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RESEARCH ARTICLE

IN VITRO ANTI OXIDANT, ANTI INFLAMMATORY AND ANTI MICROBIAL ACTIVITY OF ETHYL ACETATE FRACTION ISOLATED FROM *AMARANTHUS LIVIDUS* L.

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Abstract

For Centuries plants have been used throughout the world as drugs and remedies for various diseases. The current study aims to evaluate the invitro anti inflammatory, anti oxidant and anti microbial activity of ethyl acetate fraction of *Amaranthus lividus* L. The anti oxidant effect was determined by DPPH and superoxide scavenging assay. Anti-inflammatory activity was determined by human red blood cell membrane stabilization (HRBC) method and antimicrobial activity was determined by well diffusion method. The ethyl acetate fraction of *Amaranthus lividus* L. shows potent anti microbial effect at a concentration of 300µg/ml. The extract also shows anti inflammatory effect at 300µg/ml concentration. The extract has high antioxidant effect at very low concentrations. The results of our study reveal the significant anti oxidant, anti-inflammatory and antimicrobial activity of ethyl acetate fraction of *Amaranthus lividus* L.

INTRODUCTION

The inflammation is an essential event; this protective response may lead to potentially damaging consequences. Various autoimmune disorders are characterized by marked inflammation and associated failure of repair process. Inflammation and oxidative stress play a major role in the pathogenesis of many diseases, such as Alzheimer's disease and Parkinson's disease, rheumatoid arthritis etc. Oxidative stress and neuroinflammation are common features of chronic neurodegenerative diseases of the central nervous system (CNS), such as Alzheimer's disease (AD), Parkinson's disease (P D), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), as well as multiple sclerosis (MS). Now a day, over and incorrect use of antibiotics was the major reason for the emergence of multi drug resistant strains. Some bacteria develop genes for drug resistance in plasmids; they are able to spread drug resistance to other strains. Over and incorrect use of antibiotics leads to major side effects such as kidney failure, nephro toxicity etc. For Centuries plants have been used throughout the world as drugs and remedies for various diseases. Natural compounds and supplemental substances have become an increasingly attractive option to treat inflammatory diseases, because there is growing evidence that these nutritional constituents have potential adjunctive therapeutic effects, be it protective or restorative, on various diseases (Hurley et al.,2013). Amaranthus plants ((*Amaranthus lividus* L.) or spinach, are used extensively as a vegetable and are known to possess medicinal properties. Amaranthus consists of several antioxidant components, such as polyphenols, flavonoids, betalains, phenolics and anthocyanins (López-Mejía, 2014; Kraujalis, 2013).Bohlooli et al. (2015) studied effects of chronic daily spinach supplementation on known markers of oxidative stress and muscle damage following half marathon in well trained healthy young men. The present study aims to evaluate the invitro anti inflammatory, anti oxidant and anti microbial effect of ethyl acetate fraction of *Amaranthus lividus* L.(EAFA).

2. MATERIALS AND METHOD

2.1 Plant material

The plants *Amaranthus lividus* L.were collected from Kottayam, Kerala, India. The whole plants of *Amaranthus lividus* L. were cleaned and air dried at room temperature in shade, away from direct sunlight and then extracted by cold maceration process. Then extract filter by using filter paper. The filtrate is placed in china disc and evaporates the filter. Finally collected the crude extract and used for further study.

2.2 Preparation of ethyl acetate fraction

Five grams of obtained crude extract was dissolved in 100 ml water and sequentially extracted thrice using 100 ml hexane and ethyl acetate. Then solvent in the each fractions were removed using rotary evaporator to obtain ethyl acetate fraction (Ratheesh et al. 2007).

