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Influence of Theobroma Cocoa Extract on the Increase of HDL Level (Good Cholesterol)

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Pradeep kumar S.V, S. B. Puranik and Nandini B. N.

Drishti Institute of Distance learning, Bangalore-560004, India.

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ABSTRACT

Aqueous ethanol extract of fruits of *Theobroma cacoa* Linn. (Family: Malvaceae) was evaluated for their *in vitro* HDL (good cholesterol) increasing and antioxidant activities. Aqueous ethanolic extract of cocoa fruits was examined for fold increase of Apo A1 level in Human hepatoblastoma cell line (HepG2) against positive control- Gemfibrozil, DPPH free radical elevation and Cholesterol, high-density lipoprotein cholesterol (HDL-C) was estimated. A dose dependent study of gemfibrozil (0-400Umol/L) and aqueous ethanolic extract of Theobroma cocoa fruit (0-500 mol/L) on Apo-A1 secretion for varying times (24 Hrs) was estimated by enzyme-linked immunosorbent assay (ELISA) and prepared the response curve. Aqueous ethanolic extract of cocoa fruit caused a significant increase in the secretion of Apo A1 indicating the increase of HDL-C at lower doses (0.1-10 µg/ml) with 1.2 fold increase compared to the positive control (Gemfibrozil- 1.3 fold increase). *In vitro* antioxidant activity (DPPH) evaluation of this aqueous ethanolic extract showing efficacy (IC50) at 128.0 µg/ml. In conclusion, aqueous ethanol extract of *cocoa fruits exerted* hypolipidemic and antioxidant effects.

INTRODUCTION:

Cacoa derived foods (cocoa powder) are phenolic-rich foods derived from the fermented, roasted and milled seeds of *Theobroma cacao* [1]. These products are consumed all over the world and largely studied because of its antioxidant and antiradical *in-vitro* properties of some of their phenolic constituents (Phenolic acids, procyanidins, and flavanoids) [2]. Cocoa extracts are rich in specific antioxidants particularly polyphenols, with the basic structure of catechins and epicatechin, and especially the polymers procyanidins, similar to those found in vegetables and tea. Metabolic epidemiological studies indicate that regular intake of such products increases the plasma level of antioxidants, a desirable attribute as a defense against reactive oxygen species [3]. The antioxidants in cocoa can prevent the oxidation of LDL-cholesterol, related to the mechanism of protection in heart disease. Antioxidants can terminate or retard the oxidation process by scavenging free radicals. These antioxidants are considered as possible protection agents for reducing the oxidative damage to human body from reactive oxygen species and retard the progress of many chronic diseases as well as lipid peroxidation [4, 5, 6]. In recent studies, polyphenols have been found to be beneficial as a strong antioxidant [7, 8]. The aim of this experiment is to evaluate the pharmacological activity mainly Antioxidant (free radical scavenging) activity and effect on ApoA1 secretion of the *Theobroma cocoa* fruit extract.

MATERIAL AND METHODS:

Plant Materials:

The vacuum dried 50% aqueous-ethanolic extract of theobroma cocoa fruits were gifted by the Phytoteck Extracts Pvt Ltd, 1564-1566, Nilgiris Supermarket Cross Road, St Thomas town PO, Kammanahalli, Banagalore-560084, India. The extracts were prepared by following procedure as described by the manufacturer. Dried fruit rinds of *Theobroma cocoa* were procured from a reputed herb supplier in Southern India. The fruit rinds were chopped, dried and milled and passed through 20 mesh. The RM powder (7 Kg) was extracted with 50% ethanol (30 L) at 70°C for 3 hours and filtered. This procedure was repeated twice and all the three filtrates were combined and vacuum evaporated.

