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Original Article

High inhibition efficacy of pancreatic cholesterol esterase and porcine pancreatic lipase from natural products

Runglawan Sudmoon[1], Sanit Kaewdaungdee[2], Unchaleeporn Ameamsri[2], Warin Wonok[2], Tawatchai Tanee[3], Pornnarong Siripiyasing[4], Arunrat Chaveerach[2*]

[1]Faculty of Law, Khon Kaen University, Thailand
[2]Department of Biology, Faculty of Science, Khon Kaen University, Thailand
[3]Faculty of Environment and Resource Studies, Mahasarakham University, Thailand
[4]Faculty of Science and Technology, Rajabhat Maha Sarakham University, Thailand

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**Corresponding author email:*
raccha@kku.ac.th

Abstract

The various properties of *Garcinia atroviridis* (*Ga*), *G. schomburgkiana* (*Gs*), *Camellia sinensis* (*Cs*) and *Morus alba* (*Ma*) were investigated, aimed at finding cholesterol lowering and lipase inhibiting effects. Their phytochemical components of the species were determined by GC-MS and HPLC. The total flavonoids, phenolics and polysaccharides were measured. The inhibition percentage of CEase and PPL were determined. Cytotoxicity and genotoxicity tests in the samples were examined. The results are as followed: The various key phytochemicals distributed in all the studied species were revealed such as stigmasterol, catechol, γ-sitosterol, β-amyrin, caffeine, and squalene. An HPLC chromatogram revealed the amount and concentration of HCA in *Ga* and *Gs*, and catechin and catechol in *Ga*, *Gs*, *Cs*, and *Ma*. The highest and second highest inhibition percentages of CEase in *Ga* and Formula I were at 77.02±0.27 and 67.61±0.26, and PPL and Formula I in *Gs* at 77.92±0.59 and 78.31±0.07 compared to orlistat inhibition percentage of 84.72±0.17 and 80.83±0.38, but in different concentrations of orlistat at 10 mg/ml and Formula 1 at 3 mg/ml. Toxicity assays exhibited no IC_{50} values in all samples, but ethanol *Gs* and *Cs* extracts and methanol *Ma* extract induced DNA damages significantly (p<0.01). However, from the LD_{50} values calculated at the concentrations used, there are no effects on humans. Therefore, the studied plants and Formulas at identical concentration, 10 mg/ml would definitely show a higher inhibition effect than orlistat in reducing cholesterol and inhibiting lipase activity leading to an innovation for weight loss and high cholesterol treatments without any side effects.

Keywords: *Garcinia schomburgkiana*, *Garcinia cambogia*, *Camellia sinensis*, *Morus alba* cholesterol lowering, Lipase activity

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Introduction

Plants and phytochemicals have long served various usages for human health. Dominant compounds at high quantities should lead plants to have an activity following the compound property. The phytochemicals act as biologically active substances, giving the plant characteristics such as color, odor, taste, and activities leading to values as medicine, food, supplements, nutraceuticals, cosmetics, perfumes, essential oils, spices, etc. Well-known substances which have long been used with varied properties and important functions in the body such as oleamide which relieves stress, improves memory, induces deep sleep, stimulates appetite and is anti-inflammatory (Hachisu et al., 2015; Ameamsri et al., 2021a; Siripiyasing et al., 2022); arbutin a whitening agent which was found in *Artocarpus lacucha* or *A. thailandicus* that could be added as a whitening agent in cosmetics and a preserving agent for fruit products (Noikotr et al., 2018; Kaewduangdee et al., 2020); α- and β-amyrin in *I. batatas* and *I. pes-caprae* whose extracts can be used as an excellent analgesic and as an anti-inflammatory (Ameamsri et al., 2021b); ethyl-α-d-glucoside (α-EG) and pinoresinol 3,3'-bisdemethylpinoresinol (pinoresinol), which have a collagen stimulating, skin whitening, and an inhibitory effect on wrinkle formation, found in *Morinda citrifolia* parts (Sudmoon et al., 2022).

There are many compounds related to this research. One compound, hydroxycitric acid (HCA) which has been found in large amounts in Garcinia species, namely in the fruit rinds of *G. cambogai*, *G. atroviridi*, and *G. indica* (Semwal et al., 2015). The HCA is a competitive inhibitor of adenosine triphosphate citrate lyase that catalyzes the extra mitochondrial cleavage of citrate to oxaloacetate and acetyl coenzyme A (Fassina et al., 2015). In other words, it suppresses de novo fatty acid synthesis, increases rate of hepatic glycogen synthesis, and promotes body weight loss (Han et al., 2016). Catechin and catechol are two substances which possesses various activities. Catechins belonging to the group of flavonoids, flavonoids are one of the most common and diverse groups of polyphenols) are polyphenol compounds found in many plants, are an important component of tea leaves (*Camellia sinensis*), are strong anti-oxidants. They function as an anti-microbial, anti-viral, anti-inflammatory, anti-allergenic, UV protective, activation of skin barrier passage, and anti-cancer agent. Additionally, they increase the penetration and absorption of healthy functional foods and bio cosmetics into the body and the skin, thus improving their utility, and are safe when applied to the human body (Bae et al., 2020; Musial et al., 2020). Catechol has valuable properties include having anti-viral, anti-bacterial, anti-aging, and hypotensive effects (Musial et al., 2020), a bioactive molecule that is an antioxidant molecule revealing a strong reducing ROS agent (Zhang et al., 2017). So, the consumption of tea or isolated substances may have anti-obesity effects, anticancer properties (Zhang et al., 2019), skin conditioning agent, can be used for cosmetic and beverage ingredients (Becker et al., 2019).

The most bioactive compounds present in the M. alba leaves alkaloids, glycosides, flavonoids, steroids, tannins, saponins and anthraquinone (Faiz and Faiz, 2021) showing varied properties such as antioxidant effect, delaying adverse changes in the lipid fraction and increasing the functionality of products (Bilska, 2021).

The substances and functions are very interesting, to apply them with the objectives of lowering cholesterol and inhibiting lipase for human health, *G. cambogia*, *G. schomburgkiana*, *C. sinensis*, and *M. alba* were investigated for essential information of some of their major compounds, their toxicity, and bioactivity testing of the inhibition percentage of pancreatic cholesterol esterase and porcine lipase.

Material and Methods

Plant materials and extract preparation

The plant samples, *G. atroviridis* and *G. schomburgkiana* fruits, *C. sinensis* and *M. alba* leaves were collected at various forest and orchard fields in Thailand and identified. There is no permission to collect the material used in this study, because of they are common species, grow widely in gardens, fields, forests, or household plantings for their edible leaves and fruits. They were washed and air-dried or hot air-dried at 60 °C, then ground into a powder. The powder was combined with hexane or ethanol, separately at a rate of 1:5, and soaked for 72 h. Each solution was filtered through a Whatman no. 1 filter paper. The filtrates were kept at -20 °C until used in all experiments mentioned below.

Gas chromatography-mass spectrometry (GC-MS)

The phytochemical screening was analyzed using types of equipment and protocols following Sudmoon et al. (2022).

HCA, Catechin and Catechol detection by High Performance Liquid Chromatography (HPLC)

The 20 µl samples and standards were analyzed by HPLC using a Shimadzu LC-20AD (Japan) model with a quaternary pump, a PAD (SPD-M20A) detector, and a column Inertsil ODS-3 C18, 4.6x250 mm, 5 microns particle size (GLSciences Inc.).

