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# Some biological activities of *Pycnanthus angolensis* (Welw.) Warb

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**Abstract**

*Pycnanthus angolensis* (family Myristicaceae) is used in a variety of herbal therapies for treating skin, oral chest and gastro-intestinal ailments. However, its use in malaria control at the larval stage and potential for anti-tumor activity are yet to be investigated. Hence the anti-microbial, larvicidal and brine-shrimp lethality studies on leaves, stem and roots were carried out. The extracts and fractions of leaves elicited good anti-microbial activity against *Bacillus subtilis, Staphylococcus aureus* and none against *Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae* but gave minimal activity against fungal isolates (*Aspergilus niger* and *Candida albicans*). Both the stem and roots however, afforded lesser activities. The larvicidal assay determined in terms of percentage mortality showed that the leaves gave very weak larvicidal activity (LA%) of 20% and 35% (at 5%w/v) while the activity was somewhat moderate at 45% and 50% (10%w/v) both at 12 and 24h incubation respectively. However, the activity displayed by both the stem and roots was insignificant. The brine- shrimp lethality assay; analyzed using the Finney probit method, showed that the leaves displayed a 'significant' $LD_{50}$ value at 2.5ppm while the stem and roots gave 'moderate' $LD_{50}$ values at 362ppm and 310ppm respectively compared with literature values below the 200ppm are generally considered 'significant'. These findings indicate a correlation in the activities of the leaves and as such serve as panacea for infectious diseases and therefore scientific justification to some of the folkloric uses of the plant.

*Keywords*: Antimicrobial; Larvicidal; Brine shrimp lethality; *Pycnanthus angolensis*

## Introduction

*Pycnanthus angolensis* (Welw.) Warb. (family Myristicaceae) is a medium- sized forest tree known as 'cardboard' especially in Cameroun (Hutchinson and Dalziel, 1954) and is employed in variety of uses. The bark is used as an enema to purify breast milk of nursing mothers, mouthwash, purgative, antidote to poisons, in treating toothaches, headache, scabies (Etukudo, 2003), chest pains, skin lesions, rashes due to river blindness (Onchocercarsis) and leprosy. The roots are used as anti-helmintic (Keay *et al*, 1964) while the infusions of the leaves and seeds are used in treating tongue thrush, wounds, skin- fungal infections and to prevent miscarriage. The oil and the wax residues are used as cattle feed and in making candles,

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soap, fuel and lubricants. Previous studies have shown that kombic acids (substituted palmitic fatty acids) and their derivatives reportedly isolated from the plant posses anti-inflammatory and anti-oxidant activities(Rios *et al*,1990) However, in spite of these findings and the multiple contributions of the plant in many therapies, its use in malaria control at the larval stage and potential for anti-tumor activities are yet to be revealed.

This study was designed with the aim of investigating into potential for larvicidal and anti-tumor activities and as well as to confirm or otherwise the susceptibility of the selected microbes to extracts and fractions.

## Experimental

The leaves, stem and roots of *P. angolensis* were collected fresh in April, 2004 at a location inside the University of Uyo (Main campus) Uyo, Akwa Ibom State. These plant parts were identified by Dr R. Nia of the Department of Pharmacognosy and Natural Medicine where voucher specimens (No H47), (NoH48) and (No H49) were deposited. Sea water (collected from Kuramo Beach, Lagos), plastic soap case, brine –shrimp eggs (*Artemia salina*. Leach) obtained from San Francisco Bay Brad Inc; Newark, CA 94560, USA and *Anopheles gambiae* larvae bred in plastic buckets.

