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ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF THE LEAVES OF SRI LANKAN VARIETY OF *Durio zibethinus*

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ABSTRACT

This study was conducted to determine the total phenolic (TPC) and total flavonoid contents (TFC), antioxidant potential, and anti-inflammatory activity of the aqueous leaves extract of the Sri Lankan variety of *Durio zibethinus* (Durian) (ALEDZ). The ayurvedic "*kasaya*" preparation method was used to prepare the extract. The TPC was 90.9±6.5 mg (PGE)/g and the TFC was 77.9±9.2 mg (QE)/g. The ALEDZ displayed the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (RSA) from 7.9% to 46.5% whereas values for ascorbic acid ranged from 64.3% to 91.5% within the same concentration range (250.0-1500.0 µg/mL). The ALEDZ showed promising reducing capacity at the concentration range of 25.0-50.0 mg/mL. In the hydroxyl radical scavenging assay, the ALEDZ and ascorbic acid exhibited the RSA between 25.9%-59.8% and 46.9%-77.4%, respectively at the concentration range of 25.0-50.0 mg/mL. The anti-inflammatory activity was determined using two assays. In the NO radical scavenging assay, the plant extract has shown the RSA between 19.8%-55.3%. Within the same concentration range (20.0-100.0 µg/mL), NO RSA of ascorbic acid varied between 44.1%-78.0%. The human red blood cell membrane stabilization assay showed the protection of 12.2%-51.7% for the ALEDZ and 36.1%-73.1% for aspirin at the concentration range of 200.0-1000.0 µg/mL. Results of the present study indicate that the aqueous extract of the Sri Lankan variety of *Durio zibethinus* (Durian) (ALEDZ) has promising antioxidant and anti-inflammatory activities.

KEYWORDS: Antioxidant activity, Anti-inflammatory Activity, *Durio zibethinus*, Aqueous extract, Durian leaves.

INTRODUCTION

Plant based remedies are increasingly sought after due to the side effects associated with synthetic drugs. Plants are considered as a rich source of bioactive compounds which are responsible for many therapeutic properties such as antioxidant, anticancer, anti-inflammatory and antimicrobial activities.

Plant secondary metabolites such as phenolics, alkaloids and terpenoids are responsible for their biological activity. According to the early published literature, the antioxidant ability of many plant species directly correlate with their phenolic contents.[1] Flavonoids, a group of phenolics have been subjected to a wide spectrum of studies due to their notable antioxidant capacity and the structure-activity relationships are well-explained.[2,3]

Reactive oxidizable substances present in the body such as free radicals and reactive oxygen species (ROS) can readily react with important biomolecules; resulting in a series of detrimental consequences, certain types of malignant tumors, cardiovascular diseases and anti-inflammatory diseases.[4] Antioxidants have the ability to scavenge ROS. Therefore, it is beneficial to boost the endogenous antioxidant system with naturally occurring bimolecular species.

Inflammation is a body's natural protective mechanism which acts against pathogens, damaged cells, or irritants. Though these responses are essential, the prolonged activation may cause detrimental conditions. Non-steroidal anti-inflammatory drugs (NSAIDS) which are used in the treatment of such conditions cause much side effects. Hence there is an increasing interest on natural substances with anti- inflammatory properties.[5]

Durian (*Durio zibethinus*), the King of fruits is a tropical plant mainly grown in Southeast Asian countries. The unique smell of this fruit is due to presence of volatile Sulphur compounds. Research studies have revealed many medicinal properties associated with durian fruits include antioxidant, anti-diabetic, anti-cancer, anti-obesity and anti-cardiovascular properties.[6]

In the present study, the aqueous leaves extract of Sri Lankan variety of *Durio zibethinus* was used to investigate the total phenolic and flavonoid contents, antioxidant and anti-inflammatory activities.

