Anti-angiogenic, Anti-inflammatory and Anti-oxidant Potential of an African Recipe: *Alchornea cordifolia* Seeds

R. Nia, D.H. Paper and G. Franz
Department of Pharmacy
University of Regensburg
93051 Regensburg
Germany

E.E. Essien
School of Pharmacy
University of Uyo
Uyo, Akwa-Ibom State
Nigeria

Keywords: CAM, HET-CAM, DPPH assays, inflammation, neo-vascularisation

Abstract
The chorio-allantoic membrane (CAM) and hen’s egg test (HET-CAM) assays are based on neo-vascularisation (angiogenesis) in fertilised hen’s egg embryo. Therefore inhibition of angiogenesis is a prime target for solutions to afflictions such as growth of solid tumours, arthritis, chronic inflammations. Natural products still represent an untapped source of interesting leads for drug development against these diseases. Serial dilutions of the methanolic extract of *Alchornea cordifolia* seeds were assayed on CAM, HET-CAM and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assays. The results revealed an inhibition of angiogenesis at 0.9 score and 86% inhibition of inflammation at 250 µg/pellet on the CAM and HET-CAM respectively. It was also very effective in the DPPH assay for non-specific hydrogen atom or electron donating activity (IC₅₀: 0.68 µg/ml). Subsequent fractionations, revealed the ethyl acetate fraction (Ea) as the most active against others in a dose response trend. Interestingly, no side effects such as embryotoxic effects and others were recorded. The findings support the folkloric uses of the plant against wounds, piles and cancer and therefore have implications on the quality of herbal drugs dispensed by the traditional medical practitioners (TMP) in Africa.

INTRODUCTION
In animal experimental models, the observation that tumour promoters recruit inflammatory cells to the site of application and in return release reactive oxidative species (ROS) is a clear indication of a close relationship between inflammation and cancer. These ROS, when released beyond the antioxidant capacity of a biological system give rise to oxidative stress, which is fundamental in the pathogenesis of a variety of human afflictions such as atherosclerosis, hypertension, inflammation, cancer and AIDS (Gutteridge and Halliwell, 1994). The neolignans magnosalin, magnoshinin and isoliquiritin isolated from *Magnolia salicifolia* and Licorice root respectively have been shown as potential inhibitors of these processes (Paper, 1998). However, more potent inhibitors are still needed for solutions to chronic afflictions. The different organs of *Alchornea cordifolia* (Euphorbiaceae) are traditionally used to cure wounds, yaws, ulcers, bronchitis, inflammations and skin infections (Iwu, 1993). The anti-microbial, the smooth muscle relaxant activities as well as the chemistry of extracts from leaves have been studied by several authors (Lamikanra et al., 1990; Ogungbamila and Samuelsson, 1990; Iruka et al., 1999). Routine anti-oxidant screening revealed the seed to contain the most active principles. However this organ is yet to be investigated both for anti-inflammatory and anti-angiogenesis activity which is a logical justification of this study.

MATERIALS AND METHODS

Collection, Extraction of Plant Materials
The different organs of *Alchornea cordifolia* (Schum and Thonn) Muell. Arg. were collected (June, 2001) in Uyo local government of Akwa-Ibom State, Nigeria and were identified by the taxonomist of the Department of Pharmacognosy of the University
of Uyo, where a voucher specimen is deposited. 300 g of each fresh plant organs: [leaves (Lvs), stem bark (Sb), root bark (Rb) and seeds] were extracted cold in methanol (100%) by percolation for 48 h. The brown organic phase was filtered through Whatman paper No 1, concentrated in vacuo and freeze dried. These extracts were analysed for the presence or otherwise of bioactive ingredients using standard methods (Harborne, 1984) and assayed. The methanol extract of the seed was selected for successive fractionation in n-hexane (He), chloroform (Ch), ethyl acetate (Ea), n-butanol (Bu) and aqueous (Aq) to yield different fractions for further assays.

**Antioxidant Activity: Rapid-TLC Screening for Anti-oxidant Activity**

The freeze-dried powder from different organs of the plant were dissolved in methanol 100% and spotted on silica gel sheets, developed in methanol:ethylacetate (2:1; v/v). The plates were air-dried and sprayed with 0.2% solution of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) radical (Kirby and Schmidt, 1997) and visualised for the presence of whitish spots, indicating anti-oxidant activity.

**DPPH Assay**

The DPPH assay was carried out as described by Kirby and Schmidt, 1997. 50 µg of various dilutions from the extract of different organs were mixed with 5 ml of a 0.004% methanol solution of DPPH, after an incubation period of 30 min, the absorbancy of the sample was read at 512 nm using a spectrophotometer. Ascorbic acid (Vitamin C) was used as a positive control. The freeze dried seed extract was later selected and the anti-oxidant activity of its fractions was evaluated as earlier described.

