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Medicinal *Lagerstroemia* dose designs following γ-sitosterol quantity and human diary need for toxicity testing before use in the antihyperglycemic treatment

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ABSTRACT

Lagerstroemia floribunda and *L. macrocarpa* were investigated for phytochemicals and toxicity. Phytochemical contents were screened via GC-MS and HPLC with ethanol and hexane extracts. MTT and comet assays were performed on human peripheral blood mononuclear cells (PBMCs). Major phytochemicals contained at higher than 10% were31.83% γ-sitosterol, 10.73% phytol in *L. floribunda*; 34.97% γ-sitosterol, 13.65% oleamide and 10.56% squalene in *L. macrocarpa*. MTT assay showed IC_{50} values as 0.54 and 0.60 mg/ml in *L. floribunda* and *L. macrocarpa* ethanol extracts, yet no IC_{50} values with hexane extracts indicating high cell viability percentage 62.77 ± 1.53-75.28 ± 3.10 and 68.45 ± 3.25-84.39 ± 1.30. The IC_{50} value predicted LD_{50} of 1097.62 and 1141.49 mg/kg in *L. floribunda* and *L. macrocarpa*, slightly hazardous category of toxic chemicals. Comet assay revealed that the two studied species induced significant DNA damage in PBMCs ($p < 0.05$). Based on the previous reports that γ-sitosterol possess antihyperglycemic activity, combinations of *L. floribunda*, *L. macrocarpa*, *L. speciosa* and *L. indica* plants which contained a sufficient amount of γ-sitosterol were evaluated. They displayed no IC_{50} values but induced significant DNA damage in PBMCs ($p < 0.05$) amid all combinations. So, consumers should concentrate on their genotoxicity.

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INTRODUCTION

Both edible and medicinal plants are present in rather high numbers in Thailand, which includes the *Lagerstroemia* species, for examples, *L. speciosa* and *L. indicia* are also ornamental plants. *Lagerstroemiaindica* extracts exhibit anti-inflammatory properties, so they have been applied for treating asthma (Yang *et al.,* 2010, Labib *et al.,* 2012), and also exhibit analgesic, antihyperglycemic, antioxidant hepatoprotective effects (Labib *et al.,* 2013), and antidiabetic activity (Ashnagar *et al.,* 2013). Additionally, *L. speciosa* leaf extract was used for diabetes treatment as it contains ursolic acid (Klein *et al.,* 2007, Hou *et al.,* 2009), ellagitannins (Miura 2012), ellagic acids, flavones, glycosides,

tannins and triterpenes (Chan *et al.,* 2014). With these supporting researches and it was listed by the Ministry of Public Health as one of the promise medicinal plants in Thailand, *L. speciosa* has been used for diabetes treatment in modified forms such as tea and capsule form. Amongst the most recent publication, Sirikhansaeng *et al.,* (2017) reported that γ-sitosterol, which was another compound possess antihyperglycemic activity (Balamurugan *et al.,* 2011, 2012), was the major component found in the four *Lagerstroemia* species including. *indica*, *L. loudonii*, *L. speciosa* and *L. villosa*.

Many more species in addition to *L. speciosa* and *L. indica* exist in Thailand including *L. floribunda* and *L. macrocarpa*. This research aims to reveal their phytochemical constituents and perform toxicity testing at the cell level by MTT assay(Berridge *et al.,* 2005) and at the genomic level by comet assay(Collins, 2004) on PBMCs. Moreover, the combination of *L. speciosa*, *L. indica*, *L. floribunda* and *L. macrocarpa* dose designs following γ-sitosterol quantity and daily human requirements in need of toxicity testing, were tested before application amid antihyperglycemic treatment.

MATERIALS AND METHODS

Plant materials

Leaves of *Lagerstroemia floribunda* and *L. macrocarpa* were collected and used for ethanol and hexane extractions. These extracts will be used for phytochemical analysis and toxicity testing.

