DNP induced oxidative stress on blood components ameliorated by Pyrrole derivative of *Tinospora cordifolia.*

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ABSTRACT

The intoxications and deaths caused by 2, 4-dinitrophenol (DNP) exposure through drugs, food supplements have been frequently reported and no specific treatment has been implemented to the toxic effects of DNP in adults and children. In this study, the purified compound (TCCP), a bioactive molecule from *Tinospora cordifolia* exhibited radical scavenging activities. Hence, we investigated the *in vitro* effect of TCCP on oxidative stress induced by DNP. Blood samples from healthy donors were treated with DNP ($100\mu M/ml$) for 2h. The DNP induced oxidative damage and effect of TCCP pre-treatment were determined by assessing the serum levels of cytoplasmic, lysosomal and oxidative stress markers. TCCP effectively inhibited DNP-induced oxidative stress on blood components, which was evidenced by the levels of endogenously generated reactive oxygen species and hydrogen peroxide. In addition, the depletion of GSH (reduced glutathione) content in DNP-treated cells was prevented by TCCP. Also, experimental results reflected the decreased expression of inflammation marker such as lipid peroxidase, catalase, LDH (lactate dehydrogenase), and phosphatase levels in cells with TCCP pre incubated DNP treated group. Therefore, these studies suggest the use of TCCP as an antioxidant and anti-inflammatory agent.

1. INTRODUCTION

2, 4-Dinitrophenol (DNP) has historically been used in the manufacture of food coloring, clothing dyes, explosives, agricultural herbicides, insecticides and fungicides [1, 2]. The compound is known to uncouple mitochondrial oxidative phosphorylation and was used as an antiobesity agent early in the past century. Because of its potentially fatal adverse effects, including hyperthermia, cataract, agranulocytosis, hepatotoxicity, nephrotoxicity and cardiotoxicity, the compound was subsequently banned by the United States Food and Drug Administration [3]. Banning DNP sale for human consumption protects the general public but DNP is still sold mostly via internet sales. DNP is purchased and used by determined users who are not dissuaded from experimenting with DNP based on health threats. However, the popularity of DNP as a slimming aid has resurfaced again in recent years [4]. DNP is used by bodybuilders and extreme dieters for its fat burning properties through inhibiting efficient energy

(ATP) production in cells [5]. Through uncoupling mitochondrial oxidative phosphorylation by facilitating proton transport across the mitochondrial membrane, DNP leads to rapid consumption of energy without generating ATP and consequently, to increased fat metabolism. DNP is sold in different names like dinosan, dnoc, solfo Black, nitrophen, aldifen and chemox. Some internet sites have DNP available in bulk quantities, allowing users to purchase kilograms of DNP powder or hundreds/ thousands of DNP-containing tablets [6]. Water contamination due to spillage of DNP during transportation or from factories and industries leads to toxic injuries on exposure [7]. Moreover, adults and children from farming communities are vulnerable to toxic injuries from the chemical DNP present in pesticides and insecticides [8]. The classic phenomena noticed by overdose of phenol-based product, DNP is a combination of hyperthermia, tachycardia, diaphoresis and tachypnoea [9]. In animal studies, DNP has been shown to be teratogenic, mutagenic and carcinogenic; including developmental and reproductive toxicity has been reported [10]. Owing to acute DNP toxicity and preceding death, the patient is often profoundly hyperthermic associated with methaemoglobinaemia, a disorder with higher level of methemoglobin [11]. Methemoglobin is a form of abnormal hemoglobin that contains iron in ferric [Fe^{3+}] form rather than the usual ferrous [Fe^{2+}] form.

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Ferric [Fe^{3+}] iron has a decreased ability to bind oxygen but the ferrous iron has an increased affinity for bound oxygen. The binding of oxygen to methemoglobin results in an increased affinity of oxygen to the three other heme sites (that are still ferrous) within the same tetrameric hemoglobin unit. This leads to an overall reduced ability of the red blood cell to release oxygen to tissues [12].

