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Research Article

Effects of the methanolic extract of the rind of *Citrullus lanatus* (watermelon) on some erythrocyte parameters and indices of oxidative status in phenylhydrazine-treated male Wistar rats.

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ABSTRACT

Background: The possible ameliorative effects of the methanolic extract of the rind of *Citrullus lanatus* on some erythrocyte parameters, serum superoxide dismutase and malonaldehyde concentrations were investigated following phenylhydrazine administration using wistar rats as models. **Methods:** Adult male Wistar rats were randomly divided into 5 groups: Group 1 were untreated controls; Group 2 received only phenylhydrazine: negative controls; Group 3 received phenylhydrazine +100 mg/kg of extract; Group 4 received phenylhydrazine +500 mg/kg of extract; Group 5 received phenylhydrazine + 0.23 ml/kg of Bioferon®: positive controls. In all groups, phenylhydrazine was administered via the intraperitoneal route at a dose of 40 mg/kg body weight on day 0, with two additional doses given on day 1 of the experiment; the extract of the rind of *Citrullus lanatus* and Bioferon were both administered orally for 14 days. On day 15, the rats were placed under chloroform anesthesia and blood collected by direct cardiac puncture. Red blood cell counts, hemoglobin concentration and haematocrit were determined using automated methods; serum superoxide dismutase and malondialdehyde concentrations were determined by standard methods. **Results:** Amongst Group 2 rats, administration of phenylhydrazine significantly reduced all erythrocyte parameters and superoxide dismutase concentrations but significantly increased malondialdehyde concentrations compared to Group 1 rats ($p<0.05$). However, administration of the extract and Bioferon significantly increased values of all erythrocyte parameters and superoxide dismutase concentration and significantly reduced malondialdehyde concentration amongst both Groups 4 and 5 rats respectively compared to Group 2 rats. Only the values of superoxide dismutase were significantly elevated amongst Group 3 rats; suggesting a possible dose dependence of the effects of the extract. **Conclusion:** Results suggest a possible ameliorative potential of the rind of *Citrullus lanatus* on the erythrocyte parameters and indices of oxidative stress and lipid peroxidation following phenylhydrazine administration. The observed effects of the rind of *Citrullus lanatus* could possibly result from summation of the effects of both its antioxidant and nutritional constituents. Our findings are essentially preliminary and could benefit from further investigations.

INTRODUCTION

Citrullus lanatus (watermelon) is a warm-season crop in the Cucurbit family. The fruit has a deep green or yellow colored smooth thick exterior rind sometimes with gray or light green vertical stripes: Inside, the fruit is pink, red or even yellow with small black seeds embedded in the middle third of the flesh (Department of Agriculture, Forestry and Fisheries; RSA, 2011). Generally, watermelon flesh is the main consumable portion; however, the outer rind is also consumed in some parts of the world (Paris 2015). The rind has been evaluated as a substitute for wheat flour in cake making (Al-Sayeed and Ahmed, 2013) and is recommended in alcoholic poisoning and diabetes (Duke and Ayensu 1985; Jiyun et. al., 2011). Amongst other compounds, the rind contains alkaloids, saponins, cardiac glycosides, flavonoids, phenol, moisture, lipids, proteins, fiber and carbohydrates (Erhirhie and Ekene

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2013; Erukainure et al., 2010). The possible ameliorative effects of the methanolic extract the rind of *Citrullus lanatus* on some semen parameters and reproductive hormones following lead acetate induced toxicity (Kolawole et al., 2014), following aspirin induced gastric ulceration (Kolawole et al., 2016a) and in alloxan induced diabetes (Kolawole et al., 2016b) in male Wistar rats has recently been described from our centre.

Anemia is defined as a clinical condition in which the number of circulating red blood cells or their oxygen-carrying capacity is insufficient to meet physiologic needs (Reid et. al., 2008). Anemia develops when the rate of red blood cell production by the bone marrow fails to keep pace with loss or destruction. (Reid et. al., 2008). The incidence of anemia is generally higher in third world countries compared to developed countries due to the presence of many predisposing and aggravating factors: deficient nutrition, low socio-economic status, natural and man-made disasters, recurrent infections and an increased prevalence of blood parasites especially Plasmodium, Trypanosomes and helminthic infestation (Korubo-Owiye et al., 1998; Sanni et.al., 2005). Anemia is one of the numerous ailments claimed to have been successfully treated with plant materials by traditional medicine practitioners (Richard, 1978).

