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Preliminary evaluation of CETP inhibition from selected Garcinia species

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Abstract
Two types of Garcinia species which are *Garcinia parvifolia* and *Garcinia atroviridis* Griff ex T. Anders were selected and being labelled as UNMC 45L, UNMC 78T and UNMC 78T based on the folklore medicine 'myths' that claiming Garcinia species has the ability to be anti-cholesterol. All of these three plant parts were evaluated for therapeutic potential as CETP inhibitors by using CETP Inhibitor drug screening kit. Extraction of crude material from plants was performed via gradient maceration in hexane, ethyl acetate and ethanol. All of the extracts show significant inhibition towards CETP activity. Ethanol extracts of UNMC 45L shows greatest inhibition as the IC50 is 15.43 ± 0.4212 mg/ml followed by Hexane extract and Ethyl Acetate extracts of UNMC 78L which are 28.70 ± 1.320 mg/ml and 28.49 ± 1.126 mg/ml respectively. However, all of the extracts of UNMC 78T shows lowest inhibition towards CETP activity and it is assumed that more bioactive compound could be present in the leaves compare to twigs. The positive findings from this study suggest that Garcinia species was effective natural inhibitors towards CETP.

Introduction

According to the World Health Organization (WHO) (WHO,2011), the leading cause of death in the developed world is coronary heart disease due to atherosclerosis and it is expected to become the leading cause of death in the developing world within the first quarter of this century. The risk factors associated with atherosclerosis are hypertension, impaired glucose tolerance (IGT), central abdominal obesity, hypertriglyceridemia, increased serum levels of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) and decreased serum levels of high density lipoprotein (HDL) (Assmann *et al*, 1996).

Atherosclerosis is a progressive disorder of the arteries and always being characterized by thickening of lipids within the vessel wall, dysfunction of endothelial and vascular inflammation (Stary *et al.*,1995). The thickening of lipids leads to serious aetiologies of this disorder such as atheromatous plaques, vascular remodelling, acute and chronic obstruction of the vessel lumen, abnormalities in blood flow and limited oxygen supply to tissues and organs (Stary *et al.*, 1995). The hypothesis about the mechanisms of this disease is that it is based on the response-to-injury theory in

which endothelial injury will cause vascular inflammation and later leads to formation of cholesterol plaque.

In Malaysia, atherosclerosis is the main silence killer since three decade ago and it became the leading cause of death in developing countries. The symptoms that associated with atherosclerosis are heart disease and stroke and this make up 14.31% of total mortality reported in Malaysia by Ministry of Health (National Heart Association of Malaysia, 2010). The high incidence of atherosclerosis in Malaysia is probably due to the unhealthy sedentary lifestyle which being adopted since years ago coupled with oily food intake, smoking and drinking alcohol.

HDL metabolism is now being regarded as therapeutic strategy of atherosclerosis since epidemiological studies has shown that decreased in the level of HDL are the strong independent risk factor for cardiovascular disease (Kannel, 1987). CETP targeted therapy is one of the alternative to resolve this matter.

Cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein that is mainly bound to HDL (Tall, 1993). It is secreted mainly from the liver and circulates in plasma and its main function is to mediate the exchange of cholesterol ester (CE) in HDL for triglycerides (TG) in very low-density lipoprotein (VLDL) (Lagrost, 1994). CETP appears to play a proatherogenic role since the ability of CETP to lowers the cardioprotective HDL. A deficiency in CETP is associated with increased HDL levels and decreased LDL levels, a situation that is called as antiatherogenic (Garber, 1999). Because of this theory, inhibition of CETP activity would elevate HDL and provide a potential therapeutic benefit for patients having atherosclerosis and CHD (Garber, 1999).

