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In vitro anti-hypercholesterolemic activity of *Calotropis procera* (Aiton) using human erythrocytes

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ABSTRACT

This study evaluates *in vitro* hypocholesterolemic and antioxidant potential of *Calotropis procera* root extracts and its diterpenoid rich fraction. *In vitro* hypocholesterolemic activity was determined by measuring cholesterol concentration in the normal and hypercholesterolemic human red blood cells (hRBCs) after incubating with the test extracts (1, 10 and 100 μg/mL) for 24 h. The bioactivity guided fractionation of crude acetone root extract yielded a fraction with EC_{50} 63.03 ± 1.10 μg/mL in relation to ascorbic acid standard (EC_{50} 39.95 ± 1.25 μg/mL). In case of isolated erythrocytes of diseased donors, the purified fraction reduced the membrane cholesterol up to 1.62 ± 0.22 μmole CH/mL (52%), whereas it was 1.01 ± 0.75 μmole CH/mL (35%) in case of quercetin standard. The whole blood, when incubated with purified fraction and quercetin standard at 10 μg/mL for 24 h, it showed significant decrease in the cholesterol level of hRBCs of both, healthy ($P < 0.001$) as well as diseased ($P < 0.05$) donors. GC-HRMS analysis of the bioactive diterpenoid rich fraction revealed cyclic diterpenoids – Phenol, 2,4 bis (1,1-dimethylethyl) ester and 1,2 benzedenedicarboxylic acid, bis(2-methylpropyl) ester. In conclusion, *C. procera* root extract and its diterpenoid rich fraction exhibited potential hypocholesterolemic effect possibly by inhibiting membrane cholesterol synthesis and improving antioxidant levels in hypercholesterolemic hRBCs.

1. Introduction

Coronary heart disease (CHDs) is the major cause of death and disability in both developed and developing countries. It accounts for more than one third of total deaths globally and is becoming a worldwide epidemic with increasing clinical and economic burden (Kreatsoulas and Anand, 2010). A number of factors such as hypercholesterolemia, hypertension and smoking contribute to the development of CHDs (Patil et al., 2010). Hypercholesterolemia has been the major contributing factor for the development of CHDs (Patil et al., 2011). The strategies for preventing and treating hypercholesterolemia include dietary management, bile acid sequestration and inhibition of cholesterol biosynthesis. Besides, the existing study also shows involvement of free radicals in the etiology of CHDs (Fruchart and Duriez, 1994). It has been observed that, antioxidant levels are significantly lowered in the patients with symptomatic CHDs (Shanmugasundaram, 1995). Several studies have revealed the important role of cholesterol content of red blood cells (RBCs) membranes in the development of atherosclerosis (Tabas, 2002). Since the erythrocyte membrane contains the large amount of cholesterol, they are the important contributing factor for the development of atherosclerotic plaques (Zhong et al., 2012).

In recent times, the need for combination of multiple therapy approaches to overcome CHDs is widely accepted by majority of health care systems (Mohammad et al., 2013). Several epidemiological studies have demonstrated that diet with fruits and vegetables rich in antioxidants, are associated with lower incidences of CHDs (Aviram et al., 2005). Hence increased intake of natural antioxidants and hypocholesterolemic substances would have beneficial effects.

Many plants in the Indian system of medicine have been reported for anti-hypercholesterolemic properties and are rich sources of natural antioxidant (Devasagayam et al., 2004). *Clerodendron colebrookianum* (Boruah et al., 2014), *Commiphora mukul* (Ramesh and Saralakumari, 2012), *Celatrus paniculatus* (Patil et al., 2010), *Terminalia arjuna* (Patil et al., 2011), *Mucuna pruriens* and *Ionidium suffruticosum* (Dharmarajan and Arumugam, 2012) are known to have cardio protective activities. The antioxidants present in these plants have been shown to lower serum cholesterol levels by varying mechanisms and thus have potential to reduce the progression of atherosclerosis (Heber, 2001).

Calotropis procera (Aiton) R.Br. Asclepiadaceae is an ancient tribe shrub grown uncultivated and widespread in tropical

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regions. The plant is known for its abundant latex which is mainly recovered from the green part of the plant. Various parts of *C. procera* possess a number of biological activities mainly proteolytic (Kumar and Jagannadham, 2003), hypoglycemic, antioxidant (Yadav et al., 2014), anti-inflammatory (Freitas et al., 2012), analgesic (Dewan et al., 2000), anti-fertility (Kamath and Rana, 2002) and anti-arthritic (Kumar and Roy, 2007). Besides this various parts of *C. procera*, its latex has some medicinal activities like insecticidal, anti-fungal and wound healing (Cahiwut et al., 2010).Several bioactive molecules with different biological activities have been reported in *C. procera.* Phenol, 2,4 bis(1,1-dimethylethyl) ester with antioxidant and antiviral activity against spot syndrome virus have been reported (Prakash and Suneetha, 2014; Velmurugan et al., 2012).

