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Proximate analysis, total phenolic content, acute toxicity, anti-oxidative and anti-inflammatory studies of *Vigna unguiculata* and *Citrus limon* using Wister rat models

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Abstract

Herbal medicines have been in use for thousands of years. It is estimated that 80% of world population rely on traditional herbal medicine for prophylaxis and therapy of different ailments. In recent years, there has been an upsurge in the use of these herbal remedies due to their cultural acceptability, availability, affordability, efficacy and safety claims. This study carried out the proximate analysis, total phenolic and Citric acid contents, acute toxicity, anti-oxidative and anti-inflammatory studies of *Vigna unguiculata* and *Citrus limon* using Wister rat models. Wister rats (135 – 140 g) were used for this study. *Vigna unguiculata* was extracted using ethanol for 72 hours. Proximate analysis, Citric acid content of *Citrus limon*, total phenolic content, acute toxicities, anti-oxidative and anti-inflammatory studies were done. The yield of the extract was 1.9%. The LD50 was greater than 5,000 mg/kg body weight. The Citric acid content of *Citrus limon* was 54.2 $\pm$ 0.003 mg/ml. The proximate analysis showed the presence of protein, fat, carbohydrate, dietary fibre, moisture, and iron while total phenolic contents of *Vigna unguiculata* and *Citrus limon* were 15.3 and 19.3 mgGAE/L respectively. The combination of *Vigna unguiculata* and *Citrus limon* exhibited greatest anti-oxidation effect and recorded malondialdehyde concentration of 3.66 ± 0.34 nmol/ml as compared to the individual herbs that recorded 6.14 ± 0.38 and 4.28 ± 0.12 nmol/ml respectively. In anti-inflammatory assay, *Vigna unguiculata* had the best percentage inhibition of heat induced hemolysis of 62.79%. In conclusion, *Vigna unguiculata* and *Citrus limon* had synergistic antioxidant and antagonistic anti-inflammatory activities.

Keywords: Anti-inflammatory; Antioxidant; *Citrus limon;* Total phenolic content; *Vigna unguiculata*

1. Introduction

Traditional herbal medicines have been in use for thousands of years. It is estimated that 80% of world population rely on traditional herbal medicine for prophylaxis and therapy of different ailments. In recent years, there has been an upsurge in the use of these herbal remedies. The increase in the use of herbal products is attributed to their cultural acceptability, availability, affordability, efficacy and safety claims [1]. The effectiveness of herbal medicines stems from their numerous phytochemicals which exhibit synergistic actions such as anti-inflammatory, antiviral, antibacterial, anti-protozoa, and antioxidant. Due to their cost effectiveness, these medicines are in great demand for primary health care. A certain study reported the effective use of Indian medicinal plants for the treatment of various types of infectious diseases ranging from COVID 19, gastrointestinal infection, viral diseases, and skin and wound infections among others [2]. With respect to their safety, herbal medicines are not completely free from the possibility of toxicity or adverse effects but they are widely considered to be of lower risk compared with synthetic drugs [3].

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Proximate analysis is usually done to estimate the quantitative components of food and food substance including moisture, crude protein, total fat, and total carbohydrate, and dietary fiber, minerals among others. In a study that determined the proximate analysis and total phenolic content of *Dracaena reflexa,* the researchers reported moisture content (3.31%), ash content (8.02%), crude fibre (1.31%), crude fat (0.97%), total protein (3.70%), total carbohydrate (86.01) and nutritive value (367.56 kcal/100 g), which would make it a potential nutraceutical [4].

Total Phenolic Content (TPC) of herbal extracts are conducted to elucidate the amount of phenolic content in the samples. Phenolic compounds are renowned for their antioxidant potentials due to their redox properties. However, these phenolic contents can depend on the solvent used for the extraction process. In a certain study that evaluated the different solvent extracts of *Pereskia bleo* leaves for total phenolic content (TPC) based on the Folin–Ciocalteu test, the methanolic extract exhibited highest TPC compared to the chloroform and hexane extracts. This result suggested that the methanolic crude extract of *P. bleo* might possess best antioxidant potentials when compared with the other tested extracts [5].