Interest in CETP as a potential drug target has waxed and waned since the late 1980s when hyperalphalipoproteinaemia was first associated with CETP deficiency in Japanese men (De-Grooth *et al.*, 2002). The rapid progress has been made since 2002 when the first positive Phase II trials linking on CETP inhibition with HDL elevation and LDL lowering were reported for JTT-705 as monotherapy (De-Grooth *et al.*, 2002). This success has rekindled interest in this field and CETP is a major headline for atherosclerosis targeted therapy. Since 2002, remarkable transformation has occurred in just three short years. In 2002, Japan Tobacco, Bayer, Pfizer and Pharmacia were the major players pursuing CETP inhibitors (Pfizer, 2006). There are a few compounds that are in the clinical phase which are anacetrapib, torcetrapib and JTT-705. The use of anti-CETP antibodies, antisense oligonucleotides and a vaccine that induces antibodies for the inhibition of CETP activity are being discovered. The new era in pharmacology study is to isolate natural plant secondary metabolite as CETP inhibitors.

Garcinia species are distributed in Southeast Asia, India and West Africa (Wiart, 2006). There are 49 species out of 400 species had been documented in Malaysia (Jabit *et al.*, 2009, Nazre *et al.*, 2007). *Garcinia Atroviridis* Griff ex T. Anders could be classified under Guttiferae family. It is a native plant in Malaysia and always being known as " asam gelugor" or asam keping (Mackeen *et al.*, 2000). The young leaves of this tree can be eaten as 'ulam and the leaves is used in cooking of Malaysian cuisine (Masak Lemak cili api). In folklore medicine, *Garcinia atroviridis* has been used as

postpartum medication agent, earache, throat irritation, cough, dandruff and any stomach ache associated with pregnancy (Amran *et al.*, 2009, Johnson 1999, Krishnamurthy & Sapna, 2006). It is also possess anti-microbial, antioxidant, antitumour and anti-inflammatory activities (Mackeen *et al.*, 2000; Mackeen *et al.*, 2002).Garcinia Parvifolia is a native plant and the common name is also known as " asam kandis". It is normally being used especially in cooking to give the aroma, flavour and colour to the dishes such as curry and acar.

Due to the cholesterol lowering properties that the Garcinia species possess, the aim of the study is to see the efficacy whether the extracts from Garcinia species, have the ability to specifically inhibit the CETP activity.

Methodology

Principle of the assay kit

The CETP inhibitor drug screening kit (BioVision, Mountain View, CA, USA) uses donor molecule containing a fluorescent self-quenched neutral lipid which is transferred to an acceptor molecule in the presence of the CETP (rabbit serum). The lipid transfers of donor molecule to the acceptor molecule mediated by CETP will results in an increase of fluorescent intensity. Whereas, in the present of the inhibitor will hinder the lipid transfer and therefore will cause the decrease in fluorescent intensity.

Plant material

The leaves of *Garcinia Parvifolia* were collected from the lowland dipterocarp, Sungai Congkak Reserve Forest in Malaysia and a voucher specimen (45L) was deposited at the Herbarium of The University of Nottingham, Malaysia Campus. The Leaves and twigs of *Garcinia Atroviridis* were obtained from Bukit Ekspo, Universiti Putra Malaysia, Serdang around November 2012and the voucher specimen named as (78L) and (78T) were deposited at The University of Nottingham Malaysia campus.

Plant extraction

The plant materials were dried at the atmospheric temperature (~30 ˚C) and shaded from sunlight for 1 month. The dried plant were pulverised into smaller parts and subjected to maceration for over 3 days by using sequential gradient extraction of different polarity of solvents starting with hexane, ethyl acetate and ethanol. Solvents were removed after 3 days of maceration by using distillation under reduced pressure at 40˚C and later the crude extracts were pooled together for every and each specific solvent and this procedure were repeated for three times. All the extracts were dried in desiccators until it is concentrated. The residues obtained were designated as aqueous extracts and stored in freezer at -20˚C until assayed.

