In vitro antioxidant activity of Lantadene A

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Abstract. Lantadenes are the pentacyclic triterpenoids present in the leaves of the plant *Lantana camara.* Pentacyclic triterpenes are often studied as their biological properties are considerable and contributes to the development of modern therapeutic drugs. In recent years various natural product based compounds are extensively studied for various pharmacological activities including antioxidant activity. Therefore, this study aims to determine the *in vitro* antioxidant activity of Lantadene A (from *Lantana camara*) as results will enhance the knowledge of the compound towards development of hepatoprotective agents. The antioxidant assays performed on the compound were the DPPH radical scavenging assay, nitric oxide assay, superoxide anions scavenging activity and iron chelating assay. Results showed promising antioxidant activities as the IC_{50} for Lantadene A in the above assays were 6.574 mg/ml, 0.098 mg/ml, 2.506 mg/ml and 0.001 mg/ml respectively as compared to the standards (BHT, ascorbic acid and EDTA) 0.027 mg/ml, 0.075 mg/ml, 1.025 mg/ml and 0.47 mg/ml respectively. The DPPH radical scavenging assay for Lantadene A was weaker than BHT while iron chelating assay of Lantadene A is much stronger than the standard. The rest of the assays were comparable to the reference standards. These results suggest that Lantadene A can be a potential cadidate to be developed as an antioxidant.
Keywords: Lantadene A, Antioxidant activity, triterpenoids, free radicals

Introduction

Lantadenes is the pentacyclic triterpenoids present in the leaves of the plant *Lantana camara* (Hart *et al*., 1976; Sharma *et al*., 1988; Sharma & Sharma 1989). Lantadenes and other triterpenoids have a number of biological activities like inhibition of activation of Epstein–Barr virus, anti-AIDS, anti-inflammatory, antimicrobial, antitumor and antimutagenic. In addition, pentacyclic triterpenes are also widely used to develop modern therapeutic drugs. The presence of antioxidants in Lantana camara has been found in previous studies (Mayee & Thosar, 2011; Bhakta & Ganjewala, 2009). Phytochemicals such as alkaloids, terpenoids, phenolics, glycosides, saponins, flavonoids, tannins and phytosterols are also present in higher amounts in younger leaves.

However, the results of antioxidant activities reported are contradicting and inconclusive. Mayee & Thosar (2011) reported high scavenging activity from the Lantana camara extract and these antioxidants found were proposed to have played a part in the antiurolithiatic properties of the plant. Mensor *et al*. (2001) reported that plants from the Verbenaceae family showed lower EC50 values than the other plant extracts. On the other hand, Saini *et al*. (2007) concluded that this plant produces severe hepatotoxicity in rats, but it also prevented lipid peroxidation, that may suggest, the Lantana camara may be acting as antioxidant. Besides the inconsistent results reported from past literature, all antioxidant studies have been done mainly on the plant extract itself, none on Lantadene A. This study is to screen Lantadene A for any antioxidant activities as it is a major compound from *Lantana camara* L.

Material and Methods

Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) reagent, butylated hydroxytoulene (BHT), methanol, nitro blue tetrazolium (NBT), phosphate buffer, nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), ascorbic acid, sodium nitroprusside, Griess reagent, iron(II) chloride ($FeCl_2$), ferrozine, ethylenediaminetetraacetic acid (EDTA). All chemicals were purchased from Sigma Aldrich.

Plant material

Lantadene A (LA) compound from the plant *Lantana camara* L. is purchased from Chemtron Biotechnology, Penang.

DPPH Radical Scavenging Assay

Lantadene A (LA) is first tested in a series of different concentration to obtain the IC_{50} value. A two-fold serial dilution is then done for LA from 10 mg/ml to 0.156 mg/ml. A volume of 10 µl of LA from different concentration is also mixed with 1 ml of 0.004% DPPH solution in 80% methanol. For the mixture without LA, it is used as a control and spiked with 10 µl of blank methanol; while the commercial antioxidant, butylated hydroxytoluene (BHT) is used as a positive control. The reaction mixture is then incubated in a dark room for 30 minutes.

Discoloration was subsequently measured at a wavelength of 517 nm, where all measurements are to be done in triplicates. The DPPH radical scavenging activity is calculated using the following equation:

$$\text{Scavenging effect (\%)}: \frac{(Ao - A1)}{Ao} \times 100\%$$

Ao = absorbance of the control reaction
A1 = absorbance in the presence of LA

Finally, the IC_{50} (concentration providing 50% inhibition) is calculated graphically using a calibration curve in the linear range by plotting the compound concentration vs the corresponding scavenging effect.