2.3 Anti-inflammatory studies by human red blood cell (HRBC) membrane stabilization method

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 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 isosaline and a 10% of suspension was made. Various concentrations of extracts were prepared (100-500 µg/ml) using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were added. It was incubated at 56^0 C for 30 minutes. After incubation the tubes cooled under running water and centrifuged at 3,000 rpm for 20 minutes. The hemoglobin content of the supernatant solution was estimated on UV spectrophotometer at 560 nm. Diclofenac (100,200 and 300 g/ml) was used as reference standard and a control was prepared by omitting the extract (Nakayoma et al.1995).

% Protection= 100-Optical density of drug treated sample/ Optical density of control*100

2.4 Antioxidant studies: DPPH radical scavenging assay

The free radical scavenging activity of the fractions was measured *in vitro* by 2, 2- diphenyl-1-picryl hydrazyl (DPPH) assay method. To the methanolic solution of DPPH (100µM) an equal volume of test drug was added, dissolved in distilled water at various concentrations (10µg to 50µg/ml). After incubation for 15 minutes at room temperature absorbance was recorded at 516 nm. Blank was carried out in the same manner without the drug. For rate kinetic study absorbance was taken immediate after addition of DPPH up to 500 seconds. The experiment was conducted in triplicate.

DPPH Scavenged (%) = **A** control - **A** test / **A** control * 100

Where **A** control is the absorbance of the control reaction and **A** test is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the *Amaranthus lividus* L. extract was expressed comparing with standard (Ames, 1998).

2.5 Superoxide radical scavenging assay

To the reaction mixture containing 0.5 ml of various concentrations of drug (10 µg to 50 µg/ml), 0.2 ml of NBT and 1 ml of alkaline DMSO were added. Blank was carried without drug, only DMSO was added. Absorbance was recorded at 560 nm. The experiment was repeated thrice in triplicate. Increased absorbance of the reaction mixture indicates stronger reducing power (Siju et al.2010; Yu-Ling et al.2012).

% increase in Reducing Power = **A** test/**A** blank - 1 * 100

2.6 Antimicrobial assay

The agar well diffusion method was used to determine the growth inhibition of the bacteria by the ethyl acetate extract of *Amaranthus lividus* L.. *Escherichia coli* (MTCC 1696), *Staphylococcus aureus* (MTCC 7443), *Pseudomonas aeruginosa* (MTCC 7454), *Klebsiella pneumonia* (MTCC 9751) were used for the study. Sterile

Mullen Hinton Agar was poured in to sterile petri dishes. After solidification the plates were incubated with the inoculums (bacterial culture after 6 hours) using sterile cotton swab under aseptic condition. Wells were made in the lawned plates using a sterile well cutter. 100, 200 µl of various concentration of different extracts were poured to each well using a sterile micropipette. The plates were incubated at 37^0 C for 24 hours to obtain inhibitory zone. The diameter of the inhibitor zones developed was measured in millimeters.

2.7 Minimum inhibitory concentration by Resazurin assay method (MIC)

This method is based on a micro broth dilution method in 96 multi-well microtitre plates with slight modifications (Sarker et al.2007). Organisms was inoculated into nutrient broth and incubated at 37^0c for 24 hours.50µl of culture was added to sterilized eppendorf containing 450µl of nutrient broth. Each fraction of extracts was added to the tubes so as to make a final concentration of 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. Control tubes were also maintained for each concentration. The control eppendorf tubes contain same volume of solvent without extract. All the tubes were incubated at 37^0c for 24hrs. After incubation resazurin indicator (0.05%) was added and tubes were then incubated at 37^0c for at least 3 hours till the colour of the indicator turns pink in the control tubes. The samples transfer aseptically to 96 well plate and absorbance can be read using a spectrophotometer set at 570 nm. Indicator resazurin of purple color reduced in the presence of living bacteria. Color change from purple to pink or to colorless. In the absence of living bacteria the color of the indicator were remain purple. The lowest concentration at which color change occurred was taken as MIC.