Following tea and capsule models, γ-sitosterol content in the plants, and daily human needs of γ-sitosterol based on experiments in rats at 20 mg/kg body weight (Balamurugan *et al.,* 2011),the combinations of *L. speciosa*, *L. indica*, *L. floribunda* and *L. macrocarpa* finely ground leaves were designed for a 300 mg capsule in accordance with the following proportions,and referred to as Drug1 (*L. speciosa* 240 mg + *L. floribunda* 60 mg), Drug 2 (*L. speciosa* 240 mg + *L. indica*60 mg), Drug 3 (*L. speciosa* 240 mg + *L. macrocarpa*60 mg) and Drug 4 (*L. speciosa* 300 mg). These drugs were further toxicity tested by MTT and comet assays.

Phytochemical extraction

Crude extractions by ethanol and hexane were performed following Sirikhansaeng *et al.,* (2017). Briefly, leaf samples were cleaned and air-dried until the water had evaporated, then finely ground. The 20 g sample powder was soaked with 120 ml of hexane or ethanol (analytical grade), separately, for 72 h in the dark, at room temperature. The mixtures were subsequently filtered through a filter paper at room temperature. Then these filtrates were aliquoted and subjected to GC-MS analysis. The volume of the remaining filtrates was measure and they were subjected to a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) at 15°C, 600 rpm for 2 h to eliminate the solvent, then redissolved in dimethyl sulfoxide (DMSO) to the original volume of each filtrate. These stock extracts were stored at -20°C for further experiments.

Gas-chromatography-mass spectrometry (GC-MS)

Phytochemical analysis was performed following Sirikhansaeng *et al.,* (2017). The filtrates were subjected to an HP 6890 GC with 5973 MSD(Agilent Technologies). Peak area normalization was calculated to show relative percentage of constituents in the samples, and the constituents were identified using the mass spectra similarity to the reference compounds (Wiley 7N.1 library).

High-performance liquid chromatography (HPLC)

The exact amount of corosolic acid in the extracts was determined using 1260 Infinity HPLC System (Agilent Technologies) with Hypersil ODS C18, 4.0×250 mm, 5 Micron, with a detection wavelength of 210 nm. The standard corosolic (Sigma Aldrich) solution was prepared at 1mg/ ml in ethanol. The 10 µl each of the samples and the standard solution were subjected and the mobile phase solutions were 0.1% phosphoric acid and acetonitrilecarried out by increasing acetonitrile ratio from 55% to 100% at 0-35 min, at the flow rate of 1 ml/min.

Preparation of working solutions of the plant extracts and the drugs

To prepare the working solutions, the stock extracts of *L. floribunda* and *L. macrocarpa* were 10-fold serial diluted in water for five levels. For the drugs, each combination (300 mg) was dissolved in 12 ml of 10% DMSO (the concentration was 25 mg/ml) and these solutions were used for toxicity testing.

Preparation of human peripheral blood mononuclear cells (PBMCs)

Pooled buffy coat samples advocated by blood bank were adopted for PBMCs preparation using-Ficoll-Paque Plus (GE Healthcare) following the method provided by the company. The cells were freshly prepared at a concentration of 10^6 cells/mL in modified RPMI-1640 medium following Sirikhansaeng *et al.,* (2017), and have at least 98% viability before using in toxicity testing.

MTT assay and comet assay

Cytotoxicity testing by MTT and comet assays were performed following Sirikhansaeng *et al.,* (2017) in 96-well plates with 125 µl of cell solution per well

Table 1: Chemical constituents of ethanol and hexane *Lagerstroemia floribunda* and *L. macrocarpa* leaves

