



Inhibitory action of dried leaf of *Cassia alata* (Linn.) Roxb against lipoxygenase activity and nitric oxide generation

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Abstract

The mechanisms of inflammation mediated by metabolites of lipoxygenase and nitric oxide play essential roles in physiological immune response. The aim of this study was to evaluate the lipoxygenase (LOX) inhibitory activity of methanolic extract of dried leaf of *Cassia alata* (Linn.) Roxb. as well as its effect on *in vitro* accumulation of nitric oxide (NO). The LOX inhibitory activity was evaluated for its ability to inhibit lipoxygenase (soyLOX) by absorbance-based assay using linoleic acid as the substrate while the influence of the extract on accumulation of NO was monitored spectrophotometrically by Griess assay method. Moreover, gas chromatographic analysis was carried out on the ether extract to identify and quantify the volatile compounds in the extract. The study showed effective soyLOX inhibitory activity correlating with the NO depletion in a concentration-dependent manner. The gas chromatographic identification of the volatile compounds in the extract showed the presence of stearic, oleic and linoleic acids. This study revealed the methanolic extract of the leaf of *C. alata* (Linn.) Roxb. as potent inhibitor of both soyLOX and nitric oxide generation. Thus, these results give indications regarding therapeutic interest of *C. alata* (Linn.) Roxb. as a potential anti-inflammatory agent.

Keywords: *Cassia alata* (Linn.) Roxb.; lipoxygenase; nitric oxide; inflammation; gas chromatography.

1. Introduction

Inflammation occurs as cellular and molecular reactions which play vital roles against pathogenic agents and tissue damage (Medzhitov, 2008). The mechanisms of inflammatory responses involve series of events in which the metabolism of arachidonic acid plays a central role by the concerted and regulated actions of cyclooxygenase (COX) and the lipoxygenase (LOX) in the syntheses of biologically active mediators such as prostaglandins, thromboxane A₂, hydroperoxyeicosatetraenoic acids (HPETE'S) and leukotrienes (LT's) (Alitonou *et al.*, 2006; Rådmark *et al.*, 2015). These eicosanoids have varied biological functions which include the enhancement of inflammatory response through promotion of oedema formation and recruitment of leukocytes to the sites of inflammation (Serhan, 2002; Dennis and

Norris, 2015). Moreover, improper regulation of these metabolites had been associated with the development of various diseases such as cancer, arthritis, chronic bronchitis, atherosclerosis and asthma (Li *et al.*, 2013; Wisastra and Dekker, 2014). Inhibition of COX and LOX had been shown to ameliorate disease progression in different experimental models (Sarah *et al.*, 2015). However, other mediators of inflammatory responses could interfere with the metabolites of LOX and the respective signalling pathways thereby sustaining or abating the cascade of events leading to their respective biological effects. One of such mediators is nitric oxide (NO) whose participation had been found to potentiate a number of pathologies that are associated with HPETE (Canals *et al.*, 2003). In addition, NO is known as a biomarker of airway inflammation greatly

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associated with cysteinyl-leukotrienes, the products of LOX (Larsson *et al.*, 2005). Moreover, inhibition of NO generation had been shown to result in anti-inflammatory activity in macrophages (Khlifi *et al.*, 2013). Hence, inflammatory disorders are complex diseases whose treatment should involve more than one target and thus development of polyfunctional drugs.

Anti-inflammatory drugs targeting inhibition of LOX and NOS have been developed for therapeutic indications. However, extensive and prolonged use of these therapeutics is usually coupled with wide range of adverse effects which had led to the exploration of botanicals with such properties in the prevention and treatment of inflammatory disorders. In the quest to search for medicinal plants with such multifunctional properties, the present study was planned to evaluate the LOX inhibitory and NO scavenging properties of the methanolic extract of the dried leaf of *Cassia alata* (Linn.) Roxb., a shrub belonging to the family Leguminosae-Caesalpiniacea with therapeutic potentials commonly cultivated as an ornamental plant and used in folk medicine for the treatment of various pathologies such as skin infection and scabies (Oso and Olowookere, 2018). The botanical has been reported to contain wide range of compounds such as phytosterols, fatty acids, terpenes, esters and alcohols with potent antioxidant activities (Chua *et al.*, 2019).

