

International Journal of Medicine and Pharmaceutical Research

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Research Article

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Antioxidant Activity of Carotenoid Extracts of the leaves and flowers of *Peltophorum Pterocarpum*

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ABSTRACT

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. *Peltophorum pterocarpum*, family Leguminosae is a tree natural to tropical South-Eastern Asia and was brought to Nigeria by immigrants. It has been used traditionally by the South Western people of the country as a memory enhancer and for anti-ageing. Continuous research have revealed many chemical constituents isolated from different parts of the this tree to exhibit several biological activities such as antimicrobial activity, antioxidant activity, cytotoxic activity, antiglycemic activity, aldose reductase inhibition activity, cardiotonic activity and choline esterase inhibitory activity. The present study aims at studying the Antioxidant activity of Carotenoid extracts of the leaves and flowers of *Peltophorum pterocarpum*.

Keywords: Natural products, Bioactive compounds, Carotenoid Extracts, Peltophorum pterocarpum, Antioxidant activity.

ARTICLE INFO

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Article History: Received 11 October 2014, Accepted 29 December 2014, Available Online 10 February 2015

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Citation: V. Judia Harriet Sumathy. Antioxidant Activity of Carotenoid Extracts of the leaves and flowers of *Peltophorum Pterocarpum. Int. J. Med. Pharm, Res.*, 2015, 3(1): 902-910.

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1. Introduction

Peltophorum pterocarpum is a very common deciduous tree grown in tropical countries and known by a variety of names such as Yellow Poinciana, Golden Flame, Copper pod, Rusty shield bearer and Yellow flamboyant (Figure 1). The plant is native to tropical southeastern Asia and northern Australia and widely grown in Sri Lanka, Thailand, Vietnam, Indonesia, Malaysia, Papua New Guinea, Philippines and the islands of the coast of Northern Territory, Australia (Edward F. Gilman and Dennis G. Watson, 1994). Leaves are large, 30-60 cm long, with 8-10 pairs of pinnae each bearing 10-20 pairs of oblong leaflets

which are 0.8-2.5 cm long with oblique bases. Flowers are orange-yellow, each about 2.5 cm in diameter, fragrant, particularly at night; inflorescence is brown-tomentose and the panicles are terminal with rust-coloured buds. Fruits are 1-4 seeded pods, flat, thin, winged, 5-10 cm long, dark red when ripe, then turning black. Peltophorum pterocarpum has a deep root system. The specific epithet 'pterocarpum' alludes to its winged seed. Carotenoids are an abundant group of naturally occurring pigments (Mohammad Shahriar et.al., 2013).



Figure 1: Tree, flowers and leaves of Peltophorum pterocarpum

They occur ubiquitously in all organisms capable of conducting photosynthesis. Carotenoids are important constituents of photosynthetic organelles of all higher plants, mosses, ferns and algae. They are also found in photosynthetic membranes of phototropic bacteria and cyanobacteria. Although not synthesized by humans and animals, they are also present in their blood and tissues. More than 600 different carotenoids from natural sources have been isolated and characterized. Physical properties and natural functions and actions of carotenoids are determined by their chemical properties and these

properties are defined by their molecular structures. Carotenoids consist of 40 carbon atoms (tetraterpenes) with conjugated double bonds (Lai Teng Ling et.al., 2010). They consist of 8 isoprenoid units joined in such a manner that the rearrangement of isoprenoid units is reversed at the centre of the molecule so that the two central methyl groups are in a 1, 6 position and the remaining non terminal methyl groups are in a 1,5-position relationship. (Joanna Fiedor and Kvetoslava Burda, 2014). The taxonomic classification of Peltophorum pterocarpum is given below in Table 1.

