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Antioxidant effects of *Ixora coccinea* Linn. in a rat model of ovalbumin-induced asthma

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Oxidative stress, specifically lipid peroxidation, contributes to the pathogenesis of asthma. A natural antioxidant could be a potential therapeutic intervention. Hydro-alcoholic extract of *Ixora coccinea* (ICE) exhibit the anti-asthmatic activity in an ovalbumin (OVA) induced asthmatic rat model. These facts led us to examine their antioxidant activities. The free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and the intracellularly antioxidant activity of ICE were determined. The protective effect of ICE against 2,2′ azobis (2-amidinopropane) hydrochloride (AAPH)-induced red blood cell lysis was also evaluated. It was found that ICE could scavenge DPPH with an IC_{50} of 283.3 µg/ml and protected red blood cell against AAPH-induced hemolysis with an IC_{50} of 72.92 versus 52.08 µg/ml for ascorbic acid. Erythrocytes obtained from the ICE-administrated rats showed an enhanced resistance to hemolysis. In OVA-induced asthma, rats were sensitized and challenged with ovalbumin. The effect of ICE at 1500 mg/kg per os on malondialdehyde (MDA) production and lung catalase activity were determined. ICE significantly reduced the lipid peroxidation and enhanced catalase activity in lung ($p < 0.05$). In conclusion, the hydro-alcoholic extract of *I. coccinea* possesses an antioxidant activity and protective effect against free-radical-induced hemolysis. This may explain the traditional use of this plant as a remedy against asthma and other diseases.

Key words: Asthma, oxidative stress, antioxidants, *Ixora coccinea.*

INTRODUCTION

Many decades of research have produced a significant amount of data showing increased oxidative stress in asthma and indicating a potential role for oxidants in the pathogenesis of the disease (Caramori and Papi, 2004). A number of studies have clearly demonstrated that oxidative stress is an important consequence of the inflammatory response in asthma (Nadeem et al., 2005; Wood et al., 2003). Detrimental effects of oxidative stress on airway function include: airway smooth muscle contraction, airway hyper-responsiveness and epithelial shedding, each of which contribute to the airway obstruction that is characteristic of asthma (Wood et al., 2003). Indeed the lung is continuously exposed to oxidants, either generated endogenously by metabolic reactions (for example, from mitochondrial electron transport during respiration or released from phagocytes) and exogenously from air pollutants or cigarette smoke (Kirkham and Rahman, 2006). These agents may cause direct tissue oxidation, release of endogenous oxidants and inactivation of antioxidant defense mechanisms (Rai

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and Phadke, 2006). Host defense against the potentially damaging effects of reactive oxygen species (ROS) is provided by a range of antioxidants. These may be endogenous, such as the antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase), thiols (glutathione) and metal-binding proteins (lactoferrin, transferrin, ceruloplasmin) or exogenous, including a variety of antioxidants obtained from the diet such as tocopherols, carotenoids, flavonoids and ascorbate. Thus, it is likely that the use of antioxidants to restore the oxidant-antioxidant balance may be effective in the treatment of asthma (Kirkham and Rahman, 2006).

Medicinal herbs have are a very valuable source for natural antioxidant products. The antioxidant activity of many extracts and constituents from medicinal herbs has been widely documented *in vivo* and *in vitro*. *Ixora coccinea* Linn (Rubiaceae) is a common flowering shrub native to Asia which can be found growing in the tropical and subtropical climates of the world (Baliga and Kurian, 2012). Leaves are given in diarrhea; flowers are used in the treatment of dysentery, leucorrhoea, dysmenorrhoea, hemoptysis and catarrhal bronchitis (Ghani, 2003). Recently, potent anti-ulcerogenic (Arunachalam et al., 2009), antidiabetic (Yasmeen and Prabhu, 2011) and anti-diarrhoeal (Prabhu et al., 2010) properties of *I. coccinea* have been reported. The extracts of *I. coccinea* were found to be chemoprotective, antiviral, antimitotic, modulatory on cyclophosphamide-induced toxicity in mice and to act as anti-inflammatory agent (Ratnasooriya et al., 2005; Latha and Panikkar, 1999, 2000). In our previous paper (Missebukpo et al*.,* 2011), we have reported that hydro-alcoholic extract of *I. coccinea* leaves exhibit the anti-asthmatic activity in an ovalbumin (OVA)-induced asthmatic rat model. These facts led us to examine their antioxidant activities.

