Chapter 4:

Abstract

Evaluation of in-*vitro* antioxidant and anti-inflammatory activity of Ethanol (EECGL) and Water extract (WECGL) of *Calotropis gigantea Linn* latex

4.1 Introduction

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Abstract

Calotropis gigantea (of family Asclepiadeaceae), a wildly growing plant, exhibits antipyretic, antifertility, anti-diarrhoeal, wound healing, insecticidal activities.The present study was undertaken to evaluate *in vitro* antioxidant and anti-inflammatory activities of the ethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex. Ethanolic and water extracts were prepared from pulverized powder obtained from shed-dried latex of *Calotropis gigantea*. The *in vitro* antioxidant activity was assessed using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide, hydroxyl radical, hypochlorous acid, superoxide anion lipid peroxidation, peroxynitrite free radical scavenging method. The anti-inflammatory activity of ethanolic and water extracts was investigated by *in vitro* methods as well as in carrageenan induced mice paw edema model for acute inflammation. The findings showed that EECGL exhibited increased 2, 2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide, hydroxyl radical, hypochlorous acid, superoxide anion lipid peroxidation, peroxynitrite free radical scavenging activities almost equal to the standard (ascorbic acid) and was far better than WECGL. EECGL showed significant HRBC membrane stabilizing activity when compared to the standard anti-inflammation drug, diclofenac sodium. EECGL and WECGL exhibited maximum 71 % and 68% inhibition in protein denaturation i.e. at 500 µg/ml. EECGL and WECGL at a dose level of 10, 20 and 50 mg/kg, prevented mice paw edema as compared to carrageenan control (at 3^{rd} and 4^{th} h) in carrageenan induced mice paw edema model while standard drug indomethacin showed prevention at the concentration of 10 mg/kg. So the results reveals that ethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex possess a prominent antioxidant, anti-inflammatory activities.So, these could be employed for developing new antioxidant and anti-inflammatory agents.

4.1. Introduction

Free radicals sometimes stated as oxidants, are chemical species containing unpaired electrons and they play a significant role in chemical reactions. Biological combustion and food spoilage reactions are carried on through free radical reactions which are important in biology and medicine (Prasad et al. 2009; Min and Boff, 2002). Up to 5% of O_2 breathed in daily may be transformed to free radicals such as hydroxyl, peroxyl, singlet oxygen and superoxide radicals (Prasad et al., 2009, Bellion et al., 2010), often termed as reactive oxygen species (ROS). ROS are endogenously produced from cellular metabolism and inflammatory reactions or by exogenous exposure to ionising radiation and xenobiotics(Bellion et al., 2010). Some antioxidant enzyme systems known to neutralize these ROS include glutathione S-transferase, glutathione peroxidase, superoxide dismutase and catalase system (Lee et al., 2007). Accumulated free radicals those are not manageable by antioxidants systems, take part in oxidation reactions that can produce damaging action to the body. Oxidations processes are essential in the energy management of all living organisms and are therefore are remained under stringent control by several mechanisms (Solomon and Fryhle, 2008). However, imbalance between the production and elimination of free radicals create cellular oxidative stress, which leads to oxidative cell damage. This damage triggers for diseases like inflammation, artherosclerosis, cancer, diabetes, Parkinson's diseases, multiple sclerosis liver diseases, muscular degeneration (Prasad et al., 2009; Min and Boff, 2002; Lee et al., 2007). The accumulated free radicals bind to DNA, RNA, protein and cell membrane and result in lipid peroxidation and the onset of various diseases (Reynertson et al., 2005; Goze et al., 2009). Oxidants trigger signal transduction pathways and modify the expression of growth and differentiation-related genes (Lee et al., 2007).

4.1.2 Natural anti-oxidants

Plants are a rich source of antioxidants which safeguard them from ultraviolet damage and against lipid peroxidation. Polyphenolic compounds, secondary metabolites of plants, such as flavonoids, phenolic acids, anthocyanidins and tannins, have noteworthy antioxidants and anticancer activities (Prasad et al., 2009).It has been found by many researchers that there is an inverse association between the mortality from age related diseases and the consumption of plant products (Sharma et al.,2012), which could be due to the presence of various antioxidant compounds, especially, phenolics, which are the most reactive compounds. Antioxidants present in plant products help in the stimulation of cellular defence system and biological system against oxidative damage.

Antioxidants provides defence to the living organism which is very crucial to survival and can prevent radical formation, repairing oxidative damage, intercepting formed radical, enhance the elimination of damage molecules and recognition of excessively damaged molecules, which are not being repaired but rather eliminated to prevent mutation.

Non-enzymatic antioxidants are classified according to the category whether they are water-soluble or lipid-soluble in the lipophilic region of cell membranes. The hydrophilic antioxidants include Vitamin C (ascorbic acid) and certain polyphenol flavonoid while the lipophilic antioxidant includes ubiquinols, retinoids, carotenoids, apocynin, procyanidins, and tocophenols (Middleton et al., 2000).Other non-enzymatic antioxidants include antioxidant enzyme cofactors, oxidative enzyme inhibitors and transition metal chelators such as ethylene diamine tetra acetic acid (EDTA). Synthetic antioxidants, like butylatedhydroxyanisole and butylatedhydroxytoluene, have been developed, but their uses are very limitated as they are toxic to living cells.

Antioxidant properties by plants species is the key source and have a wide range of applications in human healthcare. Knowledge of potential antioxidant compounds present within a plant species does not necessarily indicate its antioxidant capacity, as the total antioxidant effect may be greater than individual antioxidant activity of one compound, due to synergism between different antioxidant compounds (Arnao et al., 1999).As plant produce a large number of antioxidantmolecules to control the oxidative stress caused by sunburns and oxygen, it is clear that plants may present a source of new compounds with antioxidant activity (Scartezzini and Speroni, 2000).

Recent investigations suggest that the plant derived antioxidants with free-radical scavenging properties may have great therapeutic value in free radical meditated disease like cancer.

Stress (Aging/Senescence /wounding /Xenobiotics/Radiation/Light Heat & cold Pathogens /Biotoxins Drought/Heavy Metals/Air pollutions (O3; SO2)/Hormones.

↓

ROS ($O2^-$, H2O2, $^{\cdot}OH$

↓

Oxidative Stress

↓

Molecular Damage (Lipids & Fatty Acids, Amino acids, Proteins, Nucleic Acids, Pigments.

