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Evaluation of the antimicrobial activity of crude extracts and chromatographic fractions of *Adenanthera pavonina* Linn (Leguminosae) seeds

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Most available reports on the biological activity of *Adenanthera pavonina* (AP) are on the bark or leaves and very few are on the seeds. In particular, there are no reports on the biological effects of the chromatographic fractions of *A. pavonina* seeds hence the present study aimed to evaluate the antimicrobial activity of the crude extract and chromatographic fractions of *A. pavonina* seeds. The methanolic extract was fractionated and all of the column chromatographic fractions as well as the crude extract were evaluated against different strains of *Staphylococcus aureus*. The methanolic extract (100 mg/ml) produced zones of inhibition on PHM 002 and PHM 005 while the 50 and 100 mg/ml of the hexane extract only produced inhibition on PHM 001. On the other hand, fractions ST 10-12 F exhibited activity PHM 002 at 50 and 100 mg/ml while fractions ST-13-15F exhibited activity at all concentrations (6.25, 12.5 25, 50 and 100 mg/ ml) against *S. aureus* PHM 002 strain from the skin. The study provides some justifications for the folkloric use of AP seed powder as an antiseptic paste and warrants further studies to determine the structure of the active compound in chromatographic fraction ST 13 -15F.

Key words: *Adenanthera pavonina*, antimicrobial activity, chromatographic fractions, methanolic extract.

INTRODUCTION

Plants are valuable as sources of medicine. The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (Rates, 2001). In the recent decades, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Vulto and Smet, 1988; Rates, 2001).

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This may be due to the fact that the conventional medicine can be inefficient (such as having side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems. Also, a large percentage of the world's population does not have access to conventional pharmacological treatment. Medicinal plants have served as valuable starting materials for drug development in both developing and developed countries. Today, more than 80% of the people living in Africa depend on medicinal plants and animal based medicines to satisfy their healthcare requirements (Yedjou et al., 2008). It has also been suggested that folk medicine and ecological awareness suggest that "natural" products are harmless. Many pharmaceutical products are from plants; though pharmaceutical industries have produced a number of new antibiotics in recent times, resistance by micro-organism remains a challenge. Action must therefore be taken to reduce this problem by controlling the use of antibiotics, developing research to better understand the mechanism of resistance and embarking on studies to develop new drugs either natural or synthetic (Ahmed et al., 1998; Hussain et al., 2011).

Adenanthera pavonina Linn (AP) is a medium to large deciduous tree of the family *Leguminosae* also called *Fabaceae*. The tree is common throughout the lowland tropics up to 300 to 400 m (Jayakumari et al., 2012). In terms of medicinal uses, it has been reported that bark and leaves are astringent, vulnerary and aphrodisiac and are used in ulcers, pharyngopathy and even the heart wood is astringent, aphrodisiac, haemostatic and is useful in dysentery, hemorrhages and gout (Vaidyaratnam and Variers, 1994). The root on the other hand is emetic in nature (Jayakumari et al., 2012). It has been reported that the plant have antiseptic and anti-inflammatory activities. The red powder is used as an antiseptic paste. Effects of *A. pavonina* include anti-inflammatory and analgesic from methanolic seed extract (Olajide et al., 2004) and ethanolic leaf extract (Mayuren and Ilavarasan, 2009); and antimicrobial from the bark extract (Ara et al., 2010). The blood pressure lowering effect of the seed extract has also been reported (Adedapo et al., 2009). The main important constituents are flavonoid compounds (Rastogi and Mehrotra, 1991). It is used as an antiseptic paste and also used to treat boils and inflammations (Chopra et al., 1956).

Most available reports on the biological activity of *A. pavonina* are on the bark or leaves and very few are on the seeds. In particular, there are no reports on the biological effects of the chromatographic fractions of *A. pavonina* seeds. Chromatography is the key to obtaining pure compounds for structure elucidation, for pharmacological testing or for development into therapeuticals. It also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals (Marston, 2007).

In this study, the chromatographic fractions of the seeds of *A. pavonina* are being explored for its antimicrobial properties with a view to determine the fraction responsible for this effect.

MATERIALS AND METHODS

Chemical and solvent

This includes: ammonia, acetone, Benedict's solution, chloroform, Dragendorff's reagents, distilled water, ethyl acetate, ferric chloride, hydrochloric acid, hexane, lead acetate, methanol, Mayer's reagent, potassium hydroxide, sodium hydroxide, sulphuric acid, toluene and Wagner's reagent.

Test organisms

Reference strains were used as suggested by Cos et al. (2006). These are different strains of *Staphylococcus aureus*; from the nose (strain 1) PHM 001, from the skin (strain 2) PHM 002, from the eye area (strain 3) PHM 003, from the armpit (strain 4) PHM 004 and the standard (strain 5) PHM 005. All microorganisms were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria.

