Preliminary Phytochemical and Cytotoxic Activity of The Aqueous and Chloroform Fractions of The Leaf of *Persea Americana* Mill (Lauraceae) .

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ABSTRACT

The preliminary phytochemical and cytotoxic effects of the chloroform and aqueous fractions of the methanol extract of the leaves of *Persea americana* were carried out. Cytotoxicity was carried out using tadpoles of *Ranniceps raninus* at 20-400 µg/ml. The methanol extract was subjected to solvent partitioning and chromatographic procedures guided by cytotoxic tests. Phytochemical screening results revealed the presence of saponins, flavonoids, tannins and cardiac glycosides .The chloroform fraction of the leaf extract produced 100 % tadpole mortality at 200 µg/ml. Chromatographic analysis of the active chloroform fraction yielded a compound (CH-B2) which gave a tadpole mortality of 96.70 ±3.33 % at 10 µg/ml. Cytotoxicity results showed that the chloroform fraction had an LC_{50} of 10.77 µg/ml while the its VLC sub-fractions 4 and 5 and isolated compound had LC_{50} of 4.67, 18.0 and 3.33 µg/ml respectively. The results support the folkloric use of the plant in treating tumor related ailments. However, further work need to be carried out to establish the identity of the isolated compound.

Keywords: Cytotoxicity, *Ranniceps raninus*, Phytochemicals, *Persea americana*

INTRODUCTION.

Throughout history natural products from the plants have played a major role in the life of human beings regarding for food source and for medicinal products (Leland *et al*., 2006).Natural products played a prominent role in ancient traditional medicine systems, like the Chinese, the Ayurvedic and the Egyptian traditional medicines, and are still in common use today for the treatment of various diseases. According to the World Health Organization (WHO), over 75 % of people still rely on plant-based traditional medicines for primary health care in underdeveloped or developing countries (Sarker *et al*, 2005).

The growing interest in plant natural products for anticancer activity is due to their secondary metabolites like terpenes, phenolics and alkaloids (Dai and Mumper, 2010). Polyphenols and flavonoids are known to prevent oxidative stress due to their antioxidant, free radical scavenging and apoptosis inducing properties (Goldman *et al*., 1996).

Persea americana Mill (Lauraceae) locally known in Nigeria languages as *Pia*-Yoruba, *Orumwu*-Bini, ubeoyibo, Efik-*ebanmbakara* (Gill, 1992) and *ewoebo in* Owan speaking tribes of Edo State, is native to tropical regions of the Caribbean, Mexico, and South America. It is a medium to large tree, 9-20 m in height. It is classified as an evergreen, although some varieties lose their leaves for a short time before flowering (Harrison, *et al*., 1969). The seeds of *P. americana* have been reported to have diverse application in ethnomedicine, ranging from treatment for diarrhoea, dysentery, toothache, intestinal parasites to the area of skin treatment and beautification (Pamplora and Roger, 1999). Other reported ethnomedical uses of the plant are as follows: It is used as an emmenagogue (hot water extract, decoction), for asthma (bark), for cough, fever, kidney and liver troubles, for diabetes, as food, for skin blemishes, as an arbotifacient, for female disorders among others (Morton,1981).

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Reported biological activity of the plant include analgesic, anti-inflammatory and anticonvulsant of the aqueous extract of the leaf (Adeyemi*et al*., 2002), hypoglycaemic and hypocholesterolaemic activities (Brai*et al*., 2007; Anita *et al*.,2005), wound healing activity (Nayak*et al*.,2008), anti-ulcer (Ukwe and Nwafor, 2004), vasorelaxant and blood pressure reducing activities in animal studies (Owolabi*et al*., 2005; Ojewole*et al*., 2007;Adeboye *et al*.,1999) .

This work is a continuation of our previous work (Ayinde *et al*, 2010) where a comparative cytotoxic and growth inhibitory studies was carried out on the methanol extracts of the three morphological parts (leaves, stem and root barks) of *Persea americana*.

MATERIALS AND METHOD

Partitioning of the methanol extract of the leaf of *Persea americana*

Having shown a higher activity in our previous work, over the other parts of the plant (stem and root bark), 90 g of the crude methanol extract of the leaf was re-dissolved in methanol-water (1:1) and partitioned exhaustively with chloroform (200ml ×4 volumes) in a separating funnel. The chloroform layer (lower) was collected first, followed by the aqueous fraction. This was repeated until a clear lower layer was obtained. The aqueous and the chloroform fractions were reduced to dryness using a rotary evaporator and their respective yields noted.

