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Research Article

ANTIOXIDANT, ANTIBACTERIAL AND ANTI-INFLAMMATORY POTENTIAL OF THE AQUEOUS EXTRACT OF THE RAW LEAVES OF SRI LANKAN VARIETY OF *PERSEA AMERICANA* MILLER (AVOCADO)

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ABSTRACT

Despite the advances in modern medicine, plant based remedies are currently sought after as alternatives to synthetic drugs. As such natural sources are being searched to use as antioxidants, anti-inflammatory, and antibacterial compounds. This study was focused on the investigation of aqueous leaf extract of Sri Lankan variety of *Persea americana* (ALEPA) which was prepared according to the method of *Kasaya* (Decoction) in Ayurvedic medicine to test its antioxidant, anti-inflammatory and antibacterial activities.

The total phenolic content of ALEPA was determined by Folin Ciocalteu assay was 681±6.8 mg (PGE) /g (PGE= Pyrogallol equivalents) and the flavonoid content was 1193±302 mg (QE)/g (QE= Quercetin equivalents) according to the $AlCl_3$ colorimetric assay.

DPPH radical scavenging activity (RSA) of ALEPA was 11.3±2.8-52.3±5.0% for concentrations of 50-500 µg/ml whereas ascorbic acid showed 28.0±0.7-90.7±0.1% in the concentration range of 200-500 µg/ml. ALEPA showed better ferrous ion reducing activity compared to ascorbic acid. Hydroxyl RSA of ALEPA was 17.4±1.3-41.5±0.5% in the concentration range of 20-50mg/ml whereas ascorbic acid showed 7.0±0.2-19.8±0.5% for the same concentration range.

Overproduction of nitric oxide free radicals are implicated in inflammation. In the NO scavenging assay, ALEPA showed activity 26.0±3.6-69.2±2.5% in the concentrations of 0.2-8 ppm whereas ascorbic acid showed activity 26.8±2.5-49.3±1.9% in the concentrations of 0.2-4 ppm. In human red blood cell (HRBC) assay, ALEPA showed 48.0±1.9-60.6±2.6% HRBC membrane protection whereas the Asprin showed 39.7±0.4-49.0±0.5% activity. The prevention of HRBC membrane lysis is taken as a measure of anti-inflammatory activity and also ALEPA showed antibacterial activity towards *Escherichia coli, Streptococcus aureus,* and *Bacillus subtilis.*

KEYWORDS: Antioxidant activity, Anti-inflammatory activity, Antibacterial activity, *Persea Americana.*

INTRODUCTION

Since ancient times, plants have been used as foods, flavors, dyes, aromatic fragrances, lubricants, as well as in medicine.[1] Sri Lanka has many endemic plants which have been used as remedies for various diseases. Use of natural plant extracts to combat, cure, and relieves symptoms of many diseases has gained significant due to either long term or short term toxicity, side effects, and developing resistance with respect to synthetic medicines.[2]

Reactive Oxygen Species (ROS) are formed in the body through normal and essential metabolic reactions such as energy generation from mitochondria or the detoxification reactions involving the liver cytochrome p-450 enzyme system.[3] There are inherent compounds known as antioxidants which are capable of preventing or delaying the formation of ROS such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, and ubiquinol.[3,4] Oxidative stress occurs when the ROS formation and degradation balance cannot be maintained by the inherent compounds. This may lead to various diseases like Alzheimer's disease, cancer, Parkinson disease, atherosclerosis, diabetes, and immune suppression.[5] Therefore, much attention is directed towards external use of antioxidants. Furthermore, due to more favorable effects of natural antioxidants researchers focus on plant based antioxidants. At present, arthritis rheumatism is well known inflammatory disease which is treated with synthetic steroidal and non-steroidal drugs. But, they are known to show various adverse side effects such as

renal problems, gastrointestinal irritations, high blood pressure, headache, and dizziness.[6] Antibacterial agents are inhibitory chemical compounds which can be either retard the growth of the bacteria (bacteriostatic) or kill the bacteria (germicides). The resistance towards synthetic antibacterial drugs favors the use of natural antibiotics.[7]

Persea americana Miller (avocado) is a tropical fruit which is originated in Mexico and Central America. In Sri Lanka, it is very common in wet zone. This belongs to the family Lauraceae. Avocado has been used to treat Alzheimer's disease, osteoarthritis, and cardiovascular effects.[8] Further, previous studies has shown that seeds have insecticidal, fungicidal, and antimicrobial properties.[8] This study was conducted to determine the antioxidant, anti-inflammatory, and antibacterial activities of aqueous extract of *Persea americana* (ALEPA).

MATERIALS AND METHODS

Collection of samples

Avocado raw leaves were collected from Kalutara, Sri Lanka (Latitude: 6.562354 | Longitude: 79.818897) and identification was carried out by a botanist at Plant herbarium of University of Colombo.