Plant material

The seeds of *A. pavonina* were collected from the Staff School Compound in the University of Ibadan, Oyo State throughout the month of September 2009. They were authenticated at the Forestry Research Institute (FRIN), Ibadan where a voucher specimen was deposited.

Sample preparation

The seeds were air dried and powdered. The powdered seeds were extracted using cold maceration. The powdered seeds sample (1335.09 g) was put in a large flask and defatted for 24 h using 1280 ml- hexane. It was stirred occasionally and filtered. The marc was air dried and placed in a flask. The plant material was covered with 2000 ml of methanol and allowed to stand for four days after which it was filtered and the filtrate concentrated to dryness to yield 30.77 g of extract. The residue was again extracted with another 1,300 ml of methanol and the filtrate was again concentrated to dryness to yield a further 20.61 g of crude methanolic extract.

Phytochemical screening

The phytochemical analysis was performed on the ground (powered) leaf of *A. pavonina* for identification of the constituents. The constituents tested for were alkaloids, tannins, saponins, anthraquinones, cardiac glycosides and flavonoids as described by Shale et al. (1999), Moody et al. (2006) and Sawadogo et al. (2006).

Chromatographic analysis

Thin layer chromatography (TLC)

The TLC plates of dimensions 20 × 20 cm were used. The thickness of the adsorbent layer was 1 mm. Thin layer chromatography was used to monitor fractions obtained from

Table 1. TLC analysis of crude methanolic and hexane extract of *Adenanthera pavonina* seeds.

Extract	Solvent system	Colour in daylight	Colour in UV 365 nm	R_f Value
Hexane extract	SS4	Yellow	Blue	0.85
Hexane extract	SS1	Yellow	Colourless	0.69
Hexane extract	SS3	Yellow	Colourless	0.12
Hexane extract	SS2	Yellow	Yellow	0.43, 0.60
Methanolic extract	SS4	Yellow	Blue	0.64
Methanolic extract	SS1	Yellow	Blue	0.73
Methanolic extract	SS3	Yellow	Blue	0.17
Methanolic extract	SS2	Yellow	Blue	0.53, 0.67, 0.84

column chromatography fractionation of the crude methanolic extract as well as that of the crude hexane extract. The solvent systems used were: SS1-chloroform: methanol 9:1, SS2 toluene: acetone: ethyl acetate 8:2:1, SS3 chloroform: methanol 19:1, SS4 hexane: ethyl acetate 8:2, SS5 chloroform: methanol 7:3, SS6 toluene: acetone 4:1 and SS7 toluene: acetone: ethyl acetate 80:40:20. The plates used were examined under daylight and ultraviolet (UV) at 365 nm and were sprayed with Dragendorff to detect the presence of any alkaloid present (Harborne, 1984).

Column chromatographic separation

The methanolic extract was further subjected to silica gel chromatographic separation using standard procedures. The chromatographic column of length 50 cm and internal diameter 3.5 cm was rinsed with acetone and allowed to dry. It was then clamped vertically with a retort stand, and a plug of cotton wool was inserted at the bottom of the column using a clean glass rod. The column was filled with hexane, and the silica gel slurry was mixed with hexane in a beaker. The slurry was poured into the column and tapped intermittently for uniform distribution of the gel and to avoid bubbles in the column. The column was allowed to drain for some minutes to stabilize it. A portion (20.61 g) of the crude methanolic extract was subjected to chromatography in column silica gel, was eluted with solvent mixtures of increasing polarity and dissolved in methanol by warming on a water bath. The cooled methanolic extract was then introduced on top of the silica gel adsorbent in the column. The column was eluted with solvent mixtures of increasing polarity. Fractions were collected and monitored by thin layer chromatography using appropriate solvent systems. Fractions showing similar TLC patterns were pooled together (Li et al., 2012). Nutrient agar (28 g) was mixed with distilled water to make up 1 L. The mixture was heated to boiling on a hot water bath. The molten agar was poured into universal bottles and autoclaved for 30 min at 121 psi. Nutrient broth (4 g) was dissolved with distilled in a conical flask and was made up to 500 ml. The mixture was measured in 10 ml aliquots into test tubes and sterilized in an autoclave for 20 min at 121 psi. The methanolic extract and pooled chromatographic fractions were tested for antimicrobial activity using the reference organisms stated earlier. The respective organisms were sub-cultured into the nutrient broth from the stock. The overnight culture (0.2 ml) was inoculated into 10 ml of sterile distilled water in a test tube. From the diluted organism, 0.2 ml was taken and inoculated into the cooled nutrient agar and shaken for homogeneity. The sterilized agar was poured into Petri dishes and allowed to set. It was dried and bore holed with a cork borer of diameter 8 mm. Six holes were made on each plate. Different concentrations of the extracts and fractions of *A. pavonina* seeds were then introduced into the holes. The plates were allowed to stand for 30 min to achieve diffusion after which they were incubated for 24 h at 37°C (Gurib-Fakim, 2006; Jimoh et al., 2011).

