Original Article

In silico Study, Protein Kinase Inhibition and Antiproliferative Potential of Flavonoids Isolated from *Bassia eriophora* (Schrad.) Growing in KSA

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ABSTRACT

Introduction: Kinase enzymes play an important role in cellular proliferation, the main target in cancer treatment is to inhibit their functions. Protein kinase inhibitors as flavonoids can be applied for prevention or treatment of cancers through inhibition of cell proliferation. **Objectives:** To isolate cytotoxic metabolites from *B. eriophora*, evaluate their antiproliferative and protein kinase inhibitory effects, as well as the *in silico* study for active compounds. **Materials and Methods:** Preparative HPLC was used for purification of the isolates. NMR, MS and UV spectroscopy were applied for characterization of the pure compounds. Sulphorhodamine-B and radiometric assay methods were employed for determination of the antiproliferative and protein kinase inhibition activities, respectively. The antiproliferative mechanism was predicted by *in silico* study using Molecular Operating Environment (MOE). **Results:** Five flavonoids; luteolin, acacetin-7-*O*-β-D-glucoside, diosmin, kaempferol-3-O-rutinoside and rutin were isolated and investigated for their antiproliferative and kinase inhibitory effects. Luteolin exhibited strong antiproliferative effect against certain cell lines including MCF-7, HepG2 and HCT-116 with IC_{50} (33.24 ± 0.83, 26.54 ±1.02 and 31.62 ±1.32 μM, respectively), while diosmin and kaempferol-3-O-rutinoside showed strong antiproliferative effect against MCF-7 with IC_{50} (26.56 ± 1.12 and 28.72 ±0.98). Luteolin showed highest inhibitory effect against Aurora B and CDK4/CyclinD1 with IC_{50} 3.16 and 4.95 m3.16 and 4.95 inhibit*in silico* study for the isolated metabolites against Aurora B and CDK4/CycD1 confirmed their cytotoxic profile. **Conclusion:** Five flavonoids were firstly isolated from *B. eriophora*. The putative antiproliferative mechanism of luteolin and kaempferol-3-*O*-rutinoside on Aurora B and CDK4/CycD1 kinases was predictable by *in silico* study.

Key words: *Bassia eriophora*, Antiproliferation, *in silico* study, Flavonoids, Protein kinase.

INTRODUCTION

Discovery of natural anticancer drugs is the main target for researchers and scholars since the last few decades. Protein kinase enzymes play an important role in phosphorylation of specific protein substrate, through transfer of the terminal phosphate moiety of adenosine triphosphate (ATP) molecule.[1,2] The genome of human body contains approximately 538 different protein kinases, which are categorized into three different families based on their selectivity for protein substrates.[3,4] To achieve phosphorylation process, a free hydroxyl moiety is required in the protein substrate, which is freely available in serine, threonine and tyrosine residues. Accordingly, serine/threonine kinase family can identify and attach a phosphate group to a serine or threonine residues, while

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the tyrosine and histidine specific protein kinases can phosphorylate a protein at a tyrosine or histidine moieties. Serine/threonine type including Aurora and CDKs kinases contribute in cell division through regulation of the mitotic progression signaling, deregulation of protein kinases is implicated in tumorigenesis.[5] Accordingly, one the major drug targeting in cancer treatment depends completely on kinase inhibition. The development and isolation of natural kinase inhibitors has been predicted to be a major object of pharmaceutical growth with more than 135 kinase inhibitors described to be in either Phase I or Phase II clinical trials, most of these drugs being tested for their potential as anticancer agents. Flavonoids are considered one of the best known naturally occurring anticancer drugs which are ubiquitous in most of plant species. Owing to their high potential as radical scavengers, flavonoids are employed as strong antioxidants, chemopreventive and chemotherapeutic drugs in various types of tumor.[6,7] The preliminary phytochemical and biological investigations of *Bassia eriophora* (Chenopodiaceae) resulted in detection of flavonoids as the major content with few data about its biological activities.[8-10] The goal of this research is to focus on isolation and exploration of the anticancer flavonoids of *B. eriophora* growing in the north of Kingdom Saudi Arabia. The antiproliferative and protein kinases inhibitory effects as well as the *in silico* study were conducted on the pure isolates.

