



Journal of
**Pharmacology and
Toxicology**

ISSN 1816-496X



Academic
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Anti-oxidant and Anti-inflammatory Activity of *Centrosema pulmieri* Benth (Leguminosae-papilionaceae)

¹H.O. Oladimeji, ²R. Nia and ³E. Oforah

¹Department of Pharmaceutical and Medicinal Chemistry

²Department of Pharmacognosy and Traditional Medicine,

³Department of Clinical and Biopharmacy, Faculty of Pharmacy,
University of Uyo, Uyo, Akwa Ibom State, Nigeria

Abstract: The aim of present study was designed to investigate the anti-oxidant activity and as well as to confirm or otherwise the anti-inflammatory activity of the *Centrosema pulmieri* Benth (Leguminosae-papilionaceae). Hence the DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) and HET-CAM (Hen's Eggs Chorioallantoic Membrane) assays were, respectively carried out. The crude extract and ethyl-acetate fraction gave significant anti-oxidant activity (IC_{50}) at 2.10 and 1.56 $\mu\text{g mL}^{-1}$ ($p < 0.05$), respectively compared with 0.66 $\mu\text{g mL}^{-1}$ displayed by ascorbic acid. Interestingly, the anti-inflammatory assay showed that the crude extract and ethyl-acetate fraction had a 100% inhibition while the hexane and chloroform fractions demonstrated a below 50% response. The ethyl-acetate fraction when serially diluted exhibited a concentration-dependent anti-inflammatory activity. These findings have revealed a correlation between the anti-oxidant and anti-inflammatory activities, hence scientific justification to the uses of the plant in folkloric medicine.

Key words: Anti-oxidant, anti-inflammatory, DPPH, HET-CAM assay, *Centrosema pulmieri*

INTRODUCTION

Pathological conditions including inflammations conditions have been found associated with the generation of Reactive Oxidative Species (ROS) and consequently the induction of several chain reactions among them, lipid peroxidation (Cross *et al.*, 1987).

These reactive oxidative species (generated by cellular metabolisms such as glycolysis, mitochondria respiration, xenobiotic detoxification and ionizing radiations), beyond the anti-oxidant capacity of a biological system gives rise to oxidative stress (Sies, 1991; Gutteridge and Halliwell, 1994; Maxwell, 1995; Laval, 1996). Also, Free Radical Oxidative Stress (FROS) has been implicated in the pathogenesis of a number of human diseases such as hypertension, diabetes mellitus, photosenscence leading to aging, pre-cancerous and cancerous lesions, wounds and parasitic infections (Halliwell *et al.*, 1987; Halliwell and Gutteridge, 1989; Halliwell, 1993; Becker *et al.*, 1994; Bonina *et al.*, 1998; Clostre, 1999).

These reactive oxidative species are also worrisome to the food industry as they cause great damage to fresh and processed foodstuffs thereby reducing the shelf-lives. Therefore, there is an urgent need to search for anti-oxidants from natural sources such as medicinal plants which have proved over the years to be potential sources for templates for drug development, dietary supplements and additives to nutraceuticals (Thomas and Wade, 2001).

Moreover, a number of previous studies have provided evidence that flavonoids, alkaloids and other bio-active compounds present in plants possess anti-oxidant activities which may interact directly

Corresponding Author: H.O. Oladimeji, Department of Pharmaceutical and Medicinal Chemistry,
Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom State, Nigeria
Tel : 08023348195/080859018149

with specific system mechanism and hence induce anti-inflammatory response (Ferrandiz and Alcaraz, 1991; Funio *et al.*, 1996; Yakoza *et al.*, 1997; Johnson and Loo, 2000). The tropical environment has abundant and diverse species of plants with medicinal values yet to be fully tapped. One of such candidate species is *Centrosema pulmieri* Benth (Leguminosae-papilionaceae), which is a twining legume used traditionally in Brazil, Mexico, Haiti and West Africa for the treatment of indigestion, stomach ache, wounds and other inflammatory conditions (Hastings, 1990; Tostes *et al.*, 1999).

