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ORIGINAL ARTICLE

Cytotoxicity, Protein Kinase Inhibitory Activity, and Docking Studies of Secondary Metabolites Isolated from *Brownea grandiceps* Jacq

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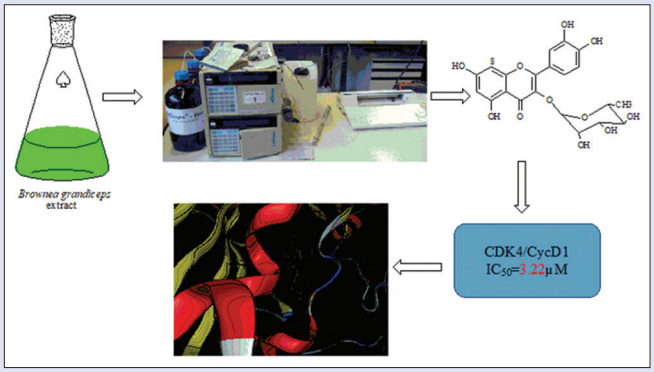
ABSTRACT

Background: Numerous kinases are excessively secreted in cancer. Consequently, inhibition of kinase enzymes has a basic role in the treatment of cancer through suppression or prevention of cancer cell multiplication. Due to its kinase inhibitory activity, flavonoids are expected to be of great importance in the discovery of new anticancer drugs. Based on the chemotaxonomic relationship among the plant genera, *Brownea grandiceps* is expected to be rich in flavonoids. **Objective:** The objective was to study the cytotoxicity, kinase inhibitory activity, and docking of the isolated metabolites. **Materials and Methods:** Ultraviolet, nuclear magnetic resonance, and mass spectroscopy were used for the identification and confirmation of the active metabolites. Ethyl acetate extract of *B. grandiceps* was chromatographed. Cytotoxicity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), protein kinase (PK) inhibitory assays, and molecular operating environment (using Molecular Operating Environment [MOE], 2016.08) were performed. **Results:** Catechin (1), quercitrin (2), trans-taxifolin-3-O-α-L-rhamnopyranoside (3), 13R-hydroxy-9Z, 11E-octadecadienoic acid (4), and 13R-hydroxy-9Z, 11E-, 15Z-octadecatrienoic acid (5) were isolated and identified. Quercitrin (2) exhibited the highest cytotoxicity against MCF-7 (inhibitory concentration for 50% [IC_{50}]: 4.24 μM) and moderate cytotoxicity against HepG2 cell (IC_{50}: 29.58 μM). Others showed moderate cytotoxicity; compounds 1–3 were tested against Aurora B, CDK4/cyclin D1, COT, IGF1-R, and FAK PKs, where quercitrin showed the highest inhibitory activity against Aurora B and CDK4/Cyclin D1 (IC_{50}: 4.78 and 3.22 μM). Docking of quercitrin against CDK4/Cyclin D1 confirmed its cytotoxic profile. **Conclusion:** The metabolites were first isolated from *B. grandiceps*. The mechanism of action against kinases enzyme was established and confirmed by docking studies of quercitrin at CDK4/Cyclin D1 using MOE program.

Key words: *Brownea grandiceps*, cytotoxicity, docking, flavonoids, protein kinase

SUMMARY

- Five known isolated metabolites were obtained from *Brownea grandiceps*, and the cytotoxicity screening of them was performed against MCF-7 and HepG2 cell lines. Quercitrin showed the highest activity against breast cancer. The postulated mechanism of quercitrin was proved by kinase inhibitory effect alongside with docking study.



Abbreviations used: MCF-7: Breast adenocarcinoma; HepG2: Human hepatocarcinoma; *B. grandiceps*: *Brownea grandiceps*; H bond: Hydrogen bond; PKs: Protein kinases.