Table 1 : Taxonomic Classification					
Kingdom	Plantae				
Sub-kingdom	Tracheobionta				
Super-division	Spermatophyta				
Division	Magnoliophyta				
Class	Magnoliopsida				
Sub-class	Rosidae				
Order	Fabales				
Family	Fabaceae				
Sub-family	Caesalpinioidae				
Genus	Peltophorum				
Species	<u>P. pterocarpum</u>				
Binomial Name	Peltophorum pterocarpum				
	(DC.) Baker ex K. Heyne				

Table 1 : Taxonomic Classificat	ion
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2. Materials and Method

Isolation of Carotenoid Pigments from Peltophorum *Pterocarpum* by Column Chromatography

Column chromatography involves the separation of compounds based on the differences in partitioning between mobile and stationary phases. The stationary phase is

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placed in a support through which the mobile phase is passed. The stationary phase serves as an adsorbent. Several types of interactions can aid in developing the desired separation (Pavia D.L., et. al., 1999). Plant material (leaves and flowers of *P. pterocarpum*), mortar and pestle, acetone, centrifuge tubes, hexane, distilled water, burette, cotton, silica gel are the materials required. About 10g of leaves are weighed, washed of impurities and dried in air. The leaves are ground in a mortar and pestle by adding acetone little by little. The leaves are ground until a fine paste is obtained. Now the mixture along with acetone is transferred to the centrifuge tube. The mortar and pestle is rinsed with acetone and again the mixture is transferred to the centrifuge tube. 5 ml of hexane is added to the centrifuge tube and capped and the mixture is shaken thoroughly. Then 5 ml of distilled water is added and shaken thoroughly with occasional venting. The mixture is centrifuged to break the emulsion. The pigment layer is the top hexane layer, which should be dark green. Most of the acetone will dissolve in the water. The pigment layer is carefully pipetted out into a clean test tube. The dark green hexane solution of the leaf pigments in the test tube may contain traces of water that must be removed before separating the components through column chromatography. To dry the solution 0.5g of anhydrous sodium sulphate is added to the hexane solution. Sodium sulphate is allowed to contact all parts of the hexane solution. After standing for 5 minutes, the liquid is transferred to another clean test tube. This extract is used for column chromatography.

A dry and clean burette is clamped onto a burette stand and a very small plug of cotton is pushed to the bottom of the burette. 15g of silica gel is taken and mixed well with 100% hexane in a slurry like consistency and the mixture is poured into the burette carefully. The silica gel must be kept wet with solvent all the time. Now the set up is allowed to stand for an hour without disturbance so that the silica gel gets packed well. After an hour, the solvent is drained till it reaches a little above the silica gel. Then the flower/leaves extract is poured gently into the burette. As the extract drains into the silica gel, the pigments begin to separate into a yellow colour carotene band and a green colour chlorophyll band. The pigment of interest here is the vellow carotenes. So, the vellow carotene band is eluted with 100% hexane and the eluant is collected separately. The carotenes are light sensitive and so care must be taken to protect the pigments from light. The fractions collected are evaporated and the thick concentrated carotene fractions are used for TLC. The total carotenoid content is calculated and the absorbance of the extracted carotenoids was measured at 450nm. The extracted carotenoids were quantified by the following formula:

Total carotenoid content $(\mu g/g) = A \times V \text{ (ml)} \times 10^4 / A^{1\%} \text{ cm} \times W \text{ (g)}$ Where A is the absorbance of the carotenoid pigment at 450 nm, V is the total extract volume, $A^{1\%} \text{ cm}$ is the absorption coefficient of carotene in hexane (2600), W is the sample weight. (Alcides Oliveira R.G., *et. al.*, 2010).

Antioxidant Activity of the Extracts

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The redox properties of antioxidants play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. In doing so, the antioxidants themselves become oxidised. This urges the constant need of antioxidants to replenish them (**Selvakumaran S.** *et. al.*, **2011**). The antioxidant properties of the ethanolic leaf and flower extracts and the carotenoid extracts from the leaves and flowers are evaluated by the following methods such as:

- 1) DPPH free radical scavenging activity
- 2) Reducing Power assay
- 3) Nitric oxide scavenging activity
- 4) Phosphomolybdenum method
- 5) Hydrogen peroxide scavenging activity

DPPH free Radical Scavenging Activity

The ability of the extracts to annihilate the DPPH radical (1, 1-diphenyl-2-picrylhydrazyl) is investigated by this method. Stock solution of leaf and flower extracts was prepared to the concentration of 10mg/5ml. From the stock solution 50, 100 and 150 μ g of each extracts were added, at an equal volume, to methanolic solution of DPPH (0.1mM). The reaction mixture is incubated for 30min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHT was used as standard controls. The annihilation activity of free radicals was calculated in % inhibition according to the following formula:

% of Inhibition = (A of control – A of Test)/A of control * 100 where A control is the absorbance of the control reaction and A test is the absorbance of the sample extracts.

