Phytochemical Analysis, Free Radical Scavenging and Antibacterial Activity of Azadirachta Indica (Margosa)

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***Abstract*—Azadirachta indica (Margosa) has been used for a long time in agriculture and alternative medicine. Margosa had been proved effective against microbial which could affect human body and high antioxidant to reduce free radical. This is a laboratory-controlled prospective study conducted at Universiti Teknologi Mara. This study aims to analyse qualitative and quantitative phytochemical and evaluate antioxidant properties of various alcoholic leaf extracts of Margosa and demonstrate the antibacterial effect of Malaysian Margosa leaf extracts against *Escherichia coli* and *Bacillus subtilis* at different temperatures and times. Preliminary phytochemical analysis for phenols and flavonoids and quantitative phytochemicals for total phenolics content and total flavonoids content were made by following standard procedures. In vitro antioxidant activity of different leaf extracts was evaluated by assessing DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The antibacterial potential of various alcoholic leaf extracts was tested using Agar well diffusion method. The quantitative phytochemical analysis of this species exhibited the presence of total phenolics content and total flavonoids content. The antioxidant activity of the species, Azadirachta indica clearly demonstrated that leaf part has prominent antioxidant properties. The leaf extracts showed inhibition against both bacteria *Escherichia coli* and *Bacillus subtilis*. From this study, it can be concluded that the species Azadirachta indica (Margosa) leaf part has the potential to be a powerful antioxidant and antibacterial activity against gram-positive and gram-negative bacteria.**

Keywords*— *Azadirachta indica, Phytochemical analysis, Antioxidant activity, Antibacterial potential

I. INTRODUCTION

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule [1]. The characteristic feature of an antioxidant is ability to scavenge the free radical due to their redox hydrogen donators and singlet oxygen quencher [2,3]. The free radicals can be scavenged by the natural (plants) and synthetic (butylated hydroxyl toluene, butylated hydroxyl anisol and tetra butyl hydro quinone) antioxidants [4]. But the usages of these synthetic antioxidants are now replaced because of the natural antioxidants could be considered as safer without any side effects [5]. In recent decades, many researchers are interested in medicinal plants for evaluation of antioxidant phytochemicals such as phenols, flavonoids and tannins which have received more attention for their potential role in prevention human diseases [6].

Herbal medicines have been known for centuries. The therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine. Azadirachta indica (Margosa) is a tree which has been used for a long time ago in agriculture and medicine [7]. Margosa is an attractive evergreen native to the Indian subcontinent. Furthermore, it cultivated throughout Southeast Asia, East, Australia and sub-Saharan Africa, Fiji, Mauritius, and many other countries in Latin America. Margosa also called as 'arista' in Sanskrit a word that means 'perfect, complete and perishable' [8]. The tree is regarded as village pharmacy in India. The importance of the Margosa tree has been recognized by US National Academy of Sciences, which had published a report in 1992 entitled 'Neem-a tree for solving global problems' [9]. All parts of Margosa tree including the leaf, seed, bark, root, flowers and fruits have medical properties [9]. Margosa leaf and its constituents have been demonstrated to exhibit antibacterial, antifungal, anti-inflammatory, antiulcer, antimalarial, immunomodulatory, hypoglycemic, antioxidant, antimutagenic and antitumor properties [10]. There are two species of Margosa under Azadirachta Indica which are Azadirachta siamensis and Azadirachta excels [11]. The taxonomic position of Margosa tree is as follows [12]:

Order	Rutales
Suborder	Retinae
Family	Meliaceae (mahogany family)
Subfamily	Melioideae
Tribe	Melieae
Genus	Azadirachta
Species	Indica

In Malaysia, Margosa leaves are commonly used as an alternative treatment to cure chicken pox, a highly contagious disease caused by primary infection with varicella zoster virus. Malaysian Margosa and Indian Margosa have about similar characteristics but the Malaysian Margosa was not explore till today even they have similar medicinal properties. The present study was carried out for qualitative and quantitative phytochemicals analysis and in vitro antioxidant activities of leaf part of Margosa using various alcoholic (methanol and ethanol) and antibacterial potential of alcoholic leaf extracts against *Bacillus subtilis*, gram-positive bacteria and *Escherichia coli*, gram-negative bacteria.