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INTRODUCTION

Plant remedies are a major source of treatment for different ailments, especially in developing countries. Their extracts may be used to treat various medical problems such as menstrual disorders, hemorrhage, wound healing, eczema, sores, and toothache. *Brownea* species have been used in traditional medicine for the treatment of hemorrhages. In Colombia and Brazil, *B. ariza* Bentham and *B. rosademonte* were mostly used in folk medicine for snake venom hemorrhages. Nevertheless, in Venezuela and Ecuador, the decoction of *B. grandiceps* flowers (commonly known as Venezuela rose) was used in traditional medicine to reduce female abnormal bleeding during menstruation.[1,2] The plant family Leguminosae is rich in various metabolites; the naturally occurring flavonoid derivatives which represent the main constituent in Leguminosae are consumed in trace amount in the daily diets and play an important role for the treatment of numerous diseases such as diabetes, cancer, and Alzheimer. Flavopiridol, a semisynthetic flavonoid, as well as flavone derivatives, is used in the treatment of different ailments and can be used as a lead for further synthesis of new drugs.[3,4] Cancer is

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a major hazard to human health; it can affect all organs of the human body leading to death. The most commonly known types are lung, liver, breast, and colorectal cancers. In the USA, cancer is considered the second cause of death next to cardiovascular diseases.[5] The high mortality of cancer patients and serious side effects of anticancer drugs oriented the researchers to focus on the discovery of new potent and safe anticancer agents. Use of natural products as a template for drug discovery, is a realistic approach to overcome this problem.[6] Hence, exploring natural anticancer drugs with high potency and few side effects is of great biological importance. All cellular processes, such as proliferation, metabolism, apoptosis, cell cycle, development, motility, and differentiation, are being regulated and catalyzed through phosphorylation by protein kinase (PK) enzymes, consisting of hundreds of members forming the large kinase family.[7] These processes can be affected by deregulation of kinase activity. Furthermore, disrupted kinases are considered oncogenic agents and can be regarded as the main cause for several diseases. They can lead to cancer by different ways: abnormal phosphorylation, chromosomal translocation, misregulated expression, epigenetic regulation, and mutation. From this point of view, PK inhibitors are considered as a promising therapeutic agent in malignancy, viral infections, and other disorders involving PKs.[8,9] Using selective anticancer drugs with oriented targets, which inhibit or kill malignant cells without affecting the normal ones, is the main approach for the discovery of new anticancer drugs.[10]

MATERIALS AND METHODS

General experimental procedures

The ^{1}H-and ^{13}C-NMR determinations were obtained with a Bruker ARX-500 nuclear magnetic resonance (NMR) spectrometer operating at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C) in DMSO-*d*6 or CD_3OD solution. The chemical shifts were expressed in δ (ppm) with reference to TMS and coupling constant (*J*) in Hertz (Hz). The spectra were recorded by the standard Bruker software (Bruker AG, Switzerland). Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) was obtained on a Thermo Finnigan LCQ DECA mass spectrometer (Waldbronn, Germany) coupled to an Agilent 1100 HPLC system (LMU, Munich, Germany) equipped with a photodiode array detector. High-performance liquid chromatography (HPLC) analysis was performed on Eurospher-100 C_{18} (5 µm) column (125 × 2 mm, i.d., Knauer, Berlin, Germany) connected to a photodiode array detector UVD 340S (Dionex, Munich, Germany) and Dionex P580A LPG pump (Knauer, Berlin, Germany) with a flow rate of 1 mL/min with Chromeleon™ (V. 6.3) HPLC program (Knauer, Berlin, Germany). Routine detection was at 235 nm in aqueous MeOH. Preparative HPLC was performed on Varian Dynamax (Palo Alto, California, USA) (250 × 4.6 mm, ID, and 250 × 21.4 mm, ID) column, prepacked with Microsorb 60-8 $C1_8$ (Palo Alto, California, USA) connected to Varian, PrepStar 218 pump and Varian, ProStar 320 UV-Vis detector (Palo Alto, California, USA) with a flow rate of 20 mL/min, using Varian Star (V.6) HPLC program (Palo Alto, California, USA). Detection was achieved with a diode array detector, and chromatograms were recorded at 235, 254, 280, and 340 nm. Column chromatography was carried out using Sephadex LH 20, 0.25–0.1 mm mesh size (Merck, Darmstadt, Germany). Vacuum LC (VLC) was carried out using Silica Gel 60, 0.04–0.063 mm mesh size (Merck).