Tinospora cordifolia from *Menispermaceae* family is one of the frequently used medicinal plants in Indian ayurvedic medicine as a tonic, vitalizer and as a remedy for various metabolic disorders [13]. The chemical constituents reported from this shrub belong to different classes, such as diterpenoid lactones, alkaloids, phenolics, glycosides, sesquiterpenoid, steroids, aliphatic compounds and polysaccharides [14]. Potential medicinal properties revealed by researchers include anti-allergic, anti-stress, anti-diabetic, antipyretic, antispasmodic, anti-inflammatory, anti-arthritic, antioxidant, anti-leprotic, antimalarial, anti-arthritic, anti-spasmodic, hepato-protective, immuno-modulatory and anti-neoplastic activities [15, 16].

Individual parts or whole *T. cordifolia* plant have been reported to improve the immune system and anti-oxidant properties in various *in vitro* and *in vivo* models [17]. Previously, the anti-inflammatory effect of *T. cordifolia* was seen on paw edema model in rats induced by carrageenan [18]. Several reports indicate anti-inflammatory activity of the decoction [19], alcohol extract [20], water extract of the stem of *T. cordifolia* [21, 22] and *T. cordifolia* extract has been proved to possess protective effect against asthmatic inflammation and oxidative stress[23]. Epicatechin from *T. cordifolia s*tem extract showed antioxidant property [24].

Number of studies has been documented on biological activities of *T. cordfolia* against various diseases, there is a paucity of information, especially against DNP induced oxidative stress with reference to lipid peroxidation and other antioxidant marker's status. As there is no specific antidote or specific management for individuals with DNP-related toxicity, the present study was conducted to evaluate the protective effect of the purified compound, a pyrrole derivative (TCCP) from *T. cordifolia* plant against the oxidative stress induced by DNP using blood components.

2. MATERIALS AND METHODS

2.1. Plant Material
2.1.1 Extraction and Isolation
Leaves of *Tinospora cordifolia* (Willd.) Miers from the family Menispermaceae, were collected from Mysuru, India. The plant was identified and authenticated by taxonomist Dr. Sudarshan, department of Botany, University of Mysore, Mysuru.

Shade dried and powdered leaves of the plant were soxhlet extracted with non-polar to polar solvent system. Since the bioactivity and the yield (1.6g/30g) obtained from butanolic fraction were high it was freed from solvent under reduced pressure to a thick green mass and used for further purification.

2.2. Column chromatography
The column chromatography (Length: 35cm; Bore: 2cm) was performed using 60-120 mesh silica gel to elute out individual components from the crude butanol extract. The mixture of ethylacetate and methanol (2:1, v/v) showed best result as eluting solvent mixture. The column was rinsed with the same solvent mixture and completely dried before use. The column was filled 3/4th with solvent mixture and the silica gel was packed approximately 2/3rd of the column length with simultaneous draining of the solvent to aid proper packing. The packing was performed after activating the silica gel at 100 °C for 1 h and gently poured on the top of the column with constant tapping to avoid air bubbles and cracks after mixing with ethylacetate and methanol. The column was run with crude butanol extract (200 mg) mixed with 2g of activated silica gel. The flow rate was maintained at 1 mL per min and the fractions collected were tested by HPTLC (High performance thin layer chromatography) using the solvent system toulene, ethylacetate, chloroform and acetic acid (6:2:5:2, v/v) for single spot. Fractions with identical spots and R$_f$ value were pooled together and named as TCCP, preceded for further analysis.

2.3. Chemicals
2, 4-Dinitrophenol (DNP), dichlorodihydrofluorescein diacetate (DCFDA), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], homovanillic acid (HVA), thiobarbituric acid (TBA), 1, 1, 3, 3-tetramethoxypropane (TMP), quercetin were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade reagents.

2.4. Evaluation of free radical scavenging activity
The free radical scavenging activity of TCCP was performed using DPPH and ABTS radical cation decolorization assay methods and IC50 was determined [25, 26].

2.5. Experimental design
Experiment was designed according to the method followed by Santhosh et al [27]. Venous blood from healthy, non-smoking human volunteers was drawn with informed consent according to the Institutional human ethical committee guidelines (IHEC–UOM No. 59/Ph.D/2011–12), University of Mysore, Mysore. Blood samples were collected in empty tubes and categorized into different groups: Group *I*- Control blood, Group *II*- DNP alone (100µM/ml), Group *III*- DNP pre-incubated with TCCP for 10min at 37˚C (1:1 ratio, w/w) and Group *IV*- TCCP alone (100µM/ml). The tubes were incubated at 37˚C for 2h and the serum samples were then separated from the cells by centrifugation at 3,000rpm for 15min and used to perform further experiments.