MATERIALS AND METHODS

Collection and preparation of plant materials

Fresh leaves of durian were collected in September 2020 from Avissawella, in Colombo district, Sri Lanka. The leaves were authenticated by the Division of Pharmaceutical Botany, Bandaranayake Memorial Ayurvedic Research Institute, Navinna, Mahargama. A voucher specimen (accession no. 2064) was deposited at the herbarium of the same institute.

The collected leaves were washed with water and air-dried for a period of two weeks at room temperature. The dried leaves were shredded into small pieces and stored in an air-tight container until required.

Preparation of water extract

The extract was prepared according to the method of Ayurvedic *kashaya* (decoction) preparation. The dried durian leaves (60 g) were mixed with 1920 mL of water and boiled until the final volume is reduced to 240 mL.[7] The aqueous extract was filtered with a fine silk cloth. Then, the filtrate was freeze-dried. The freeze-dried sample was stored at -4 °C in an air-tight container until required.[8] The prepared aqueous leaves extract of *Durio zibethinus* is abbreviated as ALEDZ.

Preparation of stock solution of sample

A stock solution with a known concentration was prepared by dissolving an appropriate amount of freeze-dried sample in a known volume of distilled water. A concentration series was prepared by diluting the stock solution with distilled water.

The prepared sample solution was used to determine the total phenolic and flavonoid contents, antioxidant and anti-inflammatory activities of the ALEDZ using seven *in-vitro* assays.

Determination of Total Phenolic Content (TPC)

The sample solution (200 µL) was mixed with 2% Na_2CO_3 (4 cm^3) and incubated in darkness for two minutes. Then, Folin-Ciocalteu reagent (FCR) (200 µL) was added and incubated again in darkness for 30 minutes. After the incubation, the absorbance measurement was taken at 750 nm. The blank was prepared by replacing FCR with distilled water. Pyrogallol was used as the standard. The TPC was expressed as milligrams of pyrogallol equivalents (PE) per one gram of freeze-dried sample using the pyrogallol calibration curve of absorbance *vs.* concentration. The higher TPC is indicated by higher absorbance values. [8]

Determination of Total Flavonoid Content (TFC)

The sample solution (0.5 cm^3) was mixed with distilled water (2 cm^3) and 5% $NaNO_2$ (150 µL) and incubated in darkness for five minutes. Then, 10% $AlCl_3$ (150 µL) was added to the reaction mixture and again kept in darkness for six minutes. After that, 1M NaOH (1 cm^3) and distilled water (1 cm^3) were added and the absorbance was measured at 510 nm. The blank was prepared by replacing $AlCl_3$ with distilled water. Quercetin was used as the standard. The TFC was expressed as milligrams of quercetin equivalents (QE) per one gram of freeze-dried sample using the quercetin calibration curve of absorbance *vs.* concentration.[9]

Antioxidant Assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

A stock solution of DPPH (240 µg/mL) was prepared by dissolving DPPH (0.006 g) in distilled methanol (25 cm^3) and stored at 20 °C until required. The working DPPH solution was prepared by diluting the stock solution with distilled methanol to attain an absorbance of about 0.98 ± 0.02 at 517 nm. An aliquot (3 cm^3) of the working DPPH solution was added to the sample solution (100 µL) and shaken well. Then, the reaction mixture was incubated for fifteen minutes in the dark at room temperature. The absorbance was taken at 517 nm. A mixture of distilled water (100 µL) and distilled methanol (3 cm^3) was used as the reagent blank. Ascorbic acid was used as the standard. The same procedure was conducted for the concentration series of ascorbic acid (100 µL) instead of the ALEDZ. The control was prepared by mixing only the working DPPH solution (3 cm^3) and distilled water (100 µL) without the plant sample or ascorbic acid. The DPPH RSA (%) was calculated for each concentration using the following equation (1).[10]

