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AMINO ACID PROFILE AND THE ANTIOXIDANT STATUS OF MISTLETOE LEAF EXTRACT IN OESTRADIOL VALERATE-INDUCED POLYCYSTIC OVARIAN SYNDROME RATS

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ABSTRACT

Viscum . species (loranthaceae) have been shown to demonstrate antioxidant activity. The biological activities of *Viscum* plant is attributed to the mixture of its pharmacologically active constituents including amino acids. Antioxidant potential of a Nigeria specie of *Viscum* (*Tapinanthus*) aqueous leaf extract was evaluated in oestradiol valerate-induced polycystic ovarian syndrome (PCOS) rats. Thirty female rats (175.84 ± 2.04 g) were assigned to groups A-F (n=5). Group A non-induced and B-F (PCOS-induced), using single dose intramuscular injection with 2 mg/kg body weight oestradiol valerate (OV), followed by oral administration with distilled water to group A (Control) and group B (PCOS-induced untreated), then metformin (standard drug) to group C. Groups D, E and F received 50, 100 and 200 mg/kg body weight of extract respectively, once daily for 30 days. The extract contained 9 essential and 6 non-essential amino acids. Glutamate (17.33 ± 0.01 mg/ml) was the highest and cysteine (1.25 ± 0.01 mg/ml) was the lowest. The extract also inhibited radicals of 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid with IC_{50} 26.86 µg/ml and 2,2-diphenyl-1-picryl-hydrazyl IC_{50} 0.41 µg/ml. The OV-treatment significantly decreased ($p<0.5$) serum activities of superoxide dismutase and catalase, vitamin C and total antioxidant capacity compared to the control. Administration with extract significantly increased the serum antioxidants similarly ($p>0.5$) to the control and metformin-treated groups. In conclusion, the *Viscum* leaf extract enhances antioxidant status of oestradiol valerate-induced PCOS rat which may combat PCOS-induced oxidative stress and protect from cellular damage.

Key words: *Tapinanthus,* oestradiol valerate, polycystic ovarian syndrome, oxidative stress.

INTRODUCTION

The exact cause of polycystic ovarian syndrome (PCOS), a common endocrine disorder in woman of reproductive age is not well understood but have been attributed to metabolic abnormality and oxidative stress (De Leo *et al.,* 2016; Bannigida *et al.,* 2018; Zhang *et al.,* 2019). Oxidative stress is a pathophysiologic imbalance in free radicals generation and the antioxidant defense systems in which the equilibrium favours formation of the free radicals (Muhammadi, 2019). Optimum concentration of free radicals and antioxidant have been shown to play important physiological processes involved in oocyte maturation, ovarian steroidogenesis and ovulation (De Leo *et al.,* 2016; Bannigida *et al.,* 2018). Free radicals, in form of reactive oxygen species (ROS) or reactive nitrogen species (RNS), contain an unpaired valence shell electron(s) which are unstable and highly reactive. Antioxidant systems (enzymatic and non-enzymatic) serve to scavenge or detoxify excess free radicals, in order to regulate the level thereby preventing resultant oxidative stress, cellular damage and apoptosis accompanying accumulation of free radicals (Zhang *et al.,* 2019). Excessive free radical generation has been reported to negatively affect reproductive stages, which in turn affect fertility in the organism (Enechukwu *et al*., 2019), some proposed possible mechanisms include elevated radical related

increased metabolism and steroidogenesis, leading to ovarian epithelium DNA damage, oocyte damage and ovarian cell apoptosis. The radicals may also be involve in regulation of ovarian mesenchyme growth, leading to mesenchyme hyperplasia that occurs in PCOS or caused Ovulation induced-inflammation and the associated inflammatory response enhancing further generation of hydrogen peroxide radical and causing oxidative stress (Farzadi *et al.* 2013). Oxidative states have been evaluated using antioxidants. Natural antioxidants from medicinal plants including the *V. album* have been reported to mitigate free radical damage in abnormal conditions (Turkkan *et al.,* 2016; Katerji *et al.,* 2019; Nwozo *et al*., 2019). This necessitated assessing antioxidant effect of the leaf extract in PCOS condition.