Recent studies reveal *in vitro* antioxidant effect of methanolic extract of the flower, leaf and stem of *I. coccinea* (Bose et al., 2008; Banerjee et al., 2011). *In vivo* antioxidant activities applicable for various diseases are experimented. For example, Bose et al. (2010) showed that *I. coccinea* extract decreased significantly lipid level in plasma and prevented hyperlipidemia, and this effect provided evidence for their antioxidant properties. Other report showed that *I. coccinea* and *I. perviflora* extract have hepatoprotective properties on CCl_4 induced liver damage in rat and this hepatoprotective effect is due to their *in vivo* antioxidant activities (Bose et al., 2011). However, the study that deals with *in vivo* antioxidant effect of *I. coccinea* extract is scarce and their antioxidant activity in animal model of ovalbumin induced asthma is not documented. In search of the mechanism of action of the extract in asthma, the aim of this study was to investigate the antioxidant activity of *I. coccinea* extract (ICE) in cell and cell-free systems, and in addition, the ability of ICE to inhibit ROS generation in model of OVA-induced asthma.

MATERIALS AND METHODS

Plant

The leaves of *I. coccinea* were collected on 24th July, 2007 in the second middle part of the day, from Lomé not far from University of Lomé (Togo). The plant was authenticated at Department of Botany, by Professor Akpagana Koffi from Laboratory of Botanic and Plant Ecology (University of Lomé). The voucher specimen (TOGO 12671) was deposited in the herbarium of this Laboratory. The dried sample was extracted in water/ethanol mixture (1 : 1) for 72 h with manual discontinue agitation. The solution was filtered evaporated using a rotary evaporator (Buchi R120) set at 45°C to obtain a dry extract which contained alkaloids, flavonoids and tannin as revealed by previously phytochemical screening.

Animals

Wistar rats (150 to 200 g body wt.) of either sex were used for the experiments described. All animals were maintained on a standard laboratory chow and water *ad libitum*. They were kept in the Animal House of the Faculty of Sciences of University of Lomé (Togo). All experiment was done following bioethics committee of University of Lomé-Togo guidelines.

Total phenols determination

Total phenolic content of the extract was determined by the Folin-Ciocalteu reaction (Lawson-Evi et al., 2011). Briefly, a mixture of *I. coccinea* extract, Folin-Ciocalteu phenol reagent, and sodium carbonate was prepared and allowed to stand at room temperature for 30 min. After that, the mixture was centrifuged and the supernatant was measured at 760 nm. Gallic acid (0 to 250 mg/L) was used as the standard for the calibration curve. The phenolic contents were calibrated using a linear equation based on the calibration curve. The contents of phenolic compounds are expressed as mg gallic acid equivalent (GAE)/g extract.

Total flavonoids content of the extract

Total flavonoids content was determined according to aluminum chloride colorimetric method (Lawson-Evi et al., 2011). The extract (0.5 ml of 1:10 g m/L) in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture was at room temperature for 30 min, the reaction mixture absorbance was measured at 415 nm with a double beam. Quercetin (5 to 100 µg/ml) was used as the standard for the calibration curve. The levels of total flavonoids contents were determined in triplicate and the result was expressed as mg quercetin equivalents (QE)/g extract.

DPPH radicals scavenging assay

Stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) solution was used to determine the free radical-scavenging activity of *I. coccinea* (Lawson-Evi et al., 2011). Different concentrations of the extract (50

to 2000 µg/ml) were added at an equal volume to methanolic solution of DPPH (100 µM) (Sigma, USA). After 15 min incubation at room temperature, the absorbance was read at 517 nm. The experiment was carried out in triplicate and quercetin was used as standard control. IC_{50} values which represent concentration required to scavenge 50% of DPPH free radicals was compared among *I. coccinea* extract and quercetin. The DPPH scavenging effect was calculated as follows:

% inhibition = (Ac – Ae) × 100 / Ac

Where: Ac = absorbance of DPPH without the sample (control), Ae = absorbance of the sample with extract or quercetin.

