

## INVESTIGATION INTO IN-VITRO RADICAL SCAVENGING AND IN-VIVO ANTI-INFLAMMATORY POTENTIAL OF *TRIDAX PROCUMBENS*

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**Summary:** *Tridax procumbens*, is a common annual weed in the West African sub-region and other tropical zones of the world and is known as "coat buttons". Traditional medical practitioners (TMPs) and the native peoples of these areas use the leaves of the plant as a remedy against several ailments ranging from conjunctivitis, diarrhoea, and dysentery to wound healing and related inflammatory conditions. Preliminary radical scavenging screenings proved positive and prompted further investigations into its anti-oxidant and anti-inflammatory activities. This, then necessitated the use of DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) and HET-CAM (Hen's egg chorioallantoic membrane) assays to investigate the radical scavenging and anti-inflammatory potentials of the plant. The results indicated that the chloroform (CHL) and the aqueous (AQU) fractions had the highest and the lowest 50 % inhibitory concentration of free radical oxidative species (FROS) at 1.92 and 4.22µg/mL in the DPPH assay. The HET-CAM (Hen's eggs test on chorioallantoic membrane) assay, a novel assay for anti-inflammatory evaluation revealed the ethylacetate (ETA) fraction as the most active: 96%: "very strong effect" in terms of restoration of an induced inflammatory condition, while the ethanol extract (ETH) showed 92%: "strong effect". The aqueous (AQU), the Hexane (HEX) and the chloroform (CHL) fractions displayed a below 25 % inhibition. Serial dilutions of the most active fraction: the ethylacetate fraction (ETA) revealed a dose dependent trend. Chemical analysis of the whole plant showed the presence of flavonoids and alkaloids in the chloroform (CHL) and ethylacetate (ETA) fractions. Interestingly, these classes of natural products have been implicated in radical scavenging and inflammation activities in other studies and it is therefore probable that, they may be playing similar functions in *Tridax procumbens* and consequently would have justified its uses in traditional medical practices.

**Key Words:** Anti-oxidant, anti-inflammatory, HET-CAM, DPPH assay, Asteraceae, *Tridax procumbens*.

### Introduction

Numerous pathological events including inflammation processes are associated with the generation of reactive oxidative species (ROS) and consequently the induction of several chain reactions among them, lipid peroxidation and others (Cross, et al., 1987). Reactive oxidative species (ROS) are either generated by cellular metabolisms such as glycolysis, mitochondrial respiration, and xenobiotic detoxification

or by exogenous factors such as ionizing radiations or chemical compounds performing red-ox reactions. Some ROS are extremely reactive and therefore interact with some "vital" macromolecules including lipids, nucleic acids and proteins. The cells have numerous defense systems to counteract the deleterious effects of ROS and small molecules such as glutathion, alpha tocopherol, melanine, vitamins C and A are anti-oxidants in the cells. However, when they are unchecked by the biological

system, they can be involved in the oxidation of low-density lipoproteins (LDL) with direct implication in atherogenesis (Grassman, et al., 2001). In addition free radicals are involved in numerous skin diseases, especially inflammation reactions, photosenscence leading to ageing and parasitic infections. (Becker, et al., 1994). These reactive oxidative species are also a great concern for the food industries as they cause great deterioration thereby limiting the shelf life of fresh and processed food stuffs. Recently, some synthetic anti-oxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been suspected to be dangerous to human health (Safer, et al., 1999). Therefore, there is an urgent need to search for novel anti-oxidants from natural sources which could be used in medicine and in the food industries as dietary supplements and additives to nutraceuticals (Thomas and Wade, 2001). It is estimated that about 400,000 plants grace the earth and less than 0.5 % have been studied exhaustively for their chemical composition and medicinal value (Hostettmann 1999). Moreover, a number of studies have suggested that, polyphenolic compounds, alkaloids and other bio-active ingredients present in the plants have radical scavenging activities and they may interact directly with specific system mechanisms and induce an anti-inflammatory response (Ferrandiz and Alcaraz, 1991). One such plant is *Tridax procumbens* (Asteraceae) commonly known as "Coat buttons" and "Ayara-utime-nse" in Ibibio tribe (southern Nigeria). The plant is a rough hairy annual herb with weak, trailing branches up to 40cm high, the leaves are simple, opposite, ovate or broadly lanceolate with a pointed apex. The plant is used traditionally for fever, cough, skin infections and wounds (Ali and Earla, 2000, Etukudo, 2000). The effectiveness of the plant to remedy ailment conditions in tradi-medical practices may not be unconnected with the natural products present in the plant and their abilities to act as radical scavengers. For these reasons, it was therefore reasonable to investigate the radical scavenging and anti-inflammatory activities of *Tridax procumbens*.

