SUPPLEMENTARY MATERIAL

Antitumor-promoting and inhibition of thermal induced protein denaturation activities of β -sitosterol and lupeol, isolated from *Diospyros lotus* L

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Abstract

The current study was designed to explore the antitumor-promoting and inhibition of thermal induced protein denaturation activities of β -sitosterol **1** and lupeol **2**, isolated from *Diospyros lotus* L. Compound **1** evoked marked concentration dependent inhibition against TPA (20 ng/32 pmol)-induced EBV-EA activation in Raji cells with IC₅₀ of 270 μ g/mL without significant toxicity (70% viability). Similarly, compound **2** showed significant antitumor promoting effect with IC₅₀ of 412 μ g/mL, without significant toxicity (60% viability). In heat induced protein denaturation assay, compound **1** elicited concentration dependent attenuation with maximum effect of 73.5% at 500 μ g/mL with EC₅₀ 117 μ g/mL. Compound **2** demonstrated significant concentration dependent protein denaturation with maximum effect of 59.2% at 500 μ g/mL with EC₅₀ 355 μ g/mL. Moreover, *in-silico* docking studies against the PI3K enzyme also shows the inhibiting potency of these compounds. In short, both the compounds caused marked antitumor-promoting effect and potent inhibition on thermal induced protein denaturation.

Keywords: *Diospyros lotus*, β -sitosterol, lupeol, antitumor-promoting and inhibition of thermal induced protein denaturation activities, docking studies against the PI3K enzyme

3. Experimental

3.1. Plant material

Diospyros lotus roots were collected from Razagram (Khall), Dir, KPK, Pakistan, in May 2009. The sample was authenticated by Dr. Abdur Rashid, Taxonomist, Botany Department, University of Peshawar, Pakistan. A voucher specimen (Bot. 20036(PUP)) has been deposited at the Herbarium, Department of Botany, University of Peshawar, Pakistan.

3.2. Extraction and isolation

Shade-dried roots of *D. lotus* (14 kg) were powdered by local grinder and repeatedly extracted with 60 L, CHCl₃ (x5) at room temperature. The combined extracts were concentrated by evaporating the solvent using rotary evaporator under reduced pressure at a temperature below 50 °C to obtain a dark red residue. The crude extract was defatted with *n*-hexane through Soxhlet apparatus (X6), at 50 °C to removed *n*-hexane soluble fraction which was subjected to column chromatography (CC); *n*-hexane-EtOAc (100:0 → 85:15) eluted gave a reddish oil residue of fatty acids. The column was further eluted with *n*-hexane-EtOAc (100:0 → 85:15) and furnished lupeol (**2**) (m.p. 210-212 °C) and β -sitosterol (**1**) (m.p. 137-140 °C). The ¹H-NMR and ¹³C-NMR spectral data of **1** and **2** (Figure1) were found identical with β -sitosterol and lupeol reported in literature (Jamal et al., 2008; Kamboj and Saluja, 2011).

3.3. In-vitro Epstein-Barr-Virus (EBV) early antigen activation induction effect

Epstein-Barr-Virus (EBV) early antigen activation assay was employed for the assessment of compounds **1** and **2**. Briefly, EBV genome-carrying lymphoblastoid cells (Raji cells) derived from Burkitt's lymphoma were cultivated in RPMI-1640 medium with 10 fetal bovine serum (FBS). Smears were made from the cell suspension, and an indirect immunofluorescence technique (Maoka et al., 2013). Spontaneous activation of EBV-EA in our subline of Raji cells

was less than 0.1%. The cells were incubated at 37 °C for 48 h in 1 mL of medium containing *n*-butyric acid (4mM), TPA [32pM= 20ng in 2 mL dimethylsulfoxide (DMSO)] and various amounts of the test compounds dissolved in 2mL of DMSO. Smears were made from the cell suspension. The EBV-EA-inducing cells were stained by means of an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compound was expressed as a ratio relative to the control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32pM). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the trypan blue staining method. The cell viability of the TPA positive control was greater than 80%. Therefore, only those compounds that induced less than 80% (% of control) of The EBV-active cells (those with a cell viability of more than 60%) were considered to be able to inhibit the activation caused by promoter substances (Tatsuzaki et al., 2010).

3.4. *In-vitro* protein denaturation assay

The protein denaturation assay was used for the estimation of antiinflammatory potential of decoction of teas. The egg albumin (0.2 mL) was mixed with 2 mL of varying concentrations of decoctions (50-500 µg/mL) and 2.8 mL of phosphate buffered saline at pH 6.4 to get a reaction mixture of 5 mL. The mixtures were incubated at 37°C±2 for 15 min and then heated at 70°C for 5 min. The resulting solutions were cooled and absorbance was taken on a spectrophotometer at 660 nm by using vehicle as blank (Chatterjee et al., 2012; Khan et al., 2014). The distilled water served as control while aspirin as a standard. Finally, the % inhibition of protein denaturation was calculated.

$$\text{Protein denaturation (\%)} = \frac{\text{Control Absorbance} - \text{Test Absorbance}}{\text{Control Absorbance}}$$

The IC₅₀ values were calculated using statistical software, GraphPad PRISM 6.

