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CARDIOPROTECTIVE ACTIVITY OF COMBINED METHANOL LEAVES EXTRACTS OF *PHYLLANTHUS AMARUS* AND *TRICHOSANTHES CUCUMERINA* ON ISOPROTERENOL INDUCED MYOCARDIAL INFARCTED WISTAR ALBINO RATS

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ABSTRACT

This present study was aimed at evaluating the cardio protective activity of combined methanol leaves extracts of *Phyllanthus amarus* and *Trichosanthes cucumerina* on isoproterenol induced myocardial infarcted wistar albino rats. Healthy 48 wistar albino rats of both sexes between 8-12 weeks of age weighing 150 -200g was used for this study. The rats were divided into 8 groups, each consisted of 6 animals. Group 1 and 2 served as the negative control and positive control while group 3 to 5 received 500:500 mg/kg, 750:250 mg/kg, 250:750 mg/kg b.w. of *Phyllanthus amarus* and *Trichosanthes cucumerina* respectively, while group 6 and 7 where treated with 1000 mg/kg of *Phyllanthus amarus* and 1000 mg/kg of *Trichosanthes cucumerina*. Group 8 received standard drug (verapamil). The treatment lasted for 30 days. At the end of the treatment, blood was collected by puncturing the retro-orbital plexus and serum was collected. Serum creatinine kinase-MB, Troponin-1, MDA, SOD and catalase were determined using standard laboratory methods. Results showed that there was significant ($p<0.05$) decrease in the troponin-1 concentration between the group 5 that was administered 250 mg/kg and 750 mg/kg body weight of *Phyllantus amarus* and *Tricosanthes cucumerina* and group 8 which was treated with standard drug (verapamil). Rsults also showed that there was marked significant ($p<0.05$) reduction in creatinine kinase in group 6 administered 1000 mg/kg body weight of *Phyllantus amarus*. Group 3 and 4 administered with 500:500 mg/kg, 750:250 mg/kg body weight of *Phyllantus amarus* and *Tricosanthes cucumerina* and group 6

administered with 1000 mg/kg body weight of *Phyllantus amarus* respectively had significant reduction ($p<0.05$) in the concentration of malondialdehyde (MDA) and there was significant ($p<0.05$) reduction in superoxide dismutase (SOD) activity in all the groups when compared to the positive control. There was significant ($p<0.05$) increase in the catalase activity in all the groups except the positive control that received no treatment. However, combination of *Phyllantus amarus* and *Tricosanthes cucumerina* at doses of 750 and 250 mg/kg and dose of 1000 mg/kg body weight of *Phyllantus amarus* are considered to have cardio protective activity and possesses free radical scavenging effect.

KEYWORDS: Cardio protective, myocardial infarction, *Phyllantus amarus, Trichosanthes cucumerina.*

INTRODUCTION

Myocardial infarction (MI) is a complex phenomenon affecting the mechanical, electrical, structural and biochemical properties of the heart (Petrich *et al*., 2006). MI and the resultant complication in cardiac function is one of the leading causes of death for both men and women (WHO, 2002). Due to changing lifestyles in developing countries, particularly in urban areas. MI is making an increasingly important contribution to mortality statistics. Acute myocardial infarction (AMI) is one of the most common diseases among the developing countries (Sathisha *et al.*, 2011). It is commonly known as a heart attack, which occurs when there is a sudden block in blood flow in one or more of the coronary arteries and this cut off blood supply to a part of the heart muscle, causing necrosis (massive cell death, a permanent damage). If the block is severe, the heart can stop beating (cardiac arrest). Myocardial infarction usually begins in the endocardium and spread towards the epicardium (Bhagwat and Padmini, 2014). There are many symptoms of acute myocardial infarction but the most common is chest pain, which may travel into the shoulder, arm, back, neck or jaw. This type of pain always starts from the center or left side of the chest and remains for few minutes. The onset of symptoms in acute myocardial infarction is usually gradual, over several minutes and rarely instantaneous (Anwar *et al.*, 2016).

Antioxidant compounds, highly present in plants have shown protective effects against diseases without reducing their therapeutic efficacy. Moreover, there is a growing interest in the usage of natural antioxidants as a protective strategy against cardiovascular related problems such as ischemia reperfusion (You *et al*., 2007). The chemo-therapeutic agents, which inhibit the free radical formation and can reduce the risk of heart diseases, have gained

imperative value in the modern medicines (Ke *et al*., 2009). Herbal medicines having antioxidant properties, may therefore, have a protective role in cardiovascular diseases (Viswanatha *et al.*, 2010).