2.6. Determination of ROS levels
The endogenous reactive oxygen species (ROS) levels were measured as described by Driver et al [28] with slight modifications. Serum samples (10µl) from each group were

incubated with Locke's solution (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO₃, 5.0mM HEPES, 2.0mM CaCl₂, 10mM glucose, pH7.4) for 30min at room temperature followed by the addition of 10μM of DCFDA. The reaction mixture was incubated at 37°C for 30min and the resulting fluorescence was measured using a Varioskan multimode plate reader (Thermo Scientific, USA) with excitation and emission wavelengths of 480 and 530nm, respectively. ROS levels were quantified from a dichlorofluorescein (DCF) standard curve and expressed as pmol DCF formed/min/mg protein.

2.7. Determination of endogenously generated H_2O_2

The rate of H_2O_2 production was estimated by measuring the increase in fluorescence due to oxidation of HVA (a specific hydrogen peroxide sensitive dye) by H_2O_2 according to the method outlined by Barja et al [29]. The serum sample (0.2mg) was added to the reaction mixture containing 200μl HEPES buffered saline (HBS), followed by the addition of 10μM HVA, and incubated for 30min at 37°C. Samples were excited at 312nm and the resulting fluorescence was measured at 420nm.

2.8. Lipid peroxidation

Induction of oxidative damage was ascertained by measuring the extent of lipid peroxidation in the serum sample by estimating thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al [30]. An aliquot of the sample (1.0mg protein) was added to tubes containing 1.5ml of acetic acid (pH 3.5, 20% v/v), SDS (8% w/v, 0.2ml) and 1.5ml thiobarbituric acid (0.8% w/v). The mixture was heated in a boiling water bath for 45min. The adducts formed were extracted into 1-butanol (3ml) and the TBARS formed was read at 532nm (Beckman Du 730 life science UV-Visible spectrophotometer, Germany) and quantified using TMP as the standard.

2.9. Determination of GSH

The GSH levels were assessed fluorimetrically as described by Chandrashekar et al [31], with minor modifications. Briefly, serum samples (0.1mg protein) were added to 1ml formic acid (0.1M) and centrifuged for 10min at 10,000g. An aliquot of the supernatant was added to sodium phosphate buffer (0.1M, pH 8.0, 5mM EDTA) containing buffered formaldehyde (1:4 v/v, 0.1M formaldehyde: 0.1M Na_2HPO_4) and 0.1ml of o-phthaldi-aldehyde. The mixture was incubated for 45min at 37°C and the fluorescence was measured at excitation and emission wavelengths of 345 and 425nm. The concentration of GSH was calculated using the standard curve and the values were expressed as mM GSH/mg protein.

3.0. Activity of antioxidant enzymes
3.0.1. Catalase & Lactate dehydrogenase (LDH)

Catalase activity was determined by measuring the rate of hydrolysis of H_2O_2 at 240nm by the method described by Aeibi et al[32]. To the reaction mixture (1ml) containing H_2O_2 (8.8mM) in sodium phosphate buffer (0.1M, pH7.0), an aliquot of serum samples (0.05mg protein) was added independently. The decrease in absorbance was monitored for 3min and the activity was expressed as μmol H_2O_2 decomposed/min/ mg protein (Є-43.6/mM/cm).

The serum LDH levels were measured spectrophotometrically by a standard enzymatic method using commercial diagnostic kit (LDH kit, Agappe diagnostics, India) at 340nm and expressed as activity/min mg protein.

3.0.2. Acid (ACP) & Alkaline phosphatase (ALP)

Acid phosphates enzyme activity was measured by the method as discussed in Tenniswood et al [33], using pNPP as substrate with slight modification. The reaction was initiated by mixing 1ml of substrate solution (20mM pNPP in 0.1M acetate buffer, pH3.8) with serum (0.2mg). After 15 min of incubation at 30°C, the reaction was stopped by adding 3ml, 1M NaOH, a blank without addition of enzyme must be run. Product formed *p*-Nitrophenolate anion (PNP) was measured at 400nm (Beckman Du 730 life science UV-Visible spectrophotometer, Germany), against blank. Activity was expressed as amount of enzyme that converts *p*-Nitrophenylphosphate (PNPP) to PNP/min/mg protein.

