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Structural characterizations of lead anti-cancer compounds from the methanolic extract of *Jatropha tanjorensis*

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Abstract

LC/ESI/MS/MS data analysis on the phytoconstituents of methanolic extract of *Jatropha tanjorensis* leaves revealed the presence of abundant flavone glucosides (homoorientin, kaempferol-3-o-rutinoside, luteolin-7-o-glucoside, 6-C-pentosyl-8-C-hexosyl apigenin, naringin and vitexin), flavonol (kaempferol and kaempferide) and flavones (baicalein and diosmetin). We, herein, demonstrated that methanolic extract of *J. tanjorensis* possess DPPH free-radical scavenging activity (IC₅₀ of 49.7 µg/mL), inhibition of lipid peroxidation activity (IC₅₀ of 189.6 µg/mL) and anti-cancer activity through MTT assay against EAC cells (IC₅₀ of 14.6 µg/mL) and Caco-2 cells (IC₅₀ of 21.0 µg/mL). *In silico* analysis indicated that cytotoxic activity of the methanolic extract of *J. tanjorensis* could be attributed to the presence of vitexin and 6-C-pentosyl-8-C-hexosyl apigenin as evidenced by exhaustive molecular docking studies carried out against 8 proteins of Bcl-2 family that play essential role in apoptosis. Moreover, drug-likeness properties of the leads and scopes to develop them as potent anti-cancer compounds are discussed.

Introduction

Jatropha tanjorensis (Family, Euphorbiaceae) also known as the "Catholic vegetable" or "Reverend Father's vegetable" is an exotic plant species found in India, Africa, and America. Ethnomedicinally *tanjorensis* is being used as a vegetable to make palatable soup. Besides it possesses antiseptic and anti-hypertensive properties (O'Hara et al., 1998). There are also reports existing on the use of this plant drug in the treatment of diabetes (Olayiwola et al., 2004). Studies carried out on the nutraceutical values of this plant drug showed the abundant presence of bioactive phytoconstituents like flavonoids (3.7%) and alkaloids (1.9%) and essential minerals such as calcium (5.7%), magnesium (4.2%) and potassium (2.2%). This data could also be useful in providing scientific evidences for its use as herbal

dietary supplement (Arun et al., 2012).

Among the main constituents identified, flavone glucosides and flavones/flavonols are of the largest group of molecules that are enriched with numerous biological activities and extensive studies are available on these bioactive molecules (Hertog and Katan, 1998). These phytomolecules besides possessing potent antioxidant property (Zhang et al., 2006; Wang et al., 1996) are also useful in combating various disorders like cancer. It was also reported that these flavonoids possess anti-proliferative (Kawaii et al., 1999) and anti-inflammatory activity (Garcia-Lafuente et al., 2009). Compounds with anti-oxidant and cytotoxic potentials could be useful in preventing the secondary damage to the biological system caused by the release of free radicals. This property makes flavonoids and its



derivatives more useful towards the development of safe and efficacious plant drugs. Present study showcases remarkable potential role of methanolic extract of *J. tanjorensis* leaves in scavenging free radicals and inhibiting the proliferation of cancer cells mainly Ehrlich Ascites Carcinoma (EAC) and Human epithelial colorectal adenocarcinoma cells (Caco-2) through various *in vitro* assays. The extract is mainly composed of several flavone glucosides and flavones/flavonols and structures of each phytochemical belonging to these classes were confirmed using MS/MS data. Principle compound(s) accounting medicinal properties of the extract were scrutinized by examining binding interactions and affinities of the phytochemicals with 8 different proteins of Bcl-2 family by means of molecular dockings and the analysis suggested that vitexin and 6-C-pentosyl-8-C-hexosyl apigenin are the lead compounds. Unique structural features of the compounds for developing them as anti-cancer drug molecules have also been brought into fore.

Materials and Methods

Plant source and chemicals

Fresh leaves of *J. tanjorensis* (Euphorbiaceae) were collected from in and around SASTRA University, Thanjavur, India during July 2012. Herbarium voucher specimens were prepared and authenticated by comparing with the deposited specimen at the Raphinet Herbarium (RHT 1291) St. Joseph's College, Trichy. All chemicals used were of HPLC reagent grade purchased from Merck (Germany).

Preparation of methanolic extract

The fresh leaves of *J. tanjorensis* were washed in running water, shade dried and pulverized to coarse powder. The powder (100 g) was then first defatted using petroleum ether for 48 hours, after which the powder was macerated for 72 hours at room temperature using methanol. The extract obtained was dried in a vacuum rotavapor at 40°C followed by lyophilization using freeze dryer (Christ Martin, USA) and stored at 4°C until use. This crude methanolic extract was used directly for identification of molecules using LC-MS/MS and to assess other biological activities through *in vitro* methods.

ESI calibrant

ESI (Electrospray ionization) Tune mix was used as an internal calibrant (Sigma-Aldrich Chemie GmbH Co. St. Louis, MO). Analyses of the internal calibrant were done as per the manufacturer's instruction. On calibrating, particular masses of the acquired mass spectrum were assigned to correspond-ding reference masses of the loaded calibrant and the deviation between reference mass and current mass in ppm (parts

per million) is calculated using the formula (Equation 1):

$$\text{Error (ppm)} = \frac{((\text{current mass} - \text{reference mass}) / \text{reference mass}) \times 10^6}{\text{Equation 1.}}$$

UV-visible spectrum

Absorption spectrum of the methanolic extract of *J. tanjorensis* was recorded in the wavelength range of 200-800 nm using NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA). 2 μL of homogenously mixed crude methanolic extract (1 mg/mL) was taken and analyzed for its UV and visible region absorption spectrum. The path length and spectral resolution were set as 1 mm and ≤ 1.8 nm respectively. Blank correction was performed using methanol. Data generated was processed as per instrument manual.

LC-MS/MS

For qualitative purpose crude methanolic extract sample was weighed and dissolved in methanol to get final concentration of 1 mg/mL was then filtered using 0.45 μm syringe filter. 500 μL of this solution was analyzed by LC/ESI/MS/MS using UHPLC+ focused (Ultra high performance liquid chromatography) RP liquid chromatography coupled to mass spectrometer (microTOF-Q II, Bruker, Germany). Liquid chromatography separations were carried out on a C18 reverse phase column (120 Å, 2.1 x 150 mm Acclaim 120, UHPLC+ Ultimate 3000 series, Dionex). UV detector was set arbitrarily at 260 nm. A discontinuous gradient elution at a flow rate of 0.2 mL/min was performed using mobile phase A represented by acetonitrile and mobile phase B represented by water (MilliQ) acidified with acetic acid (1%). The gradient started from 95% of B for 10 min, followed by achieving 90% B in 1 min, to 60% B in the next 9 min, next 10 min B reaches 80%, next 10 min to reach 40% B, 5 min to reach 0% B and was maintained for another 10 min until the run ends. Mass spectrometer with ESI ionization at negative mode equipped with HyStar 3.2 software was optimized to detect the exact mass and mass fragmentation pattern of each eluted compound. TIC spectra were acquired and elaborated using the HyStar software data analysis module. MS/MS experiments were carried out by means of auto scanning mode, where the mass spectrometer software made a choice in real time about the selection of ion to fragment based on the intensity of each peaks with a threshold set above 1500 absolute counts. Optimized parameters consisted in collision energy 10 eV, focusing potential of 350 Vpp (Voltage per peak), transfer time of 800 μs , pre pulse storage of 5 μs the instrument was operated in the negative ion mode with a capillary voltage of 3.5 KV, capillary temperature was 280°C, sheath gas (N_2) flow rate was 6 L/min and the data were acquired in the AutoMSn scanning modes. Scan range was m/z 50-

1500; number of microscan was set at 3.

