



RESEARCH ARTICLE

# Evaluation of phenolic content, anti-oxidant and modulation of blood indices of *Pavetta crassipes* K.Schum

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## Abstract

*Pavetta crassipes* K.Schum is a medicinal plant with various documented biological activities. It has a folkloric use as an anti-viral therapy and pharmacologically documented anti-microbial activity. However, its immune modulatory property has not been explored. The study is designed to evaluate the phytochemical profile as well as the anti-oxidant and immunomodulatory potential of the methanol extract of *Pavetta crassipes* leaf. *In vitro* antioxidants activity was determined by nitric oxide inhibitory assay. phenolic and flavonoid content of the extract was also determined. The immunomodulatory activity was evaluated using cyclophosphamide induced immunomodulation model in mice. Phytochemical evaluation of leaf extract was done using HPLC analyses. Results were expressed as the mean  $\pm$  SEM, IC<sub>50</sub> value for the antioxidant activity was determined, One-way ANOVA was used for differences in mean followed by Dunnett's test for multiple comparison for the immunomodulatory activity. To test for level of significance, P < 0.05 was considered significant. The extract had phenolic and flavonoid contents of 17.6  $\pm$  0.1 mg/GAE/g and 8.4  $\pm$  0.3 mg/QE/g respectively. The extract also showed nitric oxide inhibitory antioxidant activity with IC<sub>50</sub> of 2.3  $\pm$  0.4 while gallic and ascorbic acid had IC<sub>50</sub> of 1.6  $\pm$  0.1 and 8.0  $\pm$  0.6 respectively. *P. crassipes* leaf extract indicated a significant (p < 0.05) countering influence to cyclophosphamide induced decrease in total white blood count, differential leucocyte counts and red blood count. HPLC analysis indicated the presence of phenolic compounds such as rutin, quercetin and chlorogenic acid. The phytochemical profile, anti-oxidant and immunomodulatory properties of the methanol extract of *P. crassipes* leaf as evaluated in this study corroborates and justifies its folkloric use.

## Keywords

immunomodulatory, antioxidants, phenolics, phytochemical evaluation

## Introduction

Plants have long been used as remedies to cure a variety of ailments. Natural products have been extensively documented to serve key roles in current medication research, particularly for immune boosting drugs (1). Although the popularity of synthetic products has grown due to lower production costs, time effectiveness, the availability of quality control methods, stringent regulation, and direct pharmacological effect and explainable mode of action, their safety and efficacy have always been questioned, resulting in

the reliance on natural products by more than 80% of the total population in the evolving world due to their time-tested effectiveness and safety (2).

Immunomodulators regulate the immune system by improving the host defense mechanism and maintaining homeostasis within the body (3). Exogenous and endogenous variables alter immune system function and efficiency, resulting in immunosuppression or immunostimulation (4). Immune system dysfunction is responsible for inflammatory diseases, cancer, parasitic and viral infections. This obstacle can be overcome by strengthening the immune system with immunomodulatory medications (5).

Immunomodulators either amplify or rebuild immune disorder to aid in the fight against infections, and have found significant applications in the treatment of AIDS, overactive immune function for the prevention of rejection or reaction against transplanted organs, autoimmune disease, and as an adjunctive therapy in treatment for cancer (6). It has recently been proposed that in severe and life-threatening COVID-19 (SARS-CoV-2) outbreaks, a high-intensity antioxidant, anti-inflammatory, and immunostimulatory therapy could be a treatment alternative (7).

Medicinal plants have been employed as immunomodulatory drugs in the treatment of a range of disorders, especially those that affect the host defense system (8). Plant compounds such as polysaccharides, lectins, peptides, flavonoids, and tannins have been proven to modify immune responses or immunity. Several phytochemicals have demonstrated anti-infective capabilities by either directly affecting the pathogen or indirectly activating the host's inherent and adaptive defensive mechanisms (9).