Equation 1- DPPH radical scavenging activity (RSA) (%)[10]

$$\text{DPPH RSA (\%)} = \left[\frac{(A_c - A_s)}{A_c} \right] \times 100\%$$

A_c - The absorbance of the control

A_s - The absorbance of the sample

Ferric Reducing Antioxidant Power (FRAP) Assay

The sample solution (2 cm^3) was mixed with phosphate buffer (2 cm^3, pH 6.6, 0.2 M) and potassium ferricyanide (2 cm^3, 10.0 mg/mL). Then, the reaction mixture was incubated at 50 °C for 20 minutes. After the incubation, trichloroacetic acid (TCA) (2 cm^3, 100.0 mg/L) was added followed by centrifugation at 3000 rpm for 10 minutes. The supernatant (2 cm^3) was collected and added to a mixture of distilled water (2 cm^3), and freshly prepared $FeCl_3$ (0.4 cm^3, 0.1%; w/v). After 10 minutes, the absorbance was measured at 700 nm using distilled water as the reagent blank. Ascorbic acid was used as the standard. The same procedure was conducted for the concentration series of ascorbic acid (2 cm^3) instead of the ALEDZ.[8]

Hydroxyl Radical Scavenging Assay

The sample solution (100 µL) was mixed with 500 µL of 2-deoxyribose (2-DR) (2.8 mM in 50 mM, pH 7.4

phosphate buffer), 200 µL of premixed $FeCl_3$ (100 mM) and EDTA (100 mM) solution (1:1; v/v), and 100 µL of H_2O_2 (200 mM). A volume of 100 µL of ascorbic acid (300 mM) was added to the reaction mixture in order to trigger the reaction and incubated at 37 °C for one hour. After the incubation, a volume of 0.5 cm^3 of the reaction mixture was added to 1 cm^3 of 2.8% (w/v) TCA solution. Then, a volume of 1 cm^3 of 1% (w/v) thiobarbituric acid was added. The mixture was heated for fifteen minutes on a boiling water bath and cooled. The absorbance was measured at 532 nm. Distilled water was used as the reagent blank. Ascorbic acid was used as the standard. The same procedure was conducted for the concentration series of ascorbic acid (100 µL) instead of the ALEDZ. The control was prepared by adding 2-DR, pre-mixed $FeCl_3$, and EDTA solution, H_2O_2, and ascorbic acid without the plant sample or ascorbic acid. The hydroxyl RSA (%) was calculated using the following equation (2).[8]

Equation 2 – Hydroxyl radical scavenging activity (RSA) (%)[8]

$$\text{Hydroxyl RSA (\%)} = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100\%$$

A_s - The absorbance of the sample

A_c - The absorbance of the control

Anti-Inflammatory Assays

Nitric Oxide Radical Scavenging Assay

Sodium nitroprusside (SNP) (10 mM) in phosphate buffer saline (PBS) (0.5 cm^3) was added to the sample solution (1 cm^3). Then, the reaction mixture was incubated for three hours at 25 °C. The Griess reagent was prepared immediately before use by mixing equal volumes of 1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NDD). After the incubation, the freshly prepared Griess reagent (1 cm^3) was added to the reaction mixture. The absorbance of the pink color solution formed due to the formation of an azo dye was measured at 546 nm using distilled water as the reagent blank. Ascorbic acid was used as the standard. The same procedure was conducted for the concentration series of ascorbic acid (1 cm^3) instead of the ALEDZ. The control was prepared by adding only SNP, PBS, and Griess reagent without the plant sample or ascorbic acid. The nitric oxide RSA (%) was calculated using the following equation (3).[11]

Equation 3 - $NO^{\bullet}$ radical scavenging activity (%)[11]

$$NO^{\bullet}\text{ RSA (\%)} = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100\%$$

A_s - The absorbance of the sample (Plant extract or standard)

A_c - The absorbance of the control

Human Red Blood Cell (HRBC) Membrane Stabilization Assay

A sample of blood (5 cm^3) was collected from myself, I had not taken any anti-inflammatory drugs for two weeks before the experiment. The collected blood sample was centrifuged at 5000 rpm for 20 minutes. After the centrifugation, the yellow color supernatant was separated and discarded. The pellet was washed three times with normal saline using a volume equal to the pellet volume. In order to prepare the 10% red blood cell suspension, pellet red blood cells (1 cm^3) were dissolved in normal saline (9 cm^3).[8]