Phenylhydrazine (PHZ) with the chemical formula: $C_6H_8N_2$, was the first hydrazine derivative characterized by Hermann Emil Fischer in 1875 (Berger, 2007). This compound is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries: PHZ has a molecular weight of 108 and exists as yellow to pale brown crystals or as a yellowish oily liquid with a freezing point of 19.6°C and a boiling point of 243.4°C (WHO, 2000). PHZ is soluble in water, miscible with alcohol, ether, chloroform, benzene and acetone (WHO, 2000). PHZ derivatives were used firstly as antipyretics; however, their possible toxic effects on red blood cells have made their continued use fraught with danger (WHO, 2000). Exposure to PHZ may cause damage to red blood cells, potentially resulting in a haemolytic anemia (experimental) with secondary involvement of other tissues such as the spleen and liver (Stern, 1989; WHO, 2000). PHZ-induced haemolytic anaemia can be used as an experimental model for study of haematinic effects of new agents or as a model for reticulocyte research (Pokhrel and Lau-Cam, 2000; Xie et al., 2003; Biswas et al., 2005; Berger, 2007). PHZ has been reported to cause increased reactive oxygen species (ROS) and lipid peroxidation of red blood cells (Clemens et al., 1984, Amer et al., 2004) and decreased glutathione (GSH) levels; these effects are reversed by N-acetyl cysteine, a known ROS scavenger (Hill and Thornalley 1982, Clemens et al., 1984, Amer et al., 2004). Hemolytic anemia has long been known to be caused by the uptake of erythrocytes by macrophages in the spleen; translocation of phosphatidylserine from the inner to the outer side of the plasma membrane identified as a signal for phagocytosis of red blood cells under programmed death by macrophages (Berger, 2007). PHZ generates ROS within both human and rat erythrocytes; although no evidence for lipid peroxidation or phosphatidyl serine externalization was detected (de Jong et al., 1997, McMillan et al., 2005). ROS production was associated with extensive binding of oxidized and denatured hemoglobin to the membrane cytoskeleton. Thus, PHZ-induced hemolytic injury seems to be derived from oxidative alterations to red blood cell proteins rather than to interactions of membrane lipids (McMillan et al., 2005).

The rind of *Citrullus lanatus* is also a rich source of a number of antioxidant molecules including the carotenoids: lycopene and beta carotene, and contains mainly citrulline (Rimando and Perkins-Veazie, 2005); a known stimulator of nitric oxide and reported to enhance oxidative stress tolerance by acting as a novel hydroxyl radical scavenger (Akashi et al., 2001). The present study describes the effects of the methanolic extract of the rind of *Citrullus lanatus* on some erythrocyte parameters and serum concentrations of superoxide dismutase and malondialdehyde following administration of PHZ using male Wistar rats as experimental models. This is part of a series of studies from our center exploring the potential beneficial effects of the methanolic extract of the rind of *Citrullus lanatus*.

MATERIALS AND METHODS

Plant material and preparation of extracts: Fresh fruits of *Citrullus lanatus* were obtained from the community market in Elele, on the outskirts of Port Harcourt, Rivers State, Nigeria. The fruits were identified and authenticated by Dr. Chimezie Ekeke of the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria. Herbarium number: UPH/V/1214 was assigned and voucher specimens deposited. The rinds were peeled off from the whole fruit, thoroughly washed, sun-dried for 15 days and milled into a fine powder. The method of extraction employed is percolation as described by Adesanya et al., 2011. 24g of the powdered sample was soaked in a beaker containing 100 ml of 98% methanol for a period of 48 hours and then filtered with Whatman No. 1 filter paper size. The volume of filtrate obtained was 150ml before concentration; the filtrate was subsequently concentrated using a rotary evaporator. The weight of residue obtained was 8.5g.

Determination of Median Lethal Dose (LD50):
Acute toxicity study (LD50) was determined using the method described by Lorke, 1983. As previously reported (Kolawole et el., 2014; Kolawole et. al., 2016b), the LD50 of the extract was found to be greater than 2000 mg/kg body weight.