Standard Analysis and Calibration Curves; the linearity of the method was evaluated by analyzing a series of the standard. When the standard solution was injected, elution was carried out at specific flow rates and peak area responses by UV wavelengths was obtained, respectively. The calibration curve was plotted with concentration of HCA, catechin and catechol (x) versus peak area (y) in order to create the linear equation (y = mx+c) by Microsoft Excel program. The HCA, catechin and catechol peak area of each sample was used for quantity analysis. The coefficient or correlation (R^2) was used to judge the linear regression.

HCA was evaluated in the ethanol *G. atroviridis* and *G. schomburgkiana* fruit extracts, the wavelength detected was 214 nm, the gradient mobile phases consisted of MeOH and 0.01 M sulphuric acid with a flow rate of 0.7 ml/min (modified from Jena et al., 2002). The samples were injected. The results were compared to the standard and absolute of ethanol was used as a control.

The HCA standard (Sigma, USA) was dissolved in methanol at concentrations of 1 mg/ml. The standard solution was 10-fold diluted for four levels including 1, 0.1, 0.01, 0.001 mg/ml.

Catechin was detected in *G. atroviridis* and *G. schomburgkiana* fruits and *C. sinensis* and *M. alba* leaves, the wavelength detected was 272 nm (Seal, 2016), the gradient mobile phases consisted of acetonitrile and acetic acid in water at a rate 10:90 with a flow rate of 0.7 ml/min.

The 1 mg catechin standard (Sigma, USA) was dissolved in methanol at a concentration of 1 mg/ml. The standard solution was 2-fold diluted for five levels.

Catechol was detected in *G. atroviridis* and *G. schomburgkiana* fruits and *C. sinensis* and *M. alba* leaves, the wavelength detected was 280 nm (Gini and Jothi, 2018), the gradient mobile phases consisted of acetonitrile with 0.1% phosphoric acid in water at a rate 8:92 with a flow rate of 1 ml/min.

The 1 mg catechol standard (Sigma, USA) was dissolved in methanol at a concentration of 1 mg/ml. The standard solution was 2-fold diluted for five levels.

Total flavonoid detection in mg quercetin standard

The total flavonoid content of the four-study species was measured using aluminum chloride colorimeter method (modified from Kaba et al., 2019; Phowichit et al., 2019), against a quercetin standard solution curve using UV-visible spectrophotometry at a 415 nm wavelength. The sample filtrate was evaporated by rotary evaporator and the re-dissolved with 10% DMSO, then the sample solution was used to total flavonoid detection. The 500 µl of the solution was added with 1 M, 100 µl potassium acetate (CH_3COOK), 10% 100 µl aluminum chloride ($AlCl_3$), and 2.5 ml distilled water. The solution was mixed by vortex, then incubated for 30 min at room temperature. After that, the solution was analyzed by UV vis-spectrophotometer (Analytik Jena, SPECORD 210 plus, Germany) wavelengths at 415 nm. The absorbance value was used to determine the total flavonoid content (TFC), mg quercetin/g crude extract) = CV/M, where C is concentration (mg/ml) calculated from standard curve, V is the volume of the sample solution, and M is the weight of the sample (g).

Total polyphenolic detection in mg gallic acid (GAE) standard

The total polyphenol content of the four-study species was measured by the method modified from Yingngam et al. (2014) and Phowichit et al. (2019) using Folin-ciocaltea reagent. The gallic acid standard solution curve was created by 0, 50, 100, 150 mg/ml concentrations with ethanol solvent using UV-visible spectrophotometry at a 415 nm wavelength. The sample filtrate was evaporated by rotary evaporator and the re-dissolved with 10% DMSO, then the sample solution was used for total polyphenol detection. The 200 µl of the sample solution was combined with 2.5 ml distilled water and 10%, 2 µl Folin-ciocaltea reagent, vortexed, and incubated in the dark for 2 min. After that, the solution was combined with 7%, 2 ml sodium carbonate (Na_2Co_3), vortexed, and incubated in the dark for 90 min. The solution was measured for absorbance at the wavelength 765 nm with a UV-vis spectrophotometer (Analytik Jena, SPECORD 210 plus, Germany). The absorbance values were then analyzed to determine the total phenolic content (TCP) in the plant extracts by TCP (mg GAE/g crude extract) = CV/M equation.

Total polysaccharide detection in mg glucose (GLU) standard

The total polysaccharide content of the four-study species was measured by the method modified from Maingam et al. (2017) using phenol-sulphuric acid. The glucose standard solution curve was created by 0, 50, 100, 150, 200 mg/ml concentrations with distilled water. The sample filtrate was evaporated by rotary evaporator and the re-dissolved with 10% DMSO, then the sample solution was used for total polysaccharide detection. The 670 µl of the solution sample was combined with 6%, 670 µl, 2 ml sulphuric acid, vortexed, incubated at room temperature for 25 min, and the absorbance values were then analyzed using a UV-vis spectrophotometer (Analytik Jena, SPECORD 210 plus, Germany) wavelength at 490 nm to determine the total polysaccharide content (TPC) in the plant extracts by TPC (mg GLU/g crude extract) = CV/M equation.

Detection of cholesterol esterase (CEase) inhibition

CEase inhibition in the species samples and formulas was measured using the Colorimetric method modified from Kaba et al. (2019) and Zhang et al. (2020). The plant extract filtrate was dissolved in 10% DMSO. The solution was 10-fold serial diluted with distilled water for five levels used to graph inhibition percentage potentiality. The solutions were tested for CEase inhibition. Orlistat is the positive control. Orlistat 10 mg/ml (dissolved by 6%, 1 ml acetonitrile) was diluted at five levels– 1, 2, 4, 8, and 10 mg/ml– used to graph inhibition percentage potentiality. The solutions were tested for CEase inhibition. Each concentration at 20 µl of both the extract and control were combined with 100 mM, 400 µl sodium phosphate buffer, 12 mM, 500 µl taurocholate. Then 6%, 40 µl acetonitrile, 20 µl p-nitrophenyl butyrate (p-NBP), then vortexed, incubated at room temperature for 10 min. After that, 5 µg/ml, 20 µl CEase was added, the absorbance value was then analyzed using a UV-vis spectrophotometer (Analytik Jena, SPECORD 210 plus, Germany) with wavelength at 405 nm to determine CEase inhibition. The 6% acetonitrile is the negative control.

Detection of pancreatic porcine lipase (PPL) inhibition

PPL inhibition in the species samples and formulas was measured using the Colorimetric method modified from Zhang et al. (2020) and Esfandi et al. (2021). The plant extract filtrate was dissolved in 10% DMSO. The solution was 10-fold serial diluted with distilled water at five levels to graph inhibition percentage potentiality. The solutions were tested for PPL inhibition. Orlistat is the positive control. Orlistat 10 mg/ml (dissolved by 6%, 1 ml acetonitrile) was diluted at five levels– 1, 2, 4, 8, 10 mg/ml– used to graph inhibition percentage potentiality. The solutions were tested for PPL inhibition. Each concentration at 20 µl of both the extract and control were combined with 5 µg/ml, 100 µl PPL, vortexed, and incubated at 37 °C for 10 min. After that, the mixture was combined with 0.1 mM (pH 7.4), 700 µl Tris-HCL buffer, 10 mM, 100 µl p-NPB, and incubated at 37 °C for 30 min. The absorbance value was then analyzed using a UV-vis spectrophotometer (Analytik Jena, SPECORD 210 plus, Germany) with the wavelength of 410 nm to determine PPL inhibition. The 6% acetonitrile is the negative control.