In silico studies

Targets selections and preparations for molecular docking studies: Three-dimensional (3D) structures of proteins such as Bcl-2 (1GJH), Bcl-B (4B4S), Bcl-W (1ZY3), Bcl-XL (2LPC), Bfl-1 (3MQP), Mcl-1 (2KBW), Bax (1F16) and Bak (2IMS) were retrieved from Protein Data Bank (PDB). Moreover, missing hydrogen atoms were added and bond orders were assigned to all proteins considered, herein, by using Schrodinger suite 9.2. The proteins were then energy minimized using 'Steepest descent' algorithm with a tolerance of 1000 KJ/mol/nm and step size of 0.01 with required number of minimization steps. The energy minimized structures were subjected to molecular dynamics (MD) simulations in near physiological conditions (pH 7.0, 1 atmospheric pressure and ionic strength of 0.1 M NaCl) for 5 ns at 310 K by Gromacs 4.5.1 (prior to productive runs, each protein was equilibrated for 200 ps and 500 ps under NVT and NPT conditions, respectively). In each case, trajectory structures were stored at every 25 ps and the data were analyzed in terms of change in potential energy and RMSD of backbone atoms of proteins. The data analyses revealed that all the proteins reached equilibrium under defined conditions well within the time span of 5 ns MD and a representative structure from the equilibrium phase of each protein was selected. The representative structures were used as 3D structures of the corresponding proteins for further analyses carried out in the present work.

High-throughput virtual screening (HTVS) and lead optimizations

3D structures of all 10 small molecular compounds identified from methanolic extract of *J. tanjorensis* leaves were generated and energy minimized (till structural conformations are converged) using ArgusLab 4.0.1. Then, the resultant structures were processed at pH 7.0 using LigPrep module of Schrodinger suite and docked on the BH3-binding grooves (which were covered by grid boxes having about 80 x 80 x 80 Å dimensions) of Bcl-2 family proteins by Glide-XP with default parameters. However, total numbers of poses per run, total numbers of generated and selected poses per ligand were set as 10,000, 5,000 and 400 respectively. The docked ligands on each protein were ranked on the basis of their GlideScores and Glide Energies. Hydrogen-bond (H-bond) and other non-covalent interactions between ligand and protein of the docked complexes were scrutinized using PyMol 1.5.0.1. Pharmacokinetic properties of the chemical compounds were assessed by subjecting each compound to QikProp module of Schrödinger software suite 9.2 and ADME-Tox prediction tool.

DPPH scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical

(DPPH) was used for determining free radical-scavenging activity of the extract (Koleva et al., 2002). Different concentrations of methanolic extract were added at an equal volume to methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated thrice. BHT was used as standard control. IC₅₀ values determined will denote the concentration of sample that is required to scavenge 50% of DPPH free radicals.

Lipid peroxidation inhibition assay

1% freshly collected RBCs in phosphate buffer (37°C, pH 7.2) was used as a source of PUFA (Polyunsaturated fatty acid) and substrate for lipid peroxidation. Triplicates of 10 µL of selected concentrations (ranging 10, 100, 250, 500 and 1000 µg/mL) of extract were mixed with 20 µL of 1.9 mmol/L FeSO₄ and 20 µL of ascorbate (15.4 µg/mL) and were subjected to inhibition of lipid peroxidation assay. The BHT was used as a positive control and saline water was used as negative control. This reaction mixture was incubated at 37°C for 1 hour, after which reaction was terminated by adding of 200 µL EDTA (0.1 mol/L). This was followed by colour generation using Thiobarbituric acid reactive substances (TBARS) reagent (1 mL) and heated in boiling water bath for 15 min to form a stable colored derivative with malondialdehyde (MDA) (Božin et al., 2008). Direct detection of MDA is challenging due to its high reactivity and water solubility, which is conquered by the addition of TBARS to generate a stable and colored product, which can be determined spectrophotometrically. After that, probes were centrifuged for 15 min at 3700 rpm and the absorbance was measured at 517 nm using NanoDrop 2000 series spectrophotometer (Thermo Scientific, USA).

Anti-cancer assay

Ehrlich Ascites Carcinoma (EAC) and Human epithelial colorectal adenocarcinoma cells (Caco-2) were grown and maintained in RPMI-1640 and DMEM high glucose media respectively supplemented with 2nM L-glutamine, 20% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin at 37°C with 5% CO₂ until 80% confluence level is reached. EAC cells are suspension cell lines hence direct collection using centrifugation at 200 rcf for 10 min were made, on the other hand Caco-2 cells are adherent type cell lines hence trypsin was used to break the cell protein interactions with surface of culture flask, detachment of cells from flask surface was observed under inverted microscope, upon complete detachment, trypsin inactivation was carried out by adding DMEM with 20% FBS followed by centrifugation at same condition. Cells counted and 0.1 x 10⁶ were treated with various concentrations of methanolic extract for 48 hours under 37°C and 5% CO₂ in CO₂ incubator. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

assay was performed in accordance with standard textual method (Mossman, 1983). After treating cells with different concentrations of methanolic extract, absorbance was read at 590 nm using Epoch microplate spectrophotometer (BioTek, USA). Every experiment included a set of negative control (untreated cells) as well as positive control (cyclophosphamide). All experiments were performed in triplicate. The results obtained were calculated and presented as a percentage of control values.

Double staining fluorescence based apoptosis study

1×10^3 EAC and Caco-2 cells were treated with various concentrations of methanolic extract of *J. tanjorensis* for 72 hours at 37°C in 5% carbon dioxide. Cells were harvested and stained with 1:1 concentration of acridine orange and ethidium bromide (100 µg/mL) and were incubated further for 15 min at 37°C in 5% carbon dioxide. Cells were then studied with a fluorescence microscope (Axio Scope. A1, Carl Zeiss, Germany) according to the following criteria: viable cells displayed a green (Acridine orange) fluorescent staining, early apoptotic cells showed yellow staining in combination with the presence of apoptotic bodies, late apoptotic cells gave a red staining (McGahon et al., 1995; Liegler et al., 1995). ProgRes software was used to analyze the data. Integrated LED fluorescence illumination served as light source. Green filter was used for acridine orange with 500 nm as excitation and 526 nm as emission and ethidium bromide red filter was set at 510 nm for excitation with emission at 595 nm.