Preparation of water extract

Collected raw leaves were air dried for 24 h in room temperature and powdered using a domestic grinder to obtain fine particles and stored in air tight container in a refrigerator. Water extract of powdered sample was prepared according to the traditional method practiced in Ayurveda to prepare '*Kasaya*'.[9] A weight of 60 g of the powdered sample was boiled simmer in 960 ml to obtain the decoction of 240 ml. This water extract was filtered through a fine silk cloth. The filtrate was freeze dried and the powdered sample was kept at -4 ^{0}C in a cold room in an air tight container.

A stock solution of known concentration was prepared for each experiment by dissolving a known amount of freeze dried sample in a known volume of water. A concentration series was prepared by diluting the stock solution.

Estimation of Total Phenolic Content (TPC)

TPC was determined following a method by Saeed *et al.* A volume of 4 ml of 2% of Sodium bicarbonate and 200 µl of sample were mixed and incubated in dark for 2 min. Then a volume of 200 µl of Folin Ciocalteu reagent was added and incubated in dark for 30 min. The absorbance was measured at 750 nm. The blank was prepared by mixing 200 µL of methanol, 4 ml of $NaHCO_3$ and 100 µl of Folin Ciocalteu reagent. The same procedure was carried out for the standard series using Pyrogallol instead of the sample. TPC was determined from the calibration curve of Pyrogallol. The TPC was expressed as milligrams of Pyrogallol equivalents (PGE) per gram of dried sample.[1]

Estimation of the Total Flavonoid Content (TFC)

TFC was determined by a spectroscopic method.[1] A volume of 0.5 mL of sample, 2.0 mL of distilled water and 150 µl of 5% Sodium nitrite solution were mixed and incubated in the dark for 5 min. A volume of 150 µl of a 10% Aluminium chloride was added to the same mixture and kept in dark for 6 min. Then a volume of 1.0 mL of 1 M Sodium hydroxide solution and 1.0 mL of distilled water were added to the reaction mixture. The absorbance was measured at 510 nm. The blank was prepared without adding $AlCl_3$. The standard curve for TFC was made using Quercetin under the same procedure. The TFC was expressed as milligrams of Quercetin equivalents (QE) per gram of dried sample.

Estimation of In Vitro Antioxidant Activity

DPPH radical scavenging activity

measured following a method Saeed *et al.* A volume of 3.0 mL of DPPH working solution and 100 µL of the sample were mixed well and incubated in the dark for 15 min. The absorbance was measured at 517nm. The control was prepared using 3.0 mL of DPPH working solution and 100 µL of distilled water. The blank was prepared using 3.0 mL of distilled methanol and 100 µL of distilled water. The capacity to scavenge DPPH radical was calculated by following equation[1].

$$SA\ (\%) = \frac{A_0 - A_S}{A_0} \times 100$$

Where A_0 is absorbance of the control and A_S is the absorbance in the presence of extracts or standard.

The same procedure was carried out for standard concentration series using 100 µL of ascorbic acid instead of the sample.

Iron reducing power

Ferrous ion reducing power was determined following a method by Thiripuranathar *et al.* A mixture of 2 mL of phosphate buffer (pH 6.6) and 2 mL of Potassium ferricyanide and 2 mL of sample were allowed to incubate at 50 ^{0}C for 20 min. After the incubation, 2 mL of Trichloroacetic acid (100 mg/mL) was added. Then the solution was centrifuge at 3000 rpm for 10 min and a volume of 2 mL of supernatant was mixed with 2 mL of distilled water and 0.4 mL of freshly prepared Ferric chloride. The absorbance readings were taken at 700 nm. Distilled water was used as the blank. The same procedure was carried out for the standard ascorbic acid solution.[10]

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined by the ability of ALEPA to scavenge the hydroxyl radicals generated by the Fe^{3+}- ascorbate-EDTA - H_2O_2 system.[1] A volume of 500 µL of 2-deoxyribose, 200 µL of premixed Ferric chloride & EDTA mixture and 100 µL of Hydrogen peroxide were added to each of 100 µL of sample. A volume of 100 µL of ascorbic acid was added to trigger the reaction. Each solution mixture was incubated at 37 ^{0}C for 1 h. A volume of 0.5 mL of the reaction mixture was mixed with a volume of 1 mL of TCA (2.8% w/v) and 1 mL of TBA (1% w/v) were added to each. The mixture was boiled for 15 min. The boiled solutions were allowed to cool to the room temperature. The absorbance readings were measured at 532 nm. Distilled water was used as the blank. The controlled was prepared using the same solutions except for adding the sample. The same procedure was carried out for the standard ascorbic acid solution. Percentage of scavenging activity (SA) was calculated by the following equation.

$$\text{Scavenging activity}(\%) = \left(\frac{1 - A_S}{A_0}\right) * 100$$

Where A_0 is absorbance of the control and A_S is the absorbance in the presence of extracts or standard.

