In vitro and *in vivo* antioxidant analysis of *Acalypha alnifolia* Klein ex Willd.

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ABSTRACT

Herbs have been utilized to treat acute and chronic disorders for thousands of years. Natural products, due to their easy availability, low cost and lack of side effects, make themselves attractive candidates for drug research. One such indigenous medicinal plant, *Acalypha alnifolia* Klein ex Willd. (Euphorbiaceae) was selected for this study. There is no systematic and biological activity screening that has been undertaken on this plant. The objective of the present study was to examine the antioxidant potential of *A. alnifolia* leaves. The free radical scavenging effect and reducing power were analyzed in methanol and aqueous extracts of *A. alnifolia* leaves. The results highlighted both extracts exhibited better antioxidant activity. The results of *in vivo* antioxidant activity showed *A. alnifolia* were effective in restoring the activities of the oxidant stressed groups to normal levels. The leaf methanolic extract showed a better response compared to leaf aqueous extract. This study is the first report on antioxidant analysis of *A. alnifolia.* Based on findings, the leaf extracts may be used as an effective antioxidant agent to combat various ailments caused by the free radicals.

Keywords: *Acalypha alnifolia*, antioxidant, crude extracts, free radicals, Euphorbiaceae.

INTRODUCTION

Oxidation and the production of free radicals are an integral part of human metabolism. Oxygen free radicals or more generally reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living system [1]. Reactive oxygen and nitrogen species can attack various substrates in the body including lipids, nucleic acids and protein. Oxidation of any of these substrates can theoretically contribute chronic diseases such as cancer, cardiovascular diseases and age related muscular degeneration and to aging [2]. Production of ROS and defense system against them are balanced well in the living body. This balance is very much important for the maintenance of physiological condition. The collapse of the balance due to pathogenic infection and inflammation will induce the production of ROS and results in ROS toxicity. To control this toxicity the endogenous defense system starts its action against those pathogens to control ROS toxicity indirectly.

In addition to the endogenous defenses, consumption of dietary antioxidants could be an important aspect of body's defense mechanism to protect against free radicals, such as superoxide anion radicals, the hydroxyl radicals and other ROS, and also many antioxidants are being identified as anticarcinogens [3]. Many antioxidant substances derived from dietary or medicinal plants are known to be effective, versatile chemopreventive and antitumor agents in a number of experimental models of carcinogenesis [4]. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Many of these phytochemicals

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possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases [5]. It has been reported that there is an inverse relationship between the antioxidative status occurrences of human diseases [6]. In addition, antioxidant compounds which are responsible for such antioxidants activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders [7,8]. Considering the above information, the present investigation was carried out to test the antioxidant potentials of pulverized leaf extracts of *A. alnifolia* through *in vitro* and *in vivo* approaches.

MATERIALS AND METHODS

Plant collection, identification and extraction of plant materials

Fresh and healthy leaves of *A. alnifolia* were collected randomly from the region of Yercaud and Kolli hills, Tamil Nadu, South India. The nomenclature of collected plant materials was identified and authenticated by Botanical survey of India, Southern circle, India [No:BSI/SRC/5/23/2010-11/Tech-1506]. The herbarium specimen was deposited in the Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem, Tamil Nadu. The collected leaves were shade-dried and coarsely powdered by using mixer grinder. These coarse powders (25 g) were taken for successive extraction in various solvents such as methanol, chloroform, ethyl acetate and petroleum ether (each 250 ml) by using Soxhlet apparatus. The collected extracts were vacuum dried and stored for further investigation. Simultaneously, aqueous was used for extraction of plant powder and the extracts were stored. The DMSO (dimethyl sufloxide) acts as dissolving agent for these extracts.

In vitro antioxidant analysis

Reducing power assay

The reducing power assay was carried out according to the method described by Yen and Chen [9]. Sample in 1ml distilled water was mixed with 2.5ml of 0.2M phosphate buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50ºC for 20 minutes. 2.5 ml of 10% TCA was added and centrifuged for 10 minutes at 1000g. To 2.5ml of the upper layer, 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ was added and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reductive potential.