Phyllanthus amarus herb has found its traditional usefulness in several health problems such as diarrhea, dysentery, dropsy, jaundice, intermittent fevers, urogenital disorders, scabies and wounds. *Trichosanthes cucumerina* is used as an abortifacient, vermifuge, refrigerant, purgative, malaria, laxative, hemagglutinant, emetic, cathartic, bronchitis and anthelmintic. A novel is flavone glucosidal, 5, 6, 6'- trimethoxy- 3', 4'- methylene-dioxyisoflavone-7- O- beta- D- (2"- O- p- coumaroylgluco -pyranoside)1 has been characterized from the seeds of *Trichosanthes cucumerina*. The positive effects of the plant are due to the presence antioxidants in it. The dried seeds in are used for various ailments because of anthelmintic and antidiarrheal properties in it. Seeds have anti- bacterial, anti- spasmodic and insecticidal properties. Hot aqueous extract (HAE) of root tubers of *Trichosanthes cucumerina* exhibited significant anti- inflammatory activity (Saboo *et al.*, 2012).

The root extract of *Trichosanthes cucumerina* L. and the fruit juice tested cytotoxicity against human breast cancer cell lines, lung cancer cell lines and one colon cancer cell line with positive effect. The root extract inhibited more strongly than the fruit juice. Crude Ethanolic extract (EE) of *Trichosanthes cucumerina* showed significant blood glucose lowering activity in alloxan diabetic albino rats. The acetone extract of leaves of *Trichosanthes cucumerina* showed moderate larvicidal.

MATERIALS AND METHODS

Preparation of Plant Material

The leaves were washed with clean running water; they were allowed to air - dry for 21days. Then the dried leaves were placed in polyethylene bags and stored at -20^{0}C until use.

Preparation of the extracts

The leaves of *Phyllanthus amarus* and *Trichosanthes cucumerina* was chopped into small pieces. The dry leaves were ground into fine powder using an electric grinder. The powdered plant materials (1 Kg) each was sequentially extracted three times with 5 litres of methanol at room temperature for 48 hours by maceration method. The extracts were filtered through cotton wool and Whatman No. 1 filter paper and was concentrated with a rotary evaporator at 40^{0}C to dryness. The dried extracts were transferred to sample bottles which was placed in a

dessicator containing anhydrous sodium sulphate to remove any traces of water that could have been present. The dry extracts were kept in tightly stoppered bottles in a refrigerator for further analysis.

Experimental Animals

Healthy wistar albino rats of both sexes between 8-12 weeks of age weighing 150 -200g were used for the study. They were purchased from the animal house of zoology, University of Nigeria, Nsukka, Nigeria. The rats were acclimatized in different cages of five per cage (standard laboratory metal animal cage) for 14 days. The animals were maintained under good laboratory practice (12 hr light and 12 hr dark cycle at uniform temperature of 28 - 33^0C). All animals had free access to food (vital feed grower, Ibadan) and water. All the investigation involving the experimental animals was conducted in accordance with the accepted principles for laboratory animal use and care.

Experimental Design

The experimental rats were divided into 8 groups, each consisted of 6 animals.

Group 1 the control group, served as negative control and received a control diet and a single intraperitoneal (i.p.) injection of normal saline (2.5 ml/kg). Group 2 the isoproterenol induced group, served as a positive control and was given a single i.p. injection of isoproterenol, 2 mg/kg of bodyweight on 14th day and a control diet. Group 3 the rats were administered with combined methanol extract of *Phyllanthus amarus* and *Trichosanthes cucumerina* (50% / 50%), and was given orally once daily up to 30 days, followed by isoproterenol administration on 14th day. Group 4 the rats was administered with combined methanol extract of *Phyllanthus amarus* and *Trichosanthes cucumerina* (75% / 25%), and was given orally once daily up to 30 days, followed by isoproterenol administration on 14th day. Group 5 the rats was administered with combined methanol extract of *Phyllanthus amarus* and *Trichosanthes cucumerina* (25% / 75%), and was given orally once daily up to 30 days, followed by isoproterenol administration on 14th day. Group 6 the rats was administered with methanol extract of *Phyllanthus amarus* (100%), and was given orally once daily up to 30 days, followed by isoproterenol administration on 14th day. Group 7 the rats were administered with methanol extract of *Trichosanthes cucumerina* (100 %), and was given orally once daily up to 30 days, followed by isoproterenol administration on 14th day. Group 8, the Isoproterenol induced group, after the development of myocardial infarction, was treated with the standard drug Verapamil (5 µmol/kg body weight) [Sigma- SO292, USA].

The duration of the treatment was 30 days. At the end of the treatment, blood was collected from all the groups by puncturing the retro-orbital plexus and was allowed to clot at room temperature and serum was separated by centrifugation at 2500 rpm for 10 min. The animals were sacrificed to remove the heart for histopathological studies.

