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PHYTOCHEMICAL SCREENING OF *IN VITRO AGLAONEMA SIMPLEX* PLANTLET EXTRACTS AS INDUCERS OF SR-B1 LIGAND EXPRESSION

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Abstract: *Aglaonema simplex* is an aquatic plant that has been widely used as ornamental plants. The genus contains polyhydroxy alkaloids that exhibit the glycosidase inhibitor activity. This paper reports a phytochemical screening of *in vitro Aglaonema simplex* plantlets and the potential compounds as alternatives of SR-B1 ligand that plays a role in reducing atherosclerosis. The phytochemical screening was conducted using Thin Layer Chromatography and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy on methanol crude extracts of leaves, stems and roots. SR-B1 ligand activities were tested on HepG2 cell line stably transfected with SR-B1 promoter. The results showed that the extracts contained secondary metabolites belonging to the terpenoids, steroids, phenolics, alkaloids and glycosides. Luciferase assay suggested that the stem and root extracts increased the expression of SR-B1 at 1.61- and 1.72-fold higher than the control, respectively. Thus, *Aglaonema simplex* is one of the potential sources of the phytochemicals for the treatment of atherosclerosis. The tissue culture technology may be applicable for sustainable production of the identified compounds from the plant.

Keywords: Atherosclerosis, polyhydroxy alkaloids, hepatocellular carcinoma, cholesterol.

Introduction

Statistics by the World Health Organization (WHO, 2014) shows that coronary heart diseases are ranked as the first cause of mortality with 36% of death in Malaysia. Coronary heart disease occurs when the deposition of lipids in arterial vessel walls leads to the formation of atheroma that occludes the vessel lumen (Imachi *et al*., 2003). Plasma levels of high density lipoprotein (HDL) and its major protein apolipoprotein A-1 (apoA-1) are inversely associated with the risk of atherosclerosis diseases (Zhang *et al*., 2005). HDL and apoA-1 play pivotal roles in the protection against atherosclerosis via reverse cholesterol transport (RCT). In the direct pathway of RCT, the transport of HDL-cholestryl esters is mediated by scavenger receptor class B type 1 (SR-B1), a cell-surface of high density lipoprotein (HDL) receptor, found abundantly in liver and steroidogenic tissues. SR-B1 not only modulates the HDL cholesterol uptake, but also stimulates the free cholesterol efflux from the cells into HDL, suggesting a major role played by SR-B1 expression in cholesterol removal (Acton *et al*., 1999). To date, statin is widely used as a drug for atherosclerosis treatment, but the adverse side effects can cause muscle toxicity and liver enzyme abnormalities (Hu *et al*., 2012). Statin is also reported to reduce the coenzyme Q10 levels that may further exacerbate oxidative stress in chronic heart failure patients (Krum & McMurray, 2002). Moreover, the production cost of synthetic drugs is exorbitantly expensive, that a new alternative approach using natural product must be explored to develop a safe, reliable and cheaper drug for the treatment of the disease.

Research on natural products capable of increasing the SR-B1 expression is still limited. Plant secondary metabolites actually offer interesting medicinal properties as remedies for various types of diseases. An *in vitro* study of ethyl acetate crude extract of *Phaleria macrocarpa* Boerl. (*Thymelaeaceae*) leaves have shown to increase the SR-B1 expression up to 95% of the control (Andriani *et al*., 2015). Genus *Aglaonema* is a medicinal plant (Chen *et al*., 2007) but is often used as ornamental and aquarium plant. It can be found in Tropical Asia, Malay Archipelago and Papua-Asia rainforest (Mayo *et al*., 1997). In folk medicine, the root

infusion of *A. hookerianum Schott* is taken orally by Chakma tribe in Bangladesh for the treatment of conjunctivitis and constipation (Rahman *et al*., 2007). The Semelai people in Tasik Bera, Pahang, Malaysia, use poultice made from the leaves and roots of *A. nebulosum N.E.Br.* (known as *kayu ulat bulu* among them) to cure skin irritation caused by caterpillar and worms (Wetlands International, 2006).