## Materials and Methods:

### *Collection, processing and phytochemical screening*

The plant, *Tridax procumbens* was collected in September 2001 in Uyo local government of Akwa-Ibom State, Nigeria. It was identified and authenticated by the taxonomist of the Department of Pharmacognosy of the University of Uyo, where a voucher specimen was deposited. 10 kg of the whole fresh plant were extracted in 96 % ethanol by percolation for 72 hours. The brown organic phase was filtered with Whatman paper No 1, concentrated *in-vacuo* and freeze-dried. The powder extract was treated and analyzed for the presence or otherwise of bio-active components using standard methods (Harborne 1985) and assayed. The crude extract was later successively extracted into n-hexane (HEX), chloroform (CHL), ethylacetate (ETA), n-butanol (BUT)

and aqueous (AQU) fractions. The different portions were processed and weighed to evaluate their yields.

### *Anti-oxidant activity: DPPH assay*

Rapid TLC screening for anti-oxidant activity: The freeze-dried powder was dissolved in methanol (100 %) and spotted on silica gel sheets and 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 (Bondet, 1997, Kirby and Smith, 1997) and visualized for the presence of white-like spots indicating anti-oxidant positive responses.

### *DPPH assay*

The assay was carried out using the method described by Bondet, 1997, Kirby and Smith, 1997. 50 µg of the crude extract and various fractions were mixed with 5ml of a 0.004 % methanol solution of DPPH. After an incubation period of 30 min, the absorbency of the sample was read at 512 nm using a JASCO 7800 spectrophotometer. Ascorbic acid (ACA) and vitamin A (VTA) were used as positive controls.

### *Anti-inflammatory activity*

HET-CAM assay: Hen's egg chorioallantoic membrane was used with modifications

Phase 1: Pellet preparation. 5 mg sodium dodecyl sulfate (SDS) was dissolved with or without 50 mg test extract or fractions or 5 mg of controls (hydrocortisone, phenylbutazone) in 1 ml of a hot (about 60 °C) 2.5 % agarose solution. 10 µl of these gelling solutions were used for the assay (Dobson, 1990).

Phase 2: Execution. The methods described by D'Arcy and Howard, 1967 and Marchesan, et al., 1999 were used with modifications. The fertile hen's eggs were incubated for 65-70 hours at 37 °C and a relative humidity of 80 %. The eggs were placed in a horizontal position and rotated several times. They were opened on the snub end for the aspiration of 10 ml of albumin from the hole on the pointed end. At two-thirds of the height from the pointed end, the eggs were traced with a scalpel and after that the shells were removed with forceps. The aperture was covered with Keep fresh papers and the eggs were incubated at 37°C at a relative humidity of 80 % for 75 hours. 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, as positive irritation controls, CAMs were treated with SDS only. As positive controls, hydrocortisone and phenylbutazone were tested at a concentration of 72.5 µg/pellet in the presence of SDS at a concentration of 50µg/pellet. As a negative control, the crude extract was assayed at a concentration of 500 µg/mL without SDS. As a blank CAM were treated with agarose solution only.

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 vessel net appear normal. The number of experiments with a positive effect was given in percentage, indicating the level of anti-inflammatory effect.

## Results

### *Plant materials, processing and phytochemical studies*

The plant materials used in this study were identified, authenticated and collected properly bearing in mind and observing basic guides in plant collection. The solvents and reagents used were of analytical grade. Phytochemical investigations to identify the secondary plant metabolites or potential bio-active ingredients were carried out on the ethanol extract using standard guidelines described by Harborne, (1984). They revealed the presence of tannins, cardiac glycosides, flavonoids in good amounts and alkaloids as shown by the intensity of the precipitate and or color reactions as the case was, however a flavonoid has been identified and isolated before from this plant (Ali and Earla, 2000). The absence of phlobatannins, anthraquinones and saponins were noticed and recorded. Nevertheless, natural products such as tannins and some flavonoids being polyphenols may be acting in the plant as radical scavengers and therefore could be implicated in the curative and/or management of many ailments such as inflammations, atherosclerosis and wound healing. Moreover, these groups of compounds were found in large quantities. Successive partitioning of the crude ethanol extract into its eluotropic fractions yielded in w/w n-hexane (HEX): 21%, chloroform (CHL): 27%, ethylacetate (ETA): 16%, n-butanol (BUT): 13% and aqueous (AQU): 23%. The chloroform and the butanol fractions gave the highest and the lowest yield respectively (Figure 1).