3. RESULTS

3.1 DPPH Radical Scavenging Assay

DPPH radical scavenging assay of ethyl acetate fraction of *Amaranthus lividus* L. illustrates increase in scavenging of DPPH radicals in dose dependent manner due to the scavenging ability of ethyl acetate fraction of *Amaranthus lividus* L. IC50 value of ascorbic acid is 25 µg/ml (Figure 1). DPPH assay of ethyl acetate fraction of *Amaranthus lividus* L. showed significant antioxidant activity [55µg/ml= IC_{50}] as compared to standard ascorbic acid [25µg/ml=IC_{50}].

3.2 Superoxide scavenging assay

Superoxide scavenging assay of ethyl acetate fraction of *Amaranthus lividus* L. illustrates increase in scavenging of Superoxide radicals in dose dependent manner. IC50 value of ascorbic acid is 15µg/ml (Figure 2). Superoxide Radical Scavenging Assay of ethyl acetate fraction of *Amaranthus lividus* L. showed significant antioxidant activity [25µg/ml= IC_{50}] as compared to standard ascorbic acid [15µg/ml=IC_{50}].

3.3 Estimation of In-vitro anti-inflammatory activity of *Amaranthus lividus* L. by HRBC assay

The HRBC Membrane stabilization method was used for the in-vitro anti- inflammatory activity of ethyl acetate fraction of *Amaranthus lividus* L. The HRBC Membrane stabilization activity of the EAFA at concentration 500µg/ml showed 84.4% inhibition of denaturation in hypotonic solution while standard Diclofenac 300µg/ml showed 95.2% inhibition of denaturation (Figure 3).

3.4 Antimicrobial activity of various extracts of *Amaranthus lividus* L.

Well diffusion method of ethyl acetate fraction of *Amaranthus lividus* L. against different bacteria shows the potent activity of *Amaranthus lividus* L. as antimicrobial agent at a concentration of 300µg/ml (table 1). The results were shown in figure 4.