*Chemicals, Micro-organisms and Media.* The chemicals (reagents) used were: Acetone, Butanol, Ethanol, Ethyl-acetate, Hexane, Methanol and Toluene (all of AnalaR grade; Aldrich Chemicals Inc. U.S.A.), Silica gel (254GF), Ampicillin and Nystatin (Fidson Healthcare Limited, Lagos, Nigeria). The microorganisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Aspergilus niger* and *Candida albicans)* clinically isolated from human specimens; urine, faeces, wounds and vaginal swabs were obtained from the Medical Laboratory, University of Uyo Health Center, collected in sterile bottles, identified and authenticated by convectional biochemical tests (Murray *et al.*, 1985; Gibson and Khoury, 1986) and then refrigerated at 0-5$^0$C at the Pharmaceutical Microbiology and Parasitology Unit, Faculty of Pharmacy, University of Uyo, Akwa Ibom State prior to use. Also used were Mueller Hinton II Agar (Biotec Laboratories, Limited, Ipswich, England), Sabouraud Dextrose Agar (International Diagnostic Group PLC, Lancashire, England) and Nutrient Broth(Oxoid Limited, Basingstoke, U.K.).

*Extraction and processing.* The plant parts were air-dried and powdered separately on an electric mill. The resultant ground powders were then extracted with cold 50% aqueous ethanol at room temperature (27 ± 2$^0$C) for 72h.The filtrates were evaporated to dryness using a rotary evaporator(Buchi CH-920, Laboratorium-Technic, Flawk/SG, Switzerland). The dried crude ethanolic extracts were then investigated for plant metabolites (alkaloids, saponins, tannins, cardiac glycosides, terpenes, anthraquinones and flavonoids) according to the laid down phytochemical methods (Brain and Turner, 1975; Sofowora, 1993; Harbone, 1984). Also, the dried crude ethanolic extracts were separately chromatographed on silica gel (254GF) column and gradient elution carried out using hexane: ethylacetate: butanol (1:1:1) mixture. Eluates which showed similar TLC profiles under UV (λ 366nm) were pooled and bulked separately to obtain hexane, ethyl-acetate and butanol fractions which were evaporated to dryness and in addition to the parent extracts then subjected to the following biological tests.

*Antimicrobial sensitivity test.* The media were prepared according to the Manufacturers' instructions, poured into sterile Petri-dishes (diameter, 13.5cm) and then allowed to set. The agar cup diffusion method was used for the antimicrobial sensitivity tests. The bacteria were cultured in nutrient agar while

the fungi were cultured in the sabouraud dextrose agar. The inoculum of each organism was introduced into each Petri-dish. Cylindrical plugs were removed from the agar plates by means of a cork borer to produce wells of approximately 6.0mm.The wells were equidistant from each other and the edge of the plate (Washington, 1995). Concentrations of 20mg/ml and 40mg/ml of the crude ethanolic extracts and the fractions at (5mg/ml) dissolved in methanol/water (1:1 v/v) were separately introduced into wells. Also, concentrations of 10µg/ml of ampicillin (a standard antibiotic), 1mg/ml of nystatin (a standard anti-fungal drug) and methanol/ water (1:1 v/v) were introduced into other wells as positive and negative controls respectively. The experiments were carried in triplicates. The plates were at room temperature (27± $2^0$C) for 2h to allow for diffusion. The plates were then incubated at 37 ± $2^0$C for 24h.

*Larvicidal test.* The larvae were bred by keeping outdoor basins of water under growing shrubs near houses for about two weeks. After this period, at least three groups of mosquitoes larvae were identified accurately in a container using classical methods( Sievers *et al,*1949). *Anopheles gambiae*, *Aedes aegypti* and *Culex piper-fatigans* responsible for the transmission of malaria, yellow fever and filariasis respectively were so identified. The fourth instar larvae of *Anopheles gambiae* were later selected, separated and the species authenticated at the Department of Entomology, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria before further work.

