

## ANTIOXIDANT ACTIVITY EVALUATION OF METHANOLIC EXTRACT AND CRUDE POLYSACCHARIDES FROM *Plumeria alba* L. leaves

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### ABSTRACT

Methanolic (MeOH) extract and crude polysaccharides of *Plumeria alba* leaves were evaluated for *in vitro* antioxidant activity. HPLC analysis for the MeOH extract revealed the presence of ten flavonoids and nine phenolic compounds with higher antioxidant capacity. Total phenolic compounds and total flavonoids were determined. The monosaccharide composition was performed on a Dionex system (Dx-120) ion chromatography. The detected polysaccharides are heteropolysaccharides, glucose and mannose were occupied the highest level followed by galactose and rhamnose. Additionally, *Plumeria alba* polysaccharides (PAPs) consisted of the protein-bound polysaccharide. Furthermore, 14 amino acids (Asp, Thr, Glu, Ser, Ala, Gly, Arg, Tyr, Cys, Ile, Lys, Val, Pro and Phe) were identified to be components of the protein-bound polysaccharide analyzed by automated amino acid analyzer. Antioxidant activity was evaluated at different concentrations ranged from 0.25 to 3 mg/ml using three different *in vitro* antioxidant activity assays. The obtained results clearly indicated that the MeOH extract and PAPs showed a strong antioxidant activity in terms of FRAP, DPPH<sup>•</sup> free radical scavenging and reducing power assay. Thus, *Plumeria alba* could be a new source of natural antioxidants for functional foods or medicine due to its higher antioxidant potential, flavonoids and phenolics contents.

**Keywords:** *Plumeria Alba* ; Methanolic extract; Polysaccharides; Ion chromatography; Amino acids and Antioxidant activities.

### INTRODUCTION

Various diseases, such as cardiovascular disease, atherosclerosis, lung diseases and cancer are resulted during oxidative damage caused by free radicals to numerous biological substances, including DNA, protein, and lipid membranes (Tsai *et al.*, 2007; Xu *et al.*, 2009). Thus, searching for new classes of compounds found in natural sources is essential to overcome these complications. Synthetic antioxidants have been commonly added to foodstuffs, for example butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertbutylated hydroxyquinone (TBHQ), but several recent studies suggest that they could raise the toxicity formation (Cheung *et al.*, 2003). Natural antioxidants present in plants have been reported to scavenge harmful free radicals from our body and provide protection against diseases (Upadhye *et al.*, 2009). The recent statistics reported that about 23% of the total deaths in the world caused by cancer (Jemal *et al.*, 2007; Siegel *et al.*, 2015). Therefore, it is essential to search for new natural antioxidant to protect the human body from reactive oxygen species (ROS) damage, especially those based on the natural resource.

*Plumeria alba* Linn (Apocyanaceae) commonly called White Champa, a large evergreen shrub has narrow elongated leaves, white flowers with a yellow center. The plant is found throughout Central America and the Caribbean, southern and southeastern Asia. The plant is used in the treatment of ulcers, scabies and seeds have haemostatic properties (Edward and Gilman, 1994; Goyal *et al.*, 2012). The fruit is reported to be eaten in West India. The bark is used as purgative, cardiotoxic, diuretic and hypertensive (Rasool *et al.*, 2008; Sibi *et al.*, 2012). Leaves used in inflammation, rheumatism, antibacterial, bronchitis, cholera, cold and cough, Antipyretic, antifungal, stimulant etc. (Radha *et al.*, 2009). Methanolic extract of leaves showed antimicrobial activity against *Bacillus anthracis*, *Pseudomonas aeruginosa* (Radha and Sivakumar, 2009).

Very great interest was directed towards polysaccharides in recent years due to their diverse biological activities (Cai *et al.*, 2008; Chen *et al.*, 2010; Cui *et al.*, 2007). Many polysaccharides have been found to play a great role in the medical field due to their biodegradation, water soluble, non-toxicity and non-immunogenicity (Yu *et al.*, 2012; Yu and Chao, 2013). Previously published data indicated that plants polysaccharides have some antioxidant activity and can be used as a novel potential antioxidant (Han *et al.*, 2011; Chen *et al.*, 2012). Based on the separation and detection method, the analytical method used for monosaccharide composition analysis of polysaccharides is ion chromatography with Electrochemical detection (IC-ECD), because of its simplicity, selectivity and low cost which provide an alternative detection method (Kissinger, 1997). Polysaccharides can be ionized in strong basic conditions and separated by using a strong anion-exchange stationary phase and hydroxide solution as mobile phase. Electrochemical detectors (ECD) (Landberg *et al.*, 1998; Schiller *et al.*, 2002), which are directly compatible with the high ionic strength of the eluent, are often used for monosaccharide composition analysis of polysaccharides.

There is no published report about the extraction and isolation of polysaccharides from the leaves of *Plumeria alba*, let alone the antioxidant activity of methanolic extract and crude polysaccharides of *Plumeria alba* leaves. Here, we describe novel water-soluble polysaccharides, isolated from the dried leaves of *Plumeria alba*. The flavonoids and phenolic fingerprint of *Plumeria alba* methanolic extract were also analyzed by HPLC Spectrometry. Then, the crude polysaccharides of *Plumeria alba* leaves were characterized by Fourier transform-infrared spectroscopy (FT-IR) and ion chromatography (IC). Also, the amino acid composition of protein fraction was determined. Finally, the antioxidant activity *in vitro* of Methanolic extract and crude polysaccharides of *Plumeria alba* were evaluated.