Inhibition percentage of CEase and PPL calculation

% inhibition of PPL or CEase = (Absorbance of blank - Absorbance of sample/ Absorbance of control) x 100

Where

Absorbance of control = absorbance of negative control

Absorbance of sample = absorbance of orlistat or sample plant

Absorbance of blank = absorbance of blank control

Cytotoxicity and genotoxicity testing via MTT and comet assays

The filtrate samples were tested for toxicity, both cytotoxicity as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay and genotoxicity as comet assay following the steps:

1. Sample extract preparation.

The sample extracts were prepared following Wonok et al. (2021) and Sudmoon et al. (2022).

2. Human peripheral blood mononuclear cell (PBMCs) preparation.

PBMCs preparation were performed following Wonok et al. (2021) and Sudmoon et al. (2022).

3. MTT assay

The stock extract was serially 10-fold diluted with water for five working concentrations using for MTT assay. The protocols, equipment, evaluation of percentage of cell viability, IC_{50} and LD_{50} calculation were performed following Wonok et al. (2021) and Sudmoon et al. (2022).

4. Comet assay

The concentration at IC_{50} value or the maximum-treated concentration, in the case of no IC_{50} value was used in the comet assay to assess the genotoxicity of plant extracts, according to Sudmoon et al. (2022).

The key phytochemicals screened by GC-MS method revealed β-bisabolene, 2(3H)-furanone, cyclopentanecarboxylic acid, stigmasterol in *G. atroviridis* fruit; catechol, 2,5-dihydro-5-oxofuran-2-acetate, stigmasterol, γ-sitosterol, n-hexadecanoic acid in *G. schomburgkiana* fruit; n-hexadecanoic acid, phytol, γ-sitosterol in *M. alba* leaves; and β-amyrin, β-amyrone, caffeine, squalene, and 2-nitrophenoxathiin-10, 10-dioxide in *Camellia sinensis* leaves. All phytochemicals and relative content were shown in the Table 1 according to chromatogram (Figure 1).

Results

Table-1. A summary of chemicals constituents indicated by relative content percentages analyzed by GC-MS in the ethanol and hexane *Garcinia schomburgkiana* and *G. atroviridis* fruit extracts and methanol and hexane *Camellia sinensis* and *Morus alba* leaf extracts.

Compound name	Chemical formula	Relative contents (%)							
		Garcinia schomburgkiana		*G. atroviridis*		*Camellia sinensis*		*Morus alba*	
		ethanol	hexane	ethanol	hexane	ethanol	hexane	methanol	hexane
β-Bisabolene	$C_{15}H_{24}$	-	-	1.91	44.7	-	-	-	-
Catechol	$C_6H_6O_2$	-	25.42	-	-	0.55	-	-	-
2(3H)-Furanone	$C_4H_4O_2$	4.22	-	22.77	-	-	-	-	-
2,5-dihydro-5-oxofuran-2-acetate	$C_6H_5O_4$	22.71	-	-	-	-	-	-	-
Cyclopentanecarboxylic acid	$C_6H_{10}O_2$	7.47	-	20.99	-	-	-	-	-
Stigmasterol	$C_{29}H_{48}O$	1.81	14.87	0.48	11.63	4.83	-	-	1.39
γ-Sitosterol	$C_{29}H_{50}O$	1.71	11.81	-	0.92	-	-	13.80	10.32
Lupeol acetate	$C_{32}H_{52}O_2$	-	-	-	-	-	-	-	21.82
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	11.5	-	4.80	-	-	-	-	-
β-Amyrin	$C_{30}H_{50}O$	-	-	-	-	9.08	22.36	-	-
β-Amyrone	$C_{30}H_{48}O$	-	-	-	-	6.53	18.03	-	-
Caffeine	$C_8H_{10}N_4O_2$	-	-	-	-	17.07	-	-	-
2-Nitrophenoxathiin-10,10-dioxide	$C_{12}H_7NO_5S$	-	-	-	-	10.99	-	-	-
Campesterol	$C_{28}H_{48}O$	0.66	7.66	-	2.99	-	-	-	1.41
Linoleic acid	$C_{18}H_{32}O_2$	10.61	-	1.41	-	-	-	-	-
Linolenic acid	$C_{18}H_{30}O_2$	12.37	-	5.41	-	-	-	3.45	-
1H-Pyrazole-3-carboxylic acid	$C_4H_4N_2O_2$	-	-	6.96	-	-	-	-	-
Butanedioic acid	$C_4H_6O_4$	3.10	-	5.81	-	-	-	-	-
1-(4-Amino-3-butoxyphenyl)ethanone	$C_{12}H_{17}NO_2$	-	-	-	-	-	7.62	-	-
Benzyl-β-d-glucoside	$C_{13}H_{16}O_7$	-	-	-	-	-	-	4.18	-
Aurantiamide	$C_{25}H_{26}N_2O_3$	-	-	-	-	-	-	4.08	-
Lupenone	$C_{30}H_{48}O$	-	-	-	-	-	-	-	2.84
3β-Acetoxy-11- oxoursan-12-ene	$C_{32}H_{50}O_3$	-	-	-	-	-	5.92	-	-
Tetramethyluric acid	$C_9H_{12}N_4O_3$	-	-	-	-	5.14	0.48	-	-
Menthol	$C_{10}H_{20}O$	-	-	-	-	4.98	-	-	-
β-Amyrin acetate	$C_{32}H_{52}O_2$	-	-	-	-	2.22	1.86	-	-
β-Amyrenonol	$C_{30}H_{48}O_2$	-	-	-	-	4.48	-	-	-
α-Amyrin	$C_{30}H_{50}O$	-	-	-	-	1.34	3.96	-	-
dl-α-Tocopherol	$C_{29}H_{50}O_2$	0.36	1.20	0.30	3.69	-	-	0.75	-
Ethyl palmitate	$C_{18}H_{36}O_2$	3.43	-	3.16	-	0.64	-	1.35	2.95
Acetic acid	CH_3COOH	-	3.36	-	-	-	-	-	-
Squalene	$C_{30}H_{50}$	0.28	3.36	-	-	2.52	15.26	-	-