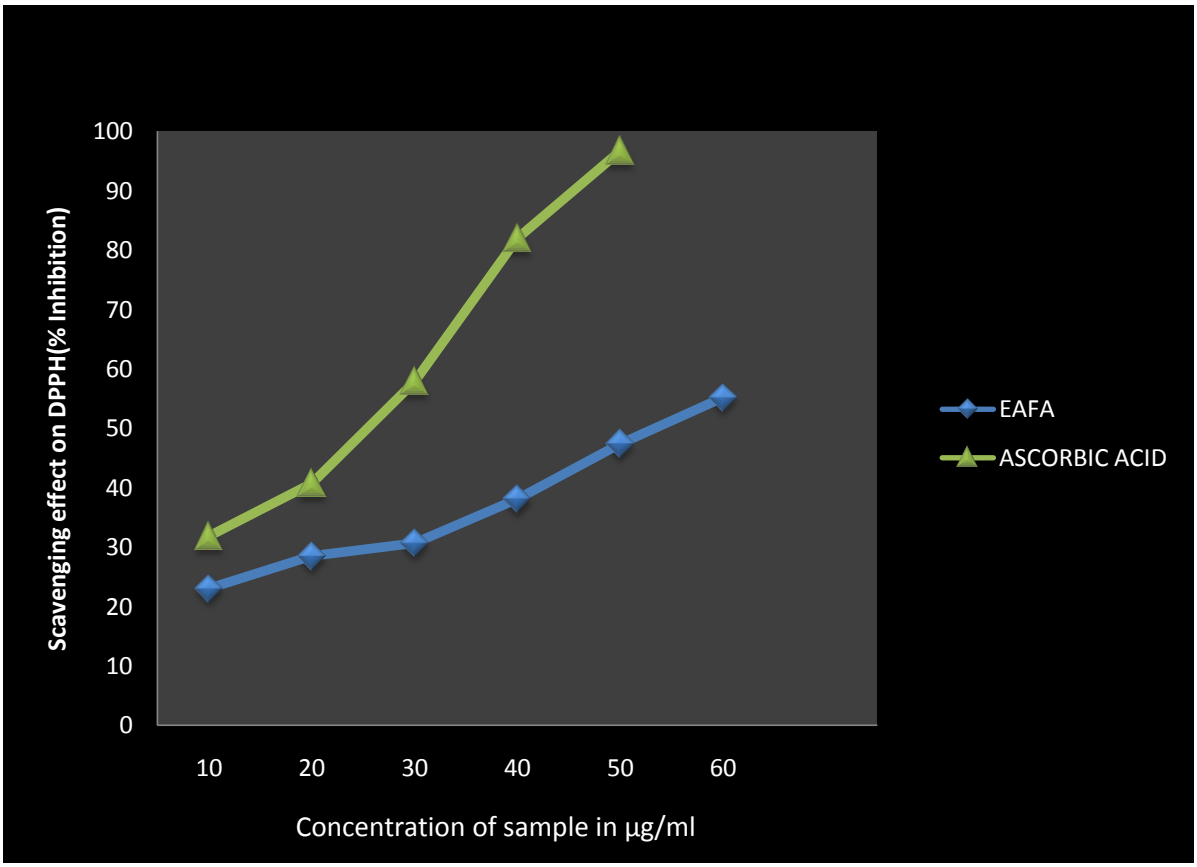

Figure 1: Dose response curve of DPPH radical scavenging assay of ethyl acetate fraction of *Amaranthus lividus* L.