MATERIALS AND METHODS

General experimental procedures

The spectral measures (MS, UV, NMR) were assessed and recorded by using the standard techniques and applying official apparatus. The NMR (^{1}H-and ^{13}C) were determined from Bruker ARX-500 spectrometer, operating at 500 MHz for 1H) and 125 MHz for ^{13}C in DMSO-d6 or CD3OD solvents. TMS was applied as reference standard, coupling constants (J-value/ Hz) and chemical shifts (δ/ ppm) were recorded by the standard Bruker software (Bruker AG, Switzerland). Recording of Mass spectra was achieved by electrospray ionization (ESI-MS), Thermo Finnigan LCQ DECA mass spectrometer (Waldbronn, Germany) supported by Agilent 1100 HPLC system (LMU, Munich, Germany) and equipped with a photodiode array detector. Preparative HPLC was performed on Agilent 1290 infinity II binary pump with prep-C_{18} (21.2 × 50mm) column with flow rate 20 mL/min, connected to Agilent 1290 infinity II prep column chamber, Agilent 1260 infinity II prepauto-sampler and Agilent 1260 infinity II prep fraction collector with tray holding 40 tubes 30 x 100 mm 50 ml (Agilent, Victoria, USA). Detection was achieved with 1260 infinity II diode array detector WR, the chromatograms were noted at different wavelengths (235, 254, 280 and 340 nm) (Agilent, Victoria, USA). Vacuum liquid chromatography column (VLC) was carried out by using normal Silica Gel 60, 0.04-0.063 mm mesh size (Merck, Germany). Sephadex LH-20 (Merck, Germany) was employed as stationary phases for open column chromatography to improve purification steps. The TLC chromatogram was visualized by UV-lamp (254 and 365 nm), VL-6.LC, 24W, France and by spraying with $AlCl_3$ reagent specific for flavonoids.

Plant material

B. eriophora (Schrad.) Asch. was collected in April 2019, around the campus of Jouf university, Sakaka, Aljouf, KSA. Identity of the plant was confirmed by Mr. Al-Hassan Hamdan, Camel Research Center, Sakaka, Aljouf, KSA. A voucher specimen (58-CPJU) has been archived in Pharmacy College, Jouf University.

Extraction and Isolation

The standard extraction method was applied by soaking the plant powder in the selective solvent.[11] The whole plant was air dried (1kg), powdered and macerated in 70% EtOH for three times till exhaustive extraction (8 L × 3). The resulting total extract (42 g) was mixed with dist. H_2O, defatted with *n*-hexane (21 g), followed by extraction with ethyl acetate to yield8 g. The EtOAc fraction was divided on VLC silica gel column chromatography, the eluent was composed of methylene chloride and methanol mixture in gradient manner, monitoring of the eluates was achieved by the pre-coated TLC plates.[12-14] Visualization was achieved by $AlCl_3$ spray reagent, after being visualized under UV-lamp (254, 365 nm). According to the TLC patterns, six fractions were collected (fr. I-VI). Fr. II was applied on normal silica gel column and eluted with MeOH in DCM, depending on the TLC plates monitoring, compound 1(22 mg) was obtained with 2% MeOH in DCM. By applying the same isolation technique, fr. III was chromatographed to on normal Si gel column and eluted by MeOH in DCM till detection of the target compound 2 (25 mh) at 8% MeOH in DCM. Where fr. V showed interesting spots on TLC, it was chromatographed by as fr. II and III with the same solvent mixture to produce 5 different sub fractions (fr. $V_{1\text{-}5}$), fr. $V_{1,2,5}$ were selected for further chromatographic separation, they were separately chromatographed on normal Si-gel column. Upon continuous elution with MeOH/DCM mixture, Fr. V_1 afforded compound 3 (18 mg) at 11% MeOH in DCM, while fr. V_2 afforded compound 4 (25 mg) at 13% MeOH