AAPH-induced hemolysis assay *in vitro*

Blood was collected from Wistar rats through retro-orbital sinus in heparinized tubes. The *in vitro* resistance of intact red blood cells to oxidation was evaluated with AAPH (Sigma-Aldrich, France) as described previously (Diallo et al., 2012). Erythrocyte and plasma were separated by centrifugation (3000 g for 10 min). The oxidation of rat erythrocyte (10% hematocrit with saline) was induced by AAPH at 37°C for 3 h under air. The extent of hemolysis was determined by measuring absorbance at 540 nm with a UV-visible recording spectrophotomer (UV-265FS, Shimadzu, Kyoto, Japan). The percentage of inhibition was calculated by the following equation:

$$\text{Inhibition (\%)} = [(A_{AAPH} - A_{I.\ coccinea}) / A_{AAPH})] \times 100$$

Where $A_{I.\ coccinea}$ is the absorbance of the sample containing *I. coccinea* extract and A_{AAPH} the absorbance of the sample without *I. coccinea.* L-ascorbic acid was used as a positive control. Four to five replicates were performed for each concentration.

Ex vivo study of anti-hemolysis activity of *I. coccinea*

Rats (n = 6) were given distilled water or extract (1 and 1500 mg kg^{-1}) orally after an overnight fast according to Zhu et al. (2002). Then the rats were anaesthetized with ether and blood was collected in heparinized tube 60 min after dosing. Erythrocytes from each rat were separated from the plasma by centrifugation at 1500 g for 20 min. The plasma was removed from the erythrocytes. After removing the buffy coat, the remaining erythrocytes were re-suspended in the plasma. 0.5 ml of the reconstituted blood was used for the haemolysis assay by adding 0.5 ml of AAPH solution and 0.5 ml of PBS followed by incubation at 37°C for 3 h. Then, 4 ml of PBS solution was added to the incubation mixture which was centrifuged at 1000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The percentage of inhibition was calculated as described earlier.

Induction of asthma in Wistar rats

The rats were actively sensitized by intraperitoneal (ip) injection of 20 mg ovalbumin with 100 mg $Al(OH)_3$ (chicken OVA, grade V, Sigma Chemicals Co., S^T Louis, MO) in physiological saline solution as described (Missebukpo et al., 2011). Control animals received ip saline with $Al(OH)_3$ on days 0, 3, 7 and 21 and intranasal (in) saline without $Al(OH)_3$ on days 24, 25, 26 and 27. Twenty-four hours after the last OVA challenge by intranasal administration of OVA, the rats were sacrificed and lungs were removed from the chest cavity.

Determination of lipid peroxidation (LPO)

Lung LPO was determined by estimating levels of malondialdehyde (MDA) using the thiobarbituric acid test (Satoh, 1978; Odabasoglu et al., 2006). Briefly, 150 mg of lung tissue were collected from each experimental rat, homogenized in 1 ml of Tris-HCl 10 mM (pH 7.4). The homogenate or standard MDA (175 µl) at 25, 31, 62.5, 125, 250, 500, 1000 ng ml^{-1} was added to a solution containing 250 µl of HCl 1 M, 100 µl of sodium dodecyle sulphate (SDS) 9.8%; 1 ml of thiobarbituric acid, 0.67% and 330 µl of distilled water. The mixture was incubated at 90°C for 1 h. Upon cooling, 2.5 ml of *n*-butanol was added. The mixture was centrifuged for 10 min at 3,000 rpm. The supernatant was measured at 535 nm (Spectra Max Molecular Device, Sunyval Corporation, California USA). The results were expressed as ng MDA/mg tissue.