Table 1. The percentage yield extracted from UNMC78L. UNMC78T and UNMC45L from different solvent used in maceration

Scientific name	Plant part	Code	Extracts	% yield (dried weight)
Garcinia atroviridis	Leaves	78L	Hexane	11.328
			Ethyl Acetate	5.059
			Ethanol	3.805
	Twigs	78T	Hexane	8.435
			Ethyl Acetate	4.312
			Ethanol	2.668
Garcinia parvifolia	Leaves	45L	Hexane	5.396
			Ethyl Acetate	4.096
			Ethanol	3.035

Sample preparation

The extracts (1000mg) were prepared by dissolving in DMSO solvent in order to obtain 1000mg/ml of stocks solutions .The stock solutions were subjected to vortex and sonication when there was difficulty in dissolving. Subsequent serial dilutions were made to the required concentrations using distilled deionized water. The final concentration of DMSO was adjusted to 0.1%.

Determination of CETP inhibitory activity

In order to assess the percentage inhibition of the crude extracts towards CETP, a fluorescence bioassay were carried out by using the CETP Drug Screening Kit (#k602-100, BioVision, Mountain View, CA, USA). The procedures of the assay can be described briefly as follows: 50 µl of the extracts were added, followed by 3 µl of rabbit serum. Then the master mix which is being provided in the assay kit (10 µl of Donor Molecule, 10 µl of Acceptor Molecule and 20 µl of CETP buffer) was added, mixed well and the volume was completed to 203 µl with the provided assay buffer. The mixture were subjected to incubation for 1 hour at 37 °C and the fluorescence intensity were measured by using fluorescence plate reader (Varioscan Flash, ThermoScientific) at Excitation wavelength of 465 nm and Emission wavelength at 535 nm. The percentage inhibition of the extracts towards CETP was determined by comparing the activity of CETP in the presence and absence of the tested compound. As a background, negative control lacking of rabbit serum was being used. Positive controls were tested in order to see the degree of inhibition by 0.1% DMSO and CETP was not being affected by DMSO. All the measurements were carried out in triplicate. The percentage inhibition of the extracts towards CETP activity was calculated as follows:

$$Percentage\ Inhibition = \left(1 - \left[\frac{sample\ read - blank\ read}{Positive\ control\ read - Negative\ control\ read}\right]\right) x\ 100$$

Analysis

Results were expressed as means ± SD of replicates. Comparison between data sets was performed using one way analysis of variance (ANOVA) followed by *t*-test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Differences were accepted as statistically significant at $p < 0.05$.

Result and discussion

Nowadays, the remarkable interest in searching for inhibitors that could inhibit the cholesterol pathway has undergone extensive research. CETP, one of the major protein that causing atherosclerosis in which it will lowers the cardio protective HDL.Inhibiting the CETP will increase the HDL level and lower down the LDL level. The rapid progress in CETP as the atherosclerosis targeted therapy has led to the development of the anti- CETP antibodies, antisense oligonucleotide and a vaccine that induces antibodies for the inhibition of CETP activity (Rittershaus *et al,* 2000,Ritsch *et al,* 1993, Davidson *et al,* 2003). However, up to date none of the research has used natural product as a new potential drug that could inhibit CETP activity.

The primary aim of the experiment is to assess the inhibitory effect of Garcinia species with different extracts against CETP inhibition as to justify the 'myths' of the used of Garcinia Species plant parts as the cholesterol lowering agent. And it is presumed that the cholesterol pathway does involve CETP pathway as well. Successful studies that used *Garcinia atroviridis* in a dietary intake of Dunkin Hartley guinea pigs which results in a decrease level of lipid profile in serum and reduce the fat deposition in the aorta of high cholesterol diet animals (Amran *et al.*,2009).