Previous study on *Aglaonema* species suggests the presence of two or more hydroxyl groups attached to the heterocyclic nitrogen atom known as polyhydroxy alkaloid or imino sugars (Asano *et al*., 1997; Rodríguez-Sánchez *et al*., 2016). Six polyhydroxy alkaloids namely α-homonojirimycin, 2(*R*), 5(*R*)-bis-(hydroxymethyl)-3(*R*), 4(*R*)-dihydroxypyrrolidine, α-*allo*-homonojirimycin, glycosyl-homonojirimycin, 2,3,4, 5-tetrahydroxy-6-hydroxymethyl piperidine have been isolated from the pressurized liquid extraction of seven *Aglaonema* species (Rodríguez-Sánchez *et al*., 2016). *Aglaonema* plant has also been reported to possess glycosidase inhibitors (Asano *et al*., 1997) and anti-bacterial activities (Ma *et al*., 2004; Roy *et al*., 2011).

The aim of this study was to determine the potential of *Aglaonema simplex* as an alternative source for SR-B1 ligand expression in the treatment of atherosclerosis. Methanol extraction of *A. simplex in* vitro culture will be carried out and the crude extracts were later subjected to phytochemicals profiling using Thin Layer Chromatography (TLC) and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). Luciferase assay was carried out to establish the interaction between *A. simplex* extracts and SR-B1 promoter region by directly measuring by the intensity of bioluminescence.

Materials and Methods

Methanol Extracts

Four month-old *in vitro* culture of *A. simplex* was used as a plant material. The plantlets were proliferated and grown in MS basic medium with conditions as described previously (Aziz *et al*., 2008). Upon harvesting, the plantlets were excised and divided into leaves (L), stems (S) and roots (R) parts. All samples were oven-dried until constant weight and ground to powder using electronic grinder. Powdered sample from leaves part was then extracted with methanol at the ratio of 1:10 (g powder/mL solvent) by cold maceration, and then filtered to give a crude liquid extract (Molyneux *et al*., 2002; Li *et al*., 2005). The extraction process was repeated four times or until the filtrate became colourless. The extracts were combined and concentrated using rotary evaporator at 40 ºC to give methanolic extracts. The same procedure was used to obtain extracts from other parts (stems and roots). The crude extracts were kept refrigerated at -20 ºC until further analysis.

Phytochemicals Profiling Using Thin Layer Chromatography (TLC)

The methanolic extracts were chromatographed on C-18 reverse phase–TLC plate with solvent system of EtOH: H_2O: AcOH (9:1:0.1). The spots on the plate were detected by different visualization reagents such as anisaldehyde-sulfuric acid 1 (with glacial acetic acid), anisaldehyde-sulfuric acid 2 (with ethanol), UV -254 nm, UV -365 nm, ninhydrin, iodine vapour, vanillin-sulfuric acid and Dragendorff (Pyka, 2004). Another Dragendorff test was conducted on silica gel 60, F_{254} TLC plate.

Phytochemicals Profiling Using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR screening was carried out according to Das *et al*. (2005). A small amount of concentrated methanolic crude extracts was directly placed on the surface of diamond crystal piece of the Thermo scientific Nicolet™ iS10, infrared spectrometer. Subsequently a constant pressure was applied to the sample. The spectra were obtained with the aid of an OMNI-sampler ATR accessory within the range of 400 – 4000 cm^{-1} wave number at 32 scans. The reference spectra were acquired from the clean blank diamond crystal. Detection was done based on

the peak value in the region of infrared spectrum and compared with literature based on Coates (2006).