↓

Cellular Effects (Membrane damage, Loss of Organelle Functions, Reduction in Metabolic Efficiency, Reduced carbon Fixation, Electrolyte Leakage, Chromatid Breaks Mutations)

↓

Cell Death

Figure 4.1 Stressors or initiators of reactive oxygen species (ROS) and the biological consequences leading to variety of physiological dysfunctions and cell death.

4.1.3 Inflammation and anti-inflammatory agents

Inflammation is derived from the Latin word '*inflammation*'which means burn, is part of the complex biological response of body tissues to harmful stimuli, such as damaged cells pathogens, or irritants (Ferrero-Miliani et al., 2007).Inflammation is a complex response to local injury or other trauma; it is characterized by heat, redness, swelling and pain. Inflammation involves various immune-system cells and many mediators. Assembling and regulation of inflammatory response is not possible without controlled migration of leukocyte populations. Inflammation has very important role to protect an organism against local injury and infections, however inflammation may turn to chronically harmful or painful state which required pharmacological treatment (Gao et al.,1996; Laupattarakasem,2003). Inflammatory diseases like asthma, rheumatoid arthritis, colitis and hepatitis are most leading diseases which cause death and disability (Jiang and Ames,2003). Chronic inflammation also leads to develope into more dangerous diseases like cancer, neurodegenerative and cardiovascular disorders (Jiang and Ames, 2003).

Inflammation is a fundamental biological response of immune system towards tissue damage and infection (Ashley et al., 2012). Acute inflammatory reactions are characterized by the increased movement of plasma and cells of innate immune system, such as neutrophils and macrophages, from the blood into the location of tissue damage. Fundamental signs of inflammation are edema, hyperalgesia, and erythema and they develop immediately

following incutaneous injection, resulting from action of proinflammatory agents like bradykinin, histamine, tachykinins, complement and reactive oxygen, and nitrogen species. Inflammation arealso expressed by increased blood flow, elevated cellular metabolism, vasodilatation, release of prostaglandins, serotonin, cytokines and extravasation of fluids (Ferrero-Miliani et al., 2007).Death and disability in the world has been increased from the typical inflammatory diseases such as rheumatoid arthiritis, asthma, colitis and hepatitis (Naik et al.,2000; Goudgaon et al.,2003 and Abreu et al.,2006).Many plants which have saponins have displayed significant anti-inflammatory activities possibly due to their non-glycosidic moiety. Many varieties of plant extracts have proved to be useful in experimental animal models of inflammation (de La Lastra and Villegas,2005; Liu et al.,2012; Lee et al.,2009).

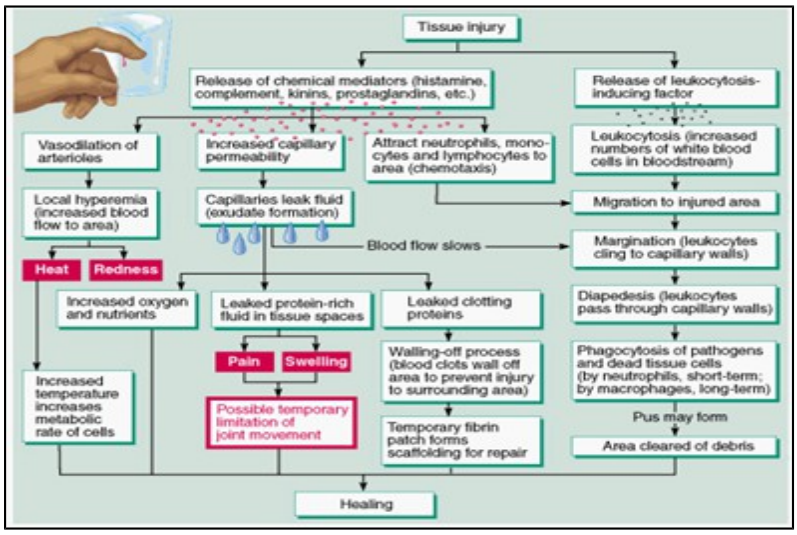

Figure 4.2 Inflammatory mediators

In recent times there is an increasing interest and awareness in medicinal plants and their preparations. Owing to safety concerns, the public prefer to use natural treatments from

natural materials such as fruits, spices, flowers, herbs, vegetables rather than using chemically synthetic agents. Thus the effective agents of natural origin are preferred to be developed and utilized. In the view of these scientific reports and the traditional usages of *C. gigantea,* the present study was aimed to investigate *in vitro* antioxidant and anti-inflammatory activities of the ethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex.

4.2 Materials and methods

4.2.1. Chemicals

Methanol, Evans blue (Lobachemie, India), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) , Taurine(Sigma-Aldrich, USA),sodium nitroprusside, ascorbic acid, orthophosphoric acid, ethylene diamine tetra acetic acid, 2-deoxy-2-ribose, hydrogen peroxide, phenazine methosulphate (SRL chemicals, India), Bovine serum albumin, sulphanilamine, orthophosphoric acid , naphthylethylene diamine-dihydrochloride,Ethylene diamine tetra acetic acid (EDTA), nitrobluetetrazolium (NBT), sodium chloride,Ferric Chloride ($FeCl_3$),sulphuric acid (H_2SO_4), 2-deoxy-2-ribose, phenazinemethosulfate (PMS),potassium chloride (KCl), ferrous sulphate ($FeSO_4$), tri chloro acetic acid (TCA),thio barbaturic acid (TBA),reduced nicotinamide adenine dinucleotide (NADH), n-butanol, pyridine,diethylene-tri-amine-pentaacetic acid (DTPA), evans blue,Folin-Ciocalteu (FC) reagent,dextrose, sodium citrate, citric acid,ascorbic acid (SRL chemicals, India), carrageenan, indomethacin, Diclofenac sodium other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; HimediaIndia, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai,India were purchased for the experimentation.

4.2.2 Collection of plant material and preparation of extracts (Maity et al., 2015)

This section has been discussed in chapter-3.

4.2.3*In-vitro* antioxidant activity

4.2.3.1 Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution spontaneously generates nitric oxide (NO) at physiological pH which interacts with oxygen to produce nitric ions and which can be estimated using Griess method. Scavengers of NO compete with oxygen, leading to reduced production of NO and pink colour chromophore is formed. One ml 10 mM sodium nitroprusside was mixed with 1 ml of each extract solution in various concentration with ascorbic acid (standard) in phosphate buffer at pH 7.4. Then this reaction mixture was incubated at 25ºC for 150 min. After that, 1 ml of incubated mixture was taken out and 1 ml of Griess reagent (1% sulphanilamine, 2 % orthophosphoric acid and 0.1% naphthylethylene diamine-dihydrochloride) was added to it(Garrat, 1964). The activity was measured spectrophtometrically at 546 nm.The experiment was carried out in triplicate. Percentage of inhibition was calculated below formula. The data were presented as Mean ± SEM .

$$Percentage\,inhibition = \frac{C - T}{C} \times 100$$

Where, C = Absorbance of the control and T = Absorbance of the test sample.