Phytochemical screening

Phytochemical screening of the aqueous and chloroform fractions of the leaf of *Persea americana* was carried out using standard methods described by Khandelwal (2006), Evans (2002) and Sofowara (1993).

Determination of cytotoxic effects of the aqueous and chloroform fractions using tadpoles (*Raniceps ranninus*).

Newly hatched tadpoles were scooped from ponds at Olomo Beach in Uhonmora village in Owan West Local Government Area of Edo State and were properly identified in the Department of Animal and Environmental Biology, Faculty of Life Science, University of Benin. Ten tadpoles of similar sizes (five days old) were selected with the aid of a broken Pasteur pipette into different beakers containing 30 ml of the natural water from the habitat of tadpoles. This was made up to 49 ml with distilled water. The mixture was made up to 50 ml with 20, 40, 100, 200 and 400 µg/ml of the chloroform fraction in 5% DMSO (Obuotor and Onajobi 2000). The experiment was repeated for the aqueous fraction and it was carried out in triplicates for all concentrations and controls. Similar procedure was repeated for subsequent chromatographic fractions.

Vacuum liquid chromatographic analysis of the chloroform fraction

About 17 g of the chloroform fraction was loaded on a silica gel G (30-70µm) in a Sinta Glass (No.3) attached to a Buchner flask connected to a vacuum pump. The eluting solvents were 200 ml of hexane (100 %), hexane-chloroform (1:1, 1:3), chloroform (100 %), chloroform-ethyl acetate (3:1, 1:1) and ethyl acetate (100 %). The seven (7) fractions obtained were concentrated and subjected to cytotoxicity test using tadpoles as stated above. Analytical thin layer chromatographic analyses of the Vacuum Liquid Chromatographic fractions of the chloroform fraction was carried out on a precoated aluminium plate of Silica gel GF_{254} using hexane- ethyl acetate (3:2). After development, the plates were sprayed with concentrated H_2SO_4 and subsequently heated for 5 min at 110°C. The coloured spots were noted and their R_f values were recorded.

Fractions CH-1-3, CH-6-7 was inactive. Based on observation made on the results obtained, coupled with the TLC profile, fractions CH-4 and CH-5 were then bulked for subsequent chromatographic analysis using 200 ml of hexane (100 %), hexane-ethyl acetate (9:1,4:1,5:2,13:7,3:2,1:1) and ethyl acetate (100 %) in increasing order of polarity on silica gel (60-120 mesh). Forty-four (44) fractions were collected. Based on the resolutions of the fractions in hexane-ethyl acetate (3:2) after analyzing the samples using thin layer chromatography and spraying with concentrated H_2SO_4, the samples were bulked into four groups (CH-A, CH-B,CH-C and CH-D) on the basis of similarities in R_f values, intensity and colours developed after heating at 110°C for 5min. The bulked fractions were subjected to biological activity using tadpoles at four concentrations of 1.25, 2.5, 5.0 and 10.0 µg/ml.

Preparative TLC of sample CH-B (1125 mg)

As a result of the high yield and conspicuous nature of its spot on TLC plate, sample 'CH-B' was subjected to preparative TLC analyses on 0.5 mm thick Silica gel G_{F254} using hexane- ethyl acetate (3:2) and developed three times.

Using UV at 254 and 356nm, four bands were scrapped, eluted, concentrated and coded as B-1, B-

1^1, B-2 and B-2^1. TLC analysis of the preparative TLC products was carried out on a commercial aluminium precoated plate of silica gel GF_{254}using chloroform-methanol (3:2) and hexane-ethyl acetate (3:2) as solvent systems.

RESULTS

The cytotoxic effects were observed to be concentration and time dependent with the chloroform fraction exhibiting higher activity in comparison to the aqueous fraction. At 40 µg/ml, the chloroform fraction produced an average % mortality of 70 ± 5.77 which increased to 100 % at 200 µg/ml. The organisms started remaining submerged within 23 mins when treated with 40 µg/ml while those treated with 200 µg/ml became motionless and remained completely submerged within 8 min of treatment. However, the highest mortality produced by the aqueous fraction was 40 ±10 % over the entire period of 24 h incubation (Figure. 1).