The objective of this study was to investigate the inhibitory action of dried leaf of *Cassia alata* (Linn.) Roxb against lipoxygenase activity and nitric oxide generation

2. Materials and methods

Preparation of plant sample

Dried leaf of *C. alata* (Linn.) Roxb. (50 g) was soaked in methanol (200 mL) for 24 hours, after which the sample was filtered and dried. The dried sample was then reconstituted in methanol to a concentration of 1 mg/mL, making a stock solution from which appropriate dilutions were prepared for subsequent analyses.

Soybean lipoxygenase (soyLOX) inhibitory activity

Crude lipoxygenase from soybean was prepared as reported by Leelaprakash *et al.* (2012). Pulverised soybean was defatted and decolorized with cold acetone. The defatted sample was dried overnight. Exactly 10 g of the pulverised sample was added into 30 mL of 50 mM sodium phosphate buffer (pH 6.8) and constantly

stirred for 5 hours at 40 °C to form a slurry. The slurry was filtered through mesh cloth. The filtrate was centrifuged at 12000 rpm for 15 minutes using a refrigerated centrifuge and the supernatant was subsequently used as LOX source. The anti-lipoxygenase activity was studied using linoleic acid as the substrate and soyLOX as the enzyme according to the method described by Shinde *et al.* (1999). Exactly 250 µL of the sample at various concentrations of 0, 25, 50, 75 and 100 µg/mL was mixed with 250 µL of 0.1 M phosphate buffer (pH 9.0) and 150 µL of soyLOX. The mixture was allowed to stand at room temperature of 30 °C for 5 minutes. Then, 500 µL of 0.6 mM linoleic acid solution was added and mixed gently. The absorbance was read at 234 nm. Indomethacin was used as reference standard. The percent inhibition was calculated using the following equation:

$$\% \text{Inhibition} = \frac{A_0 - A_1}{A_0} \times 100\%$$

Nitric oxide Accumulation Assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964). The principle is based on the reaction between nitrite (generated spontaneously from sodium nitroprusside in aqueous solution at physiological pH) with sulfanilic acid at low pH to form diazonium ion, which couples with α -naphthylamine producing a red violet azo dye measurable at 540 nm. Exactly 200 µL of 10 mM sodium nitroprusside prepared in phosphate buffer saline (pH 7.4) was mixed with 500 µL of the extract (or Trolox) at various concentrations of 0, 25, 50, 75 and 100 µL in an 8 x 12 deep-well plate. The plate was allowed to stand at room temperature of 30°C for 60 minutes. Then, 500 µL of the incubated mixture was added to 1.0 mL of sulfanilic acid reagent (prepared by mixing 33% sulfanilic acid in 20% glacial acetic acid) in a separate deep-well plate and incubated at room temperature for 5 min. Then, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed with the incubated mixture and allowed to stand at room temperature for additional 15 minutes. Finally, 200 µL of each mixture was transferred into 8 x 12 microplate and the absorbance was read at 540 nm was measured using a multi-well plate reader (Molecular Devices, Spectramax Plus). The percentage reduction in NO accumulation was calculated as percentage of absorbance of wells with samples (A1) compared to wells without sample (A0) using this equation:

$$\% \text{Reduction in NO} = \frac{A_0 - A_1}{A_0} \times 100\%$$

Chromatographic analysis identification of volatile components

Gas chromatographic analysis was performed using Agilent Technologies GC-FID (flame-ionization detection) (Model 7890) equipped with Agilent 19091S-433HP-5MS 5% Phenyl Methyl Silox column (30 m × 250 µm × film thickness 0.25 µm). Helium was used as carrier gas at a constant rate of 1.5 mL/min. The injector temperature was 250 °C. The concentrations of the identified fatty acids from the resulting chromatogram were determined by the ratio of peak area of the internal standards and the area of the respective fatty acid.