In spite of these ethno-botanical uses, its potential for anti-oxidant activity and scientific justification for anti-inflammatory activity are yet to be revealed. This present study was designed with the aim of investigating into anti-oxidant activity and as well as to confirm or otherwise the anti-inflammatory activity of the plant.

MATERIALS AND METHODS

Collection of Plant

The fresh plant of *Centrosema pulmieri* Benth (Leguminosae-papilionaceae) was collected in September 2004 in Uyo Local Government Area of Akwa Ibom State, Nigeria. It was identified by A. Williams, the taxonomist at the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmacy, University of Uyo where a voucher specimen NoH53 was deposited.

Chemicals and Reagents

The chemicals and reagents used were: Butanol, Chloroform, Ethyl-acetate, Hexane and Methanol (all of AnalaR grade; Aldrich Chemicals Inc; U.S.A.), DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate, SDS (Sodium dodecyl sulfate) and silica gel (254GF) obtained from the British Drug House Limited, England and ascorbic acid (Unique Pharmaceuticals, Lagos).

Extraction and Processing

The whole fresh plant was extracted in 100% methanol by percolation for 72 h. The brown organic phase was filtered with Whatman paper No.1, concentrated *in vacuo* and freeze-dried on a rotary evaporator (Buchi CH-920, Laboratorium-Technic, Flawk/SG, Switzerland). The resultant dried crude extract was investigated for plant metabolites (alkaloids, saponins, tannins, cardiac glycosides, terpenes, anthraquinones and flavonoids) according to standard phytochemical methods (Harborne, 1984; Sofowora, 1993). The dried crude extract was chromatographed on silica gel (254GF) column and gradient elution carried out using hexane: chloroform: ethyl-acetate: butanol: water (1:1:1:1:1). Eluates which showed similar TLC profile under UV (λ 366 nm) were pooled and bulked separately to obtain the hexane, chloroform, ethyl-acetate, butanol and aqueous fractions. The resultant fractions were then evaporated to dryness and subjected to the anti-oxidant and anti-inflammatory tests.

Anti-oxidant Test

Rapid TLC Screening for Anti-oxidant Activity

The dried crude extract and fractions were separately dissolved in 100% methanol, spotted on silica gel plates (in duplicate) and developed in ethyl-acetate: methanol (1:2 v/v) mixture. The plates were air-dried and sprayed with 2,2-diphenyl-1-picrylhydrazyl, i.e., DPPH (0.2% w/v in methanol) and visualized for the presence of white-like spots indicating positive anti-oxidant activity (Kirby and Schmidt, 1997; Bondet *et al.*, 1997).

DPPH Assay

Fifty microgram of the crude and fractions were separately mixed with 5 mL DPPH (0.004% w/v in methanol) and incubated for 30 min (Kirby and Schmidt, 1997). The absorbency of each sample was taken at λ 512 nm using a Jasco7800 spectrophotometer, the anti-oxidant activity computed and expressed as IC₅₀ (μ g mL⁻¹). Ascorbic acid was used as a positive control.

Anti-inflammatory Test

Hen's egg chorioallantoic membrane (HET-CAM) assay.

A modified HET-CAM assay according to Dobson *et al.* (1990) was used.

Phase 1: Pellet Preparation

Five milligram sodium dodecyl sulfate was dissolved with or without 50 mg test extract or fractions or 5 mg of controls(hydrocortisone and phenylbutazone) in 1 mL of a hot (60°C) 2.5% agarose solution. Ten microliter of these gelling solutions were used for the assay.

