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Full Length Research Paper

Bioautographic determination of the antistaphylococcal components of the stem bark of *Parkia biglobosa* (JACQ) BENTH (MIMOSACEAE)

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Bioautography, a scientific method which combines chromatography with bioassay was used to attempt to determine the bioactive components in the methanolic extract of the stem bark of *Parkia biglobosa.* Results obtained confirmed 4 compounds of different R_f values. Each of them showed activity against *Staphylococcus* confirming previous results reported and the method also showed the components were acting in synergy. *Pseudomonas* and *Bacillus subtilis* were not as susceptible as *Staphylococcus* to the components. *P. biglobosa* remains a hopeful source of compounds that can be effectively used against even resistant *Staphylococcus.*

Key words: Bioautography, methanolic, Parkia, *Staphylococcus*, synergy.

INTRODUCTION

Bioautography is a technique that combines Thin Layer Chromatography (TLC) with bioassay *in situ* (Shittu et al., 2006). It can be used for the screening of separated components of natural product extracts (Cannel, 1998). Bioautography combines TLC with bioassay in such a way that it is able to locate the active constituents in a complex matrix such as a plant extract (Oyi, 2001).

Plants have the ability to produce and store a wide range of chemical substances. Most of these substances are secondary metabolites which in most cases serve to defend the plant against attacks from microorganisms and other predators. A lot of them are also responsible for other characteristics like plant flavours (e.g. the terpenoid capsaicin from chili peppers) while others give plants their characteristic odour. Yet, others are respon-sible for the kind of pigments seen in plants (Cowan, 1999). The antimicrobial properties of plants are known to be traceable to these substances.

Parkia biglobosa (Jacq) Benth (Mimosaceae), is a popular tree in the West African Sub region which is used for a variety of purposes among which is the treatment of a wide range of disease situations. Recently, the attention of researchers has been drawn to the great potentials in *P. biglobosa* as an antibacterial agent. Ajaiyeoba (2002), Banwo et al. (2004), Millogo-Kone et al. (2006) and El Mahmood and Ameh (2007) have all reported the presence of plant secondary metabolites which are known to exhibit antibacterial activity against a wide range of organisms in this plant. El-Mahmood and Ameh (2007) reported the presence of tannins, saponins, Glycosides and phenolics with trace quantity of alkaloids while Millogo-Kone (2006), Banwo et al. (2004) and Ajaiyeoba (2002) confirmed the same. Only Ajaiyeoba (2002) and Millogo-Kone (2004) differed slightly by reporting the absence of alkaloids.

All researchers who have reported the results of their work on *P. biglobosa* activity agree on the efficacy of their extracts with polar solvents especially against Gram positive e.g *Staphycococcus aureus*. Ajaiyeoba (2002) reported the activity of aqueous and ethanolic extracts against Gram positive organisms and not Gram negative organisms while the hexane extract had no activity at all.

This work presents the report of the effort to identify the bioactive compounds in the parts of *P. biglobosa* using

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the more complex but more precise bioautographic method especially against the problematic *S. aureus.*

MATERIALS AND METHODS

Plant sample collection and authentication

The plant materials were collected from Samaru, Zaria in Kaduna state of Nigeria. They were authenticated in the herbarium section of the Biological Science Department of Ahmadu Bello University Zaria and a voucher specimen numbered 2846 was therein deposited for reference purposes.

Sample preparation

Plant materials were air dried in the shade for five days (on the laboratory bench) (Oboh et al., 2007), ground into powder in a mortar and extracted to exhaustion with different solvents viz (Methanol, Ethanol, Diethyl ether, Chloroform) using a Soxhlet apparatus (with appropriate care taken to avoid decomposition of the secondary metabolites sought after). Afterwards, the solvents were removed and the extracts obtained were stored in the desiccators at ambient temperature until needed. From the results of the antibacterial activity, methanol was chosen as the solvent of choice.

Each crude methanolic extract (Leaf, stem bark and root) was therefore fractionated into water, petroleum ether (b.p. 60 - 80°C) and chloroform portions (using solvents of varying polarity).

Fractionation of extract

Each dried extract (20 g) was dissolved in water (200 ml) before being shaken vigorously in a separating flask and the mixture obtained filtered. Petroleum ether (b.p. 60 - 80°C) (200 ml) was added to the mixture, shaken vigorously and allowed to settle. The petroleum ether (b.p. 60 - 80°C) layer (on top) was decanted and concentrated. Chloroform (200 ml) was then added to the aqueous layer and also vigorously shaken and allowed to settle. The aqueous and the chloroform layers were further separated and concentrated to dryness in vacuo.