in DCM. Additionally, fr. V_5 yielded compound 5 (17 mg) at 14% MeOH in DCM. All isolates were purified by elution with MeOH on Sephadex LH-20, final purification was achieved by injection of the individual isolates on Agilent infinity II prep HPLC system with one-hour program; 10% methanol (in nano pure water) for 5 min, 11 to 85% methanol for 45 min, isocratic 100% methanol for 5 min and finally gradient to the initial condition for 5 min at flow rate of 20 mL/min. The retention time for elution of each individual was; (compound 1; R_t=21.5, compound 2; R_t=20.2, compound 3; R_t=19.6, compound 4; R_t=19.1 and compound 5; R_t=18.5). More details are found in the supporting material.

Antiproliferative assay

The pure isolates have been evaluated for their cytotoxicity against MCF-7, HepG2 and HCT-116, cell lines. The sulphorhodamine-B (SRB) assay method was conducted in triplicate as reported by Skehan *et al.*[8] results were expressed relative to the untreated control cells. DMSO was applied as negative control while doxorubicin as positive standard. More details are found in the supporting material.

Kinase inhibitory assay

Aurora and CDKs are belonging to serine/threonine protein kinase type that control and regulate the mitotic cell progression; their deregulation is implicated in tumorigenesis. Therapeutic inhibition of these kinases brings great promise as probable anticancer regime due to their importance in the signaling complex of cellular division. A radiometric protein kinase assay was employed to detect the inhibitory effect of the isolated flavonoids on the selected kinases. The procedure depends on incorporation of the radioactive isotope ^{33}P with ATP, which can be determined by using a microplate scintillation counter (Microbeta, Wallac, Finland) as previously described.[15] Estimation of the IC_{50} values were conducted in triplicate and calculated by detecting ^{33}P bound to the substrate, if the amount of radioactive ^{33}P bound to substrate decreases, the activity of the tested compounds increases.

Docking study

The crystallographic structures of CDK4/CycD1 and Aurora B were obtained from Protein Data Bank [PDB ID: 2W96 with resolution 2.3 Å and 4C2V with resolution 1.49 Å, respectively]. MOE, version 2016.08 (Molecular Operating Environment) was applied for analysis of docking study.[16-19] Estimation of the scoring energy (s), binding approaches and root means of the pure isolates along with the kinases were obtained from docking of the co-crystallised ligands. Preparatory steps were performed before docking including protonation of the structures, energy minimization, running conformational analysis using a systemic search and selecting the least energetic conformer. The interactions of the most active flavonoids with amino-acids and hydrogen bond lengths were detected (Figures 2-5).

RESULTS AND DISCUSSION

Phytochemical isolation

The EtOAc fraction was chromatographed on VLC silica gel and Sephadex LH20, followed by prep HPLC to produce five pure isolates. Characterization of the isolated metabolites was achieved by various spectroscopic methods that resulted in (Figure 1).

Luteolin: yellowish-white amorphous powder, two absorptions maxima at λ_{max} (259 and 362 nm) were observed in the UV spectrum indicative to flavone nucleus. ESI-MS exhibited peak line at m/z=287 denoting $(M + H)^+$ for the molecular weight 286 and molecular formula $C_{15}H_{10}O_6$. Fragments at m/z=153 and 135 are characteristic for ring A and ring B, respectively. The characteristic ABX-spin pattern of B-ring was detected in ^{1}H-NMR at δ 7.35 (1H, d, *J*=2.1 Hz, H-2'), δ 6.90 (1H, d, *J*=8.0 Hz, H-5') andδ 7.36 (1H, dd, *J*=8.1/ 2.0 Hz, H-6'), the other two aromatic protons of A-ring appeared at δ6.20 (1H, d, *J*=1.9 Hz, H-6) and 6.43 (1H, d, *J*=1.9 Hz, H-8), the singlet peak at δ 6.53 with one proton integration (H-3), confirmed the flavone type flavonoid. The ^{13}C-NMR spectrum revealed the presence of 15 carbon atoms of Luteolin.[20]