Lauric acid	$C_{12}H_{24}O_2$	-	-	-	-	1.71	-	-	1.33
Furan	C_4H_4O	-	-	2.75	-	-	-	-	-
Ethyl linoleate	$C_{20}H_{36}O_2$	2.50	-	1.45	-	-	-	-	-
Methyl palmitate	$C_{17}H_{34}O_2$	-	1.24	-	1.62	0.35	1.46	-	-
Ethyl stearate	$C_{20}H_{40}O_2$	1.51	-	0.25	-	-	-	-	-
β-Famesene	$C_{15}H_{24}$	-	-	-	1.28	-	-	-	-
Caryophyllenyl alcohol	$C_{15}H_{26}O$	-	-	-	1.12	-	-	-	-
Phytol	$C_{20}H_{40}O$	0.69	0.43	-	-	6.92	1.81	14.93	16.04
α-Gurjunene	$C_{15}H_{24}$	-	6.30	-	-	-	-	-	-
Glycerol-β-palmitate	$C_{19}H_{38}O_4$	-	-	-	-	-	-	1.11	-
β-Eudesmol	$C_{15}H_{26}O$	-	0.58	-	-	-	-	-	-
2,6,10-Dodecatrien-1-ol	$C_{17}H_{28}O_2$	-	3.64	-	-	-	-	-	-
Phthalic acid	$H_2C_8H_4O_4$	-	1.18	-	-	-	-	-	-
Methyl linoleate	$C_{19}H_{34}O_2$	-	1.03	-	-	-	-	-	-
Methyl linolenate	$C_{19}H_{32}O_2$	-	3.48	-	1.41	-	-	4.32	-
Vitamin E	-	-	-	-	-	2.23	3.24	2.83	-
Friedoolean-6-ene	$C_{30}H_{50}$	-	3.67	-	-	-	-	-	1.87
Phenylethyl alcohol	$C_8H_{10}O$	3.17	-	-	-	-	-	-	-
Palmitoleic acid	$C_{16}H_{30}O_2$	1.50	-		-	-	-	-	-
Palmitic acid	$C_{16}H_{32}O_2$	-	-	-	-	4.85	2.41	14.22	19.32
Ethyl linolenate	$C_{20}H_{34}O_2$	3.98	-	5.41	-	-	-	-	-
Ethyl oleate	$C_{20}H_{38}O_2$	4.46	-	-	-	-	-	-	-
Caryophyllene	$C_{15}H_{24}$	-	-	-	3.62	-	-	-	-
β-Selinene	$C_{15}H_{24}$	-	-	-	3.05	-	-	-	-
α-Selinene	$C_{15}H_{24}$	-	-	-	3.50	-	-	-	-
9-Octadecenal	$C_{18}H_{34}O$	-	-	-	1.21	-	-	-	-
Oleic acid	$C_{18}H_{34}O_2$	-	-	-	1.08	-	-	-	-
Crinosterol	$C_{28}H_{46}O$	-	-	-	1.03	-	-	-	-
2-Pentacecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	-	-	-	0.70	-	-	1.42	0.82
Humulene	$C_{15}H_{24}$	-	-	-	0.61	-	-	-	-
Methyl stearate	$C_{19}H_{38}O_2$	-	0.57	-	-	-	-	-	-
Globulol	$C_{15}H_{26}O$	-	-	-	-	0.18	0.23	-	-
Friedelin	$C_{30}H_{50}O$	-	-	-	-	0.32	-	-	-
Simiarenol	$C_{30}H_{50}O$	-	-	-	-	-	-	-	2.83
Dibutyl phthalate	$C_{16}H_{22}O_4$	-	0.66	-	-	-	-	-	-
Tetradecanoic acid	$C_{14}H_{28}O_2$	-	-	0.91	-	-	-	-	-
Stigmasta-5,22-dien-3-ol	$C_{29}H_{48}O$	0.49	-	-	-	-	-	-	-
Phytol, acetate	$C_{22}H_{42}O_2$	0.91	-	-	-	-	-	-	1.48
Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}AsO_3Si_3$	-	-	-	-	0.74	4.72	-	-
Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$	-	-	-	-	0.88	4.96	-	-
γ-Tocopherol	$C_{28}H_{48}O_2$	-	-	-	-	0.86	-	0.56	0.88
Noruns-12-ene	$C_{29}H_{48}$	-	-	-	-	0.66	0.92	-	-
3-phenylphthalimidine	$C_{14}H_{11}NO$	-	-	-	-	0.65	0.91	-	-
5-(Phenoxy)-methyl-2-amino-1,3,4-thiadiazoles	$C_9H_9N_3OS$	-	-	-	-	0.65	1.64	-	-
5-Hydroxymethylfurfural	$C_6H_6O_3$	-	-	2.35	-	-	-	-	-
γ-Eudesmol	$C_{15}H_{26}O$	-	-	-	0.52	-	-	-	-
ethyl 4-ethoxybenzoate	$C_{11}H_{14}O_3$	-	-	-	-	-	-	2.05	0.98
Loliolide	$C_{11}H_{16}O_3$	-	-	-	-	-	-	1.79	-
Vomifoliol	$C_{13}H_{20}O_3$	-	-	-	-	-	-	1.68	-
(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	$C_{22}H_{20}OS$	-	-	-	-	-	-	-	0.63
1,3,2-Benzodioxaborole, 2-hydroxy	$C_6H_5BO_3$	-	-	-	-	0.63	-	-	-
Coniferyl alcohol	$C_{10}H_{12}O_3$	-	-	-	-	0.54	-	-	-
4-Vinylguaiacol	$C_9H_{10}O_2$	-	-	-	-	0.47	-	-	-
trans-2-methyl-4-n-pentylthiane, S,S-dioxide	$C_{11}H_{22}O_2S$	-	-	-	-	0.41	-	-	-
5-hydroxy-4,7,7-trimethyl-norbornan-2-one	$C_{10}H_{16}O_2$	-	-	-	-	0.35	-	-	-
Tetradecanol	$C_{14}H_{30}O$	-	-	-	-	0.37	-	-	-

Undeca-3,4-diene-2,10-dione, 5,6,6-trimethyl	$C_{14}H_{22}O_2$	-	-	-	-	0.36	-	-	-
2-nonadecanone 2,4-dinitrophenylhydrazine	$C_{25}H_{42}N_4O_4$	-	-	-	-	0.23	-	-	-
2-Aminoethanethiolsulfuric acid	$C_2H_7NO_3S_2$	-	-	-	-	0.23	-	-	-
10-Methoxy-N(b)-α-methylcorynantheol	$C_{21}H_{29}N_2O_2$	-	-	-	-	0.47	-	-	-
3-Hydroxy-2-methyl-2-nitrocyclohexyl acetate	$C_9H_{15}NO_5$	-	-	12.46	-	-	-	-	-
Glycyl-L-glutamic acid	$C_7H_{12}N_2O_5$	-	-	1.60	-	-	-	-	-
Ergost-5-en-3-ol, acetate	$C_{30}H_{50}O_2$	-	-	0.29	-	-	-	-	-
Methyl 7,8-octadecadienoate	$C_{19}H_{34}O_2$	-	-	-	-	2.02	-	-	-
Dotriacontane	$C_{32}H_{66}$	-	-	-	-	-	-	-	0.94
2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	$C_{15}H_{24}$	-	-	-	0.99	-	-	-	-
Stigmastan-3,5-diene	$C_{29}H_{48}$	0.58	-	-	-	-	-	-	-
N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)anilin	$C_{11}H_{18}N_2OSi$	-	-	-	-	-	1.30	-	-
Pyrogallol	$C_6H_6O_3$	-	-	-	-	2.52	-	-	-
Stigmastan-5,22-diene-3-ol, acetate	$C_{31}H_{50}O_2$	-	-	0.71	-	-	-	-	-
Cycloheptasiloxane, tetradecamethyl	$C_{14}H_{42}O_7Si_7$	-	-	-	-	-	0.30	-	-
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	-	-	-	-	-	0.31	-	-
2,2-Dideutero heptadecanal	$C_{17}H_{32}D_2O$	-	-	-	-	-	0.27	-	-
Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$	-	-	-	-	-	0.27	-	-
Unknown	-	-	9.52	-	14.34	-	-	27.48	12.15

*Vitamin E refers to a class of compounds, not analyzed as individual compound by the Wiley.7N.1 library"