Statistical analysis

The results were expressed as mean ± SD (standard deviation). Differences between the means were compared by one-way analysis of variance followed by Turkey-Kramer test for pairwise comparisons at $p < 0.05$

3. Results and discussion

Results of the *in vitro* soyLOX inhibitory assay of the extract and the standard (indomethacin) are shown in Figure 1. The extract and the standard had noticeable effects on the percentage inhibition of soyLOX range of 28.39 ± 1.36 (at 25 µg/mL) to 67 ± 4.43 (at 100 µg/mL) and 69.86 ± 2.05 (at 25 µg/mL) to 83.73 ± 0.85 (at 100 µg/mL), respectively. Moreover, soyLOX inhibitory potential of the indomethacin solution only increased moderately but significant ($p < 0.05$) at tested concentrations of 75 and 100 µg/mL compared to the increase at 25 and 50 µg/mL. The study showed a similar pattern in the depletion of NO in NO accumulation assay (Figure 2). The extract and the standard significantly ($p < 0.05$) reduced the NO content at the tested concentrations. The methanolic extract at a concentration of 100 µg/mL had the highest influence in reducing the NO levels by up to $39.91 \pm 1.21\%$ (0.1127 ± 0.0022) compared to the control (0.1876 ± 0.0123). Moreover, at concentrations of 25, 50 and 75 µg/mL, the influence of the extract was correspondingly significant ($p < 0.05$), decreasing the NO accumulation by $23.30 \pm 4.22\%$ (0.1401 ± 0.0079), $33.19 \pm 0.83\%$ (0.1253 ± 0.0016) and $37.00 \pm 0.42\%$ (0.1182 ± 0.0008), respectively (Table 1). Moreover, trolox, which was used as the standard generated a considerable reduction in the NO levels by up to $70.67 \pm 6.04\%$ at 100 µg/mL compared to the control and decreased correspondingly at all tested concentration. The result of the GC/FID identification and quantification of volatile compounds is shown in Table 2 and the

chromatogram is presented in Figure 3. The identified compounds comprise stearic, oleic and linoleic acids.

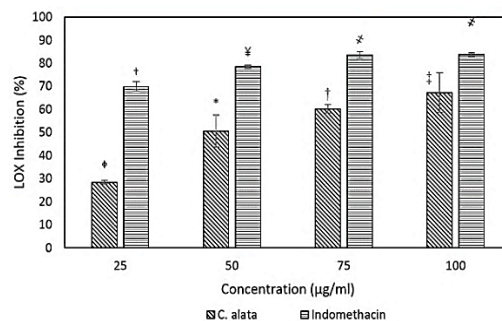


Figure 1. Lipoygenase inhibitory effect of *C. alata* (Linn.) Robx.

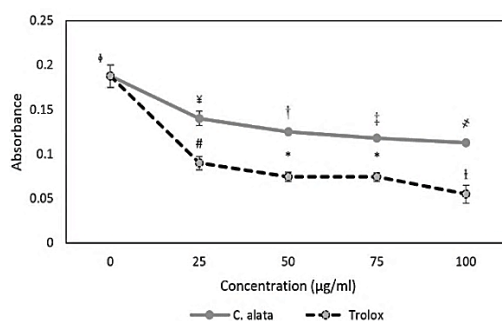


Figure 2. Effect of methanolic extract of the leaf of *C. alata* (Linn.) Robx. on NO accumulation.