DPPH radical scavenging activity

The scavenging of DPPH radical was carried out as per the method of Hsu et al. [10]. Aliquots of 1 ml of the extract and 5 ml of freshly prepared 0.1mM DPPH methanolic solution were thoroughly mixed and kept for 50 minutes in dark. The absorbance of the reaction mixture at 517nm was read in a spectrophotometer. Methanol (1ml) replacing the extract serves as positive control. Methanol was used as blank. The percentage of free radical scavenging effect was calculated as, scavenging effect (%) = [(A517control – A517test)/A517control] × 100, where A517 control = absorbance of the control at 517nm and A517test = absorbance of the test at 517nm.

In vivo antioxidant analysis

The animals were randomly divided into 5 groups and the experimental period was 21 days. All the treated rats received 10% ethanol in drinking water throughout the experimental period to induce cytochrome P450 2E1, the isoenzyme that metabolizes CCl_4 to its oxidant form. CCl_4 was

administered as a single subcutaneous injection on 21 alone (2ml/kg body weight) diluted 1:1 in paraffin oil. Animals were divided into 5 groups, viz. group I: untreated control, group II: ethanol+CCl_4, group III: ethanol+CCl_4+leaf methanol (LM), group IV: ethanol+CCl_4+leaf aqueous (LA), group V: ethanol+CCl_4+Silymarin (standard antioxidant).

The aqueous and methanolic extracts of the leaves of *A. alnifolia* was administered at 200 mg/kg body weight/day, while silymarin was administered at a dose of 100mg/ kg body weight/day. Both the plant extracts and silymarin were administered by gastric intubation tube for 21 days. On day 21, CCl_4 injection was given subcutaneously and the zero animals were sacrificed by cervical dislocation on day 22. The response elicited by *A. alnifolia* leaf extracts was compared with a standard antioxidant silymarin. The animals were dissected quickly and the blood was collected by cardiac puncture. The collected blood was centrifuged, serum was separated and the samples were stored at -85ºC, until analysis. Oxidative stress markers as thiobarbituric acid reactive substances (TBARS) [11], reduced glutathione [12], glutathione-S-transferase [13] and catalase [14] were also assayed. All the results are statistically analyzed.

RESULTS AND DISCUSSION

The leaf aqueous extract was very potent when compared to other form of extracts. The leaf aqueous extract reduces the ferric cyanide to ferrous cyanide. The aqueous extract of the leaf shows higher reductive activity than other solvent extracts (Table 1). The quantitative scavenging activities of aqueous and various solvents extract of leaves of *A. alnifolia* were tested against DPPH by quantitative spectrophotometric assay. DPPH, a stable free radical with a characteristic absorption at 517 nm was used to study the radical scavenging effects of extracts. The concentration of DPPH was highly decreased in the aqueous extracts of the leaves (Table 1). The antioxidants activities such as TBARS, CAT, GST and GSH are induced by the use of ethanol with CCl_4 exposure and the plant extract (*A. alnifolia* leaf extracts) was found to be certain protection against these tests in wistar albino rats. Ethanol stress caused a significant ($P<0.05$) increase in the activities of total protein, which was further augmented by CCl_4 administration. The treatment with the LM and LA of *A. alnifolia* were effect in restoring the activities of the oxidant stressed groups to normal levels. The LM showed a better response compared to LA.