4.2.3.2 DPPH scavenging activity

DPPH has a deep violet colour in solution and it becomes colourless or pale yellow when neutralized. This property allows visual monitoring of the reaction.Different concentrations of each extract (2.8 ml) in methanol was mixed with 0.2 ml of 1,1-Diphenyl-2-Picrylhydrazine(DPPH)(100 µM in methanol), and standard ascorbic acid (50,100,150,200 µg/ml in methanol) was mixed together. The mixtures then left in the dark at room temperature for 30 min. Absorbance then measured using Shimadzu, Japan spectrophotometer at 517 nm(Brand-Willians et al., 1995). The percentage inhibition of DPPH radical was calculated using previously mentioned formula.

4.2.3.3 Hydroxyl radical scavenging activity

The hydroxyl radical, (·OH), is highly reactive, short lived neutral form of hydroxide ion (OH-).In living organism two major types of reactive oxygen species (ROS) is present, i.e. superoxide radicals and hydroxyl radical which continuously generates in oxygen to water conversion process in living body. At first 0.1 ml Ethylene diamine tetra acetic acid (EDTA),0.01 ml of Ferric Chloride ($FeCl_3$), 0.1 ml hydrogen peroxide (H_2O_2),0.36 ml of 2-deoxy-2-ribose, 1.0 ml of various concentrations of both latex extracts and 0.1 ml of ascorbic acid (50,100,150,200µg/ml) was dissolved together in distilled water along with 0.33 ml of phosphate buffer in sequence to perform the assay. The mixture was then incubated at room temperature for 1 h. The pink color developed when 1.0 ml of trichloroacetic acid was added and incubated in boiling water bath for 30 minutes (Hussein, 2011). The absorbance was

measured at 412nm and the percentage of inhibition is calculated by using the formula previously.

4.2.3.4 Hypochlorous acid scavenging activity

Myeloperoxide (MPO) catalyzes the formation of powerful antioxidant hypochlorous acid (HOCl) from H_2O_2 and Cl- . HOCl can inactivate α1-antiprotteinase (α1-AP) which can increase proteolytic activity at sites of pulmonary inflammation and other inflammatory disorders mediated by neutrophils. Freshly HOCl was prepared using 10% (v/v) of pH-6.2 solution of NaOCl and 0.6 M sulphuric acid (H_2SO_4). The concentration was determined by measuring the absorbance at 235 nm using molar extraction coefficient of 100 M^{-1} cm^{-1}. The reaction mixture contained 1.5 mM of HOCl and different concentration of the extract and ascorbic acid and was incubated for 1 hour at 37ºC.Taurine (30mM) was added to the mixture after incubation and incubated again for 30 min at 37ºC followed by the addition of 5-thio,2-nitro benzoic acid (TNB). Absorbance was measured at 412 nm against blank (Kutter et al.,2000)and percentage scavenging was calculated according to the previously mentioned formula.

4.2.3.5 Superoxide anion scavenging activity

Superoxideanion (O2-) is a very important paramagnetic anti-oxidant, with one unpaired electron and is a free radical like dioxygen. It is the product of the one-electron reduction of dioxygen (O_2), which is also, occurs in nature widely. The superoxide radicals generated from dissolved oxygen by phenazinemethosulfate-nicotinamide adenine dinucleotide (PMS/NADH) coupling which can be measured by their ability to reduce nitrobluetetrazolium (NBT).Measurement of superoxide anion scavenging activity was done

based on the previously described method with some modifications. All the solution was prepared in 100 mM phosphate buffer. Reaction mixture contains 1 ml of nitrobluetetrazolium (NBT), 1 ml of reduced nicotinamide adenine dinucleotide (NADH) and 3 ml of test samples in different concentrations. Then 100 µl of phenazinemethosulfate (PMS) incubated at 25ºC to initiate the reaction and observed at 560 nm to measure the percentage inhibition by previously mentioned formula(Dolai et al., 2012).

4.2.3.6 Lipid peroxidation scavenging activity

Lipid peroxidation was introduced by Fe2+ ascorbate system in rat liver homogenate by Ohkawa et al., 1979.Young adult male brain was dissected and homogenated with an ice-cold Tris buffer (20mM, pH -7.4) to produce a1/10 homogenate and centrifuged at 12,000 rpm at 4ºC for 15 min and the supernatant was used for *in-vivo* lipid peroxidation assay.The reaction mixture contain 30 mM potassium chloride (KCl), 0.16 mM ferrous sulphate ($FeSO_4$), 0.06 mM ascorbic acid and the latex extract in different concentration and incubated at 37 $^{\circ}C$ for 1 h. Lastly 1.0 ml of tri chloro acetic acid (TCA) (10% w/v) and 1.5 ml of thio barbaturic acid (TBA) (1% w/v) was added and the final volume was make to 4 ml by adding distilled water and kept it in water bath for 30 min(Ohkawa et al., 1979). On cooling 1 ml of distilled water and 5 ml of n-butane: pyridine (15:1v/v) was added to the reaction mixture and centrifuged at 4000 rpm for 10 min. Organic layer formed due to coloured malondialdehyde (MDA)-TBA complex, which was measured at 532 nm (Liu et al., 2000) .The percentage inhibition was calculated using previously mentioned formula.

4.2.3.7 Peroxynitrite scavenging activity

Heavy molecular weight cytotoxic intermediate peroxynitrite ($ONOO^-$) is produced by the reaction between superoxide anione ($O2^-$) and nitric oxide (NO). Synthesis of $ONOO^-$ is carried out according to the described method by Backmanet al. (Backmanet al.,1994).Five ml H_20_2 (prepared in 0.6 M HCl solution) and mixed with 5 ml of 0.6 M potassium nitrite (KNO_2) on an ice bath for few minutes and again of ice cold 1.2 M sodium hydroxide (NaOH) was added to the reaction mixture. Excess H_2O_2 was absorbed by granular manganese dioxide (MnO_2) and reaction mixture was set at -20ºC. The concentration of peroxynitrite ($ONOO^-$) was measured at 302 nm ($\acute{\varepsilon}=1670^{-1}cm^{-1}$).