Viscum species called mistletoe in English, locally as *Afomo, Kauchi and Apari* by the Yoruba, Hausa, and Igbo languages of Nigeria respectively has oblong evergreen leaves and is hemi-parasitic, usually found attached to the trunk of various broad leaf trees like citrus, cocoa and kola-nut trees (Brahma *et al.,* 2016). It is traditionally used in south-east and south-west parts of Nigeria to manage metabolic diseases (diabetics, hypertension and stroke). Crude leaf extract of *Tapinanthus globiferus* a specie of the *Viscum* has been reported to contain antioxidant compounds that include zinc, selenium, vitamin C, phenols and flavonoids (Brahma *et al.,* 2016, Oseni *et al*, 2022). Other species have also been reported to reduce oxidative stress and increases antioxidant status in diabetic rats (Turkkan *et al.,* 2016; Nwozo *et al*., 2019). However, antioxidant effect of the *Viscum species* in chemically induced PCOS condition is not well documented. This study therefore, assessed the antioxidant potential of a Nigerian specie of the *Viscum* in oestradiol-valerate-induced polycystic ovarian syndrome rats using the selected *in-vitro* radical scavenging assays and *in –vivo* enzymatic and non-enzymatic antioxidant biomarkers.

MATERIALS AND METHOD

Chemicals and Reagents

Reagent kits used to assay superoxide dismutase and catalase activities, vitamin C and the total antioxidant capacity status were products of Agappe Diagnostics, Knonauerstrasse 54-6330 Cham-Switzerland GmbH. All other reagents used were products of Sigma Aldrich Ltd. Busch, Canada.

Plant Material and the Preparation of Plant Extract

Leaves *of Tapinanthus* grown on kola nut host tree in Osun grove, Osogbo, South-western Nigeria after duly identified and authenticated in the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria, (voucher specimen number UILH/ 001/1210) were collected, handpicked from their stem branches, rinsed under running tap water and air dried under shade. The dried leaves were pulverized using a mechanical grinder. A 100 g of the powdered sample were extracted in 500 ml of distilled water for 48 hours, filtered through whatman no 1 filter paper and the filterate was freeze dried. A 10.87g of the residue which correspond to 7.61 % yield of the extract was obtained, this was reconstituted in distilled water to 50, 100 and 200 mg/kg body weight that were administered (Oseni, 2018).

Experimental Animals

Thirty female wistar rats with average weight range of 175.84 ± 2.04 g used for the study were obtained from Department of Biochemistry, University of Ilorin, Nigeria. The animals were put in perforated plastic cages, placed in well ventilated animal house maintained with normal atmospheric condition and 12 hours dark-light cycle. The rats were placed on standard pellet feed (Top Feeds Grand **C**ereals; Premier Feed Mills Co. Ltd, Nigeria) and free acess to tap water throughout the experimental period. Animals were handled using (NAC. 2011) guidelines and as approved by university of Ilorin ethical review committee (UERC/ASN/2015/222).

Experimental Design

Following one week of acclimatization, the thirty rats were assigned to six groups (A-F) of five animals each. Animals in group (A) served as control that received only 0.5 ml of distilled water throughout the experiment. Groups B-F constituted the polycystic ovarian syndrome (PCOS-Group), which were induced using single

intramuscular injection with 2 mg/kg body weight oestradiol valerate (Amini *et al.,* 2016) and were then treated as follow: Group-B were given 0.5 ml of distilled water, Group-C received 2.4 mg/kg body weight of metformin (a PCOS reference drug). Groups D, E and the F were administered with 50, 100 and the 200 mg/kg weight of *Viscum* extract respectively. The distilled water, metformin and extract were administered orally using plastic cannula once daily and for thirty days.

Blood Collection and Preparation of Serum

At the end of the treatment period, animals were sacrificed under diethyl ether anaesthesia. The rats were quickly removed from the jar and the neck region was cleared of fur. Jugular veins were cut off and a 5 ml of the venous blood were collected from each animal into appropriately labelled plain sample bottles. The blood was allowed to clot and after full clot retraction, the supernatant serum required for analysis was obtained by standard procedure (Burtis and Ashwood, 2001)