Natural antioxidants notably polyphenols and carotenoids are widely distributed in food and medicinal plants and are well known to exhibit a wide range of biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis and anticancer. In the study that assessed the antioxidant activity at chemical and cellular based levels and their main resources from food and medicinal plants, assessment of the antioxidant activities of plant materials were done with several assay methods such as the determination of total polyphenolic content by FCR, scavenging free radical ability by TEAC, metal-reducing activity by FRAP, and a kind of cellular-based assay [6]. In a more recent study, the increasing interest in medicinal plants, due to their content of health-promoting compounds, such as caffeic acids derivatives was reported. The researchers therefore set to study the antioxidant activity of extracts obtained from the following medicinal plants: caraway (*Carum carvi* L.), coltsfoot (*Tussilago farfara* L.), dandelion (*Taraxacum officinale* F.H.Wigg.), lovage (*Levisticum officinale* L.), tarragon (*Artemisia dracunculus* L.) and white mulberry (*Morus alba* L.), characterized by their high content of caffeic acid derivatives. The water-ethanolic extracts were characterized on average by about nine times higher contents of caffeic acid derivatives level than water extracts. The researchers stated that there was a positive correlation between caffeic acid derivatives and antioxidant activity sequel to radical cation scavenging activity (ABTS) and radical scavenging activity (DPPH)) assays, especially in water-ethanolic extract of medicinal plants [7].

On the other hand, anti=inflammatory properties are conferred to plants by virtue of the phytochemical compounds contained in them. A study carried out with the species *Myracroduo nurundeuva* Allemão, *Schinus terebinthifolius* Raddi, *Spondias mombin* L., *Spondias purpurea* L. and *Spondias tuberosa* Arruda, belonging to the Anacardiaceae family, detected the presence of several secondary metabolites. The most abundant were phenols, triterpenes, flavonoids, and cinnamic acid, which were responsible for their anti-inflammatory action. Furthermore, the plants that make up the Euphorbiaceae family, such as the species *Euphorbiaceae acalypha* hispida Burm. f., *Acalypha indica* L., *Phyllanthus niruri* L., were found to be rich mainly in phenolic compounds, saponins, tannins, and triterpenes, which were responsible for their anti-inflammatory action. Researches with other plant species including *Ruellia asperula* (Mart. Ex Ness) Lindau (family Acanthaceae), Achyranthes *aspera* L., *Zingiber officinale Roscoe* (family Zingiberaceae) among others also indicated the existence of compounds with anti-inflammatory activities [8]. This present study therefore demonstrated the proximate analysis, total phenolic contents, acute toxicity, antioxidant and anti-inflammatory studies of *Vigna unguiculata* and *Citrus limon* using Wister rat models.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Wister rats (135 – 140 g) were used for this study. All the animals were obtained from the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria. The animals were housed in standard laboratory conditions of 12 hours light, room temperature, and 40 - 60% relative humidity and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to food and water. Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123 were strictly adhered to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology. There was additional approval by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes.

2.2. Chemicals and Reagents

Wash Buffer Concentrate (Sigma Aldrich Germany), Assay Buffer (Alpco USA), TMB Substrate (Cayman Chemical USA), Stop Solution (Cayman Chemical USA), Hydrochloric acid (Prime laboratories, India); Dragendoff reagent (Sigma Aldrich, United States of America); Ammonia (Shackti Industrial Gases, India), sodium hydroxide (Treveni Chemical Pvt., India); Ferric chloride (AkashPurochem. Pvt., India); Fehling's solution (Lab care Diagnostics, India); Million reagent (Interlab Chemical Pvt., India): Ethanol (TAJ Pharmaceutical Ltd., India); Acetic anhydride (Ashok Organics Industries, India); Concentrated sulfuric acid (Navin Chemical Pvt., India), Acetic acid (Kayla Africa Suppliers, South Africa); Molisch reagent (Interlab Chemical Pvt., India); alcoholic alpha naphatol (Prat Industry Corcopation, India).