The other species of the genus *Calotropis* such as *C. gigantea* is reported for its antioxidant and hypocholesterolemic potential (Jaiswal et al., 2014). However, to the best of our knowledge, no report is available on *in vitro* hypocholesterolemic potential of the root extracts of *C. procera*. In the present study, the methanol, acetone root extracts and purified fraction of *C. procera* were further investigated for its *in vitro* hypocholesterolemic, membrane stabilizing and antioxidant activities. The attempt has also been made to characterize and identify its active ingredient by chromatographic and spectroscopic methods. The structural confirmation of the compound has been done by GC-HRMS analysis.

2. Materials and methods

2.1. Plant material and preparation of extract

The roots of plant *C. procera* were collected from the agricultural out field near the campus of R. C. Patel Arts, Commerce and Science College, Shirpur, India. Plant was identified and authenticated by the expert taxonomist and the voucher specimen was deposited (RCP-02/2015) at Department of Botany, R. C. Patel Arts, Commerce and Science College, Shirpur, India. The roots were thoroughly washed, shade dried at 37 °C and grounded in grinder to obtain fine powder of it. The powdered roots (500 g) were subjected to successive extraction in the Soxhlet extractor for 48–78 h at 65 °C with increasing polarity of solvents like acetone and methanol. The extracts were filtered and subjected to dryness in rotary vacuums evaporator (Equitron, India) and stored in refrigerator until used.

2.2. Phytochemical screening

The total ash, acid soluble and water soluble ash values were calculated according to method described in the Indian Pharmacopoeia (2007). The preliminary phytochemicals like alkaloids, flavonoids, glycosides, tannins and triterpenoids were investigated as per previously reported methods (Aiyegoro and Okoh, 2010). Concentration of phenolic in the test extracts was calculated by linear regression analysis and the results were expressed as gallic acid equivalents (GAE).

2.3. In vitro antioxidant activity

2.3.1. DPPH radical scavenging assay

The DPPH radical quenching ability of the acetone, methanol crude extracts and purified fraction was performed as per the previously reported method (Aiyegoro and Okoh, 2010). Briefly, 1 mL of methanolic DPPH (2 mM) solution was mixed with different concentrations of test extracts (10–100 μg/mL) and ascorbic acid standard, after 30 min incubation in dark, absorbance was measured at 510 nm. The ability to scavenge the DPPH radical was calculated using the following formula:

$$\text{DPPH radical scavenging assay}(\%) = (A_0 - A_1) / A_0 \times 100$$

where A_0 is the absorbance of control, A_1 is the absorbance of sample.

2.3.2. Reducing power assay

The reducing power of the acetone, methanol crude extracts and purified fraction was determined as per the method of Aiyegoro and Okoh (2010). Different concentrations of the test extracts (10–100 μg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, cooled at room temperature, and then 2.5 mL of 10% tricarboxylic acid was added to the mixture and centrifuged at 600*g* for 10 min. The supernatant solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$) and the absorbance was measured at 700 nm. Increase in the absorbance of the reaction mixture indicated the reducing power. Ascorbic acid and phosphate buffer were used as standard and blank, respectively.

2.4. Membrane stabilization assay

Membrane stabilizing activity of acetone, methanol crude extracts and purified fraction was performed using human red blood cell membrane stabilization (hRBCs) assay performed as per the previously reported method (Oyedapo et al., 2010). Fresh human blood sample were collected into an anticoagulant (0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl) solution and centrifuged at 300*g* for 10 min at room temperature. Supernatant was removed and packed red blood cells were washed in normal saline (0.84% NaCl). Hematocrit (2%) was prepared and used for membrane stabilization assay as reported previously (Oyedapo et al., 2010). Test extracts (0.5–2.5 mg/mL) and ibuprofen standard was mixed with 2 mL hypo-saline, 1 mL sodium phosphate buffer (pH 7.4), and 0.5 mL hematocrit (2%). The reaction mixture was incubated at 56 °C for 30 min in water bath. After incubation, reaction mixture cooled at room temperature and again centrifuged at 600*g* for 10 min, the supernatant content was separated and released hemoglobin was estimated spectrophotometrically at 560 nm. The percent hemolysis was calculated by using following formula.

$$\%\ \text{hemolysis} = 100 - (A_1 - A_2)/A_3 \times 100$$

where, A_1 is absorbance of test, A_2 is absorbance of drug control and A_3 is absorbance of blood control.

2.5. In vitro hypocholesterolemic activity

In vitro hypocholesterolemic activity of all test extracts was carried out according to the method described in earlier report with slight modifications (Duchnowicz et al., 2012). Blood samples from hypercholestermic and healthy donors (sample size $n=6$) were collected from clinical pathological laboratories of Shirpur city. The study protocol was approved by the institutional research ethics committee (RCPREC 2/2014) and informed consent was obtained from each participant. Study was carried out on whole blood and isolated erythrocytes. The hRBCs were washed twice with phosphate buffer (pH 7.4) and centrifuged. The isolated erythrocytes (5% hematocrit) and whole blood were suspended separately in the incubation medium containing 140 mM NaCl, 10 mM KCL, 1.5 mM $MgCl_2$, 10 mM glucose, 100 μg/mL streptomycin, 5 mM Tris–HCl buffer (pH 7.4). Whole blood and hematocrit were incubated with and without test extracts, purified fraction and quercetin standard and for 24 h at 37 °C at concentrations