Estimation of Anti-inflammatory Activity

Nitric Oxide Radical Scavenging Activity

NO radical scavenging activity was determined following a method by Boora *et al.* A volume of 0.5 mL of Sodium nitroprusside (SNP) and 1.0 mL of ALEPA were mixed and incubated at 25 ^{0}C for 180 min. A volume of 1 ml of Griess reagent (equal volumes of 1% Sulphanilamide and 0.1% Napthylethylene diamine dichloride) was mixed with the reaction mixture. The absorbance was measured at 546 nm. The control was prepared using SNP, Phosphate buffer saline (PBS), and Griess reagent. Distilled water was used as the blank. The same procedure was carried out for the standard concentration series using 1.0 mL of ascorbic acid instead of the sample. Percentage SA was calculated using the following equation.[11]

$$\text{SA}\ (\%) = \left(\frac{A_0 - A_S}{A_0}\right) * 100$$

Where A_0 is absorbance of the control and A_S is the absorbance in the presence of extracts or standard.

Human Red Blood Cell Assay (HRBC)

HRBC assay was carried out according to previously published method.[12] A healthy volunteer (self) who had not taken anti-inflammatory drugs for a period of 2 weeks before obtaining blood sample was selected as the donor of human blood. A volume of 5 mL of blood was collected and it was centrifuged at 3000 rpm for 20 minutes. The pellet was washed using a volume of normal saline equal to the pellet volume. Centrifugation was carried out until yellowish color of the supernatant became clear. A volume of 1mL of ALEPA and 0.1 mL of red blood cell suspension were combined and incubated at 56 ^{0}C for 30 minutes. The mixture was cooled and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured at 540 nm. The control was prepared using the red blood cell suspension only. Distilled water was used as the blank. The same procedure was carried out for standard concentration series using aspirin instead of the sample. Percentage of protection was calculated using the following equation.

$$Protection\ (\%) = 100 - (\frac{A_S}{A_O} \times 100)$$

Where A_0 is absorbance of control at 540 nm and A_S is the absorbance in the presence of extract or standard.

Estimation of Antibacterial Activity

Plant extract was tested against three bacterial strains; *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (MRSAATCC33591), and *Bacillus subtilis* which were obtained from the pharmacy laboratory of the Faculty of Science, University of Colombo, Sri Lanka. Bacterial strains were grown on LB agar (Lysogenyl broth) medium and incubated overnight at 37 ^{0}C. The bacterial cell suspension was prepared by adjusting the turbidity against 0.5 % McFarland standards. Bacterial spread plates were prepared by inoculating fresh cell suspension (200 µL) on LB agar plates.[13,14] The antibacterial activity of ALEPA was evaluated using agar well and disk diffusion methods.

Disc Diffusion Method

Three sterilized filter paper discs (diameter of 6 mm) were immersed in 40 µL of ALEPA solution. Discs were allowed to dry at room temperature and these dried discs were placed on the bacterial spread plates. One disc was immersed in a volume of 10 µL of the Ciprofloxacin solution (1 mg/mL) as the positive control and the other disc was immersed in a volume of 10 µL of distilled water as the negative control. Both were placed on the bacterial spread plates. The plates were inverted and kept overnight. The average diameter of the resultant inhibition zones were measured.[13]

Agar Well Method

Using a sterilized cork borer 5 wells were made on the spread plate. Three wells were filled with ALEPA (about 50 µL), other with positive control (Ciprofloxacin) and the remaining well was filled with negative control (distilled water). The

plates were sealed, labeled and incubated overnight at 37 ^{0}C. The antibacterial activity was evaluated by measuring the diameter of the inhibition zones.[15,16]

RESULTS AND DISCUSSION

Phenolics and flavonoids are well known secondary metabolites in plants. They exhibit high radical scavenging activity against most of the oxidizing molecules such as singlet oxygen, and various free radicals.[1,2,17] TPC of ALEPA was determined using Folin Ciocalteu colorimetric method. ALEPA gave a value of 681 ± 6.8 mg (PGE)/ g for the TPC. TFC was estimated using Aluminium chloride colorimetric method. ALEPA gave a value of 1193 ±3.2 mg (QE) / g.

Free radicals are involved in damaging almost every molecule in Pathophysiological processes which ultimately cause various diseases. Anti-oxidants are capable of protecting bio molecules against these free radicals which means they can act as free radical scavengers.[1,2] DPPH assay is the widely used method to determine antioxidant properties. This assay measures the ability to donate electrons from the plant extract to the DPPH radical. The hydrogen atom donation causes the purple color to diminish in the test mixtures. The potency of antioxidant and the concentration of antioxidant will determine the degree of color change.[9] According to the Fig. 1, ALEPA showed radical scavenging activity of 11.3±2.8% to 52.3±5.0% within the concentration range of 50 µg/mL to 300 µg/mL. Ascorbic acid showed 28.0±0.7-90.7±0.1% in the concentration range of 200-500 µg/mL.

Therefore, this study shows that, ALEPA has the potential to donate a hydrogen atom to a free radical to scavenge the potential damage.