The prepared red blood cell suspension (0.1 cm^3) was added to the sample solution (1 cm^3). Then, the reaction mixture was incubated at 56 °C for 30 minutes. After the incubation, it was cooled and centrifuged at 3000 rpm for ten minutes. The absorbance readings of the supernatant were taken at 540 nm using distilled water as the reagent blank. Aspirin was used as the standard. The same procedure was conducted for the concentration series of aspirin (1 cm^3) instead of the ALEDZ. The control was prepared without adding the plant extract or aspirin. The percentage of hemolysis and protection were calculated using the following equations (4 and 5).[8]

Equation 4 – Hemolysis (%)[8]

$$\text{Hemolysis (\%)} = \frac{A_s}{A_c} \times 100\%$$

Equation 5 – Protection (%)[8]

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

A_c - The absorbance of the sample (Plant extract or standard)

A_s - The absorbance of the control

RESULTS AND DISCUSSION

Determination of Total Phenolic Content (TPC)

Phenolics are the main contributors of the antioxidant activity of fruits, vegetables and grains.[1] These compounds have at least one aromatic ring connected to hydroxyl groups. Antioxidant activity of phenolics increases with increasing the number of free OH groups in the structure and with increasing the conjugation of side chains to the aromatic ring.[12,13]

The Folin-Ciocalteu assay was used in this study to determine the TPC of durian leaves. According to the results, the ALEDZ has exhibited a TPC of 90.9 ± 6.5 mg(PGE)/g. It has been reported that there is a good linear correlation between the TPC and antioxidant activity.[14] Therefore, natural sources rich in phenolics can be suspected to have the antioxidant potential.

Determination of Total Flavonoid Content (TFC)

Flavonoids are one of the largest groups of naturally occurring phenolics. Due to the promising antioxidant properties of flavonoids, they have been extensively studied.[2] In the present study, the aluminium chloride colorimetric assay was used to estimate the TFC from which the ALEDZ exhibited a TFC of 77.9 ± 9.2 mg(QE)/g. Based on this result, durian leaves can be

considered as a good source of flavonoid and that indicates their promising antioxidant potential.

From the above-mentioned outcomes, the ALEDZ has moderate total phenolic and flavonoid contents. It is evident that phenolics and flavonoids are widely contributed to the antioxidant activity of plant species.[10,11]

Antioxidant assays

DPPH Radical Scavenging Assay

In this assay, a stable free radical, DPPH is used which is purple in color with the maximum absorption at 517 nm in methanol. This can undergo reduction (DPPH• to $DPPH_2$) by accepting an electron or a hydrogen atom from antioxidants. Hence, the scavenging of DPPH radicals through the addition of a radical scavenging species or an antioxidant is the basis behind this assay.[10] The color of the reduced form is yellow. Due to the decolorization of purple color, absorption values at 517 nm were decreased and that can be spectroscopically measured.[15]

The plot of DPPH RSA (%) *vs.* concentration of the sample and the standard ascorbic acid is shown in Figure 1. In this study, the DPPH RSA of the ALEDZ varied in the range of 7.9% - 46.5% whereas ascorbic acid varied in the range of 64.3% - 91.5% between the concentrations of 250.0- 1500.0 µg/mL.