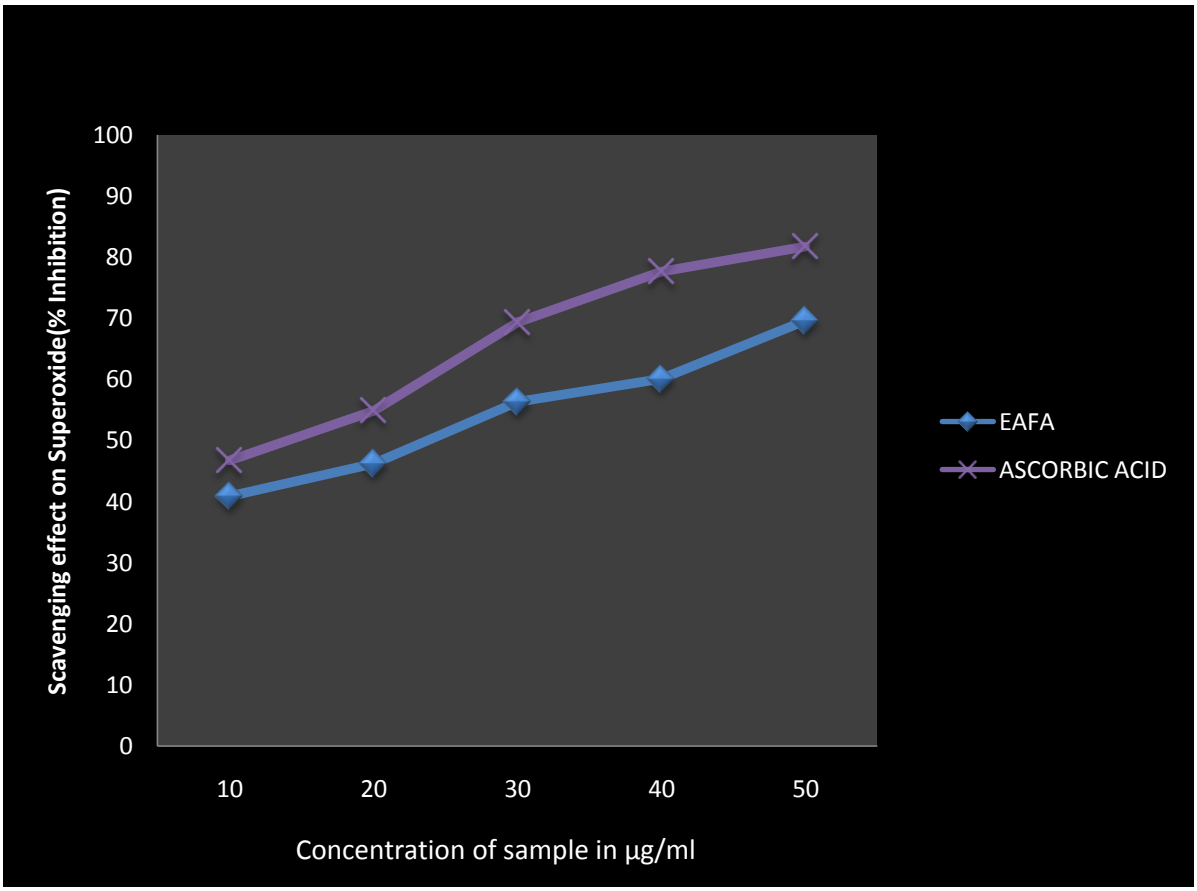

Figure 2: Dose response curve of Superoxide scavenging assay of ethyl acetate fraction of *Amaranthus lividus* L.

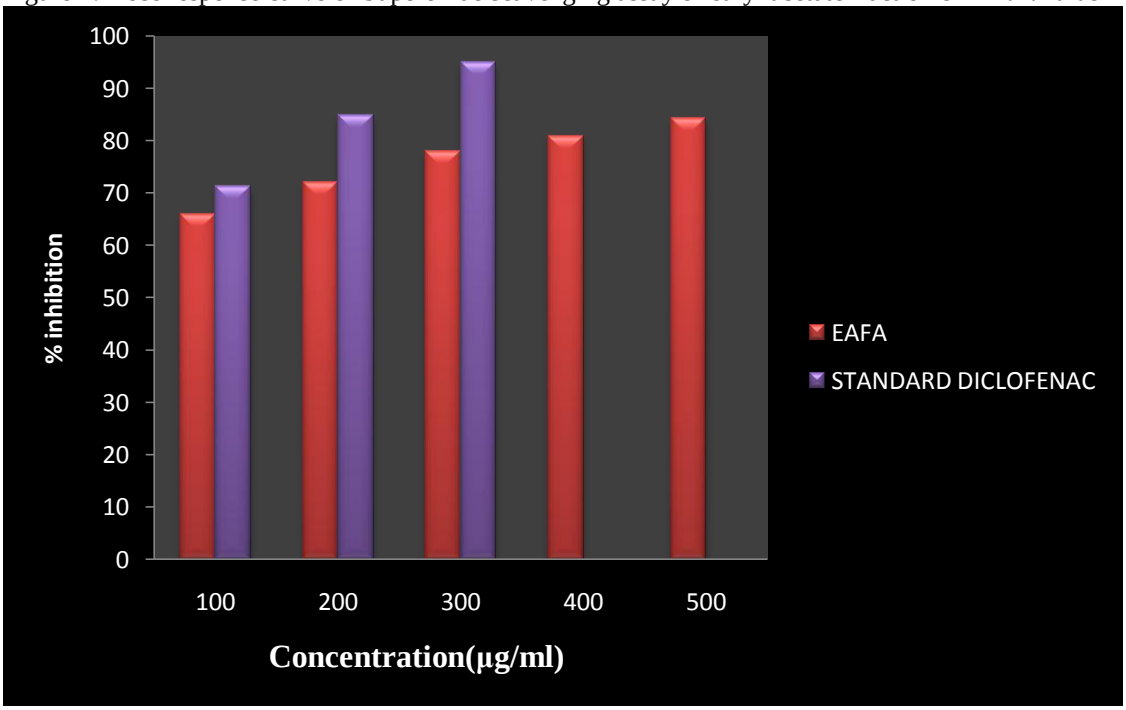

Figure 3: In-vitro anti-inflammatory activity of ethyl acetate fraction of *Amaranthus lividus* L. by HRBC assay