Alkaline phosphates enzyme activity was measured by diethanolamine [N, N-Bis (2-hydroxyethyl) amine] method with slight modifications [34]. In a reaction mixture of 3ml, 2.9ml of 1M diethanolamine of pH 9.8 containing 0.50mM magnesium chloride buffer was mixed with 50μl, 0.67M of substrate (PNPP) and equilibrated for 5min at 37°C. 50μl of 0.1mg of serum was added, immediately it was mixed by inversion and increase in absorbance was recorded for 5 min at 410nm. Difference in A410 was obtained and enzyme activity was expressed as amount of enzyme that converts *p*-Nitrophenylphosphate (PNPP) to *p*-Nitrophenol/min/mg protein.

3.1. Protein estimation

The protein concentration was determined by the Lowry's method [35] using BSA as standard, at 660nm.

3.2. Statistical Analysis

The experiments were carried out in triplicates (n=3) and analyzed by applying one way analysis of variance (ANOVA). Values are expressed as mean ± SEM. Statistical significance between control and treated blood samples were determined by Dunnett's test. For all tests, $P<0.01$ was considered statistically extremely significant, $P<0.05$ significant and non significant when $P>0.5$.

4. RESULTS AND DISCUSSION

4.1. Effect of TCCP on radical scavenging activity and endogenous antioxidant markers- ROS and H_2O_2

For many years, a large number of natural compounds of varied structures derived from medicinal plants have been suggested as the major source of anti-oxidants and are capable of exerting protective effects against oxidants and inflammation in

biological systems. *T. cordifolia* has been traditionally used as herbal medicine for the treatment of various illnesses that involve inflammation and oxidative stress. It is well known that the anti-inflammatory and antioxidant activities are closely interrelated. The free radical scavenging activity of TCCP was found to increase with increase in concentration. The IC50 values assessed by DPPH and ABTS methods were 100μM and 89μM respectively.
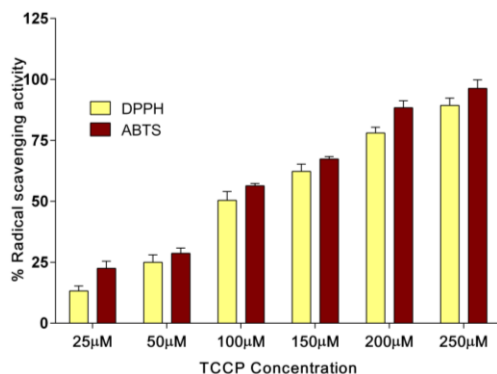

Fig. 1: Radical scavenging activity of TCCP by DPPH and ABTS methods.

Generally in RBCs, at normal physiological conditions, the reaction of heme with peripheral environment is controlled since it resides inside the pockets of heme proteins. However, during oxidative stress by pro inflammatory chemicals like DNP, hemoglobin tends to release its heme prosthetic groups. For this reason, the free heme produced becomes highly toxic to the cells by creating free radicals due to reduction of ferric (+3 ion) form to ferrous (+2) form which take part in Fenton reaction contained within its protoporphyrin IX ring. Within the RBC, deoxygenation of haemoglobin (Hb) is followed by the generation of O^{-2}. This reaction occurs to a lesser extent in normal RBC. But during stress, much more O^{-2} is generated leading to denaturation, precipitation, haemichrome formation and ROS production [36, 37]. Aqueous extract of *T. cordifolia* inhibited fenton reaction in a dose dependent fashion with an IC50 value of 700 mg/ml [38].

A significant enhancement in the levels of ROS (137 %) compared to control (p<0.01) was evident in the serum when the whole blood was incubated with DNP (100 μg/ml blood). However, the levels of ROS were significantly reduced by 38% when DNP was preincubated with TCCP and 50% in TCCP-alone group (Fig. 2a). On the other hand, a slight elevation in the levels of H_2O_2 was observed in the DNP-treated group. In case of group III, in which DNP was preincubated with TCCP, the levels of H_2O_2 were significantly reduced by 62% compared to control and in the case of TCCP alone, TCCP reduced the endogenous H_2O_2 levels by 68 % compared to the control group (Fig. 2b).