Reducing Power Assay

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm (R. Sutharsingh et. al., 2011). Samples, Ascorbic acid, 0.2M Sodium phosphate buffer, 1% Potassium ferricyanide, 10% trichloroacetic acid and 0.1% Ferric chloride are the reagents required. A stock solution of the ethanolic extracts, carotenoid extracts and the standard ascorbic acid are prepared in the concentration of 10mg/100ml (100µg/ml). From the stock solution 1ml, 2ml, 3ml, 4ml and 5ml of the solution is taken. With the same solvent the final volume of the extracts are made to 5ml. Now the concentrations are 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml. In this assay 1ml of the test samples of the extracts in different concentrations were mixed with 1 ml of 0.2M sodium phosphate buffer (ph 6.6) and 1 ml of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated in a temperature controlled water bath at 50° C for 20 minutes, followed by addition of 1ml of 10% trichloroacetic acid. The mixtures were then centrifuged for ten minutes at room temperature. The supernatant obtained was added with 1ml of deionised water and 200µl of 0.1% Ferric chloride. The blank was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by

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distilled water. The absorbance of the reaction mixture was measured at 700nm. The reducing power was expressed as an increase in the A_{700} after blank subtraction. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation: $A = (c \times V)/m$

Where, A = total content of antioxidant compounds in ascorbic acid equivalents,mg/dl, c = the concentration of ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.

Nitric Oxide Scavenging Activity Assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by Griess Illosvoy reaction. Samples, Ascorbic acid,10M Sodium nitroprusside, Sulphanilic acid reagent (33% in 20% glacial acetic acid) and 0.1% Naphthylethylenediamine hydrochloride are the reagents required. 2ml of 10M sodium nitroprusside in 0.5 ml of phosphate buffer saline (ph 7.4) was mixed with 0.5 ml of the extracts at various concentrations and the mixture incubated at 25°C for two hours. From the incubated mixture 0.5 ml was taken out and added into 1ml sulphanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 minutes. Finally, 1ml of naphthylethylenediamine hydrochloride (0.1% w/v)was mixed and incubated at room temperature for 30 minutes. The absorbance at 540nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated using the below equation: % of Inhibition = (A of control – A of Test)/A of control * 100 where A of control is the absorbance of the control reaction and A of test is the absorbance of the sample extracts.

Total Antioxidant Activity by Phosphomolybdenum Method

The phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of Mo (VI) –

3. Results and Discussion

Isolation of Carotenoid pigments by Column Chromatography



Figure 2: Isolation of pigments by Column Chromatography

Carotenoid pigments were effectively separated from the leaves and flower extracts separately in a silica gel column with 100 % hexane. The yellow band that separates when

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Mo (V) by the antioxidants and subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Pilar Prieto et al, 1999). Samples, Ascorbic acid, 0.6M sulphuric acid, 28mM Sodium phosphate and 4 mM Ammonium molybdate are the reagents required. 0.3 ml of test sample is taken in a tube and mixed with 3 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95°C for 90 min. Ascorbic acid is utilised as a reference standard. The absorbance of the mixture is then measured at 695 nm with blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation: $A = (c \times V)/m$ Where, A = total content of antioxidant compounds in ascorbic acid equivalents, mg/dl, c = the concentrationof ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.

Hydrogen Peroxide Scavenging Activity

The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2 . Samples, Ascorbic acid, Hydrogen peroxide and Phosphate buffer are the reagents required. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of H_2O_2 was determined by absorption at 230 nm using a spectrophotometer.