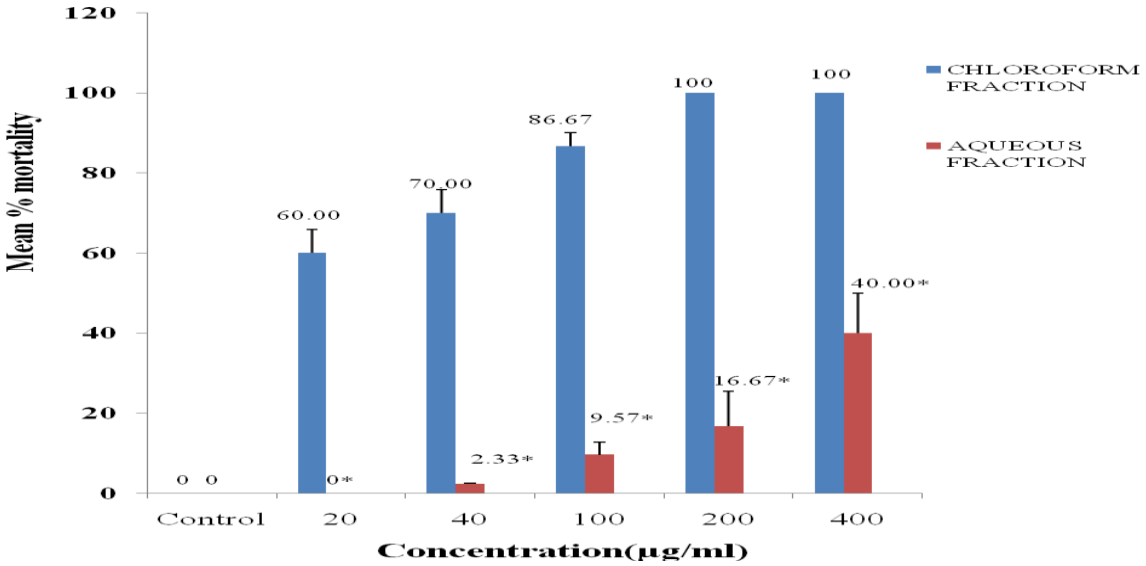

Fig. 1: Cytotoxic effects of the chloroform and aqueous fractions of the leaf extract on tadpoles. The chloroform fraction imparted 100% mortality on the tadpoles at 200 µg/ml. Values are Mean ± S.E.M, n = 10. ∗Significantly different from chloroform fraction. P< 0.05

The result of the cytotoxicity assay of the VLC sub-fractions on the tadpoles at concentrations of 2.5, 5.0, 5.0, 20, 40, 100, 200 and 400 µg/ml showed that sub-fractions CH-F4 and CH-F5 were the most active. Sub-fraction (CH-F4) gave 100% mortality at concentrations of 40, 100, 200 and 400 µg/ml at an average time of 15, 10, 7 and 5 minutes respectively. Also, at 200 and 400 µg/ml, fraction 5 (CH-F5) showed 100% mortality at an average time of 12 and 9 min respectively (Figure 2).

The results of the cytotoxic effects of the bulked VLC sub-fractions CH-A, CH-B, CH-C and CH-D showed fraction CH-B to be most active as it produced remarkable tadpole mortality in less than 2 h. At 1.25, 2.50, 5 and 10 µg/ml, the fraction was observed to give mortality of 43.33 ± 6.67, 63.33 ± 5.77, 86.67 ± 6.67 and 100 ± 0.0 % (Figure 3) at an average time of 76, 57, 38 and 20 min respectively. At the same concentrations, fractions CH-A, CH-C and CH-D showed no mortality over a period of 24 h (Figure 3).