Compound	Formula	% Relative content			
		L. floribunda (ethanol)	*L. floribunda* (hexane)	*L. macrocarpa* (ethanol)	*L. macrocarpa* (hexane)
γ-Sitosterol	$C_{29}H_{50}O$	20.23	31.83	17.02	34.97
(Z)-9-Octadecenamide (Oleamide)	$C_{18}H_{35}NO$	5.13	8.45	11.71	13.65
5-Hydroxymethylfurfural	$C_6H_6O_3$	8.98	-	13.74	-
Phytol	$C_{20}H_{40}O$	10.73	0.91	6.81	6.52
Phytol, acetate	$C_{22}H_{42}$	4.85	6.66	3.29	1.57
Squalene	$C_{30}H_{50}$	8.85	-	10.56	0.74
24-Methylenecycloartanol	$C_{31}H_{52}O$	-	1.74	-	-
Stigmast-5-en-3-ol,oleate	$C_{47}H_{82}O_2$	-	-	8.28	9.80
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	7.48	4.93	2.80	1.94
Stigmastan-3,5-diene	$C_{29}H_{48}$	-	5.46	-	7.22
Campesterol	$C_{28}H_{48}O$	3.78	7.11	2.60	5.80
3,7,11,15-Tetramethyl-2-hexadecene -1-ol	$C_{20}H_{40}O$	2.74	3.92	2.20	0.75
α-Tocopherol	$C_{29}H_{50}O_2$	0.96	4.10	1.87	6.08
Acetic acid, 7-isopropyl-3a,3b, 9b-trimethyl-2-phenyl-3a,3b,4,5,5a,7,8, 9,9a,9b,10,11-dodecahydro-6-oxa-cyclopenta[a]phenanthren-3-yl ester	$C_{30}H_{40}O_3$	-	5.97	-	1.70
cis-11-Eicosenamide	$C_{20}H_{39}NO$	0.28	-	0.58	-
7-Dehydro diosgenin	$C_{27}H_{40}O_2$	-	5.93	-	1.91
Linoleic acid	$C_{18}H_{32}O_2$	4.18	-	-	-
1,2,3-Benzenetriol	$C_{13}H_{18}O_4$	3.47	-	3.54	-
Clindamycin	$C_{18}H_{33}ClN_2O_5S$	3.44	-	1.37	-
Hexadecanamide	$C_{16}H_{33}NO$	0.70	0.78	1.40	1.18
16,16, O-Trimethyl-11β-methoxy androsterone	$C_{23}H_{38}O_3$	-	2.99	-	-
1,2,4-Trimethoxybenzene	$C_9H_{12}O_3$	-	2.87	-	-
Glycerol β-palmitate	$C_{19}H_{38}O_4$	1.51	-	0.83	-
Linolenic acid	$C_{18}H_{32}O_2$	2.69	-	-	-
Vitamin E	$C_{29}H_{50}O_2$	2.53	-	2.28	-
Ethyl α-d-glucopyranoside	$C_8H_{16}O$	1.22	-	2.40	-
Malic acid	$C_4H_6O_5$	2.40	-	-	-
A-Neooleana-3(5),12-diene	$C_{30}H_{48}$	-	2.36	-	-
Lupeol	$C_{30}H_{50}O$	-	-	-	2.30
Stigmasterol	$C_{29}H_{48}O$	-	-	0.14	2.05
Octadecanamide	$C_{18}H_{37}NO$	0.34	0.33	0.97	0.45
Pyranone	$C_6H_6O_3$	1.74	-	1.21	-
Ergot-5-en-3-yl acetate	$C_{30}H_{50}O_2$	-	-	1.60	-
Olean-12-en-3-one	$C_{30}H_{48}O$	-	1.31	-	1.37
β-Glyceryl monostearate	$C_{21}H_{42}O_4$	0.22	-	0.14	-
Tetratetracontane	$C_{44}H_{90}$	-	-	0.99	-
Dianhydromannitol	$C_6H_{10}O_4$	-	0.88	-	-
Benzene, 1,2,3-trimethoxy-5-methyl-	$C_{10}H_{14}O_3$	-	0.80	-	-
Dihydroactinidiolide	$C_{11}H_{16}O_2$	-	0.67	-	-
4-O-Hexopyranosylhexose	$C_{12}H_{22}O_{11}$	0.67	-	-	-
β-Amyrin	$C_{30}H_{50}O$	-	-	0.46	-
Cholesta-4,6-diene-3-ol	$C_{27}H_{44}$	-	-	0.44	-
Stigmata-5,24(28)-diene-3β-ol	$C_{29}H_{48}O$	-	-	0.40	-
Sakuranin	$C_{22}H_{24}O_{10}$	-	-	0.37	-
Oleic Acid	$C_{18}H_{34}O_2$	0.31	-	-	-
α-Glyceryl linoleate	$C_{21}H_{36}O_4$	0.30	-	-	-
Octadecanoic acid	$C_{18}H_{36}O_2$	0.27	-	-	-