2.3. Equipment

Glass column, flasks, beakers, test tubes, measuring cylinders, surgical blade, forceps, scissors, graph paper, white transparent paper, rotary evaporator, Analytical Weighing Balance (Metler H30, Switzerland), Electric Oven (Gallenkamp, England), Spectrophotometer (B. Bran Scientific &Instrument Company, England), Water Bath (Techmel & Techmel, Texas, USA), National Blender (Japan), Micropippete (Finnipipette® Labsystems, Finland), Plethysmometer (Biodevices, New Delhi, India) and Intubation tubes. Precision pipettes (25, 50, 100 and 300 µl, 1,000 µL) (Labcompare USA); Disposable pipette tips (Labcompare USA); Distilled or deionized water (SnowPure Water Technologies USA); Plate shaker (Biocompare USA); Microwell plate reader (BioTek India); Centrifuge (Sharplex Filters Pvt., India); Vortex mixer (Bionics Scientific Technologies (P) LTD, India); Graduated cylinder for 500 ml (Boenmed Healthcare Co. Ltd, Hong Kong); Stop watch (Avi Scientific India); EDTA containers (Sure Care Corporation), heparinized capillary tube (Thomas Scientific, USA), disposable hand gloves (Supermax Malaysia), toilet tissue.

2.4. Drugs

Iron supplement was purchased from a Pharmacy shop in Enugu state of Nigeria.

2.5. Plant materials

Vigna unguiculata and *Citrus limon* were procured from a market in Enugu state.

2.6. Extraction

Vigna unguiculata 1000 g seed powder was weighed using a weighing balance (Camry EK5350 Model, China) and extracted using cold maceration in ethanol for 72 hours with intermittent shaking. The resulting solution was filtered using Whatman filter paper and the filtrate concentrated to dryness *in vacuo* using rotary evaporator (RE300 Model, United Kingdom) at 40 °C. The extract was stored in refrigerator between 0-4 °C.

2.7. Methods

2.7.1. Determination of citric acid content of the Citrus limon juice

Ripped *Citrus limon* fruits were purchased from Ogbete Main Market Enugu State, Nigeria. The fruits were washed with clean tap water and rinsed with distilled water. The fruits were extracted using electronic juice extractor. The juice was filtered using vacuum funnel and the filtrate subjected to the analysis. Titration method was applied using sodium hydroxide as base to titrate the Citric acid in the lemon juice. Phenolphthalein was used as the indicator. The unknown concentration of Citric acid in the lemon juice (25 ml) was titrated with 0.1 M of NaOH with constant swirling until a permanent appearance of pink color was observed in the solution. The volume required to achieve this was recorded from the biuret as endpoint and subtracted from the starting volume to determine the actual volume of titrant. This procedure was repeated three times and the mean volume of titrant calculated. The amount/concentration of Citric acid in the juice was calculated using the following equation:

$$n = CV$$

where n is the amount of Citric acid in moles converted to mass using molar mass of Citric acid (192) and C is the concentration of the sodium hydroxide (0.1 M), V is the mean volume of titrant used to neutralize the Citric acid in the juice. Result was presented in mg/ml

2.7.2. Proximate analysis

Proximate analysis was done using standard methods described by Association of Official Analytical Chemists (AOAC 1990) [9].

2.7.3. Moisture Content

A petri-dish was washed and dried in the oven. Approximately 1-2 g of the sample was weighed into the petri dish. The weight of the petri dish containing the sample was noted before drying. The petri-dish and sample were put in the oven and heated at 105 °C for 2 hours; the result noted. They were heated for another 1 hour until a steady result was obtained and the weight was noted. The drying procedure was continued until a constant weight was obtained

% moisture content = (w1-w2 / Weight of sample) x 100

Where w1 = weight of petri-dish and sample before drying

W2 = weigh of petri-dish and sample after drying.