Table 1
Influence of methanolic extract of *C. alata* (Linn.) Robx. on percentage reduction of NO accumulation

Concentration (µg/mL)	<i>C. alata</i> (Linn.) Robx.	Trolox
25	23.30±4.22 ^a	52.09±3.88 ^a
50	33.19±0.83 ^b	60.20±2.75 ^b
75	37.00±0.42 ^c	60.41±2.90 ^b
100	39.91±1.21 ^d	70.67±6.04 ^c

Values are expressed as Mean ± SD of percentage reduction in NO accumulation. Value with within a column with different superscript are significantly different at $p < 0.05$.

Table 2
Composition of compounds of ether extracts from methanolic extract of *C. alata* (Linn.) Robx.

Compound	Retention time (min)	Area	Height	Concentration (g/100g)
Stearic acid	10.131	7.23	1.49	0.03
Oleic acid	14.373	4.18	0.82	0.02
Linoleic acid	14.684	7.36	1.78	0.03

Many natural products of plant origin possess antioxidants that act in response to the endogenous generation of reactive oxidant species and other free radicals (Oso et al., 2018). There has been increasing interests recently in discovering natural products with medicinal benefits which can inhibit oxidative damage and prevent disorders that are related to inflammatory responses (Zeldin, 2002).

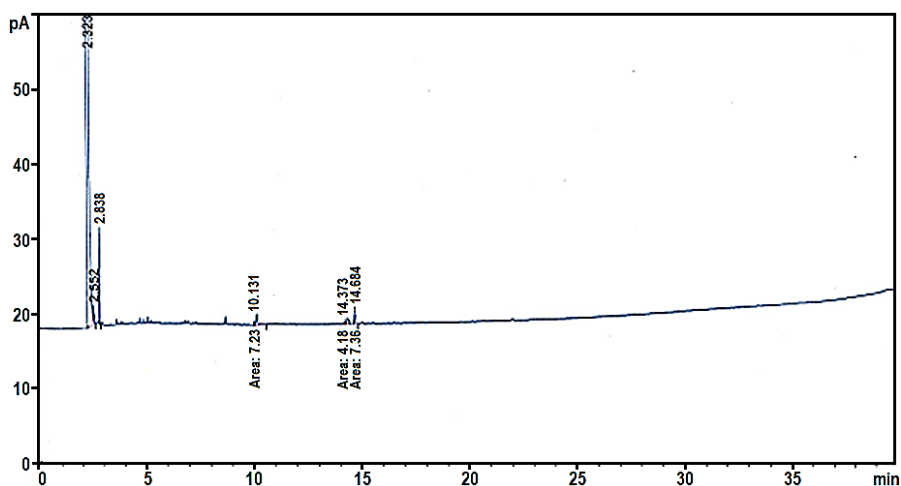


Figure 3. Gas chromatogram of ether fraction of methanolic extract of *C. alata* (Linn.) Roxb.

This study examined the soyLOX inhibitory activity of methanolic extract of *C. alata* (Linn.) Roxb. as well as its influence on the accumulation of NO. Excessive generations of metabolites of LOX such as leukotrienes play important roles in the progress of acute inflammation (Ivanov *et al.*, 2010). LOXs could be inhibited by a number of chemicals which bind to or near the iron co-factor (Skrzypczak-Jankun *et al.*, 2007). *C. alata* (Linn.) Roxb. has been used in traditional medicine in the treatment of pathologies that are associated with inflammatory disorders (Lewis and Levy, 2011). The methanolic extract of *C. alata* (Linn.) Roxb. showed the LOX inhibitory activity ranges from 33.69 ± 0.85 to 77.40 ± 8.61 at the concentrations range of $25 \mu\text{g/ml}$ to $100 \mu\text{g/ml}$. The beneficial effect of the plant has been related to its contents of kaempferol, emodin, chrysoeriol, quercetin, rhein, propelargonidins and phenols (Singh *et al.*, 2012; Duong *et al.*, 2013; Ramsay and Mueller-Harvey, 2016; Oso and Olowookere, 2018). The inhibition of soyLOX activity used in the present work could be presumed as predictive inhibition of the mammalian LOX system (Gundersen *et al.*, 2003). The involvement of LOXs in progression of several pathologies has been supported by a number of literatures, suggesting that the inhibition of LOXs may denote promising therapeutic approaches in the regulation of arachidonic acid (Sarah *et al.*, 2015). This result could validate the traditional use of the plant as remedy for inflammatory disorders. The structural features of plant polyphenols widely distributed in nature have been suggested to specially interfere with the LOX pathway (Hussain *et al.*, 2005); thus, the anti-inflammatory properties of this extract