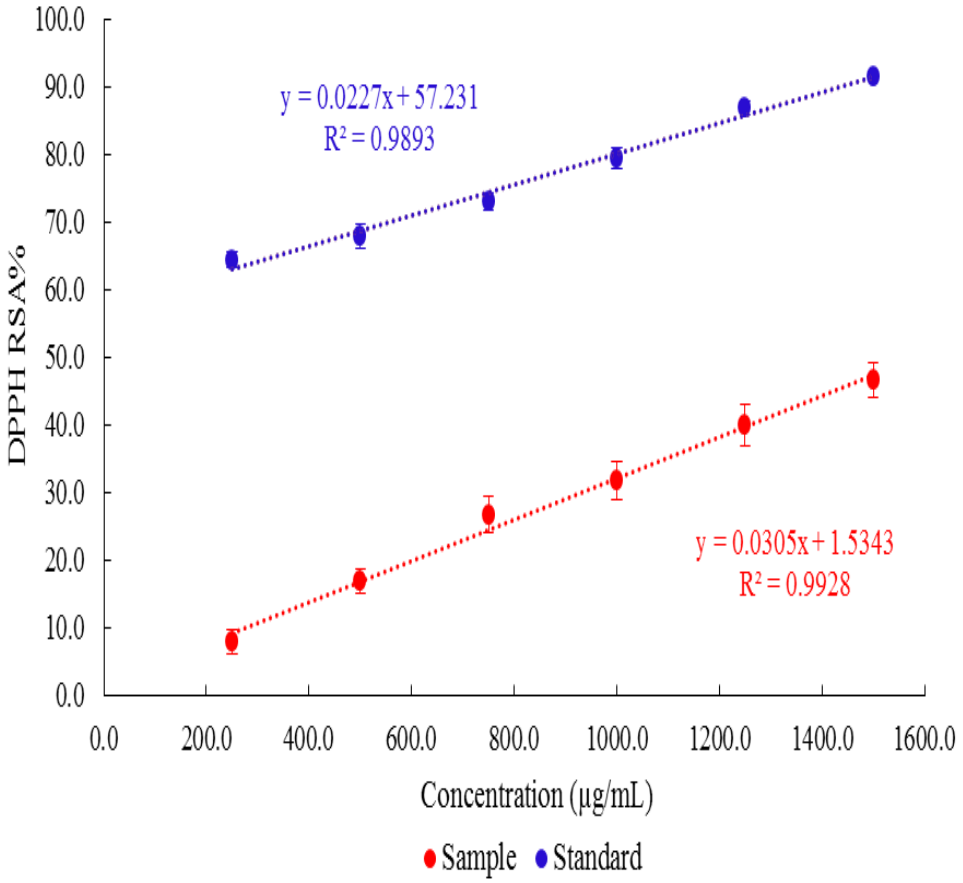

Figure 1: The plot of DPPH RSA (%) *vs.* the concentration of the plant sample.

Ferric Reducing Antioxidant Power (FRAP) Assay

In this assay, ferric ions are reduced to ferrous ions by the donation of electrons from the antioxidants forming the complex, $Fe_4[Fe(CN)_6]_3$. Due to this complex formation, the initial yellow color of the reaction mixture changes to pale green/blue color based on the degree of reducing capacity of the sample. The higher reducing capacity is indicated by the higher absorbance values at 700 nm.[16]

Figure 2 shows the plot of absorbance *vs.* concentration for the plant sample and the standard ascorbic acid. From the results of this assay, the ALEDZ has shown promising reducing activity in the concentration range of 25.0-50.0 mg/mL when compared to ascorbic acid. Previous reports suggested that the reducing activity can be used to express the antioxidant potential by the donation of a hydrogen atom to break the free radical chain.[10]

