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ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITIES OF LEAVES OF *GARCINIA ZEYLANICA*

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ABSTRACT

Plants are being used to treat many diseases despite the development of synthetic drugs due to their low side effects. This study involves the determination of the antioxidant, anti-inflammatory, and antibacterial activity of *Garcinia zeylanica* leaves. Aqueous leaf extract of Sri Lankan variety of *Garcinia zeylanica* (Kaha goraka/Ela goraka), prepared according to the method of "Kasaya" (ALEGZ) in Ayurvedic medicine was used for this study. ALEGZ gave a Total Phenolic Content of 696.1 mg (PGE) g^{-1} in Folin-Ciocalteu assay and Total Flavonoid Content of 214.9 mg (QE) g^{-1} in $AlCl_3$ colorimetric assay. The 2, 2- diphenyl-1-picrylhydrazyl radical scavenging activity of ALEGZ (0.10-4.00 mg/ml) ranged between 28.4%-80.7% whereas for ascorbic acid the percentage was 40.5%-87.1% at the same concentration range. The ALEGZ also showed a considerable amount of ferric ion reducing ability at the concentration range of 0.25-5.00 mg/ml. Hydroxyl radical scavenging activity of the ALEGZ (0.5-5.0 mg/ml) was 21.3%-59.4% and ascorbic acid showed 69.7%- 84.3% activity. In the anti-inflammatory assay, ALEGZ (20-70 µg/ml) gave nitric oxide scavenging activity of 9.7%-63.6% whereas for ascorbic acid it was 28.0%-72.7%. Human red blood cell membrane assay showed a protection of 18.7%-60.8% for the ALEGZ (100-1000 µg/ml) and aspirin showed its protection between 19.1%-65.4%. The ALEGZ also showed antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*. According to these results, the ALEGZ has proved that it has the ability to combat diseases in which radicals are implicated as well as inflammation and bacterial infestation by *Bacillus subtilis* and *Staphylococcus aureus*.

KEYWORDS: *Garcinia zeylanica*, antioxidant, anti-inflammatory, antibacterial.

INTRODUCTION

Plants have been used for health and medicinal purposes for several thousands of years since ancient times for the treatment of many diseases and illnesses. Despite the advances in modern medicine, dependence on plant-based natural products has flourished due to its time tested safety and efficacy. Bioactive compounds which are present in plants possess numerous health-related effects including antimicrobial, anti-inflammatory, antioxidant, anticancer properties etc. Therefore plants account for a significant percentage of the pharmaceutical market and their utilization have been increasing rapidly in recent years.[1,2]

Herbal preparations including decoctions for treatments are used in Ayurvedic traditional methods. Decoctions are complex mixtures of plant materials containing hundreds of important chemical components. Therefore drug development based on decoctions has gradually improved and currently, this method is being used in many areas of research.[3]

Garcinia is popular among many South Asians as it can be used to make foods more filling and satisfying and used historically in many South Asian countries to support the treatment of various health conditions. *Garcinia zeylanica* or commonly referred to as Ela goraka or Kaha goraka, is a terrestrial species of angiosperm in the family of Clusiaceae and found only in Sri Lanka. Endemic *G. zeylinica* species is being used in indigenous medicine for its antiseptic and purgative properties.[4]

Free radicals are natural byproducts of chemical processes like metabolism. However, when generated in excess or not appropriately controlled, radicals can cause damages to important biomolecules in the body acting as oxidants and reductants and they are associated with many human diseases including cancers, atherosclerosis, Alzheimer’s disease, Parkinson’s disease, etc. Reactive Oxygen Species (ROS) comprises both free radicals and non-free radical oxygen intermediates such as hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{-\bullet}$), oxygen singlet ($^1O_2$) and peroxide radicals ($O_2^{\bullet -2}$), etc. The presence of two unpaired electrons in separate orbitals in outer shell of oxygen makes it susceptible to radical formation.[5,6] These highly reactive species present in the body are capable of damaging biologically important molecules such as DNA, proteins, carbohydrates, and lipids leading to cell damage and homeostatic disruption. Oxidative stress is a state caused by the imbalance between the production and accumulation of reactive oxygen species in cells and tissues and the potential of the biological systems to detoxify these reactive products.[6] Antioxidants are substances that can prevent or slow down the damage caused by free radicals to cells and they are sometimes called "free radical scavengers". Many defense mechanisms have evolved within the organisms in order to limit the levels of damage done by free radicals including reactive oxygen species. In addition to these endogenous antioxidant defenses, consumption of dietary antioxidants is recommended.[7] Fruits and vegetables are the main sources of dietary antioxidants which could lower the risk of degenerative diseases.[8]