In ferrous reducing assay, Fe^{3+}/ ferricyanide convert into ferrous ion in the presence of antioxidants which leads to change the color from yellow to green. The antioxidant reducing power will determine the degree of the color change. Reducing power properties exerts antioxidant activity by donating a hydrogen atom to break the free radical chain.[3] According to the Table 1 ALEPA showed 0.26 to 1.06 absorbance values within the concentration range of 5-20 mg/mL whereas, ascorbic acid showed 0.41 to 0.64 absorbance values within the same concentration range. Higher absorbance indicates higher reducing power. According to the study, ALEPA showed its reducing power by reducing Fe^{3+}/ ferricyanide complex to ferrous form. This reduction is caused by the antioxidants present in the ALEPA.

Table 1: The Absorbance Values of ALEPA And Ascorbic Acid At Different Concentration In The Reducing Power Assay

Concentration mg/ml	Absorbance of ALEPA±SD	Absorbance of ascorbic acid ±SD
20	1.06±0.03	0.64±0.04
18	1.01±0.04	0.52±0.01
15	0.75±0.02	0.57±0.01
10	0.51±0.04	0.57±0.01
5	0.26±0.02	0.41±0.01

Hydroxyl radical is one of the potent radical species which cause damage to biological cells by reacting with polyunsaturated fatty acid moieties of the cell membrane.[1] In this hydroxyl scavenging assay OH radicals are formed via a reaction mixture of H_2O_2 and ferrous ions which leads to the reaction with 2-deoxyribose. This will generate an intense red color solution. The red color intensity goes down with the addition of ALEPA due to its ability scavenge OH radicals. The degree of the red color intensity depends on the potency of the scavenging activity.[3] Fig. 2, shows hydroxyl radical scavenging activity of ALEPA and ascorbic acid at different concentrations. Both show linear relationship between OH scavenging activity and concentration. According to the Fig. 2, the ALEPA shows OH radical scavenging activity from 17.4±1.3% to 41.5±0.5% within the concentration range of 20-50 mg/mL whereas, ascorbic acid shows 7.0±0.2% to 19.8±0.5% within the same concentration range.

NO is a neurotransmitter and a neuromodulator. However, problems can arise due to overproduction or with the involvement of oxygen to produce peroxynitrile which ultimately decompose into hydroxyl and nitric oxide free radicals.[11,12] In nitric oxide scavenging assay sodium nitroprusside generates nitric oxides in aqueous medium at physiological pH. The formed nitric oxides react with oxygen to generate nitrite ions. These nitrite ions can be estimated using Griess reagent. The scavenging effect is indicated by reduction of the pink color.[1,18] According to the Fig. 3, No radical percentage scavenging activity of ALEPA shows 26.0±3.6% to 69.2±2.5% for the concentration from 0.2-8 ppm whereas, ascorbic acid shows 26.8±2.5% to 49.3±1.9% within the concentration range of 0.2-4 ppm.

During inflammation, lysis of lysosomal membrane may occur. It leads to release their enzyme components that produce a variety of disorders. Therefore, lysosomal membrane stabilization is important in anti-inflammatory action. Since RBC membrane is similar to lysosomal

membrane the inhibition of heat induced RBC membrane lysis will be taken as a measure of the mechanism of anti-inflammatory activity.[19] According to the Fig. 4, the protection of HRBC membrane of ALEPA was from 48.0±1.9% to 60.6±2.6% within the concentration range of 6-40 ppm whereas, aspirin (standard) shows 39.7±0.4% to 49.0±0.5% within the same concentration range.

The diameter of the inhibition zones of ALEPA was taken as the parameter of antibacterial activity in both Agar Well and Disk Diffusion methods. According to the disk diffusion method data (Table 2), ALEPA shows antibacterial properties towards *Escherichia coli, Bacillus subtilis,* and *Streptococcus aureus.* The highest inhibition zone was given against *Bacillus subtilis.*

Table 2: Inhibition Zones with ALEPA, Positive Control and Negative Control during Disk Diffusion Method and Agar Well Method

Strain	Disk diffusion method		Agar well method	
	Positive control (mm)	Inhibition zone of ALEPA (mm)	Positive control (mm)	Inhibition zone of ALEPA (mm)
Escherichia coli	22.5 ±1.4	4.5 ±0.4	22.3 ±0.4	9.0 ±0.5
Bacillus subtills	14.2 ±0.05	6.3 ±0.2	24.0 ±0.8	9.0 ±0.3
Streptococcus aureus	12.0 ±0.07	3.1 ±1.6	24.0 ±0.4	9.0 ±0.3

According to the agar well method and disk diffusion method (Table 2), ALEPA shows antibacterial properties towards *Escherichia coli, Bacillus subtilis,* and *Streptococcus aureus.*

CONCLUSION

The aqueous raw leaf extract of *Persea americana* (Avocado) prepared according to the method of '*Kasaya*' in Ayurvedic medicine showed good antioxidant, anti-inflammatory, and antibacterial activity. Therefore, this concludes that the decoction of the raw leaves of avocado is a rich source of natural antioxidants, anti-inflammatory and antibacterial compounds.