2.7.4. Carbohydrate Determination

100 – (*%Protein + %Moisture + %Ash + %Fat + %Fibre*)

2.7.5. Ash content

The ash of foodstuff is the inorganic residue remaining after the organic matter has been burnt away. It should be noted however that the ash obtained was not necessarily of the composition as there may be some from volatilization. For the determination of ash content, empty platinum crucible was washed, dried and the weight was noted. Approximately 1-2 g of sample was weighed into the platinum crucible and placed in a muffle furnace at 550 °C for 3 hours. The sample was cooled in a desiccator after burning and weighed. The ash content was calculated as follows:

% Ash content = (W3 - W 1 / W2 - W 1) x 100

W1 = weight of empty platinum crucible

W2 = weight of platinum crucible and sample before burning

W3 = weight of platinum and ash.

2.7.6. Crude Fibre

About 2 g of material was defatted with petroleum ether and boil under reflux for 30 minutes with 200 ml of a solution containing 1.25 g of H_2SO_4 per 100 ml of solution. The solution was filtered through linen and washed with boiling water until the washings are no longer acidic. The residue was transferred into a beaker and boiled for 30 minutes with 200 ml of a solution containing 1.25 g of carbonate free NaOH per 100 ml. The final residue was filtered through a thin but close pad of washed and ignited asbestos in a Gooch crucible and dried in an electric oven and weighed. It was **i**ncinerated, cooled and weighed. The lost in weight after incineration x 100 was the percentage of crude fibre.

% crude fibre = (Weight of fibre / Weight of sample) x 100

2.7.7. Crude fat

Soxhlet Fat Extraction Method

This method was carried out by continuously extracting a food with non-polar organic solvent such as petroleum ether for about 1 hour or more. A 250 ml clean boiling flasks was dried in an oven at 105 – 110 °C for about 30 minutes and transferred into a desiccator and allowed to cool. Correspondingly labeled, cooled boiling flasks were weighed. **T**he boiling flasks were filled with about 300 ml of petroleum ether (boiling point 40 – 60 °C). The extraction thimble was plugged lightly with cotton wool. The soxhlet apparatus was assembled and allowed to reflux for about 6 hours. The **t**himble was removed with care and petroleum ether collected in the top container of the set–up and drain into a container for reuse. When flask was almost free of petroleum ether, it was removed and dried at 105 – 110 °C for 1 hour. It was transferred from the oven into a desiccator and allowed to cool; then weighed.

% fat = (wt of flask + oil - wt of flask) / Wt of sample x 100

2.7.8. Crude Proteins

Exactly 0.5 g of sample was weighed into a 30 ml kjehdal flask (gently to prevent the sample from touching the walls of the side of each and then the flasks were stoppered and shaken. Then 0.5 g of the kjedahl catalyst mixture was added. The mixture was heated cautiously in a digestion rack under fire until a clear solution appeared. The clear solution was then allowed to stand for 30 minutes and allowed to cool. After cooling, it was made up to 100 ml with distilled water to avoid caking and then 5 ml was transferred to the kjedahl distillation apparatus, followed by 5 ml of 40% sodium hydroxide. A 100 ml receiver flask containing 5 ml of 2% boric acid and indicator mixture containing 5 drops of bromocresol blue and 1 drop of methylene blue was placed under a condenser of the distillation apparatus so that the tap was about 20 cm inside the solution and distillation commenced immediately until 50 drops gets into the receiver flask, after which it was titrated to pink color using 0.01M hydrochloric acid. Crude protein content was calculated as:

% Nitrogen =Titre value x 0.01 x 14 x 4

% Protein = % Nitrogen x 6.25

2.7.9. Total Phenolic Content by Folin Ciocalteu's Assay

The total phenolic content of the extract and fruit juice were determined using the method described by Kim *et al*., (2003) [10]. One milliliter of the samples (100 µg/ml for V. unguiculata) was mixed with 0.2 ml of Folin-Ciocalteu's phenol reagent. After 5 minutes, 1 ml of 7.6% Na_2CO_3 solution was added to the mixture followed by the addition of 2 ml of distilled water. The mixture (in duplicate) was incubated at 40 °C for 30 minutes, after which the absorbance were read at 760 nm using UV-VIS spectrophotometer (Model 752, China). The total phenolic content was estimated from the calibrated curve which was made by preparing gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extracts.

2.7.10. Methods for the heavy metal (iron analysis)

Iron (Fe) analysis was conducted using Agilent FS240AA Atomic Absorption Spectrophotometer according to the method of APHA 1995 (American Public Health Association) [11]. Atomic absorption spectrometer's working principle was based on the sample being aspirated into the flame and atomized when the AAS's light beam was directed through the flame into the monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wavelength, a source lamp composed of elemental iron was used, making the method relatively free from spectral or radiational interferences. The amount of energy of the characteristic wavelength absorbed in the flame was proportional to the concentration of the element in the sample. A working solution was prepared by dissolving 1000 mg/l, of stock iron solution in a minimum volume of (1+1) HNO3. Diluted to 1 liter with 1% (v/v) HCL, appropriate dilution were carried out to produce 2, 4 and 6 ppm working solution. To digest the sample, a 5 g of the dried sample was weighed and transferred into a digestion flask and 20 ml of the acid mixture (650 ml conc HNO_3; 80 ml perchloric acid; 20 ml conc H_2SO_4) was added. The mixture was heated in a digesting flask until a clear digest was obtained. It was diluted with distilled water to the 50 ml mark.

2.8. Acute toxicity studies (LD50) of co-administration of *Vigna unguiculata* and *Citrus limon*

2.8.1. Acute Toxicity Studies

Acute toxicity analysis of the combined extract of *V. unguiculata* and *C.limon* was performed using Lorke's method (1983) [12]]. This method has two phases (Phase 1 and Phase 2).

PHASE 1: Nine adult albino mice were weighed, marked and randomized into three groups of three mice each. Each group of animals were administered different doses (10, 100 and 1000 mg/kg) of the extract combined with *C. limon* (5 ml/kg). The mice were observed for 24 hours for signs of toxicity as well as mortality.

PHASE 2: Four mice were weighed, marked and randomized into four groups of one mouse each. They received 2000, 3000, 4000 and 5000 mg/kg of the extract combined with *C. limon* (10 ml/kg). Observation for 24 hours for obvious signs of toxicity and death was recorded accordingly. The LD_{50} was calculated using the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

2.8.2. Antioxidant assay (Determination of the Lipid Peroxidation (LPO) in Serum)

The level of thiobarbituric acid reactive substance (TBARS) and malondialdehyde (MDA) production was measured in serum by the modified method as described by Draper and Hadley (1990) [13]. The serum (50 μL) was deproteinized by adding 1 mL of 14% trichloroacetic acid and 1 mL of 0.6% thiobarbituric acid. The mixture was heated in a water bath for 30 minutes to complete the reaction and then cooled on ice for 5 minutes. After centrifugation at 2000 g for 10 minutes, the absorbance of the colored product (TBARS) was measured at 535 nm with a UV spectrophotometer. The concentration of TBARS was calculated using the molar extinction coefficient of malondialdehyde (1.56×10^5 mol/L/cm) using the formula, A = ΣCL, where A = absorbance, Σ = molar coefficient, C = concentration, and L = path length. The results were expressed in nmol/ml.

