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ORIGINAL ARTICLE

Exploration of Aurora B and Cyclin-Dependent Kinase 4 Inhibitors Isolated from *Scorzonera tortuosissima* Boiss. and their Docking Studies

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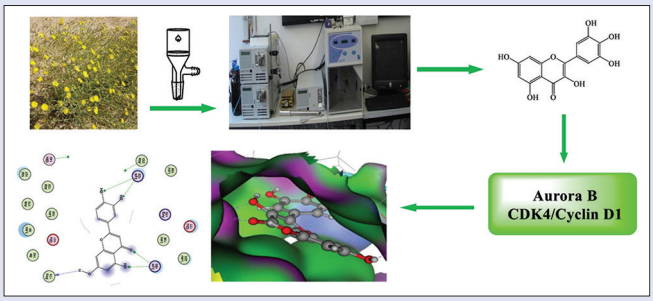
ABSTRACT

Background: Flavonoids are components of the daily human diet (fruits and vegetables), and it has been shown to inhibit several kinase enzymes. Due to its kinase inhibitory activity, they are expected to be of great importance in the discovery of new anticancer drugs. **Objectives:** The objective was to study the cytotoxicity, kinase inhibitory activity, and docking of the isolated aglycones. **Materials and Methods:** Ultraviolet, high-performance liquid chromatography (LC), nuclear magnetic resonance, and LC-mass spectrometry were used for the identification of the isolated metabolites. Antiproliferative (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and radiometric protein kinase (PK) assays were used to measure the cytotoxicity and PK inhibitory effect of the isolated flavonoids. The docking study on both Aurora B and cyclin-dependent kinase 4 (CDK4)/CycD1 was performed by molecular operating environment (MOE). **Results:** Luteolin (1), quercetin (2), myricetin (3), apigenin-7-*O*-β-D-glucopyranoside (4), and kaempferol-7-*O*-β-D-glucop yranoside (5) were isolated from *Scorzonera tortuosissima*. Quercetin and myricetin exhibited the highest cytotoxicity against the Michigan Cancer Foundation-7 (MCF-7) (IC_{50}: 5.56 and 7.14 μM, respectively) and against human hepatocellular carcinoma (HepG2) (IC_{50}: 8.61 and 10.31 μM, respectively), while compounds luteolin and apigenin-7-O-β-D-glu copyranoside showed the least cytotoxicity compared to doxorubicin against to MCF-7 and HepG2 (IC_{50}: 2.24 ± 0.85 and 1.82 ± 0.34 μM, respectively). The radiometric PK assay was applied for measurement of kinase inhibitory activity against Aurora B, CDK4/D1, cancer Osaka thyroid, IGF1-R, and FAK kinases, where the aglycone myricetin showed the highest inhibitory activity against Aurora B (IC_{50}: 2.82 μM) and against CDK4/cyclin D1 (IC_{50}: 3.16 μM), while the isolated glycosides 4 and 5 revealed the lowest activity. Docking of the most active compounds 1, 2, and 3 against Aurora B and CDK4/cyclin D1 confirmed its cytotoxic profile. **Conclusion:** The isolated flavonoids were firstly isolated from *S. tortuosissima*. The hypothetical mechanism of cytotoxic activity of 1, 2, and 3 on Aurora B and CDK4/cyclin D1 kinases was estimated by *in silico* study with these enzymes using MOE program.

Key words: Aurora B, cyclin-dependent kinase 4/cyclin D1, cytotoxicity, docking, flavonoids, *Scorzonera tortuosissima*

SUMMARY

- Five flavonoids (luteolin, quercetin, myricetin, apigenin-7-O-β-D-glucopyrano side, and kaempferol-7-*O*-β-D-glucopyranoside) were isolated and identified for the first time from *Scorzonera tortuosissima*; the cytotoxic activity of the isolated metabolites was screened on three different cell lines Michigan Cancer Foundation-7, human hepatocellular carcinoma, and human colorectal carcinoma. Myricetin and quercetin showed the highest activity. *In silico* study of the highly active molecules on Aurora B and cyclin-dependent kinase 4 (CDK4)/cyclin D1 confirmed its antiproliferative effect.