Drugs and standards

The following chemicals were obtained from the source specified: Apo A1 Kit (Pointe Scientific, Inc. 2449 Research Drive, Canton, MI 48188), Bradford reagents (BioRad Protein Assay Reagent: Cat#500-0006 Kept at 4°C), Phosphate buffer (pH 7.4), EMEM (Lonza Walkersville Inc, USA), Sodium pyruvate Gemfibrozil, 1% glutamine-penicillin-streptomycin and 1% fungizone, FBS (Life Technologies, 3175, Stanley Road, NY 14072, USA), plant extract.

DPPH (SigmaCat.No.A2174), Ascorbic acid (Sigma), Methanol (Merck) Microplates, Plate reader, Micropipettes, Eppendorf's tubes, Incubator, timer etc

Routine maintenance of cell cultures:

All cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. HepG2 (American Type Culture Collection, Manassas , VA20108,USA) cell lines was grown in a T-75 flasks, fed fresh growth medium with 15 ml of EMEM (American Type Culture Collection, Manassas, VA20108, USA) containing 10% FBS (Flowlab, Australia), 1% Glutamine-penicillin-streptomycin (Flowlab, Australia) and 1% fungizone (Flowlab, Australia). Cells were subcultured when trypsinization with PBS containing 0.5 mmol/L EDTA when it reaches 80% confluency. Cells were treated with plant extract in serum-free EMEM (SF-EMEM). Immediately before use, TC hydroalcoholic extract was solubilized in ethanol. Final ethanol concentrations that were added to the cells did not exceed 0.5%. Untreated control cells received 0.5% (v/v) ethanol without extract.

Cell viability assay

A cell viability assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described by Mosmann [9], with minor modifications. Briefly, HepG2 cells at a density of 5,000 cells per well were seeded in a 96-well ELISA microplate. The cells were incubated at 37°C in 5% CO_2 for 24 h. After 24 h, the TC hydroalcoholic extract, at various concentrations (1–500 µg/ml), were added into the wells. The cells were left to grow in the incubator for 24 h. After 24 h, MTT reagent (Merck) was added, and the mixture was further incubated for 4 h. Next, the mixture in each well was removed, and formazan crystals formed were dissolved in 10 µl of 75% isopropanol. Spectrophotometric measurement of the mixture

was performed in a microplate reader (Bio-Rad) at wavelengths of 570 and 620 nm. A log plot of cell viability (%) against the concentrations of plant extracts was plotted.

Treatment of HepG2 cells with TC Hydroalcoholic extract:

Confluent HepG2 cells maintained in DMEM were treated with methanol extracts at 300 µg/ml, a non-toxic concentration determined from the MTT assay [10]. The cells were then incubated at 37°C for 24 h. As a control, cells were incubated in the absence of the ethanol extracts. After 24 h, cells were trypsinized and then precipitated by centrifugation at 1,300 rpm for 5 min. Cells were washed with PBS twice before total cellular RNA (tcRNA) was extracted from the cells.

DPPH scavenging activity:

The antioxidant activity of the extract was estimated on the basis of the radical scavenging effect of the stable DPPH [11]. Various concentrations of the *Theobroma cocoa* extract were added to an ethanolic 0.4 mM DPPH solution (0.1 ml) in a 96 well plate. The reaction mixture was shaken vigorously and allowed to stand for 30 min at 37°C. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was determined at 517 nm using UV-Vis microplate reader and ascorbic acid was served as a positive control. The lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The scavenging activity against DPPH was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1 - (A1 - A2) / A0] \times 100\%$$

Where $A0$ was the absorbance of control (DPPH solution without the extract), $A1$ was the absorbance of DPPH solution in the presence of the extract and $A2$ was the absorbance without DPPH solution

***In vitro* Apo A1 level Modulation study on HepG2 cell lines:**

ApoA1 is a major protein of HDL, initiates cholesterol efflux and thereby facilitates removal of excess tissue cholesterol by the process of reverse cholesterol transport. The *in vitro* Apo A1 level Modulation study was done by using an adapted method described by Naito, H. K 1986 [12], with slight modifications. Briefly, HepG2 cells were grown in T75 flasks with 15 ml of