Acacetin-7-*O*-β-D-glucoside: yellowish-white amorphous powder, it showed two absorptions maxima at λ_{max} (280 and 338 nm) in the UV spectrum, together with the chemical shift at δ 6.80 (1H, s, H-3) in ^{1}H NMR and at δ 103.51 (C-3) in ^{13}C-NMR spectra, indicating the flavone type flavonoid. ESI-MS exhibited peak line at m/z=447 denoting $(M + H)^+$ for the molecular weight 446 and molecular formula $C_{22}H_{22}O_{10}$. The characteristic AA`BB` spin system of B-ring was detected in ^{1}H-NMR at δ 7.58 (2H, d, *J*=8.6 Hz, H-3,5`) and δ 7.68 (2H, d, *J*=8.6 Hz, H-2`, 6`), δ6.53 (1H, d, *J*=2.5 Hz, H-6) and δ 6.75(1H, d, *J*=2.5 Hz, H-8). Additionally, at δ 3.90 and δ 55.17 are indicative to O-CH_3 group. The ^{1}H-NMR resonances at δ5.42 -3.34 ppm and six signals in the ^{13}C-NMR spectrum at δ 99.74 - 63.07 indicated the presence of one sugar moiety. The other ^{13}C-NMR signals confirmed acacetin-7-*O*-β-D-glucoside structure.[20]

Diosmin: The UV spectrum showed two absorptions maxima at λ_{max} (269 and 372 nm), together with the appearance of sharp singlet signal for H-3 proton at δ 6.80 ppm, confirmed the flavone skeleton. The ESI-MS

exhibited a peak line at m/z=609 denoting $(M + H)^+$ and 608 $(M)^+$ for the molecular weight 608and molecular formula $C_{28}H_{32}O_{15}$. The characteristic ABX spin system of B-ring was detected in ^{1}H-NMR at δ 7.43 (1H, d, *J*=2.1 Hz, H-2`), δ7.10 (2H, d, *J*=8.30 Hz, H-5`) and δ 7.56 (1H, dd, *J*=2.12, 8.0 Hz, H-6`), δ 6.46 (1H, d, *J*=1.92 Hz, H-6) and δ6.75 (1H, d, *J*=1.91 Hz, H-8).The OCH_3 proton signal appeared at δ 3.88 as sharp singlet in ^{1}H-NMR and integrated for 3 protons, confirmed by the correspondent peak at δ 55.84 in ^{13}C-NMR.[21]

Kaempferol-3-*O*-rutinoside: The UV spectrum showed two absorptions maxima at λ_{max} (260 and 356 nm), together with the absence of H-3 proton signal suggested its flavonol type. The ESI-MS exhibited a peak line at m/z=595 denoting $(M + H)^+$ for the molecular weight 594 and molecular formula $C_{27}H_{30}O_{15}$. A peak at m/z=617 $(M + Na)^+$ and m/z=287 $(aglycone + H)^+$ were also detected. The characteristic AA`BB` spin system of B-ring was detected in ^{1}H-NMR at δ 6.91 (2H, d, *J*=8.87 Hz, H-3`, 5`) and d H-3 (2H, d, *J*=7.93 Hz, H-2`, 6`), δ6.21 (1H, d, *J*=2.12 Hz, H-6) and δ6.42 (1H, d, *J*=2.15 Hz, H-8). The anomeric proton of glucose and rhamnose moieties resonated at δ 5.33 (1H, d, *J*=7.41 Hz, H-1``) and δ5.36 (1H, d, *J*=2.94 Hz, H-1```), respectively. The CH_3 signal was clearly observed at δ1.01(1H, d, *J*=6.21). the ^{13}C-NMR signals were compatible with the elucidated structure.[17]