II. METHODOLOGY

A. Chemicals

In the present study, all the chemicals were used from Industrial Biotechnology, Food and Chemical laboratories in the Faculty of Chemical Engineering, Universiti Teknologi Mara, UiTM Shah

Alam.

B. Collection and sample preparation method

Margosa leaves was collected. The leaf that collect must in healthy condition without any bacterial or fungal infection. The leaves then washed thoroughly with water for three times and lastly washed with distilled water to remove dust. Then, the leaves will dry under shade at room temperature until it dried completely. It is better method than using oven-dried to maintain the biological compound presence in the Margosa leaves. Finally, the leaves samples were grinded using a grinder machine to smaller course powder form and stored in plastic bag contained silica gel and stored in desiccator full of silica gel.

C. Sample extraction method

The powdered plant samples (50g/ 250mL) were extracted with 70 % methanol and ethanol at range 40-100 ºC for range 1.59-4.41h on a hot plate. The extract then filtered through filter paper into a sealed glass container and stored at 4ºC.

D. Qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in the various alcoholic extracts of leaf part Margosa.

a) Phenols

By using dilute iodine solution test, formation of transient red color indicates the presence of phenolic compounds. It is observed by using 2-3 ml of extract then a few drops of dilute iodine solution were added.

b) Flavonoids

By using alkaline reagent test, the extract was treated with few drops of sodium hydroxide solution separately in a test tube. Then, the formation of intense yellow color will become colorless by addition of few drops of dilute acid indicates the presence of flavonoids.

E. Quantitative estimation of chemical constituents

a) Total phenolics content

The total phenolic contents of Margosa leaves was tested by using Folin-Ciocalteu reagent by method of Sidduraju and Becker. Then, about 50 µl of leaf extracts was taken and it was made up to 1 ml with distilled water. 500 µl of diluted Folins-phenol reagent which is 1:1 ratio with water and 2.5 ml of sodium carbonate 20%, Na2CO3 were added. The mixture then well shaken and incubated in dark condition for 15 minutes for the development of color. After the incubation process, the absorbance was measured at 725 nm. A calibration curve of gallic acid was constructed and linearity was obtained in the range of 10-50 µg/ml. Finally, the total phenolics content in the Margosa leaves extracts were expresses as mg of gallic acid equivalent (mg GAE/g extract) by using the standard curve.

Standard calibration curve of gallic acid was prepared. 30 mg of gallic acid was dissolved in 100 ml of 70 % methanol. The solution was further diluted to 1.5, 3.0, 3.5, 6.0, 7.5, 15.0, 30.0, 45.0, 60.0, 75.0 µg/ml. Then, 1 ml of aliquot of the gallic acid solution at different concentrations was taken in sealed glass container. The mixture was added with 500 µl of the Folin-Ciocalteu solution and allowed to incubate at room temperature for 5 minutes. Next, 1 ml of the sodium carbonate solution was added in each glass container. Then, the final volume should be adjusted to 10 ml with distilled water and left to stand for 30 minutes at room temperature. Finally, absorbance of the standard was measured at 765 nm using UV-spectrophotometer against blank 70 % methanol.

b) Total flavonoids content

The total flavonoids content was estimated using the procedure designated by Zhishen et al. A total of 500 µl of Margosa leaves extracts were diluted with 2 ml of distilled water and the addition of 150 µl of sodium nitrite 5% solution. This mixture the incubated for 5 min and 150 µl of aluminium chloride 10% solution was added and allowed to stand for 5 min. after that, 2 ml of sodium hydroxide 4% solution was added and made up to 5 ml with distilled water. The mixture then shaken well and it is left for 15 min at room temperature. The absorbance was measured at 510 nm. The appearance of pink color showed the presence of flavonoids content. Finally, the total flavonoids content was expressed as rutin equivalent (mg RE/g extract) on a dry weight basis using the standard curve.

The standard stock solution (1 mg/ml) of rutin were prepared by dilution in 70 % methanol. These stock solutions were stored in light resistant containers. The dilute standard solutions of concentration 1.5, 3.0, 3.5, 6.0, 7.5, 15.0, 30.0 µg/ml were prepared from above stock solution and used for calibration curve of rutin.