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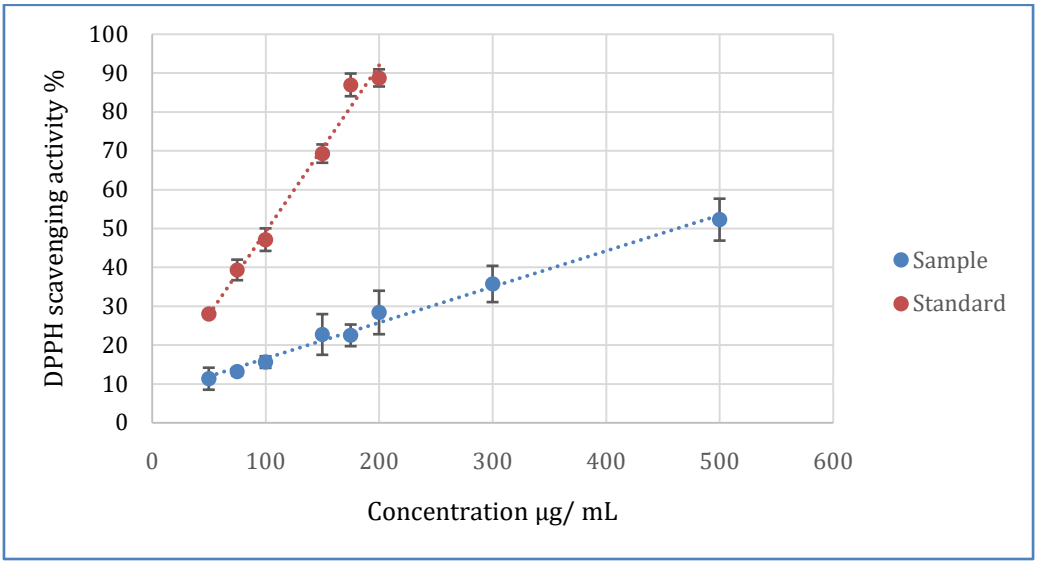

Figure 1: DPPH scavenging activity vs concentration of ALEPA and ascorbic acid

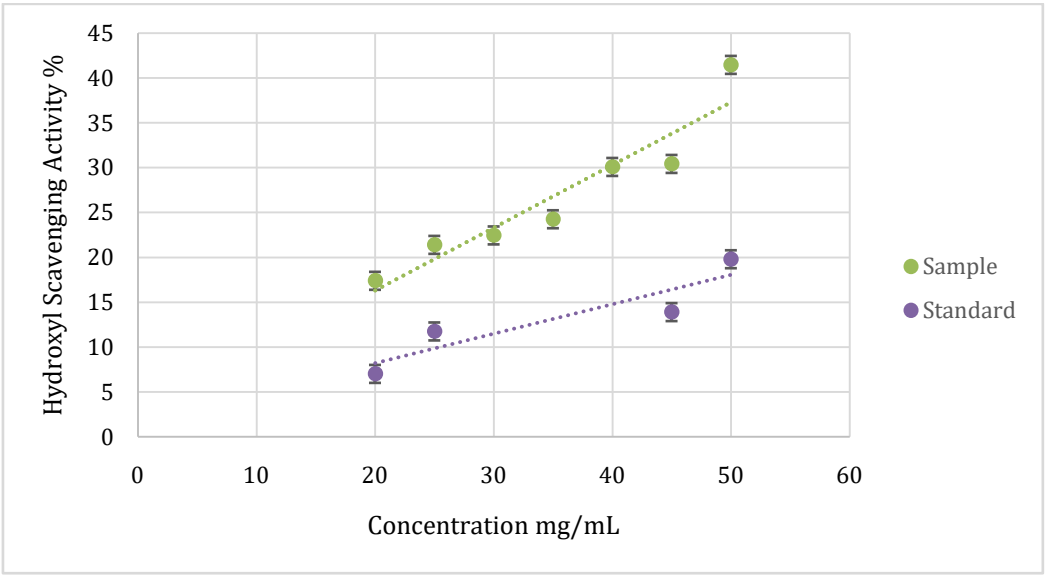

Figure 2: Percentage hydroxyl scavenging activity vs concentration of ALEPA and ascorbic acid