Table 2: The contents of corosolic acid (mg/ml) determined by HPLC from leaf extracts of *Lagerstroemia floribunda* and *L. macrocarpa*

Plant sample	Amount in each type of solvent (µg/mL)	
	Ethanol	Hexane
L. floribunda	3.50	not detected
L. macrocarpa	not detected	not detected

Table 3: Mass concentration with ethanol and hexane solvents, IC50 values and % cell viability of *L. floribunda* and *L. macrocarpa*

Plant sample	Solvent	Maximum extract concentration (mg/mL)	IC_{50} (mg/mL)	% Cell viability (mean ± S.D.)
L. floribunda	Ethanol	7	0.54	-
	Hexane	1		62.77 ± 1.53-75.28 ± 3.10
L. macrocarpa	Ethanol	11	0.60	-
	Hexane	1		68.45 ± 3.25-84.39 ± 1.30

Table 4: Viability percentages of cells treated with the Drugs via MTT assay showing high values, without IC_{50} values

Drug sample	Combination	% Cell viability (mean ± S.D.)
Drug 1	*L. speciosa* 240 mg + *L. floribunda* 60 mg	97.33 ± 0.83
Drug 2	*L. speciosa* 240 mg + *L. indica*60 mg	95.50 ± 3.82
Drug 3	*L. speciosa* 240 mg + *L. macrocarpa*60 mg	92.93 ± 1.94
Drug 4	*L. speciosa* 300 mg	87.04 ± 1.84

treated with 12.5 µl of the prepared working solutions of the plant extracts and the drugs. The cells were incubated in a humidified incubator with5% CO_2 at 37°C for 4 h. Negative control cells were untreated. Positive control cells were exposed to UV light for 20 min. Vehicle control cells were treated with 1% DMSO. After 4 h of incubation, the medium was removed by pipetting.

For MTT assay, 10 µl of 0.5 mg/ml MTT (Sigma, USA) was added in each well, then immediately incubated in the dark for 4 h at 37°C for formazan crystallization. The crystal was dissolved in 100 µl of DMSO for 2 hin the dark, then read for absorbance at 570 nm using a microplate reader (SpectraMax M5 series, Molecular Devices). Percentage of cell viability was calculated using the following equation: cell viability (%) = (average viable of treated cells/average viable of negative control cells) × 100. Doses inducing 50% inhibition of cell viability (IC_{50} value) were determined from a cell viability graph and the IC_{50}value was employed for the LD_{50} calculation (Walum 1998) to categorize hazardous levels of the tested solution (World Health Organization, 2009).

For comet assay, the treated cells were dropped on agarose gel-coated glass slides and subjected to electrophoresis (Singh *et al.,* 1988) with the power supplying at 26V, 300 mA for 25 min. The comet images were recorded at 200 magnification by employing a fluorescence microscope (Nikon, Japan), equipped with a 560 nm excitation filter, 590 nm barrier filter and a CCD video camera PCO (Germany). At least 150 cells (50 cells for each of triplicate slides) were examined for each experiment. To quantify the level of DNA damage, Olive Tail Moment (OTM), was analyzed by CASP software. The nonparametric Mann-Whitney test was used for statistical analysis of the comet assay results and statistical significance was set at $p<0.05$.

RESULTS

Phytochemical screening of the filtrate from *L. floribunda* and *L. macrocarpa* ethanol and hexane crude extracts (Figure 1, 2) showed that were several phytochemicals were dividing into two groups,i.e. major and minor groups. The major phytochemicals containing higher than 10% were 31.83% γ-sitosterol and 10.73% phytol in *L. floribunda*; 34.97% γ-sitosterol, 13.65% oleamide and 10.56% squalene in *L. macrocarpa*. The minor substance group exhibiting lower than 10% is shown in Table 1.