Statistical analysis

Data of the anti-oxidant and anti-cancer assays are expressed as the mean \pm standard deviation (SD) of three independent measurements. GraphPad software package was used for statistical analysis. IC₅₀ values

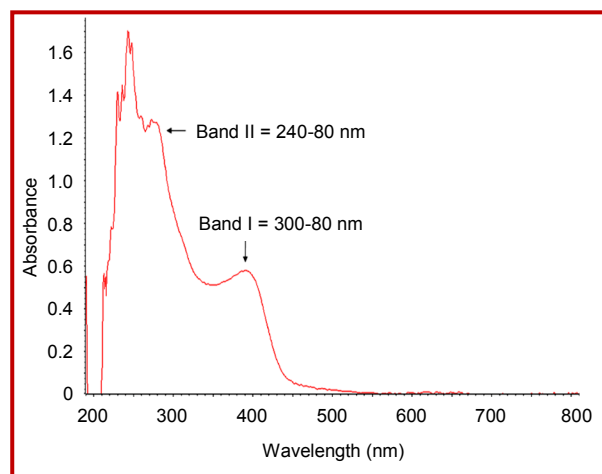


Figure 1: UV-visible absorption spectra of *Jatropha tanjorensis* leaves crude methanolic extract confirming the presence of flavonoids

were calculated using \log (dose/inhibitor) vs normalized response - Variable slope in a non-linear regression curve fit model.

Results and Discussion

UV/Vis absorption spectrum of flavonoids gives two characteristic peaks commonly referred to as bands, band I arises due to B ring in the 300 to 550 nm range and band II in the 240 to 285 nm range, arising from the A ring (Bohm, 1998). The UV-visible absorption spectra of methanolic extract also showed two strong absorption bands as band I (300-380 nm) and band II (240-80 nm) (Figure 1).

This data can be used as an indicative tool for the presence of flavones/flavonols and related flavone glucosides in the studied plant extract, whereas the MS/MS spectra provide additional significant information on the structure of identified molecules.

Characterization of plant molecules by advanced analytical methods is an important apprehension in phytochemistry, which in the present study was applied to identify different molecules and their derivatives present in *J. tanjorensis* leaf extracts. Mass spectrum analysis was performed in the sample using LC-MSMS system equipped with an ESI-Q-II analyzer and TOF (*time-of-flight*) detector calibrated at a maximum mass error of 0.6 ppm. In this study a stationary phase with hydrophilic endcapping, which has been demonstrated to be suitable for the separation, identification and characterization of flavonoids and its derivative compounds is used. Electrospray ionization mass spectrometry (ESI-MS) allows a soft ionization and provides information on structural properties. Distinction can be made between the various closely related flavonoids and information on the site of glycosylation can be obtained by LC-MS/MS. Searchable MS/MS spectra libraries such as Mass Bank, Metlin-Scripps, MS/MS fragment viewer were used to interpret the results of liquid chromatography interfaced with electrospray ionization (ESI-Q-II TOF) mass spectrometer.

Phytochemical investigation on methanolic extract of the leaves of *J. tanjorensis* led to the identification of a complex mixture of flavonoids predominantly containing flavone glucosides like Vitexin (apigenin-8-C-glucoside), luteolin-7-O-glucoside, Homoorientin (luteolin-6-C-glucoside), kaempferol-3-O-rutinoside, 6-C-pentosyl-8-C-hexosyl apigenin and naringin. Apart from this, flavones like baicalein and diosmetin and flavonols like kaempferol and kaempferide were also identified in the methanolic extract. Total Ion Chromatogram (TIC) MS and MSMS chromatogram of the methanolic extract showed several peaks with their

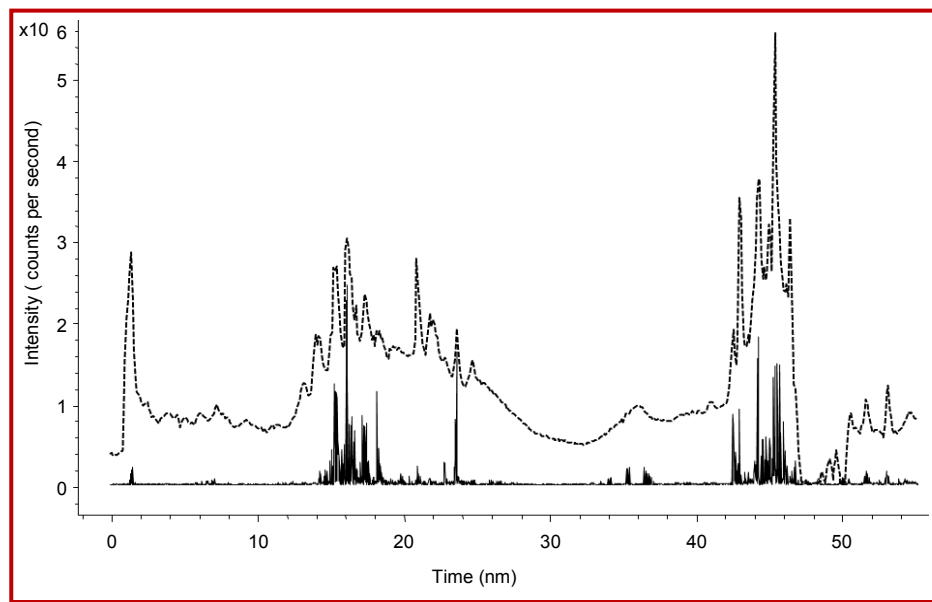


Figure 2: LC-MS/MS spectrum of crude methanolic extract of *Jatropha tanjorensis* leaves. Spectrum reveals various compounds eluted at different time point with Total Ion Chromatogram (TIC) for MS and MSMS

Table I						
ESI-MS and ESI-MSMS product ions of fractions from <i>Jatropha tanjorensis</i> leaves methanolic extract						
SL. No.	Identified compound	Rt [min.]	Mass	Parent ion [M-H] ⁻	MSMS	
					[Product ion]	
1	Luteolin-7-O-glucoside	14.4-14.5	448	447	284, 256	
2	Vitexin	16.3-16.4	432	431	283, 311, 323, 341, 269, 295, 239, 117, 161, 149	
3	Homoorientin	17.3-17.5	448	447	327, 298, 299, 285, 311, 357, 339, 269, 133, 163, 193	
4	6-c-Pentosyl-8-c-hexosyl apigenin	17.5-18.0	564	563	339, 369, 459, 425, 311, 297, 283, 269, 175, 191, 160, 214	
5	Kaempferol-3-O-rutinoside	19.1-19.2	594	593	311, 297, 282, 341, 431, 473, 353, 323, 269, 206, 164	
6	Diosmetin	17.8-18.0	300	299	254, 269, 285	
7	Naringin	23.6-23.7	580	579	181, 166, 387, 402, 417, 323, 357, 205, 151, 311	
8	Kaempferide	35.4-35.7	300	299	183, 117, 213	
9	Baicalein	43.3-43.5	270	269	156, 182, 99, 166, 166, 129, 119, 109	
10	Kaempferol	44.8-45.0	286	285	269, 205, 255, 189	

corresponding mass spectra (Figure 2).

Using negative ionization mode these base peaks were identified as deprotonated molecule [M-H]⁻. The retention time and the mass to charge ratios (m/z) of the base peaks and its respective fragmented peaks are listed in Table I. Most of the phytochemicals identified were eluted at 14-24 min and then later at 35-45 min. It was also observed that all the identified flavone glucosides were eluted with 21% ACN to 32% ACN in acidified water (14-24 min), similarly majority of identified flavones/flavonols were eluted with increase in acetonitrile content nearing to 100% (35-45 min).