It is known that iron catalyzes the formation of ROS and thus alters the cellular redox state. Another plausible cause for oxidative stress in RBC may be the high concentrations of iron and iron-containing compounds, such as heme and Hb, in the plasma owing to haemolysis and blood transfusions [39].
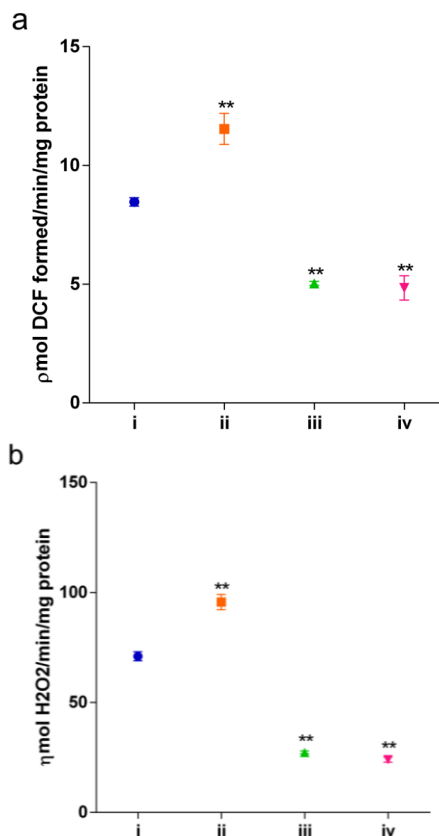

Fig. 2: Effect of TCCP on DNP- induced ROS generation and H_2O_2 levels in blood samples. i. Control, ii. Blood treated with DNP, iii. Blood treated with DNP that was pre- incubated with TCCP and iv. Blood treated with TCCP alone. Values are expressed as mean ± SEM of three replicate analyses. **p<0.01 considered significantly different compared to control.

Exposure of RBCs, platelets and polymorphonuclear leukocytes (PMN) to iron or heme increases their ROS level. The chronic oxidative stress of RBC, PMN and platelets in DNP treated blood renders them more susceptible to endogenous ROS mediated damage. Oxidative stress triggers eryptosis which outlines cell shrinkage, membrane blebbing and phospholipid damage of the cell membrane leading to phosphatidylserine translocation to the erythrocyte surface [40]. Thus, the free radicals generated affects the neighboring cellular components by peroxidation of unsaturated fatty acids in membranes, protein degradation and DNA fragmentation [41]. Several groups have suggested that molecules, which stimulate formation of ROS, can trigger apoptosis [42-44]. The exact mechanisms involved in cell death induced by ROS are not fully understood, and the protective effect mediated by some antioxidants remains controversial. The results in the present study show that pretreatment with TCCP substantially reduced ROS and H_2O_2 induced by DNP compared to DNP alone treated RBCs.

4.2. Effect of TCCP on intracellular lipid peroxidation and GSH status

Lipid peroxidation is considered to be the key event that indicates marked oxidative stress. Lipid peroxidation refers to the oxidative degradation of lipids. Assessing the end product of lipid