Polyphenolic compounds are naturally occurring secondary metabolites and are reported to show radical scavenging activity.[9] They contain one or more acidic hydroxyl residues attached to an aromatic arene ring and are potential candidates for being used as drugs to treat diseases like AIDS, bacterial infections, ulcer formations, heart ailments, mutagenesis, neural diseases, etc.[5,9] They show high reactivity against hydrogen and electron donors by stabilizing and delocalizing the unpaired electron over its polyphenol derived radical and from their potential to chelate metal ions.[7] The ability of these compounds to interact with metal ions such as Fe^{2+}, that are capable of generating free radicals, has significance in their bioactivity. Furthermore, phenolic compounds are reported to have antitumor, antimicrobial, and anti-inflammation properties.[5,9] Flavonoids are the main group of compounds that falls under the category of polyphenols and they show a potent antioxidant activity by scavenging free radicals, chelating metal ions including iron, copper, and inhibiting the enzymes responsible for free radical generation. Depending on the structure, flavonoids are capable of scavenging practically all known ROS.[10] Many studies have shown that the flavonoids also contain anti-inflammatory, anti-cancer, anti-tumor, and antibacterial properties.[11]

Nitrogen oxide ($NO^{\bullet}$) and reactive nitrogen species (RNS) are free radicals derived from the interaction of NO with oxygen or reactive oxygen species. Even low concentrations of NO radicals are able to perform its physiological functions in biological systems. However, its toxicity increases when it reacts with superoxide forming highly reactive peroxynitrite radical ($ONOO^{\bullet}$). Chronic exposure to nitric oxide radicals is associated with inflammations and cancers.[12]

Inflammation is a part of immune response that depends both on the physical actions of white blood cells and the chemicals that they produce including antibodies, cytokines, etc. Lysosomal enzymes that are released during inflammation produce a variety of disorders by damaging biomolecules and causing lipid peroxidation of membranes, finally resulting in certain pathological conditions including heart attacks, rheumatoid arthritis and septic shocks.[10] Therefore stabilization of the lysosomal membrane is important, in order to inhibit the release of lysosomal constituents from the activated neutrophil including enzymes and proteases, thus limiting the inflammation. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are taken externally to inhibit the release of lysosomal enzymes or stabilize the lysosomal membranes.[13] Human Red Blood Cell (HRBC) or erythrocyte membrane is similar to the lysosomal membrane and its stabilization implies that the extract may also stabilize lysosomal membranes. Hence in this assay stabilization of the HRBC by hypotonicity-induced membrane lysis is taken as an *in vitro* measure of anti-inflammatory activity of the plant extract.[14]

Antimicrobial agents are natural or synthetic substances that kill or inhibit the growth of micro-organisms and they work at the cellular level to disrupt the vital life processes and prevent the reproduction of micro-organisms. The Antibacterial Susceptibility Tests (ABST) are used in pathology to determine resistance of bacterial strains to different antimicrobials and also to determine the efficacy of novel antimicrobials from biological extracts against different microorganisms.[15] Anti-microbial activity can be assessed by the growth response of various micro-organisms to plant extracts. Plants contain a huge range of complex and structurally diverse compounds that can act as potential anti-microbial agents including phenolic compounds, glycosides, saponins, flavonoids, and alkaloids.[16] Agar well diffusion method and agar disk diffusion methods are commonly used to evaluate the susceptibility of antimicrobials. The antimicrobial agents diffuse in the agar medium and inhibit the reproduction of microbial strains. The diameters of the resulting inhibition zone are measured in order to determine the antimicrobial activity of the plant extract.[15,17]

MATERIALS AND METHODOLOGY

Collection of plant materials

The leaves of *G. zeylanica* were collected in March 2019 from Galle, Sri Lanka (6°4’6.956” N, 80°13’22.155” E). The plant was botanically identified by the Division of Pharmaceutical Botany, Bandaranayake Memorial Research Institute, Navinna, Maharagama. A voucher specimen (accession acc. number 2044) is deposited there.

Preparation of plant extract

Leaves were washed in order to remove contaminants and they were air-dried for 5 days at room temperature. The air-dried leaves were then powdered using a domestic grinder and water extract of the powdered Garcinia leaves was prepared according to Ayurvedic traditional method which is used to prepare "Kasaya". A mass of 60 g of powdered Garcinia leaves was mixed in 960 ml of distilled water and it was boiled until 240 ml volume of decoction was obtained. The water extract was filtered through a fine silk cloth and the filtrate was freeze-dried using a vacuum collector. The freeze-dried compound was kept at -4 ℃ in air-tight containers until required.[18,19]

Preparation of sample concentration

A stock solution of known concentration was prepared by dissolving a known mass of freeze-dried sample in a known volume of solvent for each assay separately. A concentration series of the sample was prepared by diluting the stock solution.

