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RESEARCH PAPER

Microarray analysis revealed different gene expression patterns in HepG2 cells treated with low and high concentrations of the extracts of *Anacardium occidentale* shoots

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Abstract In this study, the effects of low and high concentrations of the *Anacardium occidentale* shoot extracts on gene expression in liver HepG2 cells were investigated. From MTT assays, the concentration of the shoot extracts that maintained 50% cell viability (IC_{50}) was 1.7 mg/ml. Cell viability was kept above 90% at both 0.4 mg/ml and 0.6 mg/ml of the extracts. The three concentrations were subsequently used for the gene expression analysis using Affymetrix Human Genome 1.0 S.T arrays. The microarray data were validated using real-time qRT–PCR. A total of 246, 696 and 4503 genes were significantly regulated ($P < 0.01$) by at least 1.5-fold in response to 0.4, 0.6 and 1.7 mg/ml of the extracts, respectively. Mutually regulated genes in response to the three concentrations included *CDKN3, LOC100289612, DHFR, VRK1, CDC6, AURKB* and *GABRE*. Genes like *CYP24A1, BRCA1, AURKA, CDC2, CDK2, CDK4* and *INSR* were significantly regulated at 0.6 mg/ml and 1.7 mg but not at 0.4 mg/ml. However, the expression of genes including *LGR5*, *IGFBP3*, *RB1*, *IDE*, *LDLR, MTTP*, *APOB, MTIX, SOD2* and *SOD3* were exclusively regulated at the IC_{50} concentration. In conclusion, low concentrations of the extracts were able to significantly regulate a sizable number of genes. The type of genes that were expressed was highly dependent on the concentration of the extracts used.

Keywords *Anacardium occidentale* shoots · Methanol extracts · Gene expression · cDNA microarray analysis · HepG2 cells

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Introduction

The cashew plant or *Anacardium occidentale L* (*A. occidentale*) has many medicinal properties that are beneficial to health. Various scientific evidences have linked the various parts of cashew plant to several biological activities. The stem bark extract had been shown to have anti-bacterial [1], anti-viral [22], anti-diabetic [32] and anti-inflammatory [29] activities. Anti-tumour activity was detected in the cashew gum [30] and nut [42] while anti-ulcerogenic was reported in the cashew leaf extracts [19]. Antioxidant activities were also detected in the nut skin extracts [16]. In addition to the medicinal properties, the fruit of the *A. occidentale* is a natural whitening agent that disrupts pigmentation through the inhibition of tyrosinase [21].

In Malaysia, the young leaves or shoots of the *A. occidentale* are widely consumed as salads, and the locals believed its benefits include diabetic control and prevention. The extracts of the shoots were found to have potent antioxidant activities [35], were able to inhibit the oxidation of LDL and up-regulated LDL receptor activity in cultured HepG2 cells [39]. The antioxidant activities observed in the *A. occidentale* shoot extracts were attributed to the reported presence of phenolic compounds such as myricetin and quercetin [19, 27]. Intact quercetin glycosides, the most common flavonoids found in human diets, were shown to be absorbed at the small intestine probably through a sodium-dependent glucose transport pathway [9, 14]. Once absorbed, quercetin circulates in the plasma in conjugated forms but its antioxidant properties were maintained [25].

Other active compounds found in the crude extracts of the *A. occidentale* leaves include catechin, epicatechin, tetramer of proanthocyanidin and biflavanoids amentoflavone [19]

and agathisflavone [20]. Agathisflavone was reported to be able to induce apoptosis in Jurkat cells (acute lymphoblastic leukaemia cell line) [20] as well as a potent, competitive inhibitor for the $GABA_A$/benzodiazepine receptor [43].

Scientific molecular studies on the effects of the *A. occidentale* shoot extracts on cells are still lacking despite its reported antioxidant properties and its use in traditional medicine. We had earlier reported that the methanol extracts of the *A. occidentale* contained the highest total phenolic content compared to ethyl acetate and hexane extracts [35]. In this study, we explored the effects of the methanol extracts of the *A. occidentale* shoots on the expression of genes which could be associated with its antioxidant and medicinal properties.

Materials and methods

Chemicals

All reagents and chemicals used in the experiments were of analytical grade and obtained mostly from Sigma–Aldrich. Solvents used for extraction of plants were purchased from Fisher Scientific. Water used was of Millipore quality (ELGA Purelab Ultra Genetic system).

Preparation of methanol extract of the shoots of *Anacardium Occidentale*

In our previous study [35], we reported that the methanol extract of the *A. Occidentale* shoots possessed significantly higher antioxidant activities compared to those of the ethyl acetate and hexane extracts. The methanol extracts was subsequently used in this study. Briefly, the shoots were washed, air-dried followed by complete drying in an oven at 40°C. The dried shoots were then ground to powder and then extracted with methanol with a mass to volume ratio of 1:20 (g/mL), at room temperature for 24 h. The resulting extract was filtered and roto-evaporated (Rotavapor R-215) to dryness at 37°C, and the residues were then redissolved in dimethylsulfoxide (DMSO). For the subsequent cell culture experiments, the final concentration of DMSO was kept below 1% to avoid toxicity to the cells.

High-performance liquid chromatography

Acid hydrolyses was conducted on the dried powder of *A. occidentale* [3]. Samples (20 mg) were mixed with 50% methanol containing 1.2 M HCl and 20 mM sodium diethyldithiocarbamate as an antioxidant, in reactive vials. The samples were hydrolysed for 2 h at 90°C. Following hydrolysis, samples were centrifuged at $5000 \times g$ for 5 min and diluted with distilled water (pH 2.5) prior to analysis on the HPLC. The hydrolysed samples contained both free flavonoids and aglycones released from conjugated flavonoids following acid hydrolysis.

The HPLC system used for the flavonoid analyses comprised a Shimadzu system consisting of a system controller, a binary pump (LC 20AC), a manual injector (Rheodyne 7725i manual injector), a column oven (CTO-10AS VP) and a dual channel UV detector (SPD-20A UV–VIS). Absorbance of the samples was monitored at a wavelength of 260 nm. Flavonoids in the samples were separated using a reversed-phase column (NovaPak C_{18}, 150 × 3.0 mm, i.d 4 μm) (Waters, USA), at a temperature of 40°C. Separation of flavonoids was conducted using a gradient system containing 7–40% acetonitrile in water (pH 2.5) at a flow rate of 0.5 ml/min over 20 min. Standard solutions containing catechin, epicatechin, rutin, genistin, myricetin, morin, quercetin, genistein, kaempferol and isorhamnetin were prepared and injected on the HPLC under the same conditions.

Cell culture

The human hepatoblastoma HepG2 cell line (ATCC, Manassas, VA, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Flowlab, Australia), 1% penicillin (Flowlab, Australia) and 1% streptomycin (Flowlab, Australia). Cells were maintained in humidified air with 5% CO_2 at 37°C.

Cell viability analysis using the MTT assay

Cell viability of HepG2 cells in response to treatment with various concentration of the *A. occidentale* shoot extracts was analysed using an MTT assay as described by Mosmann, 1983 [28] with slight modifications [36]. Briefly, HepG2 cells at a density of 5000 cells per well were seeded in a 96-well ELISA microplate. The cells were incubated at 37°C in 5% CO_2 for 24 h. After 24 h, increasing concentrations of the shoots extracts (0.2–5.0 mg/ml) were added into the wells, and the cells were further incubated for 48 h. Following this, MTT reagent (Merck) was added, and the mixture was incubated for 4 h. Next, the mixture in each well was removed, and formazan crystals formed were dissolved in 10 μl of 75% isopropanol. Spectrophotometric measurement of the mixture was performed in a microplate-reader (Bio-Rad) at 590 and 620 nm wavelengths. A linear plot of cell viability (%) against the concentrations of plant extracts was constructed.

Treatment of HepG2 cells for the microarray analysis

For the gene expression analysis, 80–95% confluent HepG2 cells maintained in DMEM were treated with shoot extracts

at 0.4, 0.6 and 1.7 mg/ml. The cells were then incubated at 37°C for 24 h. As a control, cells were incubated in fresh DMEM, in the absence of the extracts. All experiments were performed in triplicate. After 24 h, cells were trypsinized and then precipitated by centrifugation at $261 \times g$ for 5 min. Following this, cells were washed with PBS twice before total cellular RNA (tcRNA) was extracted from the treated and untreated cells.