An evans blue bleaching assay was used to measure peroxynitrite scavenging activity (Karmakaret al., 2011) with some modification. In the reaction mixture 50 mM of phosphate buffer (pH 7.4),0.1mM ofdiethylene-tri-amine-pentaacetic acid (DTPA), 90 mM of sodium chloride (NaCl), 5 mM of potassium chloride (KCl), 12.5µM of Evans blue were mixed with various doses of extract (50, 100, 150 and 200 µg ml^{-1}),1 mM freshly prepared peroxynitrite and final volume was made to 1 ml. All the above mixture was incubated at 25ºC for 30 min and observed at 611 nm spectrophotometrically. The percentage scavenging of $ONOO^-$was calculated using previously mentioned formula.

4.2.3.8 Determination of total phenolic content

Folin-Ciocalteu (FC) reagent was used to determine total phenolic content according to the reference method Lin and Tang, 2007 with slight modification.Oneml of FC mixed with 1 ml of latex extract and 45 ml distilled water in a conical flask shaken for 30 min. Around 3 ml of 2 % Na_2CO_3 was added to the mixture and shaken for 2 h at room temperature. At 760 nm the absorbance was measured against distilled water as blank. Total phenol value was obtained

from the regression equation: y = 0.00048x + 0.0055 and expressed as mg/g gallic acid equivalent using the formula, C = cV/M; where C = total content of phenoliccompounds in mg/g GAE, c = the concentration of gallic acid (mg/ml) established from the calibration curve, V =volume of extract and m = the weight of plant ethanolic/water extract.

4.2.4Anti-inflammatory activity

4.2.4.1*In-vitro* anti-inflammatory activity

4.2.4.1.1 Human red blood cell (HRBC) membrane stabilization assay

The blood was collected from healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with normal saline and a 10 % suspension was made. Various concentrations of extracts were prepared (50,100,150,200 and 500 µg/ml) using distilled water and to each concentrations, 1 ml of phosphate buffer, 2 ml of hypo saline and 0.5 ml of HRBC suspension were added. It was incubated at 37°C for 30 minutes and centrifuged at 3,000 rpm for 20 minutesand the supernatant solution was estimated spectrophotometrically at 560 nm(Gopalkrishnan et al., 2009). Diclofenac sodium was used as reference standard. The experiments were performed in triplicates and mean values of the three were considered.

The percentage (%) of HRBC membrane stabilization or protection was calculated using the following formula

$$Percent\ Protection(\ ) = \frac{100 - OD\ of\ drug\ treated\ sample \times 100}{OD\ of\ Control}$$

4.2.4.1.2 Inhibition of Protein denaturation

The method as prescribed was followed with slight modifications. The reaction mixture was consisting of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37°C for 20 minutes and thenheated to 51°C for 20 minutes after cooling the samples the turbidity was measured spectrophotometrically at 660 nm (Deshpande et al.,2009). Diclofenac sodium was taken as a standard drug. The experiment was performed in triplicates.

Percent inhibition of protein denaturation was calculated as follows:

$$Percent\ Inhibition = 100 - \frac{(OD\ of\ test - OD\ of\ product\ control) \times 100}{OD\ of\ control}$$

4.2.4.2*In-vivo* anti-inflammatory activity

4.2.4.2.1 Animals

Adult male Swissalbino mice weighing 18-25 g were used for the *in vivo*anti-inflammatory study. The mice were housed in poly acrylic cage (38x 23x10 cm). The animals were kept on a 12 h light: 12 h dark regime at 25 °C for 7 days before commencement of the experiment. The animals had free access to standard diet and water. Mice were deprived of food but not

water prior to administration of the test extracts. The study was approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests & Climate Change, and Govt. of India and performed in compliance with the relevant laws and guidelines of the CPCSEA.

4.2.4.2.2 Treatment Schedule

For screening *in vivo* anti-inflammatory activity for each of the extracts

Group I: Treatment with control (Distilled water); 10 ml/kg body wt.

Group II: Treatment with control (Ethanol); 10 ml/kgbody wt.

Group-III: Treatment with carrageenan

Group IV: Treatment with carrageenan induced ethanolic extract of latex of *C. gigantea* (Test); 5,10,20,50 and 75 mg/kg body wt.

Group V: Treatment with carrageenan induced aqueous extract of latex of *C. gigantea* (Test); 5,10,20,50 and 75 mg/kg body wt.

Group VI: Treatment with standard drug, indomethacin (10mg/kg body wt.)

4.2.4.2.3 Carrageenan-induced edema in rats

Paw swelling was induced by sub-plantar injection of 0.1 ml1% sterile carrageenan in saline into the right hind paw. The solventextracts of plant at dose of 10,20 and 50 mg/kg body wt.were administeredorally 60 minutes before carrageenan injection. Indomethacin (10 mg/kg) was used as reference drug. Control group received thevehicle only (10 ml/kg). The inflammation was quantified bymeasuring the volume displaced by the paw, using vernier calipers at time 0, 1, 2, 3, and 4 h after carrageenan injection(Winter et al.,1962).

The *in vivo*anti-inflammatory activity was calculated by using the relation T, Thickness of paw in control group; T0, Thickness of paw edemain the test compound treated group

$$of\ inhibition\ of\ edema = \frac{T - T0}{T} \times 100$$

4.2.5 Statistical analysis

Results are expressed as Mean±SEM and 50 % Inhibition concentration (IC_{50}) were calculated by plotting the data in graphs as concentration verses percentage of inhibition using Microsoft office Excel 2007.

Results of anti-inflammatory activity were expressed as Meanincrease in paw diameter ± SD. Results were analyzed using oneway ANOVA. Differences were considered as statistically significant $p<0.05$ are compared to control.

4.3 Results

4.3.1*In-vitro* antioxidant activities

The free radical scavenging activity of EECGL and WECGL was tested by nitric oxide scavenging assay using ascorbic acid as a reference standard. The test concentrations ranged from 50–200 µg/ml for EECGL, WECGL and ascorbic acid. The results are shown in Table 4.1 (Fig. 4.3) that revealed nitric oxide scavenging activity having IC_{50} values of 97.56±1.4 and 140.90±2.35 µg/ml for EECGL and WECGLrespectively.IC_{50} value of EECGL was nearer to IC_{50}value of ascorbic acid (standard) which was 62.95±1.25µg/ml.