EMEM containing 10% FBS, 1% glutamine-penicillin-streptomycin and 1% fungizone in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells were subcultured when cells become 80% confluent by trypsinization with PBS containing 0.5 nmol/L EDTA. Cells were plated in 96 well plate at a concentration of $3X10^6$ cells/ml. cells were allowed to grow until it reached a confluency of 80%, a dose response curve was prepared for hydroalcoholic extracts of *Theobroma cocoa* fruit at 0.1 to 500 µg/ml concentration for determining ApoA1 secretion at varying times till 24 hrs. At the termination of incubation, 50 µl of cell supernatant was collected to estimate Apo A1 concentration by ELISA. After terminating the reaction, the cell supernatant was removed and cells were washed twice with PBS. Cells were lysed with 100 µl of 0.1% SDS, from this 10 µl of sample was removed and 250 µl of Bradford reagent was added [13, 14]. At 590 nm readings were taken.

RESULTS:

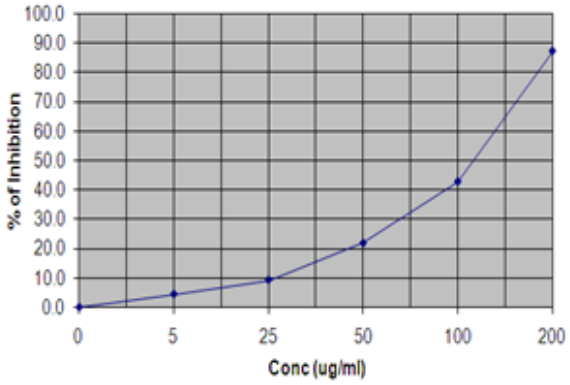
DPPH Scavenging Activity:

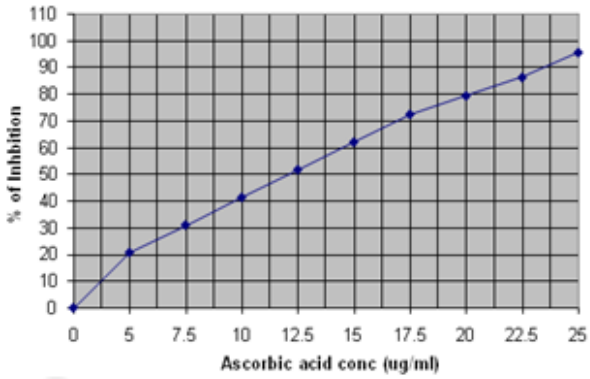
The DPPH assay was used as a preliminarily screen for antioxidant activity of the extract. The proton radical scavenging action is known as an important mechanism of antioxidants. Theobroma ethanol extract decolorized the purple of DPPH to the yellow of DPPH in a dose-dependent manner with an IC50 value of 108.2 µg/ml (Table 1). DPPH radical-scavenging ability of ascorbic acid in terms of IC50 value was 12.4 µg/ml.

Table 1:

Method	**Sample**	**Concentration (µg/ml)**	**Activity (%)**	**IC50 (µg/ml)**
DPPH	TCE	5	4.41 ±1.43	108.2
		25	9.93 ±1.21	
		50	21.80 ±1.02	
		100	42.72 ± 2.32	
		200	87.15 ± 3.42	
	Ascorbic acid	5	19.9 ± 1.03	12.4
		25	81.2 ± 2.46	
		50	83.42 ± 2.76	
		100	83.37 ± 3.12	

Antioxidant (DPPH) Activity of *Theobroma cocoa* fruit Aqueous ethanolic extract





***In vitro* Apo A1 level Modulation study of extract in human hepatoblastoma cell line (HepG2):**

Hydro-alcoholic extract of cocoa fruits showed a statistically significant increase in the Apo A1 level at lower doses of 0.1-10 µg/ml with 1.2 fold increase when compared to that of the positive control (Gemfibrozil) exhibiting 1.3 fold increase. However, on treatment with higher doses of 100 µg/ml, no increase in the level of ApoA1 was observed, whereas at concentration of 500 µg/ml, an inhibition was observed.