Test organisms

Purification of test organisms

The purity of the test organisms was confirmed by sub-culturing onto nutrient broth incubated at 37°C for 18 h. They were then streaked onto sterile nutrient agar plates and incubated at 37°C for 18 h. The developed colonies were observed under the microscope after simple staining. Pure cultures were kept on agar slopes at 4°C until needed.

Inoculum preparation

When needed, the test organisms were grown for 18 hours in nutrient broth at 37°C. They were further and appropriately diluted in normal saline to obtain about, one million cells/ml

Susceptibility testing

The agar cup diffusion method was used for this test. Sterile nutrient agar plates were flooded with appropriately diluted organism, the excess was aseptically drained and the surface, allowed to dry at ambient temperature. Wells were bored into the agar plates using a 4 mm sterile cork borer and 0.1 ml each of different concentrations of the extract and fraction ranging from 200 to 25 mg/ml was introduced into each well. The plates were allowed a diffusion time of one hour at ambient temperature and then incubated at 37°C for 24 h after which zones of inhibition were read to the nearest millimeter.

Chromatographic studies

Thin layer chromatography

The aqueous fractions of the methanolic extracts of the root, stem bark and leaves of *P. biglobosa* were chromatographically analysed using the thin layer chromatography method. This was with the intention of selecting an appropriate solvent system for the bioautography proper.

The thin-layer chromatography silica gel was made into slurry by mixing to ensure homogeneity before being spread on glass sheets according to the required dimensions. They were left to dry at ambient temperature before reactivation in the oven at 110°C for 1 h.

The plates were spotted with a solution of the fraction using spotting tubes about 1 – 2 cm above the bottom of the plate and then placed in a chromatography tank with selected solvents of different polarities just enough to wet the lower edge of the plate before the part where the spotting was done. The plates were left in the solvent for some time during which the solvent would have moved across the plate from bottom to top. The plates were removed from the tank, allowed to dry and then visualized using: (1) Iodine vapour. (2) Observation under U.V. light. (3) Fehling's solution (spray).

Bioautographic studies

The fraction was first run on a microscopic slide (as the TLC plate) with n-butanol: acetic acid: water (6:1:2 v/v) as the solvent system and spots on the plate developed using iodine vapour.

Nutrient agar (19 ml) was seeded with 1 ml of appropriately diluted overnight culture (to give 10^6 Cfu/ml). A nutrient agar base containing sterile nutrient agar (20 ml) prepared and poured into a petri dish and allowed to set. The TLC plate was then placed on the nutrient agar base and the seeded medium (which contains the test organism) was poured over it, allowed to solidify and pre-diffuse for between 1 - 2 h and then incubated at 37°C for 24 h. Inhibition around the chromatographic spots on the slide in the form of clear zones was noted. The plates were further sprayed on the surface with a 2.5 mg/ml aqueous solution of a hydrogenase inhibitor [Methyl thiazolyl tetrazolium chloride (M.T.T)]. Zones of inhibition were observed as clear zones against a purple background.

RESULTS

Chromatographic studies

The Thin layer chromatography (TLC) studies using different solvents of varying polarities revealed the presence of different compounds, (Depicted by number of spots) (Table1).

The TLC results of the aqueous fraction of the methanolic extract of the stem bark of *P. biglobosa* using

Table 1. Different components of WS revealed by TLC.

Solvent system	Sample	No of spots
n-BuoH: Acetic acid: Water (6:1:4)	WS	2
“ “ “ “ (6:1:2)	WS	4
“ “ “ “ (3:1:1)	WS	2
Ethanol: Chloroform (4:1)	WS	1
Methanol: ethyl acetate (9:3)	WS	1
” “ (9:2)	WS	1
Methanol: chloroform (4:1)	WS	1

Table 2. R_f Values of Bioactive components (compounds) of the aqueous fractions of WS.

Bioactive components (compounds)	R_f
A	0.92
B	0.72
C	0.62
D	0.56

n-butanol, acetic acid and water at a ratio of 6:1:2 as solvent system revealed 4 bands of different R_f values depicting 4 different compounds (A-D) (Table 2).

