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Research Article

Amino acid, Antioxidant and Ion Profiles of *Carpolobia lutea* Leaf (Polygalaceae)

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Abstract

Purpose: *To evaluate the amino acid, antioxidant and ionic profiles of Carpolobia lutea leaf (Polygalaceae) extract (CLL).*
Methods: *The powdered leaf was macerated and subjected to gradient solvent extraction with n-hexane, chloroform, ethyl acetate and ethanol for 72 h to obtain their respective fractions. Amino acid analysis was by cation-exchange chromatography using automated amino acid analyser. Antioxidant potential was obtained by spectrophotometric assay using 2, 2-Diphenyl-1-picrylhydrazyl DPPH while elemental and ionic analyses were carried out by atomic absorption spectrophotometry and potentiometric titration, respectively.*
Results: *Proline, alanine, serine, valine, glycine, glutamate and lysine were found in the ethanol fraction while lysine, phenyl alanine, glycine and serine were present in the ethyl acetate fraction but not in the non-polar fractions, n-hexane and chloroform. The ethyl acetate fraction contained more lysine, phenyl alanine, glycine and serine the other leaf fractions. Minimal radical scavenging activity of all the fractions was recorded. The most abundant cations in the extract were potassium and phosphorus (2.16 ± 0.05 and 1.90 ± 0.06 mg/g, respectively) while the most abundant anion was phosphate with a concentration of 23.23 ± 4.61 mg/g for the aqueous leaf fraction*
Conclusion: *The study shows that CLL fractions contain variety of amino acids which could promote wound healing, as well as major and minor elemental ions which, as essential body electrolytes, are required for various metabolic processes in the body.*

Keywords: *Carpolobia leaf, Polygalaceae, Amino acid, Antioxidant*, *Ionic profile.*

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INTRODUCTION

Plant exudates and organ extracts often contain amino acids (AA) and ions as solutes [1]. Certain amino acids and ionic contents of plant are known to influence pharmacological activity. Amino acids are required for adequate collagen synthesis which is fundamental for wound healing, and collagen synthesis depends on the local availability of amino acid pool found in the organism [2]. Healing of dermal lesions require synthesis of newly formed collagen at different times.

Antioxidants, such as polyphenols are secondary metabolites; they are ubiquitous in plants and therefore abundant in human diet. They have been shown to exert beneficial influence on human health [3]. Most phenolic compounds are potent antioxidants, and epidemiological studies have suggested a direct correlation between their high intake in diet and reduced risk of coronary heart disease and mortality by suppressing the oxidation of low-density lipoprotein [4]. Free-radical-mediated cell injury and lipid peroxidation in various pathological phenomena have been reported [5].

Trace quantities of certain elements such as copper, zinc, cobalt and others may play an important role in the functioning of various enzymes present in biological systems [6]. Physiological active substances (PAS) and elements accumulated at high doses by medicinal plants potentiate the effects of each other and this, in turn, enhances their pharmacological activities [7].

Carpolobia lutea leaf (CLL) is used traditionally among the Efik, Ibibio and Yoruba ethnic groups in Nigeria for wound healing and in the management of diarrheal fever, headache, leprosy, snake bite and venereal diseases [8]. Reported pharmacological investigations on the activities of the material include anti-inflammatory and anti-arthritis [9], antiulcerogenic and antidiarrhoeal [10] anti-hemorrhoid property [11], gastroprotective [12]; antinociceptive [13]; antidiarrhoeal mechanism [14] and antimicrobial [15]. Two new cinnamoyl 1-deoxy-glucosides and cinnamic acid have been isolated by semi-preparative HPLC and the structures established by NMR [13].

In this study, we sought to evaluate the amino acid, ionic content and antioxidant profiles of solvent/fractions of CLL.