Catalase activity

Catalase activity was measured based on the ability of the enzyme to break down H_2O_2. Decomposition of H_2O_2 in the presence of catalase was measured at 250 nm (Odabasoglu et al., 2006). Prior to the catalase measurement, 150 mg of lung of control and sensitized rats were homogenized in 1 ml of Tris-HCl 10 mM (pH 7.4). The homogenate was centrifuged and supernatants were diluted with phosphate buffer (1:20). At 25°C, 100 µl H_2O_2 (0.66 M) were added to 120 µl supernatant. The rapid decomposition of H_2O_2 was followed during 7 s from the decrease in absorbance at 250 nm. Catalase (CAT) activity was calculated by $K = \Delta DO/\Delta t \times 1000/\varepsilon$ tissue, where ε = 43.6 M^{-1}/cm molar extinction coefficient at 25°C. The results were expressed as enzymatic unity/mg lung tissues.

Statistical analysis

The data are expressed as the mean ± SEM. The statistical significance of any difference was performed by one-way analysis of variance (ANOVA) followed by Tuckey's significant difference test. A significant value was defined as $p < 0.05$. All statistical analysis were carried out using the Instat statistical package (GraphPad prism 5.0 software, Inc. USA)

RESULTS

Total phenolic and flavonoid content

The total phenolic content of ICE was 243 mg GAE/g extract and the total flavonoid content was 72.5 mg QE/g extract. The results show that *I. coccinea* has relatively high flavonoid content.

DPPH radical scavenging activity

Table 1 presents the results of DPPH radical scavenging activity of ICE. This assay provided information on the reactivity of the samples with a stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the

Table 1. IC_{50} values of hydro-alcoholic extract of the leaves of *I. coccinea* for antioxidant tests *in vitro* by AAPH and DPPH and those of quercetin and ascorbic acid.

Parameter	AAPH IC50 (µg/ml)	DPPH IC50 (µg/ml)
I. coccinea	72.92	283.3
Quercetin	-	20.00
Ascorbic acid	52.08	-

resulting decolorization is stoichiometric with respect to the number of electrons taken up. The DPPH• scavenging ability of the ICE is lower than quercetin (IC_{50} value of 20 µg/ml) enough to remove the DPPH• (IC_{50} of 283.3 µg/m), which may answer for its medicine use.

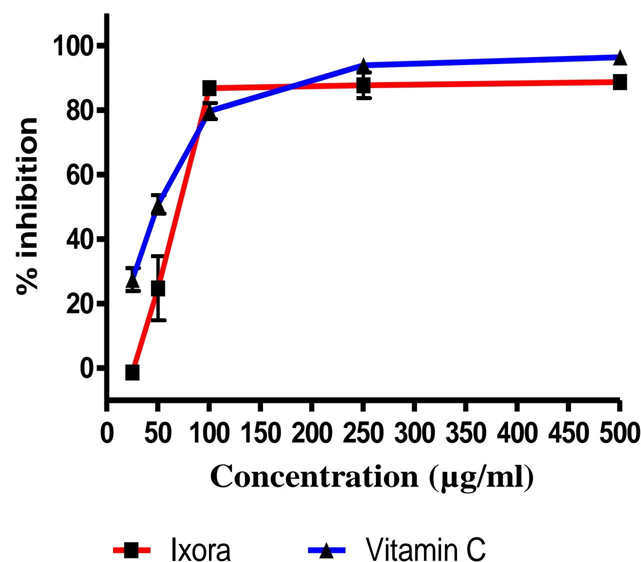

Figure 1. AAPH radical scavenging activity of hydro-alcoholic extract of the leaves of *I. coccinea* and ascorbic acid. AAPH induced in vitro Red cells membrane lipid peroxidation which leads to membrane lyse. The absorbance is a function of the color of the solution itself depends on the concentration of hemoglobin released. Inhibition percentage was calculated using mathematic formula described in the text. Values are expressed as mean ± S.E.M (n = 4).

Effect of ICE on AAPH induced-hemolysis

After 3 h of incubation with AAPH, erythrocytes were lysed. The protective effects of ICE and ascorbic acid on the hemolysis induced by AAPH are shown in Figure 1 and Table 1. IC_{50} of the ICE and ascorbic acid were 72.92 and 52.08 µg/ml, respectively. The extract had a maximum inhibitory effect of 88.72 ± 1.79%.