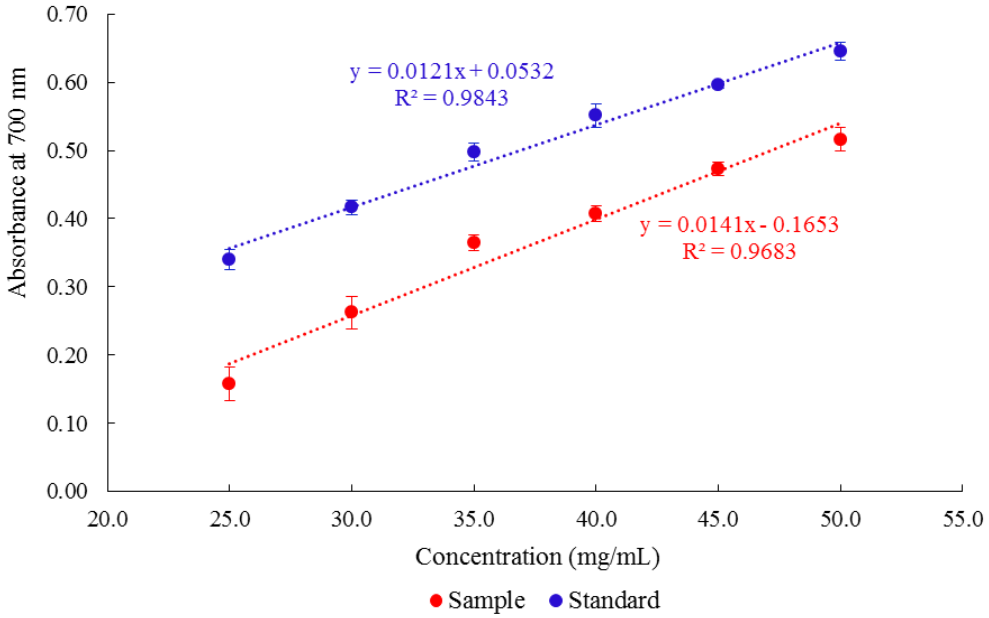

Figure 2: The plot of absorbance *vs.* concentration for the sample and the standard ascorbic acid.

Hydroxyl Radical Scavenging Assay

In this assay, hydroxyl radicals are produced from a reaction, called the Fenton reaction. Then, the produced OH radicals oxidize 2-deoxyribose (2-DR) and the reaction products form a pink chromogen after heating with TBA.[17] When the plant sample containing the hydroxyl radical scavengers is added to the medium, it competes with 2-DR and diminishes the chromogen formation. The hydroxyl radical scavenging ability can be calculated by measuring the absorbance at 532 nm. The higher radical scavenging capacity is indicated by the lower absorbance values.[18]

Hydroxyl radicals are considered as one of the powerful reactive oxygen species in biological systems. It has the ability to cause damages to almost all the biomolecules thereby contributing to carcinogenesis, mutagenesis, and cytotoxicity.[10] Antioxidants play a crucial role in scavenging these radicals. Figure 3 shows the plot of hydroxyl RSA (%) *vs.* concentration of the sample and the standard ascorbic acid. In this study, the hydroxyl RSA of aqueous extract of the durian leaves was evaluated. The results exhibited the percentage of hydroxyl RSA of the ALEDZ varied between 25.9%-59.8% whereas, for ascorbic acid, it varied between 46.9%-77.4% within the concentration range of 25.0-50.0 mg/mL.

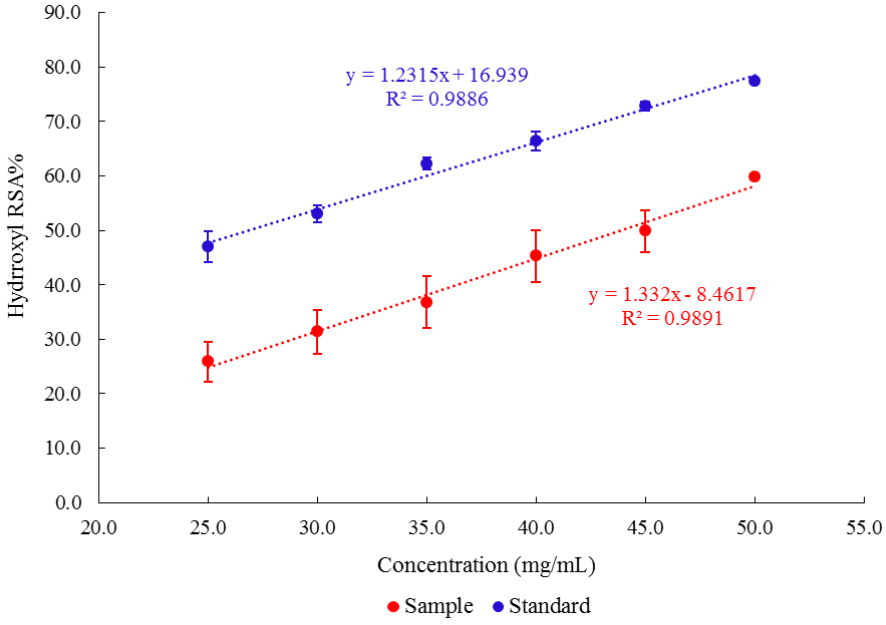

Figure 3: The plot of hydroxyl RSA (%) *vs.* concentration.

