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The Study of Antioxidant and Antibacterial Properties of Skin, Seeds and Leaves of The Sri Lankan Variety of Pumpkin.

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Abstract : Reactive oxygen species and free radicals are implicated in numerous disorders, diseases as well as in the aging process. Natural antioxidants in the form of raw extracts or chemical constituents are sort after in order to combat destructive processes due to oxidative stress. The antioxidant and antibacterial activity of acetone, ethyl acetate and methanol extracts of skin, leaves and seed of the Sri Lankan variety of Cucurbita maxima was investigated. The ethyl acetate extracts of leaves gave the highest rate of change of reducing power with concentration (0.1625 ml/mg) in Fe^{3+} reducing power assay and the highest rate of change of radical scavenging activity with concentration (7.016 ml/mg) during DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The highest hydroxyl radical activity was observed in methanol extract of seeds (82.7±3.0 %). The Folin-Ciocalteu assay showed the highest phenol content in the ethyl acetate extract of leaves (526.2±2.0 mg (PGE)/g). The highest flavonoid content was in the acetone extract of leaves (35.0±0.5 mg (QE)/g).The methanol and acetone extracts of skin and ethyl acetate extract of seed showed antibacterial activity against *Staphylococcus aureus*. Methanol extract of skin and ethyl acetate extract of leaves showed activity against *Bacillus subtilis*.

Keywords: Antioxidant capacity,Antibacterial activity, *Bacillus subtilis, Cucurbita maxima, Staphylococcus aureus*

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I. INTRODUCTION

From the beginning of human civilizations plants have been used to cure various kinds of diseases and their symptoms. Even though modern medicine has shown greater advancements, the role of plants in medicine and healthcare is still highly recognized. Due to toxicity of synthetic drugs much research attention is dedicated towards natural sources to combat many diseases [1]. Free radicals and reactive oxygen species (ROS) are implicated in many diseases such as cancer, Parkinson's disease, Alzheimer's disease and cardiovascular diseases [2]. Therefore, antioxidants play a vital role in preventing such diseases by stabilizing or deactivating free radicals. Natural antioxidants are regarded as raw extracts or their chemical constituents which are capable of preventing harmful effects caused by oxidative stress [3]. Also due to the side effects of synthetic antibiotics alternative sources such as plant derived compounds with antibacterial activity is of much intrest [4].This study involves the determination of antioxidant and antimicrobial activities of pumpkin seeds, skin and leaves. It has been shown that in food processing industry only fleshy part of the pumpkin is used and 18-21% of the fruit is generated as pumpkin waste [1]. Therefore, determination of beneficial health properties of these discarded plant parts can increase the economic value of the plant.The botanical name of pumpkin is *Cucurbita maxima* and it belongs to family Cucurbitaceae. Pumpkin is native to Central America and around 26 species of *Cucurbita* have been identified. *ANK Ruhuna*, *Arjuna*, *Meemini*, Samson, *Lanka* and squash are some of the common varieties of pumpkins in Sri Lankan market [5]. Among them the Lankan variety (which is traditionally known as *Hēn wattakka/ Lanka wattakka)* was used for this study.

II. MATERIAL AND METHDOLOGY

2.1 Collection of plant material

Fresh fruits and leaves of the Lankan variety of *Cucurbita maxima* were collected from Anuradhapura district in North Western province of Sri Lanka. They were identified at the Institute of Postharvest Technology, Anuradhapura, Sri Lanka. Skin, seeds and leaves were sun dried for two weeks and grounded into a fine powder.

2.2 Preparation of the plant extracts

The extraction was done according to a previously published procedure [2]. To each of the plant material (10 g) distilled acetone (50 mL) and 2% acetic acid(1 mL) was added and sonicated for 1 hour. After centrifugation at 800 rpm for 10 minutes the supernatant was separated out and distilled acetone (50 ml) was added to the pellet.This process of sonication and workup was repeated twice. Similar workup was done to the pellet using distilled methanol and distilled ethyl acetate respectively. The solvents of the combined supernatants were then evaporated to obtain the respective extracts.