Flavonoid derivatives present in the plant were identified using m/z values and MS/MS pattern obtained in the total ion chromatogram and by comparing them to those of the molecules available in various online databases. Figure 3 represents the MSMS pattern of identified flavonoids and its derivatives. Identification of flavonoids in first instance was made due to the presence of 182/183, 269, 284/285, 299/300 and/or 311 m/z peaks. The parent and glycone ions for all of the monoglycosylated and diglycosylated flavonoids were also clearly seen. On the basis of mass differences between the flavone glucosides and the

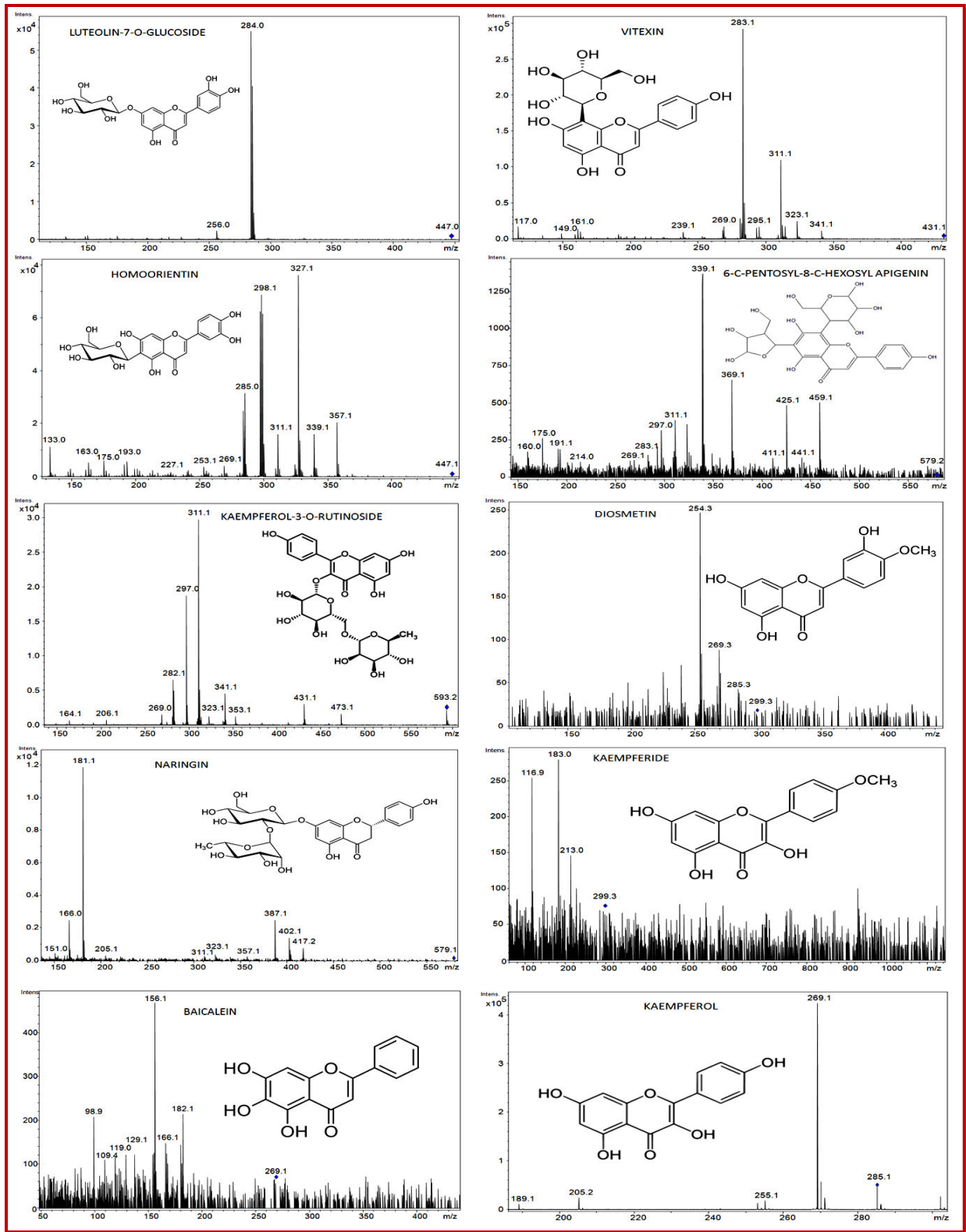


Figure 3: MSMS Spectrum of various flavonoids identified in methanolic extract of *Jatropha tanjorensis* leaves using LC-MSMS

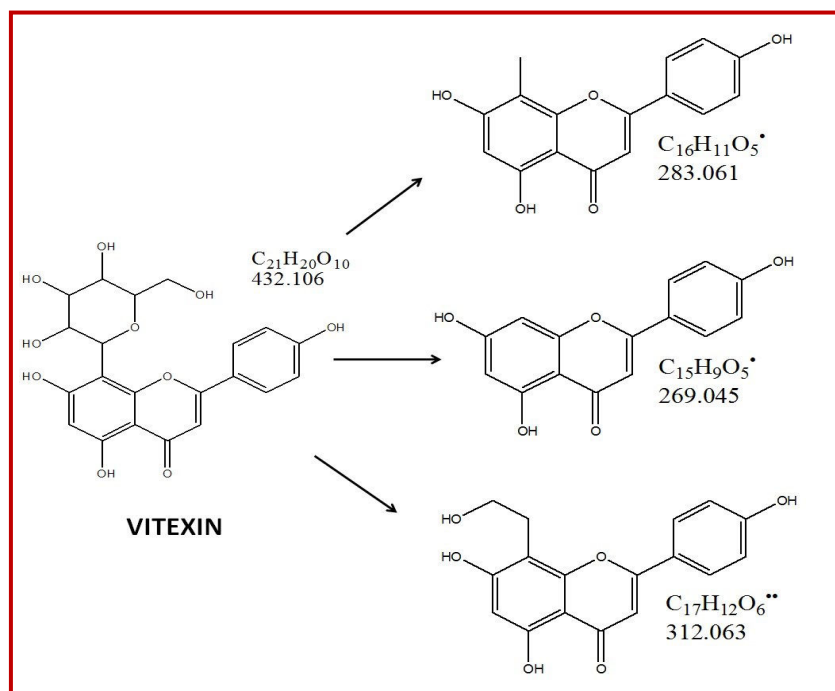


Figure 4: Typical ion fragmentation pattern of vitexin showing major fragmented ion as 283, 269 and 311/312 as consistent with that reported for vitexin

flavones/flavonols, the type of sugar present is established for example a difference of 132 amu is for pentose, 146 amu is for deoxyhexose, 162 amu is for hexose, 248 amu for malonylhexose, and 308 amu for deoxyhexosylhexose (Cuyckens and Claeys, 2004). The glycone ions, in conjunction with the UV/Vis spectra, helped in the provisional identification of most flavonoids. For illustration, the ESI-MS spectrum gave a deprotonated molecule at m/z 431.2 was characterized as vitexin. Its MS/MS data in negative ion mode are presented in Table I.

