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Full Length Research Paper

Nitric oxide scavenging ability of ethyl acetate fraction of methanolic leaf extracts of *Chromolaena odorata* (Linn.)

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The nitric oxide ($NO^{\cdot}$) scavenging activities of *Chromolaena odorata* (Linn) King and Robinson, was investigated and compared with standard Plant Phenolic antioxidant Quercetin. Disodium pentacyanonitrosyl ferrate (2-) dihydrate (sodium nitroprusside) was used as a nitric oxide donor. On disintegration at a physiological pH (7.2), 5 mmol/l of sodium nitroprusside generated a time dependent nitric oxide concentration which was scavenged in vitro by the extract. Inhibition of nitrite formation by Ethyl Acetate Fraction of Methanolic Extract of *C. odorata* (EAFCO), showed a dose-dependent response. Ability of EAFCO to scavenge nitric oxide radicals in vitro was compared to a standard Plant phenolic compound (Quercetin). Quercetin and EAFCO had IC_{50} = 50 and 380 µg/ml respectively and an IC_{100} of 2000 and 2800 µg/ml respectively. The overall result showed that the plant *C. odorata* is a good nitric oxide radical scavenger. The preliminary phytochemical analysis of *chromolaena odorata* extracts revealed the presence of alkaloids, glycosides, flavonoids, saponins and tannins. Quantitative determination of total phenolic content shows that the EAFCO contains an appreciable amount of phenolic compounds and may be responsible for the observed potential.

Key words: *Chromolaena odorata,* nitric oxide scavenger, ethyl acetate fraction.

INTRODUCTION

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the body during normal metabolism. Reactive oxygen species and reactive nitrogen species (RNS, e.g. nitric oxide, NO•) are well recognized for playing a dual role as both deleterious and beneficial species (Marian et al., 2007). NO• is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolizes arginine to citrulline with the formation of NO• *via* a five electron oxidative reaction (Marletta, 1989, Moncada et al., 1989; Ghafourifar and Cadenas, 2005; Virginia et al., 2003; David, 1999). Overproduction of reactive nitrogen species is called nitrosative stress (Klatt and Lamas, 2000; Ridnour et al., 2004). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Inactivation and nitration of human superoxide dismutase (SOD) by fluxes of nitric oxide radicals have been shown (Verónica et al., 2007). It is also clear that excessive production of free radicals causes damage to biological material and is an essential event in the etiopathogenesis of various diseases (Juranek and Bezek, 2005).

The production of these reactive species in healthy organism is approximately balanced by antioxidant defence systems. However, an organism can be suffering from so – called 'oxidative stress' while it is experiencing disturbance in the pro-oxidant – antioxidant balance in favour of the former, leading to potential damage (Halliwell and Gulteridge, 1999). It is well known that free radicals cause cell damage (Albina, 1998; Ridnour et al., 2005) through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury (Brattin and Glenda, 1985). Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals (Osawa et al., 1990;

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Houghton et al., 1995). Increased fruit and vegetable consumption is associated with a decreased incidence of cardiovascular diseases, cancer, and other chronic diseases associated with oxidative stress. The beneficial health effects of fruits and vegetables have been attributed, in part, to antioxidant flavonoids present in them. (Verlangieri et al., 1985; Joshipura et al., 1999; Riboli and Norat, 2003; Bosetti et al., 2005 ; Peterson et al., 2003; Graziani, 2005). Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics, are increasingly of interest because they retard oxidative degradation of lipids (Kähkonen et a., 1990; Rice – Evans et al., 1995; Hung et al., 2002; Lee, 2003) by different mechanisms (Soobrattee et al., 2005). Natural antioxidants (that is, tocophenols, ascorbic acid and flavonoids) are of interest because of their antitumor, antimutagenic and anticarcinogenic activities.

Reactive nitrogen intermediates, such as nitric oxide ($NO^{\cdot}$), peroxinitrite ($ONOO^{-}$) and nitrogen dioxide (NO_2) has also been shown to play an important role in the inflammatory processes (Clancy and Abramson, 1995) and possibly in carcinogenesis (Tamir and Tennenbaum, 1996). The adoption of crude extracts of plants, such as infusions, for self-medication by the general public (Houghton, 1995), has arisen in the possibility that the impact of several diseases may be either ameliorated or prevented by improving the dietary intake of natural nutrients with antioxidant properties, such as vitamin E, vitamin C, B-carotene and plant phenolics such as tannins and flavonoids (Haslam, 1996).