Total Phenolic Content (TPC) Assay

The total phenolic content was measured by Folin-Ciocalteu spectrophotometric method. A volume of 200 µl of the sample from each concentration was mixed with 4.00 ml of 2% $NaHCO_3$ and they were incubated in darkness for 2 minutes. After incubation 200 µl of Folin-Ciocalteu Reagent (FCR) was added to each reaction mixture. Then the mixtures were incubated again in darkness for 30 minutes and the absorbance was read at 750 nm. TPC was determined from extrapolation of the calibration curve which was obtained by pyrogallol concentration series, following the same procedure instead of using the plant extract. The blank was prepared by replacing the FCR with 200 µl of distilled water. [13] The total phenolic content was expressed as milligrams of Pyrogallol Equivalents (PGE) per a gram of freeze-dried sample; mg (PGE) g^{-1}.[10]

Total Flavonoid Content (TFC) Assay

In a test tube, 0.50 ml of the plant sample, 2.00 ml of distilled water, 150 µl of 5% $NaNO_2$ were mixed and incubated in darkness. After 5 minutes a volume of 150 µl of $AlCl_3$ was added to the reaction mixture and again the sample was incubated in dark for 6 more minutes. Finally, 1.00 ml of 1 mol dm^{-3} NaOH and 1.00 ml of distilled water were added. The solution was mixed well and the absorbance was taken at 510 nm against the reagent blank. The reagent blank was prepared by replacing $AlCl_3$ with distilled water. Quercetin solutions with different concentrations ranging from 1500 ppm to 400 ppm were used to plot the standard curve for TFC assay under the same procedure described earlier. The final result of the TFC assay was expressed in milligrams of Quercetin Equivalents (QE) per one gram of freeze dried sample; mg (QE) g^{-1}.[10]

Antioxidant Assays

2, 2'- Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Assay

A stock solution of DPPH was prepared by dissolving 0.006 g of 2, 2'- diphenyl-1-picrylhydrazyl in 25.00 ml of distilled methanol in a volumetric flask, and it was stored at 20 ^{0}C until required. The working DPPH solution was obtained by diluting it with distilled methanol to attain an absorbance of 0.98 ± 0.02 at 517 nm using the UV-spectrophotometer.[13] A volume of 3.0 ml of the working DPPH solution was mixed with 100 µl of the plant sample with various concentrations. The reaction mixture was shaken well and incubated in dark for 15 minutes at room temperature. Absorbance was taken at 517 nm after incubation. Ascorbic acid solutions with different concentrations were used to plot the standard curve for this assay under the same procedure described earlier.[42] The control was prepared by mixing 3.00 ml of working DPPH solution with 100 µl of distilled water and the blank was prepared by mixing 3.00 ml of distilled methanol with 100 µl of distilled water.[10]

The percentage of DPPH Radical Scavenging Activity (DPPH RSA) was determined using Equation 1.[20]

Equation 1.

$$\text{DPPH RSA (\%)} = \left[1 - \frac{As}{Ac}\right] \times 100\%$$

A_s-absorbance of the sample

A_c-absorbance of the control

Ferric Ion Reducing Power Assay

A volume of 2.00 ml of the plant sample with various concentrations was mixed with 2.00 ml of 0.2 mol dm^{-3} phosphate buffer (pH 6.6) and 2.00 ml of $K_3[Fe(CN_6)]$ (10 mg/ml) and it was incubated for 20 minutes at 50 °C. After incubation 2.00 ml of TCA; Trichloroacetic acid (100 mg/ml) was added and centrifuged at 3000 rpm for 10 minutes to collect the supernatant. To a volume of 2.00 ml of the collected supernatant, 2.00 ml of distilled water and 0.40 ml of freshly prepared $FeCl_3$ were added and mixed well. The absorbance values of each reaction mixture were taken at 700 nm after keeping for 10 minutes. Distilled water was used as the blank. The same procedure was carried out with ascorbic acid which was used as the positive control. Higher absorbance values indicate a higher reducing power.[10]

Hydroxyl Radical Scavenging Assay

An aliquot of 100 µl of the plant sample was mixed with 500 µl of 2-deoxy ribose in phosphate buffer (2.8 mM), 100 µl of H_2O_2 (200 mM) solution 200 µl of premixed $FeCl_3$ (100 mM), and EDTA (100 mM) solution (1:1; v/v). The reaction was triggered by adding 100 µl of ascorbic acid (300 mM) and incubated for one hour at 37 ^{0}C. To a volume of 0.50 ml of this reaction mixture, 1.00 ml of TCA (2.8 % w/v) and 1.00 ml of 1% TBA; Thiobarbituric acid were added. This was heated for 15 minutes in a boiling water bath. After the mixture being cooled, the absorbance at 532 nm was obtained using distilled water as the reagent blank. The same procedure was carried out for the concentration series of ascorbic acid as the standard.[10]

The percentage of the Hydroxyl Radical Scavenging Activity (HRSA) was determined using Equation 2.[21]

Equation 2.