Extraction of total cellular RNA (tcRNA)

tcRNA from both treated and untreated (control) HepG2 cells was isolated and then purified using RNAEasy kit and RNase-free DNAse set (Qiagen) according to the manufacturer's instructions. The quality of the tcRNA was estimated by measuring the absorbance ratio of 260–280 nm while its integrity was analysed using denaturing gel electrophoresis. An A260/A280 ratio above 1.8 indicated that the tcRNA was of good quality. The integrity of the tcRNA was indicated by the presence of two distinct bands corresponding to the ribosomal 28S and 18S subunits, with the intensity of the larger, 28S band approximately twice than that of the smaller, 18S band.

Microarray analysis

Affymetrix Human Gene 1.0 S.T (sense target) arrays were used for the gene expression analysis according to the conventional Affymetrix eukaryotic RNA labelling protocols (Affymetrix). Briefly, freshly extracted tcRNA (100 ng) isolated from the treated and untreated HepG2 cells was reversed transcribed to single-stranded sense strand DNA (cDNA) in two cycles using the Whole Transcript (WT) cDNA synthesis, amplification kit and sample clean-up module. The sense strand cDNA was then cleaved into small fragments using a mixture of UDP and apurinic/apyrimidinic endonuclease 1 or APE1. Following this, the fragments were end-labeled with biotinylated dideoxynucleotides using the WT Terminal Labeling kit. The biotinylated fragments (5.5 μg) were then hybridized to the Affymetrix Human Gene 1.0 S.T array at 45°C for 16 h in hybridization Oven 640. After hybridization, the arrays were stained and then washed in the Affymetrix Fluidics Station 450 under standard conditions. The stained arrays were scanned at 532 nm using an Affymetrix GeneChip Scanner 3000, and CEL files for each array were generated using the Affymetrix GeneChip® Operating Software (GCOS). The data were pre-analyzed using Affymetrix Expression Console software.

Microarray data normalization and analysis

The CEL files generated were converted to text files and exported to Partek Genomic Suite software to get the whole list of up-regulated and down-regulated genes. Probeset IDs without any annotation in the Partek software were filtered out. The filtered, whole gene list was then subjected to a one-way analysis of variance (ANOVA) in the Partek Genomic software, to determine significantly expressed sets of genes which was set according to P values less than 0.01 ($P < 0.01$) instead of $P < 0.05$ to avoid false positive results. Significantly expressed genes were then re-filtered to include only those with fold change difference of equal to or greater than 1.5. Additional information on the biological functions of the genes and the genes products was determined from the Gene Ontology (GO) Enrichment tool in the Partek Genomic Suite Software. Information on function of genes can be derived from the Gene Ontology database which provides a structured annotation of genes with respect to molecular function, biological process and cellular component. Further information on GO could be retrieved from http://www.geneontology.org/.

Validation of the DNA microarray data using qRT–PCR

The microarray data were verified using real-time relative quantitative RT–PCR (qRT–PCR) which was performed in a StepOne™ Real-Time PCR System (Applied BioSystem). The same cDNA and primer pairs for the selected up-regulated and down-regulated genes as well as a housekeeping gene, *GADPH*, as listed in Table 1 were used. The PCR amplification was carried out in 0.2 ml MicroAmp® Optical 8-tube strips in a final volume of 20 μl containing a mixture of cDNA (30 ng), reverse and forward primers (1 μM), pre-prepared Power SYBR® Green PCR master mix containing SYBR® Green 1 dye, Ampli*Taq* Gold® DNA Polymerase dNTPs, dUTP, Passive Reference 1 and optimized buffer components. The PCR parameters consisted of 40 cycles of amplification with initial denaturation at 95°C for 15 s, annealing of primers and elongation of the newly synthesized strands at 60°C for 60 s. The PCR mixture was initially held for 10 min at 95°C for Ampli*Taq* Gold® DNA polymerase activation. The Comparative C_T Method ($\Delta\Delta C_T$) was chosen for the relative quantitation of gene expression. Each sample type was run in triplicate. mRNA levels of the selected genes were normalized against that of *GADPH*.

Results

Cell viability analysis

MTT assays were performed to measure the viability of the cells in response to the treatment with different concentrations of the shoot extracts. The dose–response curve of

Table 1 Primer sequences for the selected genes used for validation of the microarray data using real-time relative quantitative PCR (qRT–PCR)

Gene name (Genebank ID)	Primer sequences	Product size (bp)
DHFR (NM_000791)	Forward: 5′ CATGGTCTGGATAGTTGGTGGC 3′	108
	Reverse: 5′ GTGTCACTTTCAAAGTCTTGCATG 3′	
TYMS (NM_001071)	Forward: 5′ ATCAAGGGATCCACAAATGA 3′	205
	Reverse: 5′ GGTCAACTCCCTGTCCTGAA 3′	
LIPC (NM_000236)	Forward: 5′ CAAGTGCCCTTGGACAAAGC 3′	130
	Reverse: 5′ TGACAGCCCTGATTGGTTTCT 3′	
CYP24A1 (NM_000782)	Forward: 5′ CTCATGCTAAATACCCAGGTG 3′	300
	Reverse: 5′ TCGCTGGCAAAACGCGATGGG 3′	
PLAUR (NM_002659)	Forward: 5′ TGCGGTGCATGCAGTGTAAGAC 3′	183
	Reverse: 5′ TCAAGCCAGTCCGATAGCTCAG 3′	
PLCXD1 (NM_018390)	Forward: 5′ ACGAGTACCTGGTCGCCTGTAT 3′	117
	Reverse: 5′ CATAGGAGACGATGACCTGTTGG 3′	
SQSTM1 (NM_003900)	Forward: 5′ CCAGTGACGAGGAATTGACAA 3′	156
	Reverse: 5′ CATCGCAGATCACATTGGGG 3′	

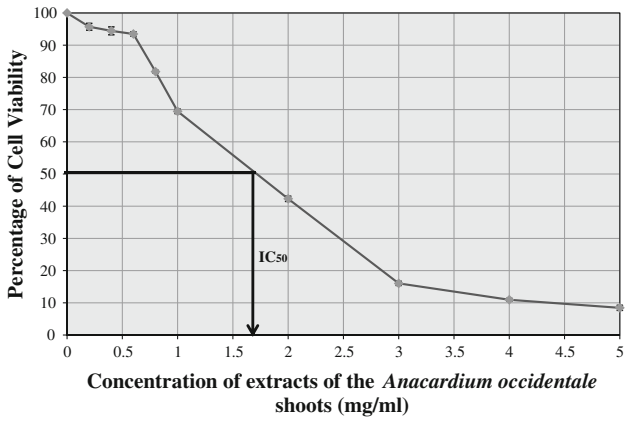

Fig. 1 A dose–response curve of percentage viability of HepG2 cells (5,000 cells/well) treated with various concentrations of the extract of the *A. occidentale* shoots (0.2–5.0 mg/ml). Analysis was done in triplicate, and the results were expressed as % of HepG2 cell viability ± std dev. From the plot, the IC_{50} was found to be 1.7 mg/ml

the viability of HepG2 cells in response to the treatment with different concentrations of the methanol extract of *A. occidentale* shoots is shown in Fig. 1. The graph shows a tri-phasal response. In the first phase, cell viability was maintained above 90% until 0.6 mg/ml of the extract concentration was reached. In the second phase, cell viability decreased steeply to below 20% from 0.6 to 3 mg/ml extract concentrations. In the third phase, the cells barely survived (viability below 10%) beyond 5 mg/ml extract concentration. The concentration of the shoot extract that reduced cell viability by 50% (IC_{50}) was 1.7 mg/ml. Low concentrations of 0.4 and 0.6 mg/ml as well as the IC_{50} concentration were subsequently chosen for the gene expression analysis.

HPLC analysis

HPLC analyses of the hydrolysed samples revealed the presence of quercetin and kaempferol (Fig. 2a) and the absence of catechin, epicatechin, rutin, genistin, genistein, myricetin, morin and isorhamnetin. The quercetin and kaempferol peaks were confirmed by comparing retention times of the peaks with the standards (Fig. 2b) which was run under the same conditions as the samples. The presence of kaempferol has not been reported previously.