ONE-Glo™ + Tox Luciferase Assay

The effect of extracts on both cell viability and luciferase activity was measured using ONE-GloTM+Tox Luciferase Reporter and Cell Viability Assay (Promega Corporation, Madison, WI, USA) (Cappato *et al*., 2016). A stable HepG2 cell line transfected with SR-B1 promoter was provided by the Malaysian Institute of Pharmaceutical and Nutraceuticals (iPharm), Penang, Malaysia. The cell-line was maintained on Minimum Essential Medium (MEM) containing 10 % (v/v) Fetal Bovine Serum (FBS), 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acid and 1% (v/v) penicillin-streptomycin (Gibco) (Andriani *et al*., 2015).

For assay, a cell culture stock was harvested when 70 to 80 % confluency was reached, and seeded into 96-wells plate (Thermo Scientific) at the density of approximately 30000 cells/well. The HepG2-SRB1 cell line was incubated for 24 hrs, washed with phosphate buffered saline (PBS) solution twice and replaced with 95 µL of new media containing 0.5 % FBS. Methanolic extracts of *A. simplex* were prepared at two-fold dilution in concentrations gradient starting from 1.56 – 100 µg/mL concentration. Five microliters of extracts diluted to various concentrations were loaded into the well, separately. The untreated cells were used as background control, while the treated cells with 0.1% (v/v) methanol as a negative control. Subsequently, the 96-well plate was incubated for 24 h at 37 °C in humidified, 5 % CO_2 atmosphere. A 20 µL of 5X CellTiter-Fluor™ reagent was added and briefly mixed in each well by orbital shaking (~ 500 rpm for 30s), and re-incubated for another 30 min at 37 ºC. The cell viability based on Fluorescence intensity was measured at 380 -400 nm_{Ex}/505 nm_{Em}. Then, 100 µL of One-Glo™ reagent was added to all wells and incubated at room temperature for 3 min, and the luminescence was measured using Glomax Microplate reader (Glomax Multi Detection System, Promega Corporation, Madison, WI, USA).

Statistical Analysis

Data were analysed in triplicates and expressed as a mean ± SD. Statistical comparison was performed using one way analysis of variance (ANOVA), followed by Tukey test using GraphPad Prism 6 software. Results with $p \leq 0.05$ are considered statistically significant among groups.

Results and Discussion

Phytochemicals Profiling

The chromatograph on RP C-18 TLC plate is shown in Figure 1 and summarized in Table 1. Due to the poor visualization of Dragendorff reagent on RP C-18 plate, another test using Dragendorff reagent was carried out on silica gel 60, F_{254} TLC plate (Figure 1j). Based on comparison with previous studies, the extracts contained the terpenes, steroids, phenolics, alkaloids, glycosides and reducing sugars. Similar findings on the ethanolic extracts of *A. hookerianum Schott* have been reported with the presence of alkaloid, glycoside, tannin, reducing sugar, saponin and gum (Roy *et al*., 2011). Some polyhydroxy alkaloids from *A. treubii,* have been identified as 2(*R*),5(*R*)-bis-(hydroxymethyl)-3(*R*),4(*R*)-dihydroxypyrrolidine, α-homonojirimycin, β-homonojirimycin, α-homomannojirimycin, β-homomannojirimycin, α-3,4-*di*-epi-homonojirimycin, 7-*O*-β-D-glucopyranosyl-α-homonojirimycin and 5-*O*-α-D-glucopyranosyl-α-homonojirimycin (Asano *et al*., 1997).

The bands and probable functional groups by FTIR analysis present in the leaves, stems and roots of crude methanolic extracts of *A. simplex,* are presented in Table 2. The data was compared with the reference bands in the Interpretation of Infrared Spectra (Coates, 2006). All crude extracts revealed the presence of hydroxy (OH), saturated aliphatic alkane (methylene C-H asymmetric/symmetric stretch), nitrogen double bond–containing compounds (thiocyanate and