Superoxide Radical Scavenging Assay

The reaction mixture consisting of 1ml of nitro blue tetrazolium (NBT) solution (156 µM NBT in phosphate buffer, pH 7.4), 1 ml NADH solution (468 µM NADH in phosphate buffer, pH 7.4), and 1ml of sample solution of Lantadene A; are mixed together. The reaction is started by adding 100 ml of phenazine methosulfate (PMS) solution (60 µM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture is then incubated at 25 ^{o} C for 5 min and following that, the absorbance is measured at 560 nm against blank sample and compared with standard, ascorbic acid. Decreased absorbance of reaction mixture indicates increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation is calculated using the following formula:

$$\text{Scavenging effect (\%)}: \frac{(Ao - A1)}{Ao} \times 100\%$$

Ao = absorbance of the control reaction
A1 = absorbance in the presence of LA

Nitric Oxide Scavenging Activity

The LA and BHT are prepared in different concentration through a series of two fold dilution. A volume of 0.5ml sample are mixed with 1ml of 5mm sodium nitroprusside and left to be incubated at 25°C for 150 minutes. To make Greiss reagent (1% sulphanilannide, 2% phosphoric acid, 0.1% N-(1-naphthyl) ethylenediamine), 2ml of phosphoric acid is to be added into 98ml of PBS, followed by 1g of sulphanilamide and 0.1g of N-(1-naphthyl) ethylenediamine dihydrochloride into that PBS solution. After incubation of 150 minutes, the solution containing LA and sodium nitroprusside is mixed with 1ml of Greiss reagent. The reaction mixture is then read at a wavelength of 546nm. The blank is the sodium nitroprusside reagent without sample. The scavenging activity and IC_{50} values were calculated as below:

$$\text{Scavenging effect (\%)}: \frac{(Ao - A1)}{Ao} \times 100\%$$

Ao = absorbance of the control reaction
A1 = absorbance in the presence of LA

The IC_{50} (concentration providing 50% inhibition) is calculated graphically using a calibration curve in the linear range by plotting the compound concentration vs the corresponding scavenging effect.

Metal Chelating Activity

First, the samples (Lantadene A and standard antioxidant, EDTA; two-fold serial dilution from 10 mg/ml to 0.156 mg/ml) are added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction is then initiated by an additional 5 mM ferrozine (0.2 ml), following which the mixture is to be shaken vigorously. After that, leave the mixture standing at room temperature for 10 mins. Subsequently, the absorbance of the resulting solution is to be measured at 562 nm. The metal chelating activities are then calculated by the given formula:

$$\text{Scavenging effect (\%)}: \frac{(Ao - A1)}{Ao} \times 100\%$$

Ao = absorbance of the control reaction
A1 = absorbance in the presence of LA

The IC_{50} (concentration providing 50% inhibition) is calculated graphically using a calibration curve in the linear range by plotting the compound concentration vs the corresponding scavenging effect.

Results and Discussion

DPPH radical is a commonly used substrate for fast evaluation of antioxidant activity because of its stability in the radical form and simplicity of the assay (Bozin *et al*., 2008). This assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang *et al*., 2005). The principle behind this assay is in the colour change of DPPH solution from purple to yellow as the radical is scavenged by the antioxidant (Karagozler *et al*., 2008).

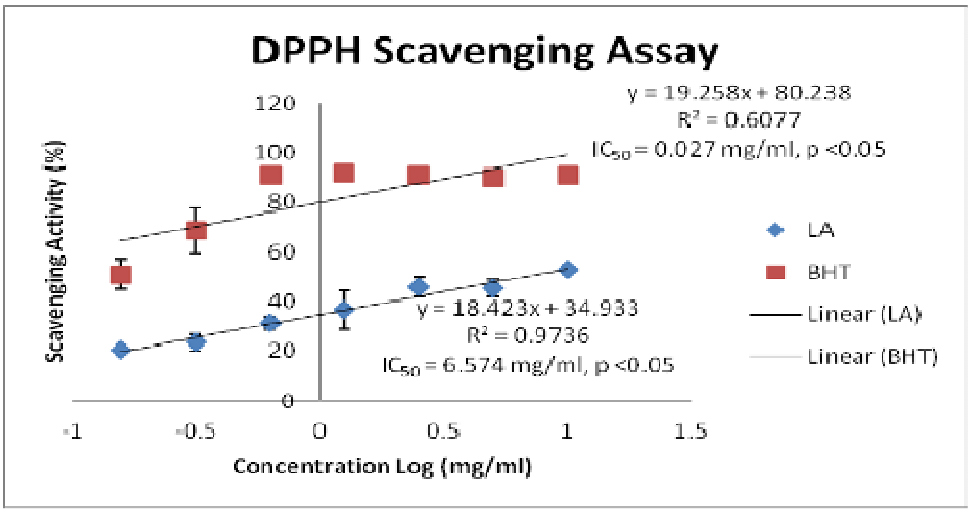

Figure 1. The IC_{50} value obtained by linear regression equation for Lantadene A and BHT.