Anti-inflammatory Assays

Nitric Oxide Radical Scavenging Assay

This assay was used to indirectly determine the amount of $NO^{\bullet}$ scavengers present in the sample using its stable decomposition product; nitrite (NO_2^-) ions. The Griess reaction, a two-step diazotization reaction was used to estimate the production of nitrites in which nitrites are converted to a pink color chromophoric azo product with the maximum absorption at 546 nm. The nitric oxide scavenging constituents in the plant sample compete with oxygen to scavenge $NO^{\bullet}$. As a consequence of this, the fewer amount of nitrite ions are generated and that will cause to reduce the production of the colored azo dye.[19,20]

Figure 4 shows the plot of NO RSA *vs.* concentration of the sample and the standard ascorbic acid. According to the results obtained in this study, the ALEDZ has shown a significant NO RSA from 19.8% to 55.3% within the concentration range of 20.0-100.0 µg/mL. The standard ascorbic acid has shown the NO RSA between 44.1% to 78.0% at the same concentration range. Therefore, it can be clearly observed that the aqueous extract of durian leaves has shown a notable amount of anti-inflammatory activity compared to the ascorbic acid.

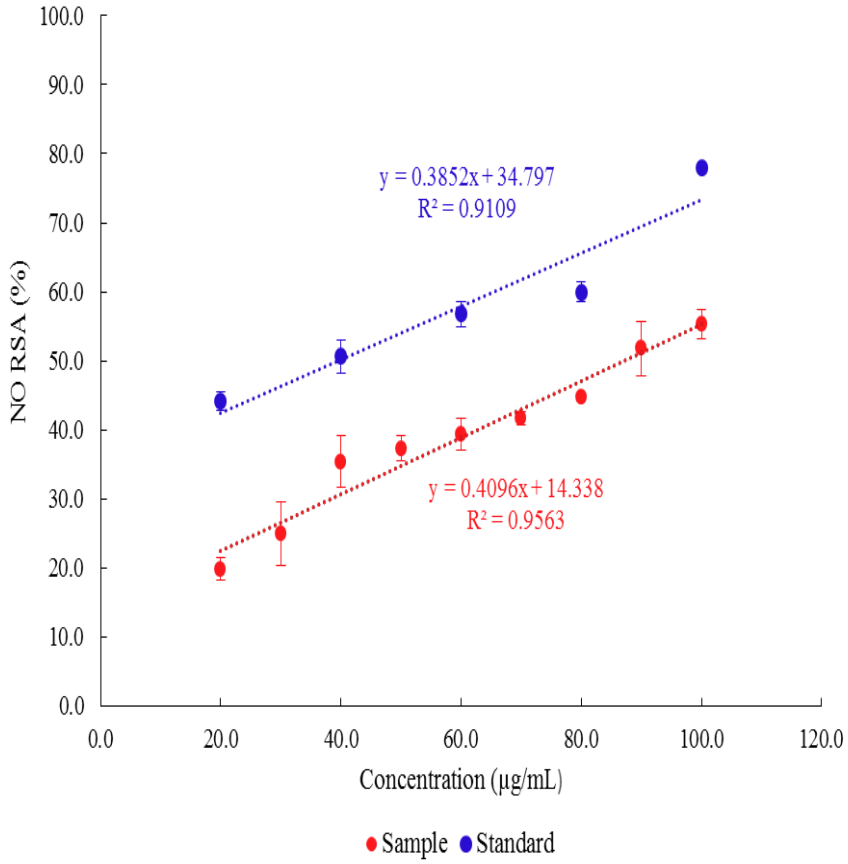

Figure 4 : The plot of NO RSA (%) *vs.* concentration of the sample and the standard.