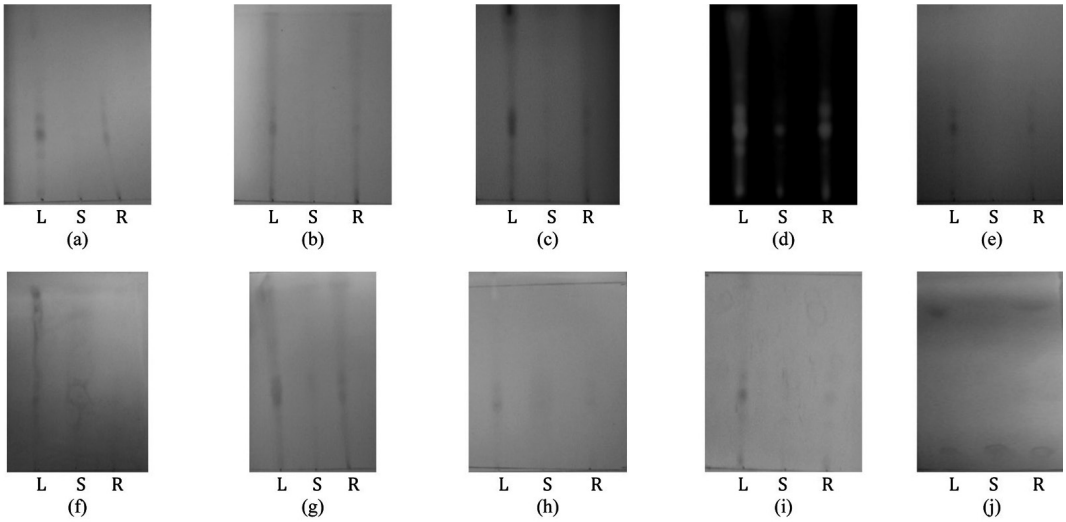

Figure 1: TLC profiling of methanolic extracts of different plant parts of *A. simplex*:- (a) anisaldehyde 1, (b) anisaldehyde 2, (c) UV-254 nm, (d) UV-365 nm, (e) naked eye, (f) ninhydrin, (g) iodine vapour, (h) vanillin-sulfuric acid and (i) Dragendorff on silica gel RP C-18, (j) Dragendorff on silica gel 60, F_{254}

isothiocyanate), aromatic ring (aryl), open chain imino (-C=N-) and cyclohexane ring functional group. A hetero-oxy compound (nitrogen-oxy) and saturated aliphatic alkane of methylene C-H bending functional group were present in the crude stems and roots extracts, but the carbonyl group was absent from the root extracts.

Cytotoxicity and SR-B1 Ligand Activity

Cytotoxicity based on MTS assay determines the ability of crude methanolic extracts to increase SR-B1 promoter. The IC_{50} for methanolic extracts of the leaves, root and stem was higher than 100 µg/mL (data not shown). Based on the guidelines by U.S. National Cancer Institute (NCI), IC_{50} < 20 µg/mL is considered as highly toxic (Vijayarathna & Sasidharan, 2012). Thus, the methanolic extracts of *in vitro A. simplex* plantlets are non-toxic for assay on the SR-B1 ligand activity against HepG2 cell lines.

The interaction between *A. simplex* extracts and SR-B1 promoter region was assayed directly by the intensity of bioluminescence. The results suggested that the stem and root extracts of *A. simplex* exhibited SR-B1 activity (Figure 2b and 2c), but not the leave extracts (Figure 2a). The leave extracts still showed a 1.11-fold increment of the luciferase activity, as compared to the negative control, and considered as weak and did not elevate the transcriptional activity of SR-B1 promoter. The gene is considered to be significantly expressed only if the genes expression with messenger RNA (mRNA) level change is higher than 1.5- fold (Fu *et al*., 2012). All of the crude extracts showed an optimum luciferase activity at the concentration of 1.56 µg/mL and slightly decreased when the concentration was higher. For the stem extracts, the highest activity was attained between 1.56 to 3.13 ug/mL, with optimal transcriptional activity of 1.61-fold at 1.56 µg/mL. The root extracts exhibited broader range of luciferase activities, from 1.56 to 50 ug/mL, where the transcriptional activity of SR-B1 promoter increased by 72% at 1.56 µg/mL concentration. For leave and stem extracts, at the concentration higher than 3.13 µg/mL, the activity decreased due to the toxicity with the reduction in cell viability observed using CellTiter-Fluor™ assay (data not shown).