HRSA (%) = $\left[1 - \frac{As}{Ac}\right] \times 100\%$

Anti-Inflammatory Assays

Nitric Oxide Radical Scavenging Assay

Nitric oxide scavenging activity was determined by the Griess Illosvoy reaction. Here a 0.50 ml volume of 10 mM Sodium nitroprusside (SNP) in standard phosphate buffer saline (PBS) was added to 1.00 ml of each concentration of the plant sample and tubes were incubated at 25 °C for 3 hours. After 3 hours incubated samples were diluted with 1.00 ml of Griess reagent. Griess reagent was prepared and used immediately by mixing equal volumes of 1% sulphanilamide (0.50 ml) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NDD; 0.50 ml). A control experiment with an equivalent amount of SNP, PBS, and Griess reagent without the test compounds was conducted in an identical manner. The absorbance of the colour developed due to the diazotization reaction of nitrite with sulfanilamide followed by its coupling to NDD was observed at 546 nm using a spectrophotometer. Distilled water was used as the reagent blank. The same procedure was carried out with the standard concentration series using 1.00 ml of ascorbic acid and instead of the plant extract.[22,23]

Nitric Oxide Radical Scavenging Activity was determined using Equation 3. [24]

Equation 3.

NO radical scavenging activity (%) = $\left[1 - \frac{As}{Ac}\right] \times 100\%$

Human Red Blood Cell (HRBC) Membrane Stabilization Assay

To prepare erythrocyte suspension, a volume of 5.00 ml of whole blood sample was collected through a vein puncture from myself without taking any NSAIDs for 2 weeks prior to the experiment. It was centrifuged at 5000 rpm for 20 minutes and the supernatant was discarded. The carefully collected pellet was washed thrice with normal saline which is equal in volume to the pellet. It was then centrifuged until the yellowish colour of the supernatant turned colourless.[23]

In order to prepare the reaction mixture 1.00 ml of the plant samples of each concentration were mixed with 0.10 ml of 10% red blood cell suspension. The reaction mixtures were then incubated at 56 °C for 30 minutes. The tubes with reaction mixtures were cooled and centrifuged again at 3000 rpm for 10 minutes. The absorbance of the supernatant after centrifugation was measured at 540 nm using distilled water as the blank. The control was prepared using only red blood cell suspension. The same procedure was carried out with the concentration series of aspirin as the positive control instead of 1.00 ml of the plant sample.[23]

The percentage of hemolysis and protection were calculated using Equations 4 and 5.[23]

Equation 4.

$$\text{Hemolysis (\%)} = \left[\frac{As}{Ac}\right] \times 100$$

Equation 5.

$$\text{Protection (\%)} = 100 - \left[\frac{As}{Ac}\right] \times 100$$

Antibacterial Assays

Disk diffusion assay,[25] and well diffusion assay[25] were conducted to test antibacterial activity against two Gram-positive bacteria, *Staphylococcus aureus,* and *Bacillus subtilis* and one Gram-negative bacteria *Escherichia coli.* Prior to the antibacterial assays, all the glassware including petri dishes, test tubes, conical flasks, volumetric flasks, etc. were sterilized according to a previously published method and the assays were carried out in a laminar hood under sterile conditions.[25] Gram-positive bacterial strains *S. aureus*, *B. subtilis*, and Gram-negative bacterial strain *E. coli* were obtained from the Pharmacy Laboratory of Faculty of Science, University of Colombo. Each culture of bacteria was isolated using a sterile inoculating loop and they were transferred to 10.00 ml of saline solution (0.9%)

separately. The turbidity of the bacterial inoculum was compared with the McFarland reagent (McFarland regent was prepared by adding 50 µl of 1.175% $BaCl_2$ dropwise to a volume of 9.95 ml of 1% sulphuric acid solution) in order to achieve the desired concentration.[26] Under aseptic conditions, a volume of 200 µl of bacterial inoculum was spread on the culture media plates containing Lysogeny Broth (LB) agar. The bacterial suspension was spread evenly along the medium using a spreader which was sterilized with 70% ethanol. After incubating the plates for 30 minutes they were used for antibacterial assays. For the agar well diffusion method, five wells were made using a sterilized coke borer on each spread plate. An aliquot of 50 µl of the plant sample (100 mg/ml) was added to three wells using a micropipette. The other two wells were filled with positive control (Ciprofloxacin; 0.25 mg/ml) and the negative control (distilled water) separately. The average diameters of the inhibition zones were measured after incubating at 37 °C for 24 hours.[27]

In agar disk diffusion method, sterilized paper disks with each 6 mm in diameter were dipped in 20 µl of the relevant solution (positive control, negative control, and plant sample) twice. The impregnated disks were allowed to air dry at room temperature and the dried disks were placed on the bacterial spread plates prepared separately using a pair of forceps. All the plates were sealed and incubated for 24 hours at 37 °C. After incubation the average diameters of the inhibition zones were measured for each bacterial strain.[27]

Data Analysis

All tests were carried out in triplicate and the data shown in the table and the graphs are the average together with Standard Deviation (SD).