Analysis of the ethanol and hexane *L. floribunda* and *L. macrocarpa* extract components via HPLC concentrated on corosolic acid findings. The results showed no detection with hexane amid all of the two studied species extracts as well as ethanol *L. macrocarpa* extract. A minimal amount in the ethanol *L. floribunda* extract was detected as shown in Table 2.

Mass of the crude extracts of the two samples derived from ethanol solvent was higher than that of hexane solvent. These extracts were subjected to serial 10-fold dilution for five levels, as used for the MTT assay. The ethanol *L. floribunda* and *L.macrocarpa*extracts showed IC_{50} values of 0.54and 0.60 mg/ml, but no IC_{50} values in hexane *L. floribunda*

Table 5: The level of DNA damage expressed as Olive Tail Moment (OTM) in PBMCs post-treatment with ethanol and hexane *Lagerstroemia floribunda* and *L. macrocarpa* leaf extracts

Sample	Combination (mg)	Olive Tail Moment (mean ± S.D.)		*p*-value
		Negative control	Drug	
Drug 1	*L.speciosa* 240 mg + *L. floribunda* 60 mg	0.11 ± 0.11	1.73 ± 0.48	< 0.0001
Drug 2	*L.speciosa* 240 mg + *L. indica*60 mg	0.30 ± 0.11	2.13 ± 0.79	< 0.0001
Drug 3	*L.speciosa* 240 mg + *L. macrocapa*60 mg	0.30 ± 0.11	2.44 ± 0.78	< 0.0001
Drug 4	*L.speciosa* 300 mg	0.11 ± 0.11	2.54 ± 0.92	< 0.0001