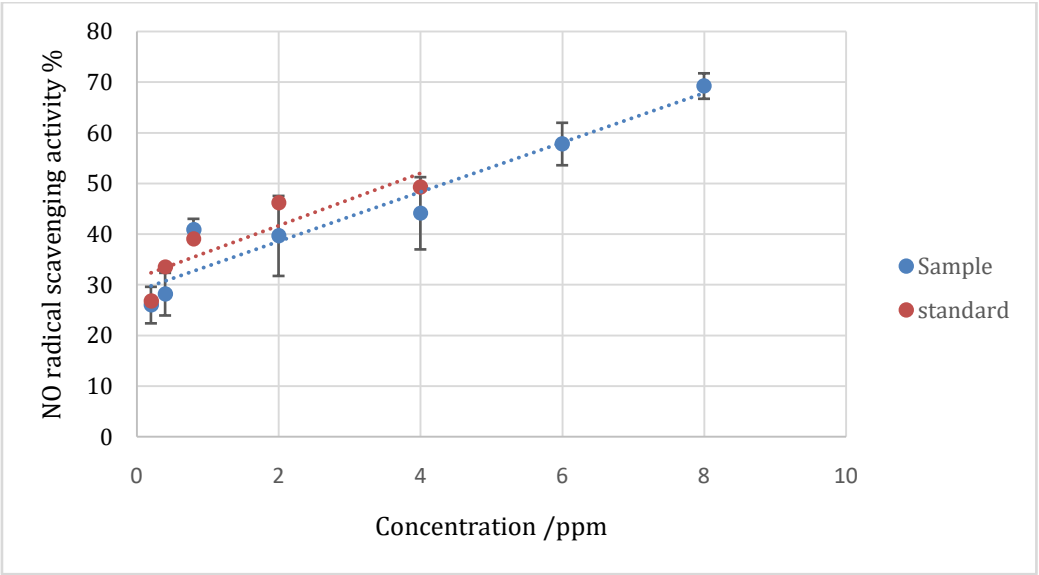

Figure 3: NO radical scavenging activity vs concentration of ALEPA and ascorbic acid

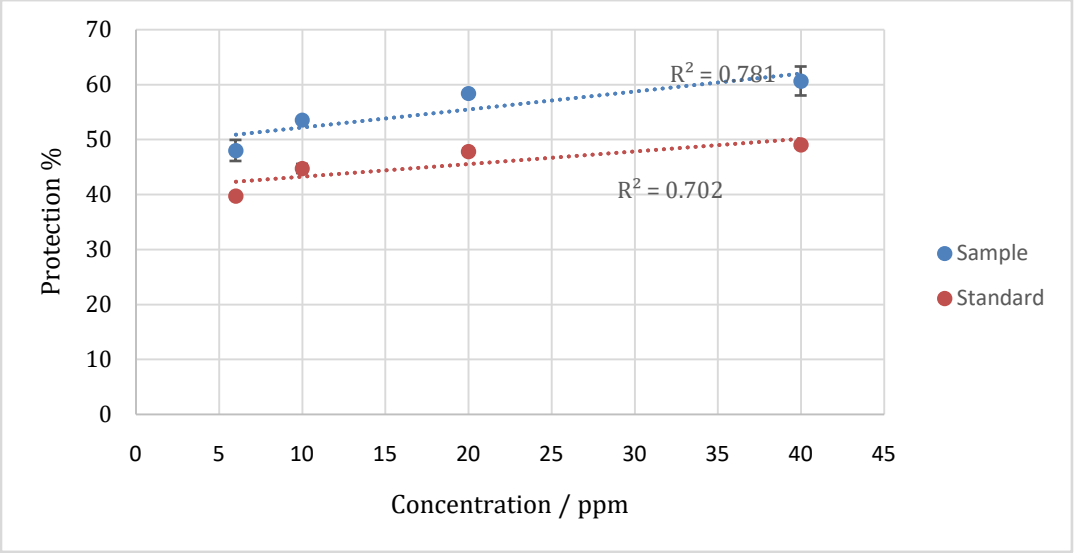

Figure 4: Percentage protection vs concentration of ALEPA and asprin standard

Figure 5: Collected raw leaves of avocado

Figure 6 a: Reaction mixture without the plant extract in TPC assay

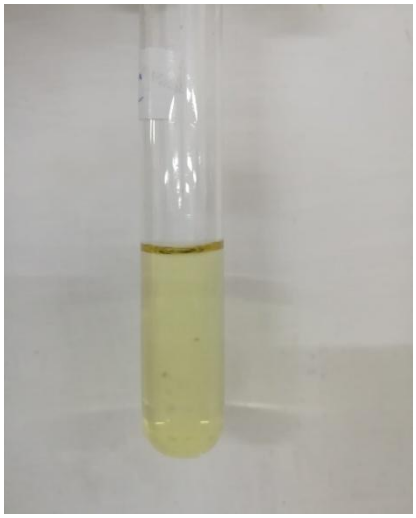

Figure 6 b: Reaction mixture with the plant extract in Total Phenolic content assay

Figure 7: Reaction mixture with the plant extract in Total Flavonoid content assay

