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TLC Profilling, Antioxidant and Toxicity Studies of Chlorophyll Extracted from Dried Carrot Greens

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Abstract

This research carried out studies on thin layer chromatography (TLC), antioxidant and toxicity of acetone extract of the pigments (chlorophyll) of dried carrot green on albino mice using LD_{50} method. The chlorophyll pigment was extracted by maceration acetone using centrifuge machine operated at 500 RPM for 20 minutes. The absorbance(s) of chlorophyll a, chlorophyll b and total carotenoids were also measured in triplicates using uv-visible spectrophotometer (absorbed at wave length of 662 nm, 645nm and 476 nm). The crude chlorophyll pigment extract was subjected to TLC using precoated (silica gel on aluminium) plate and a solvent system consisting of petroleum ether, cyclohexane, ethyl acetate, acetone and methanol. Antioxidant study was determined using the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical. LD_{50} of the extract was carried out by intraperitoneal administration of different doses (2000, 1500, 1000, 500 and 100 mg/kg to albino mice. The concentrations were calculated using the equations of Lichtenthalor and Wellburn. Result of TLC of showed five spots. Spots 1, 2, 4 and 5 (Rf: 0.17, 0.25, 0.67 and 0.82 respectively) showed orange-red colour under UV light whereas spot 3 (Rf: 0.50) showed bright-green colour. Highest scavenging ability on DPPH radical of 55.02% was recorded for the lowest extract concentration at 5µg/ml, and 53.73% at highest concentration (500µg/ml). LD_{50} result calculated was 1500 mg/kg implying that pigments extracted from carrot green is not toxic for use as a drug/food supplement. The presence of chlorophyll a, b, and carotenoid, antioxidant activities implies that the carrot greens can be valuable in reducing the risk of many diseases.

Keywords: Carrot greens, Chlorophyll, TLC, LD_{50}, Toxicity and Antioxidant

1. Introduction

Plants have been in existence from time in memorial and are the only organisms that exhibit apical dominance. Some of them can be considered practically immortal. Majority of them keep on growing throughout their life time [1].

In recent times, much research interest has been shifted to plants because of their importance and relevance to man and animals.

The green plants and some other plants pigments have been reported to possess medicinal properties such as promoting healing process in wounds, help in fighting cancer, improving liver detoxification, improving digestion

and weight control and protecting skin health [2]. An acetone extract of the leaves of carrot (*Daucus carota L*) has been reported to contain secondary metabolites possessing biological activities against some gram positive and negative bacteria: against *Pseudomona aeruginosa, Escherichia coli, Staphylococcus aureus* and *Salmonella pneumoniae* [3]. Aside the bountiful medicinal importance, the nutritional benefits of carrot leaves cannot be overlooked; like magnesium to promote healthy blood pressure, strong bones and muscles, and also to purified lymph nodes and adrenal glands, also contain potassium and calcium which make them capable of lowering blood pressure, support metabolism and help prevent osteoporosis as reported by [3].

In addition to the vast uses of chlorophyll and the green pigments of carrot as highlighted above, the knowledge and experience of using 'Edmark Splina Liquid' [4], which is a chlorophyll liquid drink supplement known to be an excellent source of nutrients which boosts immune system, increases blood count and oxygen supply in the blood, reduces the appearance of wrinkles and slows down aging, etc [5] led to our interest in this research into the carrot greens [6]. This present work is geared toward determining the concentration, TLC characterization, antioxidant and toxicity studies of green pigments (chlorophyll) extracted from dried carrot leaves and stems. This study is meant to ascertain if chlorophyll extracted from the carrot greens could be safe for use therapeutically. If so, then the carrot greens, which hitherto have been thrown away as waste by farmers, carrot sellers and consumers in Nigeria [3], could now serve as a possible source of drugs or food supplement.

The plant material (the greens of *Daucus carota L*) was collected from a farm at Naraguta Village, Jos North Local Government Area, Plateau State, Nigeria. Collection was done by manually uprooting the whole plant and separating the greens, (stems and leaves) from the roots manually, in the month of December. The carrot greens were washed with tap water and rinsed with distilled water. They were dried at room temperature under shade for 3 weeks. The plant sample was pulverized using a mortar and pestle and the powder kept in a black polythene bag for further use.