2.3 Reducing power assay

In reducing power assay the reduction of Fe^{3+} to Fe^{2+} by the reductants in the solution causes the yellow color of the test sample to turn into Perl's Prussian blue color. The reducing ability is determined by taking the absorbance of the test sample at 700 nm. Various concentrations of the plant extract (2 ml) were mixed with 2.0 mL of phosphate buffer (0.2 M, pH 6.6) and 2.0 mL of potassium ferrricyanide (10 mg/ml). This mixture was heated in a water bath of 50℃ for 20 minutes. To this solution of 10% trichloroacetic acid (2.0 mL) was added and the mixture was centrifuged at 3000 rpm for 10 minutes. From the supernatant 2.0 mL was taken and it was added with 2.0 mL of distilled water and freshly prepared 0.1% (w/v) ferric chloride solution (0.4 mL). The absorbance of this test sample was measured at 700 nm [3].

2.4 DPPH radical scavenging assay

The radical scavenging activity (RSA%) was examined using previously published method [3]. An aliquot of 3.9 mL of DPPH reagent was added to 100 µL of the plant extract at varying concentrations. The reaction mixture was shaken well and incubated in dark for 15-30 min at room temperature. The control sample was prepared by replacing the plant extract with methanol. The absorbance of the test solution was measured at 517 nm. The percentage radical scavenging activity (RSA %) was calculated using the following equation (1),

Equation 1

$$\text{RSA}(\%)\text{or \% inhibition}=\left(\frac{\text{Absorbance of the control - Absorbance of the sample}}{\text{Absorbance of the control}}\right)\times 100$$

2.5 Hydroxyl radical scavenging assay

An aliquot of 100 µL of the plant extract (100 µg/ml) was mixed with 500 µL of 2-deoxy ribose (2.8 mM) on phosphate buffer (50 Mm, pH 7.4), 200 µL of premixed $FeCl_3$ (100 mM) and EDTA solution (100 mM) (1:1; v/v) and 100 µL of H_2O_2 (200 mM) solution. To the same reaction mixture 100 µL of ascorbic acid (300 mM) was added in order to trigger the reaction and incubated for 1 hour at 37 ˚C. From this mixture 0.5 mL was taken and added with 1 mL of trichloroacetic acid (2.8 %; w/v) and 1 mL of 1% thiobarbituric acid solutions. This mixture was heated in a water bath for 15 minutes. The mixture was allowed to cool down and the absorbance was measured at 532 nm [3]. The radical scavenging activity was calculated using the equation (1).

2.6 Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method. In brief, 4 mL of 2% sodium bicarbonate was mixed with 200 µL of the extract and incubated in darkness for 2 minutes. To this solution 200 µL of the Folin-Ciocalteu reagent was added and incubated in darkness for 30 minutes. The absorbance was taken at 750 nm [6]. The total phenolic content was determined using calibration curve which was plotted using pyrogallol solutions at varying concentrations.

2.7 Total flavonoid content

The plant extract (0.5 mL) was mixed with 2.0 mL of distilled water and 150 µL of 5% $NaNO_2$ solution and incubated in the dark for 5 minutes. To this solution 150 µL of 10 % $AlCl_3$ was added and the reaction mixture was incubated for 6 minutes. Then an aliquot of 1.0 mL of 1 M NaOH solution and 1.0 mL of distilled water were added to the reaction mixture and the absorbance was measured at 510 nm r [7]. The total flavonoid content was determined from the quercetin calibration curve.

2.8 Antibacterial activity

Plant extracts were 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. These bacterial strains were grown in LB (Lysogeny Broth) agar medium and incubated overnight at 37 ˚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. The antibacterial activity of the *Cucurbita maxima* extracts were evaluated using agar well diffusion method. On the surface of the bacterial spread plate 5 wells were made using the sterilized cork borer. An aliquot of 50 µL plant extract was added to three wells. For the positive control 50 µL of the antibiotic solution was used while 50 µL of distilled methanol was used as the negative control. The plates were sealed, appropriately labeled and incubated overnight at 37 ℃.[8] The antibacterial activity was evaluated by measuring the diameter of the inhibition zones.

III. RESULTS

The Figure 1 shows that the reducing power of each extract increases progressively with the increasing concentration.