Rutin: The UV spectrum showed two absorptions maxima at λ_{max} (256 and 359 nm), together with the absence of H-3 proton signal suggested its flavonol type. The ESI-MS exhibited a peak line at m/z=611denoting $(M + H)^+$ for the molecular weight 610and molecular formula $C_{27}H_{30}O_{16}$. The characteristic ABX spin system of B-ring was detected in ^{1}H-NMR at δ 7.53 (1H, d, *J* = 2.21 Hz, H-2`), δ 6.83 (2H, d, *J* = 8.2 Hz, H-5`) and δ 7.55 (1H, dd, *J*=2.2, 8.19 Hz, H-6`), δ6.19 (1H, d, *J*=2.1 Hz, H-6) and δ 6.39 (1H, d, *J*=2.1 Hz, H-8). The anomeric proton of glucose and rhamnose moieties resonated at δ5.32 (1H, d, *J* = 8.2 Hz, H-1``) and δ5.07 (1H, d, *J* = 1.8 Hz, H-1```), respectively. The CH_3 signal was clearly observed at δ0.91 (1H, d, *J* = 7.2). the ^{13}C-NMR signals were compatible with the predicted structure.[22]

It is well established that polyphenolics particularly flavonoids are characterized by their anticancer potential against various cell lines. Owing to their great radical scavenging activity, flavonoids are regarded as protectors to the livings against radicals and carcinogenic reactive oxygen species. The Antiproliferative activity of the flavonoidal isolates has been estimated against three human cell lines; MCF-7, HepG2 and HCT-116. The SRB assay was applied as standard method. The IC_{50} for the tested isolates was expressed in μM. The tested compounds exhibited moderate to strong activity as antiproliferative agents as Table 1.

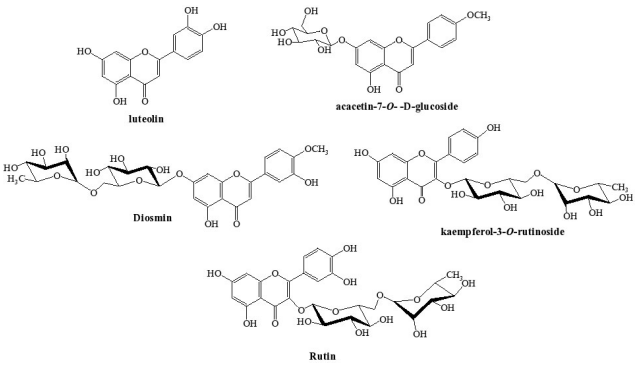

Figure 1: Structures of the isolated flavonoids.

Results of Antiproliferative assay

Table 1: Antiproliferative activity of the isolated compounds on the selected cell lines.

Isolated compounds (50 μL)	IC_{50} ± SD (μM)[a]		
	MCF-7	HepG2	HCT-116
Luteolin	33.24 ±0.83	26.54 ±1.02	31.62 ±1.32
Acacetin-7-*O*-β-D-glucoside	45.18 ±0.87	>100	>100
Diosmin	25.56 ±1.12	84.61±1.36	>100
Kaempferol-3-*O*-rutinoside	28.72 ±0.98	53.38 ±0.96	>100
Rutin	>100	>100	>100
Doxorubicin	1.97 ± 0.46	1.75 ± 0.29	1.64 ± 0.43

[a] Cell proliferation was determined by SRB assay; IC_{50} ±SD values as μM after 48 h incubation;

Table 2: Radiometric assay of the isolated compounds.

Compound name	Kinase type	
	Aurora-B	CDK4/CycD1
Luteolin	3.16	4.95
Acacetin-7-*O*-β-D-glucoside	34.12	29.56
Diosmin	26.25	31.74
Kaempferol-3-*O*-rutinoside	13.54	14.76
Rutin	41.62	52.31

IC_{50}: Inhibitory concentration for 50%

Protein kinase inhibitory activity

In order to study the mechanism of action of the isolated flavonoids as anti-proliferative molecules, they were tested for Aurora B and CDK4/CycD1 kinases inhibitory activity. A radiometric method was used to measure the inhibitory activity of these molecules against CDK4/CycD1and Aurora B. *In vitro* kinase assay revealed that compound 1has significant inhibitory activity on Aurora B (IC_{50}= 3.16 μM) more than compounds