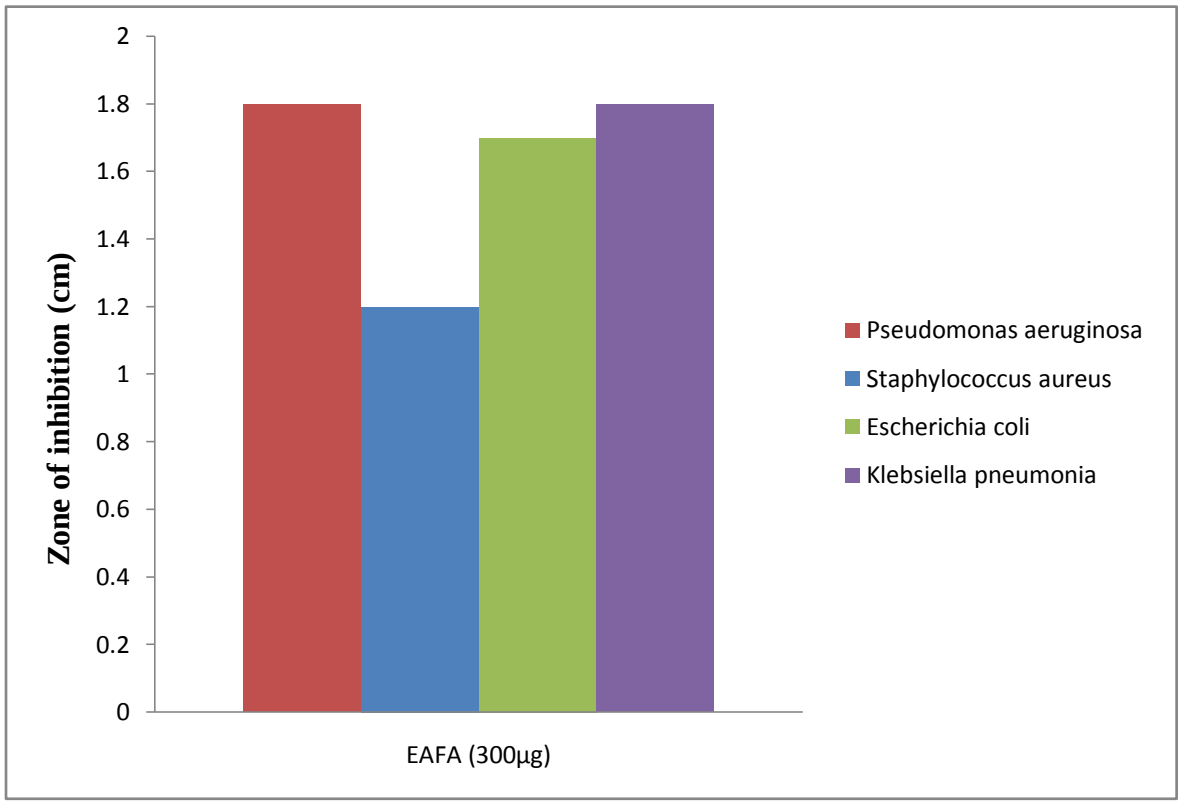

Figure 4: Antimicrobial activity of ethyl acetate fraction of *Amaranthus lividus* L.

Microorganism	Concentration of sample (µg/ml)				
	100	200	300	400	500
Escherichia coli	+	+	-	-	-
Staphylococcus aureus	+	+	-	-	-
Pseudomonas aeruginosa	+	+	-	-	-
Klebsiella pneumonia	+	+	-	-	-

+ Presence of microorganism
-Absence of microorganism
Table 1: Resazurin assay of various microorganisms

4. DISCUSSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Numerous studies demonstrated that medicinal plants are sources of nutrient and non-nutrient compounds, many of which display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus, it is important to characterize the different types of medicinal plants for their antioxidant and antimicrobial potentials, and such plants should be investigated to better understand their properties, safety and efficacy.

Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The free radicals are involved in the pathogenesis of a large number of diseases. The result of the present study showed that the high scavenging property of ethyl acetate fraction of *Amaranthus lividus* L. may be due to the phenolic compounds. The ethyl acetate fraction of *Amaranthus lividus* L. have indicated strong antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress induce diseases such as diabetes,

liver diseases, cancer, ageing etc which would be beneficial to the human health. This may be related to the high amount of phenolic and flavanoid compounds present in this plant extracts. Free radical scavenging activity of the extracts was assessed using the stable free radical DPPH. Plant extracts which reduce DPPH by donating hydrogen or an electron are considered as antioxidants having free radical scavenging activity. In the present study, DPPH and superoxide scavenging activity was found in the ethyl acetate fraction of *Amaranthus lividus* L. It is obvious from the study, that the investigated extracts have the ability to quench free radicals. This indicates that the screened plant extracts are a potential source of natural antioxidants.

The investigation is based on the need for newer anti-inflammatory agents from natural source with potent activity and lesser side effects as substitutes for chemical therapeutics. Ethyl acetate fraction of *Amaranthus lividus* L has significant anti-inflammatory activity which may be due to presence of chemical profile such as Flavones, Tri-Terpenoids, Flavonones and Phenols. The HRBC Membrane stabilization method was used for the in-vitro anti-inflammatory activity of the ethyl acetate fraction of *Amaranthus lividus* L. The HRBC Membrane stabilization activity of the EAFA at concentration 500µg/ml showed 84.4% inhibition of denaturation in hypotonic solution while standard Diclofenac 300µg/ml showed 95.2% inhibition of denaturation. Ethyl acetate fraction of *Amaranthus lividus* L exhibited membrane stabilization or heat induced hemolytic effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogues to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membrane (Chatterjee et al.1996) .Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituent of activated neutrophil such as bacterial enzymes and proteases which cause further tissue inflammation and damage. From the above study it was concluded that the ethyl acetate fraction of *Amaranthus lividus* L has significant membrane stabilization property.

A new micro dilution method has been developed for determining the minimum inhibitory concentration (MIC) of plant based compounds. The redox dye resazurin was used to determine the MIC of ethyl acetate fraction of *Amaranthus lividus* L. against different pathogens. The measurement and monitoring of cell viability is an essential technique in any laboratory focused on cell-based research. The reducing environment of the cells in the resazurin assay is measured through the conversion of resazurin (oxidised form) to resorufin (reduced form) (Lancaster et al. 1996) results in colorimetric (absorbance) and fluorescence changes. Resazurin is blue and non-fluorescent whereas resorufin is red and highly fluorescent. It is a sensitive assay if working with higher than $5x10^3$ cells per 100 µL, relatively non-toxic and provided that it is carried out carefully, the same replicates can be followed over several time points. A comparison of visual and photometric reading of the microtitre plates showed that the MIC value of ethyl acetate fraction of *Amaranthus lividus* L. was 300µg/ml. This concentration was used for further anti microbial studies.

Plants are important sources of potentially useful substances for the development of new therapeutic agents. Various phytochemical compounds as secondary metabolites have been implicated in plants as the conferment of antibacterial activities (Sule et al.2011). Moisture content of drugs could be at a minimal level to discourage the growth of bacteria, yeast or fungi during storage (Cao et al.2010). The antimicrobial activity of ethyl acetate fraction of *Amaranthus lividus* L. might be related to the action of its antibiotic compounds or to the presence of metabolic toxins. At the concentration of 300µg, EAFA shows zone of clearance against *Escherichia coli* (MTCC 1696), *Staphylococcus aureus* (MTCC 7443), *Pseudomonas aeruginosa* (MTCC 7454), *Klebsiella pneumonia* (MTCC 9751). This result indicates the efficacy of ethyl acetate fraction of *Amaranthus lividus* L. as antimicrobial agent.

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