Figure 4.4 showed the dose-dependent DPPH radical scavenging activity by the extracts and ascorbic acid. The IC_{50} values of EECGL and WECGL and ascorbic acid(Table 4.1) were found to be 100.68±1.58, 103.26±1.6 and58.42±1.12µg/ml respectively.

As in evident in the fig. 4.5, both the extracts showed dose-dependent hydroxyl radical scavenging activity. The IC_{50} value of EECGL and WECGL was found to be 119.80±1.85 and139.40±2.15 µg/ml respectively, which was comparable to that of standard ascorbic acid that showed IC_{50} of 117.80±1.92µg/ml.

Dose-dependent hypochlorous acid scavenging activity of both the extracts and ascorbic acid was found in this study (Fig 4.6). The IC_{50} values of EECGL and WECGL and ascorbic acid (Table 4.1) were found to be 105.95±1.35, 112.7±1.53 and 52.55±1.02 µg/ml respectively.

Superoxide radicals scavenging activity is shown in Fig. 4.7.EECGL and WECGL and ascorbic acid showed IC_{50} values of 147.95±2.67 and 170.00±2.8 and 59.85±1.21µg/ml respectively.

EECGL and WECGL extracts also inhibited lipid peroxidation in a dose-dependent manner (Fig4.8) as compared to ascorbic acid. The IC_{50} value of EECGL and WECGL (Table 4.1) was found to be 154.80±2.52 and 169.80±2.9 µg/ml respectively, which was comparable to standard ascorbic acid that showed IC_{50} of120.80±1.5µg/ml.

The peroxynitrite scavenging activity is shown in Figure 4.9. EECGL and WECGL showed IC_{50} values of153.53±2.25 and 165.40±2.8µg/ml respectively, which was comparable to standard ascorbic acid that showed IC_{50} of 120.58±1.15µg/ml.

Total phenolic content was determined using the Folin-Ciocalteu (FC) reagent and it was found to be 8.33 mg GAE/g and 5mg GAE/g in case of EECGL and WECGL respectively.

Table 4.1 The IC_{50}values of EECGL, WECGL and ascorbic acid for *in-vitro* antioxidant activities

In-vitro antioxidant activities	*IC_{50}(µg/ml)*		
	Ascorbic acid	*EECGL*	*WECGL*
NO Scavenging assay	*62.95±1.25*	*97.56±1.4*	*140.90±2.35*
DPPH Scavenging assay	*58.42±1.12*	*100.68±1.58*	*103.26±1.6*
Hydroxyl radical scavenging assay	*117.80±1.92*	*119.80±1.85*	*139.40±2.15*
Hyprochlorous scavenging assay	*52.55±1.02*	*105.95±1.35*	*112.7±1.53*
Superoxide anionscavenging assay	*59.85±1.21*	*147.95±2.67*	*170.00±2.8*
Lipid peroxidation scavenging	*120.80±1.5*	*154.80±2.52*	*169.80±2.9*
Peroxynitrite scavenging assay	*120.58±1.15*	*153.53±2.25*	*165.40±2.8*

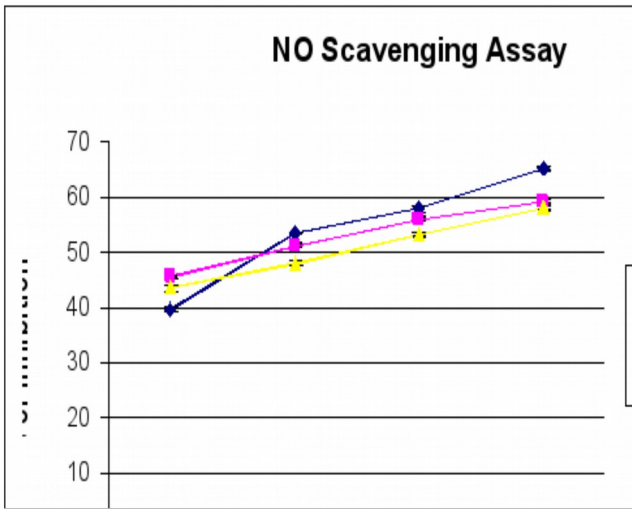

Figure 4.3 shows NO scavenging activity ofethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex and ascorbic acid. Each point represents the values obtained from three experiments and expressed as Mean±SEM.

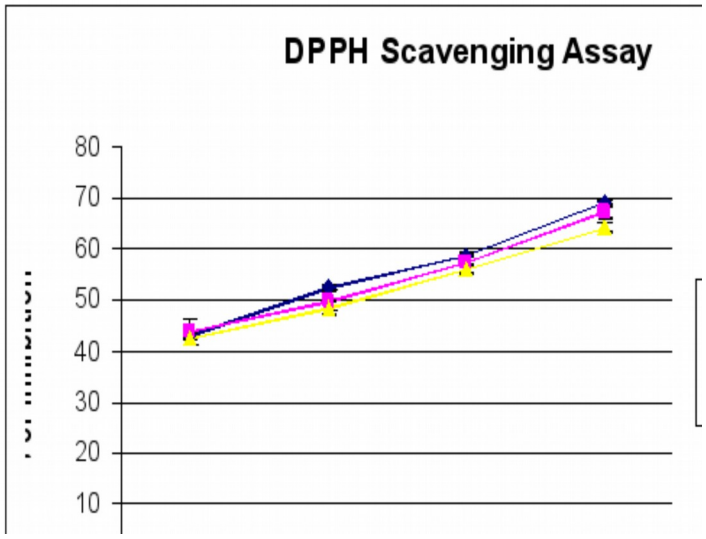

Figure 4.4 exhibits DPPH scavenging activity of ethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex and ascorbic acid. Each point represents the values obtained from three experiments and expressed as Mean±SEM.

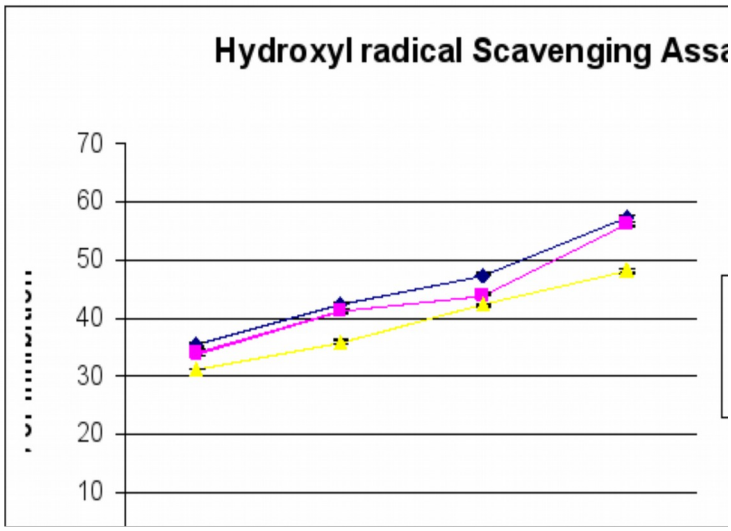

Figure 4.5 shows hydroxyl radical scavenging activity of EECGL, WECGL and ascorbic acid. Each point represents the value obtained from three experiments and expressed as Mean±SEM.