EXPERIMENTAL

Plant material

CLL was collected from the wild in Itak Ikot Akap-Ikono LGA, Akwa Ibom State, Nigeria in the month of October 2006 by Mr Okon Etefia, a traditional herbalist, affiliated to the Pharmacognosy Department of University of Uyo. The plant was authenticated by Dr Margaret Bassey of Botany Department, University of Uyo, Nigeria. A voucher specimen (UUH 998) was deposited at the herbarium of the University of Uyo. The CLL material was shade-dried for 4 days, powdered in a mortar and stored in air-tight container at room temperature.

Extraction, fractionation and HPLC analysis

The procedure for gradient solvent extraction described in a previous article [16] and fractionation method described by Nwidu *et al* [13] were adopted. The powdered leaf was macerated and subjected to gradient solvent extraction with *n*-hexane, chloroform, ethyl acetate and ethanol for 72 h to obtain their respective fractions. The volume of the liquid extract was reduced in a rotary evaporator as described previously [16]. Fractionation was carried out by thin layer chromatography using (silica gel plates on glass, 20 × 20 cm × 0.25 mm, Fluka) eluted with: $CHCl_3$/MeOH/H_2O (43:37:20, v/v/v), $CHCl_3$/ MeOH/H_2O (80:18:2, v/v/v), $CHCl_3$/MeOH/H_2O/acetic acid (43:37:30:1, v/v/v/v), and $CHCl_3$/MeOH/*n*-PrOH/H_2O (310:380:60:250, v/v/v/). An aliquot (4.0 g) of the ethyl acetate fraction (EAF) was dissolved in 10 ml methanol and

centrifuged at 2,500 g for 15 min. This procedure was performed three times. The supernatant was filtered and submitted to gel permeation chromatography using Sephadex LH-20 (Pharmacia) in a glass column (700 × 35 mm, i.d.) and methanol (98.98 %) as described previously [13].

Quantification of amino acids

Amino acid determination was carried out before and after hydrolysis of the extract. The extract (5 mg) was added to a mixture containing 6 mol L^{-1} of HCl (1 ml) and 5 % phenol/water (0.08 ml) and heated in a Pyrex tube with plastic Teflon-coated screw caps (13 × 1 cm) for 72 h at 110 ˚C to ensure complete hydrolysis of the peptide bonds. The hydrolysed sample (5 mg) was dried in an oven at 70 ˚C, diluted with 1.0 ml of sodium citrate buffer pH 2.2 and filtered through a GV Millex Unity filter (Millipore). Analysis for amino acids was performed by cation-exchange chromatography using an automated amino acid analyser, Shimadzu LC-10A/C47A. Sodium was used as eluents and post-column derivatisation achieved with o-phthaldialdehyde (OPA). Identification and quantification of the amino acids were carried out by comparison of retention time and area obtained by each amino acid with 16 amino acid standards (100 $nmoL^{-1}$), respectively. The results were expressed in µM/ml extract/fraction.

Antioxidant activity with DPPH

Antioxidant potential was evaluated spectrophotometrically. A solution of 0.004 % DPPH in methanol (99.8 %) was prepared. A stock solution of the extract was prepared from 2.5 mg of the extract in 1ml of 20 % Tween 80 and added to 9 mL methanol to give a stock solution of 250 µg/ml. A range of dilutions were made by adjusting 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mL of this stock solution to 5 ml in a volumetric flask to give stock solutions with concentrations of 5, 10, 20, 40, 80 and 160 µg/ml, respectively. One ml of each concentration of extract was added 2.0 mL of DPPH solution in a test tube and allowed to react for 30 min prior to spectrophotmetric (Hach Spectrophotometer, Japan) determination at 517 nm. The reference solution was prepared by dissolving 4 mg of DPPH in 20 mL of 20 % Tween 80 which was then made up to 200 mL. Reduction of DPPH radical was determined at 517 nm using the method of Abe *et al* [16]. Inhibition of the DPPH radical (RSC) by the samples was calculated as in Eq 1.

$$RSC = \{(Ac - Ax)/Ac\} \times 100 \quad \ldots\ldots\ldots\ldots\ldots(1)$$

where Ac is absorbance of the control and Ax is absorbance of the sample after 30 min of incubation. The values obtained were plotted on a graph of % change in absorbance versus concentration of samples.