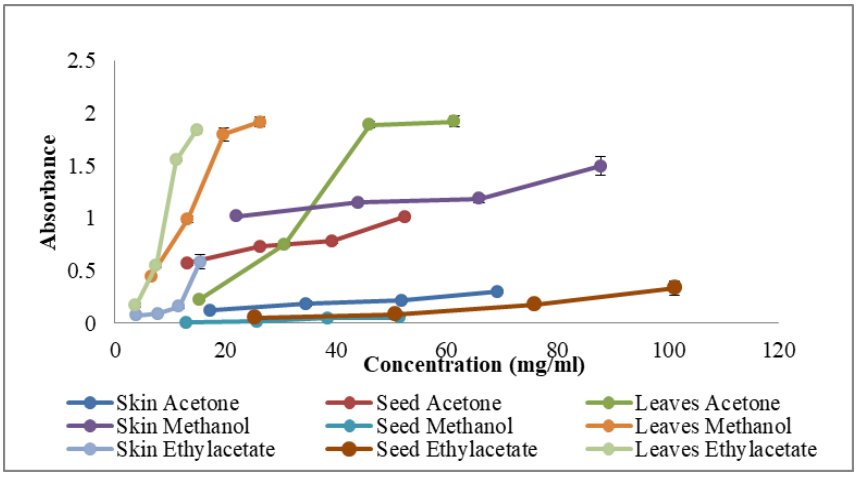

Figure 1 Absorbance at 700 nm *vs.* Concentration

In reducing power assay the absorbance values are directly related to the reducing power ability of the sample. The slope of each graph shows the change of absorbance per unit concentration. Therefore, the highest gradient indicates the plant extract with the highest reducing power and *vice versa*. In order to interpret the results clearly the gradients of the above graphs in Fig. 1 were taken and compared with each other (Fig.2). The line corresponding to the ethyl acetate extract of the leaves has the highest gradient. This shows that leaves in ethyl acetate has the reducing ability even at very low concentrations.

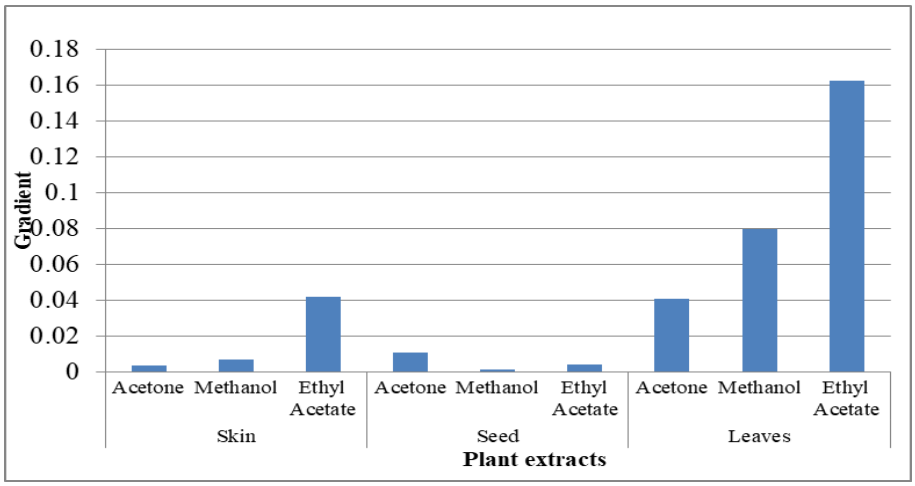

Figure 2 Rate of change of reducing power with concentration (gradients of Fig.1) of each plant extract.

Figure 3 shows the change of radical scavenging activity of the extracts in the DPPH assay with the concentration.