## MATERIALS AND METHODS

### Plant Material Collection and Extraction

Fresh leaves of *Plumeria alba* were collected from Mansoura University farm, Egypt in April-May 2013. It was authenticated by a plant taxonomist from the Department of Botany, Faculty of Science; Mansoura

University, air dried, ground into powder and sieved (60 mesh). About 100 g of dried leaves powder were soaked with 98% methyl alcohol (1:10 w/v) at 25°C for 24 hours. The extract was filtered using Whatman No.1 filter paper and concentrated to dryness under reduced pressure in a rotary vacuum evaporator. The final extract (MeOH extract) was stored in air tight containers at 4°C till further use.

#### **Extraction of *Plumeria alba* crude polysaccharides (PAPs)**

About 500 g of dried leaves powder of *Plumeria Alba* were extracted with petroleum ether (60-80 °C) for 6 h to remove lipids. After drying at room temperature, residue was extracted 3 times each for 2 h with deionized water (water-material (ml/g) 10:1), extraction temperature 70 °C) (Luo *et al.*, 2011). Each extract was left to cool at room temperature, filtered; the aqueous solution was concentrated in a rotary evaporator under reduced pressure at 50°C. The concentrated extract was precipitated by the addition of ethanol to a final concentration of 80% (v/v) and kept overnight at 4°C. The precipitates, were collected by centrifugation at 12000 rpm for 10 min, and washed three times with absolute ethanol, then vacuum-dried at 40°C to afford crude *Plumeria alba* polysaccharides (PAPs).

#### **Determination of total Phenolic Content**

Total phenolic in MeOH extract and PAPs of *Plumeria alba* leaves were determined according to the Folin-Ciocalteu method according to (Li *et al.*, 2007). Gallic acid was used as a standard. The reaction mixture was prepared by mixing 1 ml of sample (concentration 0.3 mg/ml), 9 ml of distilled water, 1 ml of Folin-Ciocalteu's reagent and 10 ml of 7% (w/v) sodium carbonate. After incubation for 90 minutes at room temperature, the absorbance was recorded at 765 nm and total phenolic content was expressed as mg gallic per gram dry extract.

#### **Determination of total flavonoid content**

Total flavonoid content in MeOH extract and PAPs of *Plumeria alba* leaves was determined by aluminium chloride colorimetric method as described by Chang *et al.*, (2002). One gram of sample was mixed with 0.1 ml of 10% aluminium chloride hexahydrate, 0.1 ml of 1M potassium acetate and 2.8 ml of deionized water. After incubation for 40 minutes at room temperature, the absorbance of the reaction mixture was recorded at 415 nm. Quercetin (QE) was chosen as a standard and the total flavonoid content were expressed as milligram (QE) per g dry extract.

#### **Identification and quantification of flavonoids and phenolic compound by HPLC**

Flavonoids of *P. alba* leaves were identified and quantified by using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) composed of a quaternary pump, an on-line degasser, a column temperature controller and a diode array detector (DAD) in National Research Center, Doki, Giza, Egypt. Separation was carried out on an Agilent Eclipse Plus™ C<sub>18</sub> column (150 mm × 3.0 mm, 3.5µm). Eluent A was acetonitrile (100%) ; eluent B was 0.05% CH<sub>3</sub>COOH aqueous solution. Gradient conditions: initial = 35% A and 65% B; 30 min = 50% A and 50% B; 40 min = 90% A and 10% B. Before injection of the next sample, the column was equilibrated with the initial elution condition for 10 min. The flow rate was established at 1.0 ml

min<sup>-1</sup> and the column temperature was at 25°C. All flavonoids were quantified using the external standard method (Mattila *et al.*, 2000). Phenolic compounds of *P. alba* leaves were identified and quantified according to the method described by Goupy *et al.*, (1999). Reversed phase HPLC (RP-HPLC)/diode array detection (DAD) (Hewlett Packard 1050) with a guard column Alltima C<sub>18</sub>, 5mm (Alltech) was used. A gradient elution was employed using the solvent system of A (CH<sub>3</sub>COOH 2.5%), B (CH<sub>3</sub>COOH 8%) and C (acetonitrile). The Solvent flow rate was 1ml min<sup>-1</sup> and the separation was performed at 35°C. Phenolic compounds were assayed by external standard calibration at 280nm and expressed in µg/100g dry matter.

#### **Monosaccharide composition analysis**

Two mg of PAPs sample were dissolved in 2 ml of 2M H<sub>2</sub>SO<sub>4</sub> in a small ampoule and hydrolyzed at 110 °C for 2 h. The hydrolyzed liquid was diluted with deionized water to 10 ml, and then filtered through a 0.2 µm nylon membrane (Nylaflo Aldrich). Chromatographic analysis was performed on a Dx-120 ion chromatography (Dionex, Sunnyvale, CA, USA) equipped with a pump, an eluent generator (EG40) with sodium hydroxide (NaOH) cartridge, a 6-port valve with a 25 µL loop and an electrochemical detector (model ED40) in The Analytical Chemistry Department, Zhejiang University, Hangzhou, China. An electrochemical flow-through detection cell composed of a 1.0-mm diameter GC working electrode, a pH-Ag/AgCl combination reference electrode and the titanium body counter electrode was used. Separations were accomplished on a Dionex column, CarboPac PA10 (250mm×4mm) coupled with a guard column (50mm×4mm i.d.) of the same filling. After each analysis, the separated column was washed by 100 mM NaOH for 10 min to remove any highly retained matrix components in column, which could cause the decrease of the retention times of analytes during prolonged chromatographic analysis. (Xi *et al.*, 2014).