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) Assay

Total phenolic content of the plant sample was determined using the Folin-Ciocalteu Reagent (FCR) method with reference to the standard curve of pyrogallol. This assay relies on the transfer of electrons from phenolic compounds in alkaline medium to phosphomolybdic and phosphotungtic acid complexes in FCR to form a blue colour complex that determined spectroscopically at 750 nm (Fig. 1).[28,29]

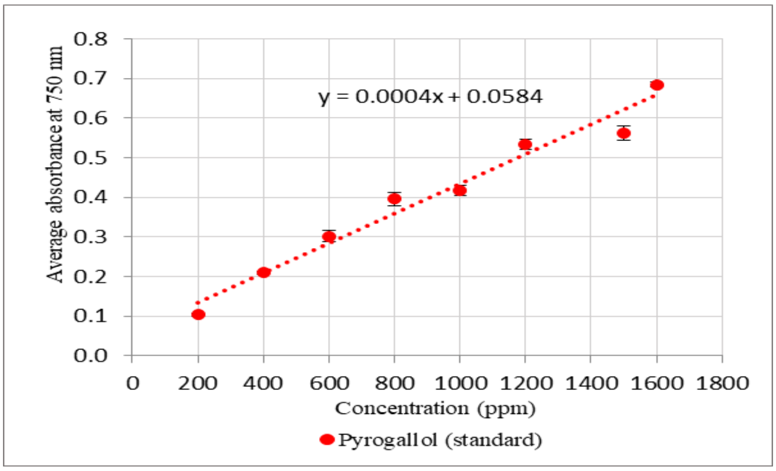

Fig. 1: Pyrogallol standard curve.

The reaction is done under alkaline conditions to make sure that FCR only reacts with phenols and decomposes rapidly to its anionic form. Since excess of reagent should be used to obtain a complete reaction it may result in precipitates and turbidity. To stop such complications lithium salts are already present in FCR reagent.[30] The final TPC obtained for the ALEGZ was 696.1 ± 71.0 mg (PGE) g^{-1}.

Total Flavonoid Content Assay

In the Aluminium colorimetric method, which is used to determine TFC; $AlCl_3$ forms acid labile complexes with the ortho-dihydroxyl groups of flavonoids. Hence with the addition of Al^{3+} ions to a solution with flavonoids, the solution turns yellow which then immediately changes to red colour after reacting with NaOH.[31]

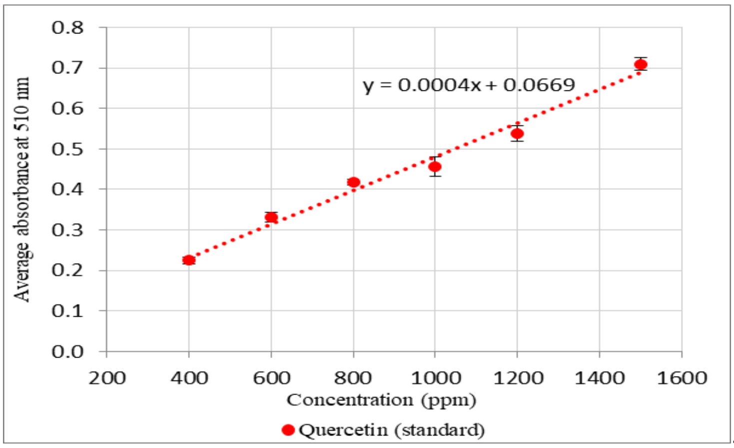

Fig. 2: Quercetin standard curve.

According to the results obtained (Fig 2); the final TFC content of the ALEGZ was 214.9 ± 15.5 mg (QE) g^{-1} with reference to the standard curve of quercetin.

Since ALEGZ showed much Total Phenol Content and Total Flavonoid Content, it was suspected that it would possess antioxidant activity as there is reported literature where the flavonoid content is proportional to antioxidant activity.[32]

A great number of *in vitro* methods including DPPH radical scavenging assay, ferric ion reducing assay, and hydroxyl radical scavenging assay have been developed to measure the efficiency of natural antioxidants in plant extracts.

Antioxidant Assays

DPPH Radical Scavenging Assay

The DPPH test is a widely used method of evaluating the free radical scavenging effect of plant extracts. The violet coloured free radical, DPPH is a stable, synthetic radical with an odd electron giving a maximum absorption at 517 nm.[33] DPPH radical solution prepared in methanol is converted into DPPH-H (Diphenylhydrazine), a non-radical resulted by the pairing of the odd electron radical with another radical obtained from the extract showing antioxidant activity. This results in the diminishing of the purple colour with the increasing concentration of antioxidants.[34,35]

