

Full Length Research Article

EVALUATION OF THE ANTI-OXIDANT AND ANTI-ANGIOGENIC EFFECTS OF *SPHENOCENTRUM JOLLYANUM* PIERRE

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The methanol extracts of *Sphenocentrum Jollyanum* organs were assessed for their anti-oxidant and anti-angiogenic activities using DPPH and CAM assays respectively. The results indicated the stem bark as the most active organ with an IC_{50} of 1.80 ± 0.25 and 1.00 ± 0.20 score (at 500 μ g/pellet) on DPPH and anti-angiogenesis assays respectively. Further fractionation of the stem bark revealed the chloroform fraction to have the highest IC_{50} 1.54 ± 0.15 and the most important score on anti-angiogenesis assay with 1.3 ± 0.10 at 250 μ g/pellet when serially diluted between 250 and 36.2 μ g/pellet. Moreover, the effects were found to be dose-dependent. These results bring to the fore the need for further studies towards confirming the anti-oxidant and anti-angiogenic potentials of the plant as well as identifying and characterizing the active principles for drug development.

Keywords: Anti-oxidant, anti-angiogenic, CAM, *Sphenocentrum jollyanum*, Menispermaceae.

INTRODUCTION

Chemical compounds with unpaired radicals such as powerful oxidants and free radicals (FR) are capable when present in the body to damage lipids, proteins and also DNA and consequently may bring about mutations (Ellnain-wojtaszek et al., 2003). Radical reactions have been implicated in the pathogenesis of chronic diseases that are life limiting such as cancer, hypertension, cardiac infarction, arteriosclerosis, diabetes etc. Overproduction of free radicals associated with A, C and E avitaminosis and the reduced level of glutathione peroxidase, catalase and superoxide dismutase seem to be the main factor leading to oxidative stress. Consequently, anti-oxidants are very important in body protection against these afflictions. The presence of anti-oxidants have been confirmed in soybean (Facino et al., 1999), garlic, red wine and green tea (Stajner et al., 1999) and in *Tridax* (Nia et al., 2003).

Angiogenesis (or neo-vascularization) may be defined as a multistep process leading to the formation of new capillaries emerging from pre-existing blood vessel systems. Any imbalance in the control of this complex system may promote numerous angiogenesis

dependant diseases (Käsbaeur et al., 2001). Inhibition of angiogenesis is a prime target to afflictions such as growth of solid tumours, arthritis, and inflammations. The search and discovery of novel anti-oxidants and anti-angiogenesis are likely to bring hope to the millions of sufferers of the above mentioned chronic diseases. Natural products still represent an important source of interesting leads for drug development. Isoliquiritin and magnoshinins isolated from *Licorice* root and *Magnolia salicifolia* respectively have been shown as potential inhibitors of these "deadly" processes (Paper, 1998).

Sphenocentrum jollyanum is an erect shrub, growing up to 1.5 m in height with very few branches. The leaves up to 20 cm long and about 5-12 cm broad are elliptical, margin entire with short and blunt apex, wedge-shaped base and smooth on both sides. The plant is distributed from Sierra Leone to Cameroon via Nigeria and is reputed against chronic wounds, cough, and other inflammatory conditions as well as tumours (Dalziel, 1985; Iwu, 1993). Routine screenings reveal the anti-oxidant activity of the stem bark of *Sphenocentrum jollyanum* and hence, the reason, we decided to study the anti-oxidant and anti-angiogenic potential.

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MATERIALS AND METHODS

Materials: collection, extraction of plant materials

The different organs of *Sphenocentrum jollyanum* Pierre. Menispermaceae. were collected (September, 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. 500 g of each plant organs: [leaves (Lvs), stem bark (Sb), root bark (Rb)] 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 (Harbone, 1984) and assayed. The chloroform fraction was selected for successive fractionation using n-hexane (He), chloroform (Ch), ethylacetate (Ea), n-butanol (Bu) to yield different fractions for further assays.

METHODS

Anti-oxidant activity: rapid-TLC screening for anti-oxidant activity:

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

CAM assay (Anti-angiogenic): 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. They were dissolved or suspended in 60 % "warm" liquid agarose solution before gelling pellets with or without test drug. 10 eggs were used per drug to be tested. The results were evaluated under the stereomicroscope. An anti-angiogenic activity exists if an inhibition to the formation of new capillaries is observed (i.e. a clear zone is observed within a capillary network) (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 were 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 as mean ± SD and the statistical significance between groups was analysed by means of an analysis of variance (ANOVA), followed by student – Newman – Keul's test. P values less than 0.05 was considered as indicative of significance.

RESULTS

Phytochemical screenings: The methanol extract of the stem bark was found to contain saponins, tannins, alkaloids and terpenes. The most active fraction contained flavonoids and alkaloids. (Table 1).

Anti-oxidant assay : Amongst the organs, the stem bark of the plant was found to have the best inhibitory concentration with IC₅₀ of 1.80 µg/mL. followed by the root bark and the leaves at 3.50 µg/mL. and 4.35 µg/mL. The purified chloroform fraction had an IC₅₀ of 1.54 µg/mL. (Table 2)

Anti-angiogenic assay: The methanol at 500 µg/pellet had a score of 1.00 while at 125 µg/pellet it was found to be 0.70. the most active fraction: the chloroform fraction scored 1.30 at 250 µg/pellet and 0.40 at 36.2 µg/pellet. (Table 3)

Table 1.
Phytochemical investigations and yields of various fractions

	Fractions from the stem bark					
	He	Ch	Ea	Bu	Aq	Ce
	Yield (%)					
	15.3	18.7	10.0	20.2	35.8	-
Bioactives	Response to phytochemical tests					
Saponins	-	-	-	+	++	++
Tannins	-	-	-	+	++	++
Flavonoids	-	++	++	+	-	++
Alkaloids	-	++	++	-	-	++
Terpenes	++	-	-	-	-	+
Phlobatannis	-	-	-	-	-	-
Cadiac glycoside	-	-	-	-	-	-

Key: + Traces ++ Present

Table 2.
Anti-oxidant response of the different organs and fractions of the stem bark.