2. Materials and Methods

Sample Collection and Preparation

The plant material (the greens of *Daucus carota L*) was collected from a farm at Naraguta Village, Jos North Local Government Area, Plateau State, Nigeria. Collection was done by manually uprooting the whole plant and separating the greens, (stems and leaves) from the roots manually, in the month of December. The carrot greens were washed with tap water and rinsed with distilled water. They were dried at room temperature under shade for 3 weeks. The plant sample was pulverized using a mortar and pestle and the powder kept in a black polythene bag for further use.

Extraction of Chlorophyll

The dried powder of the carrot greens (300 g) was used for the extraction. The sample (2 g) was measured, weighed on a digital balance and transferred into several test tubes and 30 mL of acetone was also measured and poured into each of the test tubes. They were transferred into a centrifuge machine for proper extraction at 500 RPM (revolutions per minute), for 20 minutes. The procedure was repeated for ten (10) more times. After this, extracts were filtered using Whatmann filter paper No. 1, into an open beaker and kept in a closed cupboard for 2 days for the acetone to evaporate. Finally, the extract was stored in a refrigerator under 40 ^{o}C for further use.

Determination of Concentrations of Chlorophyll

Absorbance of the extract was measured at 663.2, 646.8 and 470 nm (Major absorption peaks of chlorophylls a, b and total carotenoids) [7]. Measurement was done in triplicates using UV-Visible spectrophotometer (JENWAY 6405). The concentrations were calculated using the equations of Lichtenthaler and Wellburn [8] for determining the amount of plant pigments using acetone solvent [9]. All determinations were carried out in the dark, only using flash light, to minimize photo-degradation.

$$C_a = 11.75A_{662} - 2.350A_{645} \ (\mu g/mL \ solution)$$

$$C_b = 18.61A_{645} - 3.960A_{662} \ (\mu g/mL \ solution)$$

$C_{x+c} = 1000A_{470} – 2.270C_a – 81.4C_b/227$ (µg/mL solution)

Where C_a=Chlorophyll a,

C_b= Chlorophll b,

C_{x+c}= Total carotenoids.

Thin Layer Chromatography (TLC)

The crude chlorophyll pigment extract was subjected to thin layer chromatography (TLC) using 3 cm by10 cm precoated (silica gel on aluminium) plate, thickness 0.1 mm. The extract was spotted on the plate 1cm above from the bottom. The plate was then placed in saturated developing tank containing solvent mixture of petroleum ether, cyclohexane, ethyl acetate, acetone and methanol in the ratio 60%, 16%, 10%, 10% and 4% respectively. The solvent was allowed to move up the plate until it was about 1cm from the top. It was removed from the tank and the solvent front immediately marked. The plate was allowed to dry. The chromatogram was viewed in UV lamp and the spots were marked. This procedure was carried out in a dark room to reduce photo-degradation of the pigments [10].

R_f values (Retention factor) of the spots were calculated as follows:

$$R_f = \frac{\text{Distance between the starting point and pigment line}}{\text{Distance between the starting point and Solvent front}}$$

Antioxidant Activity

Qualitative Method by DPPH

The crude chlorophyll extract was spotted onto TLC plate and dried. The plate was developed with a mobile phase of n-hexane and ethyl acetate (8:2). The developed chromatogram was sprayed with 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) reagent (0.2% methanol) and viewed in UV light [11].

The DPPH Assay

The radical scavenging ability of the crude chlorophyll extract was tested by preparing different concentrations. Ascorbic acid was used as positive control. The reduction ability of DPPH was determined by the decrease in its absorbance at 517 nm caused by antioxidants. DPPH (0.01 g) was measured and dissolved in 250 mL of ethanol in a volumetric flask which gives 0.004%. Ascorbic acid (0.05 g) was dissolved in 50 mL of ethanol to obtain 500 µg/mL of the standard. Using dilution formula ($C_1V_1 = C_2V_2$) other concentrations were prepared by serial dilution. To each concentration of freshly prepared crude chlorophyll extract and ascorbic acid standard, 3 mL of DPPH reagent was added and allowed to stand for 30 minutes the absorbance was measured using UV- spectrophotometer at 517 nm.