#### **FT-IR spectroscopy**

PAPs sample (1mg) was milled with 300 mg of KBr, and compressed into a disk for transmission infrared spectroscopy (Guo *et al.*, 2010). IR spectra of polysaccharides were recorded on a Fourier transform infrared spectrophotometer (FTIR) BRUKER VECTORE 22 in the frequency range of 4000–400 cm<sup>-1</sup>.

#### **Protein content and amino acids composition**

The protein content of PAPs was determined by the Kjeldahl Nitrogen Determination method (Pearson, 1976). Crude protein was subsequently calculated by multiplying the nitrogen content (expressed as % N) by a factor of 6.25. The amino acids in polysaccharide sample were determined with a Hitachi L-8900 automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan). The PAPs sample (5 mg) was hydrolyzed with 6 N HCl at 110°C for 22 h under vacuum in sealed tube. The hydrolyzate was evaporated with a speedvac concentrator and the dried residue was redissolved in 0.02 M HCl solution. The samples were passed through a 0.45-µm Nylon filter before being injected into the amino acid analyzer (Xin *et al.*, 2012).

#### **Analysis of glycan–peptide linkage**

The carbohydrate–peptide linkage of PAPs was analyzed by the β-elimination reaction (Zhu and Zhou, 2005). The PAPs (10 mg) were

incubated in 10 ml 0.2 M NaOH containing 1.0 M NaBH<sub>4</sub> at 45 °C for 24h, then the samples were scanned (from 200 to 400 nm) using UV- 2550 spectrophotometer (UV-Visible spectrophotometer SHIMADZU, JAPAN). The obtained data were compared with that of the sample without alkali treatment.

#### **Evaluation of *in vitro* antioxidant activity**

The antioxidant activity evaluation of methanol extract and crude polysaccharides (PAPs) was determined by different *in vitro* methods including Ferric reducing antioxidant power (FRAP), DPPH<sup>•</sup> radical scavenging assay and to the reducing power. All the assays were carried out in triplicates and average values were considered.

#### **Ferric reducing antioxidant power (FRAP)**

The FRAP assay was previously described (Vasco *et al.*, 2008). Fresh FRAP reagent was freshly prepared, by mixing 10 volumes of acetate buffer (300 mM, pH 3.6), with one volume of TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM TPTZ in 40 mM HCl), and one volume of FeCl<sub>3</sub>.6H<sub>2</sub>O solution (12 mM) at 10:1:1 volume ratio. The reagent was warmed to 37 °C, and each 95 µl was mixed with the PAPs sample solution to a total volume of 1 ml in water. The change in absorbance was measured at 593 nm after incubation for 15 min at room temperature and converted to a FRAP value (µmol Fe (II)/g dwt of polysaccharide sample) by calibration with ferrous sulfate (FeSO<sub>4</sub>.7H<sub>2</sub>O) assayed in the same condition as for MeOH extract and PAPs.

#### **DPPH<sup>•</sup> radical scavenging assay**

The DPPH method was chosen to estimate the antioxidant property of MeOH extract and PAPs as it is one of the most efficient methods for evaluating the radical-scavenging action by a chain-breaking mechanism. This assay is based on the ability of DPPH<sup>•</sup> to be decolorized in the presence of antioxidants. The DPPH<sup>•</sup> radical scavenging activity was measured as described by Hussain *et al.*, (2008), with slight modification. Briefly, 0.1 mM solution of DPPH<sup>•</sup> in ethanol was prepared and 3.8 ml of this solution was added to 0.2 ml of MeOH extract and PAPs in water. The mixture was shaken and incubated at 25 °C for 60 min in the dark, then the absorbance was measured at 517 nm against a blank (water instead of samples and DPPH<sup>•</sup> solution). The lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percent scavenging was calculated by the following equation:

$$\text{Scavenging percentage activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100$$

Where A<sub>0</sub> is the absorbance of the DPPH solution without sample; A<sub>1</sub> is the absorbance of the test sample mixed with DPPH solution and A<sub>2</sub> is the absorbance of the sample without DPPH<sup>•</sup> solution.

#### **Reducing power assay**

The reducing power of MeOH extract and isolated PAPs of *P. alba* leaves was evaluated according to the method of Zhanyong *et al.* (2014). Different concentrations (0.25 - 3 mg/mL) were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v). The mixture was incubated for 20 min at 50 °C. The reaction was terminated by trichloroacetic acid solution (10%). The mixture was then centrifuged (1200 × g, 10 min). The solution was mixed with 2.5 mL distilled

water and ferric chloride (0.1%, w/v); After incubation at room temperature for 15 min, the absorbance was measured at 700 nm and Trolox was used as a positive control. A higher absorbance indicated a higher reducing power.

#### Experimental statistics

All treatments and assays were performed in triplicates, and the results were represented by their mean  $\pm$  SD (standard deviation) and processed using Microsoft Excel 2007 and Origin 7.0.