The CID-MS of vitexin showed characteristic fragment ions, which corresponded to the glucosyl ring fracture: m/z 341 and 311; other two were benzyl ion m/z 283 and aglycone ion m/z 269. In addition, few low abundance ions, namely m/z 117, 161 and 149 m/z generated by RDA (retro Diels-Alder) cracking ions were also identified as characteristic ions for vitexin (Figure 4) (Li et al., 2011). Similarly other molecules were also identified based on their characteristic fragmentation pattern available in various literature and online databases.

This finding is very interesting as it has been recently reported in literature that flavonoids rich extracts will exert multiple therapeutic activity (Cao et al., 1997). Present observations also further confirmed this statement, as the selected plant drug also exhibited multiple activities such as antiseptic, antihypertensive, antidiabetic besides being used as a nutraceutical source.

Apart from various mechanisms, apoptosis induction in cancerous cell is considered to be the best way of tackling cancer (Rozalski et al., 2006). Vast number of natural products have been identified which possess the activity of inducing apoptosis in cancerous cells (Amit et al., 2001). Identifying the best active compounds from plant extract has gained huge importance in cancer treatment. Anti-cancer efficacy of flavonoids is reported earlier by Iwashita et al (2000). Hence *in silico* docking studies were performed on flavonoids and its derivatives identified in selected plant against various apoptosis related proteins, especially Bcl-2 family proteins which determine the cell fate by overexpression of pro- or anti-apoptotic proteins.

GlideScores of 10 small molecular compounds on the surface grooves of 8 Bcl-2 family proteins playing essential roles in cell homeostasis processes of *Homo sapiens* have been enumerated in Table II. Surprisingly, the ligand molecules showed differential binding preferences towards anti-apoptotic and pro-apoptotic proteins.

On the basis of their binding preferences with those proteins, the chemical molecules could be classified into three groups. Group 1 consists of baicalein, diosmetin, kaempferide and kaempferol and these ligands are interacting with all the Bcl-2 family proteins. Chemical molecules such as naringin, homoorientin, kaempferol-3-rutinoside and luteolin-7-O-glucoside are belonging to Group II and they are also interacting with all protein targets except Bak, a pro-apoptotic protein. Interesting-

Table I

GlideScore of various phytochemical identified from the methanolic leaf extract of *J. tanjorensis* on interacting with the BH3-binding grooves of 8 different proteins of Bcl-2 family as calculated by means of Glide-XP molecular docking tool

Name	Bcl-2	Bcl-XL	Bcl-B	Bcl-W	Bfl-1	Mcl-1	BAK	BAX
Baicalein	-3.1	-2.5	-4.9	-6.8	-3.7	-4.4	-3.2	-6.6
Diosmetin	-4.2	-1.7	-4.6	-6.1	-3.8	-4.4	-1.5	-3.6
Homoorientin	-4.2	-5.3	-4.7	-10.0	-7.1	-5.3	---	-7.4
Kaempferide	-2.6	-1.9	-3.9	-5.3	-3.4	-3.8	-1.3	-3.0
Kaempferol	-3.9	-1.8	-4.6	-5.0	-3.7	-3.97	-2.2	-6.0
Kaempferol-3-o-rutinoside	-7.1	-6.5	-6.8	-10.7	-7.0	-7.9	---	-6.6
Luteolin-7-O-glucoside	-4.9	-6.2	-5.9	-8.4	-5.1	-5.7	---	-5.2
6-c-Pentosyl 8-c-hexosyl apigenin	-4.0	-3.0	-4.6	-8.5	-4.9	-6.6	---	---
Naringin	-3.8	-3.0	-4.9	-8.6	-5.9	-6.5	---	-4.7
Vitexin	-3.4	-3.9	-5.8	-7.7	-6.1	-4.9	---	---

ly, vitexin (VN) and 6-C-pentosyl-8-C-hexosyl apigenin (AN) belonging to Group III interact with anti-apoptotic proteins but showed bereft of interaction with pro-apoptotic proteins implying that these compounds may act as lead anti-cancer compounds of the plant extract. It should also be mentioned that the compounds of Group II may play significant role in a unique Bak-mediated apoptosis on retarding the process of cell proliferation. Of the 4 compounds, kaempferol-3-O-rutinoside (KFR) was ranked as top lead compound to 5 (Bcl-2, Bcl-XL, Bcl-B, Bcl-W & Mcl-1) of 8 proteins considered in the present study on the basis of GlideScores of the Bcl-2 family proteins-KFR complexes (Table II). The compound was ranked to second position on interacting with Bfl-1 and Bax, which depicted subtle stronger interaction with homo-orientin than their affinities with the KFR. Strikingly, the binding affinities of KFR are stronger with the Bcl-2, Bcl-B, Bcl-W, Bfl-1 and Mcl-1 than that of pro-apoptotic protein Bax and the KFR interactions with Bcl-XL (-6.5) and BAX (-6.6) are also comparable. Thus, the KFR may also partly account for the cytotoxic potential of the plant extract against EAC and Caco-2 cells.

By tie together, all flavones/flavonols studied in the present study from the *J. tanjorensis* are interacting with all the 8 proteins of Bcl-2 family and only flavone glucosides present in the source are depicting differential binding preferences with those protein targets. Interestingly, flavone glucosides of Group II and Group III are especially differing from each other in their glycosidic linkages. Flavone glucosides of Group III have glycosidic bond between 8th carbon atom of flavonoid moiety and their corresponding sugar groups, whereas different hexose groups are attached to various positions (exclusively other than 8th carbon atom) of flavonoid structures of flavone glucosides classified into Group II. While the flavone glucosides of Group II are interacting with all the anti-apoptotic proteins and Bak, the flavone glucosides (VN and AN) of Group III are interacting only with anti-apoptotic

proteins (they neither interact with Bak nor with Bax). It implies that the unique structural architectures of the VN and AN are crucial to accounting their specific interactions with anti-apoptotic proteins over pro-apoptotic proteins. BH3-binding grooves of all the 8 proteins of the Bcl-2 family and binding modes of the VN and AN on the surface grooves of the 6 anti-apoptotic proteins are shown in Figure 5.

A quick inspection to the figures clearly suggests that the surface grooves of Bax and Bak are with incompatible geometries to accommodate structures of the VN and AN (Figure 6).

In the case of the anti-apoptotic proteins-AN/VN interactions, it could be noted that the sugar moieties of the leads were tightly surrounded by a few hydrophobic residues (such as alanine, leucine, methionine, phenylalanine and valine) and at least one polar residue (such as arginine, asparagine, lysine, glutamic acid, histidine, serine, threonine and tyrosine) from the proteins to which they showed bindings. It could also be observed that at least anyone of 4 hydroxyl groups (at 3'', 4'', 5'' and 7'' of VN and 3'', 4'', 5'', 7'', 2'', 3'' and 6'' of AN) of sugar moieties of the leads invariably established hydrogen-bonding (H-bond) interactions with anyone type of polar residues that are within 4 Å proximity to the corresponding groups. However, these chemical complementarities were not apparent features in the grooves of pro-apoptotic proteins and consequently they were unable to accommodate the leads in their BH3-grooves. Thus, the unique structural and chemical features of the lead compounds (AN and VN) to interact with anti-apoptotic proteins as figured-out herein may pave a way to develop highly efficient and specific inhibitors to the anti-apoptotic proteins. Moreover, toxicities and bioavailability of the two lead compounds were predicted by using ADME-Tox and QikProp computational tools, respectively and inferred that both leads are non-toxic in nature and qualitative human oral absorption of both compounds are medium