Human Red Blood Cell (HRBC) Membrane Stabilization Assay

In this assay, anti-inflammatory activity was expressed in terms of the membrane stabilization ability of the plant extract. Due to the structural similarity between the lysosomal membrane and the red blood cell membrane, HRBCs were used and the heat-induced hemolysis of HRBCs was examined. Figure 5 shows the plot of the protection (%) *vs.* concentrations of the sample and aspirin which was calculated according the equation 5. For durian leaves extract, the protection percentage has varied from 12.2 % to 51.7 %. At the same concentration range (200.0 – 1000.0 µg/mL), the protection percentage of aspirin has varied from 36.1% to 73.1%. This result shows that the ALDEZ has the anti-inflammatory activity.

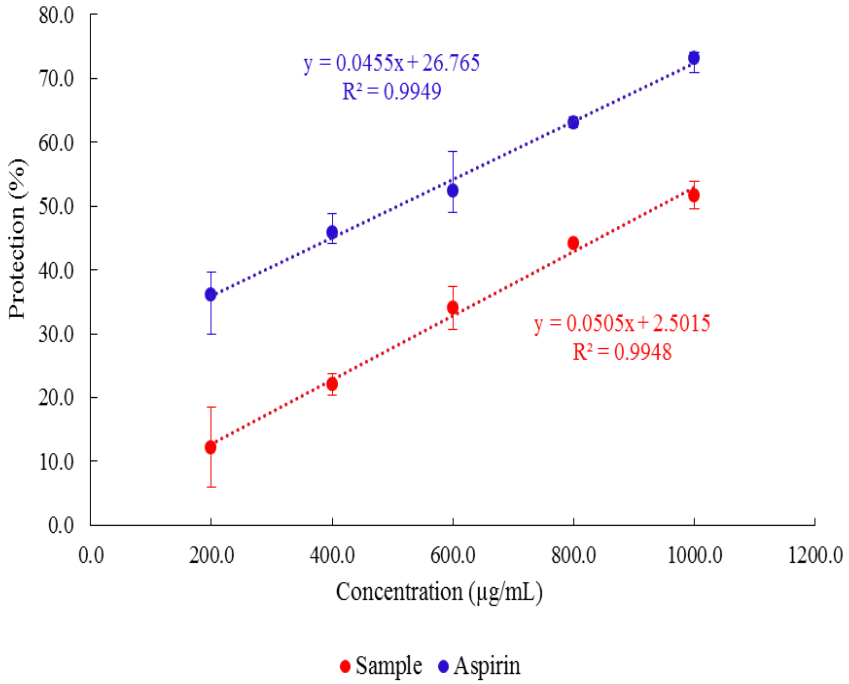

Figure 5: The plot of percentage of protection *vs.* concentrations for the sample and aspirin.

CONCLUSION

It is noteworthy that the decoctions prepared from durian leaves are believed to have antipyretic effect as well as they are used in the treatment of malaria, fatigue, phlegm, colds, skin diseases, jaundice, and swellings in traditional medicine in Malaysia[6] which provide strong evidences to their non-toxic nature.

In the present study, the aqueous leaves extract of the Sri Lankan variety of *Durio zibethinus* (durian) prepared according to the method of "*kasaya*" in Ayurveda system of medicine has shown promising antioxidant potential as evaluated by DPPH radical scavenging assay, ferric reducing power assay, and hydroxyl radical scavenging assay. In addition, the durian leaves extract has exhibited a prominent anti-inflammatory activity as evaluated by NO radical scavenging assay and human red blood cell membrane stabilization assay. Therefore, it can be concluded that the aqueous extract of durian leaves possesses the antioxidant activity and anti-inflammatory activity.

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