### RESULTS AND DISCUSSION

The use of *Plumeria alba* in folk medicine is well known. This is important for the pharmaceutical point of view. The present study has provided the experimental evidence of the antioxidant properties of MeOH extract and crude polysaccharides of *Plumeria alba* leaves. The study is designed to identify the flavonoids and phenolic compound of *Plumeria alba* methanolic extract by HPLC analysis in addition to the characterization of the crude polysaccharides (PAPs) by Fourier transform-infrared spectroscopy (FT-IR) and ion chromatography (IC), Also amino acids composition of PAPs bound protein was determined and the antioxidant activity *in vitro* of MeOH extract and crude polysaccharides from *Plumeria alba* leaves were evaluated. Previous studies have reported that methanol is the best reagent for extraction because it can easily go through the cell wall and, many useful compounds have been detected in methanolic extracts (Henriques *et al.*, 2007).

#### Phenolic and flavonoid contents

Phenolic compounds have considerable free-radical scavenging activity and their content in plants is an important parameter as antioxidant components (Subramanian *et al.*, 2011). It is usually measured spectrophotometrically by the Folin-Ciocalteu method using gallic acid as an internal standard.

Data in table (1) revealed that methanolic extract and PAPs contained average total polyphenols values of 89.7 and 11.16 mgGAE/g dry weight, respectively. PAPs yielded lower total phenols content than MeOH extract. This might be due to some structural or chemical change during the drying process. In another study, Hafizur *et al.*, (2014) reported that *P. alba* flower had a total polyphenols of 173.9  $\mu\text{g ml}^{-1}$ . Flavonoids are polyphenolic secondary metabolites which responsible for the radical scavenging effects of most plants (Lotito and Frei, 2006). It was found as 74.7 and 3.18 mg QE/g for MeOH extract and PAPs, respectively (Table 1). The number of hydroxyl groups and substitution with electron-donating alkyl or methoxy groups of flavonoids cause an increase in their antioxidant activity (Awaad et al, 2012).

**Table 1. Total polyphenols and flavonoids in methanolic extract and crude polysaccharides of *Plumeria alba* leaves.**

<i>Plumeria alba</i>	Total polyphenols (mg gallic/g dry extract)	Total flavonoids (mg quercetin /g dry extract)
MeOH extract	89.7	74.7
Crude polysaccharides	11.16	3.18

### HPLC separation of flavonoids and phenolic compounds

High-performance liquid chromatograph equipped with a diode array detector was used to determine the flavonoids and phenolic compounds in the methanol extract of *Plumeria alba* leaves.

Table 2 shows the amount of nine phenolic compounds, including, pyrogallol, rosmarinic, ferrulic acid, vanillic acid, *p*.coumaric acid, ellagic acid, salicylic acid, cinamic acid and protocatechuic and ten flavonoids, including catechin, Epicatechin, rutin, hisperedin, narenigin, quercetin, hesperetin, apigenin, kaempferol and Diosmitin in 100 g dry matter of MeOH extract of *Plumeria alba* leaves.

The obtained results showed that pyrogallol was the predominant identified phenolic compound (1027.87 µg/100 g dry weight) followed by ellagic acid (980.10 µg/100 g dry weight), while rosmarinic was the lowest amount (15.62 µg/100 g dry weight).

From the same table (2) it was clear that catechin was the major identified flavonoid component in methanolic extract of *Plumeria alba* leaves (102.87 µg/100 g dry weight), while diosmitin was the lowest value (2.51 µg/100 g dry weight).

Flavonoids and other phenolic compounds have been suggested to play a preventive role against the incidence of some common diseases like cancer, cardiovascular and neurodegenerative disorders (Hussain *et al.*, 2008). Flavonoids and hydroxycinnamic acids have been widely distributed in plants and among them, flavonoids are of particular importance in the human diet as antioxidants.

**Table 2. Polyphenol and flavonoid contents of Methanolic extract of *Plumeria Alba* leaves.**

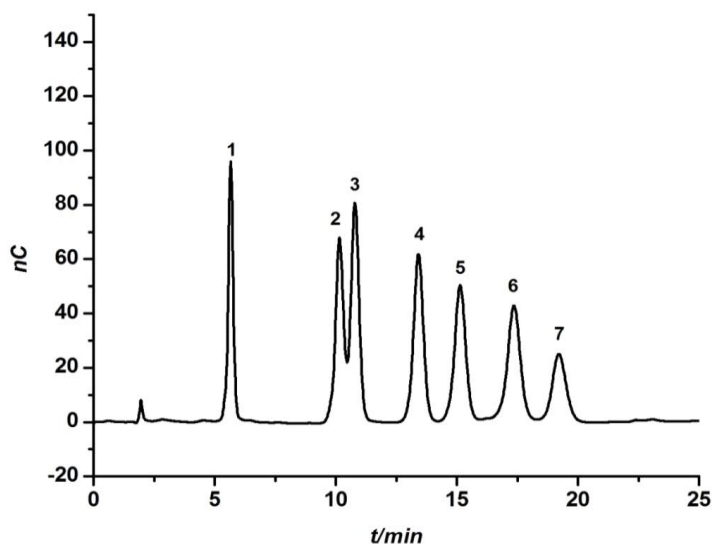
<b>Polyphenols</b>	<b>µg/100 g*</b>	<b>Flavonoids</b>	<b>µg/100 g*</b>
Pyrogallol	1027.87	Catechin	102.87
Rosmaric	15.62	Epicatechin	89.12
Ferrulic acid	47.24	Rutin	22.51
Vanillic acid	63.17	Hisperedin	11.27
<i>p</i> .coumaric acid	22.47	Narenigin	17.38
Ellagic acid	980.10	Quercetin	26.91
Salicylic acid	88.51	Hesperetin	21.18
Cinamic acid	24.28	Apigenin	8.17
Protocatechuic	17.49	Kaempferol	4.29
		Diosmitin	2.51