Biochemical Assay

Amino acid profile

Amino acid composition of *V. album* was estimated by Ninhydrin colorimetric method described in Sadasivam and Manickam, (2008). In its principle, alpha-amino acid is decarboxylated to Hydrindantin by Ninhydrin, which in presence of more Ninhydrin and ammonia yield a bluish purple colour product whose absorbance was read at 570 nm. Briefly, 10 ml of 80% ethanol was added to 10 g of the pulverized leaves, centrifuge at 4000 *g* for five minutes and the supernatant was evaporated. A 0.1 g of the resulting extract was added to 1 ml of Ninhydrin solution, made up to 2 ml with distilled water in a test tube and the tube was heated in a boiling water bath for 20 minutes. A 5 ml of the diluent was added and mixed, after 15 minutes, the absorbance of the purple colour developed was read against a reagent blank at 570 nm. The amino acid concentrations was extrapolated from a standard curve and expressed as percentage equivalent of leucine.

The 2, 2- azinobisethylbenzothiazoline-6-sulphuric acid

The 2,2 azinobis-3ethylbenzothiazoline-6-sulphuric acid (ABTS) assay was carried out Re *et al.* (1999). An equal volume of 7mM ABTS solution and 2,4 mM potassium persulfate solution were mixed and incubated at 37 in the dark for 12 h. The mixture was diluted by mixing 1 ml of ABTS solution and 60 ml of methanol. Fresh $ABTS^+$ solution was prepared for each assay. A 1 ml of the plant extract was allowed to react with 1 ml of the $ABTS^+$ solution in the tube, incubated for 7 minutes at room temperature and the change in absorbance, if the blue – green chromophore $ABTS^+$ radical reduces in presence of an antioxidant to colourless non-radical ABTS was read at 734 nm. The $ABTS^+$ scavenging capacity of the extract was compared with that of butylated hydroxyl toluene (BHT) standard and the percentage inhibition was calculated as follow:

$$\% \text{ Inhibition} = \frac{A\text{Sample} - A\text{blank} \times 100}{A\text{Standard}}$$

Where percentage inhibition is the ABTS radicals scavenged and A is the absorbance for sample, blank and standard respectively.

The 1,1-Diphenyl-2-picrylhdrazyl radical scavenging assay

Inhibition of 1,1-Diphenyl-2-picrylhdrazyl **(**DPPH) by the extract was determined Vicas *et al* (2008). The method is based on reduction of the violet DPPH radical to a colourless non-radical DPPH-H in presence of a reducing agent and absorbance of disappearance of the purple colour read at 517nm. Varying concentrations (25 - 250 µg/ml) of the extract were prepared, 2 ml each of the concentrations was mixed with 2 ml of 0.1 mM DPPH solution in methanol. The mixture was incubated at room temperature in the dark for 30 minutes. The absorbance was read against blank at 517 nm. Values obtained were converted to percentage antioxidant activities using:

$$\% \text{ Inhibition} = \frac{A\text{Sample} - A\text{blank} \times 100}{A\text{Control}}$$

Where percentage inhibition is the DPPH radicals scavenged and A is the absorbance for sample, blank and control respectively. The IC50 of the extract were obtained by linear regression analysis of dose–response curve plotting between the different concentrations and % inhibition

Determination of superoxide dismutase activity

Activity of superoxide dismutase (SOD) in serum was determined by spectrophotometric method previously described Katerji *et al* (2019). A 2.5 ml of 0.05 M phosphate buffer (pH 10.2), 0.2 ml of the serum sample and 0.3 ml adrenalin were added in the test tube. Blank was reconstituted by replacing the serum with distilled water. The solutions were mixed and increase in absorbance at 480 nm by inhibiting the oxidation of adrenaline to adrenochrome per minutes was read every 30 seconds for a period of 150 seconds. One unit of the enzyme was defined as that amount of the enzyme which causes 50% inhibition for the reduction of adrelanaline, determined by

$$\text{Increase in absorbance / minutes} = \frac{A_3 - A_0}{2.5}$$

Where, A_0 = Absorbance after 30 seconds

A_3 = Absorbance after 150 seconds

$$\% \text{ inhibition} = \frac{\text{Increase in absorbance of substrate x 100}}{\text{Increase in absorbance of blank}}$$