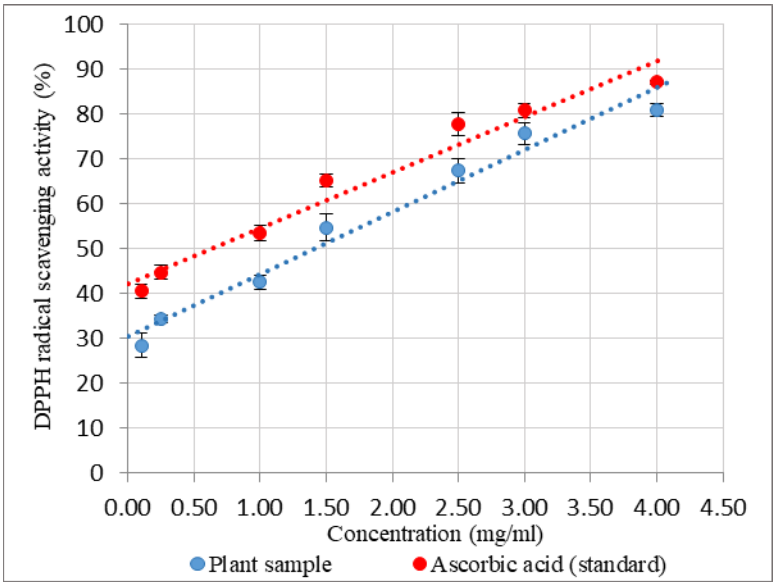

Fig. 3: DPPH radical scavenging activity (%) vs. concentration.

According to the calculated radical scavenging activity from DPPH assay, ALEGZ showed the percentage scavenging activity from 28.4% to 80.7% within the concentration range of 0.10 mg/ ml to 4.00 mg/ml whereas standard ascorbic acid showed its radical scavenging activity from 40.5% to 87.1% (Fig.3) at the same concentration range, under equal experimental conditions. Therefore these results suggest that the ALEGZ contain a considerable amount of phytochemical constituents capable of donating free radicals which could neutralize harmful radical entities.

Ferric Reducing Power Assay

The reducing ability of a compound is decided by the presence of reductones or antioxidants, which can exhibit its antioxidant activity by breaking free radical chains by donating hydrogen atoms. In this reducing power assay Fe^{3+} reduction is used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. With the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}); the yellow colour of the sample changes to different shades of green and blue depending on the concentration of antioxidants present.[13]

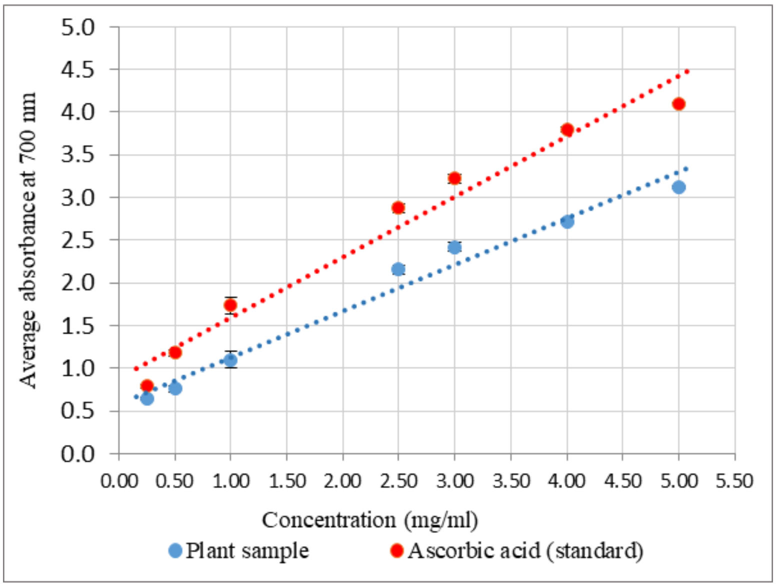

Fig. 4: Average absorbance vs. concentration for FRAP assay.

The potency of the antioxidant is estimated using the colour intensity of the formed complex. Larger colour intensity indicates higher antioxidant activity.[36] According to the present study ALEGZ showed a considerable amount of ferric reducing ability in a concentration dependent manner around the concentration range of 0.25 mg/ml to 5.00 mg/ml when compared to the standard ascorbic acid (Fig 4).

Hydroxyl Radical Scavenging Assay

Hydroxyl radical is one of the most reactive oxygen centered species and it can cause severe damages to biomolecules. It can react with polyunsaturated fatty acid moieties, thus damaging the cell membranes. Therefore these radicals may contribute to carcinogenesis, mutagenesis, and cytotoxicity.[10] In the present study, the hydroxyl radical scavenging activity was determined by measuring the competition between deoxyribose and the ALEGZ for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system.[21] Percentage Hydroxyl radical scavenging activity vs. concentration of the plant sample and the standard is given by Fig 5.

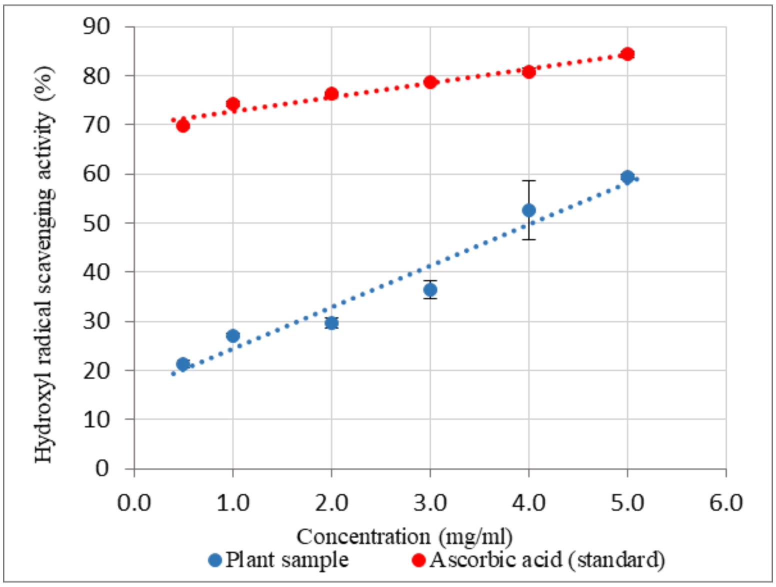

Fig. 5: Hydroxyl radical scavenging activity (%) vs. concentration.