\* µg/100 g of dry matter

### Monosaccharide composition of PAPs

The hot-water extract from the dried leaves of *P. alba* was precipitated with 80% ethanol to give the crude *Plumeria alba* polysaccharides, named PAPs which gave 2.15% yield of the dried raw material. The hydroxyl group in sugars can be ionized to be anions under a higher pH (>12.0) according to the ionization constant. The separation of

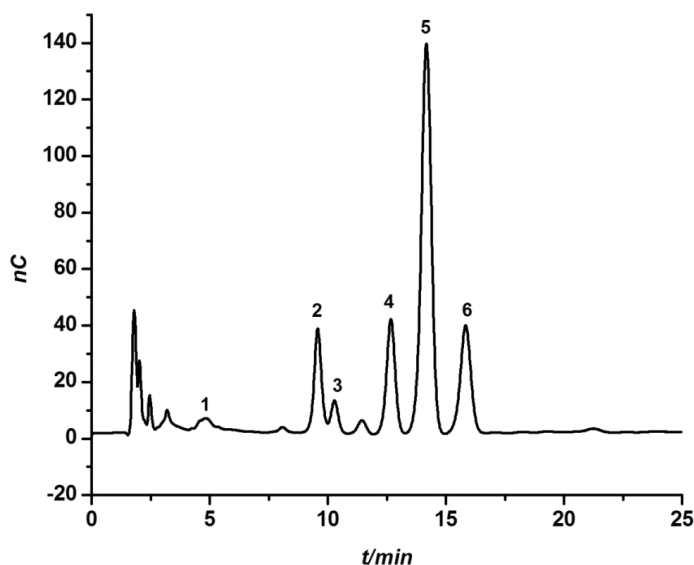
different sugars can be achieved by ion chromatography (IC) using  $\text{OH}^-$  as the mobile phase (eluent) due to their weak acidic properties (Jing *et al.*, 2007). Therefore, the common eluent used in IC was sodium hydroxide (or potassium hydroxide). To achieve the ionization of sugar hydroxyl groups, 15 mM NaOH solution was chosen as the eluent. Fig. (1) Shows a typical chromatogram of a mixture of 5.0 mg/L for each fucose, rhamnose, arabinose, galactose, glucose, mannose and fructose standard solutions detected under the optimum conditions. Good chromatographic separation of the seven sugars was obtained within a short time nearly 25 min.



**Fig.1. The chromatogram of a standard solution containing monosaccharides (concentration: 5 mg/L) Peaks: fucose (1), rhamnose (2) arabinose (3), galactose (4), glucose (5), mannose (6) and fructose (7). Column: CarboPac PA10 (250mm×4mm) coupled with a guard column (50mm×4mm i.d.). Eluent: 15mmol/L NaOH). Flow-rate: 1 mL/min.**

Monosaccharide composition of polysaccharide isolated from *P. alba* leaves was determined and validated by hydrolyzing samples of crude polysaccharides in 2M  $\text{H}_2\text{SO}_4$ , using a Dionex system (Dx-120) equipped with electrochemical detector (model ED40) and eluting by a 15 M NaOH solution. Analytical chromatograms of hydrolyzed polysaccharide sample of PAPs are shown in Fig. 2. The peaks identification of the analyzed sugars was based on the retention times in Fig. 1 and was further confirmed by adding authentic standard solutions to dilute samples. And then the relevant concentrations were expressed as the monosaccharide compositions (mg/L) of PAPs polysaccharides (Table 3). The result clearly demonstrated that glucose and mannose have the highest level followed by galactose and rhamnose.





**Fig.2.**The chromatogram of monosaccharide composition of polysaccharide isolated from *Plumeria alba* leaves. The conditions are the same as in Fig.1. Peaks: fucose (1), rhamnose (2) arabinose (3), galactose (4), glucose (5), mannose (6).

**Table 3.** The monosaccharides composition of polysaccharide isolated from *Plumeria Alba* leaves.

Plant	Monosaccharide composition (mg/L)					
	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose
Plumeria alba	0.253	4.766	1.014	5.01	15.990	8.750

**FT-IR analysis of polysaccharide PAPs**

FT-IR has been shown to be a potent tool for identification of characteristic organic groups in the polysaccharides. The FT-IR spectra of PAPs were recorded in the range of 4000-400  $\text{cm}^{-1}$  (Fig. 3). It could be seen that the IR spectra of PAPs have displayed the broad stretching peak at 3406  $\text{cm}^{-1}$  characteristic of hydroxyl (OH) groups, and the small C-H stretching of the  $\text{CH}_2$  groups at 2931  $\text{cm}^{-1}$ . A strong anisomeric stretching peak at around 1631  $\text{cm}^{-1}$  and a weak peak at around 1384  $\text{cm}^{-1}$  are attributed to the stretching vibration of the carbonyl bond C=O of the amide group and the bending vibration of the N-H bond in peptides or proteins. The small peak at 1275  $\text{cm}^{-1}$  and 1202 were observed in the PAPs sample, which implied the presence of carboxyl groups. Specifically, the PAPs spectra displayed a strong peak at around 1074  $\text{cm}^{-1}$ , which indicated the presence of alduronic acids, such as guluronic acid or mannuronic acid. In addition, the small absorption peaks at about 875  $\text{cm}^{-1}$  could be related to the  $\beta$ -glycosidic

linkages between the sugar units (Chen *et al.*, 2010). The peaks in the range of 350–600  $\text{cm}^{-1}$  are assigned to skeletal modes of pyranose rings (Yang and Zhang, 2009).