Plant material

The leaves of *B. grandiceps* Jacq were collected from Al-Orman garden, Giza, Egypt, in July 2015, and were identified by Prof. Dr. Moneer Abd El-Ghany, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Egypt. A voucher specimen was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Extraction and isolation

Air-dried powdered leaves of *B. grandiceps* (1 kg) were subjected to exhaustive extraction with methanol (3 × 5 L). The combined methanolic extract was concentrated *in vacuo* at 40°C to dryness (82 g). The concentrated methanolic extract was then suspended in distilled water (500 ml), filtered, and extracted with ethyl acetate (3 × 1 L) to yield 17 g of extract. The ethyl acetate fraction was chromatographed on VLC using silica gel 60, with 0.04–0.063-mm mesh size (Merck). Gradient elution with 5%–70% methanol in dichloromethane yielded seven fractions (Fr_1–Fr_7). Fr_2 (1.1 g) was further fractionated on Sephadex LH-20 and eluted with methanol to give five subfractions (Fr_{2a}: Fr_{2e}). Fr_{2b} (320 mg) was dissolved in methanol (HPLC grade) and filtered through 0.45-µ (millipore nylon membrane, Merck) filter and injected in preparative HPLC with 1-h program (5%–50% methanol for 35 min, 50%–100% methanol for 10 min, and isocratic 100% methanol for 10 min, gradient to the initial condition for 5 min) at a rate of 20 mL/min. The eluted peak was detected by an ultraviolet (UV) detector and collected in Erlenmeyer flasks to afford compound 5 (15 mg, t_R = 31.54). Compound 4 (12 mg, t_R = 30.88) was isolated from Fr_{2d} (235 mg) by the same method as compound 5 [Figure 1]. Fr_4 (1.5 g) was chromatographed on Sephadex LH 20 eluted with methanol to give five subfractions (Fr_{4a}–Fr_{4e}). Fr_{4d} (460 mg) was injected (400 µL) into preparative HPLC with 1-h program, to give compounds 2 (31 mg, t_R = 20.18) and 3 (25 mg, t_R = 20.88), respectively. Fr_7 (1.1 g) was eluted from Sephadex LH 20 with methanol to give four subfractions (Fr_{7a}–Fr_{7d}). Fr_{7d} (175 mg) was injected into preparative HPLC, using the same elution program described above to give compound 1 (22 mg, t_R = 17.18).

Cytotoxicity assay

A stock solutions of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were prepared in in ethanol 96% (v/v) for colorimetric assay of the tested samples. Exponentially growing cells were harvested, counted, and diluted appropriately.[11] MCF-7 and HepG2 cell lines were grown in DMEM medium supplied with 10% fetal bovine serum and 1% penicillin-streptomycin solution and kept for 2 days in cell culture flask till 70% confluence. Thereafter, the cells were detached, and a suspension (50 µL, 2 × 10^4 cells/well) of each cell line was transferred to a 96-well plate. Subsequently, the tested compound solution (50 µL) containing the appropriate concentration was added to each well. The concentration range was 3–10 µg/mL. The test plates were incubated under 5% CO_2 at 37°C for 72 h. A solution of MTT was prepared at 5 mg/mL in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution,

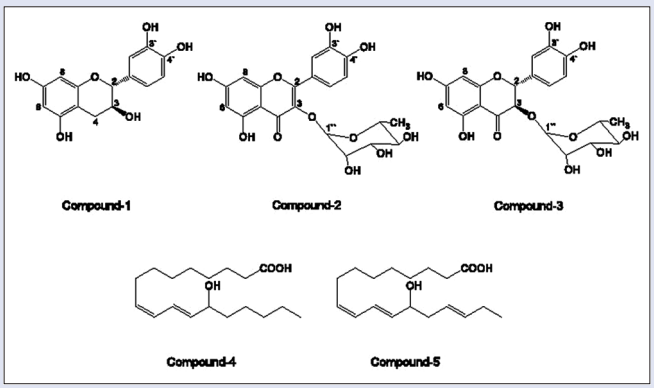

Figure 1: The structures of the isolated compounds

20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is converted to its blue formazan complex. After an incubation period of 3 h and 45 min under 5% CO_2 at 37°C in a humidified incubator, the medium was centrifuged (15 min, 20°C, 210 ×g) with 200 µL Dimethyl sulfoxide (DMSO), and the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter well spectrophotometer. The color intensity correlated with the number of healthy living cells [Table 1].