According to the results obtained, the hydroxyl radical inhibition by ALEGZ and ascorbic acid was shown in a concentration dependent manner from 21.3% to 59.4% and from 69.7% to 84.3% respectively at the concentration range of 0.5 mg/ml to 5.0 mg/ml. Results imply that the leaf extract of *G. zeylanica* possesses the ability to scavenge hydroxyl radicals when added to the reaction mixture by preventing the degradation of 2-deoxyribose.

Anti-Inflammatory Activity

Nitric Oxide Radical Scavenging Assay

In nitric oxide radical scavenging assay, the nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. These nitrite ions are diazotized with sulphanilamide acid which then couple with NDD, turning the solution to pink colour with an absorbance at 546 nm. As nitric oxide radicals are scavenged by the antioxidants, colour intensity of the reaction mixture is reduced, decreasing the absorbance. Nitric oxide radicals that are associated with inflammations are scavenged by antioxidants present in the ALEGZ, thus reducing the toxicity.[12]

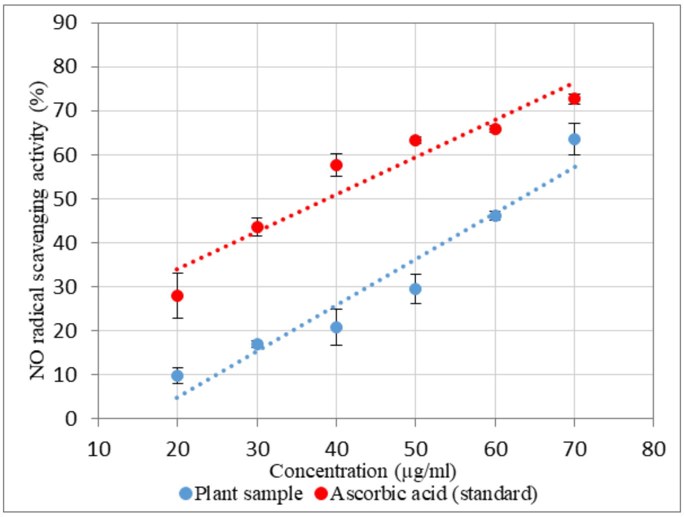

Fig. 6: Nitric oxide radical scavenging activity vs. concentration.

According to the results obtained, a considerable increase in Nitric Oxide Radical Scavenging activity from 9.8% to 63.6% was shown by the ALEGZ at the concentration range of 20 µg/ml to 70 µg/ml whereas the NO radical scavenging activity for ascorbic acid was from 28.0 % to 72.7 % at the same concentration range (Fig.6). The degree of inhibition of NO free radicals was found to be increasing with increasing concentrations of the ALEGZ, and the standard. This indicates that the ALEGZ contains compounds, capable of inhibiting the generation of nitric oxide radicals.

Human Red Blood Cell (HRBC) Membrane Stabilization Assay

Stabilization of the HRBCs membrane was studied to further evaluate the anti-inflammatory action of leaf extract of *G. zeylanica*. The percentage of membrane stabilization for the ALEGZ was tested at different concentrations ranging from 100 µg/ml to 1000 µg/ml at 540 nm and aspirin was used as the positive control. The percentages of hemolysis and protection calculated for the ALEGZ are given in Table 1.

Table 1: Percentage of hemolysis and protection for the concentration series of plant sample and aspirin.

Concentration (µg/ml)	Average percentage of hemolysis ± SD (%)		Average percentage of protection ± SD (%)	
	Plant	Aspirin	Plant	Aspirin
100	81.3 ± 0.2	80.9 ± 0.6	18.7 ± 0.2	19.1 ± 0.5
250	72.2 ± 0.9	54.1 ± 2.3	27.8 ± 0.9	45.9 ± 2.3
500	50.8 ± 0.3	44.2 ± 0.5	49.2 ± 0.3	55.8 ± 0.5
750	39.9 ± 0.4	37.3 ± 0.3	60.0 ± 0.5	62.8 ± 0.3
1000	39.3 ± 0.2	34.7 ± 0.2	60.8 ± 0.2	65.4 ± 0.2