Abbreviations used: MCF-7: Michigan Cancer Foundation-7; HepG2: Human hepatocellular carcinoma; HCT-116: Human colorectal carcinoma; *S. tortuosissima*: *Scorzonera tortuosissima*; H bond: Hydrogen bond; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKs: Protein kinases; COT: Cancer Osaka thyroid

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INTRODUCTION

The risk of various chronic diseases such as cancer, cardiovascular, chronic inflammation, diabetes, atherosclerosis, and Alzheimer's disease has been reported.[1-4] The reduction of that risk may be achieved by consumption of beverages and foods rich in natural antioxidants.[1-4] Recently, there has been an upsurge of interest in the therapeutic potential of plants which might be due to their phenolic constituents, particularly flavonoids. *Scorzonera* genus, which belongs to the sunflower family (Asteraceae), is one of the largest flowering families with a broad diversity of numerous classes of secondary metabolites, and it is distributed in Europe and northern Africa with about 160 species.[5] In folk medicine, some species of *Scorzonera* are used for the treatment of snakebites and chest problems as they have antinociceptive, anti-inflammatory, and wound healing

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effects.[5,6] Previous phytochemical investigations of *Senna alexandrina* and *Sarcoscypha austriaca* resulted in the presence of flavonoids, lignans, phenolic acids, dihydroisocoumarins, sesquiterpenes, and triterpenes.[5,6] However, no data have been reported about the chemical constituents of *Scorzonera tortuosissima*. Flavonoids are polyphenols of benzo-γ-pyrone skeleton.[7] They have concerned much attention due to their remarkable pharmacological activities *in vitro* and *in vivo* including antioxidant, anticancer, antiallergic, and anti-inflammatory activities.[8] In addition, they have an inhibitory effect on various enzymes such as yeast glucosidase and alkaline phosphatase.[9,10] They also possess antiproliferative properties targeting protein kinases (PKs) through interaction with the ATP-binding pocket at the hinge region of the kinases.[11,12] Cellular processes as proliferation, apoptosis, development, cell cycle, and differentiation are being regulated and catalyzed by PKs, so inhibition of certain kinases is very helpful in the treatment of cancer.[11] Flavonoids modulate the kinase activity which intern affects the phosphorylation of kinase substrates. The semisynthetic flavopiridol which is flavone type exhibited potent inhibitory effect on CDKs that compete with ATP of the kinase, and it has shown promising activity in preclinical and clinical trials.[13,14] Consequently, the isolated flavones, luteolin and apigenin-7-*O*-β-D-glucopyranoside, are expected to behave the same action. Furthermore, certain species of the genus *Scorzonera* have anticancer activity.[6] The present study aims to investigate flavonoid fraction from the aerial parts of *S. tortuosissima* and to evaluate its kinase inhibitory activity and cytotoxic potentials using *in vitro* assay systems.

MATERIALS AND METHODS

Plant material

The aerial parts of *S. tortuosissima* were collected from Al-Jouf region in April 2017, in the north of Saudi Arabia. The plant sample was identified by Mr. Hamdan Ogereef Al-Hassan, M. Sc. (Camel and Range Research Center), Al-Jouf, KSA. A voucher specimen (46-CPJU) was deposited at the Pharmacy College herbarium, Jouf University.