3.5. Statistical analysis

The resulting data were expressed as the mean $\pm$ SEM ($n=3$) in each group. To determine the differences between groups, one-way analysis of variance (ANOVA) was performed (GraphPad PRISM 6, San Diego, CA, USA) using the least significant difference (LSD) test at $P<0.5$ or $P<0.01$.

3.6. Molecular docking studies

The crystal structure of Phosphoinositide 3-kinase (PI3K from *Mus musculus*) was retrieved from the protein data bank (PDB) with an accession code of 4XEO. Enzyme and ligands structures were prepared for docking by using swiss pdb viewer v4.1.0 (Guex and Peitsch, 1997), Chem sketch (Li et al., 2004) and Avogadro's software's (Hanwell et al., 2012). Docking studies were carried out through Autodock Vina (Trott and Olson, 2010) and i-GEMDOCKv 2.1 softwares (Hsu et al., 2011). The docking procedure of software's was optimized by already co-crystallized ligand of receptor (PI3K). Autodock vina was connected with PyRex tools (Yellamma et al.), all the default parameters were utilized for the docking of PI3K. Another docking studies were also carried out through i-GEMDOCKv2.1 software (Mohan; Kumar et al., 2012) by using default parameters. The docked complex were analyzed by Discovery studio visualizer version 4.0 (Umamaheswari et al., 2011) PyMOL version 1.7.2 (DeLano, 2002) and LIGPLOT+ version v.1.4.5 (Laskowski and Swindells, 2011) softwares.

4. Conclusions

In conclusion, the isolated compounds, β -sitosterol **1** and lupeol **2** from *D. lotus* L demonstrated marked antitumor-promoting and inhibited thermal induced protein denaturation. Moreover, *in-*

silico docking studies against the PI3K enzyme also shows the inhibiting potency of these compounds. Thus, we can conclude that, they could be useful natural healing agents in the effective management of tumor and/or accompanied by inflammatory conditions.

Competing interests

The authors declare that they have no competing interests.

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Table S1. Inhibitory Effects on the Induction of Epstein-Barr Virus Early Antigen (EBV-EA) by Compounds Isolated from *Diospyros lotus*

Concentration(mol ratio/TPA)					
% to control (% viability)					
Samples	1000	500	100	10	IC ₅₀
Compound 1	0 (70)±0.5	25.8±1.1	68.6±2.1	94.9±0.6	270
Compound 2	5.6(60)±0.4	40.3±1.5	72.5±2.5	98.0±0.6	412
Curcumin	2.1(70)±0.6	29.3±1.2	71.6±2.2	98.2±0.5	301

TPA(32 pmol)=100 % Negative cont. 0%

Values represent the relative percentage to the positive control, with TPA (32 pmol, 20 ng) representing 100% induction at four different concentrations in terms of molar ratio/32 pmol TPA. Data are expressed as mean ±S.D (*n*= 3). IC₅₀ Values represent the mole ratio of compounds, relative to TPA, required to inhibit 50% of the positive control activated with 32 pmol TPA. Values in parentheses are percentage viability of Raji cells.

Table S2. The Docking energies of Compounds (β-sitosterol and Lupeol) along with standard (Curcumine) against PI3K enzyme

Compound Name	Autodock vina score (kcal/mol)	i-GEMDOCK score (kcal/mol)			
	B. Affinity	Total Energy	VDW	HBond	Elec
β-sitosterol	-7.2	-98	-89	-9	0
Lupeol	-7.6	-103	-97	-6	0
Curcumine (standard)	-7.1	-91	-77	-14	0

Figure S1. Chemical structures of compounds

Figure S2. Percent inhibition of compounds (1 and 2) isolated from *Diospyros lotus* L. in thermal induced protein denaturation at various concentrations. Data are expressed as mean $\pm$ SEM ($n=3$).

Figure S3: The predicted docked poses of β -sitosterol and lupeol (shows by green color ball and sticks) as well as their superimposition on standard Curcumin (cyan color only sticks) and co-crystallized ligand (red color with sticks) against phosphoinositide-3 kinase

Figure S4: 2D and 3D interactions presentation of β -sitosterol in the active site of PI3Ks

Figure S5: 2D and 3D interactions presentation of Lupeol in the active site of PI3Ks