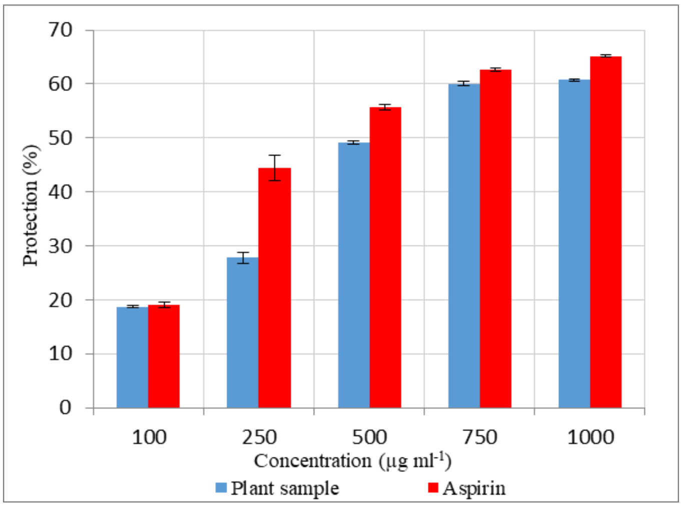

Fig. 7: Percentage of protection vs. concentration.

The results indicate that a concentration dependent increase in the HRBC membrane stabilization was observed at 100-1000 µg/ml. At this concentration range ALEGZ produced 81.3% to 39.3% inhibition of RBC hemolysis as compared to 80.9% to 34.7% of inhibition produced by the standard drug, aspirin. The percentages of protection increased with increasing concentrations of the samples. ALEGZ showed an increase in protection percentage of 18.7% to 60.8% whereas it is 19.1% to 65.4% for aspirin at the same concentration range (Fig.7). With the increasing concentrations, membrane hemolysis is decreased and membrane stabilization or the protection is increased, which implies that the anti-inflammatory activity of the ALEGZ is concentration dependent.

Antibacterial Assay

The total antibacterial activity of ALEGZ was determined using the procedure of agar well diffusion method and agar disk diffusion method. The diameters of inhibition zones created by different bacterial strains were measured to determine the antimicrobial activity (Table 2).

Table 2: Average diameters of inhibition zones in Agar Well Diffusion method and Agar Well Diffusion method.

Test method	**Bacterial strain**	**Mean diameter of the inhibition zone ± SD (mm)**		
		Plant extract	Positive control	Negative control
Agar well diffusion method	*Escherichia coli*	0.0	28.5 ± 1.3	0.0
	Bacillus subtilis	16.3 ± 0.8	20.0 ± 1.4	0.0
	Staphylococcus aureus	11.0 ± 1.4	32.0 ± 1.4	0.0
Agar disk diffusion method	*Escherichia coli*	0.0	31.7 ± 0.5	0.0
	Bacillus subtilis	10.3 ± 0.8	21.0 ± 0.9	0.0
	Staphylococcus aureus	8.3 ± 0.5	26.7 ± 1.2	0.0

Multiple drug resistance is a common issue that has emerged as a result of the indiscriminate use of commercial antimicrobial drugs utilized in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression, and allergic reactions.[37] Therefore, there is a huge demand for the development of alternative antimicrobial drugs, using medicinal plants for the treatment of infectious diseases. Several studies have been carried out in different parts of the world to screen the antimicrobial activity of different herbal extracts.[15]

According to the results of antibacterial assays done using the agar well diffusion method and agar disk diffusion method, the ALEGZ showed promising antibacterial activity towards two Gram-positive bacteria *S. aureus* and *B. subtilis*. The highest diameter of inhibition zone in both agar disk diffusion method (10.3±0.8 mm) and well diffusion method (16.3±0.8 mm) was given by *B. subtilis* whereas the diameter of inhibition zones created by *S. aureus* for these diffusion assays were 8.3±0.5 mm and 11.0±1.4 mm respectively. However, *E.coli* did not show any inhibition zone either in disk diffusion assay or well diffusion assay. *E-coli* is a Gram-negative bacteria with an impenetrable cell membrane which results in higher resistance to antibiotics. Hence, ALEGZ did not show any antibacterial activity against E-coli. According to these results it is clear that the ALEGZ can be used to develop formulations with further modifications.

CONCLUSION

The aqueous leaf extract of Sri Lankan variety of *G. zeylanica* (Kaha goraka/ Ela goraka) prepared according to the method of “Kasaya” showed promising antioxidant potential as determined by DPPH radical scavenging assay, ferric reducing power assay and hydroxyl radical scavenging assay. The aqueous leaf extract indicated good anti-inflammatory activity as determined by Nitric Oxide Radical Scavenging assay and Human Red Blood Cell Membrane stabilization assay. The extract also showed a prominent antibacterial activity against two pathogenic bacteria species, *S. aureus* and *B. subtilis*. Based on these *in vitro* studies it can be concluded that the leaves of *G. zeylanica* are a rich source of antioxidant, anti-inflammatory, and antibacterial phytochemicals and hence can be developed as a remedy to combat diseases in which free radicals are implicated and for inflammatory conditions as well as to counteract infectious diseases caused by *S. aureus* and *B. subtilis*.

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