World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Anti-inflammatory and antioxidant activities of *Commelina diffusa* (Commelinaceae)

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Received: 27-08-2014 / Revised: 19-09-2014 / Accepted: 23-09-2014

ABSTRACT

The leaves of *Commelina diffusa* are used traditionally for the treatment of a variety of disease conditions in Ghana, including inflammation and wound healing. In this study, the ethanolic extract of the leaves of *Commelina diffusa* was investigated for its anti-inflammatory and antioxidant properties. Carrageenan induced foot oedema in 7-day old chicks was used to investigate the anti-inflammatory properties of the extract while DPPH radical scavenging test, total phenolic content and total antioxidant capacity assays were used to investigate the antioxidant property of the extract. *Commelina diffusa* ethanolic leaf extract significantly ($P<0.05$) reduced the total foot oedema in a dose dependent manner. The maximum percentage inhibition of inflammation given by the extract was 43.55% (300 mg kg^{-1}). The extract also showed potent DPPH scavenging effect with an IC_{50} of 11.35 µg/ml. Total phenolic content was found to be 193.7 mg/g of extract calculated as tannic acid equivalent. Total antioxidant capacity of the extract was found to be 130.5 mg/g of (ascorbic acid equivalent). Thus, the study justifies the traditional use of the leaves of *Commelina diffusa* in the treatment of various inflammatory conditions.

Keywords: *Commelina diffusa*, anti-inflammatory, total phenol, total antioxidant capacity**,** reactive oxygen species

INTRODUCTION

Inflammation appears to be responsible for a number of human diseases [1]. It may occur as a result of oxidative stress, infection or trauma. Inflammation resulting from oxidative stress is the cause of diseases such as dyslipidemia [2], thrombosis [3-4], type 2 diabetes [5] and neurodegenerative diseases such as Alzheimer's [6]. It also plays a key role in all aspects of coronary disease including the initiation and progression of atherosclerotic plaque, plaque rupture and thrombosis.

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), play a major role in the development of oxidative stress that can lead to these inflammatory diseases [7]. To this end, a number of synthetic antioxidant agents, such as butyl hydroxyanisole and butyl hydroxytoluene, have been developed to mitigate oxidative stress. However, factors such as mutagenicity, cost and availability have limited their use in combating oxidative stress [8]. In this direction, natural antioxidants have assumed greater prominence as they are often free from side effects, less expensive and abundant in many plant sources [7]. Plant based antioxidant compounds play a defensive role by preventing the generation of free radicals and hence are extremely beneficial to alleviate inflammatory diseases [9-10].

Many plant derived substances have been shown to present with significant anti-inflammatory effects making them a potential source of molecules for the development of new drugs especially those designed for the treatment and/or control of chronic inflammatory states such as rheumatism, asthma and inflammatory bowel diseases [11]. As a result of the problems associated with current anti-inflammatory agents, such as gastric ulceration, hemorrhage, bronchospasm, kidney dysfunction, dyslipidaemia, Cushing's syndrome, hypertension and immunosuppression, there is a continuous search especially from natural sources for alternative agents [12]. Investigation of the efficacy of the large repository of plants used in folklore

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medicine for the treatment of inflammation may provide lead compounds for the discovery of new anti-inflammatory agents. *Commelina diffusa* also known as 'spreading day flower' is a herbaceous plant in the day flower family, Commelinaceae [13-14]. In ancient Chinese traditional medicine, the leaves were generally used to reduce swellings. Subsequently, *C. diffusa* received wide spread attention in Africa, America and other parts of Asia for other medicinal uses including treatment of urinary and respiratory tract infections, diarrhoea, enteritis and haemorrhoids. Topically, the crushed leaf is applied on boils, abscesses, wounds and on painful joints. It is also used in cases of dermatitis, burns, on snake bites and insect stings [15]. In previous studies, the methanol leaf extract of *C. diffusa* exhibited good antimicrobial activity against a range of Gram positive and Gram negative bacteria as well as fungi [16-17]. The methanol leaf extract has shown significant antioxidant activity by inhibiting lipid peroxidation caused by malondialdehyde (MDA) through its reaction product with thiobarbituric acid (TBA) as well as peroxidation of lipid membranes. It also demonstrated protection of MRC-5 cells against oxidation by reactive oxygen species [17-18].