RESULTS

The phytochemical screening of methanolic extract of the seeds of *A. pavonina* showed the presence of tannins, saponins, alkaloids, flavonoids and cardiac glycosides while Tables 1 to 6 show the TLC analysis of crude methanolic and hexane extract of *A. pavonina* seeds; column chromatographic separation of the crude methanolic extract of *A pavonina* seeds; TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS5 (chloroform: methanol 7:3); TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS7 (toluene: acetone: ethylacetate 80:40:20); TLC analysis of column chromatographic fractions of the methanolic extract of *A. pavonina* seeds using mobile phase SS6 (toluene: acetone 4:1) and anti-microbial screening of the crude methanolic extract, hexane extract and chromatographic fractions of *A. pavonina* seeds, respectively. Column chromatographic analysis of the methanolic extract of *A. pavonina* seeds resulted in 15 fractions on elution with different solvent mixtures. Similar fractions were bulked on the basis of thin layer chromatographic analysis. Fraction 15 was found to be the one with highest yield providing enough material for anti-microbial evaluations. TLC analysis of the fractions was done using three different solvent systems. Solvent system 6 (toluene: acetone 4:1) was found to be the most appropriate among the solvent systems as it gave the best resolution of the components. The most suitable solvent system for TLC analysis of the crude methanolic and hexane extracts, solvent system 2 (toluene: acetone: EtOAc 8:2:1) which found to be most appropriate gave 3 spots (Rf values: 0.53, 0.67, 0.84) for the methanolic extract and 2 spots (Rf values: 0.43, 0.60) for the hexane extract. The methanolic extract (100 mg/ml) produced zones of inhibition on PHM 002 and PHM 005 while the 50 and 100 mg/ml of the hexane extract only produced inhibition

Table 2. Column chromatographic separation of the crude methanolic extract of *Adenanthera pavonina* seeds.

Fraction collected	Solvent mixture	Volume collected (ml)	Yield (g)
ST 1F	Hex: Chloroform 70:30	25	
ST 2-4F	Hex: Chloroform 60:40	25	1.955
	Hex: Chloroform 60:40	25	
	Hex: Chloroform 60:40	25	
ST 5-7F	Hex: Chloroform 60:40	25	3.731
	Hex: Chloroform 60:40	25	
	Hex: Chloroform 60:40	25	
ST 8-9F	Hex: Chloroform 60:40	25	3.275
	Hex: Chloroform 40:60	25	
ST 10-12F	Methanol: Chloroform 10:90	25	3.451
	Methanol: Chloroform 10:90	25	
	Methanol: Chloroform 20:80	25	
ST 13-14F	EtoAC: Methanol 80:20	25	1.437
	EtoAC: Methanol 50:50	25	
ST 15F	100% Methanol	25	4.344

Table 3. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS5 (chloroform: methanol 7:3).

Fraction	Colour in daylight	Colour in UV 365 nm	R_f Value
ST 2-4F	Colourless	Reddish-brown	
ST 5-7F	Blue	Reddish-brown	0.87
ST 8-9F	Reddish-brown	Yellow	0.80
ST 10-12F	Reddish-brown	Yellow	0.95
ST 13-14F	Reddish-brown	Yellow	0.87
ST 15F	Reddish-brown	Yellow	-

Table 4. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS7 (toluene: acetone: ethylacetate 80:40:20).

Fraction	Colour in daylight	Colour in UV 365 nm	R_f
ST 2-4F	Reddish-brown	Yellow	0.93
ST 5-7F	Yellow	Colourless	
ST 8-9F	Reddish-brown	Colourless	
ST 10-12F	Reddish-brown	Colourless	
ST 13-14F	Yellow	Colourless	
ST 15F	Yellow	Colourless	

on PHM 001. On the other hand, only fractions ST 10-12 F and ST 13-15 F produced inhibition. The results of chromatographic analysis and antimicrobial assay are as shown in the Tables 1 to 6.

Table 5. Thin layer chromatography (TLC) analysis of column chromatographic fractions of the methanolic extract of *Adenanthera pavonina* seeds using mobile phase SS6 (Toluene: Acetone 4:1).