2.9. In vitro Anti-inflammatory study

2.9.1. Heat-induced hemolysis

Portion of 5 ml of the isotonic buffer containing 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml extract – *V. unguiculata* separately and in combination with *C. limon* (in the ratio of 2:1 v/v) was put into two duplicate sets of centrifuge tubes. The same amount of vehicles was added up in another tube as control. 50 µl of RBC suspension was added to each tube and gently mixed by inverting the test tube. One pair of tubes were incubated at 54 °C temperature for 20 minutes in water bath. Other pair was preserved at temperature 0–5 °C in ice bath. Centrifugation of the mixture was done at 1000 rpm for 5 minutes and the absorbance was taken at 540 nm by using a spectrophotometer. Dexamethasone was used as reference standard.

The percent inhibition of hemolysis was calculated according to the equation:

Inhibition of hemolysis = $100\ x\ (1 - \frac{OD2-OD1}{OD3-OD1})$ (Ranasinghe *et al.*, 2012) [14].

Where, OD1 = Test Sample Unheated; OD2 = Test Sample Heated and OD3 = Control Sample Heated.

3. Results

3.1. Result of extraction

The yield of the extract was 19 g representing about 1.9% yield

3.2. Results of acute toxicity study

No mortality or obvious sign of toxicity was observed at both phases of the acute toxicity of the combined extract and juice. The LD50 is therefore estimated to be above 5000 mg/kg in combination with 10 ml/kg of the juice.

3.3. Result of citric acid content determination of *Citrus limon*

The Citric acid content of *Citrus limon* was 54.2 $\pm$ 0.003 mg/ml

Table 1 Results of proximate analysis and total phenolic content

Proximate analysis (%)	*Vigna unguiculata* (%)	*Citrus limon* (%)
Protein	31.5	1.2
Fat	1.3	0.35
Carbohydrate	35.9	10.4
Dietary fibre	28.4	0.52
Moisture	7.9	87.6
Total phenolic content	15.3 mgGAE/g	193 mgGAE/L

3.4. Results of lipid peroxidation

Table 2 Results of lipid peroxidation; malondialdehyde (MDA)

Groups	Mean MDA concentration ± SEM (nmol/ml)
Normal control (Naive)	3.18 ± 0.10
Anaemia induced control	9.44 ± 0.48
Vigna unguiculata	6.14 ± 0.38
Citrus limon	4.28 ± 0.12
Combination of *V. unguiculata* and *C. limon*	3.66 ± 0.34
Ferrous ascorbate (9 mg Fe/kg)	4.12 ± 0.23

Table 3 Results of heat induced hemolysis

Dose (μg/mL)	%inhibition by dexamethasone	%inhibition by *Vigna. unguiculata*	%inhibition of combination of *V. unguiculata* and *C. limon*
62.5	41.01	27.81	18.35
125	49.71	35.42	25.55
250	85.28	41.74	30.19
500	87.50	54.55	39.13
1000	98.22	62.79	48.65

4. Discussion

The yield of the extract obtained after extraction was 1.9%. Extract yield can be influence by factors such as solvent used and extraction method. In a study in which extract yield, phytochemical constituents of water and water-alcohol extracts prepared using two most commonly used extraction techniques, ultrasound and microwave assisted solvent extraction, were compared with the conventional extraction method. Extract yield, chemical composition of the extracts (total phenol and withanolide content) and antioxidant activity of the extracts varied with the extraction process as well as solvent composition [15]. In the acute toxicity study, no death of mice nor obvious signs of toxicity was recorded in both phase 1 and phase 2. The median lethal dose (LD50) was > 5,000 mg/kg body weight. This implies that the *Vigna unguiculata* ethanol extract in combination with *Citrus limon* juice had good safety profile. The Citric acid concentration of *Citrus limon* was 54.2 ± 0.003 mg/ml. Several studies have shown the richness of citrus species in valuable compounds, namely organic acids. In a work intended to investigate orange and lime peels as renewable sources of citric acid, UPLC-PDA analysis showed that orange peel presented a higher citric acid content than lime. For lime and orange peels, the extraction yield was maximized by sonication at low power for 5.8 or 35.5 minutes, using a low ethanol proportion or only water as a solvent, respectively [16].