The objectives of the present studies were to determine the possible anti-inflammatory property of the leaves of *C. diffusa in-vivo*, using the carrageenan-induced foot oedema in chick model and to evaluate the free radical scavenging activity, total antioxidant activity and total phenol content of the leaf extract.

MATERIALS AND METHODS

Plant collection and extraction: Fresh leaves of *Commelina diffusa* were collected from Kotei, a suburb of Kumasi in the Ashanti Region of Ghana in September 2013. The plant was authenticated at the Department of Pharmacognosy, KNUST, where a herbarium sample (KNUST/HM 4/2014/L11) has been deposited. The leaves were washed with water, air dried for one week and coarsely powdered. One hundred and twenty grams (120 g) of the powdered material was cold macerated with 500 ml of 70 % ethanol for 3 days with occasional shaking. The mixture was filtered under reduced pressure to a small volume by means of rotavapor (R-114, Buchi, Switzerland) at a temperature of 40ºC, and evaporated to dryness on a water bath to obtain a green syrupy concentrate (Percentage yield= 6.33 $\%^{w}/_{w}$).

Phytochemical Screening: Preliminary screening of the dried powdered leaves for the presence of plant secondary metabolites was carried out using standard procedures [19].

Anti-Inflammatory Assay

Chemicals: Organic solvents used, were of analytical grade and purchased from BDH Laboratory Supplies (England). Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively.

Animals: Day old Cockerels (*Gallus gallus*) were obtained from Akate Farms, Kumasi, Ghana. The chicks were fed on chick mash obtained from AGRICARE LTD, Tanoso, Kumasi, Ghana and water *ad libitum*. Temperature was kept at 24 - 29 °C, and overhead incandescent illumination was maintained on a 12 hour light-dark cycle. Chicks were experimented at 7 day- old and were randomly divided into groups of 5 throughout the study.

Carrageenan-induced foot oedema in the chicks: The anti-inflammatory property of the extract was evaluated by the carrageenan-induced foot oedema model of inflammation in the chick with slight modifications [20]. Carrageenan (10 µl of a 2% suspension in saline) was injected sub-plantar into the right footpads of the chicks, one hour before oral administration of the extract (30, 100 and 300 mg kg^{-1} body weight) and thirty minutes before intraperitonial injection of standard reference drugs, dexamethasone (0.3, 1, 3 mg kg^{-1} body weight) and diclofenac (10, 30, 100 mg kg^{-1} body weight). Foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described by Fereidoni *et al* [21]. The oedema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at an hourly time interval. The control animals received only saline which served as vehicle.

All experimental protocols were in compliance with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST Ethics Committee.

Statistical analysis: The raw scores for foot volume increase at each hour (T_1, T_2, T_3, T_4 and T_5) for each animal was normalized as the percentage difference from the initial foot volume at time zero (T_0) and was determined as follows:

$$\%\,Increase of foot volume = \frac{(FootVolume at T1 - FootVolume at T0)}{(FootVolume T1)} \times 100$$

These values were then averaged for each treatment group and the total foot volume for each treatment group calculated in arbitrary unit as the area under the curve (AUC). Percentage inhibition of oedema

for each treatment group was determined as follows:

$$\%\ Inhibitionofoedema = \frac{(AUCcontrol - AUCtreatment)}{(AUCcontrol)} \times 100$$

Differences in AUCs were analyzed by one way analysis of variance (ANOVA) followed by Student- Newman Keuls' post test. $P < 0.05$ was considered statistically significant.

Antioxidant Assays

DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay: The free radical scavenging activity of the ethanolic extract was determined by a method described by Govindaraja *et al* [22]. To 1 ml of different concentrations of the extract prepared in methanol, 3 ml of 0.002 $\%^{w}/_{v}$ methanol solution of DPPH was added and incubated in the dark for thirty minutes. Blank methanol and ascorbic acid (25 - 0.78 µg/ml) were treated in the same way and served as negative and positive controls respectively. After 30 min of incubation in the dark, absorbance was measured at 517 nm using the UV- spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). The percentage reduction of DPPH was calculated using the following equation: DPPH scavenging activity (%) = $[(A_0-A_1)/A_0]$ x 100. Where A_0 = absorbance of negative control, A_1 = absorbance of different concentrations of extract or standard drug.