Peroxidation i. e., malondialdehyde (MDA) is a convenient tool for sensitive detection of oxidative damage. MDA, together with 4-hydroxynonenal (4-HNE), is a natural bi-product of lipid peroxidation and its quantification is generally used as marker for lipid peroxidation. One of the major endogenous antioxidant produced by the cells is GSH, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms. Data from this study clearly indicated that TCCP could inhibit lipid peroxidation and elevate GSH content in DNP treated RBCs. On the other hand, DNP treated RBCs showed elevated lipid peroxidation and drastic depletion in GSH content. Likewise, oral administration of aqueous extract of the roots from *T. cordifolia* resulted in a significant reduction in thiobarbituric acid reactive substances (TBARS) and an increase in GSH, catalase and SOD in alloxan diabetic rats [45].
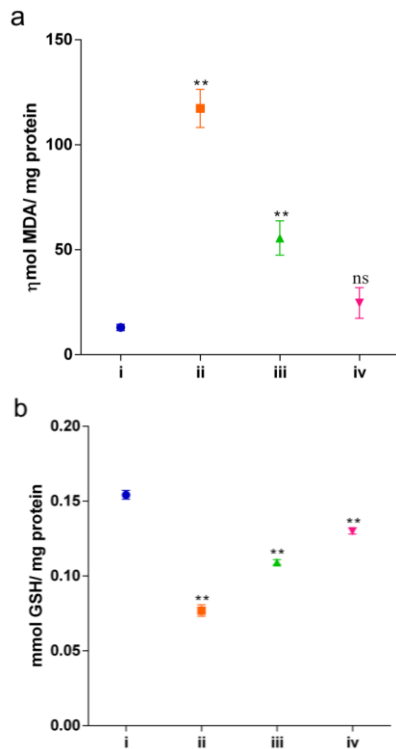
reverted to normality by 34 % when pre-incubated with TCCP (1:1 ratio, w/w). In addition, a slight decrease in the endogenous levels of GSH was seen in the TCCP-treated group (Fig. 3b).

4.3. Effect of TCCP on enzyme levels- Catalase, LDH, ACP and ALP

On the other hand, a dramatic elevation in the activity of catalase was seen in the TCCP- treated group (18.5-fold). In contrast, TCCP was able to restore the activity by 40% when preincubated with DNP. No significant alteration in enzyme activity of catalase was observed in group IV, TCCP alone (Fig. 4a).
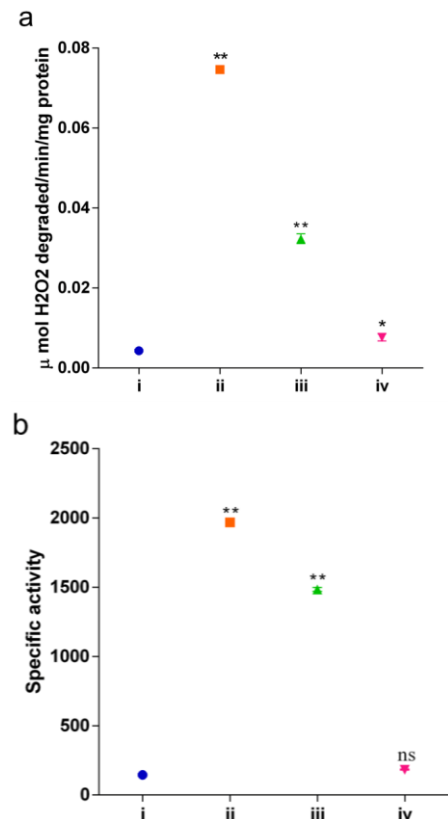

Fig. 3: Effect of TCCP lipid peroxidation and GSH in blood samples treated with DNP. i. Control, ii. Blood treated with DNP, iii. Blood treated with DNP that was pre- incubated with TCCP and iv. Blood treated with TCCP alone. Values are expressed as mean ± SEM of three replicate analyses. P > 0.05 = ns (not significant), **p<0.01 considered significantly different from control.

Fig. 4: Effect of TCCP on catalase activity and LDH level in DNP treated blood samples. i. Control, ii. Blood treated with DNP, iii. Blood treated with DNP that was pre- incubated with TCCP and iv. Blood treated with TCCP alone. Values are expressed as mean ± SEM of three replicate analyses. P > 0.05 = ns (not significant), **p<0.01, *p<0.05 considered significantly different compared to control.

In the present study the extent of lipid peroxidation was measured in terms of MDA levels and a considerable increase (1:9) was observed in the DNP treated group compared to control. Interestingly, in the pre-incubation group did not show any significant alterations but complete normalization of lipid peroxidation was observed in the TCCP-alone group (Fig. 3a). In the DNP-treated group, there was a drastic decline (66 %) in serum GSH level compared to control. The decreased levels of GSH

Similarly, there was a robust elevation of LDH (Fig. 4b), ACP (Fig. 5a) and ALP activities (Fig. 5b) in the DNP treated groups. TCCP exhibited significant protective effects by diminishing LDH, ACP and ALP levels by 25%, 46% and 24%, respectively, compared to the DNP-treated group. TCCP alone did not show any significant alterations in enzymatic activities.