Estimation of serum creatine kinase-MB (CK-MB)

Methods and calculations used for estimation of serum creatine kinase-MB are as outlined in Hans (1974). The creatine kinase (CK) activity is dependent on the ATP/Mg2+ ratio and the optimum molar ratio is 1/1, each should be as specified in the reagent below. The auxiliary and indicator enzymes are pyruvate kinase (PK) and lactate dehydrogenase (LDH).

(i) Creatine + ATP CPK creatine phosphate + ADP

(ii) ADP + phosphoenolpyruvate PK ATP + pyruvate

(iii) Pyruvate + NADH + H + LDH Lactate + NAD+

A quantity, 0.50 ml of the serum, 0.70 ml of buffer/coenzyme mixture, 0.05 ml of LDH/PK suspension, 0.10 ml of GSH solution was pipetted into a cuvette, mixed and allows to stand for 15 min at 25°C and 1.75 ml of creatine-glycine buffer was added to it, mixed and the absorbance at 365 nm was measured.

Estimation of serum cardiac Troponin-I

A quantity, 100 µl of the sample and 100 µl of MAB-HRP conjugate was pipetted into a burette, mixed and incubated for 6 h at 4°C under gentle shaking. 200 µl of the substrate 0-phenylenediamine/H202 was added to it, mixed and incubated in the dark for 15 min, then 50 ml of 2 mol/L H2S04 was again added, mixed and the absorbance at 492 nm was measured.

Estimation of Lipid Peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) concentration formed, using the method of Okhawa *et al*., (1979). Malondialdehyde (MDA), the end product of lipid peroxidation, is a good marker of free radical–mediated damage and oxidative stress. The principle of this method consists in the reaction of MDA with thiobarbituric acid (TBA) in acidic conditions and at a higher temperature (90-100°C) to form a pink MDA-(TBA)2 complex, which can be quantified spectrophotometrically at 530 nm. In the procedure, 0.5mL of 20% TCA was added to 0.5mL of the tissue homogenate, then there was an addition of 1 mL of 0.67% TBA. The mixture was incubated at 100^{o}C for 15 min in a water bath, cooled and then added with 4 mL of n-butanol and centrifuged at 3000rpm for

15min. The absorbance of the clear pink supernatant was then read against a blank at 532 nm spectrophotometrically. The concentration of MDA is expressed in nmol / g of the tissue.

Determination of SOD activity

Determination of the activities of antioxidant enzymes (SOD and CAT) Superoxide Dismutase (SOD) activity in the liver homogenates of the rats was determined according to the method described by Sun and Zigma (1978).

Determination of catalase activity

The enzymatic activity of catalase was determined by the method of Clairborne, (1985). The principle is based on the hydrogen peroxide H_2O_2 degradation in the presence of the enzyme. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.4, 19mM H_2O_2 and 16.5 uL tissue homogenate. The consumption of H_2O_2 was monitored spectrophotometrically at 240 nm for 1 min. and the enzymatic activity is calculated according to the formula: K = 2.303/T x log (A1/A2) (4)

Where: K: Rate of reaction

T: Time interval (minutes)

A2: Absorbance at 60 seconds interval

A1: Absorbance at time zero

Statistical analysis

Data generated from the study were presented as mean ± SEM. Statistical analysis was done by One-way analysis of variance using the SPSS version 21.0. The mean difference at $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Table 1: Results of Cardiac biomarkers.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Troponin (µ/l)	31.0±1.41^{ab}	39.5±4.94^{a}	36.0±4.24^{b}	36.5±3.53^{b}	30.5±2.12^{d}	33.5±0.70^{c}	36.0±1.41^{b}	30.0±1.41^{d}
CK-MB (µ/l)	16.9±0.40^{a}	113.0±0.38^{d}	630±0.43^{a}	246±0.31^{c}	303.5±3.11^{b}	84.9±0.05^{e}	71.3±0.48^{e}	84.9±0.06^{e}

Values are expressed in mean±sem, values in the same column with different superscript vary significantly ($p<0.05$).

Table 2: Results of Antioxidants parameters.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
MDA (µmol/l)	2.0±0.07^{d}	3.5±0.70^{b}	1.5±0.07^{e}	5.8±21.2^{a}	1.5±0.70^{e}	3.9±0.70^{b}	2.9±0.07^{c}	1.8±1.40^{d}
SOD (µmol/l)	155.5±53.44^{c}	308.0±1.41^{a}	243.0±2.82^{b}	254.5±10.6^{b}	230.0±0.00^{b}	267.0±53.74^{b}	306.0±2.82^{a}	229.5±0.70^{b}
Catalase (µ/l)	99.0±0.01^{a}	57.9±1.01^{b}	91.4±1.03^{a}	84.32±0.03^{a}	83.45±1.41^{a}	83.21±0.45^{a}	83.11±0.00^{a}	81.23±0.23^{a}

Values are expressed in mean±sem, values in the same column with different superscript vary significantly ($p<0.05$).