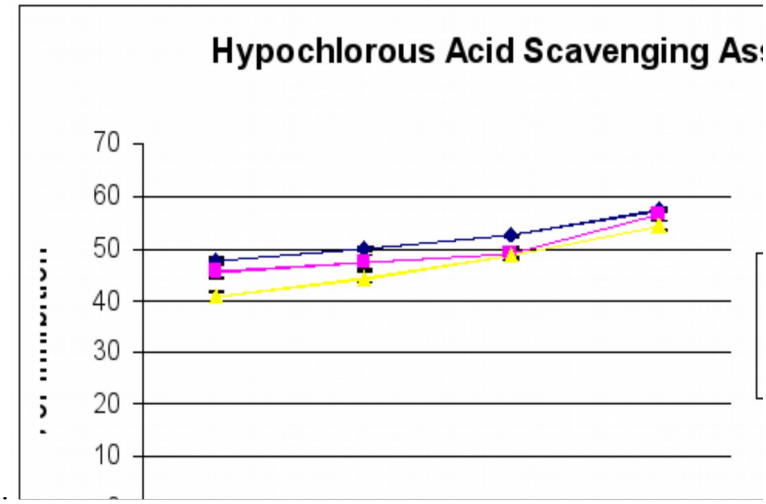

Figure 4.6. The bar diagram shows hypochlorous acid scavenging activity of EECGL, WECGL and ascorbic acid. Each point represents the value obtained from three experiments and expressed as Mean±SEM.

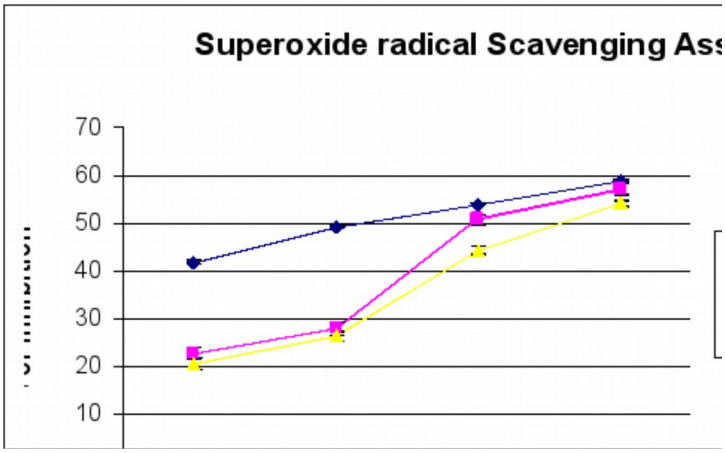

Figure 4.7 shows superoxide anion scavenging activity of EECGL, WECGL and ascorbic acid. Ea experiments and expressed as Mean

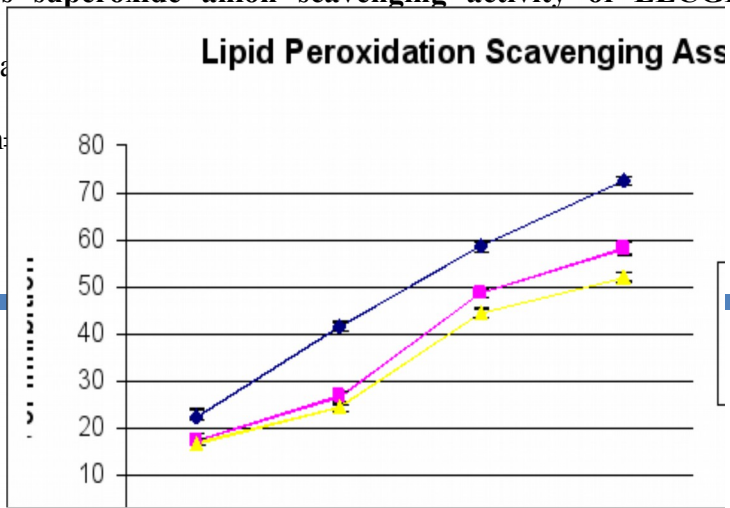

Figure 4.8 exhibits Lipid peroxidation scavenging activity of EECGL, WECGL and ascorbic acid. Each point represents the value obtained from three experiments and expressed as Mean±SEM.

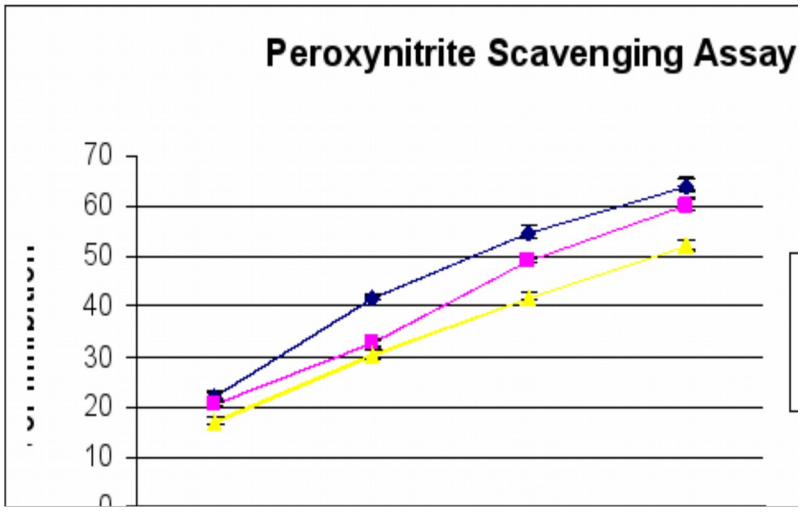

Figure 4.9 Peroxynitrite scavenging activity of EECGL, WECGL and ascorbic acid. Each point represents the value obtained from three experiments and expressed as Mean±SEM.