Figure 1. Yields of the purified fractions

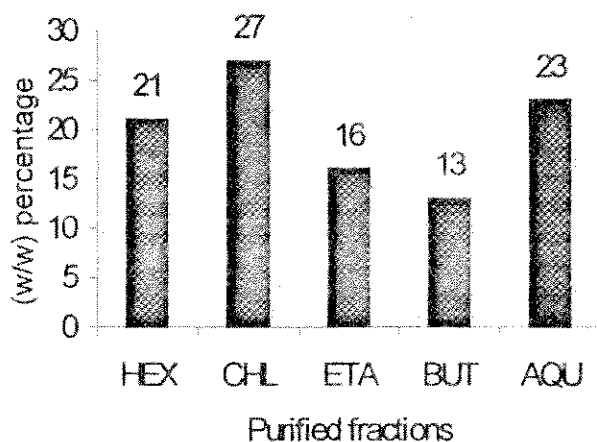
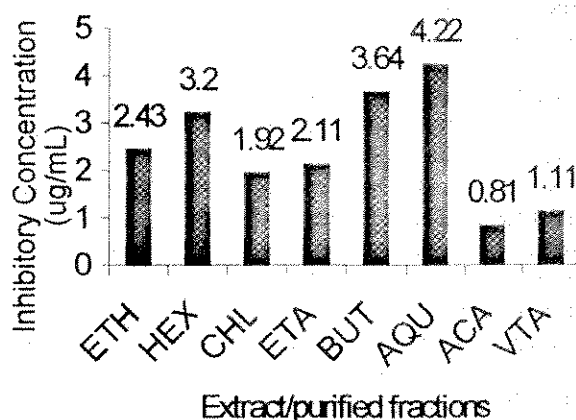


Figure 2: DPPH assay: Radical scavenger activity



### *DPPH assay: Radical scavenging*

The crude ethanol extract of the whole plant and its successive portions were assayed to evaluate their potential as scavenger of free radicals which are the origin of free radical oxidative stress (FROS) found to be fundamental in the pathogenesis of several conditions such as hypertension, atherosclerosis, diabetes mellitus, AIDS and skin problems such as acceleration of skin ageing and formation of pre-cancerous and cancerous lesions (Bonina, *et al.*, 1998 and Clostre, 1999). Therefore using a combination of chromatographic and spectrophotometric analyses, the chloroform fraction (CHL) displayed the best prospect in respect to the number and quality of DPPH positive spots and the 50 % inhibition concentration ( $IC_{50}$ : 1.92 µg/ml), indicating its radical scavenging potential. The "mother" ethanol extract gave an  $IC_{50}$ : 2.40 µg/ml, the lowest, 50 % inhibition was found in the aqueous (AQU) and n-butanol (BUT) fractions with  $IC_{50}$ : 4.22 µg/ml and  $IC_{50}$ : 3.64 µg/ml respectively (Figure 2). These results were comparable to standard radical scavenger agents, used in the study such as ascorbic (ACA) acid and vitamin A (VTA) which displayed  $IC_{50}$ : 0.81 µg/ml and  $IC_{50}$ : 1.11 µg/ml respectively. The chloroform (CHL) with the highest 50 % inhibition and the ethylacetate (ETA) fractions were subsequently investigated for the presence of bio-active ingredients contained therein (Harborne, 1985) and obviously were found to contain medium polar natural products such as alkaloids and flavonoids. These products have been implicated in radical scavenging activities in other studies (Fumio *et al.*, 1996, Yokozawa, *et al.*, 1997, Jonhson and Loo, 2000) and consequently could contribute in palliating the conditions for which the plant is used in trado-medical practices. These conditions are free radical generated pathological conditions such as inflammation, wounds, etc. The spectrum produced by the ethanol and chloroform fractions may also be interesting to the nutraceuticals.

food and spice industries, where the need for substitutes to butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are in great demand. This could only be achieved by furthering this study to the isolation and characterization of the alkaloids and flavonoids therein.