Microarray analysis: normalization and visualization of data

Figure 3 shows the principal component analysis (PCA) plot of the microarray data for each of the array for the control and samples treated with 0.4, 0.6 and 1.7 mg/ml of the shoot extracts. The three arrays for the control samples were grouped together but separately from those of the treated samples. Figure 4 shows hierarchical clustering of the microarray data generated from each of the arrays where the reproducibility pattern correlates with that shown by the PCA plot.

Venn diagram in Fig. 5 shows the number of genes that were significantly regulated ($P < 0.01$) by at least 1.5-fold. The total number of genes regulated by 0.4, 0.6 and 1.7 mg/ml of the methanol extracts of *A. occidentale* were 246, 696 and 4,503, respectively. An increase in concentration by 1.5-fold from 0.4 to 0.6 mg/ml was able to increase the number of regulated genes by threefold (65%). In addition, increasing the concentration from 0.4 to 1.7 mg/ml increased the number of regulated genes by 18-fold (95%). Figure 5 also shows that a total of 94 genes were mutually regulated in response to the three

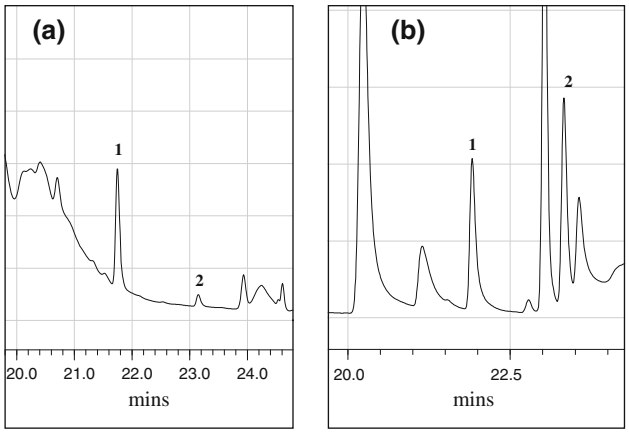

Fig. 2 Gradient reverse phase HPLC analysis of flavonoids in the shoots of *A. occidentale* and the flavonoid standards. HPLC analysis was performed on the hydrolysed samples of *A. occidentale* (Fig. 2a) and the flavonoid standards (Fig. 2b). Flavonoids were separated on a NovaPak C_{18} reversed-phase column (150 × 3.0 mm i.d, 4 µm), using a linear gradient system of 7–40% acetonitrile in water (pH 2.5), at a flow rate of 0.5 ml/min. Absorbance was measured at a wavelength of 260 nm. 1:quercetin; 2:kaempferol

concentrations where 5 were up-regulated and 89 were down-regulated. Ninety-eight genes were mutually regulated in response to the 0.4 and 0.6 mg/ml extracts. On the other hand, 178 were mutually regulated in response to the 0.4 and 1.7 mg/ml extracts. A larger number of genes, with a total of 571, were regulated when cells were treated with 0.6 and 1.7 mg/ml of the extracts.

Mutually regulated genes in response to the three concentrations included *CDKN3, LOC100289612, DHFR, VRK1, CDC6, AURKB, CYP2S1* and *GABRE* (Table 2). Genes like *CYP24A1, CDH2, E2F5, BRCA1, BRCA2, AURKA, CDC2 (CDK1), CDK2, CDK4, CHECK1, CCNA2, ACAT, IGFBP1, DUSP5* and *INSR* were significantly regulated at 0.6 and 1.7 mg/ml but not at 0.4 mg/ml. In addition, the expressions of genes such as the *LGR5*, *IGFBP3*, *RB1*, *IDE*, *LDLR, MTTP*, *APOB, SCP2*, *MTIX, SOD* and *SOD3* were exclusively regulated at the 1.7 mg/ml dose. Amongst the highly significantly suppressed genes were *CYP24A1, LGR5*, *CDH2* and *DHFR* by 27.8-, 16.4-, 15.5-, 10.0-fold, respectively. On the other hand, amongst the highly induced genes were the *DUSP5*, *IGFBP 3, IGFBP1*, *LDLR* and *INSR* by 9.1-, 8.0-, 4.2-, 3.6- and 2.6-fold, respectively (Table 2).

Other genes that were being significantly regulated in response to the 1.7 mg/ml shoot extracts were those associated with cell cycle check points either directly or indirectly. These included the *CDK5, CDK6, CCNB1* and *2, CCNE1* and *2*, *CCNH, CDKN1A* (p21/Cip1), *CDKN1C* (p57/Kip2), *CDKN2B* (p15), *CDKN2D* (p19), *CDKN3*, *RBL1, RBL2, NSUN6, NOP2, DAPK1, PAK2, HDAC2* and *G2E3.* Genes coding for other ubiquitin ligase isoforms, *UBE2C* and *UBE3B,* were also aberrantly expressed. In addition, genes associated with the Wnt/*β*-catenin signalling pathway including the *Wnt6*, *FZDs 1, 4*, *6, 7, 8, 9* and *10, CDH1, CTNNA2, DKK1, APC, NUCKS1, CSNK1G2*, *CSNK1G3* and *TCF* were all aberrantly expressed. In addition, genes associated with cancers, *BRIP1, BAP, BRCC3, RAS, SOS1, STAT2,* were all down-regulated (data not shown).

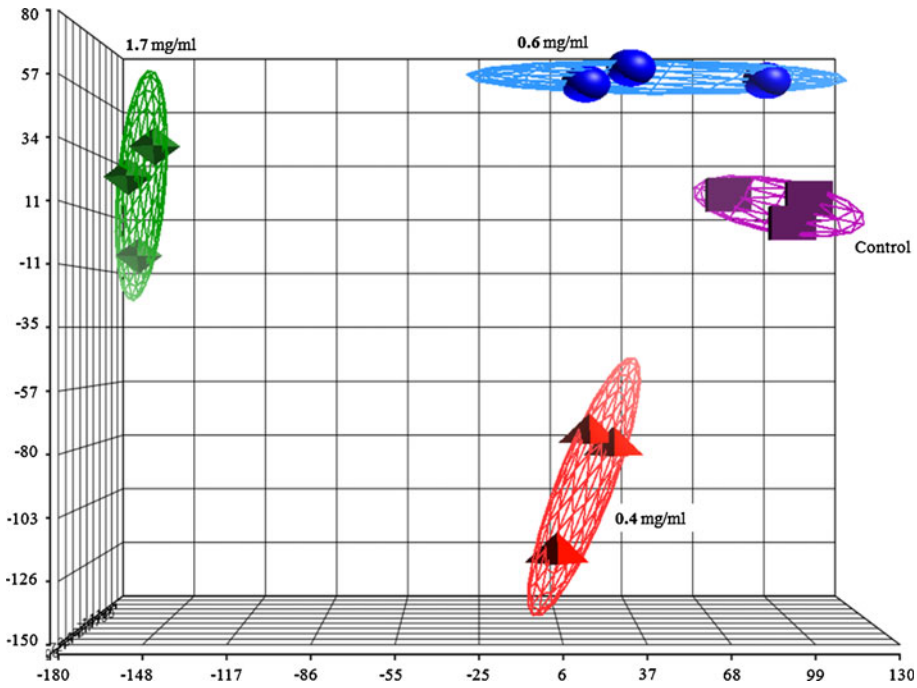

Fig. 3 A principal component analysis (PCA) plot derived from biological replicates ($n = 3$) of HepG2 cells grown in 0.4, 0.6 and 1.7 mg/ml of the extracts of the *A. occidentale* shoots

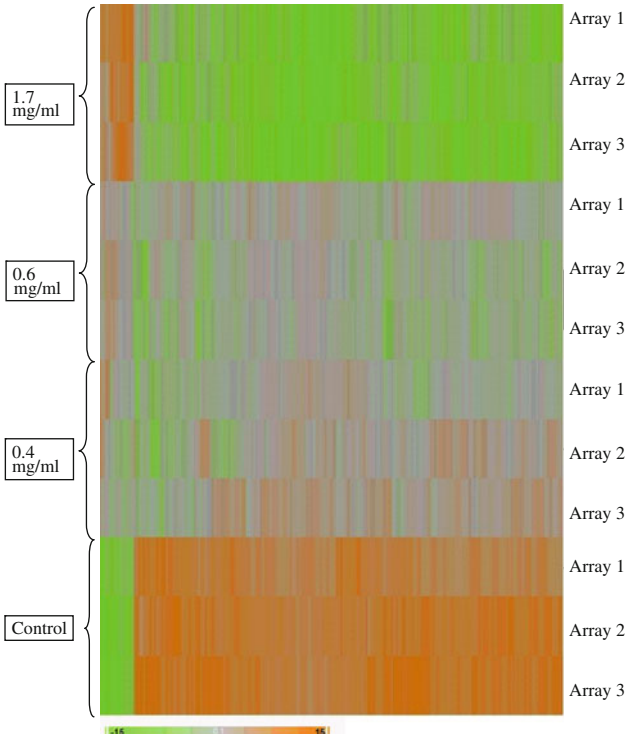

Fig. 4 Hierarchical cluster analysis of genes showing the differential expression of genes in HepG2 cells in response to the treatment with 0.4, 0.6 and 1.7 mg/ml of extracts of *A. occidentale* shoots