Protein kinase

The growth and differentiation of cells are regulated by PK enzymes through signal transduction pathways. Five PK enzymes were used for the determination of the inhibitory potency of the isolated compounds. The IC_{50} of compounds 1–3 showing an inhibitory potency of ≥40% with at least one of the five kinases at an assay concentration of 1×10^{-06} g/mL was determined. IC_{50} values were measured by testing ten concentrations of each sample in singlicate ($n = 1$) ten serial dilations per each compound and tested one time.[7,8]

Sample preparation

The compounds were provided as 1×10^{-03} g/mL stock solutions in 100% DMSO (1000 or 500 µL) and stored at −20°C. Prior to the assays, 100 µL of the stock solutions was transferred into separate microtiter plates. Subsequently, they were subjected to serial, semi-logarithmic dilution using 100% DMSO as a solvent resulting in ten different concentrations. 100% DMSO was used as control. Subsequently, 7 µL × 5 µL of each concentration was aliquoted and diluted with 45 µL H_2O only a few minutes before the transfer into the assay plate to minimize precipitation. The plates were shaken thoroughly and then used for the transfer of 5 µL compound solution into the assay plates.

Protein kinase assay

A proprietary PK assay was used for measuring the kinase activity of the PKs. All kinase assays were performed in 96-well Flash Plates™ from Perkin Elmer/NEN (Boston, MA, USA) in a 50 µL reaction volume. The reaction mixture was pipetted in the following order: 20 µL assay buffer, 5 µL ATP solution in H_2O, 5 µL test compound in 10% DMSO, and 10 µL substrate/10 µL enzyme solution (premixed). The assay for all enzymes contained 60 mM HEPES-NaOH (pH 7.5), 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 50 µg/mL PEG20000, and 1 µM [γ^{33}P]-ATP. The reaction mixtures were incubated at 30°C for 80 min and stopped with 50 µL 2% (v/v) H_3PO_4. The plates were aspirated and washed two times with 200 µL of 0.9% (w/v) NaCl or 200 µL H_2O. All assays were performed with a Beckman Coulter/Sagian robotic system (Beckman, Coulter, USA) [Table 2].[7,8]

Table 1: Cytotoxic activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Compounds	IC_{50}±SD (µM)	
	MCF-7	HepG2
Catechin	28.86±2.71	70.24±1.51
Quercitrin	4.24±0.84	29.58±0.85
Trans-taxifolin-3-O-α-L-rhamnopyranoside	27.01±2.13	>200
13R-hydroxy-9Z, 11E-octadecadienoic acid*	>200	>200
3R-hydroxy-9Z, 11E-, 15E-octadecatrienoic acid*	>200	>200
Doxorubicin	2.24±0.85	1.82±0.34
Erlotinib	ND	12.86±2.56

Values are the average of three independent experiments run in triplicate. *Compounds 4 and 5 have kinase inhibitory activity more than 200 µM. ND: Not determined; SD: Standard deviation; IC_{50}: Inhibitory concentration for 50%

Docking study

Instruments: Dell Precision T3600 Workstation (Intel Xeon E5-1660 3.3 GHz, 16 GB 1600 MHz DDR3 ECC RDIMM 1 TB [7200 RPM], 1 GB Nvidia Quadro 2000, Windows 7 Professional [64 Bit]). Software: Molecular Operating Environment, package version 2016.08 (Montreal, Quebec, Canada) was used in the docking experiments. The crystal structures of the cyclin-dependent kinase 4 (Code: 2W9Z) were obtained from the Protein Data Bank.[12]

The study of the scoring energy(s), root mean, and amino-acid interactions was carried out through docking of the co-crystallized ligand. The compounds for docking were processed via their three-dimensional (3D) structure. Certain processes were taken before docking, which included running conformational analysis using a systemic search, 3D protonation of the structures, selecting the least energetic conformer, and applying the same docking technique used with ligand.[13] The hydrogen bond lengths and interactions of amino acids were detected [Figure 2].