F. DPPH free radical scavenging assay

The free radical scavenging activity (antioxidant capacity) of Azadirachta indica leaves extract on stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was estimated by method Brand-Williams et.al., [13]. Briefly, 2 ml of extracts at varying temperature and time was mixed with 2 ml of DPPH solution in methanol (0.004% w/v). The mixture allowed to stand at room temperature in dark for 20 minutes. Then the mixture was shaken well and absorbance was recorded at 517nm using uv-spectrophotometer. Ascorbic acid was used as reference standard and control consisted of DPPH solution without leaves extract. The test was performed in triplicate and percentage scavenging of DPPH free radical by extract was calculated using the equation: (Acontrol-Atest)/ Acontrol x 100%. Acontrol is the absorbance of control and Atest is the absorbance of extract or standard.

G. Determination of Antimicrobial activity

The microbial activity of the Margosa leaves extracts were determined by using agar well diffusion method. LB broth agar was inoculated with the *Escherichia coli* and *Bacillus subtilis*. Then, the bacterial inoculums were spreading on the media. Wells were punched in the both agar and filled with the Margosa leaves extracts and control. The plates then were incubated at 27 ºC for 24 hours and the microbial activity was obtained by measuring the diameter of the zone of inhibition. Then, the microbial potential of the different extracts was evaluated by comparing their zone of inhibition.

III. RESULTS AND DISCUSSION

A. Qualitative phytochemical analysis

The present study revealed that the various alcoholic of leaves part of Margosa contained phenols and flavonoids (Table 1). Both phenols and flavonoids present in ethanol and methanol extracts. All the phenols present in ethanol extracts are highly. It is due to the boiling point of ethanol which is 78.37 ºC, make the active ingredients in the extracts in better condition. While, in methanol extracts it boiling point is 64.7 ºC. Therefore, the active ingredients slowly decrease and make moderately to highly present. Also, same in the situation for flavonoids in both alcoholic extractions.

Table 1: Preliminary qualitative phytochemical analysis.

Temperature, ºC	Time, hour	Ethanol		Methanol	
		P	F	P	F
50	2.00	+++	+++	++	+
90	2.00	+++	+++	++	++
50	4.00	+++	++	+++	++
90	4.00	+++	++	+++	+
40	3.00	+++	++	+++	++
100	3.00	+++	++	++	+
70	1.59	+++	++	+++	++
70	4.41	+++	++	++	+
70	3.00	+++	++	+++	++
70	3.00	+++	++	+++	++
70	3.00	+++	++	+++	++
70	3.00	+++	++	+++	++
70	3.00	+++	++	+++	++

+++: highly present, ++: moderately, +: low, -: absent, P: phenols, F: flavonoids.

B. *Quantitative estimation of chemical constituents*

a) *Phenols*

Table 2: Total phenolics content of different alcoholic extracts.

Factor		Total phenolics (mg/g)	
Temperature, ºC	Time, hour	Ethanol	Methanol
50	2.00	0.1838	0.1324
90	2.00	0.2780	0.1897
50	4.00	0.2030	0.1662
90	4.00	0.1838	0.1324
40	3.00	0.1471	0.1044
100	3.00	0.2500	0.1794
70	1.59	0.1706	0.1706
70	4.41	0.1794	0.1471
70	3.00	0.2133	0.1956
70	3.00	0.2133	0.1956
70	3.00	0.2133	0.1956
70	3.00	0.2133	0.1956
70	3.00	0.2133	0.1956

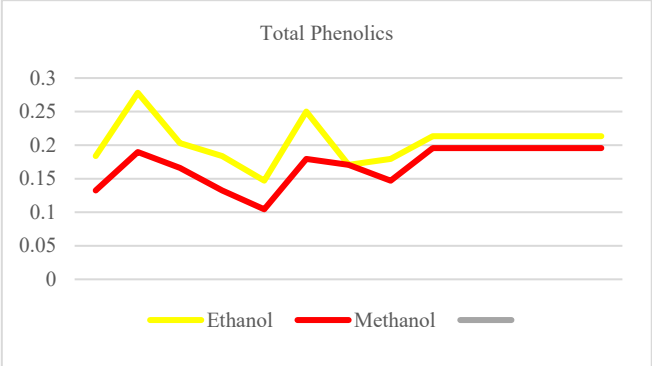

Fig.1: Pattern of total phenolics for ethanol and methanol extracts.