Total antioxidant capacity: Different concentrations of the extract (100 – 6.25 µg/ml) and ascorbic acid (25 – 0.78 µg/ml) (positive control) were prepared in methanol. The reaction mixture consisted of 1ml of plant extract or standard drug and 3 ml of reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4, and 4 mM ammonium molybdate). The mixtures were incubated at 95 ºC for 90 minutes. After cooling to room temperature, the absorbance of the supernatant liquid was measured at 695 nm using a UV-spectrophotometer. A blank solution containing 3 ml of reagent solution and 1ml of methanol was processed under the same conditions to serve as negative control. The total antioxidant capacity was expressed in terms of ascorbic acid equivalent of the extract (mg/g of dry mass of extract)[23].

Total phenol content determination: To determine the total phenol content of extract, 0.5 ml of extract (100 - 6.25 µg/ml) was mixed with 0.1 ml of Folin-Ciocalteu reagent (0.5 N) and incubated at room temperature for 15 minutes. 2.5 ml of saturated sodium carbonate was added and further incubated at room temperature (28 ºC) for 30 minutes. Absorption at 765 nm was measured against the blank which contained 0.5 ml methanol. Tannic acid was used as the positive control and the total phenol content expressed as mg/g of tannic acid equivalents (TAEs) [24].

RESULTS

In the chick carrageenan assay, the leaf extract of *C. diffusa* (ECD) exhibited a dose dependent anti-inflammatory activity (Figure 1) with maximum inhibition of 43.55% at 300 mg/kg body weight. Similarly, diclofenac and dexamethasone, used as positive controls, showed dose dependent anti-inflammatory activities (Figure 2) with respective inhibitions of 49.39 and 49.78% at the highest dose used. In the antioxidant assays using the DPPH radical scavenging method, the extract exhibited concentration dependent anti radical activity (Figure 3) with IC_{50} value of 11.35 where as ascorbic acid, the standard antioxidant used, gave an IC_{50} value of 2.64. The total antioxidant capacity assay of the extract was determined using ascorbic acid, a known antioxidant, as the standard and reference drug. The antioxidant activity was calculated in mg of ascorbic acid equivalent per gram of plant extract (AAE). The calibration graph for ascorbic acid showed a good linearity with a correlation coefficient (r^2) of 0.9645 (Figure 4b). The total antioxidant capacity of *C. diffusa* was found to be 130.5 mg of ascorbic acid per gram of extract. Similarly in the total phenol assay, tannic acid was used as the standard phenolic substance and the total phenol calculated as mg of tannic acid per gram of extract. Tannic acid also showed a good linearity with a very high correlation coefficient (Figure 4a). The total phenolic content was found to be 193.7 mg of tannic acid per gram of plant extract.

DISCUSSION

C. diffusa leaves are traditionally used for the treatment of various inflammatory conditions as well as a wound healing agent [15]. In this study, the anti-inflammatory activity of the ethanolic leaf extract (ECD) was tested using the chick carrageenan- induced foot oedema model. The free radical scavenging, total antioxidant capacity and total phenol content were also investigated. In the anti-inflammatory assay, injection of 10 µl of 1% carrageenan caused a time dependent increase in the footpads of 7-day old chicks. The observed effect peaked two hours after carrageenan injection and reduced gradually from the third hour (Figure 3) and throughout the period of observation indicating the body's inherent ability to fight inflammation. However, from the time course curves for diclofenac (Figures 2a & 2b) and dexamethasone (Figures 2c & 2d), the oedema response of treated animals reduced at a significantly faster rate compared with the negative

