovered that, the ethanol extract (AP-W) and ethyl acetate extract (AP-X) demonstrated promising activity by inhibiting the growth of all bacterial and fungal species used in the study. From the results, both solvent-extracts of ethanol (AP-W) and ethyl acetate (AP-X) displayed promising activity, while the solvent extracts of chloroform (AP-Y) and n-hexane (AP-Z) displayed poor activity. This activity was noted through the observation of visible zones of inhibition around the 6 mm wells. Gentamicin and ketoconazole used as reference groups also displayed good activities

against all the bacterial and fungal species used in this assay. These two reference groups validated the test methods and also proved that, the results obtained were only due to the activity of the extracts and the effects of other factors were nullified. These can be observed clearly from Figures 1 and 2 that follow.

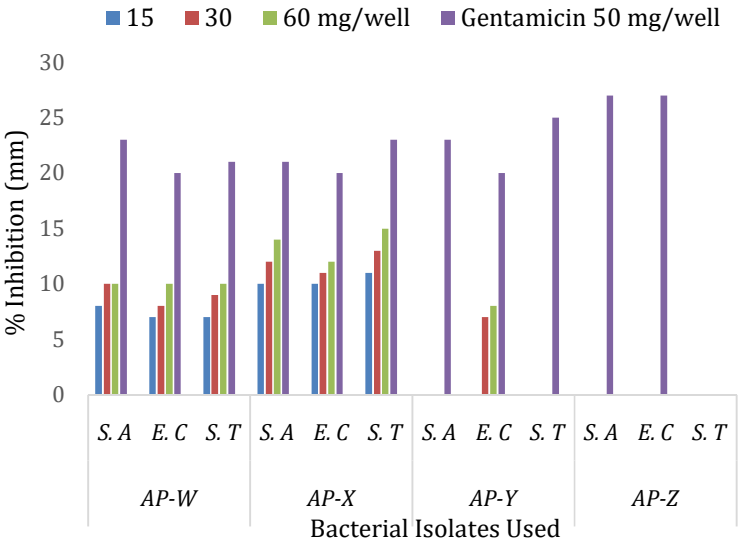

Figure 3. Activity of four different extracts and gentamicin on bacteria.

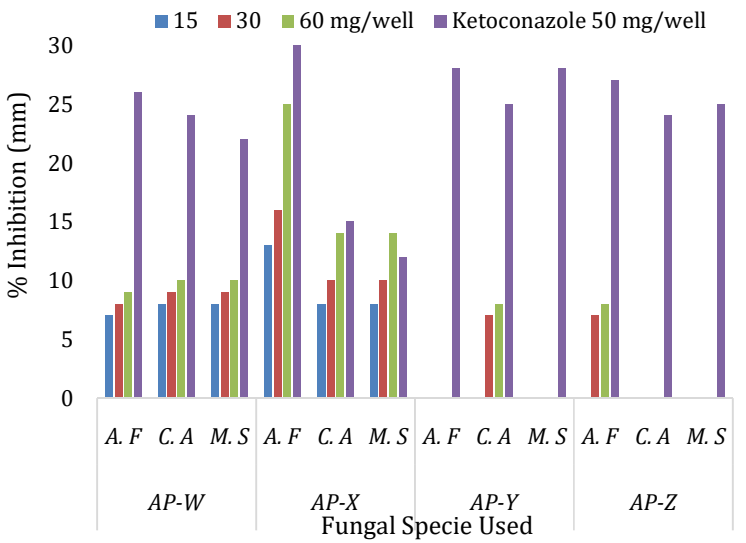

Figure 4. Activity of four different extracts and ketoconazole on fungi.

These findings are in agreement with those reported by Hussain et al. [20], Renilda et al. [29], Mujahid et al. [14] and Abdu and Adamu [7]. The promising activities of the two extracts (AP-W and AP-X) suggested the presence of active antimicrobial secondary metabolites like alkaloids, phenols, flavonoids, sterols, tannins, glycosides among others imbedded in the stem bark of *A. pavonina* (Abdu and Adamu [7]).