The method employed for the determination of larvicidal activity was adopted from that described by several authors( Ojewole *et al,*2000) and WHO directives on such assay with modifications (WHO,1970).Thirty (30) *Anopheles gambiae* larvae in their fourth stage were put in recovery cups (250ml plastic jars) containing 10ml water (pH 7.0) at room temperature (27±$2^0$C). Three (3ml) volume each of the graded concentrations of the extracts (5 and 10 % w/v) were added to 90ml water, mixed thoroughly and then poured into exposure cups (250ml plastic jars containing larvae food). Each aqueous solution of the extract was set up in triplicates. Negative control (containing 90ml water and larvae food) as well as positive control (containing 3ml absolute alcohol, 90ml water and larvae) were also set up in triplicates. Both the test controls set up were maintained in room temperature (27±$2^0$C). The *Anopheles* larvae in each recovery cup were scooped and transferred by means of small nets into the test exposure cups containing the sample solutions and or control larvae food and water. The larvae in the test and controls set up were incubated for a period of 12 and 24h at room temperature (27±$2^0$C). The larvae were gently scooped into small nets, washed with water, transferred into recovery cups containing 100ml of water, maintained at pH 7.0 and allowed to settle. Prior to mortality determinations, the larvae in recovery cups were gently disturbed and made to go below the water surface by agitating the water with a sterile pipette. The dead and dying larvae, which started to float on the water surface, were pushed down the recovery cups. The living larvae which were able to swim to the water surface were allowed to do so within 5minutes following agitation. The larvae remaining and or staying at the bottom of the recovery cups unable to swim to the water surface were regarded as dead.

*Brine shrimp lethality assay.* Some sea- water was placed in a small plastic tank with perforated dividing dam which was fabricated from plastic soap case. Some shrimp eggs were added to one side of the divided tank. This side was darkened by covering it with a plastic lid while the other compartment was exposed. The set-up was left for 48h for the

shrimp to hatch and mature as nauplii. Mature nauplii usually swim to the exposed compartment.

A stock solution of the sample was prepared by dissolving 20mg of the material in 2ml of methanol/water (1:1 v/v). To obtain the desired final concentrations such as 1000µg/ml, 100µg/ml and 10µg/ml; 0.5ml, 0.05ml and 0.005ml of the stock solution were transferred into the three vials respectively. The solvent was then evaporated by leaving the vials in a vacuum desiccator for 24h. Ten (10) shrimp nauplii were counted into each vial (i.e.30nauplii per dilution). The total volume of solution in each vial was adjusted to 5ml by adding the sea- water (5ml/vial). The control (methanol/water 1:1 v/v) was prepared in the same way except that the sample of the extract was omitted. The vials were maintained in the laboratory with normal fluorescent illumination and the set-up left for 24h.The number of survivors, usually swimming was counted with the aid of a magnifying lens for each of the vials at the end of 24h.Thus the number of the dead was computed; hence the $LD_{50}$ in p.p.m. (parts per million) was determined using the Finney probit analysis software (McLauglin,1988).

**Results and Discussion**

The phytochemical screening revealed the presence of saponins, cardiac glycosides and terpenes (trace) in the extracts of leaves, stem and roots. This confirms previous studies as contained in (Vander Schyf and Swanepoel, 1990). However, alkaloids, tannins, anthraquinones and flavonoids were absent in the extracts (Table 1). The extracts and the fractions (obtained from the chromatographic purification of the extracts) were screened for antibacterial and anti- fungal activities using *B. subtilis* and *S. aureus*, *E. coli*, *S. typhi* and *K. pneumoniae* and *A. niger* and *C. albicans* to represent a desirable spectrum of microbes. The extracts were tested at 20mg/ml and 40mg/ml and the fractions (5mg/ml). The results presented in Table 2 show that the activities elicited were concentration-dependent. Generally, the extracts were active against *B. subtilis* and *S. aureus* but were inactive against *E. coli, S. typhi* and *K. pneumoniae*. This is not surprising because gram- negative bacteria posses sophisticated cell-wall which does not allow the permeation of external agents (Brown, 1975).Also the anti-fungal activity was somewhat insignificant probably because the cell walls of fungi resemble those of higher plants and hence limits the permeation of substances into them. The antibacterial and anti-fungal activities of fractions were slightly higher than those of the crude extracts most probably because of level of purity associated with them. Looking at the results critically, one can infer that the antibacterial and anti-fungal activities were generally minimal. This is not surprising because the phytochemical screening carried out on the extracts revealed the absence of tannins and flavonoids which have been reported in previous studies (Adesina *et al.*, 2000; Burapadaja and Bunchoo, 1995; Lamikanra *et al.*, 1990) to be antimicrobial. It is very probable that the absence of these bioactive compounds might have been responsible for the low level of activities observed.