Figure 8 a: DPPH working solution

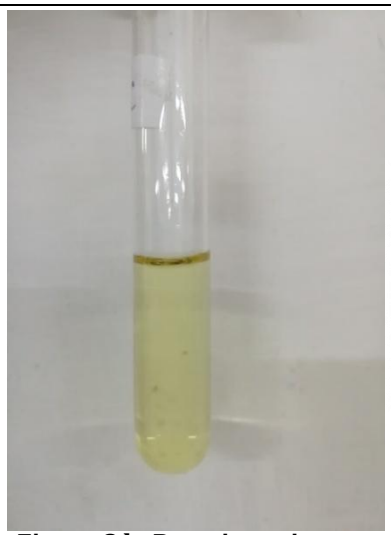

Figure 8 b: Reaction mixture with ALEPA in DPPH assay

Figure 9: Plant extract with $FeCl_3$ in ferrous ion reducing assay

Figure 10 a: Reaction mixture without the plant extract in Hydroxyl radical assay

Figure 10 b: With the plant extract in Hydroxyl radical scavenging assay

Figure 11: Color of the reaction mixture with the plant extract in Nitric oxide radical scavenging assay

Figure 12 a: Collected blood sample

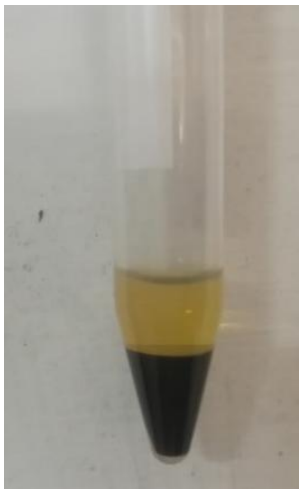

Figure 12 b: Yellowish supernatant after centrifugation at 300 rpm for 20 min

supernatant of HRBC assay

Figure 12 b: 10% RBC suspension in Human red blood cell assay

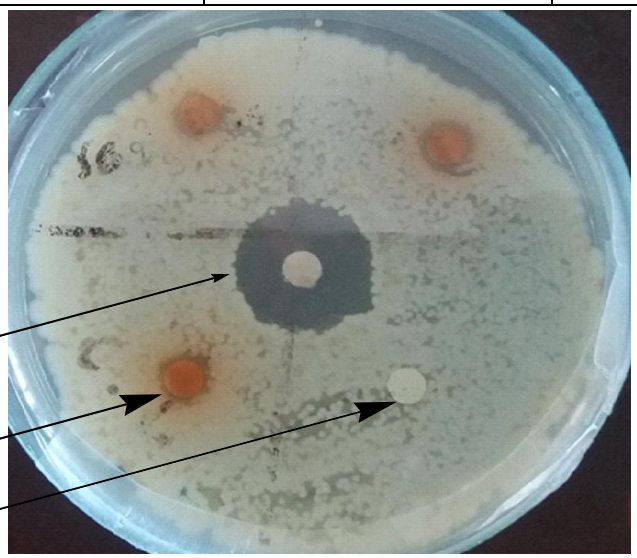

Figure 13: Inhibition zones of ALEPA against *E. coli* during disk diffusion assay

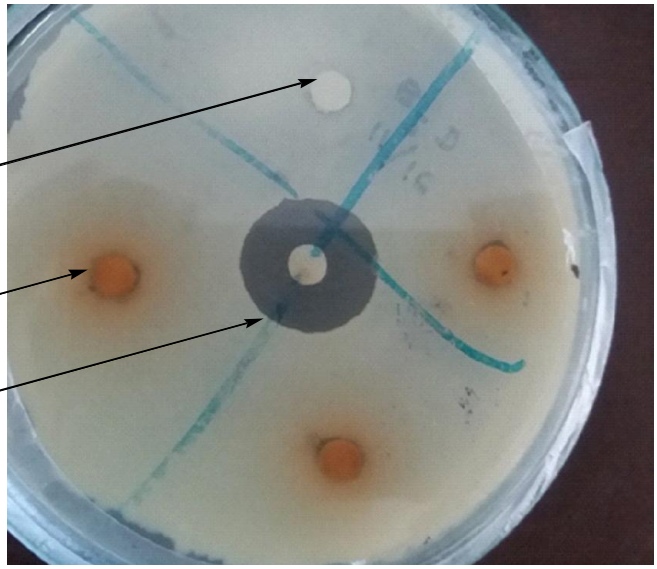

Figure 14: Inhibition zones of ALEPA against *Streptococcus aureus* during disk diffusion assay