A number of polyphenolic phytochemicals such as resveratrol and quercetin (Kawada et al., 1998) α - tocophenol (Arroyo et al., 1992) and catechin (Pannala et al., 1997, Middleton et al., 1981) have been found to inhibit the RNS effect. Kim et al. (1998) in screening edible Japanese plants for nitric oxide generation inhibitory activities in RAW 264.7 cells also found some herbs and vegetables expressing inhibitory activities. Utilization of these significant sources of natural antioxidant to prevent or improve ROS – or RNS mediated injury becomes very important. *C. odorata* (L.) R. King and H. Robinson (formerly *Eupatorium odoratum* L.), a perennial belonging to the plant family Asteraceae (= Compositae), is a diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures of southern Asia and western Africa. The common plant is known as Siam weed, 'Elizabeth', 'Independence leaf' and 'Awolowo' among the Igbos of the South-Eastern Nigeria. Phenolic compounds from the plant *C. odorata* has been reported to protect cultured skin cells from oxidative damage (Phan et al., 2001). Thang et al. (2001) have also shown that extracts from the leaves of *C. odorata* can protect human dermal fibroblast and epidermal keratinocytes against hydrogen peroxide and hypoxanthine – xanthine oxidase induced damage. However, there are no reports on the nitric oxide scavenging activity of the plant. Hence, nitric oxide scavenging activity of *C. odorata* was investigated.

MATERIALS AND METHODS

Materials

Disodium pentacyanonitrosyl ferrate (2-) dihydrate (Sodium Nitroprusside) – (SNP) Sulfanilamide, N – (1 – naphthyl) – ethylenediamine dihydrochloride [NED]), Quercetin, Catechin, Dimethyl sulfoxide (DMSO) were from the Sigma Chemical Co. (St Louis, MO).

Sample preparation

Fresh leaves of *C. odorata* used in this study were collected from Ihiagwa, in Owerri West L.G.A. of Imo State, Nigeria. The plant was identified by J. M. C. Ekekwe, plant kingdom scientific analyst, Department of Botany, University of Nigeria, Nsukka. Leaves were sun dried for 2 days, allowed to dry completely at room temperature under a fan for another 2 days and ground into powder in a mill – (BL 335 Kenwood). The powder 400 g was soaked in 3.0 l methanol and left to stand for 1 week at room temperature. The extract was filtered, and the residue was re – extracted under the same conditions. The combined filtrate was concentrated in a rotary evaporator at 48°C to obtain the methanolic extracts of *C. odorata* (MECO). The MECO were dispersed into water and then partitioned between ethyl acetate and water. The ethyl acetate layer was concentrated in a rotary evaporator at less than 50°c to obtain the ethyl acetate fraction of *C. odorata* (EAFCO). The EAFCO used in this study were dissolved in Dimethylsulfoxide (DMSO).

Phytochemical screening

Chemical tests were carried out on the extracts and on the powdered specimen using standard procedures to identify the constituents as described by Trease and Evans (1989).

Determination of the content of total phenolics

Total phenolic content were determined using the method of Swain and Hilis as described by Wettashinghe and Shaidi (2000). Briefly, extracts were dissolved in methanol to obtain a concentration of 0.5 mg/ml. Folin-Denis reagent (0.5 ml) was added to centrifuge tubes containing 0.5 ml of the extracts. Content were mixed and 1ml of a saturated sodium carbonate solution was added into each tube. Volume was then adjusted to10ml by the addition of 8 ml of deionized water and the content were mixed vigorously. Tubes were allowed to stand at ambient temperature for 25 min and then centrifuged for 5 min at 4000 xg. Absorbance of the supernatants was measured at 725 nm. A blank sample for each extract was used for background subtraction. Content of total phenolics in each extract was determined using a standard curve prepared for (+) catechin. Total extracted phenolics were expressed as mg (+) catechin equivalent/g extract.