In firefly, a decrease in reporter gene expression with corresponding reduced number of cells at certain concentrations has been reported, suggesting that the tested compounds or extracts may not be effective in inducing luciferase expression (Worzella *et al*., 2012).

Table 1: The potential phytochemicals present in the *A.simplex* methanolic extracts from the TLC analyses

Visualization reagent	Colour of the spot	Suggested phytochemicals	Reference	Potential chemical constituents
Anisaldehyde-sulfuric acid 1,2	Yellow, green, grey	L-rhamnose (Green)	Krebs *et al*. (1969)	Reducing sugar, glycosides
		D-xylose (Grey)	Krebs *et al*. (1969)	
		L-arabinose (Yellow-green)	Krebs *et al*. (1969)	
		D-mannose (Green)	Krebs *et al*. (1969)	
		D-galactose (Green-grey)	Krebs *et al*. (1969)	
		Pyrethrins (Grey-black)	Stahl & Pfeifle (1965)	Mono- or sesquiterpenes
		Steroid (Grey or green)	Lisboa (1964)	Steroid
		Hydroxylated polyacetylenic ketones (Dark-grey)	Bauer & Remiger (1989)	Polyacetylene
UV 254 nm	Dark grey	Phenolic (Dark zone)	Klimczak *et al*. (1972)	Phenolic
UV 365 nm	Orange-red	Artemisinin (Orange)	Gabriels & Plaizier-Vercammen (2003)	Sesquiterpene
		Cupreine, Dihydrocuprine, Cupreidine, Dihydrocupreidine (Orange-red)*	Svendsen & Verpoorte (2011)	Chinchona alkaloid
		Anthraquinone of Frangulae and Rhamnus cortex (Bright orange-red)	Wagner *et al*. (2009)	Anthraquinone
Ninhydrin	Green, grey	Enteramine (Green – blue green)	Harris & Pollock (1953)	Amino acid
Iodine vapour	Yellow-brown	All lipophilic compounds containing conjugated double bonds give yellow-brown colour	Wagner *et al*. (2009)	Double bond
		Phenolic (Yellow)	Sharma *et al*. (1998)	Phenolic
		Racemic mixtures of (±)-hyoscyamine atropine and (±)-colchicine (Yellow-brown)	Bhushan & Ali (1993)	Colchicine alkaloid
Vanillin-sulfuric acid	Yellow, green, grey	Terpenes, sterols and alkaloids (Dark colour zones)	Wagner *et al*. (2009)	Terpene, sterol, alkaloid
		Taxisterone (Grey-green)	Horn & Bergamasco (1985)	Steroid
Dragendorff	Orange	Steroidal alkaloids (Orange-brown)	Ogbuanu *et al*. (2014)	Alkaloid

*After spraying with sulfuric acid

Table 2. The FT-IR bands and functional groups of *A. simplex* methanolic crude extracts

Bands, wave number (cm^{-1})			Functional group
Leaves	Stems	Roots	
3281.49 (broad)	3365.09 (broad)	3346.30 (broad)	Hydroxy
2918.24, 2850.04	2918.15, 2850.62	2915.98, 2849.05	Saturated aliphatic alkane
2160.72	2161.73	2161.80	Nitrogen multiple and cumulated double bond
2033.75	2034.95	2037.36	
1978.53	1979.71	1979.97	Aromatic combination bands
1713.58	1716.21	-	Carbonyl
1615.34	1651.59	1634.21	Open chain imino
-	1515.97	1515.62	Nitrogen-oxy
-	1455.04	1454.99	Saturated aliphatic alkane
1367.32	1346.51	1347.19	Hydroxy or amino
1030.63	1058.05	1057.53	Cyclohexane ring vibration
891.29	707.54	815.67	Aromatic ring (aryl)