General experimental procedures

The nuclear magnetic resonance (NMR) determination was obtained with Varian Mercury (USA) 400 MHz spectrometer at 400 (^{1}H) and 100MHz (^{13}C) in a DMSO-*d*6 or CD_3OD solution (Mansoura University, Mansoura, Egypt). The spectra were recorded by the standard Bruker software. Liquid chromatography–mass spectrometry (LC-MS) was recorded on a Thermo Finnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 high-performance liquid chromatography (HPLC) system equipped with a photodiode array detector. HPLC analysis was performed on Eurospher-100 C_{18} (5 µm) column (125 mm × 2 mm, Knauer, Berlin, Germany) connected to a photodiode array detector UVD 340S (Dionex, Munich, Germany) and Dionex P580A LPG pump with flow rate 0.5–1 mL/min with Chromeleon (V. 6.3) HPLC Program. Routine detection was at 235 nm in aqueous methanol. Preparative HPLC was performed on Agilent prep-C_{18} (250 mm × 21.2 mm) column, prepacked with Microsorb 60-8 C_{18} connected to Agilent 1260 infinity II preparative LC system, 1260 infinity II diode array detector with flow rate 10–20 mL/min (Agilent, Victoria, USA). Detection was achieved with diode array detector, and chromatograms were noted at different wavelengths (235, 254, 280, and 340 nm). VLC was carried out using normal Silica Gel 60, 0.04–0.063 mm mesh size (Merck, Germany). Column chromatography was carried out using Sephadex LH-20, 0.25–0.1 mm mesh size (Merck, Germany).

Extraction and isolation

The dried aerial parts of *S. tortuosissima* (1200 g) were defatted several times with pets. Ether (18 g) was then subjected to extraction by 8 L ethyl alcohol (70%) for three times. The total ethanolic extracts were evaporated at 40°C in vacuo yielding 35 g of dark green residue. It was suspended in 500 ml distilled water and shaken with ethyl acetate 3 times to yield 6 g residue. The EtOAc residue was subjected to a vacuum liquid column packed with silica gel stationary phase and eluted with CH_2Cl_2-MeOH (100:0–65:35). The eluted fractions (100 mL/each) were tested by TLC using 2%–20% methanol in chloroform, and those that showed similar chromatographic patterns were combined to give 6 major fractions (Frs.: 1–6). Frs. 2, 4, and 6 were selected for purification of their contents by passing each over Sephadex LH-20, eluted with methanol. Subfractions 2c (600 mg), 4b (125 mg), and 6c (470 mg) were dissolved individually 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% methanol for 5 min, 6%–90% methanol for 45 min, isocratic 100% methanol for 5 min, gradient to the initial condition for 5 min at flow rate of 20 mL/min. The resultant peaks were recorded by ultraviolet (UV) detector and collected in Erlenmeyer flasks to afford compounds: 1 (35 mg, t_R = 23.42, from 2c), 2 (63 mg, t_R = 22.51, from 2c), 3 (13 mg, t_R = 22.01, from 4b), 4 (25 mg, t_R = 20.54, from 6c), and 5 (20 mg, t_R = 20.18, from 6c).

In vitro antiproliferative assay

Antiproliferative activity of the isolated flavonoids was evaluated against human breast adenocarcinoma (Michigan Cancer Foundation-7 [MCF-7]), human hepatocellular carcinoma (HepG2), and human colorectal carcinoma (HCT-116) cell lines, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method as previously described.[9] The color intensity is associated with the number of healthy living cells [Table 1].

Kinase inhibitory assay

A radiometric PK assay depends on the incorporation of the longest-lived radioactive isotope ^{33}P with ATP, which can be determined

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

Isolated compounds (50 µL)	IC_{50}±SD (µM)[a]		
	MCF-7	HepG2	HCT-116
Luteolin	34.16±2.29	23.61±1.35	24.34±1.51
Quercetin	7.14±0.88	10.31±1.02	61.42±2.11
Myricetin	5.56±2.34	8.61±1.43	39.42±2.11
Apigenin-7-*O*-β-D-glucopyranoside	45.62±1.59	76.78±2.12	>100
Kaempferol-7-*O*-β-D-glucopyranoside	13.28±1.52	19.73±2.03	57.34±2.62
Doxorubicin	2.24±0.85	1.82±0.34	2.47±0.56

[a]Cell proliferation was determined by MTT assay. IC_{50}±SD or IC_{50} values as µM after 48-h incubation. Values are the average of three independent experiments. SD: Standard deviation; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}: Inhibitory concentration for 50%; MCF-7: Michigan Cancer Foundation-7; HepG2: Human hepatocellular carcinoma; HCT-116: Human colorectal carcinoma

with a microplate scintillation counter (Microbeta, Wallac, Finland), as previously described.[9] The IC_{50} values were calculated by microplate scintillation counter that can detect ^{33}P bound to the substrate. If the radioactive ^{33}P amount bound to substrate decreases, it means that the activity of the tested compounds increases.