Table 1. Mean values of IC_{50} of Lantadene A and standard antioxidants in antioxidant assays. Mean IC_{50} values in the same row followed by a different superscript indicate significant differences (P< 0.05).

Crt. No.	Antioxidant Assays	IC_{50} value of LA (mg/ml)	IC_{50} value of standard (mg/ml)
1.	DPPH radical scavenging activity	6.574[a]	0.027[b]
2.	Superoxide Anion Scavenging Assay	2.506	1.025
3.	Nitric Oxide	0.098[a]	0.075[b]
4.	Metal Chelating Activity	0.470[a]	0.001[b]

From the graph shown in Figure 1, BHT has a higher scavenging activity as compared to LA. The DPPH radical scavenging activity was found to be increasing as dose increases. At 10 mg/ml, BHT's scavenging activity of the DPPH radical is 90.78% while LA gives a value of 53.11%. This shows that LA has lower DPPH radical scavenging abilities than the commercial antioxidant. Moreover from table 1, the IC_{50} of LA is 6.574 mg/ml while BHT gives an IC_{50} of 0.027 mg/ml. Bascially, a higher DPPH radical scavenging activity is associated with a lower IC_{50} value. The scavenging activity of LA is significantly lower than BHT as $p<0.05$. Despite that LA has weaker antioxidant activities from BHT, LA still shows quite a potential to be a natural antioxidant and can be used as a therapeutic source.

J.G. de Melo *et al*. (2010) reported that the IC_{50} in the leaves extract of *Lantana camara* L. was 114.63±6.16 ug/ml for DPPH radical scavenging assay. Sanjeeb Kalita *et al*. (2012) performed a similar test and IC_{50} in the leaves extract of *Lantana camara* L. was found to be 42.66 µg/ml. Mayee *et al*. (2011) showed that the IC_{50} of the extract was 578.63 µg/ml while Bhakta and Ganjewala (2009) measured the DPPH scavenging activity in *Lantana camara* L. leaves from different positions of the tree to be in the range of 54% - 62%.

Bhakta and Ganejewala's findings support this study's results where at 10mg/ml, DPPH scavenging activity was reported to be 53.11%. Past literature showed inconsistent IC_{50} values for *Lantana camara* L. but even so, the leaves extract had higher values of IC_{50} as compared to the pure compound of Lantadene A from the plant. This means that there are other antioxidant compounds from the plant extract that contributes to the high antioxidant activity as reported by J. Patel *et al*. (2011) where the presence of other phytochemicals such as saponin, steroids, alkaloids, tannins, polyphenols, terpenoids and flavonoids were confirmed. Lantadene A even though a weaker antioxidant still contributes to the overall free radical scavenging purported by oxidation and stress.

Superoxide anion radicals are produced endogenously by flavoenzymes like xanthine oxidase, which converts hypoxanthine and subsequently to uric acid (Basha *et al*., 2011). Superoxide radicals are generated by PMS-NADH coupling from dissolved oxygen. Subsequently, superoxide radicals are capable of being measured on their ability to reduce NBT. Accordingly, through the decrease in absorbance at 560nm with the compound, that indicates their ability to quench superoxide radicals in the reaction mixture (Gulcin *et al*., 2005). This thus exhibits a dose dependent increase in superoxide scavenging activity.