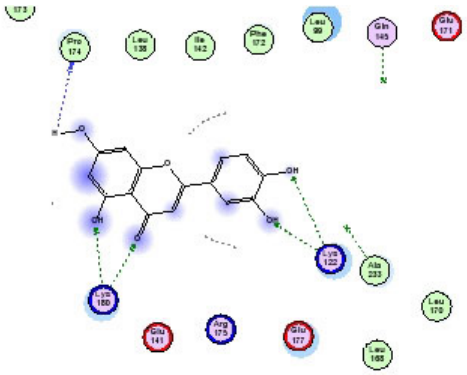

Figure 2a

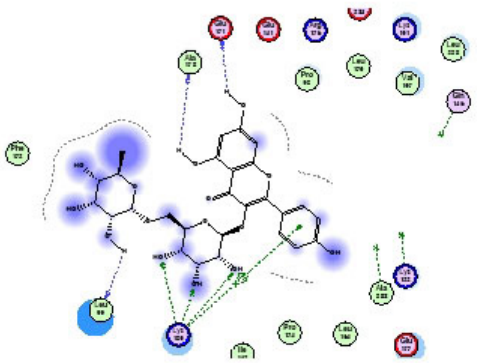

Figure 3a

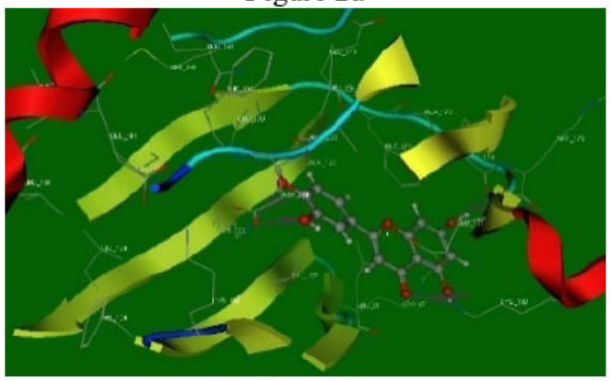

Figure 2b

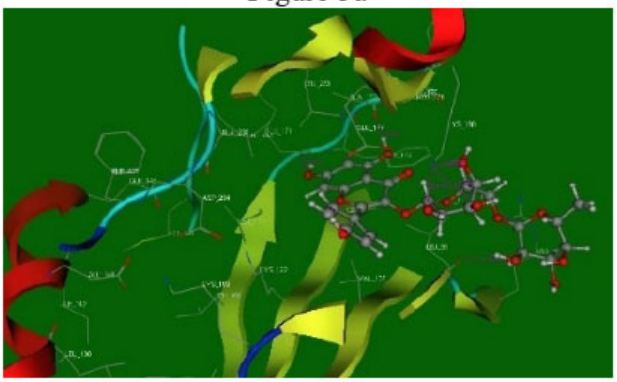

Figure 3b

Figure 2-3: (A) 2D; Binding modes and (B) 3D; mapping surface of compounds 1 and 4 with Aurora B.

4 and 5 with IC_{50}= 13.54 and 41.62 µM, respectively (Table 2). Furthermore, the inhibitory effect of 1 on CDK4/CycD1 (IC_{50}= 4.95 µM) also more active than other compounds, this suggesting that the presence of sugar moiety has an adverse effect on the activity of compounds against protein kinases.