Docking study

Retrieving of the Aurora B and CDK4 crystallographic structures was obtained from Protein Data Bank (PDB ID: 4C2V, resolution 1.49 Å and PDB ID: 2W96, resolution 2.3 Å, respectively) (https://www.rcsb.org), which were used as simulating targets for docking. The Molecular Operating Environment (MOE, version 2016.08) was applied for the analysis of docking study.[15-18]

Estimation of the free score energy(s), binding approaches, and root means of the isolated metabolites with Aurora B and CDK4 were obtained through docking of the co-crystallized ligands. The isolated compounds for docking were processed through their 3D structure using MOE software package. Certain processes were taken before docking, which included protonation of the structures, energy minimization, running conformational analysis using a systemic search, and selecting the least energetic conformer;[19,20] the hydrogen bond lengths and interactions of amino acid were detected.

RESULTS AND DISCUSSION

Structure elucidation

Structure elucidation of the isolated flavonoids was established by LC-MS, NMR, UV, and mass spectra and comparison with literature data [Figure 1]. They are characterized as:

Luteolin

It is a yellowish-white amorphous powder, and it gives greenish-brown color with methanolic $FeCl_3$ on TLC. The UV spectrum recorded in methanol showed two absorption maxima at 265 (band II) and 344 (band I) nm, characteristic for flavone nucleus. The pseudomolecular ion fragment at m/z 287 $[M+H]^+$ and the ^{13}C NMR indicated $C_{15}H_{10}O_6$ as the molecular formula for the expected structure. The ^1H NMR spectrum clearly showed typical signals for flavone, all data together with the reported literatures confirmed Luteolin structure.[21]

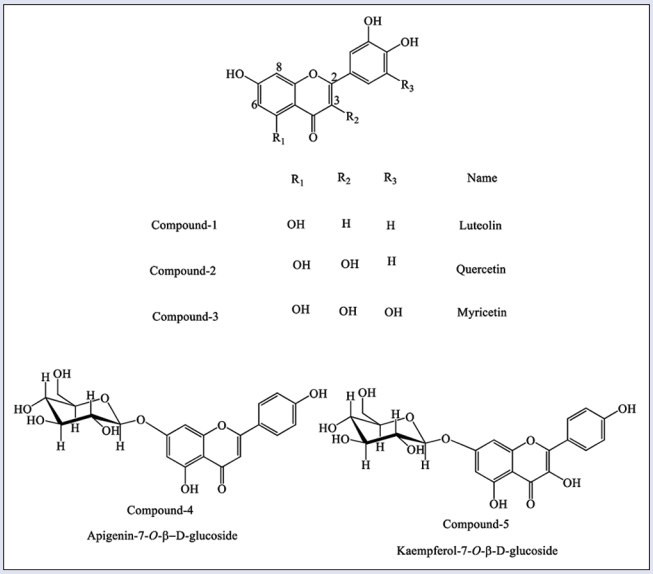

Figure 1: Structures of the isolated flavonoid

Quercetin

It is a yellow amorphous powder; the UV spectrum recorded in methanol showed that two absorption maxima at 256 (band II) and 348 (band I) nm, obtained from UV spectrum, were typical for flavonols. Positive ESI-MS mode showed quasimolecular ion peaks at m/z 303 $[M+H]^+$ and 325 $[M+Na]^+$, corresponding to the molecular formula $C_{15}H_{10}O_7$. In addition, ion peaks at m/z 604 $[2M]^+$, 627 $[2M+Na]^+$, and 603 $[2M-H]^+$ were observed in the positive and negative ESI-MS spectral modes, respectively. The establishment of quercetin was furtherly confirmed by ^1H and ^{13}C NMR spectral data and by comparison with those reported in literatures.[22,23]