Fraction	Colour in daylight	Colour in UV 365nm	R_f
ST 2-4F	Yellow	Blue	0.27
ST 5-7F	Yellow	Reddish-brown	0.28
ST 8-9F	Yellow	Reddish-brown	0.15
ST 10-12F	Yellow	Reddish-brown	0.38
ST 13-14F	Yellow	Reddish-brown	0.13
ST 15F	Yellow	Reddish-brown	0.07

Table 6. Anti-microbial screening of the crude methanolic extract, hexane extract and chromatographic fractions of *Adenanthera pavonina* seeds.

Extract/fraction	Microorganism / Zone of inhibition (mm)				
Concentration mg/ml	***Staph aureus* PHM 001**	***Staph aureus* PHM 002**	***Staph aureus* PHM 003**	***Staph aureus* PHM 004**	***Staph aureus* PHM 005**
Methanolic extract					
100	-	9	-	-	14
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	28	18	12	28	20
Hexane extract					
100	12	-	-	-	-
50	10	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	28	13	12	24	22
ST 2-7 F					
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin	22	13	10	11	15
ST 10 -12 F					
100	-	4	-	-	-
50	-	3	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
6.25	-	-	-	-	-
Gentamicin					
ST 13 -15 F					
100	-	20	-	-	-
50	-	11	-	-	-

Table 6. Contd.

25	-	10	-	-	-
12.5	-	8	-	-	-
6.25	-	7	-	-	-
Gentamicin	22	20	5	11	10

Gentamicin 500 ug/ml – Positive control. Diameter of cork borer – 8 mm.

DISCUSSION

Phytochemical screening of *A. pavonina* seed showed the presence of tannins, alkaloids, flavonoids and cardiac glycosides. Saponins are of great pharmaceutical importance because of their relationship to compounds such as sex hormones, cortisones, diuretics, steroids, vitamin D, and cardiac glycosides. Flavonoids have influence on arachidonic acid metabolism, thus could have anti-inflammatory, anti-allergic, anti-thrombotic or vasoprotective effects. Flavonoids and tannins are phenolic compounds and plant phenolics are also a major group of compounds that acts as primary antioxidant or free radical scavengers (Ayoola et al., 2008). Tannins and saponins are also found to be effective anti-oxidants, antimicrobial and anti-carcinogenic agents (Lai et al., 2010). Flavonoids are known to target prostaglandins which are observed in the late phase of acute inflammation and pain perception (Chakraborty et al., 2004). Cardiac glycosides are steroidal glycosides which exert a slowing and strengthening effect on a failing heart. In fact cardiac glycosides are being tested for their anticancer properties (Chen et al., 2006; Frese et al., 2006; Newman et al., 2008). Thin layer chromatographic screening of *A. pavonina* seed crude extract and column chromategraphic fractions showed the presence of several alkaloid substances on spraying with Dragendorff reagent. Alkaloids are generally quite bitter and many are toxic (Takimoto and Calvo, 2008). Some alkaloids such as vinca alkaloids have been used to treat diabetes, high blood pressure, and the drugs have even been used as disinfectants. However, the vinca alkaloids are most famous for being cancer fighters (Takimoto and Calvo, 2008). The implications of all these is that this plant is of great medicinal importance.

Column chromatographic analysis of the methanolic extract of *A. pavonina* seeds resulted in 15 fractions on elution with different solvent mixtures. Similar fractions were bulked on the basis of thin layer chromatographic analysis. Fraction 15 was found to be the one with highest yield providing enough material for anti-microbial evaluations. Anti-microbial activities of the extract and components from *A. pavonina* seeds showed that only fractions ST 13 - 15 F was able to inhibit the growth of *S. aureus* strain 2 isolated from the human skin as all concentrations used. The methanolic extract was effective only at the highest concentration (100 mg/ml) against *S. aureus* strain 2 and *S. aureus* strain 5. It was ineffective at all other concentrations against other strains. The hexane extract was effective at concentrations 50 and 100 mg/ml against *S. aureus* strain 1 isolated from the nose alone, while it was inactive against all other strains of *S. aureus* at all concentrations. Fraction ST 2-7F showed no activity against any of the *S. aureus* strains, while fraction ST 10-12F was active against *S. aureus strain* 2 only at 50 and 100 mg/ml. Flavonoids, alkaloids, saponins and tannins have been reported to be active against several bacteria and fungi (Hassan et al., 2004). The presence of these in the crude extract of *A. pavonina* could be responsible for the antibacterial activity exhibited by the crude extract and components from *A. pavonina* in this study. The standard reference, gentamicin 500 µg/ml was able to inhibit all strains of *S. aureus*. Based on the results obtained from this study, there was preliminary evidence that there reside in the extract and components from *A. pavonina* seed, compounds with antibacterial activity against isolates of *S. aureus*. There is a need for further studies to determine the ED_{50}, as well as to isolate and characterize the constituents from the active fractions of this plant.

Conflict of interests

The authors did not declare any conflict of interest.

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