Determination of catalase activity

Catalase activity was measured using spectrophotometry. The reaction mixture (1.5 ml) contained 1 ml of 0.01 M phosphate buffer, pH 7.0, 0.1 ml of test serum sample and 0.4 ml of 2 M H_2O_2. A 2 ml of dichromate acetic acid reagent (5% potassium dichromate: glacial acetic acid in ratio 1:3) was added in order to stop the reaction. Decrease in absorbance due to decomposition of H_2O_2 was monitored and the change in absorbance was read at 620 nm. One unit of catalase will decompose 1.0 µmole of H_2O_2 under specified assay condition. Catalase activity was determined as µM of H_2O_2 consumed/ min/mg/protein and expressed in (U/L) international unit per liter (Burtis and Ashwood, 2001)

$$\text{Catalase activity (units/ mg)} = \frac{\Delta\text{Absorbance / min} \times 1000}{43.6 \times \text{mg protein/ml reaction mixture}}$$

Determination of vitamin C concentration

Vitamin C concentration was estimated using colorimetric method (Burtis and Ashwood 2001). Vitamin C in the serum was oxidized to its de-hydro form by cupric sulphate. The dehydroascorbic acid reacted with 2, 4 – dinitrophenylhydrazone (DNPH) in presence of concentrated sulphuric acid solution to form a red colour. Absorbance of the colour which was read at 520 nm is proportional to the concentration of vitamin C in the serum. Ascorbic acid was used as standard. Concentrations of vitamin C in the sample were determined using;

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 2.0 \text{ mg/dL [standard]}$$

Total antioxidant capacity in serum

The total antioxidant capacity (TAC) was determined using spectrophotometric method by Apak *et al*. (2004). A known volume (20 µL) of trolox standard with varying concentrations of 0, 0.26, 0.58 and 0.90 µM were pipetted in test tubes. A 20 µL of the test serum samples were pipetted into the corresponding tubes. A 100 µL of the cupric working reagent (constituted by adding 100 µL of Cu^{2+}- neocuproine in phosphate buffer (pH 7.4) and 8 µL of the hydroxylated probe) was also pipetted to all reaction tubes. The contents in the tubes were thoroughly mixed by gently tapping the tubes and then incubated at room temperature for 30 minutes. The Cu^{2+} was reduced to Cu^{+} in presence of antioxidants. The resulting Cu^{+} reacts with a chromogenic compound to form a coloured complex. The intensity of the colour at 570 nm is directly proportional to total concentration of antioxidant in the sample. The total antioxidant concentration was determined from the interpolation of the change in absorbance for trolox the standard calibrator using the following equation:

$$[\text{TAC } (\mu M)] = \frac{\text{A sample} - \text{A blank x dilution}}{\text{Slope } (\mu M^{-1})}$$

Data Analysis

Data are presented as mean of five determination ± standard error of mean (SEM). The significance of difference among groups was determined by One-way Analysis of Variance (ANOVA), Duncan's Test was used for the Post Hoc analyses of the multiple comparisons and $p < 0.05$ was accepted as significant. The charts were drawn using graph pad prism 5.

RESULTS AND DISCUSSION

Results of the selected antioxidants that were assessed to evaluate antioxidant potential of mistletoe leaves aqueous extract in oestradiol

valerate-induced PCOS animals revealed presence of nine essential and six non-essential amino acids (75 %) of total naturally occurring amino acids in the extract (Table 1). Amino acids can serve as antiradical compound by donating electron (s) which may reduce or scavenge free radicals (Zou, 2016; Tejpal, 2017). Therefore the presence of amino acids can confined redox balance potential on the extract. For instance, imidazole, phenol, and the pyrrolidine rings of histidine, tyrosine and proline respectively, may serve as hydrogen donors with the NH and OH groups. The thiol (SH) group in cysteine and methionine may provide chelating sites for pro-oxidant metal ion radicals. Furthermore, glycine, isoleucine, valine and phenylalnine may be involved in formation of hydrophobic peptide molecules capable of scavenging free radicals

The extract also inhibited and reduced radicals of ABTS with an IC_{50} of 26.86 µg/ml and the DPPH (IC_{50} 0.28 µg/ml) in a concentration depended manner (Table 2). The DPPH and ABTS radicals scavenged, forming non-radical 2,2-diphenyl-1-picrylhydrazine (DPPH-H) and the 2,2′-Azino-bis-3-ethylbenz-thiazoline-6-Sulfonic acid non radical cation ($ABTS^{+)}$ which is an indication of electron migration or hydrogen atom transferred action, a major role of reducing property (Zou, 2016; Katerji *et al*., 2019) and the IC_{50} values of <50µg/ml reported for a strong and potent antioxidant as shown in the extract can justify an antioxidant potential for the plant. Amino acids content, % inhibition of DPPH and ABTS radicals, our observations in this study is similar to the range reported in mistletoe species collected from different host trees elsewhere (Tejpal, 2017; Oseni *et al* 2022; Ulla *et al* 2022).