In vivo antioxidant activity of ICE

Oral administration of the ICE reduced the extent of AAPH-induced hemolysis. The extract at 1.5 g/kg had an inhibitory effect of 47.202 ± 7.57% (Figure 2). Figure 3 shows the results of the level of the control, Ova-sensitized and treated rats. MDA, a marker for the oxidant stress, was significantly increased in sensitized rats com-pared with saline ($p < 0.05$) (Figure 3). ICE at 1.5 g/kg significantly reduced the MDA level of the lung ($p < 0.05$). Activity of catalase in tissue homogenate did not show any significant change in OVA-sensitized and challenged rats compared with control. Figure 4 illustrates that the activity of catalase in treated rats demonstrated twice higher levels of lung catalase activity (0.467 ± 0.042 µcat/mg lung) compared with saline rat (0.203 ± 0.032 µcat/mg lung).

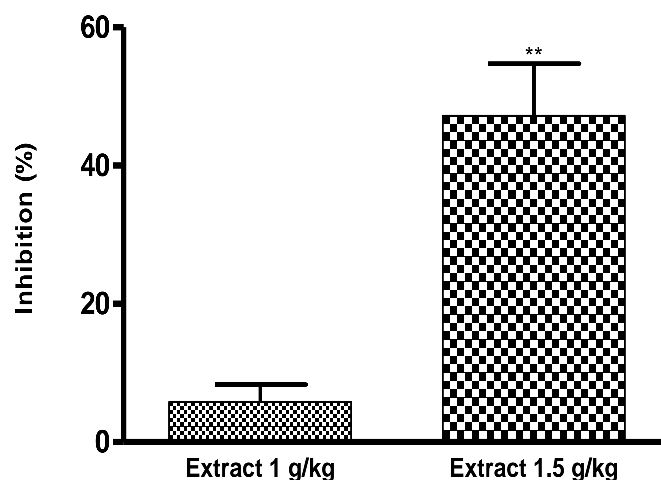

Figure 2. Inhibitory effect of hydro-alcoholic extract of the leaves of *I. coccinea* on AAPH-induced lysis of rat red blood cells. Rats were treated before blood collection. Test was done as described above in figure 1. The results are expressed as means ± SEM (n = 6) **p < 0.001 as compared with control group (0% inhibition).

DISCUSSION

Asthma is a chronic inflammatory disease of the respiratory tract where inflammation is often associated with an increased generation of ROS (Nadeem et al., 2008; Caramori and Papi, 2004). A wealth of studies identifies that ROS and loss of antioxidant defenses participate in the pathogenesis of asthma. Therefore, radical scavengers or antioxidants could play a useful role in role in therapy because antioxidants can mobilize and up-regulate the anti-oxidative capacity of cells to annihilate excessive ROS formation. This can be achieved through

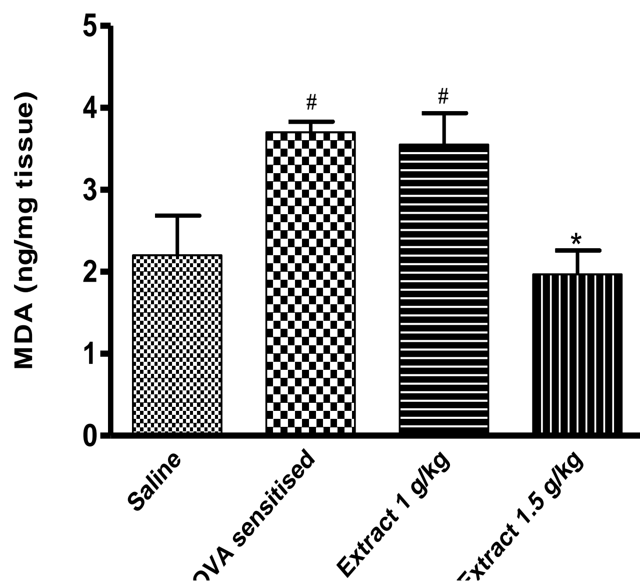

Figure 3. Content of malondialdehyde in lung homogenate of control (saline), Ova-sensitized and treated groups. Rats were made asthmatic using 28 days murine model. After 28 days rats were sacrificed and lungs were removed, 150 mg of tissues were homogenized to perform the test. Each point represents the mean ± S.E.M. (n = 4). $^{\#}p < 0.05$ vs saline rats; $^{*}p < 0.05$ vs Ova sensitized rats.