4.3.2 *In-vitro* anti inflammatory activities

HRBC membrane stabilizing activity

From the *in vitro* anti-inflammatory activity screening (Table 4.2), it wasobserved that EECGL showed significant HRBC membrane stabilizing activity when compared to the standardanti-inflammation drug, diclofenac sodium. The percentage of stabilization of diclofenac sodium, EECGL and WECGL at the concentration of 500 µg/ml were found to be 88.28%, 75.28% and 72.18% respectively. With the increasing concentration, the activities of the extracts were also increased.

Percentage of inhibition of protein denaturation

EECGL and WECGL exhibited 71 % and 68% maximum percentage of inhibition of protein denaturation i.e. at 500 µg/ml and its effect may be compared with the standard anti-inflammation drug,diclofenac sodium which showed 83% maximuminhibition at the same concentration.

4.3.3 *In-vivo* anti-inflammatory activity

EECGL, WECGL and standarddrug indomethacin as compared to carrageenan control (at 3rd and 4th h)in carrageenan induced paw edema model using verniercallipers. Both extracts administered at a dose level of 10, 20 and 50 mg/kg, prevented carrageenan induced paw edema at 1–4th h in a dose-dependent manner while indomethacin showed prevention at the concentration of 10 mg/kg (Table 4.3).

Table 4.2 Effect of EECGL and WECGL on HRBC membrane stabilization method

Concentration (µg/ml)	*HRBC membrane stabilizing activity*		
	Diclofenac sodium (%)	*EECGL (%)*	*WECGL (%)*
50	*75.61±1.08*	*58.15 ±0.98*	*55.2 ±0.67*
100	*80.2 ±1.72*	*62.27 ±0.88*	*60.27 ±0.89*
150	*83.45 ±1.45*	*65.78 ±1.05*	*62.18 ±0.78*
200	*85.18 ±1.8*	*70.12 ±1.23*	*68.24 ±0.93*
500	*88.28 ±1.2*	*75.28 ±1.12*	*72.18 ±1.23*

Each value represents the mean ± SEM. N=3.

Figure4.10 shows the percentageinhibition of protein denaturation by EECGL, WECGL and diclofenac sodium. Each value represents the mean ± SEM. N=3.

Table 4.3Effect of EECGL and WECGL and indomethacin as compared to carrageenan control in carrageenan induced paw edema model

Groups	*0 hr*	*30 min*	*1hr*	*2hr*	*3hr*	*4hr*
Carrageenan	*1.6±0.02*	*3.42±0.08*	*4.02±0.1*	*4.02±0.1*	*3.95±0.08*	*4±0.1*
Carrageenan + Indomethacin (10mg/kg bwt)	*1.61±0.02*	*3.02±0.06*	*2.98±0.05*	*2.67±0.05*	*2.2±0.01**	*1.81±0.01**
Carrageenan + EECGL (10mg/kg bwt)	*1.65±0.023*	*3.3±0.08*	*3.38±0.07*	*3.2±0.1*	*3±0.15*	*2.52±0.27**
Carrageenan + EECGL (20mg/kg bwt)	*1.56±0.01*	*3.5±0.09*	*3.35±0.08*	*3.1±0.15*	*2.85±0.1**	*2.83±0.2**
Carrageenan + EECGL (50mg/kg bwt)	*1.6±0.03*	*3.4±0.075*	*3.3±0.062*	*3±0.1*	*2.5±0.15**	*2.32±0.27**
Carrageenan + WECGL (10mg/kg bwt)	*1.75±0.023*	*3.38±0.05*	*3.2±0.07*	*3.12±0.15*	*3±0.09*	*2.85±0.27**
Carrageenan + WECGL (20mg/kg bwt)	*1.58±0.025*	*3.2±0.058*	*3.13±0.07*	*3±0.1*	*2.9±0.15**	*2.55±0.29**
Carrageenan + WECGL (50mg/kg bwt)	*1.68±0.027*	*3.1±0.08*	*3.1±0.07*	*3±0.1*	*2.8±0.15*	*2.5±0.2**

Values are expressed as mean ± SEM. * denotes p< 0.05-significant compared to carrageenan treated group. ** denotes p< 0.01-significant compared to carrageenan treated group.

4.4 Discussion

Nowadays, natural products are used intensively as potential drugs for the treatment and prevention of various diseases and conditions including cancer, cardiovascular disorders, aging and these diseases are generally arisen from oxidative stress and sometimes are happened through inflammation. For these reasons, worldwide researches are going on for the utilization of biological and pharmacological active compounds from plant origins to develop suitable antioxidant and anti-inflammatory agents.

NO, DPPH, hydroxyl radical, hypochlorous acid, superoxide radical, lipid peroxidation and peroxynitrite scavenging assay showed the concentration-dependent *in-vitr o*antioxidant activity of EECGL and WECGL compared to the standard antioxidant,ascorbic acid.

Nitric oxide is an important bio-regulatory molecule having many physiological effects including neural signal transduction, control of blood pressure, platelet formation, antitumor and anti-microbial activity (Jagetia et al.,2004). NO has slow toxic property after reaction with superoxide radicals and oxygen. These reaction products are able to cause much cellular damage (Vriesman et al., 1997). Nitric oxide (NO) acts as potent pleiotropic inhibitor of physiological processes like neuronal signaling, inhibition of platelet aggregation, smooth muscle relaxation, and regulation of cell mediated toxicity (Park et al.,2012).The extracts showed potent NO scavenging activity. Presence of more phenolic components in EECGL most probably made the difference in NO scavenging activity of EECGL than that of WECGL.

DPPH radicals react with suitable reducing agents as electrons become paired-off and the solution change color stochiometrically with the number of electrons taken up (Chidambara et al.,2003). Such reactivity has been commonly used to test the ability of plant extracts to act

as free radical scavengers. EECGL showed higher IC_{50} values for DPPH scavenging assay than WECGL and may be considered as a potent DPPH scavenging agent.

Hydroxyl radicals ($OH^{\cdot}$) are major reactive oxygen species causing lipid peroxidation and enormous metabolic damage in a biological system. The oxygen derived hydroxyl radicals along with the added transition metal ions causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid. Upon the addition of EECGL and WECGL to the reaction mixture, it removed the $OH^{\cdot}$from the sugar and prevent the reaction. The hydrogen peroxide scavenging activity of EECGL and WECGL were noticed prominently like ascorbic acid. The ability of the extracts to quench $OH^{\cdot}$is directly linked to the prevention of lipid peroxidation and appears to be moderate scavenger of reactive oxygen species, thus reducing rate of chain reaction (Mustafa et al.,2010).