Preliminary larvicidal assay was carried out on the crude extracts of the leaves, stem and roots at 5%w/v and 10%w/v and at 12 and 24h incubation. The larvicidal activity (LA %) was calculated in terms of percentage mortality. The lethality furnished after 12 and 24h incubation was concentration and time - dependent (Tables 3 and 4). At 5%w/v(12 and 24h) and 10%w/v (12 and 24h), the leaves demonstrated some level of lethality(LA) at 20% and 35%; 45% and 50% respectively compared with those given by the stem (15%and 25%;25% and 35%) and roots (0%and 10%;15% and 20%) (Tables 3 and 4). Also the crude extracts of the leaves, stem and roots tested positive to saponins, which have

been shown in separate studies (Ojewole *et al.*, 2000; Bentley *et al.*, 1984; Oladimeji *et al.*, 2006; Nia *et al.*, 2006) to be lethal to the fourth instar larvae of *Anopheles gambiae*) thereby preventing the emergence of adult mosquitoes responsible for the transmission of malaria which still ravage large numbers of people around the world.

**Table 1**. Phytochemical screening of extracts of leaves, stem and roots of *P. angolensis*.

| Plant metabolite | Test | Leaf | Stem | Root |
|---|---|---|---|---|
| Alkaloids | Dragendorff's | - | - | - |
| | Mayer's | - | - | - |
| Saponins | Froth | + | + | + |
| | Emulsion | + | + | + |
| Tannins | Ferric chloride | - | - | - |
| Cardiac glycosides | Salkowski's | + | + | + |
| | Liebermann-Burchard's | + | + | + |
| Terpenes | Sulphuric acid | + | + | + |
| Anthraquinones | Borntrager's | - | - | - |
| Flavonoids | Shinoda's | - | - | - |

+ = Present, - =Absent (not detected).

**Table 2.** Antimicrobial sensitivity of extracts and fractions of *P. angolensis* at different concentrations in methanol/water (1:1). *Zone of inhibition ±0.5(mm)

| Test organism | Leaf | | Stem | | Root | | Lh | Le | Lb | Sh | Se | Sb | Rh | Re | Rb | A | N | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mg/ml→ | 20 | 40 | 20 | 40 | 20 | 40 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | | | |
| *B. subtilis* | 11 | 12 | 9 | 10 | 10 | 10.5 | 11 | 14 | 11 | 9 | 10 | 9.5 | 10 | 11 | 10 | 20 | Nt | - |
| *S. aureus* | 12 | 13.5 | 6 | 6.5 | 10 | 10.5 | 12 | 14 | 12 | 7 | 8 | 7 | 10 | 11 | 10 | 19 | Nt | - |
| *E. coli* | 2 | 2.5 | 3 | 3.5 | 3 | 3.5 | 2 | 3 | 2.5 | 3 | 3.5 | 3 | 3 | 3.5 | 3 | 17 | Nt | - |
| *S. typhi* | 5 | 5.5 | 3 | 3.5 | 3 | 3.5 | 5 | 6 | 5 | 3 | 3.5 | 3 | 4 | 4 | 3 | 18 | Nt | - |
| *K. pnuemoniae* | 5 | 5.5 | 3 | 3.5 | 3 | 3.5 | 5 | 5.5 | 5 | 3 | 3.5 | 3 | 3 | 3.5 | 3 | 18 | Nt | - |
| *A.. niger* | 6 | 6.5 | 6 | 6.5 | 6 | 6.5 | 6 | 7 | 6 | 6 | 6.5 | 6 | 6 | 7 | 6 | Nt | 20 | - |
| *C. albicans* | 6 | 7 | 6 | 6.5 | 6 | 7 | 6 | 7 | 6 | 6 | 7 | 6 | 6 | 7 | 6 | Nt | 22 | - |