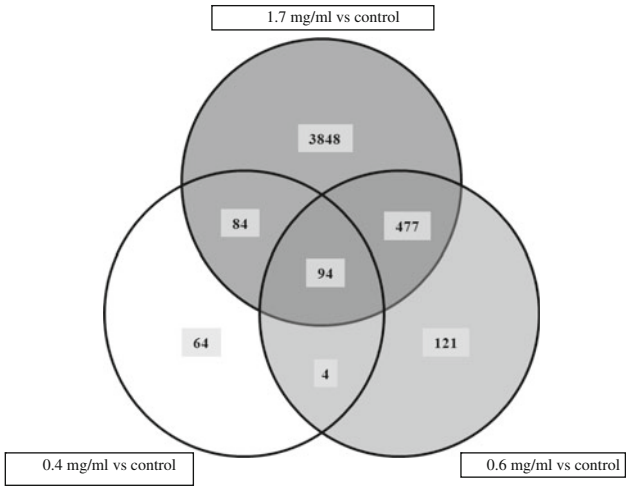

Fig. 5 A Venn diagram illustrating the number of genes in HepG2 cells that were mutually regulated at 0.4, 0.6 and 1.7 mg/ml of the shoot extracts. The total numbers of genes regulated at 0.4, 0.6 and 1.7 mg/ml of the extracts of *A. occidentale* were 246, 696 and 4503 respectively. An increase in concentration by 1.5-fold from 0.4 to 0.6 mg/ml was able to increase the number of regulated genes by threefold (65%). Increasing the concentration from 0.4 to 1.7 mg/ml increases the number of regulated genes by 18-fold (95%). A total of 94 genes were mutually regulated in response to the 3 concentrations, 5 were up-regulated and 89 were down-regulated. Ninety-eight genes were mutually regulated in response to the 0.4 and 0.6 mg/ml extracts. On the other hand, 178 were mutually regulated in response to the 0.4 and 1.7 mg/ml extracts. A larger number of genes of 571 were mutually regulated when cells were treated with 0.6 and 1.7 mg/ml extracts

Gene ontology (GO): biological interpretation

Gene ontology analysis of the products of the significantly regulated genes in response to the 3 concentrations of the *A. occidentale* shoot extracts is shown in Figs. 6, 7 and 8. The data are presented according to the following categories; biological process (Fig. 6), molecular function (Fig. 7) and cell component (Fig. 8). Selected significantly down-regulated and up-regulated genes in each of the category are shown in Table 3.

Figures 6, 7 and 8 show that most of the genes (and the subsequent gene products) are involved in cellular processes, biological regulations and metabolic processes in response to the three concentrations of the extracts. A total of 18 genes that are involved in localization, growth, locomotion and pigmentation were regulated in response to the 0.6 mg/ml but not the 0.4 mg/ml extracts. As the concentration was increased from 0.6 to 1.7 mg/ml, the number of genes rose from 18 to 88 in the same 3 subcategories. In addition, 3 genes that are involved in reproduction were regulated only in the presence of 1.7 mg/ml of the extracts. Genes like *CYP24A1* and *DHFR* are involved in cellular as well as metabolic processes (Table 3A). In addition, under the "Molecular function" category, the majority of genes that were regulated in response to the 3 concentrations were involved in binding, followed by catalytic activity and transcriptional regulation activity (Fig. 7). Table 3B lists selected genes such as *INSR* and *LDLR* that are involved in binding, MAOB in catalytic activity and *RB1* in transcription regulator activity. The majority of the gene products were found as cell part, in extracellular region/part and in synapses (Fig. 8), and the selected genes are listed in Table 3C.

Validation of the microarray data using qRT–PCR

The microarray data were validated by quantitating selected significantly regulated genes, *PLAUR, PLCXD1, SQSTM1, CYP24A1, DHFR, TYMS* and *LIPC* using real-time RT–PCR (qRT–PCR). All data were normalized to the reference gene, *GAPDH*. As shown in Fig. 9, the expressions of the *PLAUR, PLCXD1* and *SQSTM1* genes were all up-regulated while those of *CYP24A1, DHFR, TYMS* and *LIPC* were down-regulated. The expression patterns obtained through qRT–PCR analysis were consistent with those of the microarray results.

Discussion and conclusion

In Malaysia, the young leaves or shoots of the *A.occidentale* are widely consumed as salads and the locals believed

Table 2 Selected significantly expressed genes in HepG2 cells in response to treatment with 0.4, 0.6 and 1.7 mg/ml of the extracts of *A. occidentale* shoots

Gene ID	Gene name	Gene product	Fold change (0.4 mg/ml)	Fold change (0.6 mg/ml)	Fold change (1.7 mg/ml)
NM_005192	*CDKN3*	Cyclin-dependent kinase inhibitor 3	−2.7	−2.6	−10.5
AY605064	*LOC100289612*	Arsenic transactivated protein 1	−3.0	−3.6	−10.2
AK293146	*DHFR*	Dihydrofolate reductase	−2.4	−2.4	−10.0
NM_003384	*VRK1*	Vaccinia related kinase 1	−2.3	−2.6	−6.7
NM_001254	*CDC6*	Cell division cycle 6 homolog (S. cerevisiae)	−2.4	−2.7	−4.3
NM_030622	*CYP2S1*	Cytochrome P450, family 2, subfamily S, polypeptide 1	+1.8	+2.3	+3.5
NM_004961	*GABRE*	Gamma-aminobutyric acid (GABA) A receptor, epsilon	+1.6	+2.1	+2.7
NM_004217	*AURKB*	Aurora kinase B	−1.6	−1.6	−2.2
NM_000782	*CYP24A1*	Cytochrome P450, family 24, subfamily A, polypeptide 1	NR	−2.9	−27.8
NM_001792	*CDH2*	Cadherin 2, type 1, N-cadherin (neuronal)	NR	−1.8	−15.5
NM_001951	*E2F5*	E2F transcription factor 5, p130-binding	NR	−1.9	−5.5
NR_027676	*BRCA1*	Breast cancer 1, early onset	NR	−2.4	−5.1
NM_000236	*LIPC*	Lipase, hepatic	NR	−3.2	−4.7
NM_198433	*AURKA*	Aurora kinase A	NR	−1.5	−4.4
NM_001786	*CDC2*	Cell division cycle 2, G1 to S and G2 to M	NR	−2.5	−4.1
NM_001798	*CDK2*	Cyclin-dependent kinase 2	NR	−2.0	−3.2
NM_001237	*CCNA2*	Cyclin A2	NR	−1.7	−3.1
NM_000059	*BRCA2*	Breast cancer 2, early onset	NR	−2.0	−2.7
NM_000019	*ACAT1*	Acetyl-coenzyme A acetyltransferase 1	NR	−1.9	−2.6
NM_000075	*CDK4*	Cyclin-dependent kinase 4	NR	−1.8	−2.6
NM_001274	*CHEK1*	CHK1 checkpoint homolog (S. pombe)	NR	−1.8	−2.3
NM_000208	*INSR*	Insulin receptor	NR	+2.3	+2.6
NM_000596	*IGFBP1*	Insulin-like growth factor binding protein 1	NR	+3.7	+4.2
NM_004419	*DUSP5*	Dual specificity phosphatase 5	NR	+3.3	+9.1
NM_003667	*LGR5*	Leucine-rich repeat-containing G protein-coupled receptor 5	NR	NR	−16.4
NM_000321	*RB1*	Retinoblastoma 1	NR	NR	−7.6
NM_031966	*CCNB1*	Cyclin B1	NR	NR	−5.7
NM_000253	*MTTP*	Microsomal triglyceride transfer protein	NR	NR	−3.7
NM_002979	*SCP2*	Sterol carrier protein 2	NR	NR	−3.5
NM_004969	*IDE*	Insulin-degrading enzyme	NR	NR	−2.1
NM_000384	*APOB*	Apolipoprotein B	NR	NR	−2.0
NM_001013398	*IGFBP3*	Insulin-like growth factor binding protein 3	NR	NR	+8.0
NM_000527	*LDLR*	Low-density lipoprotein receptor	NR	NR	+3.6
NM_005952	*MTIX*	Metallothionein 1X	NR	NR	+2.8
NM_003102	*SOD3*	Superoxide dismutase 3, extracellular	NR	NR	+2.1