The extracts in various concentrations were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2 . The percentage of H_2O_2 scavenging by the extract and standard compounds was calculated as follows: % of Inhibition = (A of control – A of Test)/A of control * 100 where A of control is the absorbance of the control reaction and A of test is the absorbance of the sample extracts.

eluted with 100% hexane is identified to be the carotenoid pigments. Thus the carotenoid pigments with hexane was collected and stored in glass vials at -20° C (**Figure 2**).

Quantification of Carotenoids

The absorbance of the extracted carotenoids was measured at 450nm. The extracted carotenoids was quantified by the following formula: Total carotenoid content ($\mu g/g$) = A x V (ml) x 10⁴ / A^{1%} cm x W (g)

Where A is the absorbance of the carotenoid pigment at 450 nm, V is the total extract volume, $A^{1\%}$ cm is the absorption coefficient of carotene in hexane (2600), W is the sample weight.

Thus, Total carotenoid content in leaves = $0.231 \times 10 \times 10^4 / 2600 \times 10 = 0.88 \mu g/g$. Total carotenoid content in flowers = $0.145 \times 10 \times 10^4 / 2600 \times 10 = 0.56 \mu g/g$.

Antioxidant Activity of the Extracts DPPH radical scavenging activity

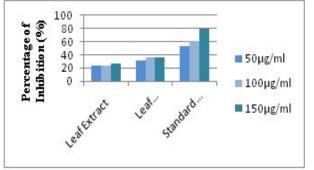


Figure 3: DPPH Scavenging Potential of Leaf Extracts

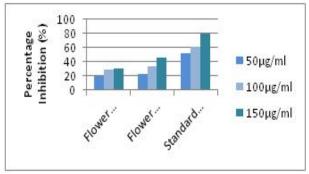


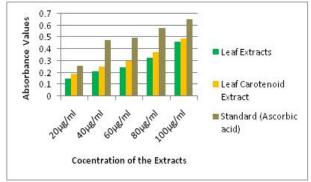
Figure 4: DPPH Scavenging Potential of Flower Extracts

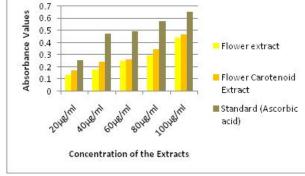
	Table 2: DPPH Scavenging Potential of Leaf and Flower extracts									
	Concentration of		Per	centage of Inhibit	ion (%)					
S.No	the samples (µg/ml)	Leaf Extract	Leaf Carotenoid Extract	Flower extract	Flower Carotenoid Extract	Standard (BHT)				
1	50	22.81	30.59	20.59	22.31	52.93				
2	100	23.18	35.54	29.41	34.12	61.22				
3	150	26.53	36.28	30.5	45.95	80.39				

The DPPH radical scavenging assay depends on the decolouration of the purple coloured methanolic DPPH solution to

yellow by the radical scavengers present in the extracts. The radical scavenger acts by reducing the stable DPPH radical in the presence of hydrogen donating antioxidant to diphenylpicryl hydrazine. The percentage of inhibition is calculated as follows, % of Inhibition = (A of control - A of Test)/A of control * 100. The percentage of inhibition increased with increasing concentration (Table 2). Compared to the leaf and flower crude extracts, the carotenoid extracts of both the leaves and flowers exhibited higher scavenging activity. The Standard BHT has the highest scavenging activity (Figures 3 & 4).

Reducing Power Assay





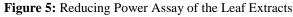


Figure 6: Reducing Power Assay of the Flower Extracts

Table 3: Reducing Power of the Crude Extracts and Carotenoid extracts								
	Concentration of	Asc	orbic Acid Equi	valents (mg/dl)±SD			
S.No	the samples (µg/ml)	Leaf Extracts	Leaf Carotenoid Extract	Flower extract	Flower Carotenoid Extract			
1	20	0.9 ± 0.25	1.3±0.31	0.5±0.35	1.1±0.47			
2	40	1.45±0.22	1.95±0.50	1.2±0.24	1.8 ± 0.58			
3	60	1.8±0.39	2.15±0.48	1.8±0.79	1.95±0.89			
4	80	2.3±0.19	2.85±0.77	2.15±0.31	2.6±0.33			
5	100	3.6±0.34	3.9±0.50	3.45±0.90	3.9±0.65			