RESULTS AND DISCUSSION

Structure elucidation

Structure elucidation of the isolated secondary metabolites was established by HPLC, NMR, UV, and mass spectra, as well as by comparison with literature data [Figure 1].

Catechin

It is yellow amorphous powder, greenish brown in color with methanolic $FeCl_3$ on TLC. The UV spectrum recorded in methanol showed maximum absorbance bands at 225 and 280 nm which were typical for a flavan-3-ol. The ESI-MS showed molecular ion peak at m/z 291 [M + H] +, with the ^{13}C NMR spectroscopic analysis indicating the molecular formula $C_{15}H_{14}O_6$. The ^{1}H NMR spectrum clearly showed typical signals for flavan-3-ols; all data together with the reported literatures confirmed the catechin structure.[14,15]

Quercitrin (quercetin-3-O-α-L-rhamnopyranoside)

It is yellow amorphous powder; the UV spectrum showed two absorption maxima at 256 and 348 nm which is typical for flavonols. Positive and negative ESI-MS showed peaks at m/z 448 [M] + and 447 [M-H] +, respectively, corresponding to the molecular formula

Table 2: Kinase inhibitory assay (inhibitory concentration for 50%)

Compounds	Kinase				
	Aurora-B* µM IC_{50}	CDK4/Cyclin D1* µM IC_{50}	COT* µM IC_{50}	FAK* µM IC_{50}	IGF1-R* µM IC_{50}
Catechin	15.85	11.25	16.44	28.25	33.20
Quercitrin	4.78	3.22	25.65	16.35	21.21
Trans-taxifolin-3-O-α-L-rhamnopyranoside	35.05	10.66	28.78	20.22	27.32

*Kinases enzymes secreted in various cancer diseases. IC_{50}: Inhibitory concentration for 50%

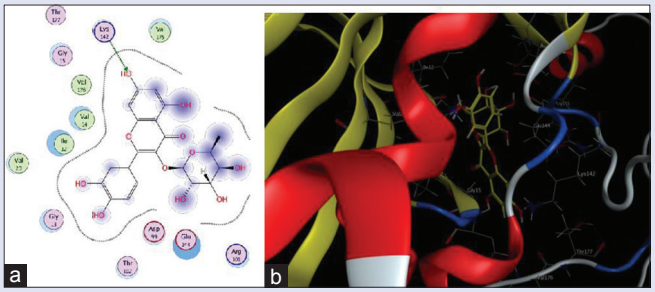

Figure 2: (a) Two-dimensional and (b) three-dimensional interactions of quercitrin with cyclin-dependent kinase 4

$C_{21}H_{20}O_{11}$. In addition, ion peaks at m/z 896 [2M] +, 919 [2M + Na] +, 303 [aglycon + H] +, and 895 [2M-H] + were observed in the positive and negative ESI-MS spectral modes, respectively. Establishment of quercitrin was further confirmed by ^{1}H and ^{13}C NMR spectral data and by comparison with those reported in literatures.[15,16]

Trans-taxifolin-3-O-α-L-rhamnopyranoside

It is whitish amorphous powder; the molecular formula has been determined as $C_{21}H_{22}O_{11}$ from the positive ESI-MS ion peaks at m/z 450 [M] +, 473 [M + Na] +, 923 [2M + Na] +, and 305 [aglycon + H] + and the negative ESI-MS ion peaks at m/z 449 [M-H] +, 899 [2M-H] +, and 303 [aglycon-H] +. The UV spectrum showed a shoulder-type signal at 330 nm for band I characteristic for dihydroflavonol and absorption maxima for band II at 290 nm. The ^{1}H and ^{13}C NMR data exhibited typical signals of dihydroflavonol-type skeleton which also confirmed the structure elucidation as *trans*-taxifolin-3-*O*-*α*-L-rhamnopyranos ide.[15,17]