The successful isolation of secondary metabolite is largely dependent on the type of solvent used during the extraction procedure. The method of extraction has been developed to represent the bioactivity guided fractionation at which the extraction occurs stepwise from low to high polarity. Thus, three types of solvents are being used for plant extractions which are hexane, ethyl acetate and ethanol. Table 2 summarise the percentage yield obtained for the extraction of G*arcinia atroviridis* and G*arcinia parvifolia*

Table 2 Yield of Extraction of *Garcinia atroviridis* and *Garcinia parvifolia*

Scientific name	Plant part	Code	Extracts	Percentage yield
Garcinia atroviridis	Leaves	78L	Hexane	9.328
			Ethyl Acetate	10.059
			Ethanol	13.805
	Twigs	78T	Hexane	8.435
			Ethyl Acetate	10.312
			Ethanol	12.668
	Fruit	78F	Hexane	5.632
			Ethyl Acetate	6.125
			Ethanol	8.013
Garcinia parvifolia	Leaves	45L	Hexane	3.396
			Ethyl Acetate	4.096
			Ethanol	5.035

Based on figure 1(a), (b) and (c) all the extracts of the plants part (Hex, EtOAc and EtOH) shows significant results against the CETP activity. At the higher concentration of the crude extract (100mg/ml) almost 100% inhibition can be seen for all of the plant parts. Almost similar patterns of inhibition can be seen at the concentration of 50 mg/ml and 100 mg/ml for every plant extracts. It is presume that the percentage inhibition at the 50mg/ml or higher that reach the plateau level may be due to the optimal condition in which the compound can produce greatest inhibition.