The percentage scavenging activity on DPPH was calculated using the formula:

$$\% \text{ Scavenging activity} = \frac{A_o - A_s}{A_o} \times 100 \qquad [12]$$

Where, A_o is the absorbance of negative control and A_s is the absorbance of the sample

Toxicological Study

Animals used

The animals (albino mice) used for this research were obtained from the Small Animal House, University of Jos, Nigeria. Thirty mice (29 – 44 g) of either sex were housed in groups of 5 per cage for 3 days prior to experimentation in an ideal standard environment as per Organization for Economic Co-operation and Development (OECD) [13]. They were fed with standard diet (Growers' feeds obtained from Grand Cereals and Oil Mill Ltd, Bukuru, Jos, Nigeria) and water was given to the mice *ad libitum*. Each experimental group consisted of five (5) animals according to protocol approved by the University and Departmental Committee for Research and Ethics University of Jos. Each animal was used only once, and all animals were sacrificed using chloroform vapour in a glass cage at the end of the study (i.e., by Euthanasia) [14].

Preparation of different doses of the carrot greens extract

Replace with: A stock solution was prepared by dissolving the extract in normal saline (4g/40 cm^3) and different doses of 2000, 1500, 1000, 500 and 100 mg/kg were prepared by serial dilution. Since chlorophyll is insoluble in water, a drop of “Tween 80” was mixed with the extract to enable solubility.

Toxicology study of acetone extract of carrot green

Doses (2000, 1500, 1000, 500 and 100 mg/kg) were administered intraperitoneally to five (5) groups of five (5) mice each. Normal saline (0.1 cm^3/10 g) was also administered to a group of five (5) mice as control. Mortality in each group within 24 hours and after 48 hours was recorded. The mice that survived (did not die) were observed for seven (7) more days for any signs of delayed toxicity.

Determination of Median Lethal Dose (LD_{50})

For each mouse, the observation was made for 24 hours and symptoms of toxicity and rate of mortality in each group were noted. At the end of study period, dead animals were counted for the calculation of LD_{50}. LD_{50} of the extract on the test animals was calculated by Arithmetic method of [15] as modified by [16] reported by [17].

$LD_{50} = LD_y - \underline{\Sigma (D_d \times M_d)}\ N$

LD_y = Highest dose

D_d = Dose difference

M_d = Mean dead

N = Number of mice per group

3. Results and Discussions

3.1 Results

Percentage yield of the extract; the absorbance(s) of chlorophyll a, chlorophyll b and carotenoids; concentrations of chlorophyll a, chlorophyll b and carotenoids; antioxidant activity; toxicological studies and LD_{50} of the carrot greens extract are shown in tables 1 to 7, respectively. The results are analysed in triplicate and presented as mean ± standard deviation (SD) of triplicate determinations. The level of association between the extract and the standard in antioxidant activity was subjected to analysis of variance (ANOVA) and T-test (5% probability) using the Statistical Program tools for Social Sciences (SPSS) version 20.0. [18]

Table 1: Percentage of Carrot Greens Extract

Weight of powdered sample (g)	Weight of extract (g)	Percentage extract (%)
300	8	2.67

Table 2: UV Absorbance(s) of Pigments in Carrot greens extracts

Pigments	UV Wavelength (nm)	Measurements			Mean ± SD
		1st	2nd	3rd	
Chlorophyll a	662	1.511	1.501	1.511	1.508 ± 0.008
Chlorophyll b	645	1.221	1.221	1.220	1.221 ± 0.001
Carotenoids	476	1.011	1.001	1.011	1.008 ± 0.005

Table 3: Concentrations f Chlorophyll Pigments in Carrot Greens Extracts

Pigments	Concentrations (µg/mL)
Chlorophyll a (C_a)	14.85 ± 0.06
Chlorophyll b (C_b)	16.72 ± 0.03
Total Carotenoid (C_{x+c})	971.29 ± 4.71

Table 4: Results of TLC analysis of the crude chlorophyll extract.