The basal specific activity of catalase was found to be higher. From the results, it can be deduced that ethanol stress caused a significant ($P<0.05$) decline in the activities of catalase, which was further augmented by CCl_4 exposure. The LM and LA extracts of *A. alnifolia* were very effective in reverting back the changes in the status of catalase activity caused by CCl_4 stress to a greater extent which can be evidenced by significantly higher activities of catalase found in leaf extracts of *A. alnifolia* treated groups than untreated control. LM extracts was more potent as evidenced by the significant increase in the activities. The specific activities of GST in the control groups were significant ($P<0.05$) reduction in the GST activity upon ethanol exposure. Administration of CCl_4 further reduced the activity of GST. Treatment with *A. alnifolia* leaf extracts at all the time points selected caused a significant elevation ($P < 0.05$) in the activity of GST compared to control rats. It is evident from the table 2 that the plant extracts help in suppressing the damage caused by CCl_4. The levels of GSH in rats were varied and depend on their exposure to ethanol and CCl_4 in the presence or absence of either *A. alnifolia* leaf extracts or silymarin drugs. The levels of GSH were significantly ($P<0.05$) decreased upon ethanol stress, which was further significantly reduced on CCl_4 assault of rats. Treatment with the leaf extracts of *A. alnifolia* increased the GSH content. The effects evoked by the *A. alnofolia* leaf extracts are comparable to those induced by silymarin.

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has

been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidant activity [15].

Table 1. Reducing power assay and DPPH radical scavenging activity of aqueous and organic solvent extracts from the leaves of *A. alnifolia.*

Leaf extract	Reducing power assay (absorbance at 770nm)	DPPH radical scavenging activity (% scavenging at 570nm)
Aqueous	0.58035	91.565
Chloroform	0.05405	52.555
Petroleum ether	0.03155	48.340
Methanol	0.47530	88.760

Table 2. Antioxidant effect of leaf extracts of *A. alnifolia* on oxidative stress markers in blood plasma of Wistar albino rats.

Group	TBARS (nmol MDA liberated/mg protein)	Catalase (µmol H_2O_2 used/min/mg of protein)	GSH (µg GSH/mg of protein)	GST (mg protein/mL of enzyme)
Group I (Control)	0.241±0.011	36.49±0.430	2.150±0.017	1.165±0.010
Group II (CCl_4)	$1.442±0.014^c$	$15.25±0.432^c$	$1.395±0.010^c$	$0.060±0.008^c$
Group III (CCl_4+LM)	$0.871±0.024^f$	$31.24±0.767^f$	$2.063±0.015^f$	$1.977±0.052^f$
Group IV (CCl_4+LA)	$0.991±0.017^f$	$24.96±0.168^f$	$1.903±0.016^f$	$1.717±0.024^f$
Group V (Silymarin)	$0.588±0.014^f$	$31.58±0.724^f$	$2.122±0.014^f$	$2.182±0.014^f$

Values are expressed as mean ± SD for five animals in each group; $^aP<0.05$, $^bP<0.01$ and $^cP<0.001$ between control and CCl_4 group values; $^dP<0.05$, $^eP<0.01$ and $^fP<0.001$ between CCl_4 and drug treated groups.

The presence of reductants in the extracts causes the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form. The yellow colour of the test solution was changed to various shades of green and blue depending upon the reducing power of the sample. The reducing power increased with increasing the amount of the extracts. The reducing power of compounds serves as indicator of its potential antioxidant activity. The absorbance of aqueous extracts of leaves of *A. alnifolia* was found to be higher. Increased absorbance of the extract indicates the increased reducing power. Antioxidant donates protons to the radical (DPPH) and thus absorption decreases. The decrease in absorption is taken as measures of extend of radical scavenging. The aqueous and methanolic extracts of leaves were subjected to screening for their possible antioxidant activity. All the extracts show some degree of free radical scavenging activity. The aqueous and methanol extracts of leaves seems to be fairly significant.

In agreement with our results, the aqueous extract of *Piper betel* leaves was found to possess maximum DPPH quenching activity [16]. The aqueous extract of herb *Artemisa campestris* and leaves of *Azadirachta indica* and the methanol extract of *Leea indica* and *Spermacoce articularis* [17] showed strong DPPH radical scavenging activity. Based on the results, the present study is strongly suggests that the plant *A. alnifolia* leaves can be used in medicinal preparations to combat the disorders caused by oxidative stress. In future studies would concentrate on the isolation and

purification of the antioxidant compounds present in *A. alnifolia* which may act as a drug to control the diseases in human beings.

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