Molecular Docking Study

The binding poses of the docked compounds 1 and 4 as the most active inhibitory effect on the selected kinase with both Aurora B and CDK4/CycD1, represented promising and valid potential binding modes with the same as for the co-crystalline ligands.[23,24] The molecular docking study revealed that compound was sitting deeply in the ATP-binding pocket of Aurora B, with binding free energy-21.3469 kcal/mol and it participates in hydrogen bonding interactions of 3`-OH and 4`-OH with Lys122. Additionally, there was hydrogen bonding of 5-OH and 4-carbonyl with Lys180 and 7-OH with Pro174 of ring A of the flavonoid moiety [Figures 2a and 2b]. The study also displayed the high affinity of compound 4 to Aurora B with score energy -17.2689kcal/mol and its binding mode showed interactions with Lys180, Leu99 and Ala173 residues [Figures 3a and 3b]. Furthermore, compounds 1 and 4 displayed the high affinity to CDK4/CycD1 with score energy-19.3755 and -23.9667 kcal/mol, respectively (Table 3) and its binding mode showed hydrogen bonding interactions with Val96 and Ala16 residues for compound 1 (Figures 4a and 4b) and with Val96, Lys35, Asp97, Thr102 for compound 4 (Figures 5a and 5b).

CONCLUSION

Previous study concerning phytochemical and biological investigations of *B. eriophora* are scarce. As a result of our continuous research on Saudi plants, five phytochemicals have been isolated and characterized by the normal spectroscopic methods. The antiproliferative study of the pure isolates revealed their impact as promising cytotoxic drugs. Diosmin, kaempferol-3-*O*-rutinoside and luteolin were the most effective metabolites against MCF-7 cell lines, while luteolin exhibited a moderate cytotoxic effect on both HepG2 and HCT-116 cell lines. Moreover, the inhibitory effect of the isolates against Aurora-B and CDK4/CycD1 kinases confirmed their antiproliferative effect against the targeted cell lines. These results reflect the high medicinal value of *B. eriophora* as cytotoxic drug, which is growing in Aljouf area as