Antioxidants are substances that suppress the oxidation process thus inhibiting the accumulation of free radicals that trigger chain reactions and eventual cell damage. Several studies have suggested that oxidative damage has great impact on immune system of humans (10-12).

*Pavetta crassipes* K. Schum. (Rubiaceae) is a shrub distributed in West and Central African savannahs and locally known as 'Gadu' in Hausa. The leaf is utilized in the therapy of respiratory ailments, hypertension and viral infections in Nigerian Ethnomedicine (13) One of the compounds isolated from *P. crassipes* include quercetin-3-O-rutinoside (14). The plant is reported to possess wide range of pharmacological effects that include antiplasmodia/malarial (15), hypotensive (16), antiprotozoal, antimicrobial, antitumor (17) and anti-tuberculosis activities (18).

There is currently a lot of interest in the use of medicinal plants as immune system modulators. According to Kotwal (19), phytochemicals responsible for the immunomodulatory activities of some medicinal plants are in the form of alkaloids, flavonoids, terpenoids, polysaccharide, lactones, and glycosides. Medicinal plants with antiviral properties have been reported to have immune boosting effects (20, 21) reported the antimicrobial activity of the leaf extract of *P. crassipes*, however, there are no reports on its immunomodulatory activity. The study is therefore

designed to evaluate the phytochemical profile as well as the anti-oxidant and immunomodulatory properties of the methanol extract of *P. crassipes* leaf.

## Materials and Methods

### Materials

All chemicals and reagents were of analytical grade unless otherwise specified. All chromatographic solvents were High Performance Liquid Chromatography (HPLC) grade and sourced from Sigma Aldrich.

### Methodology

#### Plant collection and Extraction

Fresh *P. crassipes* leaves were obtained in Chaza, Suleja local government area, Niger state, Nigeria. A Taxonomist at the National Institute for Pharmaceutical Research and Development (NIPRD) herbarium in Abuja authenticated the plant. A reference specimen NIPRD/H/7132 was also submitted. After 2 weeks of air drying at room temperature, the samples were crushed, packed, and stored in an airtight container until needed for analysis. 100 g of crushed plant material was macerated in 99.9% methanol for 72 hrs. The obtained extract was concentrated in vacuo at 40°C to yield a methanol extract of *P. crassipes*.

#### Estimation of Phenolic Content and Antioxidant activity

##### Total Phenolic Content

The spectrophotometric approach previously described by (22) was employed at a concentration of 1 mg/mL extract. Folin-ciocalteu in water (10%, 2.5 mL) and Na<sub>2</sub>CO<sub>3</sub> 7.5%, 2.0 mL, were added to 0.5 mL of extracts in a test tube. Blank was made by substituting the equivalent volume of water for the extract. After 30 min. incubation in the dark, the reaction mixture was measured at 765 nm. This technique was also utilized to generate the calibration graph for the reference gallic acid. Phenolic content was reported as mg of gallic acid equivalent per gram sample (mg GAE /g).

##### Total Flavonoid Content

The flavonoid content was assessed using slightly modified methods of Esievo, Adamu (23). In a test tube, 1mL of extract was mixed with 4mL of distilled water; 0.3mL of 5% sodium nitrite was added and incubated for 5 minutes; and 0.3mL of 10% aluminum chloride was added and incubated for another 5 minutes. In the tube, 2mL of 1M sodium hydroxide was introduced, followed by 10 mL of distilled water. The standard agent was quercetin (QE). At 510nm, absorbance was measured. A calibration curve was created and utilized to quantify flavonoid levels in extracts. Flavonoid concentration was measured in milligrams of quercetin equivalent per gram of sample (mg QE/g).