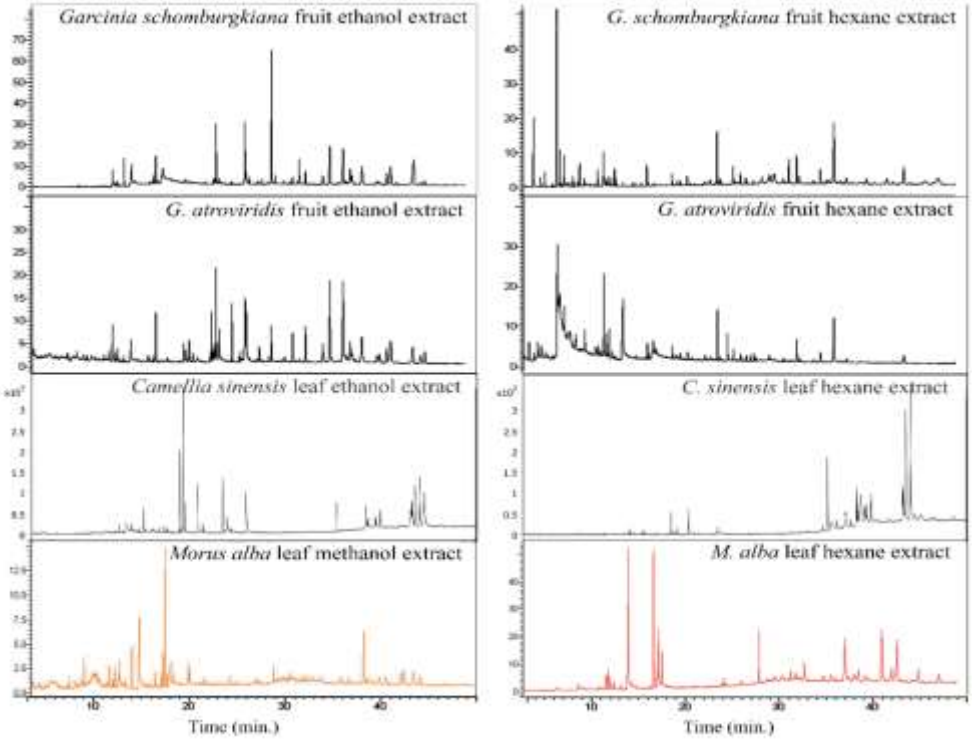

Figure-1. GC-MS chromatograms of the ethanol and hexane fruit extracts of *Garcinia schomburgkiana* and *G. atroviridis*; ethanol and hexane leaf extract of *Camellia sinensis*; and methanol and hexane leaf extract *Morus alba* showing retention time and peak area

Table-2. Details of the raw materials used, a g of leaf powder per 5 ml of ethanol and hexane solvents, and the results from HPLC analysis including peak areas, concentrations and amounts of HCA, catechin and catechol.

Plant samples	Solvents	Filtrate volume (ml)	HCA			Catechin			Catechol		
			Peak area (mAU)	Conc. (mg/ml)	Amounts (mg/g sample)	Peak area (mAU)	Conc. (mg/ml)	Amounts (mg/g sample)	Peak area (mAU)	Conc. (mg/ml)	Amounts (mg/g sample)
Garcinia schomburgkiana	ethanol	4.5	4438853	1.48	0.06	3499976	0.57	1.28	1946602	0.17	0.38
	hexane	19.0	-	-	-	4234	ND	ND	1479044	0.12	1.16
G. atroviridis	ethanol	6.1	8710269	2.91	0.12	1488261	0.23	0.71	3194517	0.29	0.90
	hexane	23.0	-	-	-	6308	ND	ND	60934	ND	ND
Camellia sinensis	ethanol	3.9	-	-	-	45715857	7.60	14.83	21542222	2.13	4.15
	hexane	16.0	-	-	-	3121	ND	ND	327775	0.01	0.06
Morus alba	ethanol	4.3	-	-	-	411902	0.23	0.12	0	ND	ND
	hexane	7.5	-	-	-	0	ND	ND	0	ND	ND
Formula 1	ethanol	5.1	-	-	-	35951286	5.98	14.64	2413060	0.22	0.55
	hexane	28.0	-	-	-	5544	ND	ND	0	ND	ND
Formula 2	ethanol	5.4	-	-	-	58119759	9.67	26.11	13651449	1.34	3.62
	hexane	25.0	-	-	-	8641	ND	ND	0	ND	ND

*ND = Not detected

The HPLC chromatogram for HCA in ethanol *G. atroviridis* and *G. schomburgkiana* fruit extracts (Figure 2A) revealed the amount and concentration, 0.12 and 0.06 mg/g, and 2.91 mg/ml and 1.48 mg/ml, respectively. Between ethanol and hexane solvents, the highest amount and concentration were mentioned here: catechin in *G. atroviridis G. schomburgkiana* fruits, *Camellia sinensis* and *Morus alba* leaves were 0.71, 1.28, 14.83, 0.12 mg/g and 0.23, 0.57, 7.60, 0.23 mg/ml following the chromatogram in Figure 2B; catechol was 0.90, 1.16, 4.15, 0.00 mg/g and 0.29, 0.17, 2.13, 0.00 mg/ml, respectively. The chromatogram is shown in Figure 2C. Amounts and concentrations of HCA, catechin and catechol are shown in Table 2.

With various protocols, total flavonoids, phenolic and polysaccharide contents extracted with hexane and ethanol solvents were measured. All values were reported in concentrations and amount as mg QE/g, mg GAE/g and mg GLU/g crude extracts, respectively shown in Table 3. QE, GAE, and GLU standard calibration curves and correlation coefficient (R^2) were y = 0.0104x + 0.0431, 0.9996; y = 0.0036x + 0.082, 0.9991; y = 0.002x + 0.0546, 0.9993, respectively. Some high amounts and concentrations of these substances in *G. atroviridis* and *G. schomburgkiana* fruits and *Camellia sinensis* and *Morus alba* leaves are mentioned at 382.16, 86.36, 65.16, 81.78 mg QE/g crude extract and 105.42, 27.63, 22.09, 29.44 mg/ml; phenolics were 460.27, 319.88, 37.044.94 mg GAE/g crude extract and 126.97, 102.36, 25.33, 12.56 mg/ml; polysaccharides were 3168.43, 2713.44, 243.38, 363.19 mg GLU/g crude extract and 874.05, 868.30, 82.50, 154.00 mg/m.