Hydro-alcoholic extract of cocoa fruits did not show any cytotoxic effect on HepG2 cells at all the studied doses (0.1 to 500 µg/ml) at 24 hrs of treatment.

Thus, the result seems to suggest that *Theobroma cocoa* fruits hydroalcoholic extract is an efficacious and potent extract in elevating the level of ApoA1 in human hepatoblastoma cell line (HepG2) at lower doses.

Table 2:

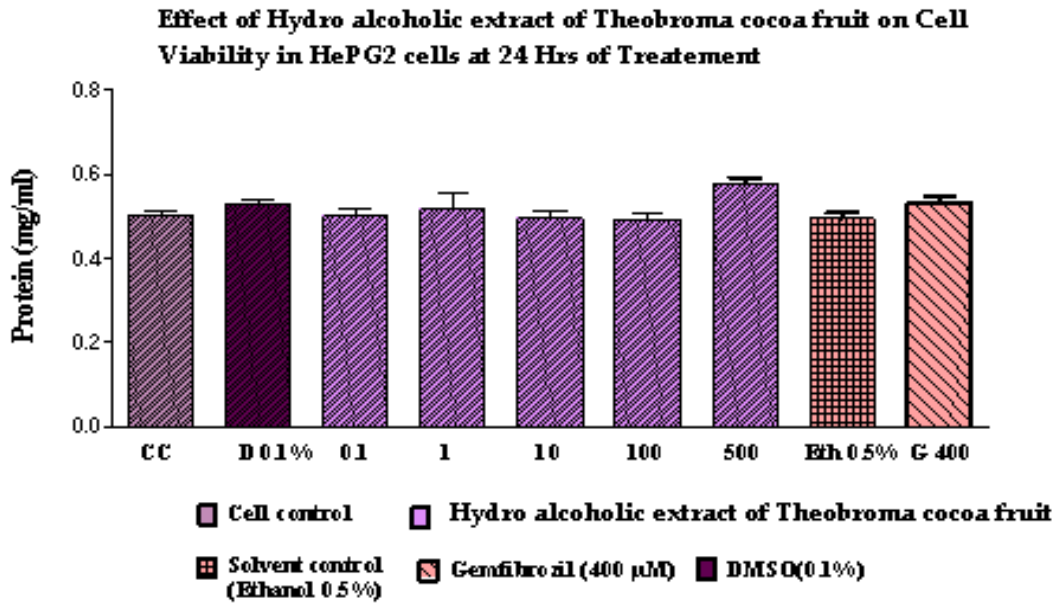
Sample*	Concentration (µg/ml)	ApoA1 (ng/ml per mg protein)	Fold increase (I) over respective controls**
Cell control	0	330 ± 10	---
DMSO	0.1%	350 ± 10	---
Hydro alcoholic extract of *Theobroma cocoa* fruit	0.1	420 ± 30	1.2**
	1	430 ± 27	1.2**
	10	410 ± 6.7	1.2**
	100	360 ± 3.4	---
	500	31 ± 2	---
Ethanol	0.5%	300 ± 0.28	---
Gemfibrozil	400 µM	380 ± 21	1.3**