##### Nitric Oxide Inhibitory Assay

The extracts' potential to suppress nitric oxide was assessed using the Greiss reaction method, as previously described (24) In a test tube, 10 mM sodium nitroprusside in phosphate-buffered saline pH (7.4) was combined with various concentrations of the extract (1-31.25mg/mL) and

incubated at 25 °C for 180 min. Ascorbic and Garlic acid (standard antioxidant agents) were also subjected to the same experiment as the extract. The absorbance at 550 nm was measured after 1 mL of Greiss reagent (1% sulphanilamide, 2% H<sub>3</sub>PO<sub>4</sub> and 0.1% naphthylethylenediamine dihydrochloride) was reacted with 1 mL of the combination of sodium nitroprusside and the extract. The inhibitory activities of the extract, ascorbic acid and garlic acid on nitrite production were computed and the IC<sub>50</sub> was established.

### Immunomodulatory Activity

#### Experimental Animals

A total of 38 healthy Swiss albino mice (20-30 g) from both sexes were obtained from the NIPRD Animal Facility Centre for both the experimental groups (5 animals/group) and the acute toxicity study groups of 4 animals/group. The mice were kept in a standard environment with a temperature of 25°C, relative humidity, and a 12-hour light/dark cycle. They were given a standard rat diet and allowed unlimited access to water. The study was conducted following the "Principles of Laboratory Animal Care" (NIH, 2011) and the NIPRD Standard Operating Procedures for whole animal studies.

#### Acute toxicity test

The oral acute limit test was carried out in accordance with the OECD (25) method for measuring oral acute toxicity. Eight young female mice were obtained from the NIPRD animal facility center and fasted overnight. The mice were weighed and divided into two groups of four. Group one received a dose of 2000 mg/kg *P. crassipes* extract and group two received normal saline in an equivalent volume of 10 mL/kg body weight. Animals were monitored for the first eight hours, with specific attention paid to the first four hours, then every 24 hours during the next 24 hours, and daily for 14 days for symptoms of toxicity.

### Cyclophosphamide induced Immunosuppressant model

#### Treatment protocol

The mice were randomized according to their body weight into 6 treatment groups (5 mice per group). Group 1 received 10 mL of normal saline and served as normal control, Group 2 received 3 mg/kg of cyclophosphamide and served as negative control, Groups 3- 5 received graded doses (250, 500 and 1000 mg/kg) of *P. crassipes* and served as the treatment group. Group 6 received 25 mg/kg dose of levamisole and served as positive control. All groups were orally treated for 14 days at a volume of 10 mL/kg body weight. On the 11<sup>th</sup>, 12<sup>th</sup> and 13<sup>th</sup> day (3 days), 3 mg/kg dose of cyclophosphamide was then administered intraperitoneally to all groups except group 1, one hr. after test drug treatments (26).

#### Hematological analysis

On day 14, one hour after the last treatment, blood samples were collected from the tail and full hematological parameters were measured using automated Hematology Analyzer (Wincom, YNH 7021).

### Chromatographic fingerprint analysis

#### Preparation of samples

0.2g of the extract was weighed into a clean beaker and dissolved with 80 % ethanol solution and made up to 10 mL with the solvent. Whatman 1 filter paper was used to filter the sample solution. After decanting the filtrate into a 10 mL volumetric flask, an 80% aqueous ethanol solution was added to make up the volume. Prior to usage, the final solution was filtered via a 0.45m Millipore membrane filter. A volume of 10 µL of each solution was introduced into the HPLC instrument for analysis.

#### Preparation of reference compounds

Reference compounds gallic acid, chlorogenic acid, rutin, catechin and quercetin were used in the HPLC analysis. The stock solution of concentration 2 mg/mL for each of the reference compound was prepared separately by weighing 0.02g (20mg) of each compound into a clean 25mL beaker and dissolving with 5mL methanol, the mixture was then transferred into a 10 mL volumetric flask and made up to mark. Prior to usage, the final solution was filtered via a 0.45 m Millipore membrane filter. 10 µL of each reference solution was used for the analysis.