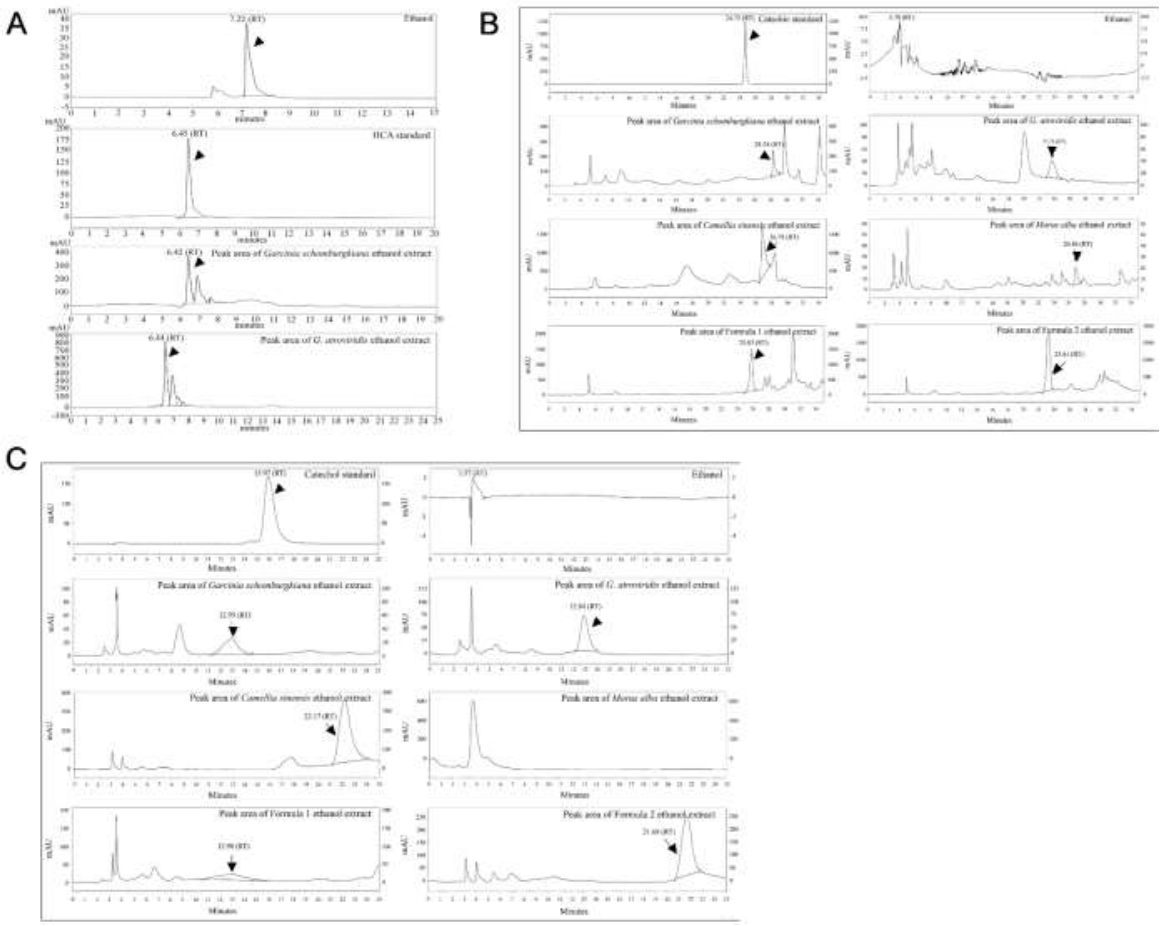

Figure-2. HPLC chromatograms showing retention time of key phytochemicals detection, (A) HCA detection in ethanol *Garcinia schomburgkiana* and *G. atroviridis* fruit extracts, (B) Catechin detection in ethanol extracts of *G. schomburgkiana*, *G. atroviridis* fruits, *Camellia sinensis* and *Morus alba* leaves, (C) Catechol detection in ethanol extracts of *G schomburgkiana*, *G. atroviridis* fruit, *C. sinensis* and *M. alba* leaves

Table-3. The results of total flavonoid, phenolic and polysaccharide contents of ethanol and hexane *Garcinia schomburgkiana*, *G. atroviridis*, *Camellia sinensis* and *Morus alba* extracts.

Plant samples	Solvents	Total flavonoid contents		Total phenolic contents		Total polysaccharide contents	
		Conc. (mg/ml)	mg QE/ g of crude extracts	Conc. (mg/ml)	mg GAE/ g of crude extracts	Conc. (mg/ml)	mg GLU/ g of crude extracts
Garcinia schomburgkiana	ethanol	27.63	86.36	102.36	319.88	868.30	2,713.44
	hexane	8.20	20.50	15.50	38.75	549.00	1,372.50
G. atroviridis	ethanol	105.42	382.16	126.97	460.27	874.05	3,168.43
	hexane	58.68	205.39	25.33	88.67	743.05	2,600.68
Camellia sinensis	ethanol	22.09	65.16	12.56	37.04	82.50	243.38
	hexane	3.83	9.57	4.94	12.36	32.40	81.00
Morus alba	ethanol	29.44	81.78	1.78	4.94	130.75	363.19
	hexane	35.78	62.61	1.36	2.38	154.00	269.50

******QE = quercetin, GAE = gallic acid and GLU = glucose standards

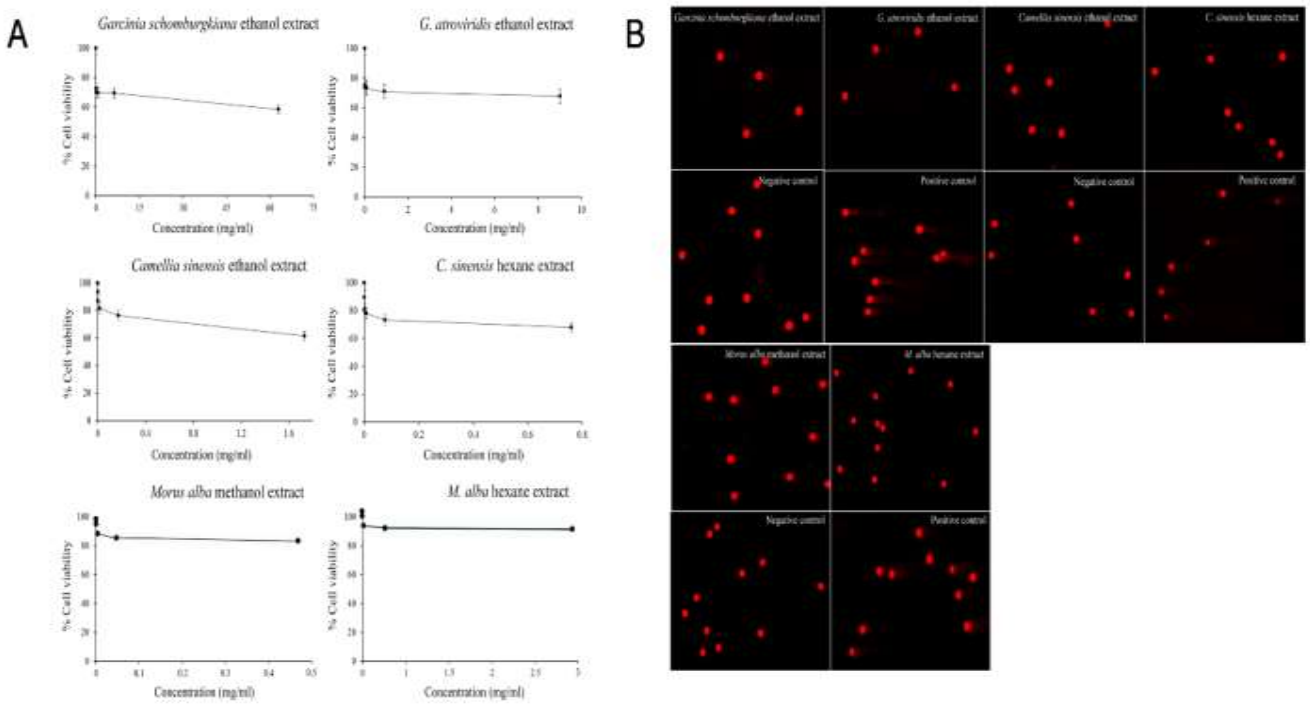

Figure-3. The toxicity evaluation of PBMCs treated with ethanol *Garcinia schomburgkiana* and *G. atroviridis* fruit extracts; ethanol and hexane *Camellia sinensis* leaf extracts; and methanol and hexane *Morus alba* leaf extracts, (A) cytotoxicity testing from MTT assay showing no IC_{50} values, high viability percentages, (B) the comet images (200X) showing significant DNA damages ($p<0.01$) in ethanol *G. schomburgkiana* and *C. sinensis* extracts and methanol *M. alba* extract, compared to negative control

The cytotoxic assay exhibited no IC_{50} values in all samples (Figure 3A), but in the genotoxic test, they induced significant DNA damages ($p<0.01$) in ethanol *G. schomburgkiana* and *Camellia sinensis* extracts and methanol *Morus* alba extract (Figure 3B) with the highest working concentration as shown in the Table 4 when lacking IC_{50} values. The values of cell viability percentage and Olive tail moment (OTM) of DNA damage expression were shown in Table 4.