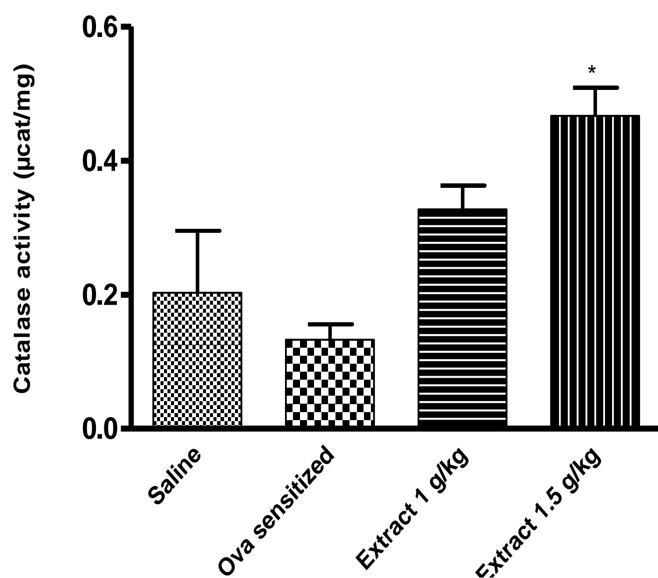

Figure 4. Catalase activity in control (saline), Ova-sensitized and treated groups. 150 mg of lung homogenized tissues were used to perform catalase activity essay. Catalase activity is expressed as enzymatic unity/mg lung. Each point represents the mean ± S.E.M. (n = 4). $^{*}P < 0.05$ compared with OVA-sensitized group.

two approaches: Either by increasing the endogenous antioxidant enzyme defenses or by enhancing the non-enzymatic defenses through dietary or pharmacological means (Kirkham and Rahman, 2006). Thus, we investigated the possible radical scavenging activity of hydro-alcohol extract of *I. coccinea* leaves by use of series of *in vitro* and *in vivo* experiments with some new methods applied to evaluate antioxidant activities of this extract.

Free radical scavenging activity was evaluated *in vitro* using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical and AAPH. In this study, ICE possessed *in vitro* antioxidant activity when tested with DPPH radical scavenging assay. The IC_{50} for the extract was 283.3 µg/ml compared with quercetin (IC_{50} = 20 µg/ml). Thus, it can act as moderate radical-scavengers which can reduce the auto-oxidation in body system or even food product that contains unsaturated lipid when compared with quercetin (Rao et al., 2007). Recently, Idowu et al. (2010) also reported that some of the phytochemicals isolated from the leaves of *I. coccinea* are effective in DPPH scavenging effects *in vitro* with gallic acid as control. These results corroborate those obtained by other researchers (Banerjee et al., 2011; Bose et al., 2011) who showed antioxidant activities of different parts of *I. coccinea* using different methods of evaluation of antioxidant activities *in vitro* like: superoxide anion scavenging activity assay, hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, Fe^{2+} chelating activity assay, hydrogen peroxide scavenging activity assay and reducing power assay.