#### HPLC analysis

The HPLC fingerprinting analysis was performed on a Shimadzu HPLC system that included an Ultra-Fast LC-20AB prominence equipped with a SIL-20AC auto-sampler, a DGU-20A3 degasser, an SPD20A UV-diode array detector (UV-DAD), a column oven CTO-20AC, a system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan). A binary gradient elution method with acetonitrile as solvent A and 0.1% phosphoric acid in HPLC grade water as solvent B was employed for the fingerprint analysis. Samples were separated using gradient elution as follows: 5 min., 20% A; 5-10 min., 20-25% A; 10-15 minutes, 25-30% A; 15-20 minutes, 30-40% A; and 20-25 minutes, 30% A. the column temperature was set at 40 C, flow rate was 0.6 mL/min and detector was optimized to 254 nm.

#### Validation of HPLC method

According to ICH requirements, the HPLC method for analyzing *Pavetta crassipes* extract was validated in terms of linearity, precision and accuracy (27). The standards were analyzed at different concentrations in triplicate to obtain a calibration plot. Correlation coefficient was determined using the linear regression model. The precisions of HPLC peak area measurements for all the reference substances was computed as the relative standard deviations (RSD) of five repeated runs of 600 µg/mL each. The recoveries of the compounds were determined on the same day using the conventional addition procedure. Three different concentrations of mixed standard solutions were spiked into the sample, and the recovery results were computed by comparing the spiked and un-spiked samples under the same conditions.

#### Statistical analysis

Data were analyzed using GraphPad Prism v.6.01. Results were expressed as mean ± SEM. One-way ANOVA was used

to compare for differences between groups and Dunnett's test for multiple comparisons. Results with  $P < 0.05$  are considered statistically significant.

## Results

### Total phenolics, flavonoids content and Nitric oxide inhibitory activity

Results from total phenols content (TPC) and total flavonoids content (TFC) quantifications revealed that the methanol extract of *P. crassipes* had TPC and TFC value of  $17.6 \pm 0.1$  mg/GAE/g and  $8.4 \pm 0.3$  mg/QE/g respectively. *P. crassipes* exhibited nitric oxide inhibitory activity with  $IC_{50}$  value of  $2.3 \pm 0.4$   $\mu$ g/mL while the standard drugs, gallic and ascorbic acid had  $IC_{50}$  values of  $1.6 \pm 0.1$  and  $8.0 \pm 0.6$  ( $\mu$ g/mL) respectively (Table 1 & 2).

### Oral acute toxicity

**Table 1.** Nitric Oxide inhibitory activity of *P. crissipes* leaves extract

Conc. ( $\mu$ g/mL)	Inhibition (%)		
	Gallic acid	Ascorbic acid	<i>P. crassipes</i>
800	81.16	89.20	85.56
400	78.77	83.67	80.67
200	75.59	81.30	78.19
100	72.40	75.78	75.76
50	70.02	67.09	73.86
25	65.24	63.15	65.46

**Table 2.** Antioxidant activity of methanol extract of *Pavetta crassipes*

Extract/standard	Nitric oxide inhibition $IC_{50}$ ( $\mu$ g/mL)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
<i>P. crassipes</i>	$2.3 \pm 0.4$	$17.6 \pm 0.1$	$8.4 \pm 0.3$
Ascorbic acid	$8.0 \pm 0.6$	-	-
Gallic acid	$1.6 \pm 0.1$	-	-