could possibly be explained by the hypothetical inhibitory effects of its phenolic compounds on arachidonic acid metabolism. This is in agreement with previous claim that hydro-alcoholic extract of the botanical could exhibit anti allergic activity through inhibition of LOX (Singh *et al.*, 2012). Nonetheless, the *in vivo* predisposition of the inhibitors might be influenced by the source of the enzyme, its isozyme and the oxidation state of co-factor (Pham *et al.*, 1998; Borbulevych *et al.*, 2004).

In addition, the potential of the extract to deplete NO was also investigated comparable to trolox, a standard antioxidant and an analogue of vitamin E. NO is a free radical and neurotransmitter with cytoprotective and cytotoxic action. It is usually formed as a product of metabolism of L-arginine by nitric oxide synthase. Despite the fact that its half-life in blood is short, due to its rapid clearance and inactivation by haemoglobin and myoglobin, NO can form complexes with other compounds that can serve as stable NO carriers thus enhancing its bioactivity (Pereira *et al.*, 2011). Moreover, it has been indicated that bioactivities of NO can be potentiated as it can be generated non-enzymatically or enzymatically from nitrate and nitrite during tissue hypoxia or by disproportionation at low pH (Zweier *et al.*, 1999; Li *et al.*, 2008). The present study indicated that the methanolic extract of *C. alata* (Linn.) Roxb. considerably reduced the accumulation of NO in the reaction wells in concentration depended manner. This observation could substantiate previous reports on the antioxidant potential of the extracts of *C. alata* (Linn.) Roxb. (Chatterjee *et al.*, 2013; Oso *et al.*, 2018). NO has been extensively

studied to explicate its role as a signalling molecule in metabolism, cell function and disease responses. NO reacts with molecules such as free radicals or transition metal ions that possess unpaired orbital electrons (Padmaja and Huie, 1993). Excessive generation of NO has been associated with various inflammatory disorders including cancer and cardiovascular diseases (Pereira *et al.*, 2011). Decreasing the NO concentration during inflammatory responses might be helpful in lessening its threats in the interplay between cellular oxidative stress and inflammation.

Furthermore, based on the identification and quantification of volatile compounds present in the methanolic extract of the plant, it could be concluded that compounds such as stearic, oleic and linoleic acids could also contribute to the professed anti-inflammatory properties (Das, 2006; Carrillo *et al.*, 2012).

Besides the suggested physiological roles of *C. alata* (Linn.) Roxb., its limiting actions on LOX and NO could also play important role in food processing. LOX have been associated with the generation of undesirable flavour and aroma during processing and storage of proteinous plant products (Robinson *et al.*, 1995). Moreover, LOX may also lead to loss of essential nutrients and natural colorants by enhancing co-oxidation of carotenoids (Robinson *et al.*, 1995). This suggests that *C. alata* (Linn.) Roxb. might also be used as a preservative agent to improve the storage quality of various food products.

4. Conclusions

In the present investigation, methanolic extract of *C. alata* (Linn.) Robx. was found to inhibit soyLOX activity in concentration-dependent manner and reduce accumulation of NO generated by sodium nitroprusside. The observations show that the extract has the potential to interact with these biological targets. This perception on the inhibition of LOXs and clearance of NO could have shifting effects in the development of anti-inflammatory drugs. Nevertheless, additional works are encouraged to recommend its potential use as an alternative therapeutic agent against inflammatory disorders.

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