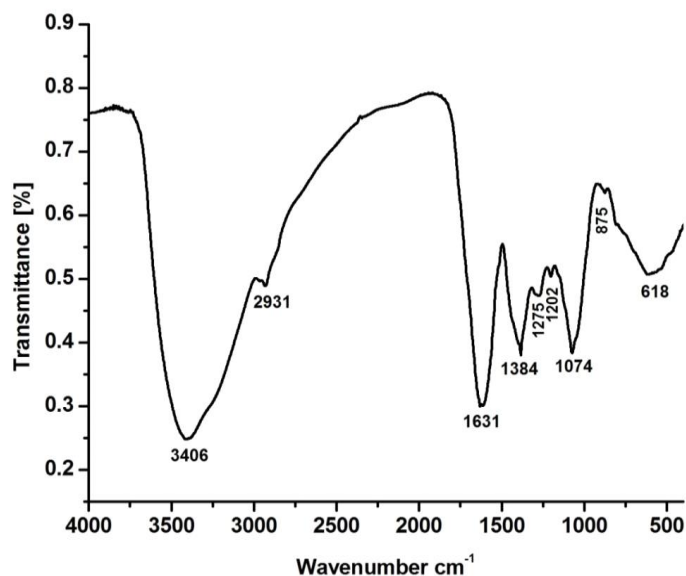


Fig. 3. FT-IR spectrum of polysaccharide isolated from *Plumeria alba* leaves

#### Protein content and amino acids composition

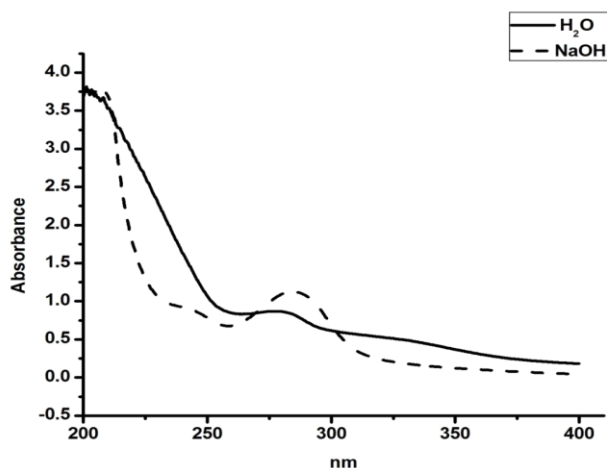
Protein content of *P. alba* polysaccharide was 3.9% and the amino acid composition in protein-bound polysaccharide was analyzed by the automatic amino acid analyzer. Table (4) shows the content of amino acids in PAPs sample. Fourteen amino acids (Asp, Thr, Glu, Ser, Ala, Gly, Arg, Tyr, Cys, Ile, Lys, Val, Pro and Phe) were found in PAPs sample, with the exception of histidine, methionine and leucine which were not detected in PAPs. The major amino acids were glutamic, aspartic and cystine which were accounted 0.481, 0.430 and 0.403 ng/100g, respectively. Isoleucine and arginine have the amino acid present in the lower amounts in PAPs which were accounted as 0.025 and 0.028 ng/100g, respectively. The presence of hydroxyl amino acids such as threonine and serine indicated the possibility of the existence of the *O*-glycosidic linkage (Chen *et al.*, 2008; Kilcoyne *et al.*, 2009) were involved in binding between protein and carbohydrate. There is no available literature on amino acid content of any *Plumeria* species for relevant comparison of the present results.

**Table 4.**The amino acid composition of protein pound with the polysaccharide of *Plumeria alba* leaves.

No.	Name of amino acid	Content ng/100g
1	Aspartic acid	0.430
2	Threonine	0.181
3	Glutamic acid	0.481
4	Serine	0.225
5	Alanine	0.050
6	Glycine	0.076
7	Arginine	0.028
8	Histidine	nd
9	Tyrosine	0.279
10	Cystine	0.403
11	Isoleucine	0.025
12	Lysine	0.028
13	Valine	0.298
14	Methionine	nd
15	Proline	0.378
16	Phenylalanine	0.059
17	Leucine	nd

#### **Analysis of glycan–peptide linkage**

Linkages in the structure of glycoproteins can be divided into two categories on the basis of their stability to alkali: O- glycosidic linkages and N-glycosidic linkages. The alkali- sensitive O-glycosidic linkages are readily split in relatively mild conditions by a  $\beta$ -elimination mechanism resulting in the release of the carbohydrate moiety. The UV scanning method has been widely employed to analyze the type of linkages in glycoproteins (Elizabeth, 1998). UV scanning spectra of the sample with and without alkali treatment are shown in Figs. (4). By comparison, the sample with alkali treatment and absorbance at 280 nm, showing that  $\beta$ -elimination reaction had taken place, which demonstrated that the protein and carbohydrate were linked by O-linkage in PAPs. This was previously reported by Fengjie *et al.*, (2013).