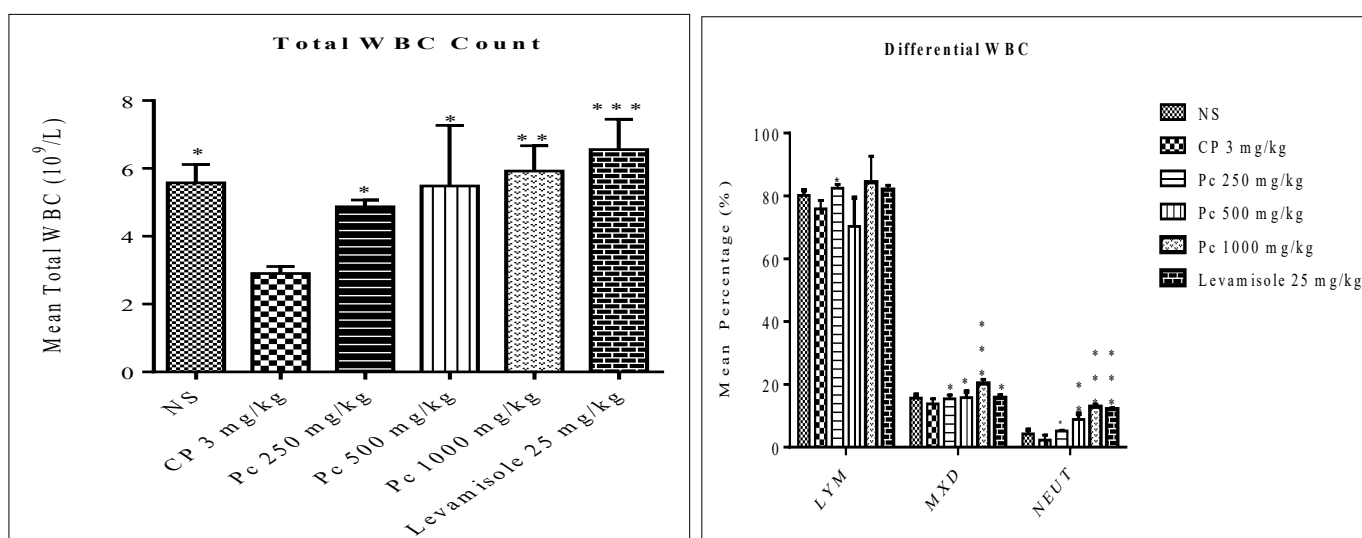
No acute or delayed toxicity signs were observed on administration of *P. crassipes*. However, there were signs of sedation and decrease locomotion one hour after administration which lasted for about an hour. The estimated  $LD_{50}$  is greater than 2000 mg/kg.

### Immunomodulatory

The immune-suppressed group (CP) showed significant ( $p < 0.05$ ) differences in blood parameters compared with the group with normal immune status. *P. crassipes* produces significant ( $p < 0.05$ ) dose-dependent differences in white blood cell count at doses of 250, 500 and 1000 mg/kg when compared to the cyclophosphamide group. There were no substantial changes between the test groups where the immunity was decreased by cyclophosphamide administration from day 11 to 13 and the normal saline group with a non-compromised immune status. This shows some kind of inhibition of the cyclophosphamide effect. The most significant effect was observed in 1000 mg/kg dose for WBC, lymphocytes and MXD (monocytes-basophils-eosinophils mixed). Levamisole significantly increased both the white blood cells and red blood cells parameters even in the presence of cyclophosphamide (Fig. 1 & 2).