Furthermore, proximate analysis and total phenolic content (TPC) results showed that *Vigna unguiculata* contain protein 31.5%; fat 1.3%; carbohydrate 35.9%; dietary fibre 28.4%; moisture 7.9% and TPC of 15.3 mgGAE/g. *Citrus limon* contain protein 1.2%; fat 0.35%; carbohydrate 10.4%; dietary fibre 0.52%; moisture 87.6% and TPC of 193 mgGAE/L. Proteins represent one of the most concentrated nutrients in legumes, and they can be easily used as components in innovative human foods. In addition, legumes have higher protein content than cereals: therefore, they represent a primary source of amino acids for humans [17].

In lipid peroxidation assay, malondialdehyde (MDA) serum concentration was used as a marker of oxidative stress in all the groups. The normal control group had the least MDA concentration (3.18 ± 0.10 nmol/ml) indicating freedom from oxidative stress. The anemia induced group recorded the most MDA level of 9.44 ± 0.48 nmol/ml which is as a result of increased oxidative stress. When compared with the anemia induced group, *V. unguiculata* and *C. limon* separately showed potentials in reduction of oxidative stress and this was more marked in the case of *C. limon*. *V. unguiculata* and *C. limon* recorded 6.14 ± 0.38 and 4.28 ± 0.12 nmol/ml respectively. The difference between *V.*

unguiculata and *C. limon* might be attributed to the fact that *C. limon* contain citric acid which is a natural antioxidant. Citric acid is a known antioxidant that acts to inhibit the oxidation of other molecules by scavenging reactive oxygen species (ROS) that are produced as by-products of normal cellular function [18]. The combination of the two herbs showed better antioxidant effect with MDA concentration of 3.66 ± 0.34 mmol/ml. this suggested a synergistic interaction between the two herbs.

Anti-inflammatory study was done by comparing the effects of different doses of dexamethasone, a standard anti-inflammatory drug; *Vigna unguiculata* monotherapy; and combination of *V. unguiculata* and *C. limon* on heat induced hemolysis. Expectedly, dexamethasone had the greatest %inhibition at all tested doses reaching 98.22% at a dose of 1,000 mg/mL. At the same dose of 1,000 mg/ml, *V. unguiculata* had %inhibition of 62.79%; an indication of good anti-inflammatory property. However, when given in combination with *C. limon*, the %inhibition reduced to 48.65% at the same dose of 1,000 mg/mL. This showed that *C. limon* might have interacted antagonistically with *V. unguiculata*. In a certain study, the anti-inflammatory effect of essential oils (EO) obtained from four different *Citrus* species. *C. limon*, *C. latifolia*, *C. aurantifolia* or *C. limonia* (10 to 100 mg/kg, p.o.) were evaluated in chemical induced inflammation (formalin-induced licking response) and carrageenan-induced inflammation in the subcutaneous air pouch model. EOs from *C. limon*, *C. aurantifolia* and *C. limonia* exhibited anti-inflammatory effects by reducing cell migration, cytokine production and protein extravasation induced by carrageenan. These effects were also obtained with similar amounts of pure limonene [19].

5. Conclusion

The combination of *Vigna unguiculata* and *Citrus limon* had greater antioxidant activities than each of the herbs given separately. This suggested a synergistic interaction between the two herbs which is also a consequence of their compositions especially Citric acid which is a natural antioxidant as well as iron and protein as obtained in *V. unguiculata*. Conversely, the anti-inflammatory effects of the individual herbs surpasses that of the combination therapy with *V. unguiculata* having the greatest anti-inflammatory activity.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declared no conflict of interest

Statement of ethical approval

Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123) were strictly adhered to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology. There was additional approval by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes.

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