Myricetin

It is yellow amorphous powder; the UV spectrum in methanol showed that two absorption maxima at 257 (band II) and 351 (band I) nm were typical for flavonols. ESI-MS-positive mode showed peaks at m/z 319 $[M+H]^+$ and 341 $[M+Na]^+$, corresponding to the molecular formula $C_{15}H_{10}O_8$. Moreover, ion peaks at m/z 636 $[2M]^+$, 659 $[2M+Na]^+$, and 635 $[2M-H]^+$ were observed in the positive and negative ESI-MS spectral modes, respectively. The obtained data (^1H and ^{13}C NMR) were in close agreement with the reported myricetin.[24]

Apigenin-7-O-β-D-glucopyranoside

It is yellow amorphous powder; the UV spectrum showed that two absorption maxima at 268 (band II) and 337 (band I) nm were typical for flavones. The ESI-MS operated at both positive and negative modes produced quasimolecular peaks at m/z 433 $[M+H]^+$ and 431 $[M-H]^+$, corresponding to the molecular formula $C_{21}H_{20}O_{10}$. In addition, ion peaks at m/z 864 $[2M]^+$, 887 $[2M+Na]^+$, 271 $[aglycon+H]^+$, and 863 $[2M-H]^+$ were obtained. By comparing the ^1H and ^{13}C NMR with the reported literature, it was assumed as apigenin-7-*O*-β-D-glucopyranoside.[25]

Kaempferol-7-O-β-D-glucopyranoside

It is yellow amorphous powder; the UV spectrum showed that two absorption maxima at 256 (band II) and 348 (band I) nm were typical for flavonols. ESI-MS spectral modes resulted in the appearance of two quasimolecular ions at m/z 449 $[M+H]^+$ and 447 $[M-H]^+$, corresponding to the molecular formula $C_{21}H_{20}O_{11}$. In addition, ion peaks at m/z 896 $[2M]^+$, 919 $[2M+Na]^+$, 287 $[aglycon+H]^+$, and 895 $[2M-H]^+$ were also obtained. Elucidation of kaempferol-7-O-β-D-glucopyranoside was established by ^1H and ^{13}C NMR spectral data and by comparison with those reported in literatures.[26]

In vitro antiproliferative activity

Evaluation of the isolated metabolites (1–5) for their antiproliferative activity was performed by the standard MTT assay method against human breast adenocarcinoma (MCF-7), HepG2, and HCT-116

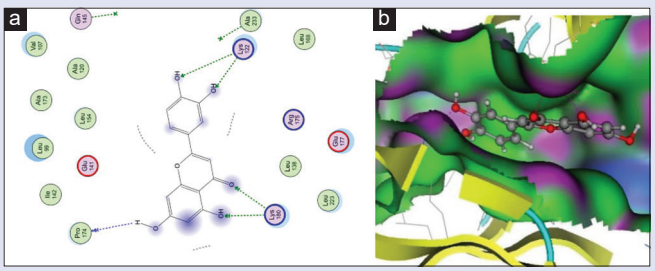

Figure 2: (a) 2D; binding modes and (b) 3D mapping surface of Luteolin with Aurora B

cell lines. IC_{50} in (μM) for the tested compounds is represented in Table 1. Compound 3 showed a strong activity on both MCF-7 and HepG2 (IC_{50} = 5.56 and 8.61 μM, respectively). Compound 2 displayed strong activity against MCF-7 with IC_{50} = 7.14 μM and moderate activity on HepG2 (IC_{50} = 10.31 μM), while compounds 1, 4, and 5 showed the least activity against the tested cell lines.