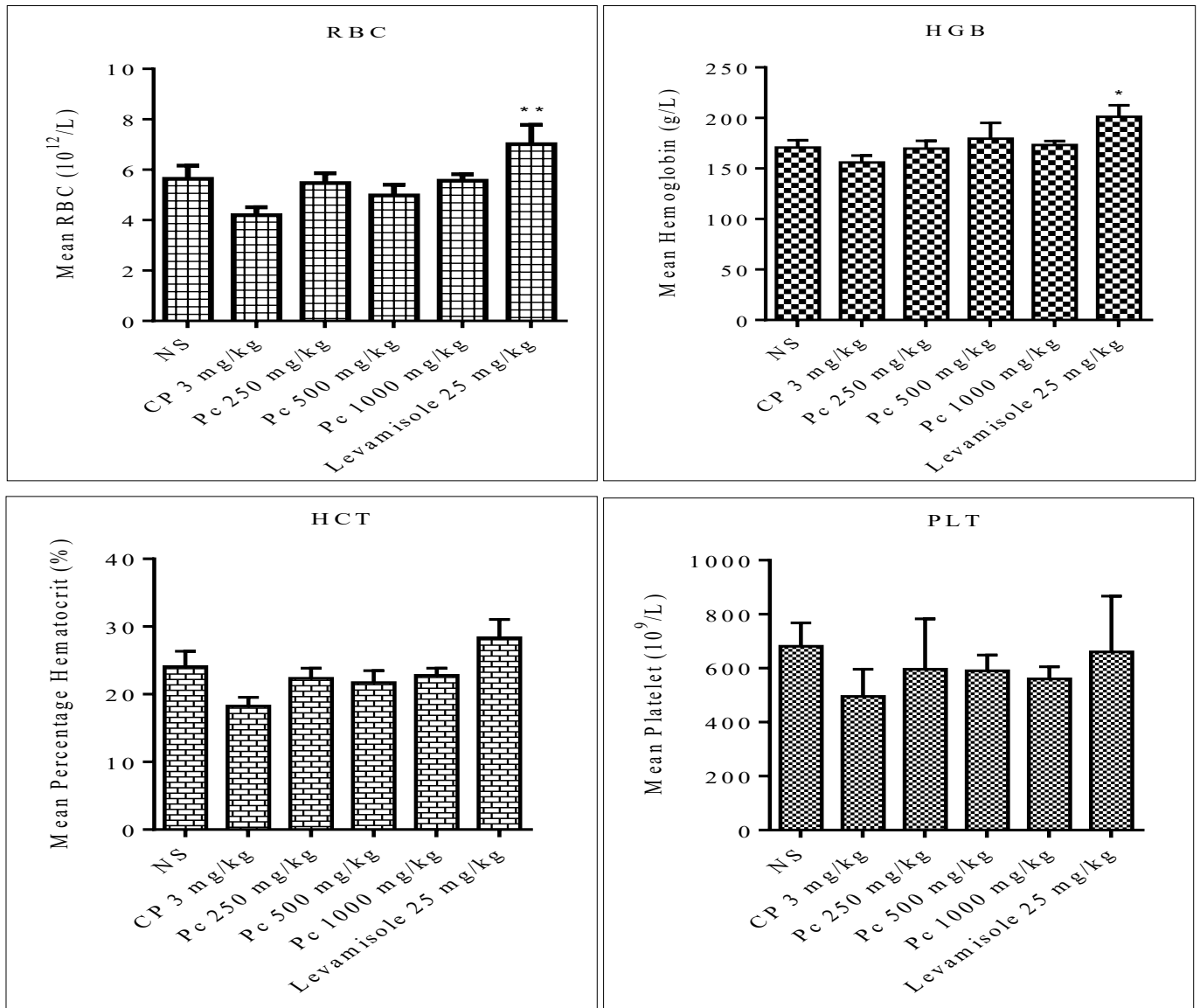
### HPLC Evaluation of *Pavetta crassipes* leaf extract

The retention periods for gallic acid, chlorogenic acid, rutin, catechin, and quercetin under the current experimental protocols are 2.88, 3.66, 9.08, 14.60 and 22.86 min, respectively. The chromatographic peaks in the samples were recognized by comparing their retention time and UV spectrum to that of each reference compound, and quantification was performed using the equation derived from the calibration curve presented in Table 3. The HPLC analysis of some phenolic acid and flavonoid shows that the sample contains; 71.77  $\mu$ g/mL of gallic acid, 35.49  $\mu$ g/mL of chlorogenic acid, 82.43  $\mu$ g/mL of rutin, 130.91  $\mu$ g/mL of catechin and 14.61  $\mu$ g/mL of quercetin (Table 4). In terms of linearity, precision and accuracy, an HPLC method for



**Fig 1.** Effects of Methanol extract of *P. crassipes* on white blood cells in immunocompromised mice. Values are represented as mean value  $\pm$  standard error of mean (SEM)  $n=5$ ; *P. crassipes* treated groups (250 mg, 500 mg and 1000mg/kg, negative CP (cyclophosphamide), positive control (levamisole) and a sham group NS (Normal saline). One-way ANOVA was used for differences in mean followed by Dunnett's test for multiple comparison \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from negative control (CP). RBC: Red blood cell. HGB: Hemoglobin. HCT: Hematocrit.