Experimental design:
Twenty-five male Wistar rats were used for this study. The rats were aged 8 to10 weeks and weighed between 175 and 210g. They were divided into five Groups: 1 to 5 consisting of 5 rats per group. Rats in each group were placed in separate cages in the Animal House of Madonna University, Nigeria under natural day and night cycles. The rats had free access to normal rat chow and tap water ad libitum. They were allowed two weeks of acclimatization to their environment. The study was conducted in accordance with the guidelines for the care and use of laboratory animals by the US Institute for Laboratory and Animal Research (1996). After acclimatization, anemia was induced in all rats except Group 1 rats via intra-peritoneal injection of 40mg/kg of PHZ on day 0 and two additional injections at 9am and 6pm on day 1 of the experiment as previously descried by Ashour, 2014. The rats were subsequently treated as follows:
Group 1: Control group. Rats in this group received only 2 ml/kg body weight of extract vehicle.
Group 2: PHZ only-Negative control group. Rats in this group were left untreated after receiving the doses of PHZ.
Group 3: PHZ + Low dose extract group. Rats in this group were treated with 100 mg/kg body weight of the extract of the rind of *Citrullus lanatus* after receiving the doses of PHZ.
Group 4: PHZ + High dose extract group. Rats in this group were treated with 500 mg/kg body weight of the extract of the rind of *Citrullus lanatus* after receiving the doses of PHZ.
Group 5: Positive control group. Rats in this group were given 0.23 ml/kg body weight of Bioferon® after receiving the doses of PHZ. This is as described by Zara et al., 2014.

The extract vehicle, extract of the rind of *Citrullus lanatus* and Bioferon® were orally administered daily to the rats for 14days using an oral cannula. The choice of the low and high dose of the extract of the rind of *Citrullus lanatus* administered in the present study was as previously described by the authors (Kolawole et al., 2016a; Kolawole et al., 2016b). The data reported in the present study was part of a larger study protocol attempting to explore the effects of the methanolic extract of the rind of *Citrullus lanatus* on reproductive functions of male Wistar rats.

Determination of erythrocyte parameters, serum superoxide dismutase (SOD) and malondiadehyde (MDA) concentrations: On day 15, the rats were placed under chloroform anesthesia and blood samples collected via direct cardiac puncture. A portion of the blood samples were immediately transferred into EDTA specimen bottles for assessment of red blood cell count, hemoglobin concentration and haematocrit scores. These parameters were determined using an auto-hematological analyzer (Beckman Coulter, USA). The remaining portion was placed into plain sample bottles, allowed to coagulate and serum carefully obtained for determination of superoxide dismutase (SOD) and malondiadehyde (MDA) concentrations. Specifically, SOD concentration was determined using the method of Misra and Fridovich (1972) as previously described (Kolawole et al., 2016a). The determination of MDA concentration was also as previously described (Kolawole et al., 2016a). Briefly, 2ml of thiobarbituric acid (TBA) reagent and 1ml of trichloroacetic acid (TCA) were mixed with the obtained serum. The mixture was heated at 60°C for 20 minutes and then cooled before centrifugation at 400 rpm for 10 minutes. The absorbance of the obtained supernatant was read at a wavelength 540nm.

Statistical analysis
Significant differences were determined using the One-Way Analysis of Variance (ANOVA) followed by the LSD post hoc tests. A p value <0.05 was considered statistically significant. Results obtained are presented in Table 1 as mean ± standard error of mean (SEM).

RESULTS
Table 1 shows the effects of the methanolic extract of the rind of *Citrullus lanatus* on red blood cell count, hemoglobin concentration and haematocrit scores and serum concentrations of both superoxide dismutase and malondialdehyde in male Wistar rats following the administration of PHZ. Amongst Group 2 (PHZ-only: negative control) rats, significantly lower values were observed for red blood cell count, haemoglobin concentration, haematocrit scores and serum superoxide dismutase concentration; while significantly higher value was observed for serum malondiadehyde concentration compared to Group 1 (untreated control) rats ($p<0.05$). Similarly, amongst Group 4 (PHZ + High dose extract) rats, the values of red blood cell count, haemoglobin concentration, haematocrit scores and serum superoxide dismutase values were found to be significantly higher; while value of serum malondiadehyde concentration was found to be significantly lower compared to values obtained amongst Group 2 (PHZ-only: negative control) rats

($p<0.05$). For Group 3 (PHZ + Low dose extract) rats, only the values of serum superoxide dismutase concentration were found to be significantly higher compared to Group 2 (PHZ-only: negative control) rats: No significant differences were observed in the values of red blood cell count, haemoglobin concentration, haematocrit scores and serum malondiadehyde concentration between Group 3 (PHZ + Low dose extract) and Group 2 (PHZ-only: negative control) rats ($p>0.05$). These findings suggest a possible dose-dependence of the effects of the methanolic extract of the rind of *Citrullus lanatus* on these erythrocyte parameters and superoxide dismutase concentration following administration of PHZ in experimental animals.