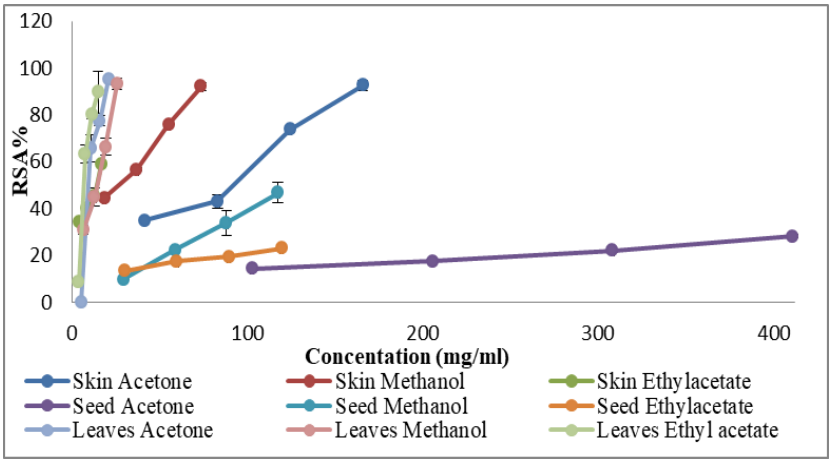

Figure 3 Radical Scavenging Activity % (in DPPH aasay) *vs.* Concentration

Figure 3 shows that the RSA% increases progressively with the increasing concentration. Here too, the line with the highest gradient of Fig.3 shows the highest DPPH radical scavenging ability (Fig.4). Accordingly the highest DPPH radical scavenging ability is shown by the ethyl acetate extract of leaves.

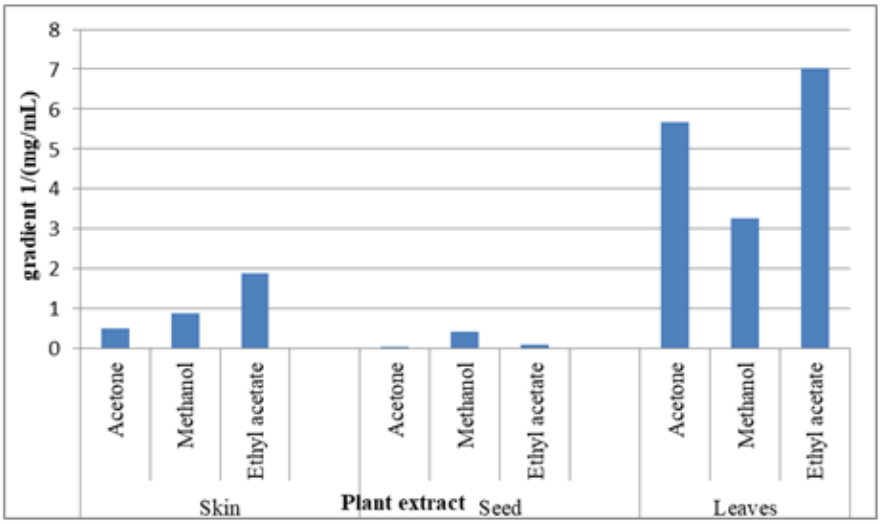

Figure 4 Rate of change of radical scavenging activity with concentration (gradients of Fig.3) of each plant extract

The hydroxyl radical scavenging activity was examined at a sample concentration of 100µg/ml (Table 1). The highest hydroxyl radical scavenging ability was shown by the methanol extract of seeds.

Table 1 Hydroxyl radical scavenging activity (%) of plant extracts

Plant extract	**Hydroxyl radical scavenging activity (%)**
Skin Acetone	68.9±0.8
Skin Methanol	66.8±1.4
Skin Ethyl acetate	68.0±0.5
Seed Acetone	36.2±1.8
Seed Methanol	82.7±3.0
Seed Ethyl acetate	68.4±2.2
Leaves Acetone	60.7±2.1
Leaves Methanol	80.6±3.1
Leaves Ethyl acetate	69.6±1.2

The total phenolic and flavonoid contents in each plant extract are given Table 2.

Table.2 Total phenolic content and Total flavonoid content of each plant extract.

Plant extract	Total phenolic content (mg(PGE)/g)	Total flavonoid content(mg(QE)/g)
Skin Methanol	6.3 ± 0.3	0.4±0.1
Skin Ethyl acetate	4.6 ± 0.2	29.9±1.7
Seed Acetone	1.4 ± 0.1	7.4±0.5
Seed Methanol	6.2 ± 0.6	3.4±0.2
Seed Ethyl acetate	11.0 ± 0.1	6.1±0.2
Leaves Acetone	77.3 ± 2.3	35.0±0.5
Leaves Methanol	7.0 ± 0.2	4.5±0.2
Leaves Ethyl acetate	526.2 ± 2.0	5.4±0.3

Phenolic compounds specifically flavonoids are associated with antioxidant activity. According to the above table the ethyl acetate extract shows correlation with the results obtained for reducing power assay and DPPH radical scavenging assay.