Details of the GenBank accession number, name of the gene and its respective gene product, fold change difference between treated and non-treated cells and *p* values are included. *NR* Not regulated

its benefits include diabetes control or prevention. The methanol extracts of the shoots were found to have potent antioxidant activities [35]. Bioactive compounds found in the *A. occidentale* shoot extracts that could be linked to the antioxidant activities and other medicinal properties included myricetin and quercetin [19, 27], amentoflavone [19] and agathisflavone [20]. Dietary antioxidants could be absorbed through the intestine, albeit in small quantities. Once in the circulation, they are quickly metabolized [14, 44] but the antioxidant properties were retained [25]. Our group had reported that low concentration of crude extract of antioxidant-rich *T. indica* (0.3 mg/ml) was able to significantly regulate a sizable number of genes in HepG2 cells [36]. In this study, based on the MTT assays, cells showed more than 90% viability at 0.4 and 0.6 mg/ml. The IC_{50} concentration was found to be 1.7 mg/ml. This study was aimed to (1) investigate the effects of low and high concentration of the *A. occidentale* shoot extracts on

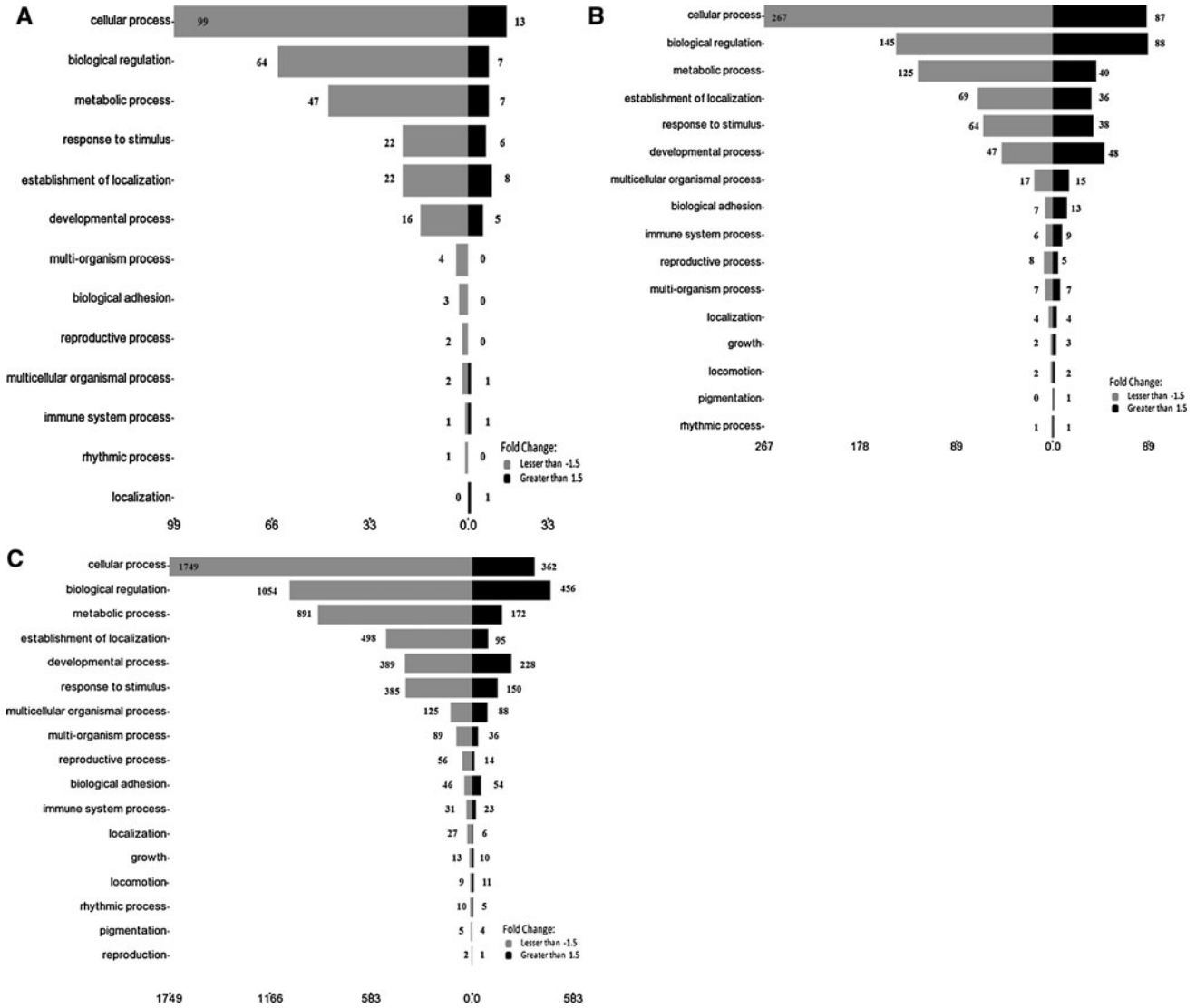

Fig. 6 Gene ontology analysis under the "Biological process" category, of significantly regulated genes ($P < 0.01$, fold change of at least ± 1.5) in HepG2 cells in response to 0.4 mg/ml (**a**), 0.6 mg/ml (**b**) and 1.7 mg/ml (**c**) of the extracts of *A. occidentale* shoots

the expression of genes in liver HepC2 cells and (2) identify genes that could be associated with the medicinal properties of the shoot extracts.

HPLC analyses showed the presence of quercetin in the extracts of the *A. occidentale* shoots that confirmed earlier findings by other researchers [19, 27]. In addition, we also detected the presence of kaempferol which has not been reported previously. Kaempferol possessed significant antioxidant activities [34] and has cancer chemopreventive properties towards several tumour cell lines including lung and leukaemic cell lines [8, 31].

In this study, cDNA microarray analysis showed that the extracts of the *A. occidentale* shoots at a low concentration of 0.4 mg/ml was able to significantly up-regulated ($P < 0.01$) a total of 248 genes by at least 1.5-fold. Amongst the down-regulated genes were those encoding arsenic transactivated protein (*LOC100289612*), vaccinia-related kinase 1 (*VRK1*), dihydrofolate reductase (*DHFR*), cell division cycle 6 homolog (*S.cerevisiae*) (*CDC6*), cyclin-dependent kinase inhibitor 3 (*CDKN3*) and aurora kinase B (*AURKB*). Increasing the concentrations from 0.4 to 0.6 mg/ml led to an increase in the number of regulated genes from 248 to 696. Amongst the 696 genes were *CYP24A1, CDH2, E2F5, BRCA1, BRCA2, AURKA, CDC2 (CDK1), CDK2, CDK4, CHECK1, CCNA2, ACAT, IGFBP1, DUSP5* and *INSR*. These genes were also expressed in response to the IC_{50} concentration, but as expected, the fold change was much larger compared to that of the 0.6 mg/ml. At an IC_{50} concentration of 1.7 mg/ml, a total of 4286 genes were significantly regulated. In addition, the expressions of genes such as the *LGR5*, *IGFBP3*, *RB1*, *IDE*, *LDLR, MTTP*, *APOB, SCP2*, *MTIX, SOD* and *SOD3*