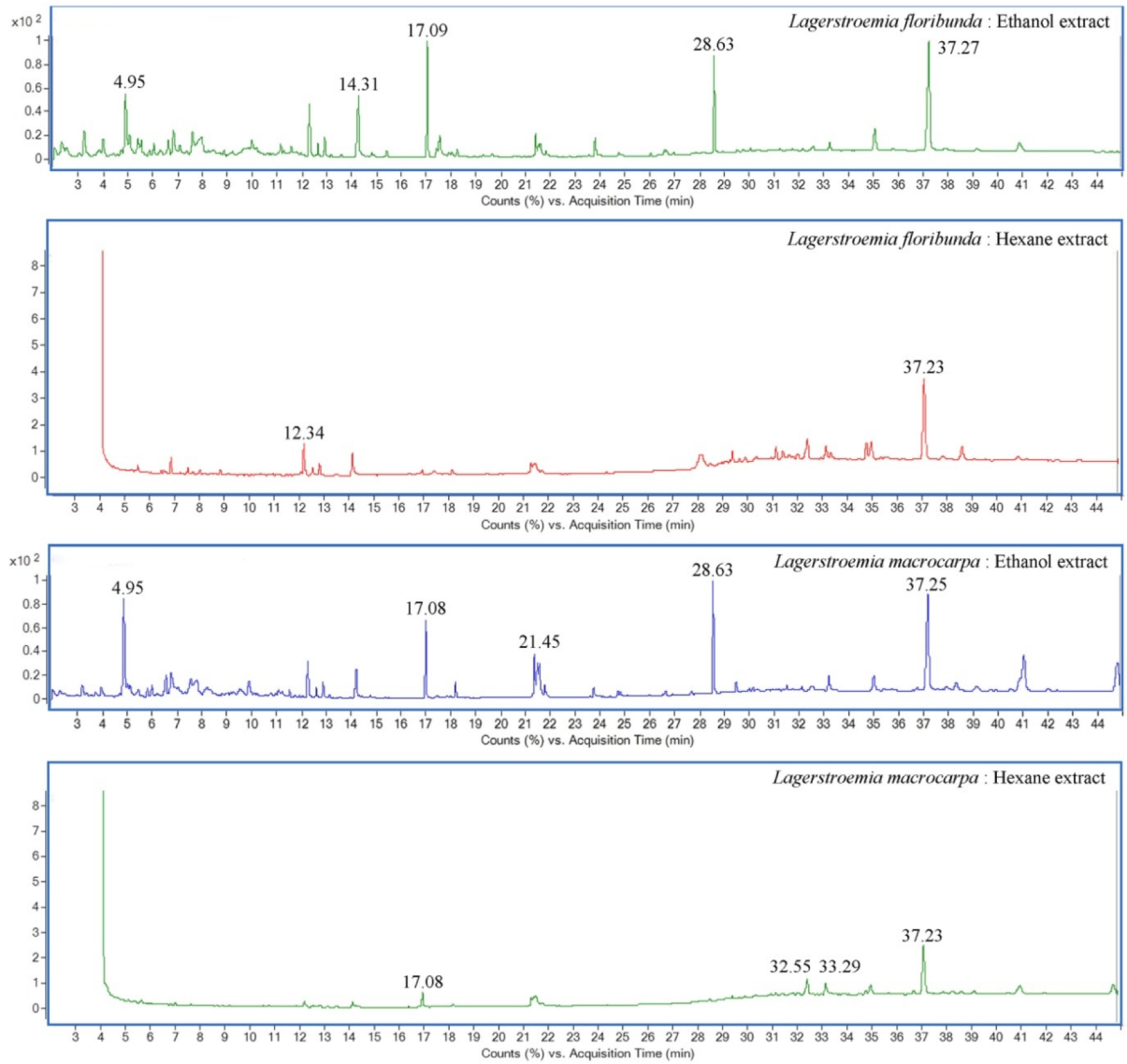

Figure 1: Chromatograms of ethanol and hexane extracts from leaves of *L. floribunda* and *L. Macrocarpa* leaves

and *L.macrocarpa* extracts. They showed percentages of cell viability at 62.77 ± 1.53-75.28 ± 3.10 and 68.45 ± 3.25-84.39 ± 1.30 (Figure 3 and Table 3).

Based on MTT assay, Drugs 1, 2, 3, and 4exhibited high percentages of cell viability at87.04 ± 1.84 to 97.33 ± 0.83 (Table 4 and figure 4), so no IC_{50} value. However, for the in-depth study of genotoxicity level with comet assay, the results showed that compared to negative control (untreated cells), the four tested Drugs induced significant DNA damage in PBMCs (p<0.05) (Table 5 and Figure 5).

DISCUSSION

The plants, *L. floribunda* and *L. macrocarpa*, contained high amounts ofγ-sitosterol (31.83% and 34.97%) which is most notable for indicating potential single bioactivity for treatment when consumed.*L. macrocarpa* contained a higher amount, (34.97%) than *L. speciosa* which exhibited 34.4% γ-sitosterol (Sirikhansaeng *et al.,* 2017). Those remaining in major and minor groups ought to have their activities supported, and include γ-sitosterol.