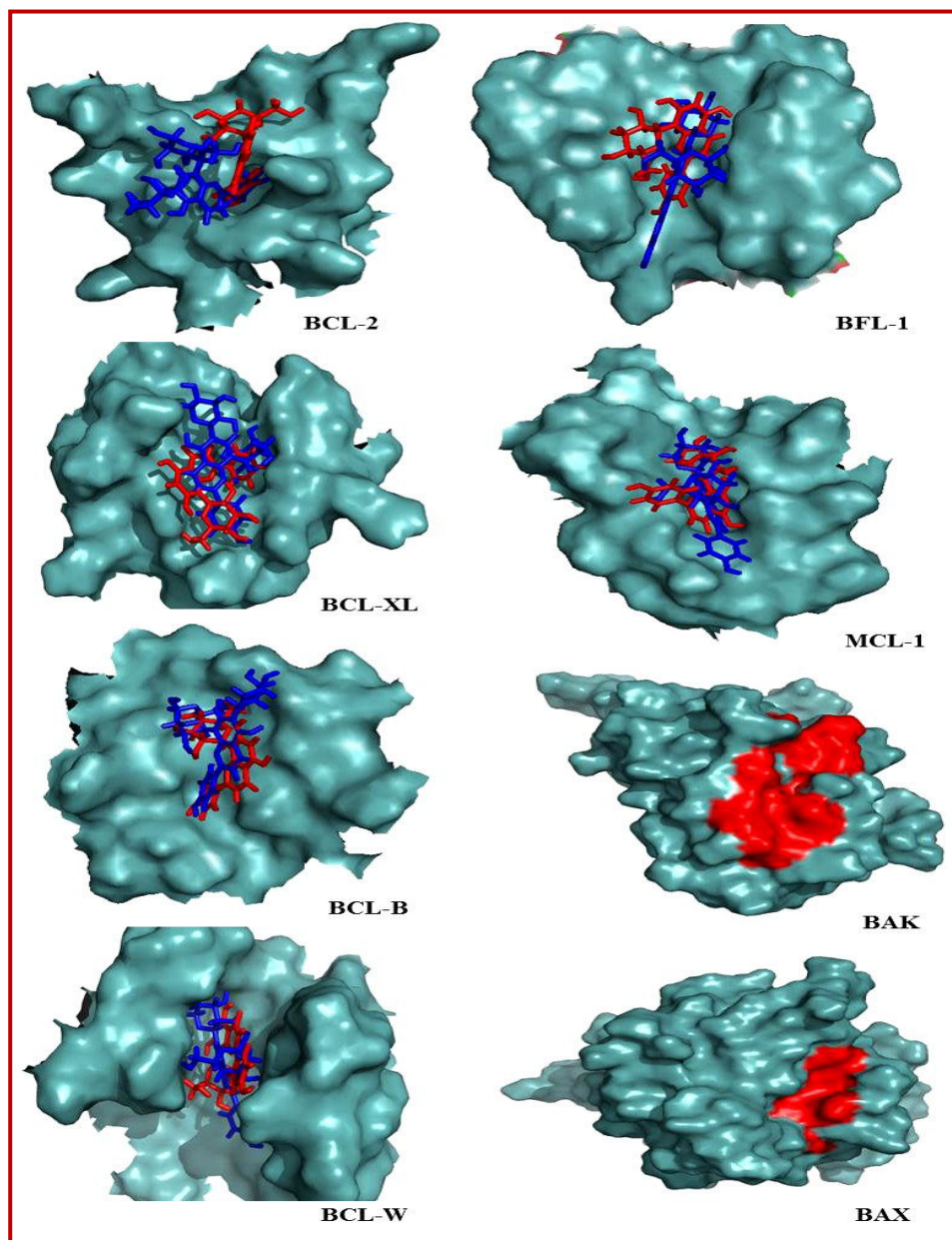


Figure 5: BH3-grooves (shown in red surface models) of 2 pro-apoptotic proteins and binding locations of the leads (VN in red sticks models and AN in blue sticks models) on the BH3-binding grooves of the 6 anti-apoptotic proteins are illustrated

suggesting that they are also promising lead compounds for developing them as therapeutic drugs against cancer.

In silico studies carried out using the molecules identified in the selected plant extract docked effectively against Bcl-2 family proteins suggesting the possible induction of apoptosis in studied cancer cells by the extract. Supporting anti-cancer and epifluorescence data obtained in *in vitro* studies are presented and discussed in sequel.

Flavonoids are known to have strong free radical scavenging activity (Pietta et al., 2000) and play vital

role in preventing the secondary damage due to the release of free radicals. DPPH stable free radical method is used in the present work, as this is a rapid and sensitive assay to assess the anti-oxidant activity of a specific compound or plant extracts (Koleva et al., 2002). The free radical DPPH has a characteristic absorption at 517 nm (purple in color), which upon treatment with anti-oxidant, decreases significantly due to hydrogen atom transfer from anti-oxidant (radical-scavengers) to DPPH. In the present assay, methanolic extract of *J. tanjorensis* leaves exhibited a noticeable free radical scavenging activity (Figure 7) with an IC₅₀ value of 49.7 µg/mL, this may be due to the presence of

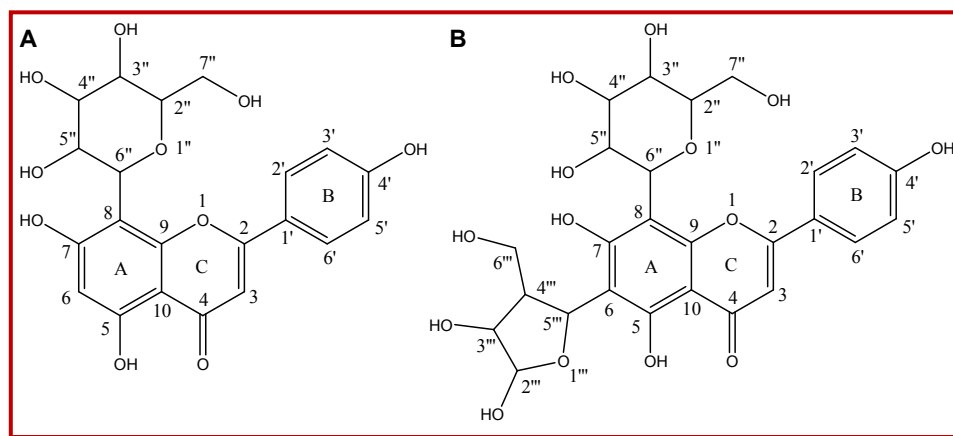


Figure 6: Molecular structure of A. Vitexin (VN) and B. 6-C-pentosyl-8-C-hexosyl apigenin (AN)