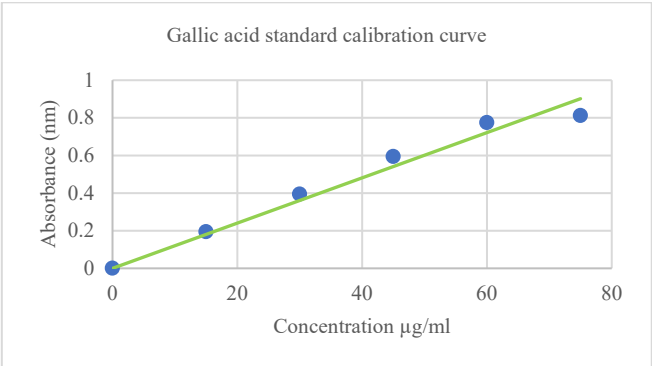

Fig. 2: Gallic acid standard calibration curve.

Total phenolics content of both alcoholic extracts, ethanol and methanol was varying widely in range between 0.1324 to 0.2780 mg/g extract (Table 2). Ethanol extract were demonstrated higher total phenolics content (0.2780 mg/g at 90ºC, 2 h) than methanol extract.

b) *Flavonoids*

Table 3: Total flavonoids content of different alcoholic extracts.

Factor		Total flavonoids(mg/g)	
Temperature, ºC	Time hour	Ethanol	Methanol
50	2.00	0.0875	0.0137
90	2.00	0.1037	0.0273
50	4.00	0.0332	0.0323
90	4.00	0.0218	0.0118
40	3.00	0.0471	0.0137
100	3.00	0.0500	0.0130
70	1.59	0.0482	0.0187
70	4.41	0.0364	0.0239
70	3.00	0.0500	0.0250
70	3.00	0.0500	0.0250
70	3.00	0.0500	0.0250
70	3.00	0.0500	0.0250
70	3.00	0.0500	0.0250

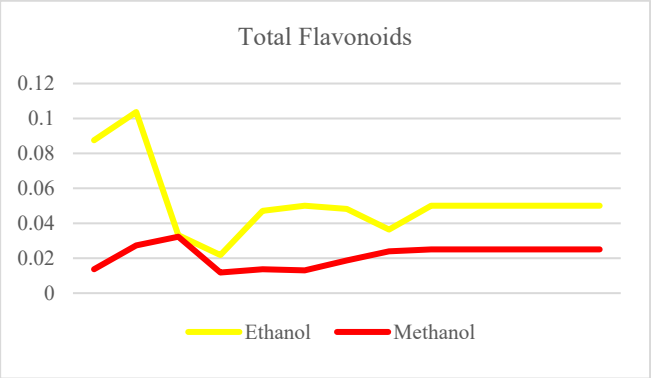

Fig.3: Pattern of total phenolics for ethanol and methanol extracts.

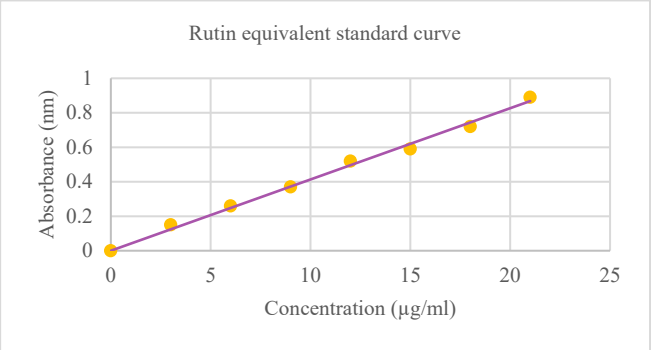

Fig. 4: Rutin equivalent standard curve.

The total flavonoids content of both alcoholic extracts, ethanol and methanol was varying widely in range between 0.0118 to 0.1037 mg/g extract (Table 3). Ethanol extract were demonstrated higher total phenolics content (0.1037 mg/g at 90ºC, 2 h) than methanol extract.

C. *DPPH free radical scavenging assay*

Table 4: DPPH radical scavenging activity.