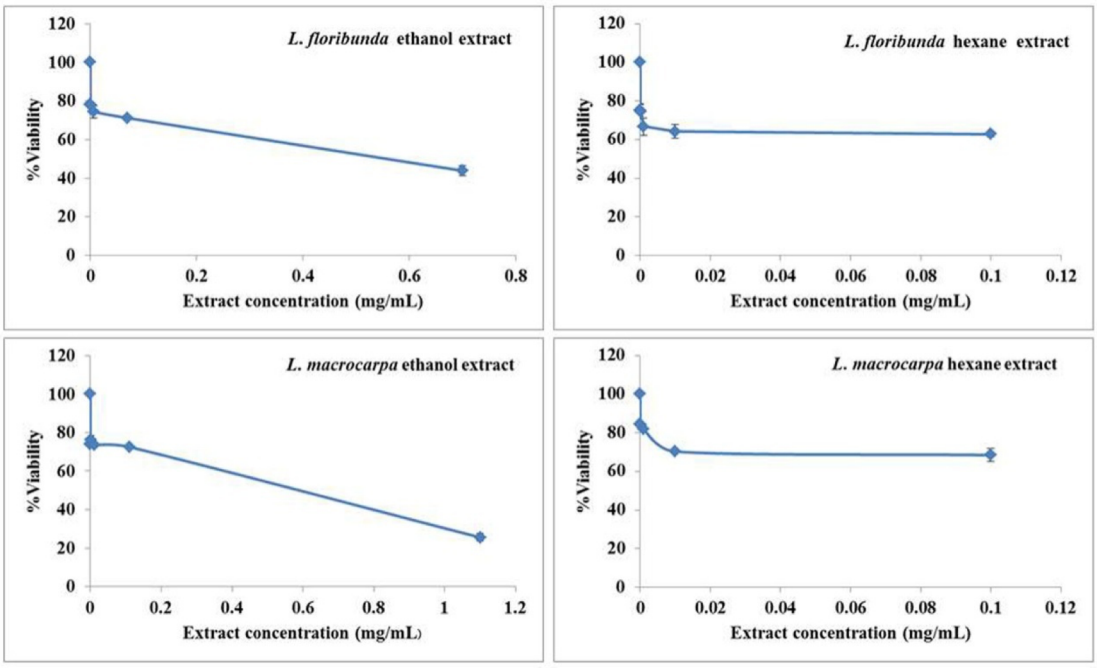

Figure 2: Cytotoxicity and IC50 values of ethanol and hexane extract from Lagerstroemia floribunda and L. macrocarpa

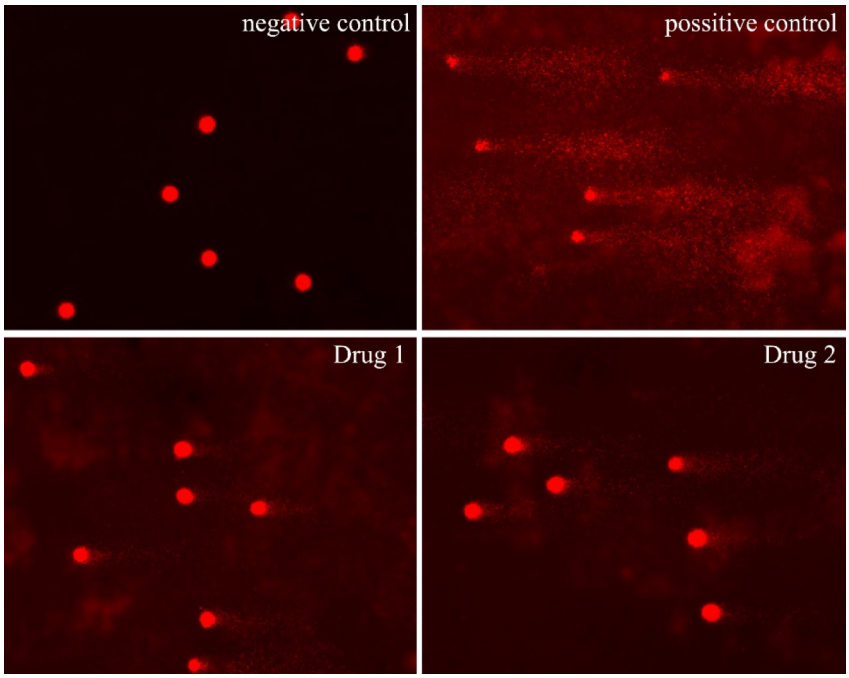

Figure 3: Comet assay images of PBMC's (200X); negative control, positive control, Drug 1 and Drug 2. Similar figures are not presented

Additionally, HPLC showed their results to be lacking or in a small amount (3.50 µg/ml in *L. floribunda*) of corosolic acid in agreement to the information from GC-MS analysis. So, *L. floribunda* and *L. macrocarpa* can effectively be employed for the activity of γ-sitosterol -just as *L. speciosa* and *L. indica*; especially amid diabetes treatment. For this benefit, their toxicity was evaluated and resulted in that: MTT assay showed IC_{50} values as 0.54 and 0.60 mg/ml in *L. floribunda* and *L. macrocarpa* ethanol extracts, yet no IC_{50} values with hexane extracts indicating a high percentage of cell viability 62.77 ± 1.53-75.28 ± 3.10 and 68.45 ± 3.25-84.39 ± 1.30. These assumptions are caused by the fact that polar phytochemicals dissolve more readily in ethanol since it is a more polar substance than hydrocarbon hexane - which is part of the non-polar group. The IC_{50} value was used to predictLD_{50} of1097.62 and 1141.49 mg/kg in *L. floribunda* and *L. macrocarpa* belonging to WHO Class III of toxic chemicals (over 500 mg/kg body weight, oral), slightly hazardous category of toxic chemicals. Accordingly, comet assay for genome toxicity testing

revealed that the two species studied induced significant DNA damage in PBMCs (p<0.05).