Determination of nitric oxide scavenging ability

The compound sodium nitroprusside (SNP) is known to decompose in aqueous solution at physiological pH (7.2) producing $NO^{\cdot}$. Under aerobic conditions, $NO^{\cdot}$ reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent (Marcocci et al., 1994). The scavenging effect of the EAFCO on nitric oxide was measured according to the method of Marcocci et al. (1994) (modified). 4 ml of extract solution at different concentrations were added in the test tubes to 1ml of sodium nitroprusside (SNP) solution (5 mM) and the tubes incubated at 29°C for 2 h. An aliquot (2 ml) of the incubation solution was

Table 1. Phytochemicals presents in MECO, EAFCO and whole plant.

Phytochemicals	Methanolic Extract (MECO)	Ethyl acetate fraction of Methanolic Extract (EAFCO)	Dry powdered leaf of *Chromolaena. odorata*
Alkaloids	+	+	+
Flavonoids	+	+	+
Tanins	+	+	+
Saponins	-	-	+
Glycosides	+	+	+
Steroidal	+	+	+
Aglycone	+	+	+
Protein	+	+	+

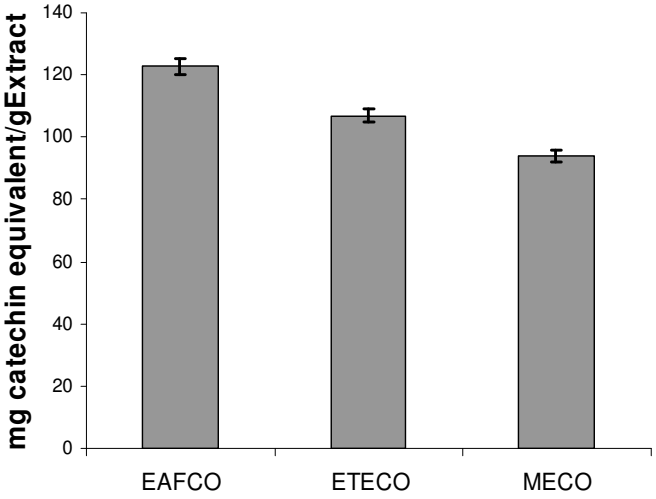


Figure 1. Phenolic content of extracts in mg catechin equivalent/ g extract

was removed and diluted with 1.2 ml Griess reagent (1% Sulfanilamide in 5% H_3PO_4 and 0.1 % Naphthylethylenediamine dihydrochloride). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm and referred to the absorbance of standard solution of sodium nitrite salt treated in the same way with Griess reagent. The amount of nitrite produced was determined from a standard dose – response curve y = 0.0054x (Linear from 0 - 500 µmol/L Sodium nitrite (BDH). Correlation coefficient ($R^{2)}$ = 0.9966).

Inhibition of nitrite formation by EAFCO and the standard plant antioxidant (Quercetin) were calculated relative to the control. Inhibition data (percentage inhibition) were linearized against the concentrations of the extract and standard plant antioxidant (Quercetin) using gamma parameter (r) as shown in the equation below (Kim et al., 1994). IC_{50} which is an inhibitory concentration of the extract required to reduce 50% of the nitrite formation was determined. The total inhibition concentrations were estimated from the %Inhibition plot or from the linear regression of log transformation plots of the dose – response data. Data which did not fit the above linear model were fitted into other models (Exponential, polynomial and logarithmic regressions) that allowed us determine IC_{50} and IC_{100}.

$$\text{Gamma parameter } (\Gamma) = \frac{\%\text{Inhibition}}{(100 - \%\text{Inhibition})} \quad \ldots\ldots\ldots\text{Eqn 1}$$

$$\text{Gamma parameter } (\Gamma) = 1 = IC_{50} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eqn 2}$$

$$\text{Log } [\%\text{Inhibition}] = 2 = IC_{100} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{Eqn 3}$$

Statistical analysis

Data were analysed using the Statistical Analysis System soft ware package. Analysis of variance was performed using ANOVA procedures. Significant differences ($P \leq 0.05$) between means were determined using Duncan's Multiple Ranged Test.

RESULTS AND DICUSSION

The compound disodium pentanonitrosyl ferrate (2-) dihydrate (5 mmol) which was used as a nitric oxide donor decomposed at a physiological pH (7.2) and generated nitric oxide which under aerobic condition produced stable nitrite (7.64 µmol/l). The griess assay used to determine the nitrite concentration is a suitable method for bio-guided fractionation of potential anti-inflammatory plant extracts (Dirsch et al., 1998). The plant *C. odorata* has been found to be a potential anti-inflammatory agent, protecting cultured skin cells from oxidative damage (Phang et al., 2001) and protecting human dermal fibroblast epidermal keratinocytes against hydrogen peroxide and hypoxanthine-xanthine oxidase induced oxidative damage (Thang et al., 2001).