Lh, Le, Lb (hexane, ethylacetate and butanol fractions of leaves);
Sh, Se, Sb (hexane, ethylacetate and butanol fractions of stem)
Rh, Re, Rb (hexane, ethylacetate and butanol fractions of roots)
A = Ampicillin (standard antibiotic or antibacterial drug); N = Nystatin (standard anti-fungal drug);
C = Methanol/water(1:1 v/v); Nt =Not tested; *Zone of inhibition less the diameter of cup (6mm).

**Table 3**.Larvicidal activity (LA %) of extracts of *P. angolensis* at 5%w/v after 12 and 24h incubation.

| Sample | LA % (12h) | LA % (24h) |
|---|---|---|
| Leaf | 20 | 35 |
| Stem | 15 | 25 |
| Root | 0 | 10 |
| PC | 100 | 100 |
| NC | 0 | 0 |

**Table 4**. Larvicidal activity (LA %) of extracts of *P .angolensis* at 10%w/v after 12 and 24h incubation.

| Sample | LA % (12h) | LA % (24h) |
|---|---|---|
| Leaf | 45 | 50 |
| Stem | 25 | 35 |
| Root | 15 | 20 |
| PC | 100 | 100 |
| NC | 0 | 0 |

PC = Positive control; NC = Negative control.

**Table 5.** Brine- shrimp lethality assay of extracts of leaves, stem and roots of *P. angolensis* concentration

| Sample | 1000µg/ml | 100µg/ml | 10µg/ml | $LD_{50}$ (ppm) |
|---|---|---|---|---|
| Leaf | 10 | 9 | 7 | 2.5 |
| Stem | 7 | 3 | 2 | 362 |
| Root | 9 | 7 | 2 | 310 |
| *Persia major*(bark) | | | | 2.6 |
| *Myrsine africana* (root) | | | | 114 |

The brine-shrimp assay determines the lethalities of materials toward brine-shrimp larvae (nauplii) and in doing so predicts the ability to kill cancer cell cultures, kill various pests and exert a wide range of pharmacologic effects. The shrimp nauplii have been used for a number of bioassay systems in which natural product extracts, fractions or pure isolates are tested at concentrations of 1000µg/ml, 100µg/ml and 10µg/ml in vials containing 5ml of brine and ten nauplii in each of the three replicates (Meyer *et al*, 1982).The $LD_{50}$ values in p.p.m. are estimated with 95% confidence using the appropriate mathematical estimates; the Finney probit analysis program being the model routinely employed. The $LD_{50}$ values of the extracts of leaves, stem and roots obtained in this study compared with those of other plants in literature (Kupahan *et al.*, 1969; Ma *et al.*, 1989; Oladimeji *et al.*, 2005) are presented in Table 5.The results show that the leaves displayed a 'significant' $LD_{50}$ value at 2.5ppm while the stem and roots gave 'marginal' $LD_{50}$ values at 362ppm and 310ppm respectively when compared with those of *Persia major* (2.6ppm) and *Myrsine Africana* (114ppm) whose values below the 200ppm are considered 'significant' (Kupahan *et al.*, 1969; Ma *et al.*, 1989; Oladimeji *et al.*, 2005).

The results of the antimicrobial assay indicate the potential of the plant as a panacea for bacterial infections especially of those of gram positive origin while the findings of the larvicidal and brine-shrimp lethality assays reveal novel potential of the plant in the fights against malaria and tumors in man.

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