However, at a certain higher concentration, the expression is unexpectedly increased before subsequently decreasing again. This inconsistency in activities can be attributed to several reasons. The number of cells seeded in the 96-well plate using manual pipetting may have contributed towards low replication efficiency (Puig-Basagoiti *et al*., 2005). Out of seven replicates carried out in this study, the three best luminescence readings with low standard deviations are selected to calculate the luciferase activity. At higher concentration, it is presumed that the more concentrated the solution is, the more likely that the compounds will be precipitated out (Popa-Burke & Russell, 2014). Precipitation occurs especially when the compound is stored in DMSO, potentially affecting the downstream thus consequently interrupting the bioluminescence signalling (Waybright *et al*., 2009). The bioactive compounds that could potentially act as ligand may be an active modifier as inhibitor or activator of *Renilla* luciferase enzyme. At higher extract concentrations, the compounds could mimic the substrate and as a result, both the mimic and the natural substrate compete to bind to the active sites of the enzyme through competitive, uncompetitive, non-competitive and allosteric inhibition mechanism (Strelow *et al*., 2012). When the concentration of the substrates increase, the possibility of the compounds to bind to the enzyme is lower and this is directly correlated to the signal intensity of the assay. This may explain the unexpectedly increment of the luciferase activity. The reduction of expression thereafter may be due to the higher concentrations of compounds as mimicking-substrate that inhibits the enzyme, thus reducing the luminescence reading.

The crude leave extracts showed lower/ no activity than the crude stems and roots, indicating that there was a synergistic interaction between the phytochemicals present in both the crude stem and root extracts that could increase the SR-B1 promoter activity. The synergistic effects among the constituents could enhance the potentiality of *A. simplex* as a ligand for SR-B1 receptor. Both active crude extracts showed their potency as an agonist of high-affinity ligand binding of SR-B1. It can be suggested that low concentration of *A. simplex* extracts is adequate to maximally occupy a ligand-binding site of SR-B1 and to trigger the expression. Phytochemicals such as phenolics and glycosides showed antiatherosclerosis potential through increasing the SR-B1 ligand expression have reported by Andriani *et al.* (2015; 2014). The SR-B1 receptor plays an important role in HDL metabolism, thus reducing the incidence of coronary heart diseases via reverse cholesterol transport. This receptor helps to dispose of excessive cholesterol in the blood stream to the liver and further secretion by the bile (Rhainds & Brissette, 2004). Our study has shown that the