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Figure 7: Reducing Power Activity of the Extracts

The reducing power assay was used to test the reducing capability of the extracts. Their ability to reduce the ferricyanide (Fe3+) complex to their ferrous form is determined by measuring the absorbance at 700nm (**Mohammad Shahriar** *et. al.* **2013**). From the **Table 3**, a concentration dependent reducing activity was observed for

Nitric Oxide Scavenging Activity

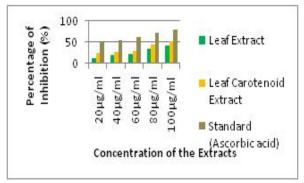
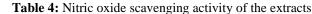


Figure 8: Nitric oxide scavenging activity of the Leaf extracts

the four extracts. The reducing power of the extracts increased with increase in concentration. However, the carotenoids extracts from leaves and flowers showed higher reducing activity compared to the crude leaf and flower extracts (**Figures 5 - 7**).

	Concentration of	Percentage of Inhibition (%)±SD				
S.No	the samples (µg/ml)	Leaf Extract	Leaf Carotenoid Extract	Flower extract	Flower Carotenoid Extract	Standard (Ascorbic acid)
1	20	12±0.76	24±0.61	11±0.72	21±0.92	50±0.72
2	40	20±0.58	28±0.66	16±0.81	34±0.64	56±0.35
3	60	22±0.72	30±0.91	26±0.68	33±0.90	62±0.75
4	80	36±0.65	44±0.75	34±0.36	40±1.1	72±0.55
5	100	42±0.55	52±0.40	40±0.51	50±0.85	80±0.85



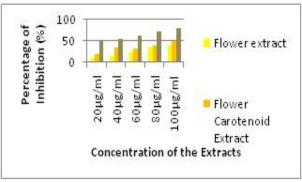


Figure 9: Nitric oxide Scavenging Activity of Flower Extracts

Nitric oxide is a very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess Reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546nm. The percentage of inhibition is calculated as follows, % of

Phospho molybdenum Method

Total antioxidant capacity of the different extracts of *Peltophorum pterocarpum* was evaluated by the phosphomolybdenum method and was expressed as International Journal of Medicine and Pharmaceutical Research



Figure 10: Nitric oxide scavenging activity of the extracts

Inhibition = (A of control – A of Test)/A of control * 100. The scavenging of NO by the extracts increased with increased concentration (Table 4). Here also the maximum activity was shown by the ascorbic acid (80% inhibition) followed by the leaf carotenoid extracts (52% inhibition) (Figures 8 - 10).

ascorbic acid equivalents (AAE) per deciliter of plant extract. Total antioxidant capacity was found to increase with increasing concentration (**Table 5**). The carotenoid extracts of the leaves and flowers were found to possess the

highest total antioxidant capacity. (Figures 11 - 13).

	Concentration of	Asc	corbic acid equiv	alents (mg/dl)	±SD
S.No	the samples (µg/ml)	Leaf Extract	Leaf Carotenoid Extract	Flower extract	Flower Carotenoid Extract
1	20	1.7±0.73	2.25±1.08	1.4 ± 0.86	2±0.86
2	40	1.8 ± 0.87	2.9±0.46	1.6 ± 0.54	2.5±0.64
3	60	2.8±0.49	3.5±0.82	1.85±0.83	3.1±0.54
4	80	3.6±0.89	4.8±0.89	2.8±1.02	3.8±0.68
5	100	4.3±0.66	6.1±1.18	3.7±0.95	5.9±0.59

Table 5: Total Antioxidant activity of the extracts by phosphomolybdenum method

Phospho molybdenum Method

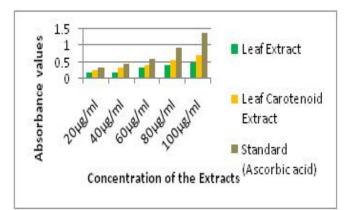


Figure 11: Phosphomolybdenum method for Leaf Extracts

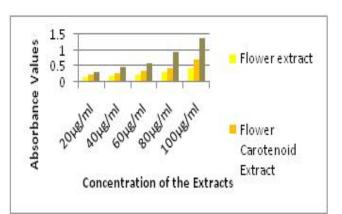


Figure 12: Phosphomolybdenum method for Flower extracts



Figure 13: Total Antioxidant Capacity of the Extracts by Phosphomolybdenum method