Elemental analysis of the plant leaf

The extract (1 g) was digested by repeated treatment with boiling hydrochloric acid:nitric acid mixture (3:1) [17]. The solution was evaporated to dryness, the residue extracted with dilute hydrochloric acid and then filtered. The filtrate was air-dried and the residue analysed for K, Na, Ca, Zn and other elements by inductively coupled argon-plasma emission spectrometry on a Thermo Jarell Ash model "Trace Analyzer" (TJA Solution, Franklin, USA) with axial plasma. Generalized shifts in background emission were determined at off-peak wavelength positions, and deducted from simultaneous measurements of intensities at on-peak wavelength for each element. Sample and standard solution matrices were matched.

Determination of ionic content of plant leaf

This determination was carried out by potentiometric titration. The pH meter (Brinkmann 632, Switzerland) was standardized by standard buffer solutions and a calibration curve drawn. For the assessment of anions, this was carried out in triplicate.

Approximately 0.2 - 0.3 g of the powdered leaf was soaked in 10 ml of water for 1 h and then filtered; the filtrate was made up to volume in a 50 ml volumetric flask. An analytical curve was prepared from the stock solution by successive dilution to give a range of concentrations (1×10^{-2} - 1×10^{-4} moL^{-1}). The various ions were estimated by plotting log concentration ion against the potential difference (E, mV).

RESULTS

Amino acid composition

The amino acid composition of the different fractions of CLL - ethanol, ethyl acetate extract and ethanol, ethyl acetate, chloroform and n-hexane - are shown in Table 1. All the fractions contain essential amino acids. A high level of proline, alanine, serine, valine, glycine, glutamate and lysine was observed in the ethanol fraction while ethyl acetate fraction contained higher levels of lysine, phenyl alanine, glycine and serine than the other fractions.

Antioxidant activity

The antioxidant activity of the CLL fractions are shown in Table 2. The results show minimal radical scavenging activity of all fractions of CLL when compared to standard compounds.

Elemental and anion profile of extract

The results of the analysis of the plant leaf (Table 3) show that it contains significant amounts of cations which ranged from 0.01 ± 0.003 mg/g (for copper) to 2.12 ± 0.05 (for potassium). Heavy metal ion content (lead and mercury) were < 0.004.

Anion contents of the plant leaf are shown in Table 4. PO_4^{2-}, SO_4^{2-}, CL^-, F^-, and NO^{3-} are shown in Table 4. The results indicate that the aqueous leaf extract contains phosphate (23.23 ± 4.61) sulphate (12.4 ± 0.62), chloride (0.33 ± 0.25), nitrate (1.35 ± 46.4) and fluoride (< 0.2) mg/g of leaf extract. The most abundant anions are phosphate and sulphate.

Table 1: Amino acid profile of *Carpolobia lutea* leaf extracts/fractions

	Amino acid (nmol.mg)					
Amino acid	***ETF***	***CHF***	***EAF***	***n-HF***	***CEAE***	***CETE***
Proline	112.5	17.1	10.1	2.9	5.5	8.1
Aspartate	20.6	7.7	3.2	4.0	4.4	6.3
Threonine	7.9	5.2	1.8	2.0	1.9	5.5
Serine	11.7	5.7	6.1	4.7	8.0	26.0
Glutamate	22.3	7.8	6.3	4.0	6.4	7.0
Glycine	24.6	13.9	8.1	8.1	5.5	19.4
Alanine	15.5	9.9	5.3	0.0	5.4	9.6
Cystine	3.9	0.0	0.0	0.0	0.0	0.0
Valine	16.9	8.9	2.5	2.2	3.3	8.3
Methionine	6.3	0.2	0.9	0.2	1.0	1.9
Isoleucine	11.9	6.7	2.7	2.0	3.1	5.7
Leucine	14.0	12.1	3.6	3.5	4.3	9.8
Tyrosine	14.0	2.3	0.0	1.2	0.0	4.0
Phenyl alanine	7.4	6.8	11.5	2.3	11.5	14.9
Histidine	0.6	0.8	0.5	0.6	0.5	1.7
Lysine	39.4	5.3	47.7	0.9	5.5	9.6
Arginine	2.9	2.9	0.7	0.0	0.8	1.4
Total proteins (g/100g)	0.72	0.27	0.16	0.1	0.18	0.34