**HET-CAM/CAM Assays**

The modified method of Marchesan et al. (1998) was used. Fertilised hens’ eggs were incubated for 75 h at 37°C and a relative humidity of 80%. The eggs were placed in horizontal position and rotated several times. They were opened on the snub side and prior to this, 10 ml albumen were sucked off through a hole pierced down by the side and sealed. Then a round piece of shell (3-4 cm diameter) was removed from the top of the blunt end and the eggs were sealed with laboratory film and incubated for further 75 h. The pellets consisting of 10 µl gelled 2.5% agarose solution were used as vehicle and sodium dodecyl sulphate (SDS) as inflammatory inducer. They were dissolved or suspended in 60% “warm” liquid agarose solution before gelling pellets with or without test drug in the presence or absence of SDS were used. 10 eggs were used per drug to be tested. The results were evaluated under the stereomicroscope. An anti-inflammatory activity exists if the irritation of the membrane induced by SDS decreases (i.e. the star-like picture around the granuloma) and the blood vessel net appears normal. The number of eggs with a positive effect is given in per cent and indicates the measure of the anti-inflammatory activity of the drug tested (Marchesan et al., 1998). The anti-angiogenic activity was evaluated by using a score system (0-2). Suramin (50 µg/pellet) was tested as positive control. As blank, CAMs was treated only with agarose solution (score 0). Score < 0.5, no anti-angiogenic effect; score ≥ 0.5, weak to strong anti-angiogenic effect (Marchesan et al., 1998).

**Statistical Analysis**

The data are expressed (Table 2 and 3) as mean ± SD and the statistical significance between groups was analysed by means of an analysis of variance (ANOVA), followed by student – New man – Keul’s test. P values less than 0.01 was considered as indicative of significance.

**RESULTS AND DISCUSSION**

**Processing of Plant Materials and Phytochemical Screening**

The plant materials used in this research were processed accordingly, all the
reagents and solvents were of analytical grade. Phytochemical screening carried out as described by Harborne (1984), revealed the presence of flavonoids and tannins in all organs of the plant, in addition the seed extract revealed alkaloids and saponins. These compounds may play vital role in the expression of activity in the plant. Further fractionation of the methanol extract of the seeds yielded in w/w, n-hexane (He) 14.25%, chloroform (Ch) 30.50%, ethylacetate (Ea) 27.02%, n-butanol (Bu) 12.50% and aqueous (Aq) 15.48%. Chloroform and n-butanol fractions had the highest and the lowest yields respectively. The main active fraction (ethyl acetate) contain flavonoids, tannins and alkaloids.

**Anti-oxidant Activity: DPPH Assay**

The anti-oxidant activity was used as a preliminary screening on all organs of the plant. The aim was to select the most active part for further assays. A combined chromatographic and spectrophotometric analysis was used and the seed extract displayed a good prospect on both systems (IC$_{50}$ 0.68 µg/ml) but the extract from the leaves was less effective (IC$_{50}$ 3.21 µg/ml) as compared to vitamin C (IC$_{50}$ 0.7 µg/ml) (Table 1). Further evaluation of its partitioned fractions revealed the EtOAc (Ea) as the most effective anti-oxidant fraction with IC$_{50}$ 0.38 µg/ml (Fig. 1) with n-hexane (He) recording an IC$_{50}$ 3.83 µg/ml. This preliminary findings encouraged anti-inflammatory and anti-angiogenesis assays on the seed extract and its fractions since the interrelation between oxidation, inflammation and cancer has been demonstrated (Lundolm and Gelin, 1994).

**Anti-inflammatory Activity: HET-CAM Assay**

The serial diluted methanol and EtOAc fractions displayed a dose effect between 500 and 72.5 µg/pellet in restoring to normal the membrane irritation induced by sodium dodecyl sulfate (SDS). Therefore, the typical picture of membrane irritation after drug application was reversed to normal after 24 h. Within the dilution zone the effect of inhibition of membrane irritation was graded and found in the average, to be in the “good effect zone” according to Marchesan et al. (1998). However total inhibition (100%) was found at 500 µg/pellet in the methanol extract and the lowest inhibition (72%) was found at 72.5 µg/pellet in the EtOAc fraction and were considered strong and good effects respectively (Fig. 2). It is noteworthy to mention that purification of the methanol extract enhanced the activity. The activities of the EtOAc fractions and the methanol extract were comparable to that of hydrocortisone (50 µg/pellet). At the concentration of 72.5 µg/pellet instead of 50 µg/pellet the EtOAc fraction had a “good effect” (72%), an indication for further dilution. These results indicate the presence of bioactive components having therapeutic benefits in alleviating inflammatory conditions.