13R-hydroxy-9Z, 11E-octadecadienoic acid

It is white amorphous powder; the UV spectrum recorded in methanol showed maximum absorbance bands at 236 nm which indicated the presence of diene. The positive ESI-MS m/z 638 [2M + 2Na] +, negative ESI-MS m/z 295 [M-H] +, 591 [2M-H] + indicated the molecular weight to be 296, and, together with the ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic analysis, indicated the molecular formula $C_{18}H_{32}O_3$. The ^{1}H NMR (CD_3OD, 500 MHz) spectrum allowed assignments of the double-bond configurations; the coupling constant of H-9 and H-10 protons was 10.7 Hz and that of H-11 and H-12 protons was 15.1 Hz. This demonstrated that the Δ^{9} and Δ^{11} double bonds had Z and E configurations, respectively. The stereochemistry of C-13 followed from the coupling constant of H-13 proton with H-12 proton indicated that it was *R*. Therefore, the structure was confirmed as 13*R*-hydroxy-9*Z*, 11*E*-octadecadienoic acid.[18,19]

13R-hydroxy-9Z, 11E-, 15E-octadecatrienoic acid

It is white amorphous powder; the UV spectrum showed maximum absorption at 238 nm which indicated the presence of diene. The positive ESI-MS m/z 634 [2M + 2Na] +, negative ESI-MS m/z 293 [M-H] +, 587 [2M-H] + indicated the molecular weight to be 294. In addition, the ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic analysis indicated the molecular formula $C_{18}H_{30}O_3$. The ^{1}H NMR (CD_3OD, 500 MHz) spectrum showed the signals and coupling patterns as in compound 4 except an additional Δ^{15} double bond in an *E* configuration from the coupling constant of H-15 and H-16 protons was 15.6 Hz. Therefore, the structure was elucidated as 13*R*-hydroxy-9*Z*, 11*E*-, 15*E*-octadecatrienoic acid.[18,19]

Cytotoxic activity

Cytotoxic activity of the isolated compounds (1–5) was evaluated against breast cancer (MCF-7) and hepatocellular carcinoma (HepG-2) cell lines, using MTT assay method. IC_{50} for all compounds was determined and represented in μM/mL [Table 1]. The results showed that compound 2 has relatively strong activity on MCF-7. Compounds 1 and 3 displayed moderate activity, whereas compounds 4 and 5 showed no activity.

Molecular docking study

The docking studies of quercitrin in cyclin-dependent kinase 4 showed a score energy (S) = −5.1991 kcal/mol with formation of hydrogen bond between 7-phenolic group of quercitrin and Lys142 amino acid [Figure 2].

CONCLUSION

To the best of our knowledge, it is the first phytochemical study of *Brownea grandiceps*. Five compounds were isolated and elucidated by different spectroscopic techniques as well as by comparison with literature data. Upon cytotoxicity screening of the isolated metabolites, quercitrin exhibited the most potent cytotoxic effect against MCF-7 cell line. Furthermore, the assay of inhibitory effect of compounds 1–3 on PKs was elucidated, and quercitrin demonstrated the most effective inhibitory activity against enzyme cyclin-dependent kinase 4. The docking study of quercitrin on the target enzyme cyclin-dependent kinase 4 confirmed the mechanism of action and proved the mode of action against MCF-7 cell line. All the isolated flavonoids had moderate-to-strong activity against HepG-2 and MCF-7 cell lines, but the fatty acids displayed no activity; this may be attributed to the hydrophobic characteristic of fatty acids in contrast to flavonoids with hydrophilic characteristics. The future plan for this work is to modify the hydrophilicity of flavonoids through semi-synthesis and screening of the semi-synthetic flavonoids against different cancer cell lines. The study of relation between kinase inhibition and cytotoxicity and polarity of isolated metabolites and semi-synthetic flavonoids will be investigated and rationale.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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