Phase 2: Execution

The methods of D'Arcy and Howard (1967) and Marchesan *et al.* (1999) were used with modifications. The fertile hen's eggs were incubated for 65-70 h at 37°C and at a relative humidity of 80%. The eggs were placed in a horizontal position and rotated several times. They were opened on the snub end after aspiration of 10 mL of albumin from the hole on the pointed end. At two-third of the height from the pointed end, the eggs were traced with a scalpel and after the shells were removed with forceps. The aperture was covered with Keep fresh papers and the eggs incubated at 37°C and at a relative humidity of 80% for 75 h. One pellet per egg was put on the formed chorioallantoic membrane (CAM) which was about 2 cm in diameter. The eggs were incubated for 1 day and then evaluated. For every test, 10-15 eggs were utilized. To evaluate the effects; CAMs treated with Sodium Dodecyl Sulfate (SDS) only served as positive irritation controls. Positive controls; hydrocortisone and phenylbutazone were tested at a concentration of 72.5 µg pellet⁻¹ in the presence of SDS at a concentration of 50 µg pellet⁻¹ while the crude extract and fractions were tested at a concentration of 500 µg mL⁻¹ without SDS. CAMs treated with agarose solution served as blank controls. The inhibition or otherwise of the membrane irritation was observed. A positive effect corresponding to anti-inflammatory activity exists if the irritation of the membrane induced by SDS decreases and the blood vessels appear normal. The number of experiments with a positive effect was given in percentage, indicating the level of anti-inflammatory effect.

RESULTS AND DISCUSSION

Processing of Plant Material

The plant materials used in this study were identified, authenticated and collected, observing basic guidelines of plant collection. The solvents and reagents used were of analytical grade. Phytochemical screening of crude extract of the plant revealed the presence of saponins, tannins and flavonoids while alkaloids, cardiac glycosides, terpenes and anthraquinones were absent (Table 1). This confirms previous findings as contained in Hastings (1990) and Tostes *et al.* (1999) and it is instructive that natural products such as tannins and flavonoids present in the plant may act as anti-oxidants and therefore could be implicated in the curative and or management of many ailments such as inflammations, atherosclerosis, wounds etc.

Table 1: Phytochemical screening of the crude extract of *Centrosema plumieri*

Plant metabolite	Test	CP
Alkaloids	Dragendoff's	-
	Mayer's	-
Saponins	Froth	++
	Emulsion	++
Tannins	Ferric chloride	+++
Cardiac glycosides	Salkowski's	-
	Liebermann-Burchard's	-
Terpenes	Sulphuric acid	-
Anthraquinones	Bomtrager's	-
Flavonoids	Shinoda's	+++

CP = Crude extract of *Centrosema plumieri*; - = Absent; ++ = Moderately present; +++ = Abundantly present

Anti-oxidant activity (DPPH Assay)

The chromatographic analysis of the crude extract and fractions when sprayed with DPPH and visualized in ordinary daylight showed white spots indicating anti-oxidant activity. The activity was most remarkably demonstrated in the ethyl-acetate fraction which gave the most intensified spots. Interestingly, the spectrophotometric determination also showed that both the crude extract and ethyl-acetate fraction gave the highest anti-oxidant activities (IC_{50}) at 2.10 and 1.56 $\mu\text{g mL}^{-1}$ ($p < 0.05$), respectively compared with 0.66 $\mu\text{g mL}^{-1}$ given by ascorbic acid (a standard anti-oxidant agent). This implies a somewhat correlation in activity especially in the ethyl-acetate fraction. However, the hexane and aqueous fractions showed the lowest anti-oxidant activities at 5.11 and 4.50 $\mu\text{g mL}^{-1}$, respectively (Table 2).

The phytochemical screening of the crude extract the plant showed the presence of saponins, tannins and flavonoids which have been implicated in other studies to scavenge free radicals (showing anti-oxidant activity) in biological systems when in excess (Funio *et al.*, 1996; Laval, 1996; Yokosawa *et al.*, 1997; Gao-zhonghong *et al.*, 1999; Johnson and Loo, 2000). The anti-oxidant activity given by the ethyl-acetate fraction may be of tremendous importance to the food and spice industries where substitutes to chemicals (butylated hydroxytoluene and butylated hydroxyanisole) currently in use are in great demand. This could be achieved by furthering this study to the isolation and characterization of the tannins and flavonoids. In addition, these free radicals have also been found in human pathological conditions such as inflammations which have reportedly been linked to oxidative processes (Lundolm and Gelin, 1994) hence the need for the anti-inflammatory assay.