Temperature, ºC	Time, hour	DPPH, %	
		Ethanol	Methanol
50	2.00	57.13	51.38
90	2.00	87.62	55.97
50	4.00	51.44	52.62

90	4.00	52.89	51.38
40	3.00	50.16	47.44
100	3.00	50.83	54.94
70	1.59	62.18	54.06
70	4.41	49.22	52.71
70	3.00	79.64	56.56
70	3.00	79.64	56.56
70	3.00	79.64	56.56
70	3.00	79.64	56.56
70	3.00	79.64	56.56

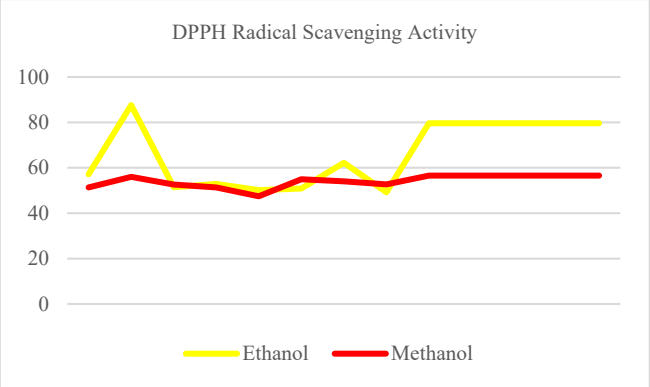

Fig. 5: Pattern of the DPPH radical scavenging activity

The data on DPPH radical scavenging activity of Margosa is presented in Table 4. The highest value from ethanol and methanol extracts for the radical scavenging activity are 87.62 % and 56.56% at 90 ºC and 2 hours and 70 ºC and 3 hours respectively. Higher percentage of radical scavenging activity (DPPH) means lower value of absorbance. From the Fig. 5, it shown that ethanol extracts probably had more antioxidant than methanol extracts.

D. *Determination of Antimicrobial activity*

Table 5: Measurement of zone inhibition on *Escherichia coli*.

Temperature, ºC	Time, hour	Zone of inhibition, mm	
		Ethanol	Methanol
50	2.00	19	11
90	2.00	21	17
50	4.00	16	9
90	4.00	15	8
40	3.00	9	3
100	3.00	13	7
70	1.59	20	12
70	4.41	17	10
70	3.00	20	14
70	3.00	20	14
70	3.00	20	14
70	3.00	20	14
70	3.00	20	14

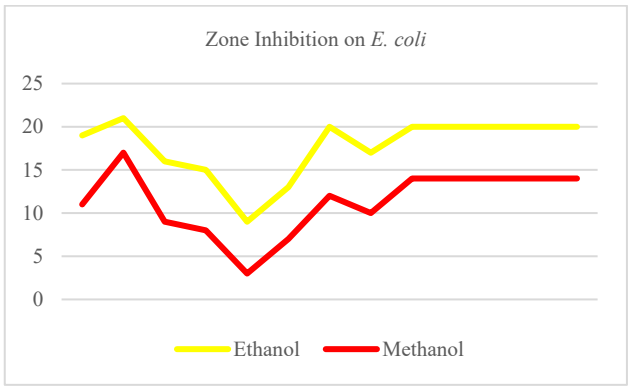

Fig. 6: Pattern of the zone inhibition on *Escherichia coli*.

The data on the measurement of zone inhibition of *Escherichia coli* on both methanol and ethanol extracts is presented in Table 5. The results showed that the zone inhibition for ethanol extract is larger than methanol extract. Also, the largest zone inhibition on methanol and ethanol extracts are 17 mm and 21 mm at temperature, 90 ºC and time, 2 hours.

Table 6: Measurement of zone inhibition on *Bacillus subtilis*.

Temperature, ºC	Time, hour	Zone of inhibition, mm	
		Ethanol	Methanol
50	2.00	24	16
90	2.00	27	22
50	4.00	23	14
90	4.00	21	13
40	3.00	13	6
100	3.00	18	10
70	1.59	25	17
70	4.41	20	12
70	3.00	26	19
70	3.00	26	19
70	3.00	26	19
70	3.00	26	19
70	3.00	26	19

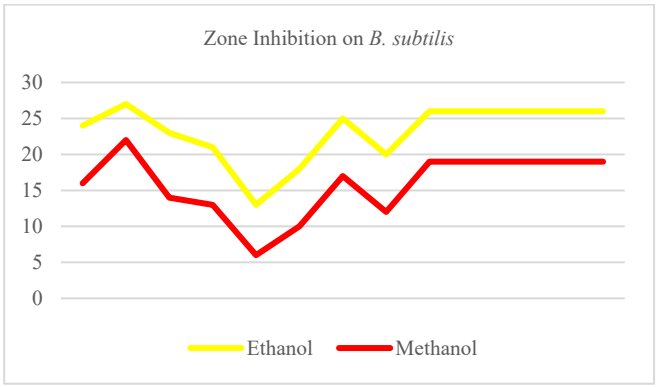

Fig. 7: Pattern of the zone inhibition on *Bacillus subtilis*.