γ-sitosterol was previously reported to possess anti-hyperglycemic activity by increasing insulin secretion in response to glucose, as confirmed by an immune histochemical study of the pancreas (Balamurugan *et al.,* 2011, Balamurugan *et al.,* 2012). As a consequence, it was composed of several factors, namely, its high content in *L. floribunda*, *L. macrocarpa*, *L. speciosa* and *L. indica*; *L. speciosa* and *L. indica* have long been used as medicinal plants amid a plethora of treatments (Klein *et al.,* 2007, Hou *et al.,* 2009, Yang *et al.,* 2010, Miura *et al.,* 2012, Ashnagar *et al.,* 2013, Labib *et al.,* 2013, Chan *et al.,* 2014). Furthermore, *L. speciosa* was registered as one of the 170 recognised medicinal plants in Thailand as listed by the Ministry of Public Health. Consequently, in Thailand, *L. speciosa* has been applied to diabetes treatment in modified forms such as tea and capsules (Figure 4). Therefore, in this research, the four plant combinations in a 300 mg ground plant form(supposed to be a capsule or a tea sac) in proportions referred to as 'Drugs', were designed to have their toxicity tested for repetition checking prior to use in humans, following γ-sitosterol quantity and human daily use amid anti-hyperglycemic treatment. They exhibited no IC_{50}, yet induced significant DNA damage in PBMCs (p<0.05) in all combinations, i.e. Drugs 1-4. Notably, both MTT for cytotoxicity and comet assay for genotoxicity showed greater toxicity in the medicinal *L. speciosa* plant alone -which was more used than the others according to authors'observation. Hence, consumers ought to concentrate on their genome toxicity even if a capsule or tea sac is being consumed.

Figure 4: Medicinal L. species plants in prepared forms as tea sacs and capsule quoted as a diabetes treatment

For other major phytochemicals including 10.73% phytol in *L. floribunda*, 13.65% oleamide and 10.56% squalene in *L. macrocarpa*, they exhibited specific functions, though were assumed insufficient in that they were unable to produce single bioactivity. However, as support, most are beneficial to humans. Oleamide is a protective agent against scopolamine-induced memory loss and is suggested use as a chemopreventive agent against Alzheimer's disease (Heo *et al.,* 2003). Additionally, it induces deep sleep (Huitron-Resendiz 2001) and the upregulation of appetite(Dewey 1986, Martinez-Gonzalez 2004). Squalene is a triterpene necessary for life. In the human body, it is a natural and essential component used for the syntheses of cholesterol, steroid hormones and vitamin D. It may also be an anticancer substance, as it possesses chemo-preventative activity (Smith 2000, Owen *et al.,* 2004). Phytol is diterpene alcohol that can be applied as a precursor for the manufacture of synthetic forms of vitamin E (Netscher 2007) and vitamin K1 (Daines *et al.,* 2003). Besides that, it is used in the fragrance industry in the manufacture of cosmetics, shampoos, toilet soaps, household cleaners and detergents. In actuality, each phytochemical possesses specific functions, yet they may potentially be unknown. Therefore, the tests for total substance contents, for safe human usage minus toxicity including cytotoxicity and genotoxicity levels, are the most appropriate experiments to be carried out.

CONCLUSION

The phytochemical γ-sitosterol is also found in *L. floribunda* and *L. macrocarpa* at much the same high amounts as found in the former two studied species, *L. speciosa* and *L. indica*. They were of great interest; however, consumers would be well advised to consider plant toxicity.

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