#### HET-CAM assay: In-vivo Anti-inflammatory activity

HET-CAM assay, is a novel model for anti-inflammatory assay. The method uses the highly vascularized Hen's egg chorioallantoic membrane as the biological test surface. The ethanol extract and its successive purified fractions were assayed at 500 µg/pellet to identify the most active fraction. An inflammatory condition was induced by sodium dodecyl sulphate (SDS) at 50µg/pellet and was observed after 24 hours. In this peculiar condition, the blood vessels form a star-like picture around the granuloma. The inhibition of the inflammatory condition was found to be over 90 % for the ethanol (ETH) extract and ethylacetate (ETA) fractions at 92 and 96 % respectively and were graded as "strong" and "very strong" according to Marchesan et al., (1999) scale. The second most active fraction was n-butanol (BUT) at 55 % "weak effect". The other fractions: aqueous (AQU) and n-hexane

Figure 3: HETCAM assay: Anti-inflammatory results

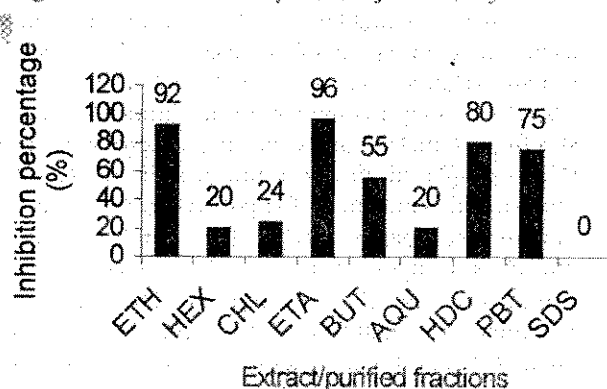
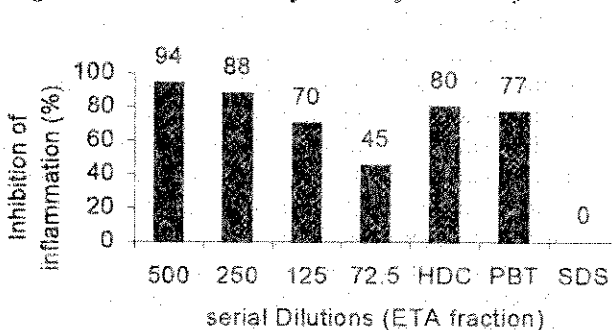


Figure 4. HET-CAM assay: Anti-inflammatory results



(HEX) recorded 20 % each while the chloroform fraction (CHL) had 24% and stood in the "very weak effect" inhibition zone. These results were compared to the restorative potential of the standard anti-

inflammatory drugs such as hydrocortisone (HDC) and phenoxy butazone (PBT) at 80 and 75 % inhibition (Figure 3). The ethylacetate (ETA) fraction was later, serially diluted between 500 and 72.5 µg/pellet, to find out the spectrum of the whole fraction and the extent of the inhibition. The results revealed a dose dependant trend (figure 4), with 94, 88, 70 and 45 % inhibition at 500, 250, 125 and 72.5 µg/pellet respectively. Since the most active fraction in respect to this assay was ethylacetate (ETA) fraction and was found to contain moderate polar natural products: alkaloids and flavonoids as earlier indicated in this study. These bioactive natural principles have been implicated in mechanisms counteracting reactive oxidative species (ROS) induced in the pathogenesis of inflammation and related ailments in biological systems. From the present research, it is therefore probable that the plant *Tridax procumbens* is in such line as a panacea against inflammatory conditions and other ailments claimed in traditional medical practices.

#### Conclusion

Chemical analysis of the ethanol extract was found to contain tannins, cardiac glycosides, flavonoids and alkaloids. The purified chloroform (CHL) and ethylacetate (ETA) fractions contained

#### of serially diluted ETA fraction

only flavonoids and alkaloids. The DPPH assay revealed the chloroform fraction, to have the highest radical scavenger activity at IC<sub>50</sub>: 1.92 µg/ml and the HETCAM displayed a restorative potential of inflammation at 96% for the ethylacetate fraction (ETA). The active ingredients in the plant and the activities observed may be playing a vital role in relation to the uses of *Tridax procumbens* in traditional medical practices (TMPs).

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