Cytotoxicity Test using Brine Shrimps (*Artemia Cyst*)

The cytotoxicity test results of ethanol and ethyl acetate extracts showed observed mortality of 70 and 100% respectively at same concentration (1000 µg/mL) and at a concentration of 100 µg/mL, mortality (40%) observed with ethyl acetate extract is higher than mortality (20%) in ethanol extract. At concentration (10 µg/mL) only the ethyl acetate extract was observed to have 10% mortality. Potassium dichromate (positive control) as a cytotoxic agent was observed to show mortality (100%) at all concentrations (1000, 100 and 10 µg/mL). This cytotoxic behaviour of the extracts (AP-W and AP-X) is a further confirmation of activity. These finding can be observed apparently from Figure 5.

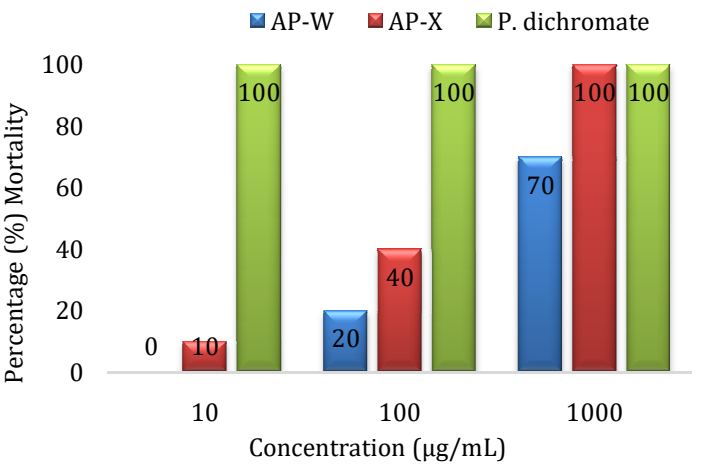

Figure 5. Cytotoxicity test results for extracts (AP-W) and AP-X) and P. dichromate.

Renilda et al. [36] carried out toxicity study on *Adenanthera pavonina Linn* and reported that, ethyl acetate extract was having LC_{50} = 602 µg and ethanol extract LC_{50} = 1387 µg. Meyer et al. [38], concluded that, LD_{50} value of less than 1000 µg/mL is toxic while LD_{50} value greater than 1000 µg/mL is non-toxic. The toxicity suggests the presence of active secondary metabolites which can be used either as antimicrobial, antitumour, antiinflamatory or insecticidal agent.

The antimicrobial and cytotoxicity results of the present study, uncovered solvent-extract of ethyl acetate (AP-X) more active than the remaining solvent-extracts (AP-W, AP-Y and AP-Z) and therefore was chosen for column chromatographic isolation of targeted antimicrobial agents.

Column Chromatography

A total of 61 fractions (AP-X_1–AP-X_{61}) were collected in the process of column chromatographic isolation of the targeted antimicrobial compounds. Two (2) out of these numbers with R_f values 0.63 (AP-X_{38}) and0.70 (AP-X_{44}) were selected for reconfirmation of activity because each of them appeared as a single spot on thin layer chromatographic plate and are much in quantity (AP-X_{38} = 800 mg and AP-X_{44} = 650 mg).

Antibacterial Activity of Isolated Compounds

The antibacterial activity of the isolated compounds appeared more promising than the highly active solvent-extract of ethyl acetate, which can be seen from Figure 5.

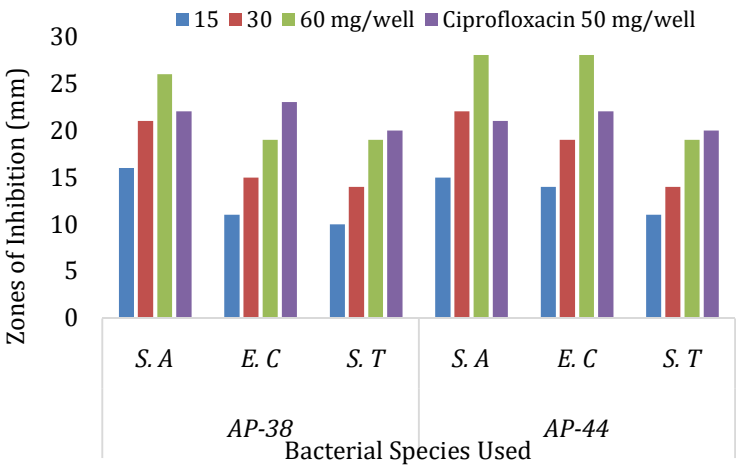

Figure 5. Antibacterial activity of isolated compounds and ciprofloxacin.