controls which received no drug treatment. The anti-inflammatory activity of *C. diffusa* extract was only 1.1 times lower than both diclofenac and dexamethasone, used as standard drugs. Previous *in vitro* anti-inflammatory evaluation of the leaves of *C. diffusa*, by testing its ability to inhibit NF-κB activation as an indicator of anti-inflammatory activity, gave negative results [17]. In the current study, the anti-inflammatory activity was tested by an *in-vivo* method to either confirm or prove otherwise the previous observation. This was necessary because though the nuclear factor NF-κB pathway has long been considered a prototypical pro-inflammatory signalling pathway based on the activation of NF-κB by pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrotic factor α (TNFα), other studies have revealed that NF-κB could be an equally difficult therapeutic target in inflammatory diseases due to the complex role of NF-κB in inflammation acting as both a pro and anti-inflammatory agents in certain instances [25]. This makes *in-vitro* studies using NF-κB in inflammation complex and difficult for its effects to be completely extrapolated in *in-vivo* studies. Thus the results obtained have shown that the leaf of *C. diffusa* moderately inhibit acute oedema induced by carageenan in the chick footpad and thus gives scientific credence for its use in the treatment of inflammation as suggested by folklore medicine. In the DPPH antioxidant assay, there was a concentration-dependent scavenging activity of the extract which was observed as a decrease in DPPH absorbance with increasing concentration of extract. Similar results were observed for ascorbic acid which was used as positive control. The radical scavenging potency of the extract was about 4 times lower than the standard antioxidant used.It is believed that antioxidant activities of medicinal plants must be evaluated by more than one method (by at least two methods) in order to take into account different modes of action of a given antioxidant [26]. Therefore in the present studies the total antioxidant capacity assay, which takes into account all measureable antioxidants in the extract, was also performed. The extract again showed a high antioxidant capacity in mg of ascorbic acid equivalent per gram of extract. The antioxidant activity of *C. diffusa* leaves may partly support it anti-inflammatory activity. This is because the excessive production of reactive oxygen metabolites by phagocytic leucocytes during theinflammatory process, as part of host defense, deregulates cellular function causing tissue injury which in turn augments the state of inflammation leading to chronic inflammatory diseases [27]. Antioxidants, which scavenge these reactive oxygen metabolites, have been found to complement the anti-inflammatory process, promote tissue repair and wound healing. The extract also gave a high phenolic content of 193.7 mg/g of tannic acid per mg of extract. Thus the antioxidant activity may be due to phenolic compounds, which are known to exhibit antioxidant activity. This is further supported by the presence of tannins and flavonoids found in the phytochemical screening of the plant. Other secondary metabolites present included alkaloids, phytosterols and triterpenoids (Table 1). These compounds may be responsible for the anti-inflammatory and antioxidant activity of *C. diffusa* extract observed in the present studies.

CONCLUSION

The leaf extract of *Commelina diffusa* exhibit anti-inflammatory and antioxidant activities and thus gives scientific credence to its use in folklore medicine.

Acknowledgement: We are highly indebted to technicians of the departments of Pharmacognosy and Phramacology, Faculty of Pharmacy, KNUST, for their invaluable technical assistance.

Table 1: Phytochemical screening of the leaves of *C. diffusa*

SECONDARY METABOLITE	RESULTS
Reducing sugar	+
Saponins	-
Athracene glycosides	-
Tannins	+
Alkaloids	+
Phytosterols	+
Flavanoids	+
Triterpenoids	+