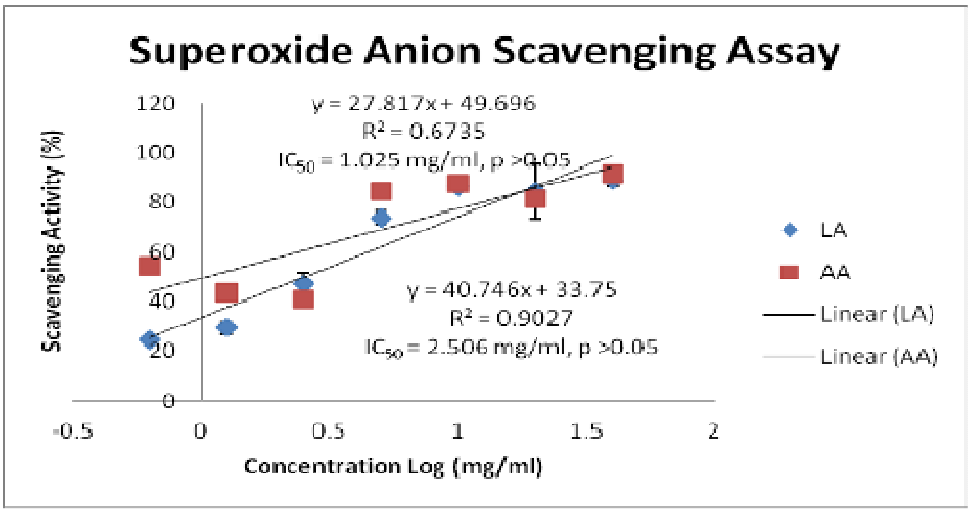

Figure 2. The IC_{50} value obtained by linear regression equation for Lantadene A and ascorbic acid.

From the graph shown in Figure 2, ascorbic acid has a higher superoxide anion scavenging activity as compared to LA. The superoxide anion scavenging activity was found to be increasing as dose increases. At 10 mg/ml, AA's scavenging activity of the superoxide anion is 87.61% while LA gives a value of 86.57%. This shows that LA has superoxide anion scavenging abilities that are comparable to the commercial antioxidant, ascorbic acid. The IC_{50} of LA obtained from table 1 is 2.506 mg/ml while AA gives an IC_{50} of 1.025 mg/ml. Using T-independent test of SPSS version 20.0 to analyze both scavenging activities, it is found that both results are not significantly different ($p>0.05$), meaning that LA's potential as an antioxidant is similar to ascorbic acid.

Superoxide anion is also very harmful to cellular components (Korycka-Dahl & Richardson, 1978). Robak and Glyglewski (1988) reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. As shown in Figure 3, the superoxide radical scavenging activities of Lantadene A and the reference standard are increased markedly with increasing concentrations. The results suggest that Lantadene A is a potent scavenger of superoxide radical as the standard ascorbic acid.

As a potent pleiotropic mediator, nitric oxide (NO) possesses smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. Apart from that, it is also a diffusible free radical capable of playing a variety of roles as an effector molecule in a range of biological systems. That includes neuronal messenger, vasodilation and antimicrobial and antitumour activities. While NO and superoxide radicals are involved in host defense, excessive production of these radicals contributes to the pathogenesis of certain inflammatory diseases. In addition, the toxicity of NO greatly increases when it reacts with superoxide radical, allowing the formation of the highly reactive peroxynitrite anion (ONOO-). Whereas its protonated form, peroxynitrous acid (ONOOH); is a very strong oxidant (Malinski T., 2007).

The main route of damage is the nitration of hydroxylation of aromatic compounds, particularly tyrosine; where under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid, responsible for oxidative damage of proteins in living systems (Szabo *et al*., 2007; Burney *et al*., 1999). Also in nitric oxide assay, NO generated from sodium nitroprusside reacts with oxygen forming nitrite (Hazra *et al*, 2008). The compound thus inhibits nitrite formation by directly competing with oxygen in reaction with nitric oxide.

From the graph shown in Figure 3, BHT has a higher inhibition activity as compared to LA. The nitrite inhibition activity was found to be increasing as dose increases. At 500 µg/ml, BHT's inhibition activity of nitrite formation is 90.03% while LA gives a value of 72.12%. This shows that LA has lower nitric inhibition activity than the commercial antioxidant. Besides that, the IC_{50} of LA in table 1 is 0.098 µg/ml while BHT gives an IC_{50} of 0.075 µg/ml. The inhibition activity of LA is significantly lower than BHT as $p<0.05$. Mayee *et al*. (2011) reported that the IC_{50} values for the inhibition of NO induced free radicals by *Lantana camara* L. extract were found to be -789.37 µg/ml compared to ascorbic acid with an IC_{50} value of -7.04 µg/ml.