From the results of the detection of these important substances, Formula 1 and Formula 2 were created by different rate combination of the studied plant combination. These plants and the formulas were used to test for biological activity in CEase and PPL inhibitions. With hexane and ethanol solvents, each studied plant showed the highest and second highest inhibition percentage of CEase in *G. atroviridis* and the Formula 1 at 77.02±0.27 and 67.61±0.26, and PPL in G. *schomburgkiana* and the Formula 1 at 77.92±0.59 and 78.31±0.07 compared to the positive control, orlistat. Graphs showing relations between extract concentrations and inhibition percentages of CEase and PPL are shown in Figures 4A and 4B, and all percentage inhibition values of all the studied samples are shown in Table 5.

Table-4. Cells viability percentage and level of DNA damage expressed as Olive Tail Moment (OTM) in PBMCs after treatment with ethanol and hexane *Gacinia schomburgkiana* and *G. atroviridis* fruit extracts, *Camellia sinensis* and *Morus alba* leaf extracts

Plant samples	Solvents	Working conc. (mg/ml)	% Cell viability±S.D.	OTM (Mean±S.D.)	*p*-value	LD_{50} for the rat mg/kg body weight	WHO class
Negative control		-	-	0.36±0.27	-	-	-
Garcinia schomburgkiana	ethanol	6.30	72.70±0.07 - 58.49±0.08	0.54±0.24	0.0001 (<0.01)	2,737.50	III, slightly hazardous when oral over 2,000 mg/kg body weight
	hexane	-	-	-	-	-	-
G. atroviridis	ethanol	0.90	75.43±0.10 - 67.95±0.10	0.38±0.24	0.3671 (>0.01)	-	-
	hexane	-	-	-	-	-	-
Negative control		-	-	0.12±0.08	-	-	-
Camellia sinensis	ethanol	1.73	61.60±0.23 - 93.65±0.44	0.21±0.14	<0.0001 (<0.01)	1,692.60	II, moderately hazardous, when oral 50-2,000 mg/kg body weight
	hexane	0.76	67.99±0.18 - 89.89±0.41	0.12±0.08	0.2359 (>0.01)	-	-
Negative control		-	-	0.03±0.16	-	-	-
Morus alba	methanol	3.00	94.91±0.11 - 99.49±0.11	0.11±0.12	<0.0001 (<0.01)	2,077.05	III, when oral over 2,000 mg/kg body weight will get slightly hazardous
	hexane	0.48	96.89±0.09 - 99.63±0.13	0.01±0.12	0.1036 (>0.01)	-	

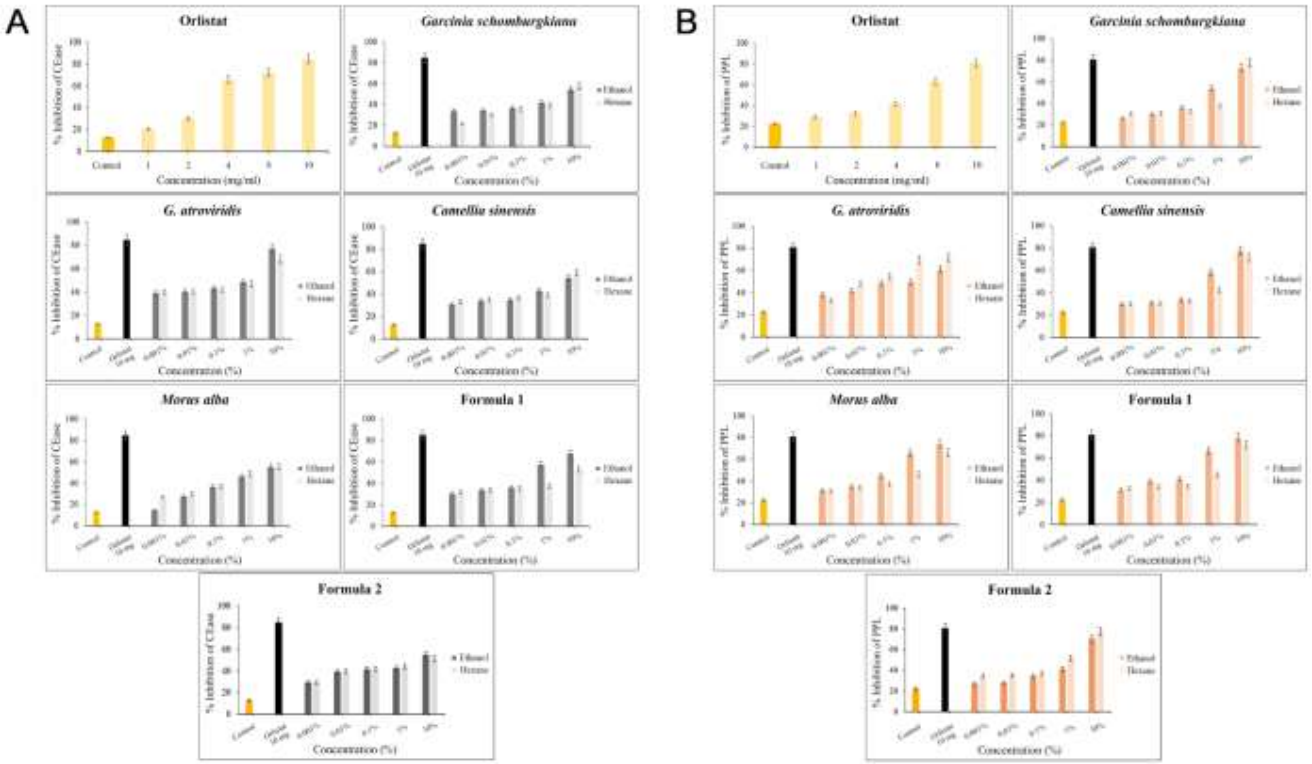

Figure-4. Inhibition percentage of (A) Pancreatic cholesterol esterase (CEase) and (B) Porcine pancreatic lipase (PPL) activities by ethanol and hexane *Garcinia schomburgkiana* and *G. atroviridis* fruit extracts, *Camellia sinensis* and *Morus alba* leaf extracts, Formula 1 and Formula 2 extracts

Table-5. Pancreatic cholesterol esterase (CEase) and pancreatic porcine lipase (PPL) inhibitory activities of ethanol and hexane *Garcinia schomburgkiana* and *G. atroviridis* fruit extracts, *Camellia sinensis* and *Morus alba* leaf extracts, Formula 1 and Formula 2 extracts.