+: present, -: not detected

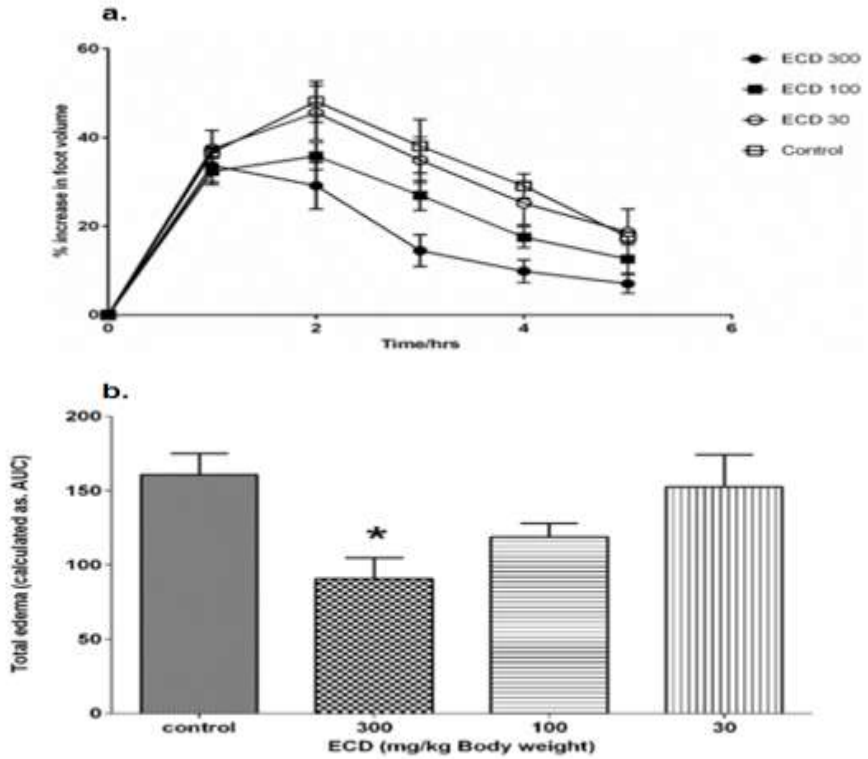

Figure 1: Effect of *C. diffusa extract* [ECD(30-300 mgkg^{-1}oral)(a-b)] on time course curve and the total oedema response, calculated as AUC's, for 5 hours, in carrageenan induced paw oedema in chicks. Values are means ± S.E.M (n=5). *P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's*post hoc* test).