n-HF = n-hexane fraction, EAF = ethyl acetate fraction, ETF = ethanol fraction, CHF = chloroform, fraction, CETE = crude ethanol extract, CEAE = crude ethyl acetate extract

Table 2: Antioxidant activity (DPPH) of *Carpolobia lutea* leaf fractions

	Antioxidant activity (%)					
Fraction	**5 µg/mL**	**10 µg/mL**	**20 µg/mL**	**40 µg/mL**	**80 µg/mL**	**160 µg/mL**
n-HF	3.7 %	6.0 %	5.9 %	5.7 %	6.0 %	7.7 %
CHF	12.9 %	8.3 %	5.0%	5.0 %	7.3 %	7.6 %
EAF	2.6 %	2.8 %	4.6 %	4.4%	6.0 %	10.4%
ETF	3.2 %	4.4 %	4.4 %	5.6 %	11.6 %	15.6 %
CEAE	5.5 %	4.3 %	3.9 %	4.6 %	5.7 %	20.1 %
CETE	7.4 %	6.5 %	13.3 %	8.4 %	8.5 %	9.2 %
CA	62.0 %	90.0 %	91.0 %	93.7 %	93.7 %	93.9 %
FA	25.9 %	0.3 %	62.9 %	85.2 %	90.5 %	92.5 %
GA	74.8 %	93.4 %	93.8 %	93.8 %	93.9 %	93.9 %
TCA	5.9 %	3.5 %	7.2 %	6.3 %	6.5 %	13.5 %

Key: *nHF = n-hexane fraction, CHF = chloroform fraction, EAF = ethyl acetate fraction, ethanol fraction, crude ethyl acetate extract, CA = cinnamic acids, FA = ferullic acid, gallic acids, TCA= transcinnamic acids*

Table 3: Cation content (mg/g) of *Carpolobia lutea* aqueous leaf extract

Cation content (mg/g)										
Na	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Cu**	**Hg**	**Pb**	**P**	**Zn**
0.494 ±0.020	2.12 ±0.05	0.51 ±0.01	0.15 ±0.004	0.24 ±0.00	0.35 ±0.008	0.011 ±0.00	<0.00	<0.00	1.90 ±0.06	0.020 ±0.00

Table 4: Anion content (mg/L) / $_{P}$H of *Carpolobia lutea* aqueous aqueous extract

Anion content (mg/g) extract				
PO_4^{2-}	SO_4^{2-}	Cl^-	F^-	NO_3^-
23.2 ±4.6	12.4 ±0.6	0.3 ±0.3	<0.2	1.3 ±46.4

DISCUSSION

Determination of amino acid constituents of the leaf fractions revealed that it has both essential and non-essential amino acids with ethanol fraction having the highest amount of protein per gram. Investigation of the effects of a new wound dressing called vulnamins that contains four essential amino acids (glycine, L-lysine, L-proline and L-leucine) for collagen and elastin synthesis in aged rats has been reported to induce wound closure and tissue regeneration [2]. Cassino and Ricci [19] showed that vulnamins induces rapid tissue regeneration and wound closure in chronic human skin lesions of various etiologies, and concluded that topical treatment may open a new frontier in the treatment of patients with chronic ulcers.