Test samples	Solvents	Mass conc. (mg/ml)	Pancreatic cholesterol esterase (CEase)		Pancreatic porcine lipase (PPL)	
			% Inhibition (mean±S.D.)	IC_{50} (mg/ml)	% Inhibition (mean±S.D.)	IC_{50} (mg/ml)
Negative control; 6% acetonitrile	-	-	12.81±0.20	-	22.57±0.13	-
Positive control; Orlistat	-	10.00	20.34±0.20 - 84.72±0.17	3.10	29.03±0.02 - 80.83±0.38	8.00
Garcinia schomburgkiana	ethanol	8.96	33.71±0.24 - 54.61±0.17	6.00	26.46±0.11 - 73.12±0.13	0.60
	hexane	1.70	21.47±0.23 - 58.19±0.26	1.02	30.74±0.18 - 77.92±0.59	0.64
G. atroviridis	ethanol	10.89	38.98±0.32 - 77.02±0.27	1.60	38.05±0.44 - 61.09±0.29	1.25
	hexane	0.14	39.92±0.24 - 64.97±0.26	0.04	32.94±0.13 - 71.47±0.15	0.001
Camellia sinensis	ethanol	1.73	30.70±0.24 - 54.24±0.17	1.14	29.87±0.07 - 77.66±0.54	0.09
	hexane	0.76	33.15±0.24 - 59.32±0.39	0.45	30.09±0.02 - 71.73±0.15	0.24
Morus alba	ethanol	1.04	14.53±0.40 - 55.18±0.15	0.48	31.13±0.18 - 73.93±0.09	0.02
	hexane	0.14	26.93±0.23 - 55.18±0.28	0.04	30.87±0.20 - 66.28±0.22	0.03
Formula 1	ethanol	3.00	30.13±0.14 - 67.61±0.26	0.02	31.13±0.11 - 78.31±0.07	0.01
	hexane	0.52	31.70±0.10 - 53.11±0.17	0.42	32.68±0.11 - 72.50±0.25	0.13
Formula 2	ethanol	2.96	29.19±0.31 - 54.61±0.34	1.90	26.98±0.11 - 70.78±0.13	0.82
	hexane	0.87	29.19±0.31 - 51.60±0.33	0.68	34.89±0.24 - 77.43±0.23	0.09

*Note: The value was expressed as median±S.D (n=3)

Discussion

The phytochemical analysis in very important as a base of further investigation that benefits practical use in people. The phytochemicals in all studied samples, many substances such as catechol, catechin have been previously reported that they were found with various functions in *C. sinensis* (Becker et al., 2019; Zhang et al., 2019; Bae et al., 2020; Musial et al., 2020) leading to the use of the plant with various advantages mentioned above in the introduction. The anti-obesity effect of *C. sinensis* could be attributed to food intake reduction, lipids absorption and inhibition of the start of digestion, lipogenesis, transportation of lipids from liver to adipose tissue, and the increasingly of lipids excretion and visceral lipid oxidation (Zhang et al., 2019). Here, there is a substance, catechol in high relative percentages as 25.42 in *G. schomburgkiana* fruit extract out of the highest relative percentages found in *G. atroviridis*, while the low relative percentage of 0.55 was found in *C. sinensis*. Therefore, the identical function in these two *Garcinia* species containing catechin and catechol were expected. This is the first report of catechol in *G. schomburgkiana* fruit extract, while the previous study reported that catechol was found in the branches and roots of *G. schomburgkiana* (Meechai et al., 2016). The other compounds which were found in high relation percentages, but are not know in their exactly biological roles including 44.7% β-Bisabolene in *G. atroviridis* fruit extract, 22.77% 2(3H)-furanone in *G. atroviridis* fruit

extract, 22.71% 2,5-dihydro-5-oxofuran-2-acetate in *G. schomburgkiana* fruit extract, and 20.99% cyclopentanecarboxylic acid in *G. atroviridis* fruit extract. For 21.82% lupeol acetate in *M. alba* leaf extract, its function as a potent antifungal compound against opportunistic human and phytopathogenic mold *Macrophomina phaseolina* was reported by Javed et al., 2021. So, *M. alba* leaf should be further tested for this activity.

The HPLC chromatograms showed the amount and concentration of HCA in the ethanol solvent of 0.12 and 0.06 mg/g, and 2.91 mg/ml and 1.48 mg/ml in *G. atroviridis* and *G. schomburgkiana*, respectively, which were not known for biological activity in human body following the HCA functions. Previous scientific research detected HCA in fruits of *G. schomburgkiana*, *G. cambogia* and *G. indica* also (Semwal et al., 2015). Chuah et al. (2013) revealed that HCA is one of the important supplements for anti-obesity and weight management. Its effect on weight management is mainly attributed to giving the feeling of being full and satisfied while the anti-obesity effect is by reduction of de novo lipogenesis and acceleration of fat oxidation. So, the further steps of this investigation are finding the inhibition activity percentages of CEase and PPL by bringing these studied plants containing high catechol, catechin and HCA together into two formulas, Formula 1 and Formula 2 (ratios follows a petty patent) which are expected to have the mentioned properties. However, the other compounds related in these experiments were also evaluated including total flavonoid, phenolic and polysaccharide contents. Flavonoids are a family of polyphenolic compounds which are widespread in vegetables and are consumed as part of the human diet. There are other types of polyphenols such as tannins, resveratrol. Flavonoids and related polyphenolic compounds have significant anti-inflammatory activity (González et al., 2011). Total polysaccharide and phenolic contents have strong correlation with antioxidant activity, so higher phenolic and polysaccharide contents could be a significant source of natural antioxidants (Birhanie et al., 2021) according to Zhang et al., 2019 report. Here, all studied samples are strongly efficient natural antioxidants with highest values of total flavonoids, phenolic and polysaccharide contents being in *G. atroviridis*, *G. schomburgkiana*, *M. alba* and *C. sinensis*, respectively (Table 3) according to all quoted references which indicated their antioxidant activity.

The research has the objective of testing the inhibition percentage of CEase and PPL for all studied samples. Orlistat was selected to be the control due to its activity. It is a weight-loss agent with a novel mechanism of action, which was approved by the Food and Drug Administration for the treatment of obesity. It inhibits gastric and pancreatic lipases in the lumen of the gastrointestinal tract to decrease systemic absorption of dietary fat (Heck et al., 2000). Therefore, the natural product activity of these studied samples has to be compared to orlistat control. The results showed efficacy of *G. atroviridis* and the Formula 1 for CEase inhibition, and *G. schomburgkiana* and Formula 1 for PPL inhibition compared to orlistat, but in different concentration. Orlistat, which was tested at about three-times higher concentration (10 mg/ml) than Formula 1 (3 mg/ml), showed higher efficacy than Formula 1. So, with identical concentrations using 10 mg/ml, the inhibition efficiency for both CEase and PPL by the Formula 1 should be higher than orlistat.

Toxicity assays exhibited no IC_{50} values in all samples, but induced significant DNA damage ($p<0.01$) by ethanol *Gs* and *Cs* extracts and methanol *Ma* extract compared to negative control. However, LD_{50} values calculated from the concentrations used, 6.30, 0.90 and 3.00 mg/ml, LD50 values for the rat were 2,737.50, 1,692.60, and 2,077.05 mg/kg body weight which are in WHO class III (slightly hazardous when oral over 2,000 mg/kg body weight), class II (moderately hazardous, when oral 50-2,000 mg/kg body weight), and class III (when oral over 2,000 mg/kg body weight will get slightly hazardous) following Table 4. That is actually a very high dosage, which is unlikely to be consumed. So, the summary is that there are no effects on humans.

Conclusion

The studied plants, *G. atroviridis*, *G. schomburgkiana*, *M. alba* and *C. sinensis* contained crucial bioactive compounds such as HCA, catechin, and catechol, as well as prototype creation as Formula 1 and 2 from these plants. From the results, not only the single plant can inhibit the CEase and PPL activities, but also Formula 1 which was formulated from the four studied species in a rate ratio, has no toxic effects on humans when consumed for reducing cholesterol and inhibiting lipase activity leading to innovation for weight loss and high cholesterol treatments without any side effects.

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Contribution of Authors

Sudmoon R: Acquired funding, performed and examined the experiments, analyzed data, and wrote original manuscript draft

Kaewdaungdee S, Ameamsri U & Wonok W: Partially performed the experiments

Tanee T: Partially supervised and performed experiments.

Siripiyasing P: Provided plant materials and performed data validation.

Chaveerach A: Conceptualized, project administration, acquired funding, supervised, wrote the manuscript, reviewed and edited.