TESTED MATERIALS								
Organs and purified fractions								
Lvs	SB	RB	He	Ch	Ea	Bu	Aq	Vit.C
Inhibitory Concentration IC ₅₀ (µg/mL)								
4.35	1.80	3.50	2.70	1.54	2.20	3.40	3.60	0.80
±1.05	±0.25	±0.32	±1.60	±0.15	±0.18	±0.25	±0.37	±0.21

Experiment carried out in triplicate and expressed as mean ± SD

Table 3.
Anti-angiogenic (CAM) response of the diluted extract and fractions

	Parameters	
	Dose (µg/pellet)	Inhibition (%) ± SD
Crude diluted Extract		
Methanol (Me)	500	1.00 ± 0.20
Methanol (Me)	250	0.90 ± 0.10
Methanol (Me)	125	0.70 ± 0.20
Diluted Purified Fractions		
Chloroform (CH)	250	1.30 ± 0.10
Chloroform (CH)	125	1.00 ± 0.20
Chloroform(CH)	72.5	0.90 ± 0.10
Chloroform (CH)	36.2	0.40 ± 0.10
Control		
Suramin	50	0.50 ± 0.10

P < 0.001; Experiments carried out in triplicate and expressed as Means ± SD

DISCUSSION

Processing and phytochemical screening of plant material

The reagents and solvents used in this study were of analytical grade. The plant materials were all processed in methanol (100%). Saponins, tannins, terpenes flavonoids and alkaloids were detected in the crude extract of the stem bark (SB) (Table 1) (Harbone, 1984). Successive partitioning reveals the aqueous (Aq) fraction to have the

highest yield 35.8% (w/w) followed by the *n*-Butanol fraction (Bu) with 20.2% (w/w) the chloroform fraction (Ch) 18.7 % (w/w) and *n*-Hexane 15.3 % (w/w). The ethyl acetate fraction had the lowest yield 10.0% (w/w). The chloroform (CH) fraction which was found to be the most active portion was screened for its bioactive content and it responded positively to the test of flavonoids and alkaloids. (Table1). These bioactive ingredients may be playing a vital role in the activity expressed by the plant.

Anti-oxidant activity: DPPH ASSAY

Preliminary anti-oxidant activity was carried out on all organs of the plant so as to select the organ with the highest activity. To achieve this aim, a combination of chromatographic and spectrophotometer analysis was used. These efforts indicated the stem bark as the most active with 50% inhibitory concentration (IC₅₀) at 1.80 µg/mL as against on IC₅₀ of 0.80 µg/mL for Vitamin C. The Leaves (Lvs) were found to have the least IC₅₀ of 4.35 µg/mL. The root bark (Rb) recorded 3.50 µg/mL. Successive fractions of the most active organ (the stem bark) were assayed and the chloroform fraction was found to be the most active with an IC₅₀ of 1.54 µg/mL. The aqueous fraction was the least active among the purified fractions and recorded IC₅₀: 3.60 µg/mL. The other fractions recorded IC₅₀ of 3.40, 2.70 and 2.20

µg/mL for *n*-Butanol, *n*-Hexane and ethyl acetate fractions respectively. These results were comparable to Vitamin C. The findings emanating from this study have pointed out the potential of the plant as anti-oxidant which could be exploited in drug development in search of powerful anti-oxidants urgently needed to challenge free radicals in biological systems and consequently prevent the body from free radicals originated ailments. Chronic afflictions such as cancer, arthritis, diabetic are all considered end-point of both free radicals and angiogenesis processes and consequently we aimed to also study the anti-angiogenic activity of the plant.

Anti-angiogenic activity

The methanol extract of the stem bark was diluted between 500 and 125 µg/pellet while the chloroform fraction (the most active fraction) was equally serially diluted (250-36.2). In both cases there was a dose dependent effect in inhibiting the formation of new blood vessels. These effects were observed and scored according to Marchesan et al., (1998). The effect of the crude extract was highest at 500 µg/pellet (score 1.00) and the reduced concentration was followed by a reduced score (Table 3). The dilution of the most active fraction revealed an increased activity at 250 µg/pellet i.e. 1.10 score and there from, it decreased down to 0.40. The other dilutions recorded scores of 1.00 and 0.9 at 125 and 32.2 µg/pellet. It is noteworthy to mention that at 36.2 the score of 0.40 showed that there was no anti-angiogenic activity since anti-angiogenic activity is graded between 0.5 – 2.0. (Marchesan et al 1998). The methanol extract of the plant and the purified chloroform fraction (CH) as well as the bio-active ingredients found therein such as flavonoids and alkaloids may represent potential targets for the search of natural products that could, inhibit angiogenesis. The flavonoids specifically have been reported to play such roles (Paper, 1998). The plant *Sphenocentrum jollyanum* is used as a powerful wound healing agent and a cough drug as well as against tumours (Iwu, 1993; Dalziel, 1985). The anti-angiogenic potential of the methanol extract (Me) at 500 µg/pellet

and the purified chloroform fraction (Ch) at 250 µg/pellet with scores of 1.00 and 1.30 respectively and the detection of alkaloid and flavonoids in the most active fraction may not be unconnected to the beneficial effects of the plant against inflammations and Tumour related ailments. Moreover, previous researchers have isolated and characterised isoquinoline alkaloids such as palmatine, columbamine and some bitter tasting diterpenes from the plant (Iwu, 1993).

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