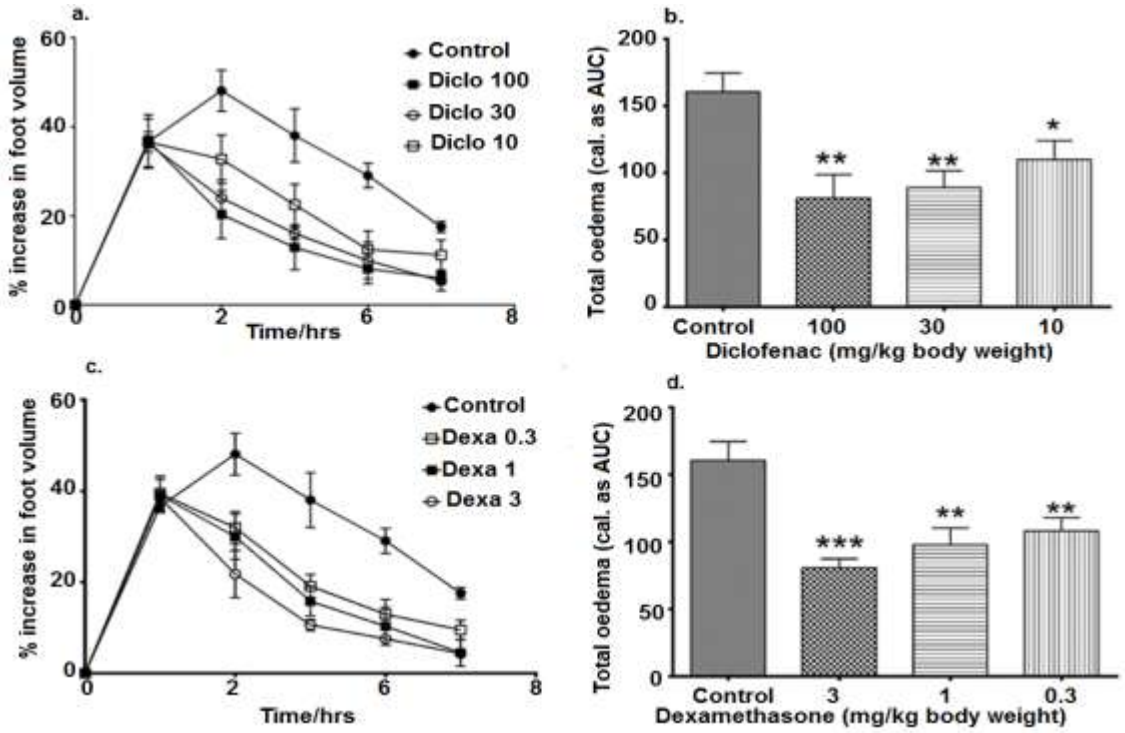

Figure 2: Effect of diclofenac [(10-100 mg/kg; *i.p*)(a-b)] and dexamethasone [(0.1-3 mg/kg; *i.p*)(c-d)] on time course curve and the total oedema response, calculated as AUC's, for 5 hours, in carrageenan induced paw oedema in chicks. Values are means ± S.E.M (n=5) *** p < 0.001, ** p < 0.01. *P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's*post hoc* test).

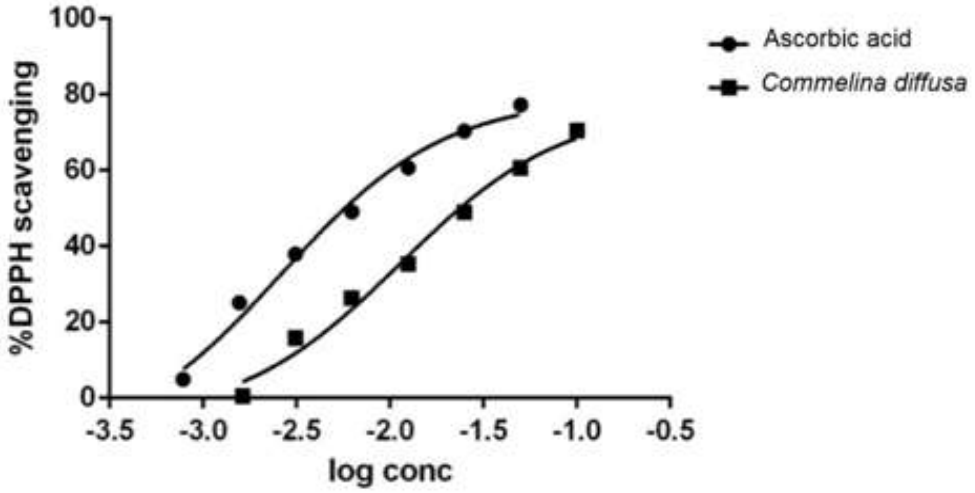

Figure 3: DPPH radical scavenging activity of extract and vitamin C

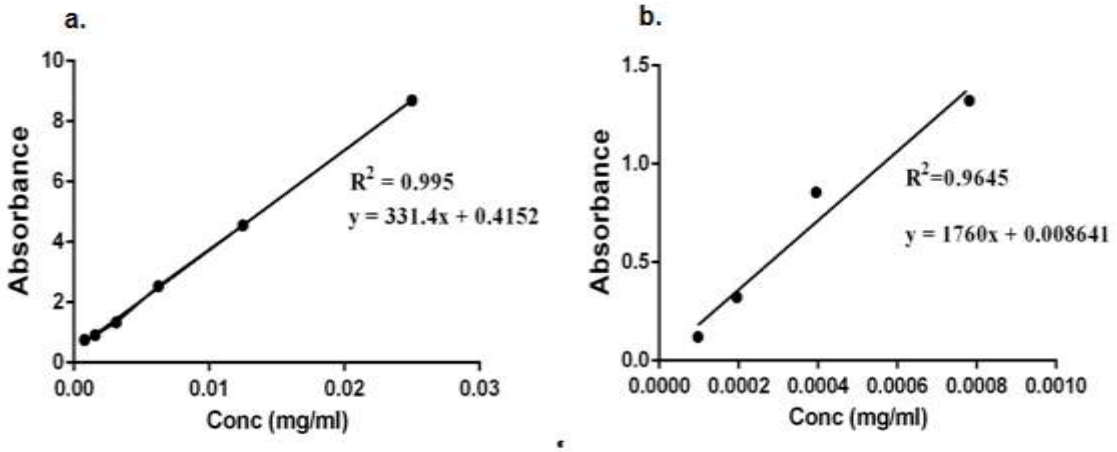

Figure 4: Calibration graph of tannic acid (a) and ascorbic acid (b)

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