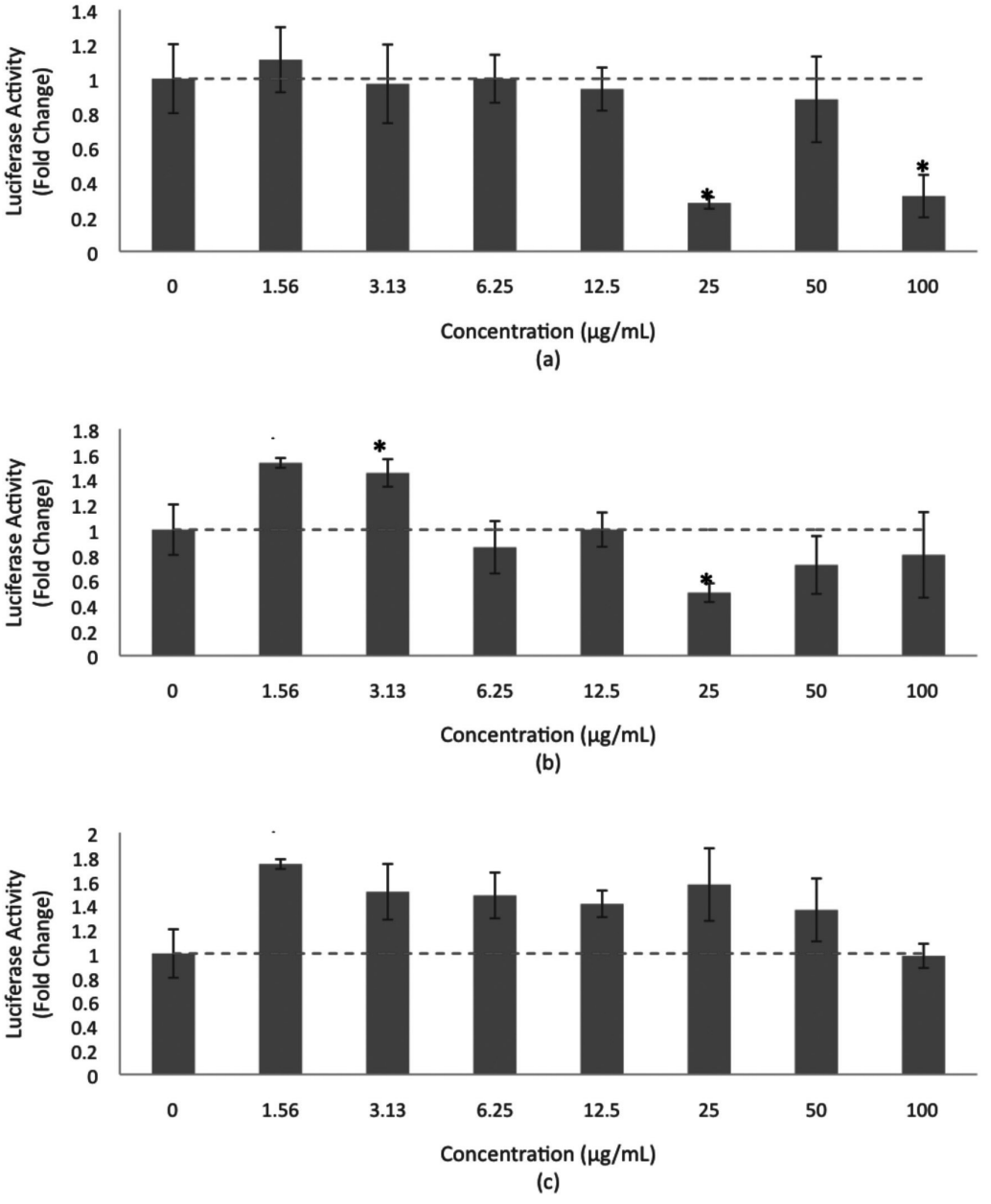

Figure 2: Luciferase activity of transfected HepG2 cells treated with two-fold dilution (1.56 to 100 µg/mL) of (a) leaves, (b) stems and (c) roots of *A.simplex* methanolic extracts. The graph bar data represents the mean value ± SD (n=3)

[a] $F(7, 16) = 11.66$, $p < 0.0001$.

[b] $F(7,16) = 24.07$, $p < 0.0001$.

[c] $F(7,16) = 4.291$, $p < 0.005$.

*denotes the significant differences in relative fold change of transcriptional activity as compared to the negative control ($p \leq 0.05$).

A. simplex extracts could act as inducer for SR-B1 receptor at the transcriptional level, thus up-regulating the free cholesterol ester uptake and subsequently increasing the apoliprotein A-1 level and HDL metabolism.

Conclusion

The methanolic extracts of *A. simplex* leave, stem and root parts contained the terpenes, steroids, phenolics, alkaloids, glycosides and reducing sugars. The stem and root extracts increased the expression of SR-B1 at 1.61- and 1.72-fold higher than the control, respectively. This finding suggested that *A. simplex* is one of the potential sources of alternative for SR-B1 ligand. The *in vitro* plantlets and the tissue culture technology may be applicable for sustainable production of the identified compounds from this plant.

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