Spots	1	2	3	4	5
R_f Value	0.17	0.25	0.50	0.67	0.82
Colour in UV	OR	OR	BG	OR	OR

Note: OR= Orange-red, BG= Bright-green

Table 5: Result of Percentage Scavenging on DPPH Radical

Concentration (µg/mL)	Scavenging Activity of DPPH (%)	
	Crude Chlorophyll Extract	**Ascorbic Acid**
5	55.02±0.07^{a}	64.19±0.06^{d}
10	18.74±0.06^{d}	59.82±0.04^{e}
50	49.25±0.31^{c}	88.82±0.01^{a}
100	18.39±0.04^{d}	87.03±0.04^{c}
500	53.73±0.07^{b}	88.63±0.03^{b}

Note: Values presented as Mean ± SD (n=3), Values in the same column having same letters are not significantly different ($p \leq 0.05$).

Figure 1 below shows a relationship in antioxidant activity (DPPH radical scavenging activities) against concentrations of chlorophyll extracted from carrot green and standard antioxidant (ascorbic acid).

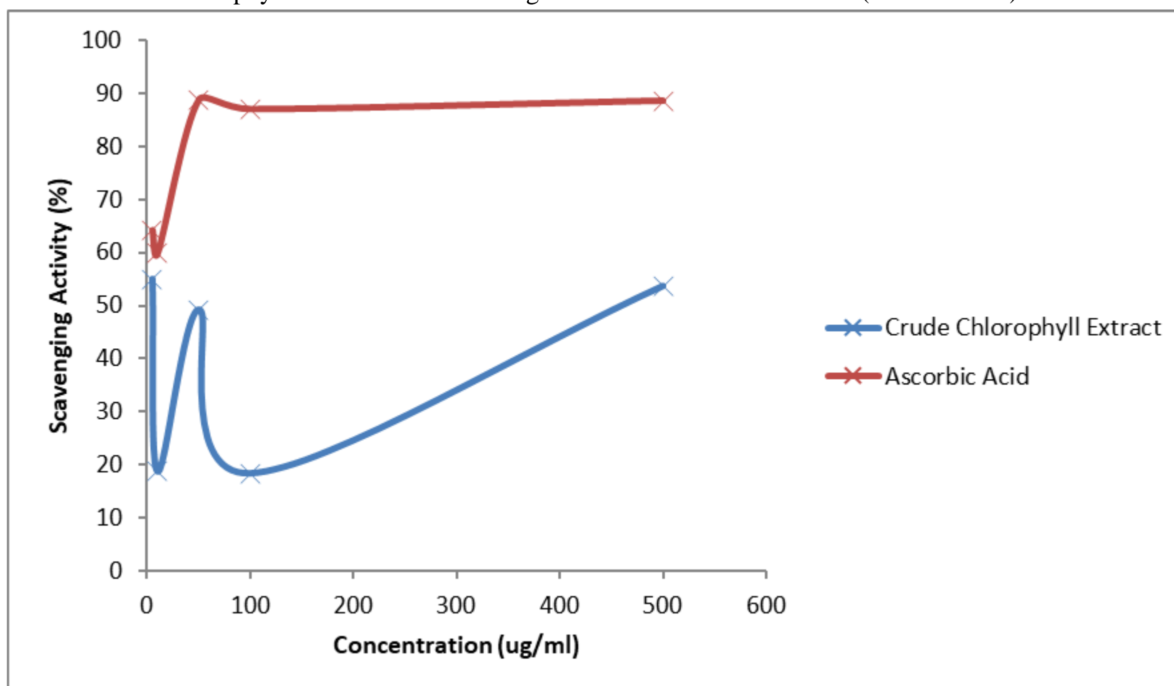


Figure 1: Scavenging Ability of Crude Chlorophyll Extract and Ascorbic acid on DPPH at Different Concentrations

Table 6: Results of Toxicological Study of Different Doses of Carrot Greens Extract Administered Intraperioneally

Groups	Doses (mg/Kg	Mortality (x /N)	Symptoms
Group I	100 mg/kg	0/5	Nil
Group II	500 mg/kg	0/5	Nil
Group III	1000 mg/kg	0/5	Corner sitting
Group IV	1500 mg/kg	3/5	Corner sitting, Drowsy
Group V	2000 mg/kg	4/5	Corner sitting, Drowsy
Group VI	Saline (10 ml /kg)	0/5	Nil.