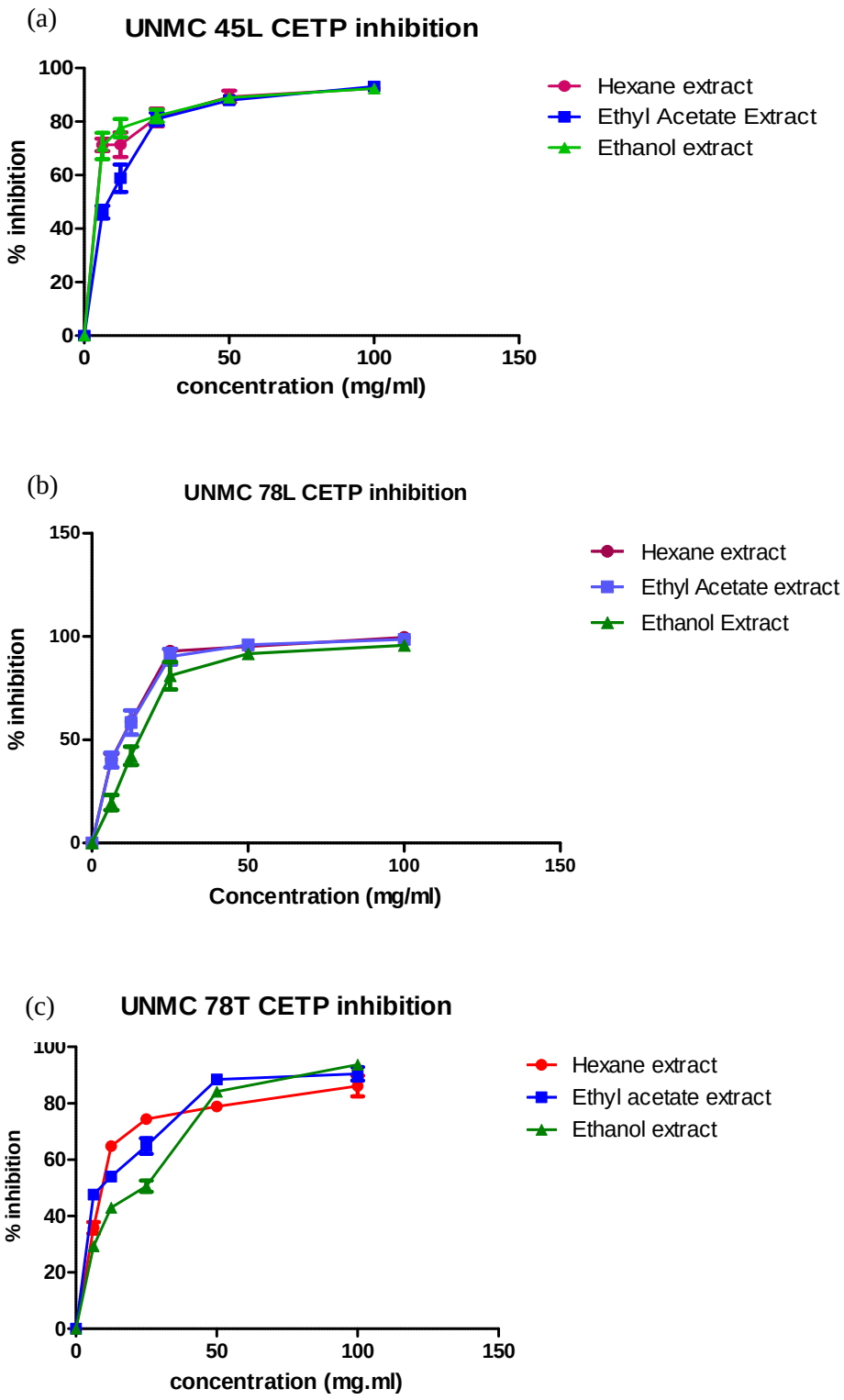

Figure 1 (a) The percentage inhibition of *Garcinia parvolia* leaves part which is denoted as UNMC45L. (b) The percentage inhibition of UNMC 78L (c) The percentage inhibition of UNMC 78T

From table 3, it is clear that the Ethanol extract of UNMC45L shows the greatest inhibition towards CETP activity followed by the Hexane extract and Ethyl Acetate extracts of UNMC78L. While the

remaining plant does show varying degree of activity towards the CETP inhibition. The IC50 of UNMC 78T shows higher concentration values compared to the leaves of UNMC 78L and UNMC 45L. From the graph 1(c),UNMC78T does show the lowest percentage inhibition towards CETP and it is speculated that more bioactive compound could be present in the leaves compare to twigs.

Table 2. The IC50 of UNMC 78L, UNMC 78T and UNMC 45L from different types of solvents used.

Solvents	UNMC78L	UNMC78T	UNMC45L
Hexane	14.53± 0.5111 mg/ml	28.70 ± 1.320 mg/ml	17.26 ± 0.4821 mg/ml
Ethyl Acetate	14.93 ± 0.5311 mg/ml	28.49 ± 1.126 mg/ml	16.07 ± 0.450 mg/ml
Ethanol	15.43 ± 0.4212 mg/ml	33.22± 0.6063mg/ml	12.30 ± 1.377 mg/ml

Conclusion

On the basis of present investigations, it is concluded that there is an interesting insight in exploring the new and potent inhibitors from natural sources. Focusing on CETP as the target protein for the inhibition to takes place is a great chance in order to decrease the chances of atherosclerosis. Besides, Malaysia is rich with its own plant diversity and the usage of the local plants in the drug discovery for CETP in reducing atherosclerosis is a great jumping stone to further use local plants as a therapeutic agents. Plant based drugs has massive therapeutic compensations as they can serve the purpose of becoming anti-atherosclerosis without any side effect which are often being associated with the synthetic drug. The tested plant extracts of UNMC 45L, UNMC 78L and UNMC 78T showed an appreciable inhibitory activity towards CETP. From this, it can be concluded that selected plants that already being acknowledge based on its ethno pharmacology effects against cholesterol has the potential effects towards the inhibition of CETP. Therefore, the traditional 'myths' claiming that Garcinia species plants have the potential in reducing weight / cholesterol can be supported by these findings. And, it is also concluded that the certain Garcinia plant species can be regarded as a good natural inhibitors towards the CETP.

As a consequence of this study, the isolation of the inhibitory compounds is underway at which it presents in the extracts that shows the large inhibitory effects against CETP. The isolation of the new and effective compounds that acts as inhibitors towards CETP is important for the drug development in decreasing the chances of having atherosclerosis in the world.

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