Furthermore, serum activities of superoxide dismutase (SOD) and catalase, vitamin C concentration and the total antioxidant capacity (TAC), which were significantly ($p<0.05$) decreased by 10.31%, 33.73%, 37.04 % and 17.96 % respectively in the oestradiol valerate-induced PCOS animals, were elevated following administration with the extract. The increase was significantly higher compared with the PCOS-induced un-treated group (Figures 1 - 4). The reduced SOD, catalase, vitamin C and the TAC in the PCOS animals may suggest enhanced generation and the release of superoxide radical (O^{*}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{*}), singlet oxygen (O_2) as well as lipid derived radicals like hydroperoxyl radical (HOO^{*}) and alkylperoxyl radical (ROO^{*}), emanating from enhanced metabolism of androgen which is associated with pathophysiology of PCOS. Elevated level of these radicals might have caused increased utilization of the antioxidants in an attempt to scavenging, removal or prevention of chain reaction and the further formation of radicals. Consequence of the depletion, might account for decrease in the antioxidants which were observed in this study. These agree with the reduced SOD, catalase, TAC and vitamin C earlier reported by Sumithra *et al.,* (2015), Sak *et al.,* (2018), and Muhammadi, (2019).

Elevated activity, an indicative of increase in the serum, our observation after administration with *Viscum*, suggests that the extract may enhance the activation of SOD in dismutation of O^{*} to H_2O_2 and O_2 and decomposition of H_2O_2 to H_2O and O_2 by catalase, therefore, enabling these enzymatic antioxidants to cope with mopping up of generated superoxide and hydroxyl radicals associated with PCOS pathophysiology, this can minimize the ROS mediated oxidative stress effect in the PCOS-induced condition. The increased activity were similar to what was observed in earlier studies Hayyan *et al*. (2016) and Muhammadi, (2019). Increased serum levels of vitamin C and TAC in the PCOS group treated with 100 and 200 mg/kg extract imply that the extract may enhance scavenging superoxide anion, oxygen radicals, water soluble free radicals and lipid peroxidation derived radicals (Satish and Dilipkumar 2015; Mostafa *et al*., 2015).

The *in-vivo* antioxidants status corroborate with the *in vitro* antioxidant effect in this study, these consistently indicate possible antioxidant potential in the extract. The possible antioxidant mechanisms may include chelation of metal ions (Fe^{2+}/Cu^{2+}) such as complexation of glycine, alanine or methionine with cupper and then inhibition of the Fenton reaction (Gharda *et al.,* 2017). Structural and chemical characteristics including molecular mass, hydrophobicity, electron and hydrogen-bonding properties of the amino acid composition as well as the steric

properties of the amino acid residues at the C- and N-termini in peptides of the enzymes, being proteins (Ohashi *et al*., 2015; Zou, *et al.,* 2016; Najjar *et al*., 2017). Low molecular mass and hydrophobic amino acid peptide residues in SOD and catalase might have enhance the enzymatic activity by their impact on the conformation and accessibility of the enzymes' active sites. Leucine, isoleucine and valine may promote induction and activation of catalase. Glutamate, cysteine and glycine may also enhance synthesis of glutathione (GSH), a tripeptide (L-γ-glutamyl-l-cysteinyl-glycine) responsible for maintaining proper antioxidants state (Sumithra *et al*., 2015). These may all contribute to antioxidant effect of the amino acids in PCOS condition.

CONCLUSION

Aqueous extract of mistletoe leaf enhances antioxidant status in oestradiol valerate-induced PCOS rats. The amino acids acting singly or combined with some other constituents in the *Viscum* via their electron rich and donor property might have conferred the antioxidant potential, which may defend oxidative stress in the PCOS animals. The antioxidant potential may be part of mechanisms by which the *Tapinanthus* would exerts ameliorative effects in PCOS condition.

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