Anti-inflammatory Assay (HET-CAM)

The HET-CAM assay is a novel model for determining anti-inflammatory activity. The method uses the highly vascularized Hen's egg chorioallantoic membrane as biological test surface. The crude extract and fractions were assayed at 500 $\mu\text{g pellet}^{-1}$.

An inflammatory condition was induced by Sodium Dodecyl Sulphate (SDS) at 50 $\mu\text{g pellet}^{-1}$ and was observed after 24 h. In this condition (before restoration), the inflammatory condition clearly showed the formation of a granuloma and in all cases the blood vessels assumed a star-like shape. The inhibition of membrane irritation (% inhibition) was found to be total for the crude extract and ethyl-acetate fraction at 100% which is graded as 'strong effect' while the hexane and chloroform fractions elicited 40 and 45% inhibition, respectively which are regarded as very 'weak effect' (Marchesan *et al.*, 19998) (Table 3).

The ethyl-acetate fraction which gave the highest anti-inflammatory response was subsequently serially diluted between 500 and 72.5 $\mu\text{g pellet}^{-1}$, assayed and the results showed a concentration-dependent trend (Table 4). The dilutions; 500, 250 and 125 $\mu\text{g pellet}^{-1}$ displayed strong anti-inflammatory responses (inhibition) at 100, 96 and 92%, respectively (Table 4) which compare favorably with inhibition displayed by hydrocortisone and phenylbutazone at 80 and 78%, respectively. These findings express a correlation between the anti-oxidant and anti-inflammatory activities given by the ethyl-acetate fraction which has been found to contain tannins and flavonoids and already implicated in mechanisms counteracting Reactive Oxidative Species (ROS) indicated in the pathogenesis of inflammations and other related ailments in biological systems hence confirming previous studies (Lundolm and Gelin, 1994).

Consequently, this study has revealed the potential for anti-oxidant activity and also lent scientific justification to the use of the plant in inflammatory conditions as claimed in ethno-medicine. However, the evaluation of toxicity and safety studies is recommended before the plant is formulated for use in especially the less privileged regions of the world.

Table 2: The anti-oxidant activity of crude extract and fractions of *Centrosema pulmieri*

Sample	IC ₅₀ (µg mL ⁻¹)
CP	2.10*
HE	5.11
CH	2.50*
EA	1.56*
BU	3.66
AQ	4.50
Ac (Control)	0.06

Refer to Table 1; HE =Hexane fraction; CH = Chloroform fraction; EA = Ethyl-acetate fraction; BU = Butanol fraction; AQ = Aqueous fraction; Ac = Ascorbic acid *Statistically significant (p<0.05)

Table 3: The HET-CAM assay demonstrating the anti-inflammatory activity of the crude extract and fractions of *Centrosema pulmieri* at 500µg pellet⁻¹

Sample	% inhibition
CP	100
HE	40
CH	45
EA	100
BU	55
AQ	55
Hc (Positive control)	80
Ph (Positive control)	82
SDS	0

Refer to Table 1 and 2; Hc = Hydrocortisone; Ph =Phenylbutazone; SDS = Sodium dodecyl sulfate

Table 4: HET-CAM assay demonstrating the anti-inflammatory activity (inhibition of membrane irritation) of the ethyl-acetate fraction using serial dilutions

Sample (µg pellet ⁻¹)	% inhibition
EA (500)	100
EA (250)	96
EA (125)	92
EA (72.5)	76
Hc (72.5)	80
Ph (72.5)	78
SDS (500)	0

Refer to Table 1-3. Irritation was induced by SDS at 500 µg pellet⁻¹ as positive control

ACKNOWLEDGMENTS

The assistance rendered by D.H. Paper (Institute of Pharmaceutical Biology, Faculty of Pharmacy, University of Resensberg, Germany) to R. Nia in the anti-inflammatory activity assay while the latter was on his post-doctoral fellowship is acknowledged.

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