Myeloperoxide (MPO) catalyzes the formation of powerful antioxidant hypochlorous acid (HOCl) from H_2O_2 and Cl^-. The hyprochlorous scavenging activity of EECGL and WECGL were seen markedly. Harmful HOCl is generated at the site of inflammation by enzyme myeloperoxidase present in neutrophil due to the oxidation of Cl^-ion which reacts with taurine and decrease the colour of 5-thio, 2-nitro benzoic acid (TNB) (Hazra et al., 2008and Carroll et al., 1995).The inhibition of this oxidation by an EECGL and WECGL is a measurement of its HOCl scavenging activity in a dose dependent manner.

Superoxide anion is very detrimental to cell and cellular components. Oxidative enzyme of the body and non-enzymatic reaction such as auto oxidation by catecholamines produces these superoxide anions (Dolai et al.,2012).With the decrease in the absorbance at 560 nm, EECGL and WECGL indicate the ability to quench superoxide radicals in the reaction mixture where EECGL shows strong antioxidant activity than WECGL.

High molecular weight peroxynitrite ($ONOO^-$) causes oxidative damage to tissue (Dolai et al.,2012).EECGL and WECGL showed peroxynitrite scavenging activity in dose dependent manner, where EECGL demonstrated more activity than WECGL in the terms of IC_{50} value.

Presence of high phenolic content in *C. gigantea* latex (EECGL and WECGL) by FC reagent test proves that the latex is rich in flavonoids and triterpenoids (MaitiChoudhury et al., 2016) Therefore it will definitely showed antioxidant activities.

Denaturation of proteins is one of the causes of inflammation. The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause tissue inflammation and leads to damage (Chou,1997).

By inhibiting hypotonicity induced RBC membrane lysis, *C. gigantea* latex (WECGL and EECGL) extractsmay show their membrane stabilization effect. The exact mechanism of the membrane stabilization by the extracts is not clear yet, hypothetically it can be said that induced hemolysis may arise from shrinkage of the cell due to osmosis of intercellular fluids and electrolyte or interaction of fluid proteins (Shinde et al., 1999; Vadivu et al.,2008).

For the assay of acute inflammation, carrageenan induced edema has been commonly used as an experimental animal model which is believed to be biphasic in nature. In the first phase (1-2 h) of the carrageenan model is mainly facilitated by biochemically important vasoactive amines like serotonin, histamine, and in the damaged tissue surroundings enhance the synthesis of prostaglandins. In the last phase, prostaglandin release is continued and is mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Winter et al.,1962).

Flavonoids are a class of group of natural substances with variable phenolic structures widely distributed in the plant kingdom. A variety of *in vitro* and *in vivo* experiments have shown

that selected flavonoids possess anti-inflammatory activity (Yuan et al, 2005). There are a number of saponins isolated from various plants which have anti-inflammatory activity (Yuan et al, 2005). The ethanolic (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex possess flavonoids and saponins (MaitiChoudhury et al., 2016) and these phytochemical may be responsible for the anti-inflammatory activity of these extracts.

4.6Conclusion

On the basis of the results obtained in the present study, it was concluded that the ethanolic and waterextracts *Calotropis gigantea* latexpossess significant *in vitro* antioxidant activity. Presence of adequate amount of phenol and flavonoid compounds may account for this fact. The ethanol and water extract of *Calotropis gigantea* latexalso possess anti-inflammatory properties. These activities may be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols. This study gives on idea that the compound of the plant *Calotropis gigantea* latexcan be used as lead compound for designing a potent anti-inflammatory drug which can be used for treatment of various diseases such as cancer, neurological disorder, aging and inflammation.

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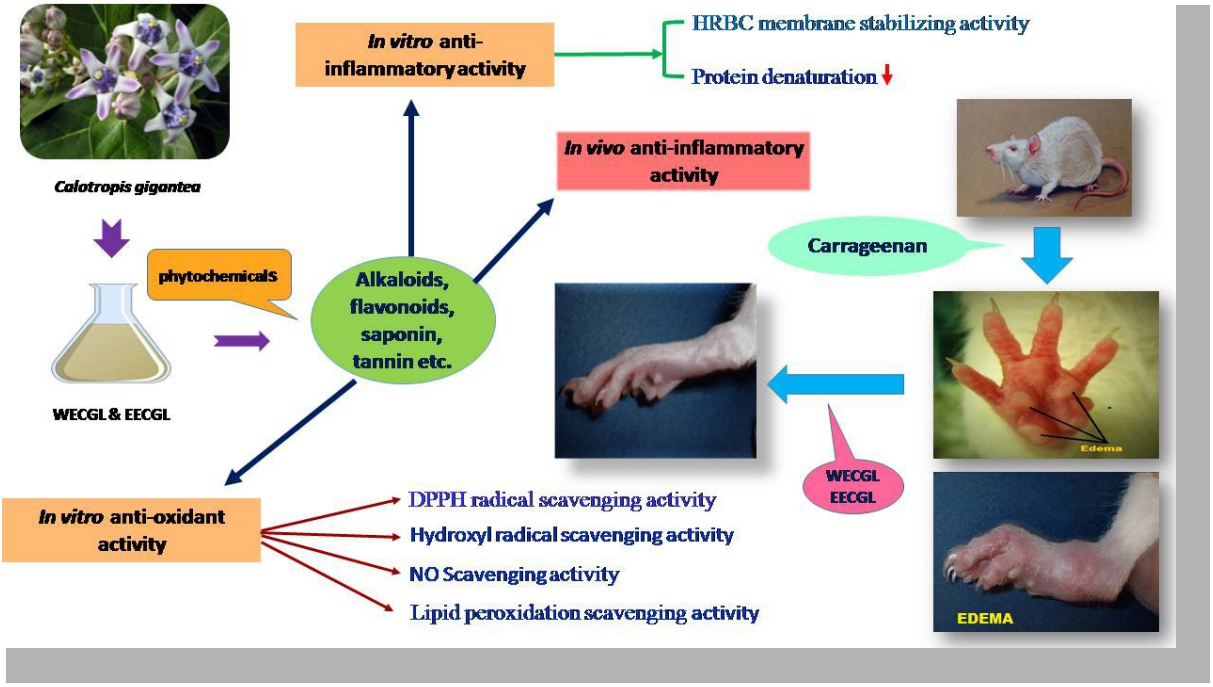

Figure 4.11 Schematic view of antioxidant and anti-inflammatory activities of *Calotropis gigantea* latex extract

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