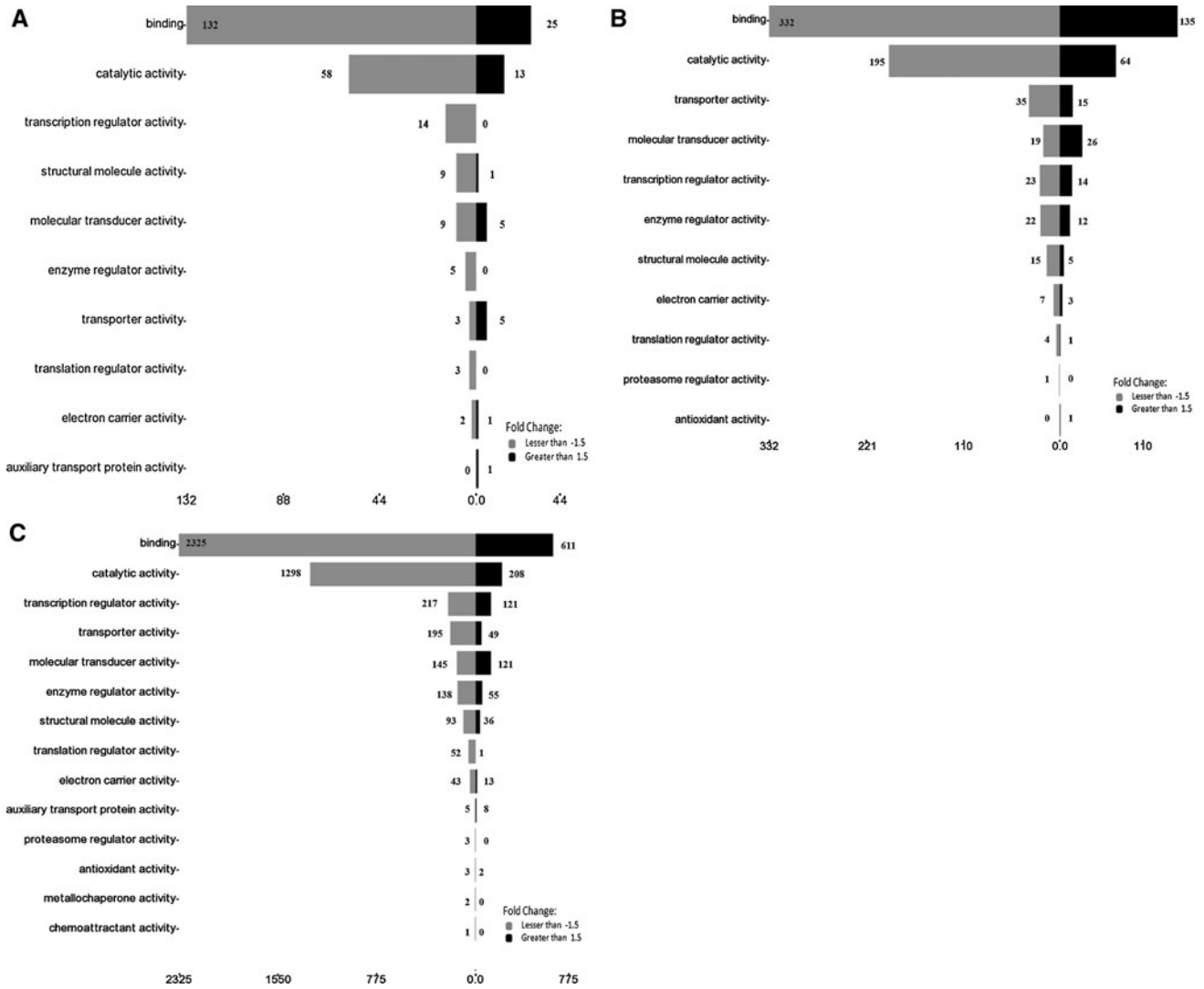

Fig. 7 Gene ontology analysis under the "Molecular function" category, of significantly regulated genes ($P < 0.01$, fold change of at least ±1.5) in HepG2 cells in response to 0.4 mg/ml (**a**), 0.6 mg/ml (**b**) and 1.7 mg/ml (**c**) of the extracts of *A. occidentale* shoots

were exclusively regulated at the 1.7 mg/ml dose. Interestingly, for all three concentrations, the down-regulated genes were three times as many as those that were up-regulated (full data are not shown) as indicated in the hierarchical analysis. Mutually regulated genes in response to the three concentrations included *CDKN3, LOC100289612, DHFR, VRK1, CDC6, AURKB, CYP2S1* and *GABRE* (Table 2). Amongst the highly significantly suppressed genes were *CYP24A1, LGR5*, *CDH2* and *DHFR* by 27.8-, 16.4-, 15.5-, 10.0-fold, respectively. On the other hand, amongst the highly induced genes were the *DUSP5*, *IGFBP 3, IGFBP1*, *LDLR* and *INSR* by 9.1-, 8.0-, 4.2-, 3.6- and 2.6-fold, respectively.

CYP24A1 gene codes for the hepatic enzyme, CYP24A1 or 24-hydroxylase which is the rate limiting enzyme in the catabolism of the active form of vitamin D, 1α,25-$(OH)_2D_3$ or calcitriol [18, 23]. 1α, 25-$(OH)_2$ D_3, synthesized in the kidney by the CYP27B1, promotes dietary absorption of calcium and phosphate as well as maintain the levels of the two minerals. There are increasing evidences that individuals with low serum vitamin D have a higher risk of developing various types of cancers [18, 23] and myocardial diseases [33]. In addition, 1α, 25-$(OH)_2$ D_3 is also important in regulating cell cycle check points as well as controlling multiple signalling pathways including those of the MAPK/ERK, PI3 K/AKT, Wnt and TGF-β [5]. The *CYP24A1* gene has been reported to act as an oncogene and its overexpression was detected in cancers of the colon, ovary and lung [2, 18, 23]. In vivo studies have indicated that exposing cancer cells to a high concentration of the active metabolites of vitamin D stopped the cells from progressing. This occurred via a mechanism affecting cell cycle and increasing apoptosis, ultimately slowing or stopping growth of the tumour [45]. The shoot extracts of