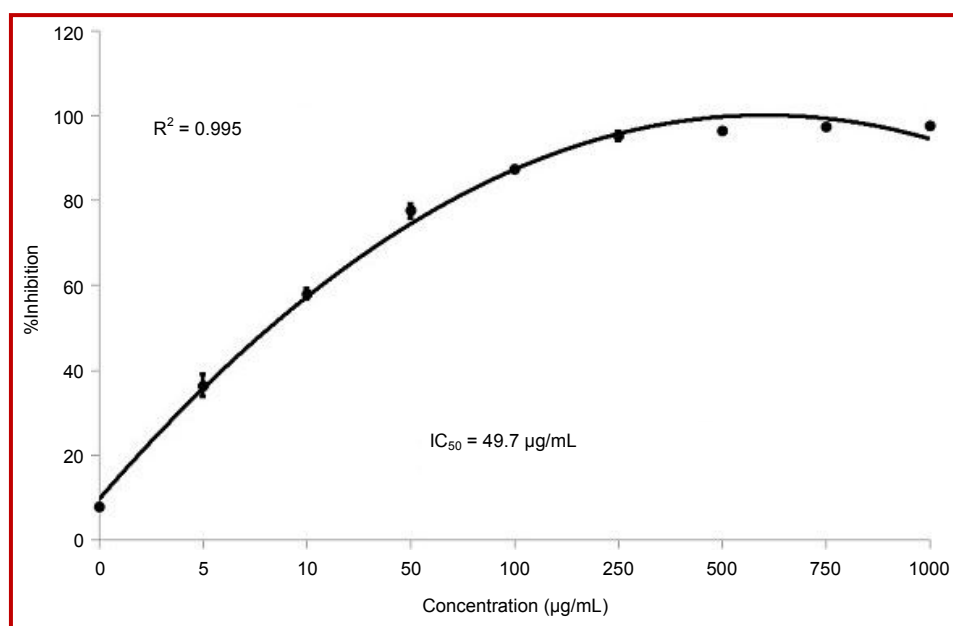


Figure 7: DPPH free radical scavenging activity of *Jatropha tanjorensis* leaves crude methanolic extract showing an IC_{50} of 49.7 $\mu\text{g/mL}$

flavonoids and their glycoside derivatives in the extract which might have acted as donors of hydrogen atoms or as electrons in the transformation of DPPH radical into its reduced form DPPH-H.

Lipid peroxidation results in biological membrane damage leading to cell death and the production of mutagenic and carcinogenic by-products. Malondialdehyde (MDA) has been used most widely as a biomarker in various studies associated with lipid peroxidation. The determination of MDA may be problematic because of its high reactivity and water solubility, and it is therefore necessary to generate stable derivatives. One of the most commonly used is thiobarbituric acid adduct, which can be determined spectrophotometrically. In the present work, freshly collected 1% RBCs solution was used as a substrate for LP due to its high content of polyunsaturated fatty acids. LP of polyunsaturated fatty acids was

triggered by Fe^{2+} (Iron^{II}) and ascorbate which, through Fenton reaction, generate $\text{OH}\cdot$ radicals (Laguerre et al., 2007). Identified flavonoids possess catechol moiety on ring B having 3-OH moiety which can function as a chelation site as a result can be oxidized. Thus, presence of 3-OH and 4-oxo group in combination with a double bond between C2 and C3, increases the scavenging activity of flavonoids. An IC_{50} value 189.6 $\mu\text{g/mL}$ (Figure 8) suggests that methanolic extract possess moderate inhibiting activity against lipid peroxidation and is efficient in inhibiting LP by both through radical scavenging and by chelating iron(II) by forming Iron-flavonoid complex to avoid its involvement in the generation of $\text{OH}\cdot$ radicals through Fenton reaction.

Di Carlo (1999) reports that hydroxyl moieties and 2-3 double bonds are important structural features of flavonoids that are associated with its biochemical and biological activities. Several classes of phytochemicals

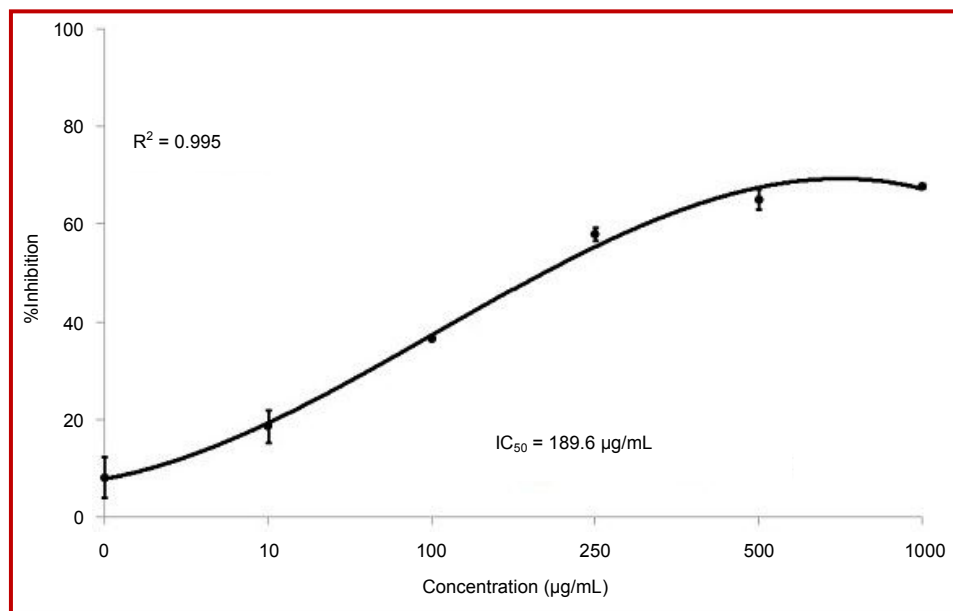


Figure 8: Inhibition of LPO activity of *Jatropha tanjorensis* leaves crude methanolic extract showing an IC_{50} of 189.6 $\mu\text{g/mL}$

have been shown to induce apoptosis in cell lines. Anti-cancer potential of flavonoids may be attributed to the presence of various hydroxyl groups and the degrees of glycosidic linkages. There are many modes of action by which phytochemicals inhibit the growth of cancerous cells, such as initiation of apoptosis through different intracellular cell signaling pathways. Anti-cancer efficacy of flavonoids rich methanolic extract of *J. tanjorensis* was evaluated through *in vitro* cytotoxicity assay against EAC and Caco-2 using MTT reagent. MTT assay has been extensively used to determine the cytotoxic potential of plant extracts against various cancerous cells. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is used to determine the cell viability in assays of cell proliferation and cytotoxicity. MTT facilitates this determination, as this gets reduced to yield an insoluble purple formazon product in metabolically active cells. Methanolic extract revealed potent cytotoxic activity against both the cancer cells with an IC_{50} value 14.6 and 21.0 $\mu\text{g/mL}$ for EAC and Caco-2 respectively.

One of the major causes for the occurrence of cancer is inadequate activation of apoptosis (Johnstone et al., 2002). Bcl-2-family proteins are important regulators of apoptosis. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL, contains a surface hydrophobic groove in which the BH3 domain of the pro-apoptotic counterparts binds (Reed, 1995). This binding is important for regulating apoptosis. Bax and Bak which belongs to pro-apoptotic proteins mediate the permeabilization of mitochondrial membrane upon oligomerization, resulting in the release of intermembrane space proteins such as cytochrome *c*, OMI/HTRA2, SMAC/DIABLO and endonuclease G leading to the formation of apoptotic bodies, condensation and fragmentation of

nuclei which brings cell death (Kuwana and Newmeyer, 2003). Bcl-XL plays its anti-apoptotic role by forming complex with Bax protein thus bringing conformational changes to Bax and hence prevents its activity of releasing apoptosis execution signaling factors. Inhibition of anti-apoptotic proteins by various natural products are the recent advancement in exploring the possible mode of action of these molecules in treating cancer. The possible role of identified compounds from *J. tanjorensis* in inducing apoptosis in cancer cells are discussed in detail through *in silico* modeling studies. Based on the extensive *in silico* experiments it was understood that methanolic extract of *J. tanjorensis* brings its anti-cancer activity through inhibiting anti-apoptotic proteins and inducing the activity of pro-apoptotic proteins.