DISCUSSION

Myocardial infarction is making an increasingly important contribution to mortality statistics. The term myocardial infarction refers to the death of cardiac myocytes. It occurs when myocardial ischemia, reduction in myocardial oxygen supply, surpasses a critical threshold and in the process overwhelms the inherent mechanisms for myocardial cellular repair (You *et al.*, 2007). Isoproterenol, a synthetic sympathomimetic β adrenergic agonist, inflicts myocardial damage through several mechanisms that include coronary insufficiency, oxidative stress, depletion of high phosphate energy, intracellular Ca^{2+} overload, and alteration of metabolism (Upaganlawa *et al.*, 2011). In this study cardioprotective effects of combined methanol extracts of *Phyllantus amarus* and *Trichosanthes cucumerina* at different ratio/inclusion was analyzed against isoproterenol induced myocardial infarction in albino rat, Group 2 rats were induced with isoproterenol without treatment showed high level of troponin and CK-mb. The findings are consistent with those of Patel *et al.*, (2010) and Khalil *et al.* (2015) who reported that ISO causes damage to the membrane sarcolemma of the myocardium and leakage of cardiac markers into the circulation. It was observed from the study that there was significant ($p<0.05$) difference in the troponin concentration between the group 5 that was administered 250mg/kg and 750mg/kg body weight of *Phyllantus amarus* and *Tricosanthes cucumerina* and group 8 which was treated with standard drug (verapamil) when compared with group 2 and other treated groups, this shows that 250mg/kg and 750mg/kg body weight of *Phyllantus amarus* and *Tricosanthes cucumerina* suggested the best dose inclusion that could ameliorate cardiac dysfunction. Troponin measurement is the gold standard in diagnosing MI, particularly the cardiac troponin T and I (Patel *et al*., 2010; Khalil *et al*., 2015). This is because troponin T and I in the cardiac muscle have different structures of amino acid than the troponin contained in skeletal muscle (Mythili and Malathi, 2015). The leakage of cardiac markers from the myocardium occurs due to the rupture of cellular membrane caused by oxidative damage (Patel *et al.*, 2010).

Creatinine kinase (CK) is an enzyme that catalyses the transformation of creatinine and ATP to creatine phosphate an ADP (McLeish and Kenyon, 2005). CK-MB rises in the serum at 4-9 hour after the onset of chest pain or cardiac cell damage (Sabesan and Narasimhan, 2015). The study also indicated that there was marked significant ($p<0.05$) reduction in creatinine kinase in group 6 administered 1000mg/kg body weight of *Phyllantus amarus* when compared to the positive control that didn't receive any treatment and other treated groups, this activity in reduction of creatinine kinase by *Phyllantus amarus* could be as a result of

phytochemicals it contains (Kiran *et al.*, 2011). This is in tandem with work done by Mtopip *et al*., (2019).

Auto-oxidation reaction in ISO generates abundant free radicals which may attack any cells in the body. However, the primary attack involves the formation of peroxyl radicals from polyunsaturated fatty acids (PUFA), which can be found in the cell membrane. The radicals attack the nearby fatty acids present in the membrane, and trigger the lipid peroxidation reaction (Patel *et al*., 2010). The oxidized product of ISO also increases the amount of ROS by interacting with sulfhydryl group to form superoxide anions, and subsequently, hydrogen peroxide (H_2O_2) (Akila and Vennila, 2016).

Increased concentration of malondialdehyde is an indication of oxidative stress, thus, the level of antioxidant defense systems is mostly dwindling in alloxan- induced diabetic experimental rats (Prince and Menon, 2000). Group 3 and 4 administered with 750 and 250 mg/kg body weight of *Phyllantus amarus* and *Tricosanthes cucumerina* and 1000 mg/kg body weight of *Phyllantus amarus* respectively had significant reduction ($p<0.05$) in the concentration of malondialdehyde when compared with the positive control and other treated groups. Superoxide dismutase acts on superoxide anion free radical (O^{2-}) and converts it into molecular oxygen and hydrogen peroxide (Younus, 2018). The study revealed that there was significant ($p<0.05$) reduction in superoxide dismutase activity in all the groups when compared to the positive control that was not treated. Any reduction in the activity of this catalase may cause deleterious effects as a result of superoxide and hydrogen peroxide assimilation (Oyedemi *et al*., 2010). This present study revealed that there were significant ($p<0.05$) increase in the catalase activity in all the groups except the positive control that received no treatment. Conversely, inclusion of *Phyllantus amarus* and *Tricosanthes cucumerina* at doses of 750 mg/kg and 250 mg/kg and dose of 1000 mg/kg body weight are considered to have cardio protective effect and reduction in free radical species.

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