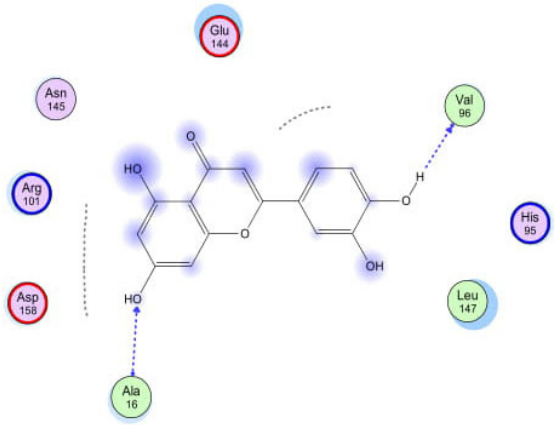

Figure 4a

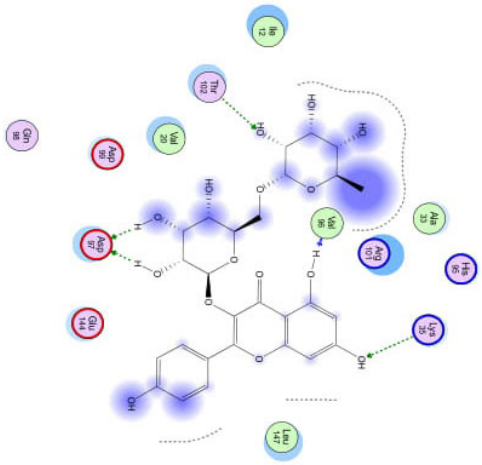

Figure 5a

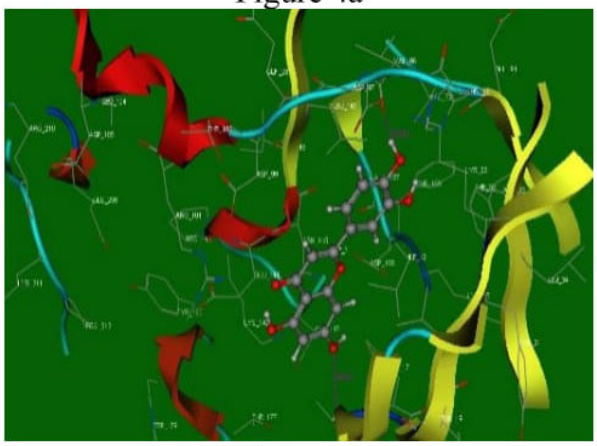

Figure 4b

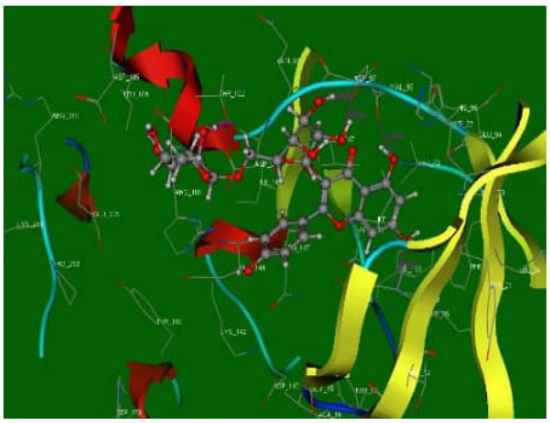

Figure 5b

Figure 4-5: (A) 2D; binding modes and (B) 3D; mapping surface of compounds 1 and4 with CDK4/Cyclin D1.

Table 3: Molecular modelling data of compounds 1 and 4 with Aurora B and CDK4/Cyclin D1 kinases.

Receptor	Compound	Binding free energy (kcal/mol)	Type of interaction	Receptor amino acid residues	Functional groups
Aurora B	Luteolin	-21.3469	H- bond	Lys122	OH
			H- bond	Lys180	OH
			H- bond	Lys180	C=O
			H- bond	Pro174	OH
	Kaempferol-3-*O*-rutinoside	-17.2689	H- bond	Lys180	OH
			H- bond	Leu99	OH
			H- bond	Ala173	OH
			H- bond	Glu171	OH
CDK4/CycD1	Luteolin	-19.3755	H- bond	Val96	OH
			H- bond	Ala16	OH
	Kaempferol-3-*O*-rutinoside	-23.9667	H- bond	Val96	OH
			H- bond	Lys35	OH
			H- bond	Asp97	OH
			H- bond	Thr102	OH

H-bond; Hydrogen bonding, OH; hydroxyl group, C=O; carbonyl group

pastoral and grazing plant and can be safely introduced to the traditional medicine.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MCF-7: Human breast adenocarcinoma; **HepG2**: Human hepatocellular carcinoma; **HCT-116:** Human colorectal carcinoma; ***B. eriophora***: *Bassia eriophora*; **PKs:** Protein kinases; **SRB:** sulphorhodamine-B; **MOE:** Molecular Operating Environment.

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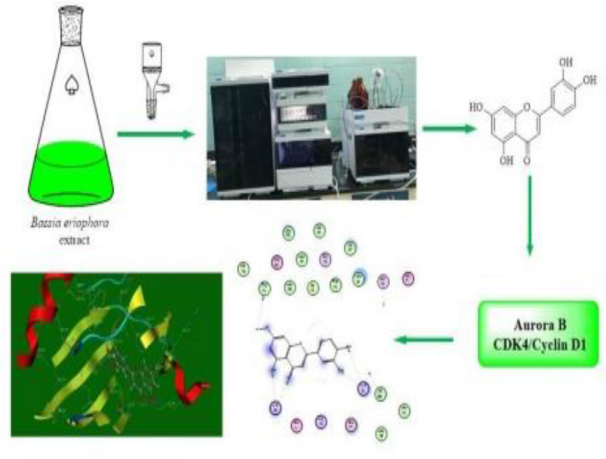
PICTORIAL ABSTRACT



SUMMARY

Five flavonoids were isolated and identified for the first time from *B. eriophora*, the cytotoxic activity of the isolated molecules was screened on three different cell lines including MCF-7, HepG2 and HCT-116, Diosmin, kaempferol-3-*O*-rutinoside and luteolin were the most effective metabolites. *In silico* study of the highly active molecules on Aurora B and CDK4/CycD1 confirmed its antiproliferative effect.

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