Taking the lead from *in silico* modeling studies, the mechanism of cell death induced was assessed through cell morphological changes by staining cells with fluorescent dyes, including acridine orange and ethidium bromide. Cell death induced EAC and Caco-2 cells were treated with test drug extract and stained with acridine orange and ethidium bromide (1:1) at a final concentration of 100 $\mu\text{g/mL}$ (AO/EB dual staining).

Two different concentrations were chosen for EAC cells based on the IC_{50} values determined by MTT assay, which were 10 and 20 $\mu\text{g/mL}$ for EAC and 20 and 30 $\mu\text{g/mL}$ for Caco-2 cell line. As a control, both cell lines were cultured in complete media and stained with AO/EB (Figure 9A and 9A'). Figure 9B, 9B' and 9C, 9C' shows that the methanolic extract from *J. tanjorensis* induced apoptosis on both cells. Cells stained with acridine orange/ethidium bromide showed green fluorescence representing viable cells, yellow staining

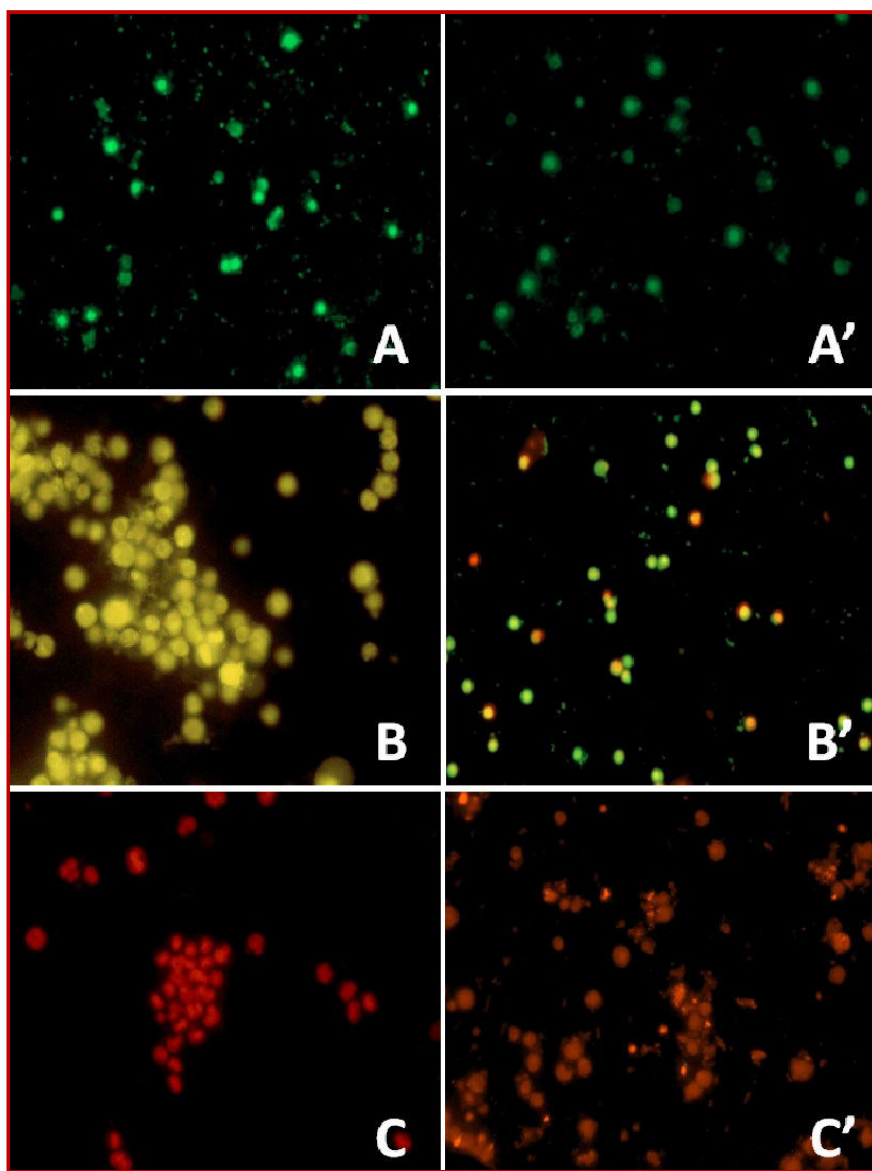


Figure 9: Morphological observation with acridine orange and ethidium bromide (AO/EB) staining at actual magnification 40x. EAC cells (A) and Caco-2 cells (A') were treated without and with *J. tanjorensis* methanolic extract, 10 µg/mL (B), 20 µg/mL (C), 20 µg/mL (B') and 30 µg/mL (C') for 72 hours. Each experiment was performed in triplicate (n=3) and generated similar morphological features

represented early apoptotic cells, whereas, reddish or orange staining represented late apoptotic cells (McGahon et al., 1995). Besides apoptotic cells can also be recognized by a set of morphological features that include loss of cell volume (Lockshin and Beaulaton, 1981), blebbing of the plasma membrane and compaction of chromatin into dense masses that lie at the periphery of the nucleus or, in other cases, condensation of the entire nucleus into a dense ball with the chromatin distributed evenly throughout the nucleus (Wyllie et al., 1980). As shown in Figure 9B and 9B', EAC and Caco-2 cells treated with 10 µg/mL and 20 µg/mL respectively of methanolic extract showed changes in cellular morphology like cell shrinkage,

membrane blebbing, chromosomal condensation and nuclear fragmentation. On the other hand, Figures 9C and 9C' showed similar features of cells treated with 10 µg/mL and 20 µg/mL of methanolic extract (Figure 9B and 9B'), but with extra features of late stage apoptotic activity such as apoptotic bodies, condensed and fragmented nuclei with increased brightness.

During apoptosis (programmed cell death), the cell's cytoskeleton breaks up and causes the membrane to bulge outward (Vermeulen et al., 2002). These bulges may separate from the cell, taking a portion of cytoplasm with them to become *apoptotic bodies* as clearly seen in Figure 9B, B', C and C'. This morphological

features of treated cancer cells revealed that a stronger apoptosis signal was induced with higher concentration of *J. tanjorensis* extract. This induction of apoptotic signal through *J. tanjorensis* leaves extract is evidenced and supported by the *in silico* studies also (Figure 5).

The present investigation constitutes the first comprehensive report on the constituents and anti-cancer activity of the methanolic extract of *J. tanjorensis* and contributes in providing scientific evidences for the traditional uses of selected plant drug *J. tanjorensis*. Hence we can predict that extract containing all these 10 compounds can act as a better oral potent drug for promoting apoptosis in cancer incidences.

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