Table 3 shows the sensitivity of *S. aureus* and *B.subtilis* to the extracts of the extracts of *Cucurbita maxima*.

Table 3 Antibacterial activity of extracts of *Cucurbita maxima*

Plant extract	Average diameter of inhibition zones during agar well diffusion method (mm)*		
	E.coli	***S. aureus***	***B.subtilis***
Skin Acetone	-	9.13±0.29	-
Skin Methanol	-	7.58±1.27	4.83±3.43
Skin Ethyl acetate	-	-	-
Seed Acetone	-	-	-
Seed Methanol	-	-	-
Seed Ethyl acetate	-	6.61±0.16	-
Leaves Acetone	-	-	-
Leaves Methanol	-	-	-
Leaves Ethyl acetate	-	-	7.75±0.11
Positive control	43.77±2.32	27.04±1.31	28.91±1.83
Negative control	-	-	-

*: The diameter of the inhibition zones are given without the diameter of thwell (6 mm) - : No activity

Different extracts of leaves skin and seed show antibacterial activity against *S. aureus* and *B.subtilis* as shown in Table 3.

IV. DISCUSSION

The main objective of this research study was to investigate the antioxidant and antimicrobial properties of plant parts of pumpkin which are generated as waste.In reducing power assay the reduction of Fe^{3+}/ ferricyanide complex to Fe^{2+} form by the reductants in the solution causes the yellow colour of the test sample to turn into blue. It is suggested that the reducing power exerted by extracts can exhibit antioxidant action by donating a hydrogen atom to break the radical chain [3]. The DPPH assay is based on the ability of the natural products to donate electron to the DPPH radical. The deep purple colour of the DPPH radical is reduced depending on the activity of the radical scavengers in the test sample [3]. Our results indicate that the ethyl acetate extract of the leaves of the Sri Lankan variety of pumpkin contains the highest reducing power and radical scavenging activity. Hydroxyl radical is regarded as a strong reactive oxygen species which can react with phospholipids in the cell membrane thus damaging the cells [9]. The deoxy ribose assay determines the ability to scavenge the hydroxyl radical by plant extracts. The hydroxyl radical that is generated from the reaction between H_2O_2 and Fe^{3+} .The hydroxyl radical then attacks 2-deoxyribose to form malondialdehyde (MDA) which gives a pink colour to the solution in the presence of thiobarbituric acid. The degree of the reduction of pink colour is proportional to the concentration and the potency of antioxidants [10]. All the extracts of leaves show a significant hydroxyl radical scavenging ability.Phenols and flavonoids are considered as two important classes of phytochemicals which can exert antioxidant activity [11]. The amount of phenols present in an extract is quantified using the Folin-Ciocalteu assay [3]. Phenols reduce the phosphomolybdate

complex in the Folin-Ciocalteu reagent which causes a change in the colour of the reagent from yellow to green [3]. The highest phenol content was observed in ethyl acetate extract of leaves thus showing a significant correlation with the DPPH assay and reducing power assay. he concentration of flavonoids in the extracts is determined by forming a complex between flavonoids and $AlCl_3$ [12]. The highest flavonoid content was observed in acetone extract of leaves according to the Aluminium chloride colourimetric method. Therefore, this research study shows that the consumption of the leaves of the Lankan variety of *Cucurbita maxima* may combat diseases and disorders arising from oxidative stress.It is noteworthy that the seeds and skin extracts of the Lankan variety of *Cucurbita maxima* also showed antioxidant activity in varying amounts. All the extracts of skin and seed showed a high hydroxyl radical scavenging activity in contrast to the activity in the DPPH and reducing power assay.The susceptibility of plant extracts towards pathogenic microorganisms was assessed using the agar well method. Skin and seed extracts gave positive results against *S.aureus* whereas skin and leaves extracts showed antibacterial activity against *B.subtilis*. None of the extract showed any antibacterial activity towards *E.coli*. Since *E.coli* is gram negative bacteria it has impenetrable cell membrane which results in high resistance towards antibiotics [13].

V. CONCLUSION

Despite the advances in modern medicine, plant based remedies are increasingly sort after in order to combat destructive processes due to oxidative stress including aging processes. This study highlights that plant constituents that are generally discarded have a potential to be used inorder to counteract such processes. These plant parts also contain antibacterial constituents.

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