On comparing the activity of the isolated compounds (AP-X_{38} and AP-X_{44}) and that of ethyl acetate extract (AP-X), the activity of isolated compounds at lowest concentration (15 µg/mL) is greater than or equal to activity of ethyl acetate extract at highest concentration (60 µg/mL). The highest zones of inhibition measured for one of the isolated compounds was 28 mm and least was 10 mm and the highest and least zones of inhibition for ethyl acetate extract were 15 mm and 7 mm at concentrations (60 and 15 mg/well) respectively. Ciprofloxacin which is used as a reference standard inhibited the

growth of all bacterial species, *S. aureus* (S.A), *E. coli* (E.C) and *S. typhi* (S.T) used, with a measurement (20 mm) as the least zone of inhibition. These phenomena can be observed clearly from Figure 6 that follows.

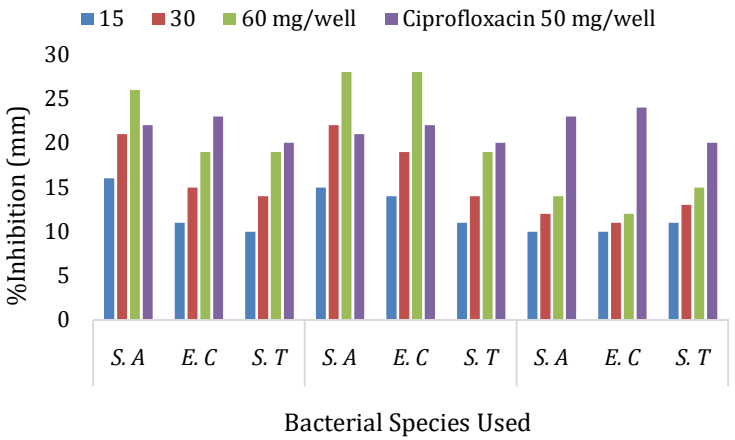

Figure 6. Activity comparison of isolated compounds, ethyl acetate extract and ciprofloxacin.

Minimum Inhibition Concentration (MIC) of Isolated Compounds

The minimum inhibition concentrations for the two isolated compounds were 0.5 mg/mL (AP-X_{38}) and 0.25 mg/mL (AP-X_{44}) against *E. coli, S. typhi* and *S. aureus*. This result was determined by observing the visible growth of bacteria (turbidity of the nutrient broth) in the glass vial. This is a promising activity when compared with minimum inhibition concentration value (12.5 mg/mL) reported by Abbas et al. [37] for isolated compound (β-sitosterol glucoside) from stem bark of *A. pavonina.* The details of this assay (MIC) are shown in Table 2 that follows.

Table 2. Minimum inhibition concentration of the isolated compounds.

Fraction	**Conc. (mg/mL)**	***S. aureus***	***E. coli***	***S. typhy***
AP-X_{38}	1.0	-	-	-
	0.5	-	-	-
	0.25	+	+	+
	0.125	+	+	+

+ve control	NB + BS	+	+	+
- ve control	NB only	-	-	-
AP-X_{44}	1.0	-	-	-
	0.5	-	-	-
	0.25	-	-	-
	0.125	+	+	+
+ve control	NB + BI	+	+	+
- ve control	NB only	-	-	-

NB = Nutrient broth BS = Bacterial Species,

+ve = Positive and –ve = Negative

Conclusion

Evidently, the results obtained for this study are indicatives that, *Adenanthera pavonina* is a medicinal plant that can serve as a source for the isolation of active compounds that may serve as leading compounds in antimicrobial drug development, and to be a relief for the increasing problem of antibiotic resistance. The local used of the plant in folk medicine in Southern Borno State, Nigeria, suggested that, it represent a cheaper and safer alternative in the treatment of mastitis and other infectious diseases.

This study justifies part of ethno medicinal claims on the plant, as effective as conventional medicine in combating pathogenic microorganisms of clinical and veterinary concern.

Recommendation

(i) Characterizations (GC-MS, HNMR, CNMR and FT-IR) of the isolated compounds are recommended in order to elucidate their structures.

(ii) Further antimicrobial and toxicological evaluations (*In vivo*) should be conducted with model animals to determine the dose dependent activity and safer usage of these isolated compounds.

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