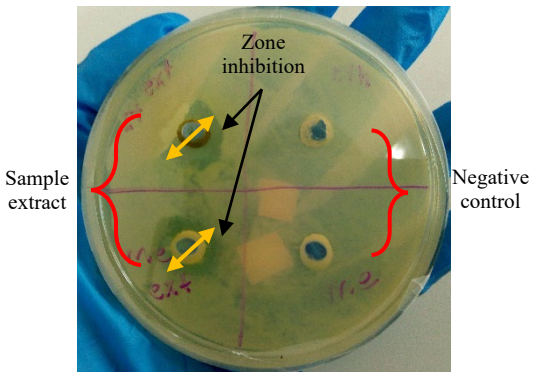

Fig. 8: Sample of alcoholic extract against bacteria.

Measurement of zone inhibition of Margosa extract for methanol and ethanol against *Bacillus subtilis* showed in Table 6 above. The present study showed the same result for measurement of zone inhibition against *Escherichia coli*. Ethanol extract had the largest zone inhibition by 27 mm and methanol 22 mm. Both values present on same temperature and time which are 90 ºC and 2 hours.

Plant essential extracts have been used by many thousands of years, in pharmaceuticals, alternative medicinal, food preservation and natural therapies. Preliminary qualitative phytochemical analysis made for the leaf of Azadirachta indica (Margosa) revealed the presence of phenols and flavonoids. These secondary metabolites are reported to have many biological and therapeutic properties [14-17], so this species is expected to have many medicinal

uses. The phytochemical properties analysis of Margosa extract had earlier been reported by Kraus [18].

Majority of the secondary metabolites studied and ascorbic acid in leaf of Margosa has present with higher amount in ethanol extract than methanol extract. Other than that, it showed reduced in results value when the temperature and time were increased. Therefore, it is explained that the polarity level, temperature, time and species nature are playing major role in extracting the secondary metabolites [19].

The biological property, antioxidant activity was determined to be effective through various assays for the leaf of Margosa. The concentration of antioxidant compounds is needed to decrease the DPPH radicals. DPPH is a stable free radical and that can accept an electron or hydrogen radical to become a stable diamagnetic molecule [20]. A freshly prepared DPPH solution is of deep purple color with absorption maximum at 517 nm and in the presence of antioxidant, this color disappears due to quenching of DPPH free radicals and converting them into a colorless product 2,2-diphenyl-1-picrylhydrazyl [21]. Hence DPPH is usually used as a substance to evaluate the antioxidant activity [22]. In the present study, the extracts had significant scavenging effects on the DPPH radical which was increasing with the increase in temperature and time of the sample. Similar trend of DPPH free radical scavenging activity was already documented well [14,23]. Among methanol and ethanol extract, present study showed that ethanol exhibited higher DPPH radical scavenging activity. This might be due to the presence of phenols and flavonoids content.

Plants extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. In this study showed that the plant extracts inhibited the bacterial growth but their effectiveness varied. Ahmad *et al.*, 1998 studied the antibacterial activity of the *V.negundo* while plant of hexane, alcoholic and aqueous extracts against *Bacillus subtilis*, *Escherichia* coli, *Proteus vulgaris*, *S.typhimurium*, *P.aeruginosa* and *S.aureus* had no activity. The antimicrobial activity of many plant extracts had been previously reviewed and classified as strong, medium or weak [24]. The inhibition produced by the plant extracts against bacteria depends upon various intrinsic and extrinsic parameters.

IV. Conclusion

It is concluded that the species of Azadirachta indica (Margosa) possessed the potential antioxidant activity due to the basis of response in terms of scavenging free radicals. It may be due to the presence of respective secondary metabolites such as phenolics content, flavonoids content *etc*. in the plant species. The strong correlation between the contents of phenolics and flavonoids compound and radical scavenging activity indicates these phytochemical constituents are major contributors to the antioxidant potential of this species. Therefore, this species can be attempted to derive drugs of antioxidant properties. However, further studies by in vitro and in vivo models are still needed to confirm this property.

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