Bioautography studies

Results of the bioautography test of WS are shown in Figures 1, 2 and 3. It revealed that all the 4 compounds separated by TLC, had good activity against *S. aureus* and very mild effect on *Pseudomonas aeruginosa* while there was no activity against *Escherichia coli* and *Bacillus subtilis.*

DISCUSSION

To obtain a valid biological data on a natural product, it is expected that the compound responsible for the biological activity must be obtained in a pure form. Bio-autography has become a veritable tool in the separation of these compounds most of which are bioactives. The bioactive compounds in the fractions studied were successfully separated using the thin layer chromate-graphy with the intention of making valid statements about each compound especially as it concerns its antistaphylococcal ability

The number of spots observed during the separation by the TLC depicted the compounds that were present in the extract. The TLC plates of WS using n-butanol: acetic acid: Water (6:1:2v/v) as solvents system showed four bands of different R_f values.

Bioautography results obtained confirmed the presence of different compounds within the fractions. All of these compounds showed good activity individually against the *S. aureus* ATCC 25923 used as test organism. The clearing observed around the spots during the test using WS confirmed that if each of these compounds is isolated and purified, it can be formulated into a drug that can be useful for the control of *S. aureus.* Worthy of note is the fact that the combined effects of these compounds gave a clearer and bigger activity (See Figures 1 and 2). It may be that the known antibacterial effect of the stem bark of *P. biglobosa* is due to the combined effect of these compounds. It is also very possible that the combined effects of any two or three of the compounds will produce a better antibacterial effect against *S. aureus* or any other organisms that has been observed. This is because it has been suggested that generally, plant antibacterials are generally weak with orders of magnitude less than that of common antibiotics from bacteria and fungi but functions in synergy (Lewis and Frederick, 2006). Results obtained using *Ps. aeruginosa* as test organism showed a very low activity of the separated compounds against this organism while for *B. subtilis* and *E .coli*, the separated compounds showed no activity at all (Fig. 3). The combined effects of these compounds may have brought about whatever original activity of the fraction noted during the antimicrobial activity and MIC tests especially against *E. coli* and *B. subtilis* and also for *Ps. aeruginosa* (Reported in other works) since such combinations are known to produce a synergistic effect (Esimone et al., 1999) and they have proved to be efficient (Fritz, 1986; Klastersky et al., 1986). The 4 compounds identified by this work have proven to be good antimicrobials against *S. aureus* which is known to be resistant to a number of antibiotics.

Plants produce compounds that can be effective antimicrobials if they find their way into the cells of pathogens especially across the double membrane