The excessive peroxidation of biomembranes is accepted as one of the processes by which tissues can be damaged during inflammation (Zhu et al., 2002). The peroxidation of erythrocyte membranes and hemolysis induced by AAPH has been extensively studied as a model for membrane-peroxidative damage (Yoshida et al., 2004). In the present study, we investigated the antioxidative activity of *I. coccinea* extract using AAPH induced hemolysis. A dose-dependent protection was demonstrated toward the hemolysis of red blood cells *in vitro* with IC_{50} = 72.92 µg/ml. The inhibitory effect of ICE was nearer to ascorbic acid (IC_{50} = 52.08 µg/ml), which has been shown to act as an antioxidant against human low-density lipoprotein oxidation and acts as a primary defense against aqueous radicals in the blood (Ma et al., 1994). To explore mechanism of action of *I. coccinea* extract in the protection with bronchic epithelial mem-brane cells in asthma, we have evaluated antioxidant effect of extract *in vivo*. For *in vivo* assays, ICE was found to increase the levels of antioxidant in plasma. The increase in plasma antioxidant capacity observed following ICE administration is suggested by our erythrocyte hemolysis data. When we mixed erythrocytes from rats given ICE with plasma collected, the degree of hemolysis inhibition was related to the concentration of extract in the plasma.

Oxidative stress is a hallmark of asthma and increased

levels of oxidants are considered markers of the inflammatory process. Most studies to date addressing the role of oxidants in the etiology of asthma were based on the therapeutic administration of antioxidants (Reynaert et al., 2007). In the current study, OVA-sensitized rats with inflammation characteristics (Missebukpo et al., 2011), had an increment in pulmonary malondialdehyde (MDA) when compared with control group (non sensitized rats). This implies that rats during sensitization are exposed to a considerable degree of lipid peroxidation. This finding is consistent with other observation (Bulani et al., 2011). The increase in ROS during sensitization, as demonstrated by significant elevation of MDA, may overwhelm endogenous anti-oxidant defenses. This is illustrated in the present work by the decrease of MDA level in the lung in treated group, accompanied by increased catalase activity. In line with our findings, Bulani et al. (2011) showed that ovalbumin significantly increased the level of lipid peroxidation and decreased the level of GSH, SOD and catalase in the OVA sensitized rats when compared with non-sensitized group.

Catalase represents an important component of the endogenous antioxidant defense system of the lung, one of the major antioxidant enzymes that prevent the biological macromolecules from oxidative damage (Zhang et al., 2003). Increased ROS lead to modification of proteins and alterations in their function that are biologically relevant to the initiation and maintenance of inflammation, among which is the loss of antioxidant capacity of catalase (Comhair and Erzurum, 2010). However our results reveal no significant decrease in catalase activity in OVA-sensitized rats compared with non-sensitized. But Ghosh et al. (2006) demonstrated the oxidative inactivation of catalase in a murine model of allergic airway disease as well as decreased catalase activity in lungs of patients with asthma.

Several epidemiological studies have been undertaken which have established a beneficial link between polyphenol intake and lower disease risk with many of the clinical benefits being attributed to both the antioxidant and anti-inflammatory properties of polyphenols (Arts and Hollman 2005). Phenolic and flavonoid compounds are recognized as material base of the antioxidant activity of plant extract (Adedapo et al., 2009). Therefore, the chemical constituents present in the extract, which are responsible for this activity, need to be investigated. High total phenolic and flavonoids content values found in the extract (243 mg/g GAE and 72.5 mg/g QE) imply the role of phenolic compounds in contributing these activities.

Some of phenolic constituents have already been isolated from this plant and some have antioxidant properties *in vivo* model (Bose et al., 2011; Sen et al., 2011; Versiani et al., 2012; Idowu et al., 2010; Lee et al., 2010). Hence, the observed antioxidant activity may be due to the presence of any of these constituents. In unpublished results, we have shown that ICE contains chlorogenic acid, caffeic acid, and scopoletin which are strong antioxidants (Sato et al., 2011; Shaw et al., 2003).

Conclusion

From the above results, it is evident that hydro-alcohol extract of *I. coccinea* possessed both *in vitro* and *in vivo* antioxidant activity. It was not only able to enhance the plasma antioxidant level, but was also able to enter into living cells in the organ and protect them from oxidative damage after 5 days of consumption. It can be used in compensating the decrease in total antioxidant capacity in lung and enhance the Catalase activity in organ and thereby reduces the risks of lipid peroxidation in asthma. This is evident with the highest total phenolic and flavonoid content.

Abbreviations

ICE, Extract of Ixora coccinea; **OVA,** ovalbumin.

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