Similarly, amongst Group 5 (PHZ + Bioferon®: positive control) rats, significantly higher values were observed for red blood cell count, haemoglobin concentration, haematocrit scores and serum superoxide dismutase concentration; while significantly lower value was observed for serum malondiadehyde concentration as compared to Group 2 (PHZ-only: negative control) rats. Overall, the pattern of changes in the parameters under investigation amongst Group 5 (PHZ + Bioferon®: positive control) rats were observed to be essentially similar to the pattern obtained amongst Group 4 (PHZ + High dose extract) rats. Furthermore, no significant differences were observed in the values of all the parameters under investigation between Group 5 (PHZ + Bioferon®: positive control) and Group 4 (PHZ + High dose extract) rats ($p>0.05$).

DISCUSSIONS

The results of the present study clearly suggest possible ameliorative potential of the methanolic extract of the rind of *Citrullus lanatus* on some erythrocyte parameters, indices of enzymatic antioxidant status (superoxide dismutase), lipid peroxidation and oxidative stress (malondialdehyde) (Singh et al., 2014) following the administration of PHZ in our experimental animals. Expectedly, the significant reduction in the values of the assessed erythrocyte indices in the present study: red blood cell count, haemoglobin concentration and haematocrit scores, following the administration of PHZ to our experimental animals, fairly confirms the previously described and reported toxic effects of PHZ on the red blood cell (Stern, 1989; Berger 2007; WHO, 2000; Ashour, 2014). Our findings are fairly consistent with previous reports of reductions in haemoglobin levels, red blood cell counts, packed cell volume and an impairment in erythrocyte deformability following PHZ administration in experimental animals (Ashour, 2014; Berger 2007). These effects of PHZ predictably results in an anaemia (usually of a hemolytic nature) consequent on red cell damage with secondary involvement of the spleen and liver (Stern 1989; WHO 2000). Noteworthy, the ameliorative effects of the methanolic extract of the rind of *Citrullus lanatus* seen in the present study on red blood cell parameters were apparently dose dependent and fairly comparable with the effects of Bioferon; a haematinic preparation containing Ferric Ammonium citrate as an active ingredient and useful for the prophylaxis of iron, zinc, vitamin and folic acid deficiencies and curative treatment for anaemia.

Whereas the effects of PHZ administration on serum concentrations of superoxide dismutase and malondiadehyde were found to be essentially contrasting: significant decrease in superoxide dismutase with corresponding significant increase in malondiadehyde concentrations; administration of the methanolic extract of the rind of *Citrullus lanatus* apparently reversed these effects causing an increase in superoxide dismutase concentrations and a corresponding decrease in malondiadehyde concentrations. Although the effects of the methanolic extract of the rind of *Citrullus lanatus* on superoxide dismutase appeared to be dose-dependent; the effects on malondiadehyde concentration were comparatively found to be independent of dose. Furthermore, these effects of the methanolic extract of the rind of *Citrullus lanatus* on both superoxide dismutase and malondiadehyde concentrations were observed to be fairly similar to the effects of Bioferon on these indices.

Reasons for the potential ameliorative effects of the methanolic extract of the rind of *Citrullus lanatus* are likely attributable to the chemical constituents of the rind. The rind of *Citrullus lanatus* has been reported to contain several phytochemicals including alkaloids, flavonoids, saponin and tannins (Erukainure et al., 2010). Specifically, the amino acid citrulline, the carotenoid lycopene and a number of phenolic compounds: 4-hydrobenzoic acid, vanillin and cuomanic acid have been described in the rind ((Al-Sayeed and Ahmed, 2013). Also found in the fleshy edible portion include water, protein, carbohydrates, various minerals and a number of vitamins including ascorbic acid and tocopherol. Citrulline and lycopene are antioxidants as well as ascorbic acid and tocopherol found predominantly in the fleshy edible portions of the fruit. Clearly, the rind of *Citrullus lanatus* contains several constituents of nutritional and antioxidant value that could contribute to the effects observed in the present study. For instance, the significant improvement in the values of the various erythrocyte parameters adversely affected by the administration of

Table 1: Effect of methanolic extract of the rind of *Citrullus lanatus* on some erythrocyte parameters, serum superoxide dismutase and malondiadehyde concentration following administration of phenylhydrazine.