**Fig.4. UV Scanning spectrum of PAPs linkage.**

#### **Antioxidants activity**

Many human diseases, such as arteriosclerosis, nephritis, diabetes, and cancers are related to the bio-oxidation. Therefore, the research on natural antioxidants isolated from plants, microorganisms and marine algae have greater interest in pharmaceutical, nutraceuticals and functional foods for health protection and disease prevention (Yan *et al.*, 2014). We herein aim to evaluate the potency of MeOH extract and crude polysaccharide of *P. alba* leaves (PAPs) as reliable antioxidants for the first time using Ferric reducing antioxidant power (FRAP), DPPH<sup>•</sup> radical scavenging assay and the reducing power.

#### **Ferric reducing antioxidant power (FRAP)**

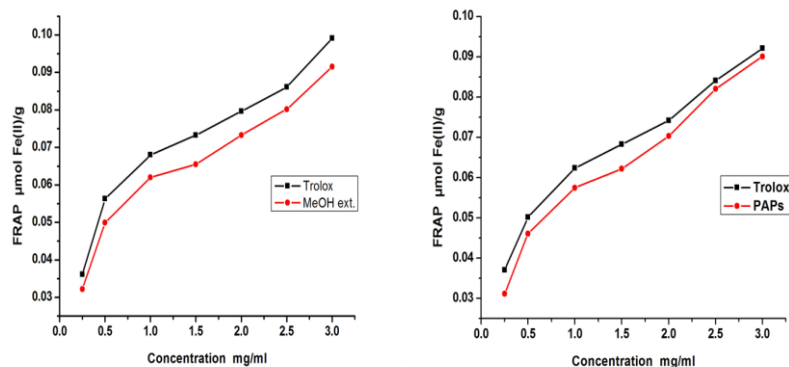
In the FRAP assay, ferric to ferrous ion reduction at low pH led to the formation of a colored ferrous-tripyridyltriazine complex, and the FRAP values of the sample were obtained by calibration with ferrous sulfate. As shown in Fig. (5), ferric reducing powers increased as the concentrations of crude polysaccharide increased (from 0.25 to 3 mg/ml). The polysaccharide at a concentration of 3mg/ml showed a FRAP value of 0.0915 and 0.0901  $\mu\text{mol Fe (II)}/\text{g}$  for MeOH extract and PAPs, respectively. Apparently, the ferric reducing power of the MeOH extract of *P. alba* was distinctly higher than that of the crude polysaccharides (PAPs).

#### **Scavenging activity of DPPH radicals**

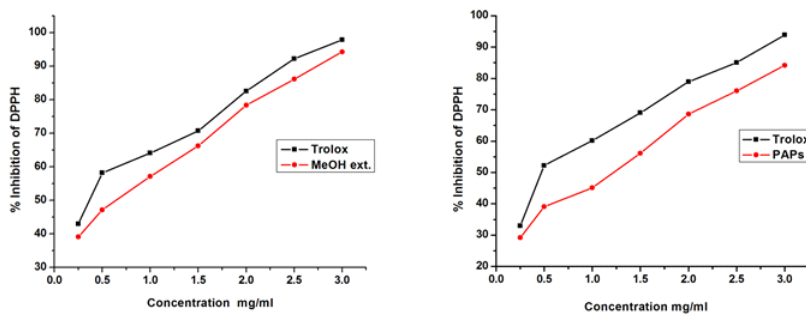
The DPPH free-radical scavenging effect of MeOH extract and PAPs were evaluated (Fig. 6). DPPH is organic nitrogen radical with an ultraviolet-visible absorption at 517 nm; it is visually noticeable as a change in color from purple to yellow (Li *et al.*, 2011). The decrease in absorbance of DPPH<sup>•</sup> radical is due to the antioxidants because of the reaction between antioxidant compounds and radical progress which results in the scavenging of the radical by hydrogen donation. MeOH extract and PAPs exhibited a comparable antioxidant activity with that of standard Trolox at different

concentration tested (0.25, 0.5, 1.5, 2, 2.5 and 3 mg/ml) (Fig. 6). Fig. 6 also shows that the DPPH radical scavenging activity increases with the increase of the concentration of the samples. At a concentration of 3 mg/ml the percentage inhibition was 94.28 and 84.18 for MeOH extract and PAPs, respectively.

The results indicated that MeOH extract and PAPs have a strong scavenging activity on DPPH<sup>•</sup> radical in a concentration-dependent manner. The antioxidant activity of methanolic extract and polysaccharides is highly related to their chemical structure (Rao and Muralikrishna, 2006), hence the hydroxyl group of the monosaccharide unit can donate proton to reduce the DPPH<sup>•</sup> radical (Yang *et al.*, 2010).