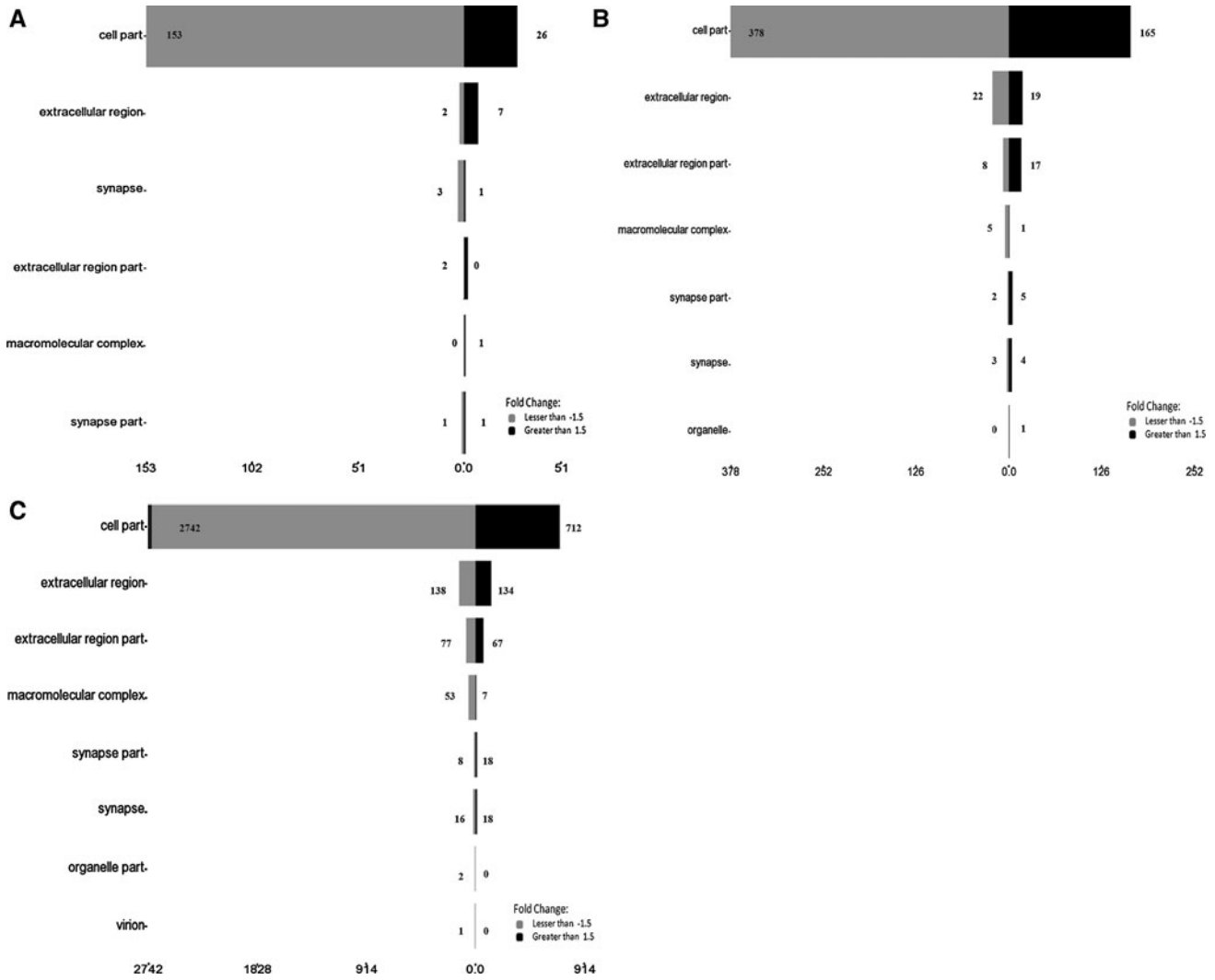

Fig. 8 Gene ontology analysis under the "Cellular component" category, of significantly regulated genes ($P < 0.01$, fold change of at least ± 1.5) in HepG2 cells in response to 0.4 mg/ml (**a**), 0.6 mg/ml (**b**) and 1.7 mg/ml (**c**) of the extracts of *A. occidentale* shoots

the *A. Occidentale* were able to highly suppress the *CYP24A1* gene; hence, it has the potential to be used synergistically with vitamin D to maintain the bioavailability and bioactivity of the latter.

Apart from the *CYP24A1* gene, *LGR5* and *CDH2* were also highly suppressed, by 16- and 15-fold respectively at the IC_{50} concentration of the shoot extracts. The leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) belongs to the G-protein-coupled receptor (GPCR) superfamily [15]. LGR5 had been reported to be a marker of adult stem cells where the *LGR5* gene transcription is under the control of the canonical or beta-catenin Wnt signalling pathway [11]. This pathway, which is involved in embryogenesis and normal physiological processes, is critical in the regulation of adult stem cells [11, 37]. Dysregulation of this pathway is linked to cancers [37]. An overexpression of LGR5 was observed in many types of cancers including those of the colon [26, 46], oesophagus [4], ovary [26] and the hepatocytes [47]. In this study, genes associated with the Wnt/β-catenin signalling pathway were also regulated at the IC_{50} concentration, albeit modestly. These included the *Wnt6*, *FZDs 1, 4, 6, 7, 8, 9* and *10, CDH1, CDH2, CTNNA2, DKK1, APC, NUCKS1, CSNK1G2*, *CSNK1G3* and *TCF* (data not shown).

One of the most interesting observations of this study was the fact that the shoot extracts were able to directly regulate a spectrum of genes involved in the G1 as well as G2 cell cycle check points. These included the *CDK1 (CDC2), CDK2, CDK4, CDK5, CDK6, CCNA2, CCNB1* and *2, CCNE1* and *2, CCNH, CDKN1A* (p21/Cip1), *CDKN1C* (p57/Kip2), *CDKN2B* (p15), *CDKN2D* (p19), *CDKN3*, *RB1, RBL1, RBL2, NSUN6, NOP2, AURKA, AURKB, DAPK1, PAK2, E2F5, HDAC2, VRK1, CREB* and *G2E3.* Genes coding for other ubiquitin ligase isoforms,

Table 3 Gene ontology analysis of selected significantly regulated genes

(A) Gene ontology (Biological process)	Selected down-regulated genes	Selected up-regulated genes
Cellular process	CYP24A1, DHFR, CDH1, CDH2, CDC2, CDK2, CDK4, CDK5, CDK6, CDKN3, CCNA2, CCNB1, CCNB2, CCNE1, CCNE2, CHEK1, RB1, AURKA, AURKB, BRCA1, BRCA2, IDE, LIPC, MTTP, SCP2, APOB, ACAT1	DUSP5, INSR, IRS2, SOD2, SOD3, LDLR
Biological regulation	CDH1, CDC2, CDK2, CDK4, CDK5, CDK6, CDKN3, CHEK1, CCNA2, CCNB1, CCNB2, CCNE1, CCNE2, E2F4, AURKA, RB1, CKS1B, PAK2, BRCA1, BRCA2, CSNK1G3, FZD4, FZD6, LIPC, APOB, APOBEC3F, APOH, MTTP, IDE	CDH4, CDKN2B, CDKN1A, APC2, IRS2, WNT6, FZD1, FZD7, FZD8, FZD9, FZD10, IGFBP1, IGFBP2, IGFBP3, IGFBP6, INSR, LDLR, SOD2
Metabolic process	CDH1, CCNH, CHEK1, CDK5, E2F4, CYP24A1, RB1, DHFR, BRCA1, LIPC, MAOB, MTTP, SCP2, ACAT1, APOB	APC2, SOD2, SOD3, INSR, LDLR
Response to stimuli	BRCA1, BRCA2, CHEK1, CDK5	IRS2, CDKN2D, SOD2, SOD3, CDKN2B, CDKN1A, MT1X
Establishment of localization	LIPC, MTTP, SCP2, APOB	LDLR
Developmental process	CDH2, RB1, CCNB2, BRCA1, SCP2, FZD6, CDK6, BRCA2, CCNF, DKK1, CDK5 E2F4, IDE, APOB, CDH1, FZD4	FZD7, CDK5R1, FZD9, IRS2, IGF2, CDKN2D, FZD10, CDKN1C, SOD2, FZD8, WNT6, FZD1, INSR
Multi-organism process	RB1	INSR, LDLR
Biological adhesion	CDH2, FZD6, CDK5, CDH1	CDH4
Reproductive process	SCP2, BRCA2, CHEK1, APOB	
Multicellular organismal process	APOH, ACAT1, CDK5, E2F4	FZD9, LDLR
Rythmic process	CDK4	–
Growth	CCNB2, BRCA2	–
Pigmentation	–	SOD2

(B) Gene ontology (Molecular function)	Selected down-regulated genes	Selected up-regulated genes
Binding	CYP24A1, CDH2, CDKN3, RB1, DHFR, CCNB1, CCNB2, BRCA1, LIPC, AURKA, CDC2, BRCC3, MTTP, SCP2, CDK2,CCNA2, APOH, CDK6, RBL2, BRCA2, ACAT1, CDK4, CHEK1, CCNE1, AURKB, CDK5, E2F4, IDE, APOB, CDH1, FZD4, CSNK1G2, CCNE2	CDH4, IGFBP2, IRS2, FZD10, SOD2, FZD8, WNT6, SOD3, IGFBP6, FOXO3, FZD1, INSR, MT1X, LDLR, IGFBP3
Catalytic activity	CYP24A1, CDKN3, DHFR, BRCA1, LIPC, AURKA, CDC2, MAOB, SCP2, CDK2, CDK6, BRCA2, ACAT1, CCNH, CDK4, CHEK1, AURKB, CDK5, IDE	SOD2, SOD3, INSR, DUSP5
Transcription regulator activity	RB1, BRCA1, BRCA2, E2F4, CDH1	FOXO3
Structural molecular activity	–	WNT6
Molecular transducer activity	FZD6, CDK5, IDE, FZD4	FZD7, FZD9, IRS2, FZD10, FZD8, WNT6, FZD1, INSR, MT1X, LDLR
Enzyme regulator activity	CDH1, CCNE1	IGFBP3
Transporter activity	LIPC, MTTP, APOB	LDLR
Electron carrier activity	CYP24A1, MAOB	–
Antioxidant activity	–	SOD3