Ethyl acetate has a concentrating effect on plant phenolic compound content due to its solvent polarity (Gow-chi et al., 2001). This agrees with our results (Figure 1) which shows that the EAFCO contains 31% more phenolic compound than MECO, thus justifying our use of the EAFCO in preference to MECO in the assay. It is well known that many phenolic compounds express antioxidant and radical scavenging activity (Kahkohen et al., 1999; Rice-Evans et al., 1997; Laughton et al., 1991; Bravo, 1998; Pannala et al., 1997; Sato et al., 1996; Robert et al., 2003; Wozniak et al., 2004).

Our result (Table 1 and Figure 1) shows that the plant contains phenolic compounds. The EAFCO ability to scavenge nitric oxide generated in-vitro by sodium nitroprusside was concentration dependent (Figure 2). The extract demonstrated a marked ability to scavenge NO and/or inhibit nitrite formation as it compared well with

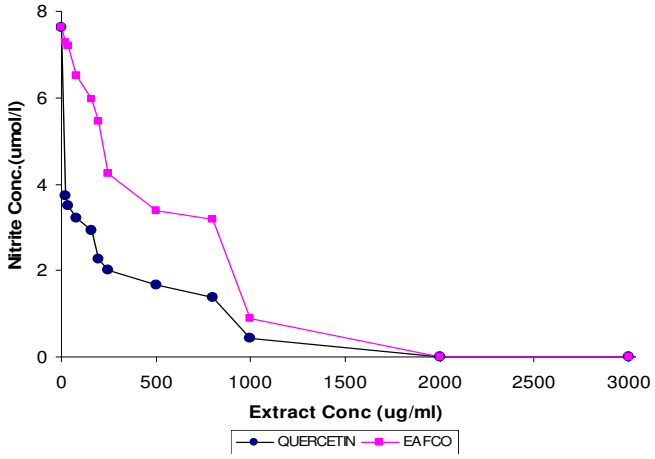
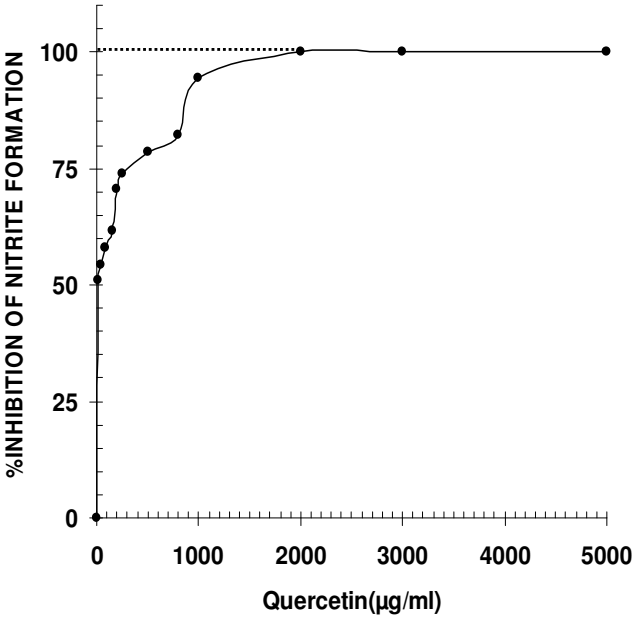
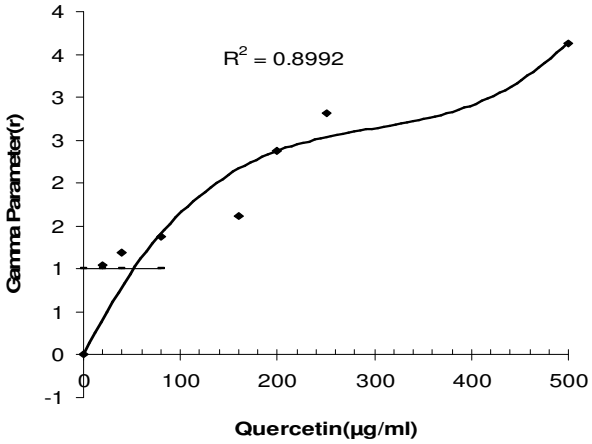


Figure 2. Nitrite concentration in response to various concentrations of Quercetin and EAFCO upon the decomposition of sodium nitroprusside (5 mmol) for 2 hour.