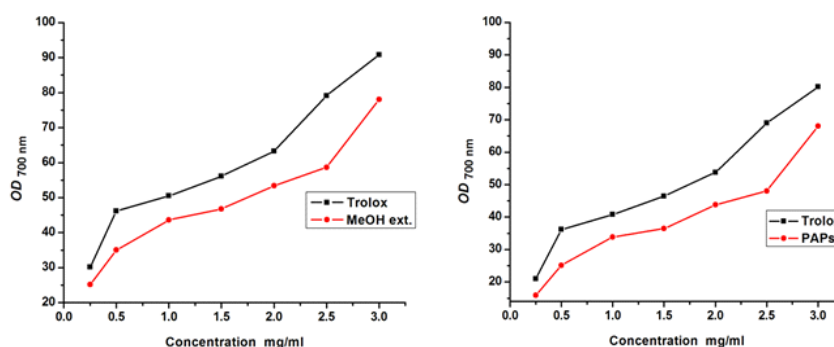
**Fig. 5. The antioxidant activity of MeOH extract and PAPs at different concentrations by FRAP assay.**



**Fig. 6. The antioxidant activity of MeOH extract and PAPs at different concentrations by scavenging activity on DPPH radicals**

### Reducing power assay

The reducing power represents as a significant potential activity key. The presence of reductant in the reaction can be monitored by the formation of Perl's Prussian blue at 700 nm (Jin *et al.*, 2012). As seen in Fig 7, the reducing capacity of MeOH extract and PAPs increased with increasing the concentration, which indicated that MeOH extract and PAPs were electron donors and could react with free radicals to convert them into more stable products (Ma *et al.*, 2012). The higher the absorbance values were, the stronger reducing power was. However, the reducing power of the MeOH extract was higher than that of PAPs at a concentration of 3 mg/ml.



**Fig. 7. The antioxidant activity of MeOH extract and PAPs at different concentrations by reducing power assay.**

The lack of correlation between the activity values measured by the three methods of the same sample has been observed in many previous studies (Cao and Prior, 1998; Schlesier *et al.*, 2002). Generally, the obtained results in this study clearly show the high antioxidant activity of MeOH extract and PAPs of *P.alba* leaves and suggest their use in diseases arising from free radicals. Methanolic extract has flavonoids and phenolic compounds that have considerable free-radical scavenging activity. The presence of compounds that contain a number of hydroxyl groups beside methoxy group cause high antioxidant activity of *P. alba*.

Some workers have studied the antioxidant activity of methanolic flower extracts of *Plumeria alba* and *Plumeria rubra*, for instance, Hafizur *et al.*, (2014) has showed that DPPH assay of methanolic extract of *Plumeria* revealed 81% and 72% inhibition by *P. alba* and *P. rubra* respectively. Also significant free radical scavenging activities were observed due to the higher phenolic content. The obtained results revealed that the MeOH extract and crude polysaccharides of *P.alba* leaves have highly antioxidant activities.

## CONCLUSION

In the present study, novel polysaccharide (PAPs) isolated from the hot water extraction of *P. alba* leaves showed highly *in vitro* antioxidant activity. This result clearly demonstrates that *P. alba* sugar is a heteropolysaccharides containing glucose and mannose as the highest level followed by galactose and rhamnose. HPLC analysis for the MeOH extract revealed the presence of nine phenolic compound and ten flavonoids with higher antioxidant capacity. Additionally, PAPs consisted of the protein-bound polysaccharide. Moreover, the three different *in vitro* antioxidant activity assays, including FRAP assay, radical scavenging activity (DPPH<sup>•</sup>), and reducing power revealed that MeOH extract and PAPs have potent antioxidant activity and could be employed as a new easily accessible source of natural antioxidants. They can be a possible to be used as dietary supplement in therapeutics and food. Further investigation for antioxidant activities *in vivo* and its mechanisms will be performed in future studies.

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## تقييم النشاط المضاد للأكسدة للمستخلص المثلي والسكريات العديدة لاوراق نبات الياسمين الهندي

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اجريت هذه الدراسة لتقييم النشاط المضاد للأكسدة للمستخلص المثلي والسكريات العديدة لاوراق نبات الياسمين الهندي . وقد تم التعرف علي المركبات الفينولية وكذلك الفلافونيدات في المستخلص المثلي للاوراق باستخدام جهاز التحليل الكروماتوجرافي عالي الاداء. وظهرت النتائج احتواء المستخلص المثلي علي 10 انواع من الفلافونيدات و 9 من الفينولات. تم ايضا تقدير الفينولات والفلافونيدات الكلية فكانت الفينولات الكلية 89.7 و 11.16 مجم/جرام للمستخلص المثلي والسكريات العديدة علي التوالي. اما الفينولات الكلية كانت 74.7 و 3.18 مجم/جرام للمستخلص المثلي والسكريات العديدة علي التوالي . وبالتعرف علي السكريات العديدة با الكروماتوجرافي الايوني Dionex system (Dx-120) ion chromatography وجد ان الجلوكوزو المانوز هم اكثر السكريات الاحادية الموجودة يليهم الجالاكتوز والرامنوز. وقد لوحظ ان السكريات العديدة تحتوي علي 3.9% بروتين كما تم تحديد 14 حمض اميني في بروتين السكريات العديدة. وكانت احماض Glutamic, Aspartic and Cystine هي اعلي الاحماض تواجد. المستخلص المثلي والسكريات العديدة تم استخدامها بتركيزات تتراوح من 0.25 الي 3 مجم/مل ومن خلال ثلاث طرق FRAP, DPPH free radical scavenging and reducing power assay لتقييم نشاطها المضاد للأكسدة. وظهرت النتائج ايضا ان المستخلص المثلي والسكريات العديدة لها نشاط مضاد للأكسدة ويمكن أن تكون بمثابة بدائل للمضادات الاصطناعية.