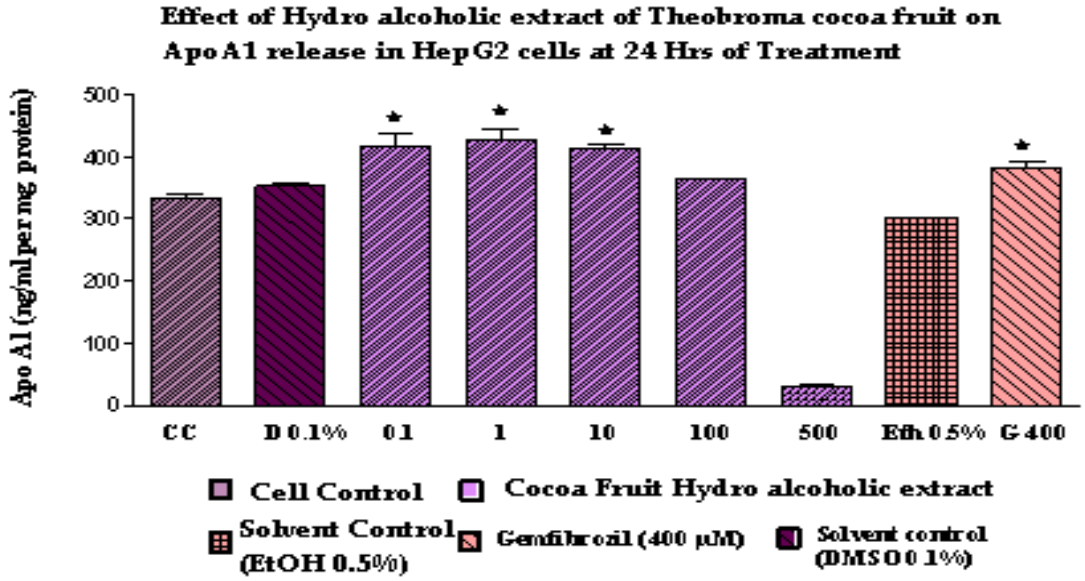
*The sample was diluted in water, sterilized through a 0.22 µ filter and used for the assay. The experiment was terminated at 24 hrs.

**Results are the mean ± SD of triplicates per sample. *Theobroma cocoa* fruit Hydro alcoholic extract is compared to the cell control while Gemfibrozil has been compared respective ethanol control.

**P < 0.01

Effect of *Theobroma cocoa* fruit hydro alcoholic extract on Apo A-I (a major protein of HDL) level in human hepatoblastoma cell line (HepG2).





DISCUSSION:

Apolipoproteins are the main components of the HDL and transporter lipoproteins play an important role in good cholesterol transport in the body. Apolipoproteins initiates cholesterol efflux and thereby facilitates removal of excess tissue cholesterol by the process of reverse cholesterol transport. We designed and conducted this study to investigate the effects of *Theobroma cocoa* hydroalcoholic extract on ApoA-I secretion. The present study provided the evidence that Hydroalcoholic extract of cocoa fruit is capable of influencing lipid and

apolipoprotein production. Initial studies examined the effects of the extract on Apo A1 secretion in HepG2 cells. Our results confirmed that *Theobroma cocoa* fruit hydroalcoholic extract is capable of increasing the HDL-C in a dose-dependent manner. Interestingly, this extract showed significant increase in the Apo A1 level at lower doses of 0.1-10 µg/ml with 1.2 fold increase when compared to that of the positive control (Gemfibrozil). Effect of the extract on the viability of HepG2 Cells was also checked at 24 hrs of treatment of cells with different extract concentration, indicating no alteration in cell viability.

Present study results also prove that *Theobroma cocoa* extract is a strong antioxidant and free radical scavenger. Antioxidant activity assessed by the DPPH method infers that, antioxidant activity of the extract increases in a dose-dependent manner with an IC50 value of 108.2 µg/ml (Table 1). DPPH radical-scavenging ability of ascorbic acid in terms of IC50 value was 12.4 µg/ml.

CONCLUSION:

Due to various pharmacological effects of the *Theobroma cocoa* fruits hydroalcoholic extract, majorly its effect on elevating the level of ApoA1 in human hepatoblastoma cell line (HepG2) at lower doses and strong Antioxidant activity, this extract could be used for the treatment of Hyper cholesterolemia. Subsequently, it can also be advisable to use consistently for the Cardiovascular health and free radical scavenging purpose.

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