**Anti-angiogenic Activity: CAM Assay**

The methanol extract as well as the serial dilutions of EtOAc fraction were found to inhibit the formation of new blood vessels (neo-vascularization) in a dose dependent manner between 500 and 72.5 µg/pellet (Fig. 3). The effects were graded according to Marchesan et al. (1998), in the score range of 0.7-1.2. The highest inhibition was found in the EtOAc fraction at 250 µg/pellet and was scored 1.2 and the lowest at 72.5 µg/pellet with a score of 0.7. These results were comparable to suramin, included as a positive control, and at 50 µg/pellet it was scored 0.7. These results indicate that the methanol extract of the seeds, and precisely its EtOAc fraction represents a potential target for the search of novel angiogenesis inhibitors. Flavonoids found present in higher quantity in the plant may be a prime target since they have been reported to play prominent role in angiogenesis inhibition (Paper, 1998).

The findings obtained from this work have justified the implications of this plant in traditional medicine in curing inflammations and tumour suspected ailments. These claims are herein substantiated by the degree of anti-oxidant activity, the percentage of inflammation inhibition and the observed anti-angiogenesis activity score. Besides, these facts indicates the presence of beneficial anti-tumour and or anti-inflammatory
ingredients which may play a vital role in the immune response as well as in cell proliferation (Ames and Gold, 1990). In addition cancer patients, when treated with anti-inflammatory drugs, not only experience less pain but also a concomitant prolongation of survival time (Lundolm and Gelin, 1994). These facts could be exploited in drug development. Moreover this research has also validated the assays used: HET-CAM and CAM which are benchtop models, highly needed in drug discovery strategies as it has tackled the problems caused by chronic conditions in inflammation and cancer. Nevertheless this does not preclude more in-depth study for its improvement.

**ACKNOWLEDGEMENTS**

One of us (R. Nia), thanks the ICSC W.L. for the financial support and the Department of Pharmacy, University of Regensburg (Germany) for the visiting scholarship and the University of Uyo (Nigeria) for a leave of absence.

**Literature Cited**


### Tables

Table 1. Anti-oxidant response of different organs and fractions from the seeds.

<table>
<thead>
<tr>
<th></th>
<th>Lvs</th>
<th>Sb</th>
<th>Rb</th>
<th>Seed</th>
<th>Me</th>
<th>He</th>
<th>Ch</th>
<th>Ea</th>
<th>Bu</th>
<th>Aq</th>
<th>VitC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50} (µg/mL)</td>
<td>3.21</td>
<td>2.45</td>
<td>1.85</td>
<td>0.68</td>
<td>0.7</td>
<td>3.83</td>
<td>1.71</td>
<td>0.36</td>
<td>262</td>
<td>2.25</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 2. Anti-inflammatory (HET-CAM) response of the tested drugs.

<table>
<thead>
<tr>
<th>Extract/Fraction</th>
<th>Dose (µg/pellet)</th>
<th>Score (%) ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH (me)</td>
<td>500</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>MeOH (Ne)</td>
<td>250</td>
<td>86 ± 2.00</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>250</td>
<td>88 ± 1.53</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>125</td>
<td>75 ± 2.52</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>72.5</td>
<td>72 ± 1.53</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate (SDS)</td>
<td>72.5</td>
<td>09 ± 2.08</td>
</tr>
<tr>
<td>Hydrocortisone (HC)</td>
<td>50</td>
<td>84 ± 1.00</td>
</tr>
</tbody>
</table>

* Values represent mean ± SD from triplicate experiments
P < 0.001

Table 3. Anti-angiogenic (CAM) response of the tested drugs.

<table>
<thead>
<tr>
<th>Extract/Fraction</th>
<th>Dose (µg/pellet)</th>
<th>Inhibition (%) ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH (me)</td>
<td>500</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>MeOH (Ne)</td>
<td>250</td>
<td>09 ± 0.10</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>250</td>
<td>1.2 ± 0.20</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>125</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>Ethyl acetate (Ea)</td>
<td>72.5</td>
<td>0.7 ± 1.10</td>
</tr>
<tr>
<td>Suramin</td>
<td>50</td>
<td>0.7 ± 1.10</td>
</tr>
</tbody>
</table>

* Values represent mean ± SD from triplicate experiments
P < 0.001
Figures

Fig. 1. Anti-oxidant response of organs and fractions from the seed.

Fig. 2. Anti-inflammatory (HET-CAM) response of methanol extract and EtOAc fractions.

Fig. 3. Anti-angiogenic (CAM) response from methanol extract and EtOAc fractions.