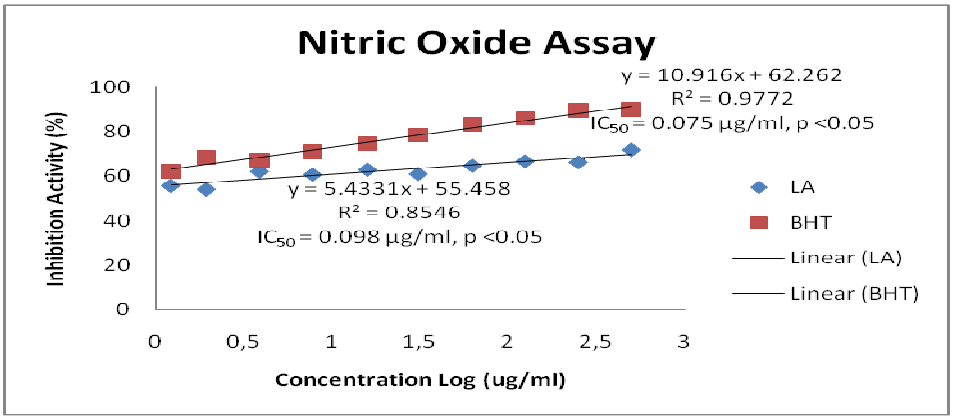

Figure 3. The IC_{50} value obtained by linear regression equation for Lantadene A and BHT.

By the Fenton reaction, lipid peroxidation can be stimulated by iron, whilst also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. These two can themselves extract hydrogen and perpetuate a chain reaction of lipid peroxidation (Gulcin *et al*., 2003; Halliwell B., 1991). The method of Dinis *et al*. (1994) was used to estimate the ferrous ions chalting activity by Lantadene A and its standard. Complexes with Fe^{2+} can be quantitatively form by Ferrozine. Complex formation is disrupted in the presence of chelating agents, resulting that the red colour of the complex decreases. Thus the measurement on the reduction of colour allows estimation of the chelating activity of the coexisting chelator. For this essay, the formation of ferrous and ferrozine complex was interfered by LA and EDTA. This suggests they possess chelating activity and captures ferrous ion before ferrozine. Metal chelating capacity was also significant since it caused the reduction on the concentration of the catalyzing transition metal in lipid peroxidation. According to a report, chelating agents forming σ-bonds with a metal, are effective as secondary antioxidants, due to their ability to reduce redox potential, thus stabilizing the oxidized form of metal ion (Gulcin *et al*., 2005).

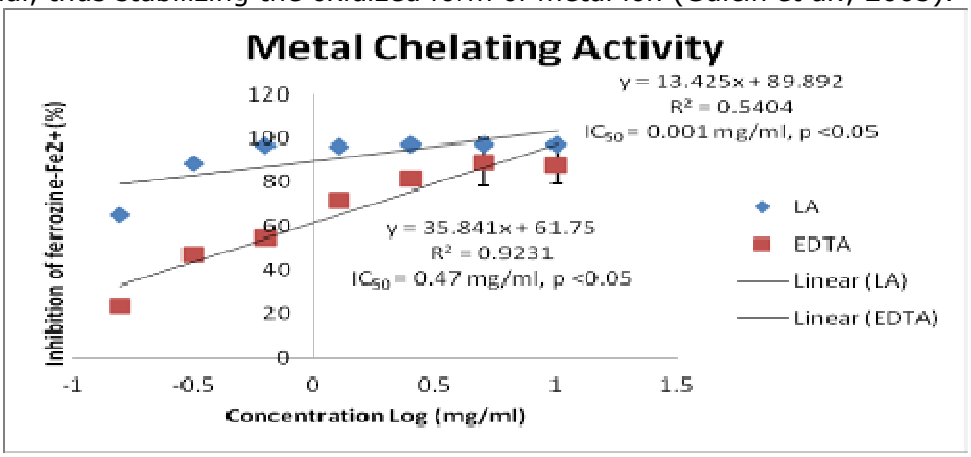

Figure 4. The IC_{50} value obtained by linear regression equation for Lantadene A and EDTA.

From the graph shown in Figure 4, LA is able to interfere with the formation of ferrozine-Fe^{2+} complex with an IC_{50} value of 0.001 mg/ml (Table 1). The chelating activity in LA is even higher than that of the standard, EDTA which has an IC_{50} of 0.47 mg/ml. The scavenging activity of the standard antioxidant is significantly lower than LA as $p<0.05$. This study shows that LA has a marked capacity for iron binding, suggesting the presence of triterpnoids that has potent iron chelating capacity.

Conclusions

On the basis of the results obtained in the present study, it is concluded that Lantadene A from the plant *Lantana camara* L. exhibits high antioxidant and free radical scavenging activities. It is also a more potent iron chelator compared to the commercial antioxidant. These *in vitro* antioxidant assay indicate that this compound is a natural source of antioxidant and might be helpful in lowering the hepatotoxic effects

from the plant itself. Therefore, further investigation is needed to understand the antioxidant activities in combating hepatotoxic effects of *Lantana camara* L.

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