**Fig. 2.** Effects of Methanol extract of *P. crassipes* on red blood cells of immunocompromised mice. Values are represented as mean value  $\pm$  standard error of mean (SEM)  $n=5$ ; *P. crassipes* treated groups (250 mg, 500 mg and 1000mg/kg, negative CP (cyclophosphamide), positive control (levamisole) and a sham group NS (Normal saline). One-way ANOVA was used for differences in mean followed by Dunnett's test for multiple comparison \* $p<0.05$ , \*\* $p<0.01$  significantly different from negative control (CP). RBC: Red blood cell. HGB: Hemoglobin. HCT: Hematocrit.

**Table 3:** HPLC Method validation: Precision (repeatability), accuracy (percentage recovery), linearity and range of gallic acid, chlorogenic acid, rutin, catechin and quercetin reference compounds.

S/N	Compounds	Retention time (min) mean $\pm$ SD	Linear equation	Correlation coefficient ( $R^2$ )	Linear range ( $\mu\text{g/mL}$ )	Precision (Repeatability) % RSD $n=5$	Accuracy (Percentage Recovery) mean $\pm$ SD ( $n=3$ )
1	Gallic acid	2.88 $\pm$ 0.1	$Y=7199.3x-336726$	0.993	37.5-600	2.22	99.9 $\pm$ 2.8
2	Chlorogenic acid	3.66 $\pm$ 0.1	$Y=9473.9x-297476$	0.996	37.5-600	4.63	96.7 $\pm$ 5.6
3	Rutin	9.08 $\pm$ 0.2	$Y=6657.4x-89816$	0.998	37.5-600	3.19	101.4 $\pm$ 6.3
4	Catechin	14.60 $\pm$ 0.1	$Y=11852x-261781$	0.999	37.5-600	0.52	98.2 $\pm$ 6.7
5	Quercetin	22.86 $\pm$ 0.3	$Y=16306x-205776$	0.998	37.5-600	0.69	99.6 $\pm$ 2.6

analyzing a mixture of gallic acid, chlorogenic acid, rutin,

**Table 4.** HPLC quantification of Gallic acid, chlorogenic acid, rutin, catechin and quercetin in *Pavetta crassipes*.

S/N	Compounds	Concentration ( $\mu\text{g/mL}$ )
1	Gallic acid	71.77 $\pm$ 0.1
2	Chlorogenic acid	35.49 $\pm$ 0.2
3	Rutin	82.43 $\pm$ 0.5
4	Catechin	130.91 $\pm$ 1.6
5	Quercetin	14.61 $\pm$ 0.2

catechin and quercetin was developed and validated. This study's settings resulted in complete peak-to-baseline resolution of the substances examined. This approach has the advantage of a significantly shorter retention time as well as very excellent resolution for mixed reference compounds. Furthermore, spiking samples with the reference substances validated the peaks' identities.

## Discussion

Flavonoids are phenolic molecules that play key roles in fighting free radicals and therefore help in avoiding oxidative stress-related diseases (28). They show anti-allergic, anti-inflammatory, anti-microbial, anti-cancer and immune boosting activity (29). In this study, the extract of *Pavetta crassipes* showed high phenolic and flavonoid content. *P. crassipes* exhibited a good nitric oxide inhibitory activity with  $IC_{50}$  of  $2.3 \pm 0.4$   $\mu\text{g/mL}$  compared to the standard drugs, gallic ( $1.6 \pm 0.1$   $\mu\text{g/mL}$ ) and ascorbic acid ( $8.0 \pm 0.6$   $\mu\text{g/mL}$ ) (Table 2). The nitric oxide inhibitory activity may be due to the high level of phenolic content in the extract which has the ability to inhibit the production of nitric oxide from sodium nitroprusside. Polyphenols have long been thought to have the ability to reduce nitric oxide and peroxynitrite production (30). Previous research has found an indirect link between the consumption of antioxidant-rich foods and the lowering of risk factors for several human diseases caused by free radical production (31, 32).