	Group 1: Untreated (Control) (n=5)	Group 2: PHZ-only. (Negative control) (n=5)	Group 3: PHZ +100 mg/kg of extract. (PHZ + Low dose extract) (n=5)	Group 4: PHZ +500 mg/kg of extract. (PHZ + High dose extract) (n=5)	Group 5: PHZ + 0.23 ml/kg of Bioferon® (Positive control) (n=5)
Red blood cell count ($x10^6 \mu L^{-1}$)	8.70±0.25*	4.00±0.55	5.05±0.31	7.50±0.20*	8.00±0.35*
Haemoglobin concentration (g/dl)	12.58±0.56*	9.28±1.42	10.76±1.61	12.42±0.44*	12.32±0.28*
Haematocrit (%)	39.60±1.47*	25.80±5.39	30.20±2.05	39.00±2.00*	43.00±2.44*
Superoxide dismutase concentration (U/mg protein)	3.15±0.09*	1.34±0.09	2.19±0.27*	3.99±0.12*	3.43±0.19*
Malondialdehyde concentration (nmol/mg protein)	4.47±0.00*	6.60±0.39	5.26±0.00	5.14±0.00*	5.18±1.11*

*All values=Mean ± SEM. * indicates significant differences compared to Group 2 rats at p<0.05.*

PHZ may be due to the presence of these phytochemicals in the rind of *Citrullus lanatus* (Erukainure et al., 2010). Alkaloids are known to inhibit cyclic adenosine monophosphate (cAMP) phosphodiesterase thereby causing an accumulation of cAMP levels (Ndem et.al., 2013). This stimulates protein phosphorylation and synthesis with a possible enhancement of erythropoiesis (Ndem et. al., 2013). Another mechanism by which the extract could possibly enhance erythrocyte parameters may be due to its content of ascorbic acid and tocopherol (Edwards et al.,2003). Ascorbic acid possesses significant antioxidant properties and could presumably deplete the free radicals presumably generated by the administration of PHZ, leading to a reduction in oxidative stress. (Dietrich, 2003). The beneficial effects of tocopherol could possibly due to its reported antioxidant potential (Claro et al., 2006). Tocopherol is a lipid soluble antioxidant which plays a major protective role against oxidative stress and prevents the production of lipid peroxides by scavenging free radicals which are toxic byproducts of many metabolic processes in biological membranes (Oyeyemi et. al., 2015). Also, both ascorbic acid and tocopherol could contribute to a decrease in oxidative stress caused by the in vitro administration of PHZ via inhibition of Heinz bodies and methemoglobin formation (Claro et al., 2006). Quercetin an antioxidant bioflavonoid compound, reportedly present in *Citrullus lanatus* (Oseni and Okoye, 2013; Adetutu, et al., 2015) also suppresses reactive oxygen and nitrogen species and partially protects against reduced malondialdehyde levels (Luangaram et al., 2007). Furthermore, it has been reported that alkaloids and flavonoids, present in the rind of *Citrullus lanatus*, acts to protect cells as antioxidants and thus prevent or repair damage to red blood cells by free radicals or highly reactive oxygen species (Asgary et al., 2005). The extract of *Citrullus lanatus* also reduces lipid peroxidation as the degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues (Davey et al., 2005). Clearly, the presence of these antioxidants in the rind extract of *Citrullus lanatus* could possibly reverse the damaging effect of PHZ administration. We suggest that the effects of the methanolic extract of the rind of *Citrullus lanatus* seen in the present study could possibly be the result of summation of the effects of its antioxidant and nutritional constituents.

In conclusion, the present study describes the potential ameliorative effects of the methanolic extract of the rind of *Citrullus lanatus* on red blood cell count, haemoglobin concentration, haematocrit scores and serum superoxide dismutase and malondiadehyde concentrations following administration of PHZ to experimental animals using male wistar rats as model. These effects of the rind of *Citrullus lanatus* could possibly be the result of summation of the effects of its antioxidant and nutritional constituents. Our findings are essentially preliminary and could benefit from further investigations.

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