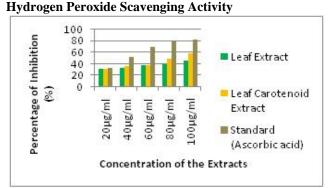


Figure 14: Hydrogen peroxide scavenging activity of the Leaf Extracts

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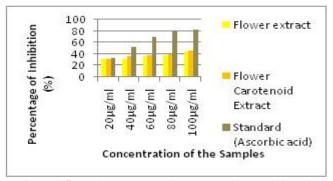


Figure 15: Hydrogen Peroxide Scavenging Activity of Flower Extracts

Table 6: Hydrogen Peroxide Scavenging Activity									
	Concentration of	Percentage of Inhibition (%)							
S.No		Leaf Extract	Leaf Carotenoid Extract	Flower extract	Flower Carotenoid Extract	Standard (Ascorbic acid)			
1	20	32.40±0.58	32.24±0.77	31.93±0.91	31.54±1.13	33.80±0.58			
2	40	33.48±0.75	36.60±0.99	32.32±0.40	35.82±0.98	51.79±0.93			
3	60	38.16±1.4	38.31±1.12	36.52±0.64	37.61±0.87	69.85±0.84			
4	80	39.33±0.41	49.76±0.94	38.24±0.51	40.42±1.21	79.82±0.96			
5	100	45.40±0.95	59.42±0.82	44.00±0.61	46.26±0.78	82.00±0.78			



Figure 16: Hydrogen peroxide scavenging activity of the extracts

Hydrogen peroxide, although not a radical species play a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally-occurring iron complexes inside the cell believed

4. Conclusion

Carotenoids were extracted from the leaves and flowers of Peltophorum pterocarpum by Column chromatography. The carotenoid pigments and the crude ethanolic leaf and flower extracts were then analysed for their antioxidant activity. The antioxidant activity was carried out using DPPH radical scavenging activity, Reducing power assay, Nitric oxide scavenging activity, Phosphomolybdenum method and Hydrogen peroxide scavenging activity. In all the five methodologies done, the carotenoid pigments from leaves and flowers showed higher antioxidant activity compared to the crude leaf and flower extracts. It is also interesting to note that Carotenoids play a very important role in human health. They are known to be very efficient physical and chemical quenchers of singlet oxygen (O₂), as well as potent scavengers of other reactive oxygen species (ROS), thus acting as very important natural

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to react with H2O2 in vivo to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects. Scavenging of hydrogen peroxide of different extracts are given in the below table. The carotenoid extracts showed better antioxidant activity than the crude extracts. Ascorbic acid exhibited higher percentage of hydrogen peroxide scavenging activity. The activity increases with increased concentration (Table 6 & Figures 14-16). In all the above antioxidant activities the carotenoids exhibited more antioxidant activity than the crude leaf and flower extracts. Thus with these specific antioxidant mechanisms, the carotenoids may be developed as specific bioactive products for their use in many food industries as food colourants. This can provide an alternative to the synthetic chemicals used as colourants or preservatives.

antioxidants. This is of special significance, because the uncontrolled generation and concomitant increase of ROS level in the body results in "oxidative stress", an essential contributor to the pathogenic processes of many diseases. They are suggested to play a protective role in a number of ROS-mediated disorders, such as, i.e., cardiovascular diseases, several types of cancer or neurological, as well as photosensitive or eye-related disorders. Carotenoids are also suggested to participate in the stimulation of the immune system, the modulation of intracellular signaling pathways (gap junction communication), the regulation of the cell cycle and apoptosis, the modulation of growth factors, cell differentiation and the modulation of various types of receptors or adhesion molecules. Thus future studies can be undertaken to study the Anti - Microbial and Anti – Cancer Activity of these carotenoids pigments.

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