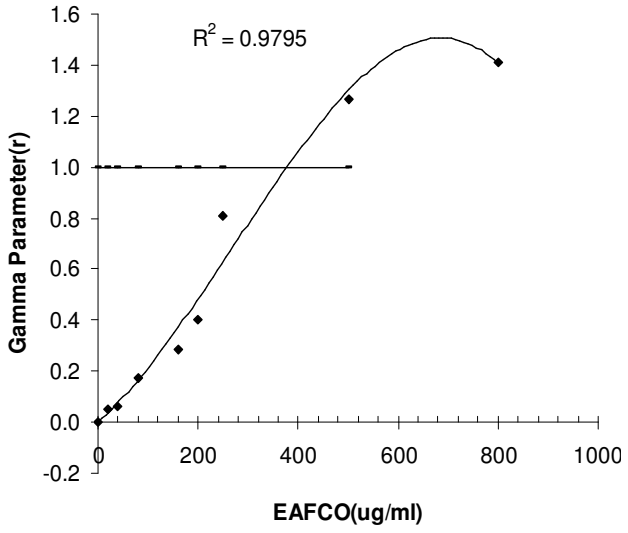


Figure 3. Plot of Γ-Parameter of %Inhibition of Nitrite formation by Quercetin (top) and EAFCO (below) on decomposition of 5 Mm SNP at pH 7.2. (IC_{50} = 50 and 380 µg/ml).

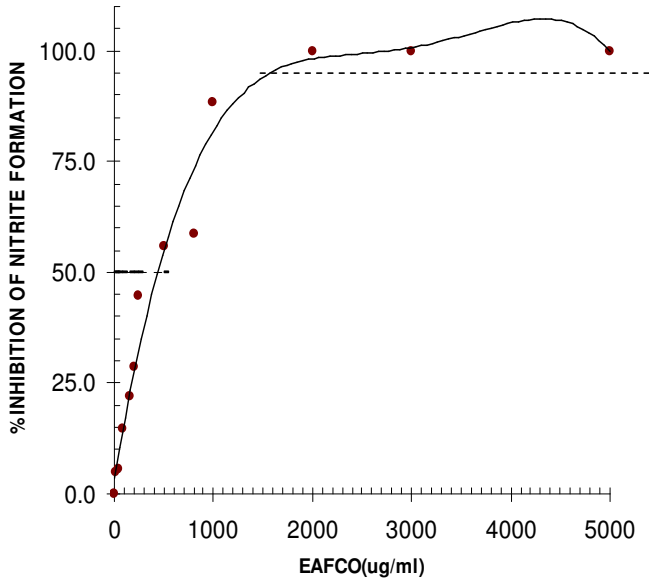


Figure 4. Plot of %Inhibition of Nitrite(µmol/l) formation by Quercetin (top) and EAFCO (below) on decomposition of 25Mm SNP at pH 7.2 (IC_{100} = 2000 and 2800 µg/ml respectively)

l with the standard antioxidant (Quercetin) (Figure 2). Comparing the EAFCO ability with the standard antioxidant using the IC_{50} and IC_{100} as our index (Figure 3 and 4), Quercetin and EAFCO had IC_{50} = 50 and 380µg/ml respectively and an IC_{100} of 2000 and 2800 µg/ml respectively. The overall result showed that the plant *C. odorata* is a good nitric oxide radical scavenger. Preliminary phytochemical analysis (Table 1) shows that the plant contains Phenolic compounds (flavonoids and tannins). These compounds have been implicated in antioxidant metabolism (Chung et al., 1998) and the nitric

oxide scavenging activity of flavonoids and phenolic compounds are known (Kim et al., 1998; Kim et al., 1999; Middleton et al., 1996; Crozier et al., 2000; Madson et al., 2000; Jagethia et al., 2004), we can speculate that these constituents might be responsible for the observed scavenging activity.

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