Table 7: Determination of Median Lethal Dose (LD_{50}) of Carrot Greens

Dose (mg/kg)	Dose difference (D_d)	Death	Mean Death (M_d)	($D_d \times M_d$)
100	0	0	0	0
500	400	0	0	0
1000	500	0	0	0
1500	500	3	1.5	750
2000	500	4	3.5	1750
Sum (Σ)				= 2500

From the values in table 4, applying the formula shown above, the LD_{50} could be calculated as follows;

$LD_{50} = 2000 – (2500/5)$

$= 2000 -500$

$= 1500$ mg/kg.

The arithmetic method of Karber [15] as modified by Aliu and Nwude [17] showed that the median lethal dose (LD_{50}) of acetone extract of carrot greens (*Daucus carota L*) in albino mice was 1500.00 mg/kg.

3.2 Discussions

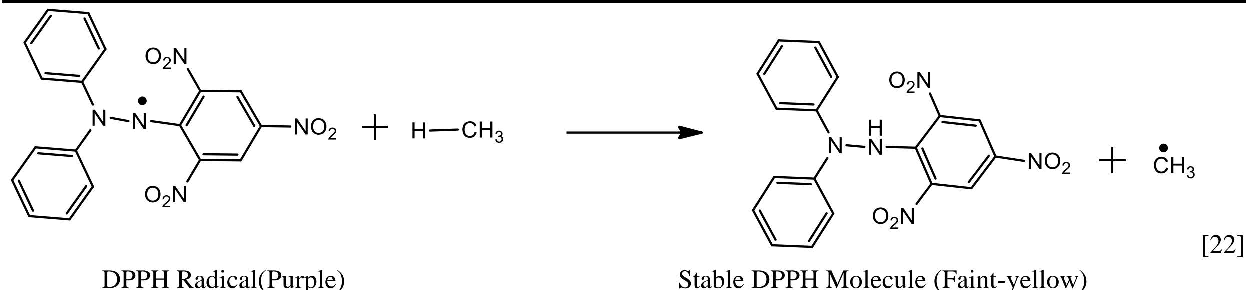
The extraction of 300 g of the powdered greens of carrot gave an extract of weight 8 g. The percentage yield of the extract was calculated to be 2.67 (see Table 1).

The pigment level of carotenoids as shown in Table 3 was determined to be highest (971.29 µg/mL) in dried carrot greens which is almost 32 times higher than the amount found in *Cladostephus verticillatus* algae extracted with acetone solvent as reported by [19]. Consuming foods containing natural pigments rich in carotenoid content have been reported to be associated with decreased risk for a number of diseases including cancers, cardiovascular diseases, age-related macular degeneration, and photosensitivity associated with UV exposure [20]. Carotenoids are thought to have good provitamin A activity, antioxidant activity, ability to regulate gene transcription, enhancement of gap junction communication, phase II-enzyme inducing activity [20]. A dried carrot green is found to content a very high amount of carotenoid (971.29 µg/mL) which suggests its use as good additive in food and beverages.

Concentration levels of Chlorophyll a and chlorophyll b in carrot greens (14.85 and 16.72 µg/mL) respectively are low compared to that found by [19] in *Codium tomentosum* algae as 49.7 and 21.6 µg/mL respectively. Chlorophyll in *Codium tomemtosum* algae was determined fresh whereas that of carrot greens was determined in dried leaves, this and other factors such as method of extraction resulted in the lower content of chlorophyll a and chlorophyll b obtained in carrot greens extract found in this experiment.

The result of the TLC analysis of the crude chlorophyll extract is shown in Table 4. The result showed five spots. Spots 1, 2, 4 and 5 with Rf values, 0.17, 0.25, 0.67 and 0.82 respectively. They showed orange-red colours in UV light which were the colours of chlorophyll pigments reported in the literature [21] Spot 3 (Rf value of 1.50) showed bright-green colour. This gives information of the number and identity of the pigments present in the dried greens of the root vegetable. Chlorophyll pigments naturally absorbs blue and red wavelengths of light, thus emitting a green colour under natural light, when in UV, electrons are promoted to excited state, pigment the absorbs only blue-violet orange wavelengths and the electrons will fluoresce and red colour is observed [21].