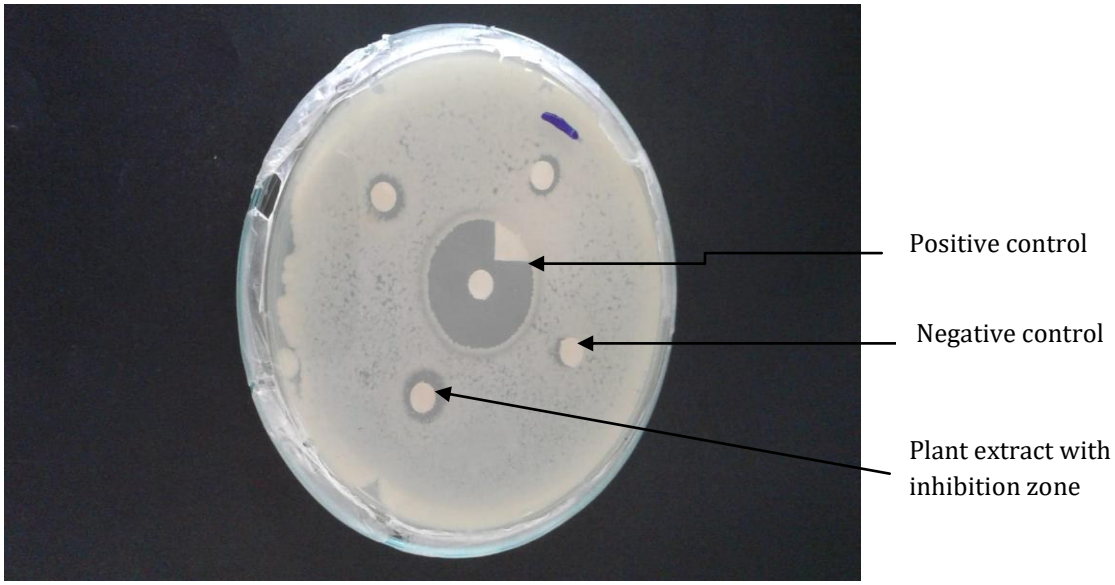

Figure 15: Inhibition zones of ALEPA against *Bacillus subtilis* during disk diffusion assay

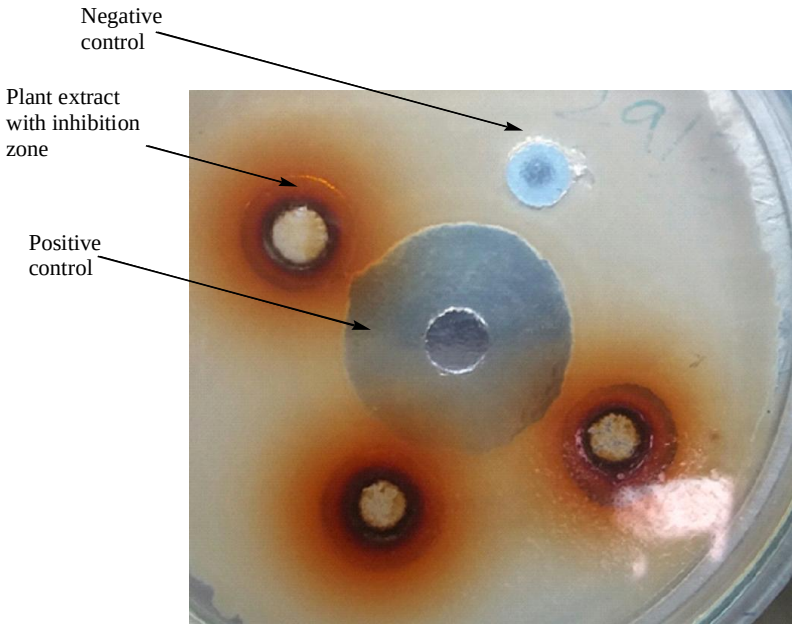

Figure 16: Inhibition zones of ALEPA in *Streptococcus aureus* during agar well assay

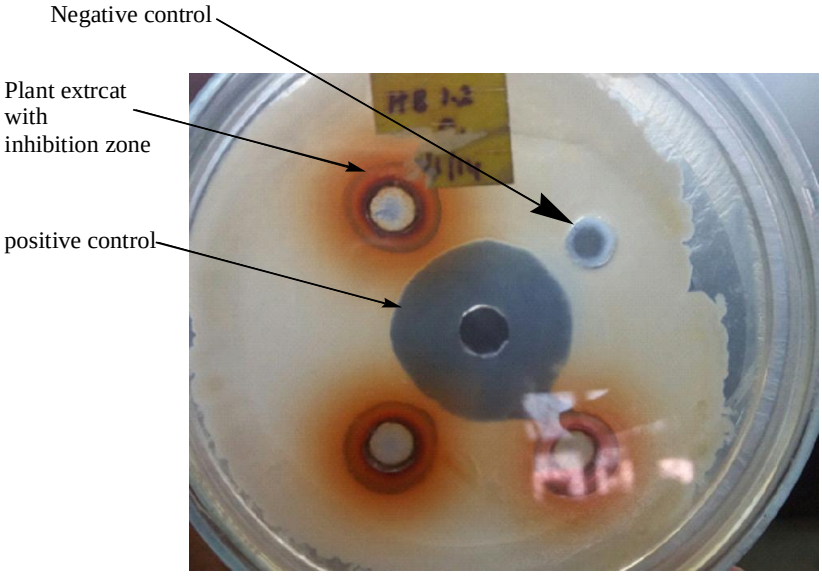

Figure 17: Inhibition zones of ALEPA in *E. coli* during agar well assay