In this study, the immune boosting effects of *P. crassipes* against cyclophosphamide-induced immunosuppression in Swiss albino mice was evaluated. It was observed that hematological parameters such as red blood count, total leucocyte and differential leucocyte count were significantly decreased after administration of cyclophosphamide to mice. *P. crassipes* showed an overall inhibitory response against the immunosuppressive effect of cyclophosphamide in a dose dependent manner (Fig. 1 & 2). Daily administration of *P. crassipes* for 13 consecutive days, produced significant ( $p < 0.05$ ) changes in White blood count, lymphocytes and neutrophils parameters when compared with control animals (CP). Also, a significant rise in MXD in the treatment groups indicates a corresponding rise in monocytes, basophils and eosinophils. The ability of the extract of *P. crassipes* to augment white blood cell count, neutrophil and lymphocyte count indicates immunostimulatory ability.

Modulators influence the activities of the immune system either by selectively inhibiting or by intensifying the specific cell production of immune responsive cells like neutrophils, lymphocytes, macrophages, natural killer cells, and cytotoxic T lymphocytes. Moreover, depending on the cellular context, red blood cells are significant natural immune system mediators. These cells either stimulate or preserve the immune system in a latent condition. Red blood cells play important immunological roles as well, such as chemokine modulation, nucleic acid binding, and pathogen elimination (33). The administration of *P. crassipes* maintained the numbers of red blood cells and hemoglobin, even in the presence of cyclophosphamide. All doses of *P. crassipes* effectively inhibited the haemato-

suppressive effect induced by cyclophosphamide. Studies have proved that levamisole, the reference drug in this study, is a good immunomodulator in animals and humans (34).

Several studies have reported immunostimulatory impact of plants constituents such as phenols against cyclophosphamide induced myelosuppression in mice (35, 36).

The finding of this study has shown that the leaf of *P. crassipes* are rich in phenolic acids and flavonoid compounds, this is in tandem with the report of earlier researchers (18). Specifically, rutin (37), chlorogenic acid, methyl chlorogenate (38) quercetin and catechin (18) are among reported polyphenolics in the leaf of *P. crassipes*. These compounds are known to have significant antioxidant and immune boosting properties, indicating that the leaf of *P. crassipes* could alleviate immunosuppression and inflammation through multiple mechanisms thus justifying its use in preparations for antiviral and immune boosting products. According to recent research, plant polyphenols can modulate distinct cell types by acting in several signaling cascades (39). Flavonoids and phenolic compounds are thought to have wellness promoting characteristics as a result of their reported antioxidant capability in both *in vivo* and *in vitro* systems. They have the ability to activate human defense enzyme systems (40).

## Conclusion

The extract of *Pavetta crassipes* K. Schum showed antioxidant and immunostimulatory potentials against cyclophosphamide induced immunosuppression. The observed activity could be attributed to the presence of bioactive phytochemicals in the plant. This study justifies its folkloric use in antiviral therapy and documented antimicrobial property.

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## Authors contributions

EKB conceptualized the study, performed the *in vitro* antioxidant experiments and wrote the original draft of the manuscript; FSA designed the experimental protocol for the immunomodulatory study and wrote draft of the manuscript; AA performed the phytochemical (HPLC) study; FOT, GIB participated in the study design and reviewed the manuscript; IJA coordinated the research and reviewed the manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interests to declare.

**Ethical issues:** Ethical approval for animal experiments was granted by the Animal Care and Ethics committee, National Institute for Pharmaceutical Research and Development (NIPRD) with approval number NIPRD/05:03:05-40.

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