Table 3 continued

(C) Gene ontology (Cellular component)	Selected down-regulated genes	Selected up-regulated genes
Cell part	CYP24A1, LGR5, CDH2, RB1, CCNB1, CCNB2, E2F5, TYMS, BRCA1, LIPC, ATG4C, SOS1, AURKA, G2E3, CDC2, CHEK2, BRCC3, MAOB, MTTP, SCP2, GAS2, MTHFD1, CDK2, CCNA2, APOH, CKS1B, RBL1, UBE2C, FZD6, BARD1, PAK2, CDK6, RBL2, BRCA2, CCNF, ACAT1, CCNH, CDK4, PAK1IP1, NUCKS1, DKK1, STAT2, CHEK1, CCNE1, AURKB, CDK5, E2F4, DAPK1, IDE, TOP2B, BCCIP, CSNK1G3, APOB, CDH1, TET1, APOBEC3F, FZD4, ACAT2, CSNK1G2, CCNE2, CDK7, LRP1	FZD7, CTNNA2, CDK5R1, CDH4, APC2, FZD9, IRS2, CDKN2D, FZD10, CDKN1C, AQP6, SOD2, FOLR3, FZD8, AQP12A, SOD3, FOXO3, FZD1, CDKN2B, CDKN1A, LRP12, INSR, LDLR, AQP3, EGLN3, IGFBP3, DUSP5
Extracellular region	LIPC, ATG4C, APOH, DKK1, APOB	APC2, IGFBP2, IGF2, FOLR3, WNT6, SOD3, IGFBP6, IGFBP4, IGFBP1, IGFBP3
Synapse	CDH2, CDK5	CDK5R1
Extracellular region part	LIPC, APOH, IDE, APOB	APC2, IGFBP2, IGF2, WNT6, SOD3, IGFBP4, IGFBP1, IGFBP3
Macromolecular complex	–	APC2
Synapse part	SOS1	–

Genes were categorized according to their involvement in biological process (A), molecular function (B) and as cell component (C)

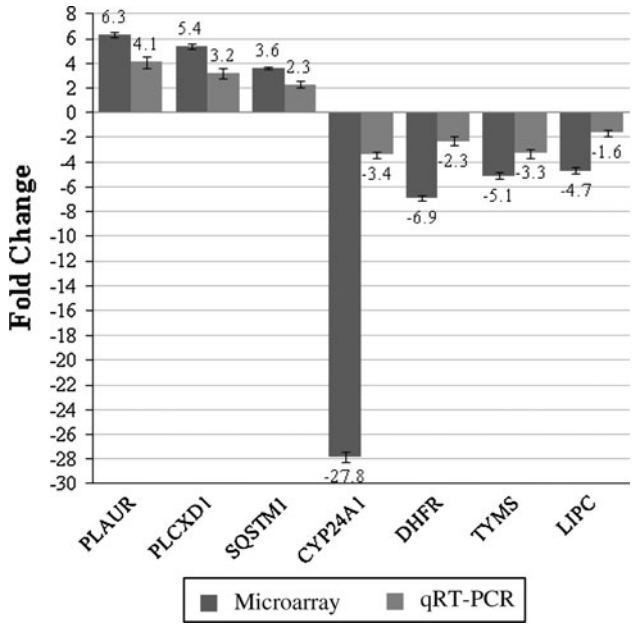

Fig. 9 Validation of the microarray data using semi-quantitative RT–PCR (qRT–PCR). A few genes that were significantly regulated in HepG2 cells in response to treatment with extracts of the *A. occidentale* shoots were selected namely *PLAUR, PLCXD1, SQSTM1, CYP24A1, DHFR, TYMS* and *LIPC* using real-time RT–PCR. All data were normalized to the reference gene, *GAPDH*. The expressions of the *PLAUR, PLCXD1* and *SQSTM1* genes were all up-regulated while those of *CYP24A1, DHFR, TYMS* and *LIPC* were down-regulated. The expression patterns obtained through real-time RT–PCR were consistent with the microarray results

UBE2C and *UBE3B,* was also aberrantly expressed. VRK1 is involved in the regulation of DNA replication through the phosphorylation of CREB leading to the regulation of *CCND1* gene expression [17].

Aurora kinases comprise three members, Aurora A, Aurora B and Aurora C. *Aurora-A* is transcriptionally regulated by E2F3 during a cell cycle. E2F3 induces *Aurora-A* expression by binding directly to *Aurora-A* promoter and subsequently stimulates the promoter activity [12]. Both could thus be an important target for cancer intervention [12]. In addition, aurora-B has been shown to be overexpressed in many cancers including breast cancers [10]. The methanol extracts of the *A. occidentale* shoots at the IC_{50} concentration, suppressed the *Aurora A* and *Aurora B*, but not *Aurora C*, by fourfold and twofold respectively. An RNA methyltransferase, NSUN2 had been shown to be a novel substrate for Aurora B, which contained a NOL/NOP/sun domain [38]. In this study, the extracts were able to suppress both *Aurora A* and *Aurora B* as well as *NOP2* and *NSUN6,* suggesting its potential as an anti-cancer agent.

Insulin-like growth factor binding protein-3 (IGFBP3) inhibits the growth of non-small cell lung cancer (NSCLC) cells. IGFBP3 overexpression inhibits the phosphorylation of Akt and glycogen synthase kinase-3 beta and the activity of MAPK, all three are activated by IGF-mediated signalling pathways that have mitogenic and anti-apoptotic properties and have been implicated in the development of

lung cancer [24]. Nuclear IGFBP3 induces apoptosis and is targeted to ubiquitin/proteosome-dependent proteolysis. IGFBP3 degradation is dependent on active ubiquitin-E1 ligase [41].

The shoot extracts also up-regulated *LDLR* gene which correlated with findings by Salleh et al. [39] who reported that the cashew shoots were able to increase LDLR activity in cultured HepG2 cells. Other genes associated with lipid metabolism including *LIPC, ACAT1, MTTP, APOB* and *SCP2* were down-regulated. LDL-R is responsible for the internalization of cholesterol-rich lipoprotein, LDL, from the blood circulation through the recognition of its ApoB by the receptor [6]. *SCP2* gene expression was reported to be enhanced by oxidized LDL [13] which could be ingested by macrophages leading to the formation of foam cells, a critical step in atherosclerosis. ACAT is responsible to convert free cholesterol to cholesteryl ester in tissues. ACAT1 is the main isoenzyme in the neuronal brain [7], and its presence is associated with certain forms of Alzheimer disease. *MTTP* encodes microsomal triacylglycerol transfer protein (MTP) which is required for the assembly of nascent chylomicrons and VLDL while hepatic lipase encoded by *LIPC* does not only hydrolyses triacylglycerols and phospholipids in circulating plasma lipoproteins, it also regulates lipoprotein uptake by cells [40]. Except for the *ACAT1*, the expression of the *LDLR, MTTP, SCP2* and *APOB* genes was only observed in response to the IC_{50} concentration, not those of 0.4 and 0.6 mg/ml suggesting a concentration-dependent expression of the genes.

The anti-diabetic properties of the shoot extracts could be linked to the up-regulation of the genes coding for the insulin receptor and the down-regulation of the insulin-degrading enzyme (IDE).

The hypolipidaemic, anti-diabetic and anti-cancer properties could be attributed to the presence of quercetin and kaempferol in the shoot extracts of the *A. occidentale*.

Quercetin and kaempferol are normally present in nature as glycosides. Free and glycoside forms of the two flavonoids are absorbed in the intestine (9, 14, 48) and are found conjugated with glucuronides or sulphates in the blood circulation. The concentrations of the extracts selected in the study were based on MTT assays whereby cell viability was maintained above 90% at the extract concentrations of 0.3 and 0.4 mg/ml. The concentrations used were probably not a true reflection of the flavonoids levels in the plasma as it was reported that the levels of free and conjugated flavonoids such as kaempferol in blood was in the range of 1.7–6.1 μg/ml after oral ingestion of 25–50 mg/kg body weight [48]. However, when using an in vitro model like HepG2 cells, sometimes it is necessary to use higher concentrations of extracts to see significant changes in gene expression [36]. Nevertheless, this in vitro study is still useful to provide preliminary information on the possible molecular mechanisms in relation to the medicinal properties of the shoot extracts of *A. occidentale*. Further confirmation using an in vivo model would need to be carried out to corroborate the in vitro findings.

Conclusion

In conclusion, low concentrations of the shoot extracts of *A. occidentale* were able to significantly regulate a sizable number of genes in HepG2 cells. However, the type of the expressed genes was highly dependent on the concentration of the extracts used. Amongst the genes that were significantly regulated were those involved in regulating cell cycle check points, apoptosis and cell proliferation, lipoprotein metabolism and insulin signalling suggesting the potential of the plant to act as anti-cancer, hypolipidaemic and anti-diabetic agents.

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