Stable DPPH radicals are being used to evaluate the antioxidant activities of many substances base on their hydrogen donating abilities. The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical gains electron or hydrogen radical and be converted to 2, 2-Diphenyl-1-picrylhydrazine to form stable molecule indicated by rapid change of its purple colour to faint-yellow. The equation of this reaction of radical scavenging ability of DPPH is shown:



[22]

DPPH Radical(Purple) Stable DPPH Molecule (Faint-yellow)

Highest scavenging ability on DPPH radical of 55.02% was recorded for the lowest extract concentration at 5 μg/mL (Table 5). When concentration of the extract was increased to 10 μg/mL, scavenging ability dropped to 18.74%. The extract revealed only 53.73% scavenging activity at highest concentration, 500 μg/mL. Trend of scavenging ability of both crude chlorophyll extract and the standard (ascorbic acid) were not concentration dependent (Figure I). The crude chlorophyll extract of the dried carrot greens can be said to possess relatively low antioxidant activity, however, scavenging ability on DPPH radical of standard ascorbic acid was higher than the crude chlorophyll extract in all the concentrations. Statistical analysis showed that the difference between the treatments is significant at 95% confidence limit. Comparing to antioxidant activity for fresh dehydrated carrot leaves by DPPH at three stages of development 40, 80 and 100 days was determined by [23], the result obtained were 73.35, 67.78, and 63.78 ug/mL respectively. In contrast, the low antioxidant activity in the dried carrot greens is attributed to stressful environmental conditions, such as temperature, humidity, UV light irradiation, air pollution, and other factors that are unfavourable during the drying periods. These invisible signs of injuries may have altered the antioxidant system [23].

The result of toxicology study by lethal median dose (LD_{50}) of the crude chlorophyll extract is presented in Tables 6 and 7. None of the animals in Group I, II and VI showed any clinical or behavioral changes throughout the observation period. However, depression, weakness and loss of appetite in the first 5hr were observed in Groups III, IV, and V animals that were treated with the higher doses of the extract. All animals in Groups, II and VI were active all through the study. In Group III the animals suffered depression, became drowsy and exhibited corner sitting but none died. Animals in group IV and V became weak, drowsy and exhibited corner sitting and some (3 and 4, respectively) eventually died within 48 hours (Table 6). It is also worth noting here that there were also no further records of death for those who survived 48 hours, even after observing for 5 more days.

The result of the LD_{50} reveals that the LD_{50} of the crude extract of carrot greens (*Daucus carota L)* is 1500 mg/kg. This implied that the extract is slightly toxic; therefore, if it is to be administered, it has to be in low dosage. If the extract LD_{50} values less than 300mg/kg is considered highly toxic. According to [24], as cited by [15], LD_{50} values between (0.0 – 50) mg/kg are highly toxic, (50 – 500) mg/kg are moderately toxic, (500 – 5000) mg/kg are slightly toxic, while 5000 mg/kg and above are non-toxic. However, it has been suggested that substances with intraperitoneal LD_{50} of above 1000 mg/kg should be regarded as safe [25, 26, 27].

The above imply that the extract could even be safer, if administered orally [28] as cited by [29].

The LD_{50} studies of the green pigments of carrot greens (*Daucus carota L*) showed that the extract is safe for use as drug or food supplement.

4. Conclusion

The result of this work shows that carrot greens contain carotenoid, chlorophyll a and chlorophyll b. carotenoids, chlorophyll a and b are good provitamin A, known for reducing risk of disease such as cancers, cardiovascular, age-related macular degeneration and photo-sensitivity associated with UV exposure. Similarly, it can be concluded that the chlorophyll extract from dried carrot greens possess antioxidant activity and found to be safe being for use as food supplement, can be used as a source of natural antioxidants for medicinal purposes/or starting material for compounding a drug.

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