The amino acid profile of the various fractions of CLL indicates the presence, in varying proportions, of these four essential amino acids - glycine, L-lysine, L-proline and L-leucine. Arginine, glutamine and proline are the immediate precursors for polyamine synthesis, which is essential to proliferation, differentiation and repair of intestinal epithelial cells [20]. This may account for the gastroprotective and antiulcer effects of CLL [9,12]. Glutamate, glycine and cysteine are precursors for the synthesis of glutathione, a tripeptide critical for defending the intestinal mucosa against toxic and peroxidative damage [20] which may play a role in the moderate acute, subacute and subchronic toxicity profile of CLL [21]. The fractions investigated were reported as effective antiulcer agents [9,12]. The same amino acids, which were also present in the fractions in our study, may exert antinociceptive effect as reported in a previous work [13]. Besides, its poor anti-helicobacter activity [15] can be attributed to the ability of *H. pylori* to grow by utilizing amino acids as biosynthetic precursors [22].

Ionic analysis reveals the presence of both major and trace elements. Potassium and phosphorus ions predominate. Phosphate and sulphate were the most abundant anions. The presence of trace quantities of certain elements such as copper, zinc and cobalt may play an important role in the functioning of various enzymes (e.g., copper is incorporated into metalloenzymes involved in hemoglobin formation, drug/xenobiotic metabolism, carbohydrate metabolism, catecholamine biosynthesis and cross-linking of collagen, elastin, and hair keratin as well as in the antioxidant defense mechanism [23]; copper-dependent enzymes, such as cytochrome c oxidase, superoxide dismutase, ferroxidases, monoamine oxidase, and dopamine *β*-monooxygenase, function to reduce reactive oxygen species (ROS) or molecular oxygen [23]; ceruloplasmin, a copper-containing glycoprotein produced in the liver binds about 95% of the copper in serum, exhibiting ferroxidase activity and catalyzes the conversion of ferrous to ferric iron which is then transferred, the absence of which could lead to ferrous iron abundance within both the reticuloendothelial system and parenchymal cells [23]; dopamine-*β*-hydroxylase (DBH), a cuproenzyme, plays a role in the production of noradrenaline, in copper deficiency, as DBH activity may be lower, inducing higher ratios of dopamine to norepinephrine [23]; peptidylglycine *α*-amidating monooxygenase and lysyl oxidase are both cuproenzymes whose activity or gene expression may be influenced by copper deficiency [23]; zinc (II) and copper (II) ions are the second and third most abundant transition metals in humans, and they are found either at the active sites or as structural components of a good number of enzymes [24]; the main biochemical role of cobalt in biological systems is its involvement in vitamin B_{12}, a co-enzyme in some biochemical processes [24]; these metals and some of their complexes have been found to exhibit antimicrobial activities [24] and the antimicrobial activity of CLL [15] could be attributed to it in biological systems [6].

Metals which are most often concentrated by medicinal plants that synthesize and accumulate PAS of various types (alkaloids, phenolic compounds, terpenoids, etc) play the role of cofactors or activators of enzymes. Such metals (Co, Zn, Fe, Cu, Mn, and Cr) display their activity at various molecular levels. Moreover, microelements present in medicinal plants can enhance the therapeutic effect of major components. Physiological active substances (PAS) and elements accumulated in high concentrations by medicinal plants potentiate the effect of each other, which enhance their pharmacological activities [7].

Soluble mineral salts widely used medicines contain microelements. However, these compounds are characterized by low assimilability after peroral administration (not more than 3 – 10 %). Therapeutic doses of soluble mineral salts are ten times higher than their biotic concentrations, which can lead to overdosing and delayed side effects associated with individual differences in the assimilability of individual elements or groups of elements. The use of medicinal plants as the source of microelements aids to prevent these adverse consequences [7].

CONCLUSION

This study shows that the leaf extract of *Carpolobia lutea* is a natural source of ingredients that could aid the management of gastric ulcers, hemorrhoid, wound healing and diarrhea. This work gives some credence to some of the ethnomedicinal uses of the plant. The pharmacological evaluation of the wound-healing potential of this plant is ongoing in our laboratory.

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