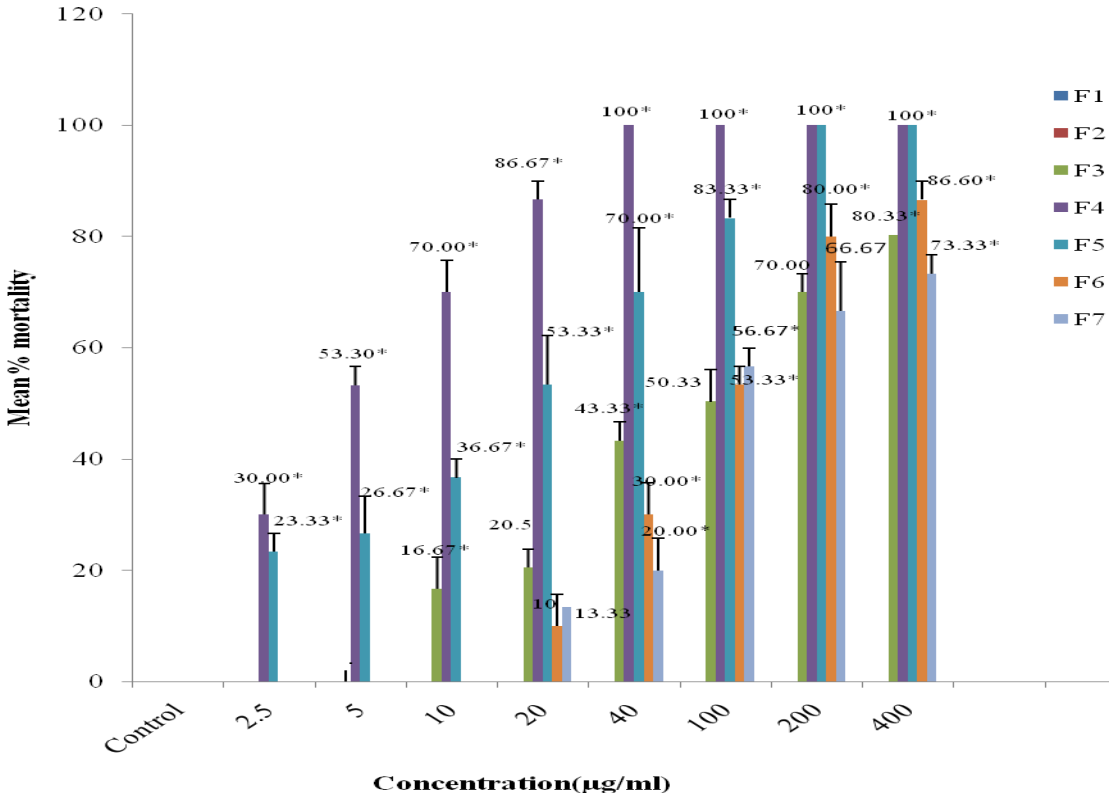

Fig 2: The Cytotoxic effects of the VLC products of the chloroform fraction of *Persea americana* on tadpoles. CLF-4 had the highest effect as it imparted 100% mortality on the tadpoles at 40 µg/ml. Values are Mean ± S.E.M, n = 10. *Values are significant at P<0.05

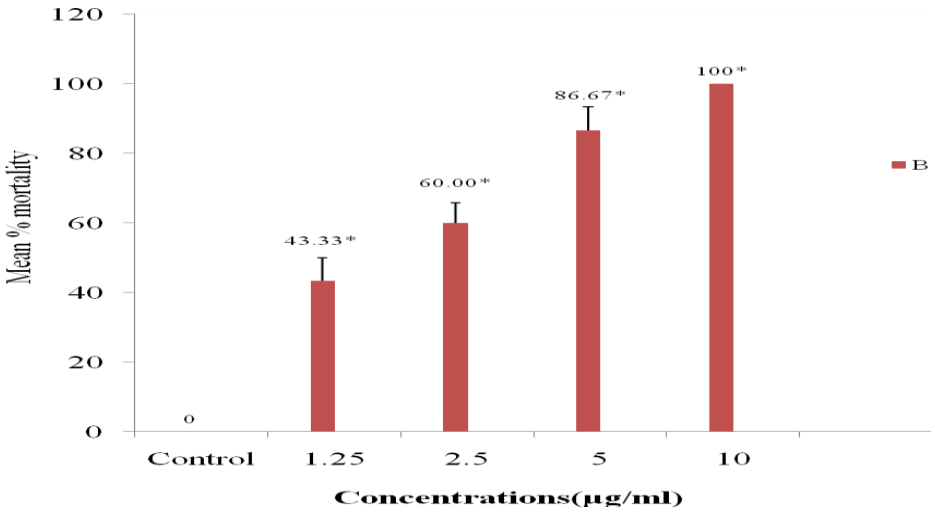

Fig.3: Cytotoxic effects of the bulked chromatographic fractions. Only sub-fraction CH-B was observed to be lethal to the tadpoles. Values are Mean ± S.E.M, n = 10. *Significantly different from control at P<0.05

Cytotoxic activity of the preparative TLC isolates was tested at concentrations of 1.25, 2.5,5 and 10 µg/ml for 24 h. Isolates B-2 and B-2[1] were observed to produce 66.70 ± 3.33 and 53.3 ± 3.33 % mortality respectively at a concentration of 5 µg/ml which increased to 96.70 ±3.33 and 67.60 ± 8.82 % respectively at 10 µg/ml (Figure 4). However, at this maximum concentration used, isolates B-1 and B1[1] showed no mortality.