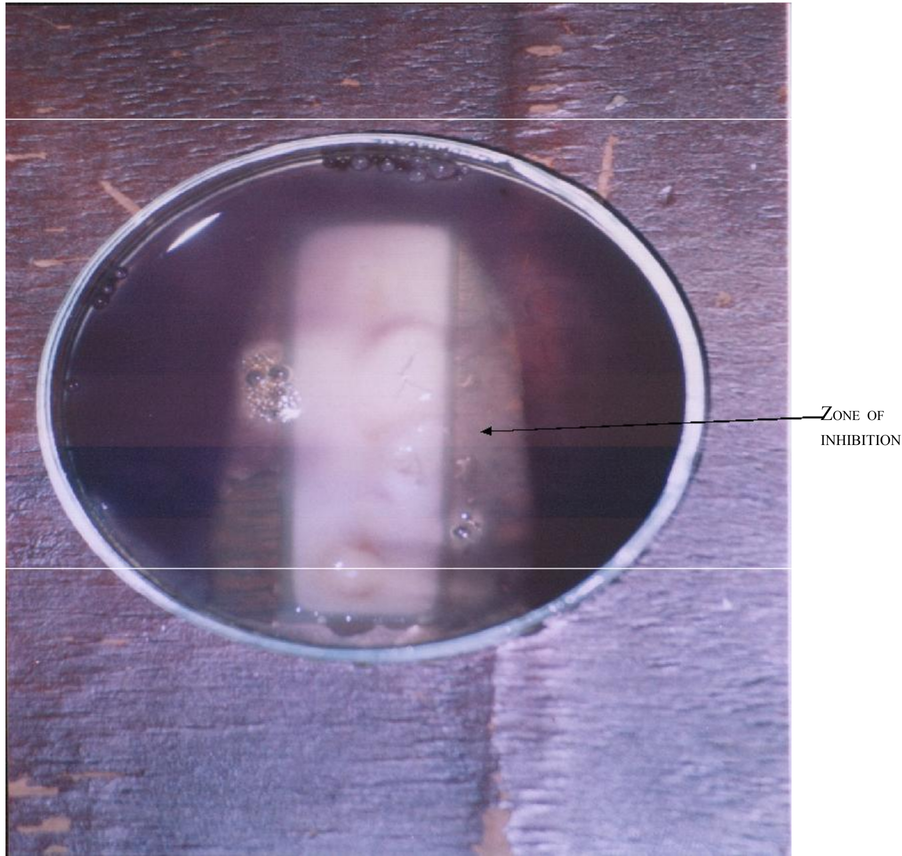

Figure 1. Bioautography result of WS against *Staphylococcus aureus* after spraying with Methyl Thiazolyl Tetrazolium Chloride (M.T.T).

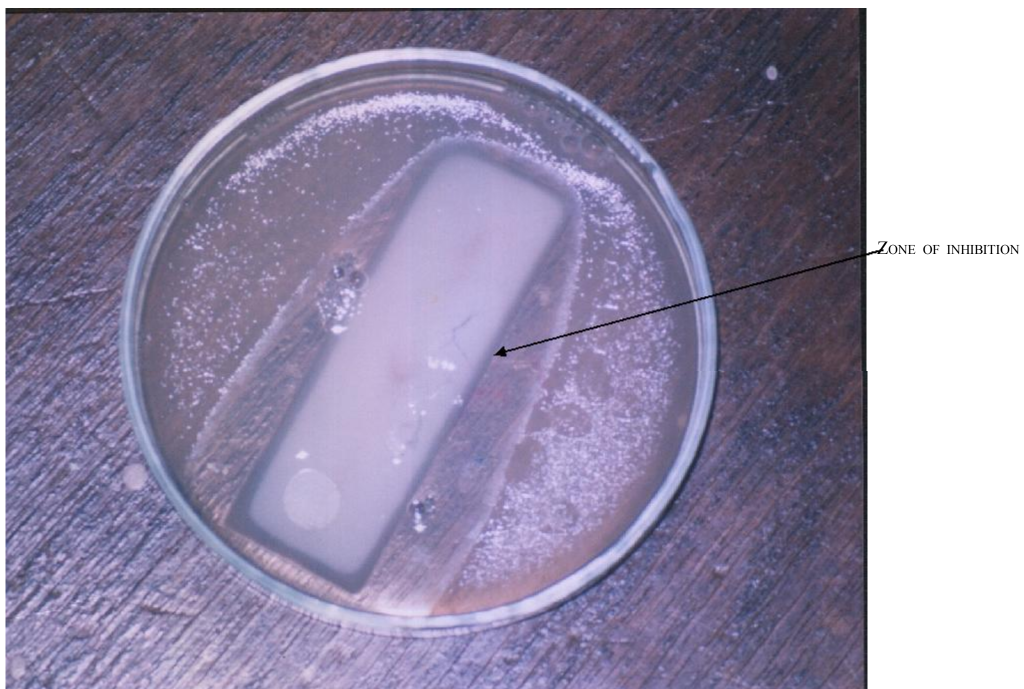

Figure 2. Bioautgraphy result of WS against *Staphylococcus aureus* before spraying with Thiazolyl Tetrazolium Chloride (M.T.T).

Figure 3. Bioautography results of WS against *B. subtilis,* (1) *E. coli* (2) *and Ps. aeruginosa* (3) after spraying with Thiazolyl Tetrazolium Chloride (M.T.T).

barrier of Gram negative bacteria (Sibanda and Okoh, 2007). The delivery of plant compounds like the ones identified in this work into the cells of pathogens can be ensured by the production of efflux pump inhibitors (which are also produced by plants). Work is continuing in our laboratory to confirm the possibilities of these compounds functioning also as efflux pump inhibitors.

Conclusion

The aqueous fraction of the methanolic extract of the stem bark of *Parkia biglobosa* (WS) contains components (compounds) which on their own exhibited remarkable activity against *S. aureus* and very mild activity against *Ps. aeruginosa*. In a combined state however, the compounds showed an even better activity against all the organisms tested. This confirms that the activity of the fraction is the combined effect of the various components (compounds) in it. Further combination studies are suggested as this may lead to an agent of better activity.

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