There was an increase in level of both catalase and LDH in DNP treated serum due to erythrocyte damage. Catalase is an intracellular, peroxisomal and antioxidant enzyme that degrades hydrogen peroxide in to water and oxygen. It is possible that intracellular catalase may be released outside of cells, if the cells

are injured and further stimulate neighboring cells to produce COX-2 and iNOS. The root extract of *T. cordifolia* has been reported to decrease the concentration of glycosylated hemoglobin, plasma thiobarbituric acid reactive substances, GSH, SOD and catalase activity in heart and brain of diabetic rats [46, 47]. LDH is abundant in RBCs and acts as a marker for hemolysis. Reports available on root extract of *T. cordifolia* that lowers hepatic glucose-6-phosphatase and serum acid phosphatase, alkaline phosphatase, and LDH in diabetic rats [45]. In the same way, the current results define the low level of catalase and LDH in TCCP pre incubated DNP treated serum.
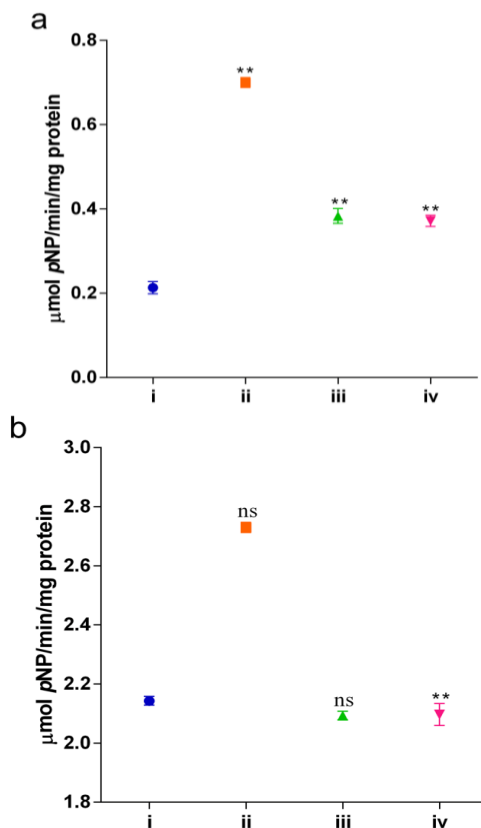

Fig. 5: Effect of TCCP on the levels of phosphotases (ACP and ALP) in blood samples treated with DNP. i. Control, ii. Blood treated with DNP, iii. Blood treated with DNP that was pre- incubated with TCCP and iv. Blood treated with TCCP alone. Values are expressed as mean ± SEM of three replicate analyses P > 0.05 = ns (not significant), **p<0.01 considered significantly different compared to control.

The induction of the enzymatic and non-enzymatic defensive mechanism on exposure to DNP could be an adaptive or a counteractive response that enables the cells to overcome the damage. From a previous clinical report, patient with symptoms of tachypnea and hyperthermia post consumption of DNP also showed increased LDH concentration (768U/L). The increase in serum LDH is an indirect evidence of hemolysis [2].

The decreased level of tissue enzymes, i.e., SOD, catalase and increased level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and acid phosphatase (ACP) were observed in mice treated with lead. Oral administration of aqueous stem extract and aqueous leaves

extract from *T.cordifolia* along with the lead nitrate (5mg/kg body weight, i.p. for 30 days) increased the activities of SOD and CAT and decreased the levels of AST, ALT, ALP, and ACP enzymes in mice [48]. Correspondingly, our results demonstrate the protective efficacy of pyrrole derivative against DNP-induced oxidative damage by stabilizing and reducing the levels of ACP and ALP in blood components.

5. CONCLUSION

In summary, the current study demonstrates the perturbances in blood components due to enhanced oxidative stress induced by DNP. The study also clearly demonstrates the efficacy of purified compound TCCP from *T. cordifolia* in neutralizing DNP induced oxidative stress in blood components. However, the molecular mechanism that is responsible for its protective activity of TCCP on blood components remains to be determined.

6. ACKNOWLEDGEMENT

Conflict of Interests: There are no conflicts of interest.

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