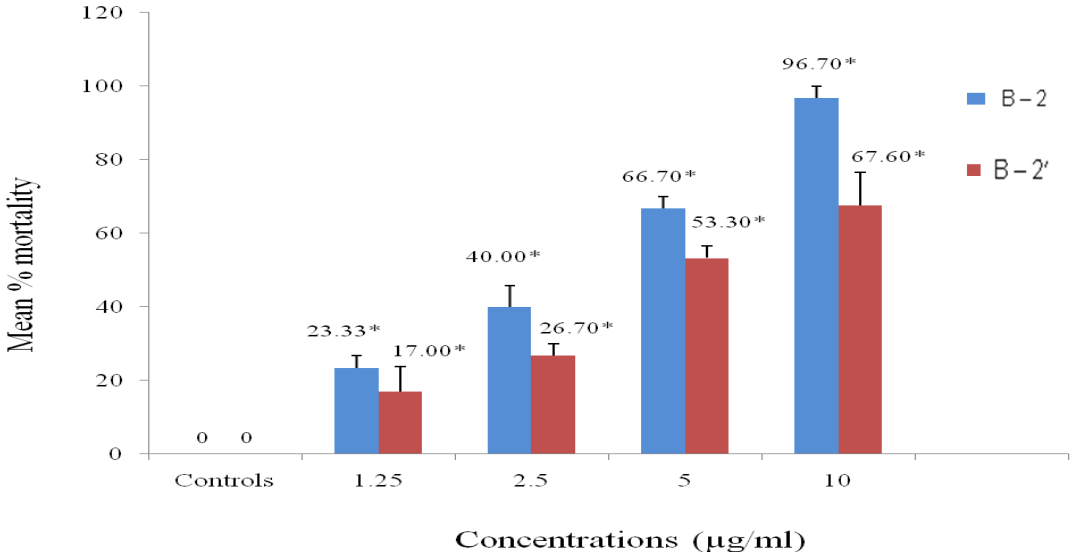

Fig. 4: Cytotoxic effects of the preparative TLC isolates of sample CH-B on tadpoles. Values are Mean ± S.E.M, n = 10. *Significantly different from control at P<0.05

Table 1: LC_{50} of the chloroform fraction and the chromatographic fractions.

	LC_{50} (µg/ml)
Chloroform fraction (100% mortality at 200µg/ml)	10.77
VLC fractions 4 (CH-4) of the chloroform fraction (100%) mortality at 40µg/ml)	4.67
VLC fraction CH-5 of the Chloroform fraction(100% mortality at 200µg/ml)	18.00
Isolated compound (B-2 ; R_f (0.52 and 0.38 in solvent systems $CHCl_3$-CH_3OH (3:2) and C_6H_{14}-$CH_3COOC_2H_5$ (3:2)(almost 100% mortality at 10µg/ml)	3.33

DISCUSION

The choice of methanol extract of the leaf of *Persea americana* for partitioning was based on the higher activities it had over the other parts of the plant in our previous work (Ayinde *et al*., 2010).

The Vacuum Liquid Chromatography (VLC) was employed to further partition the chloroform fraction to rapidly yield bioactive sub-fractions. This approach was observed to further enhance the activities of some of the fractions obtained from the parent fraction. For instance, the chloroform sub-fraction 4 (CH-4) obtained from the VLC analyses of the chloroform fraction was observed to produce 100 % mortality at 40 µg/ml (in 15 min), which implies that it is five times more active than the chloroform faction. This was further established by the LC_{50} of 10.77 µg/ml calculated for the chloroform fraction while 4.67 and

18 µg/ml for the VLC chloroform sub-fraction 4 (CH-4) and CH-5 (Table 1).

The thin layer chromatographic analysis of the seven fractions obtained in hexane-ethyl acetate (3:2) gave a clear resolution of the components in the solvent system. CH-4 and CH-5 were observed to have common constituents which were more conspicuous than the others. Similarly, they were observed to show almost similar biological activity on tadpoles and also similar R_f value, hence they were bulked together. Because of the clear resolution seen on the chromatogram, the bulked active VLC fraction (CH-4 and CH-5) was then subjected to preparative TLC. TLC analyses of the preparative tlc isolates was carried out using chloroform-methanol (3:2) and hexane-ethyl acetate (3:2) to confirm their purity. The TLC profile of the isolates revealed one spot each. Biological activity of the pure isolates (B2 and $B2^1$) showed a concentration dependent activity. From this study, it can be inferred that *Persea americana* leaf may likely have effects on tumour-producing cells as claimed in ethnomedical uses of the plant among the Owan people of Edo State. However, further research work need to be carried out to establish the identity of the isolated compound and determine its biological activity using human cell lines *in vitro* to confirm the folkloric anti-tumour activity of the plant.

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