Protein kinase activity

Aurora B and CDK4 are serine/threonine kinases that play a critical role in regulating many of the processes that are pivotal to mitosis. CDKs are the key components of cell-cycle initiation and control. Numerous genetic and epigenetic changes may induce the overactivation of CDK regulatory pathways. In this study, three flavonoids, luteolin, quercetin, and myricetin, were characterized as inhibitors of Aurora B and CDK4. They are common flavonoids often found in dietary sources including vegetables, fruits, wines, and dietary oils. Previous studies reported that the semisynthetic flavopiridol has shown the highest inhibitory activity against CDK4 (IC50: 0.10 μM) with high antiproliferative potential in many cancers.[27] Due to the pharmacophoric feature similarity between flavopiridol and the isolated metabolites, the current study was focused on the exploration of the PK inhibitory activity for the isolated flavonoids. In order to study the mechanism of action of the isolated flavonoids as cytotoxic molecules, the target compounds were screened for Aurora B and CDK4 kinase inhibitory activity. A radiometric PK assay method was used to measure the action of these molecules against some kinases as Aurora B and CDK4. *In vitro* kinase assay revealed that compound 3 has a significant inhibitory activity on Aurora B (IC_{50} = 2.82 μM) more than compounds 1 and 2 with IC_{50} = 3.25 and 3.81 μM, respectively [Table 2], in addition to the action of 3 on CDK4/CycD1 (IC_{50} = 3.16 μM).

Molecular docking study

Docking study of the active compounds with both Aurora B and CDK4/CycD1 showed the same binding modes of the co-crystalline ligands. For Aurora B, the key amino acid residues of the protein hinge region are Lys122, Lys180, Ala173, and Leu99 and for CDK4/CycD1 are His95, Phe93, Val96, and Asp97.[27,28]

In silico study of the most active aglycones [Figures 2-7] showed that compound 3 was sitting deeply in the ATP-binding site of Aurora B [Figure 4a and b] with binding free energy = −24.2757 kcal/mol [Table 3], and it participates in hydrogen-bonding interactions of 3‘-OH and 4‘-OH with Lys122, which also showed hydrogen bonding of 3-OH with Ala173 and hydrophobic interaction of ring

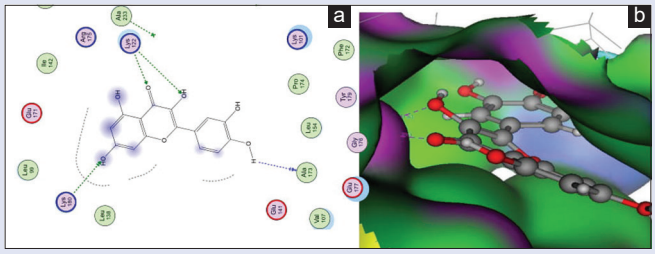

Figure 3: (a) 2D; binding modes and (b) 3D mapping surface of Quereetin with Aurora B

Table 2: Kinase inhibitory assay of the isolated compounds

Compounds	IC_{50} values (μM)[a]				
	Aurora-B	CDK4/CycD1	COT	FAK	IGF1-R
Luteolin	3.25	5.22	>100	>100	83.4
Quercetin	3.81	4.27	33.25	51.80	64.40
Myricetin	2.82	3.16	16.42	35.91	23.71
Apigenin-7-*O*-β-D-glucopyranoside	11.17	9.80	>100	56.14	>100
Kaempferol-7-*O*-β-D-glucopyranoside	9.01	8.62	52.16	45.08	71.42

Values are the average of three independent experiments. IC_{50}: Inhibitory concentration for 50%; COT: Cancer Osaka thyroid; IGF1-R: Insulin-like growth factor-1 receptor; FAK: Focal adhesion kinase

Table 3: Docking of the active compounds against 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	−23.1332	H-bond	Lys122	OH
			H-bond	Lys180	OH
			H-bond	Lys180	C=O
			H-bond	Pro174	OH
	Quercetin	−22.0159	H-bond	Lys122	OH
			H-bond	Lys122	C=O
			H-bond	Lys180	OH
			H-bond	Ala173	OH
	Myricetin	−24.2757	H-bond	Lys122	OH
			Hydrophobic	Lys180	Aromatic
			H-bond	Ala173	OH
			H-bond	Leu99	OH
CDK4/ cyclin D1	Luteolin	−17.5018	H-bond	Val96	OH
			H-bond	Arg101	OH
	Quercetin	−18.4496	H-bond	Val96	OH
			H-bond	Ala16	OH
	Myricetin	−16.4051	H-bond	Val96	OH
			H-bond	Ala16	OH
			H-bond	Lys35	OH

H-bond: Hydrogen bonding, OH: Hydroxyl group, C=O: Carbonyl group

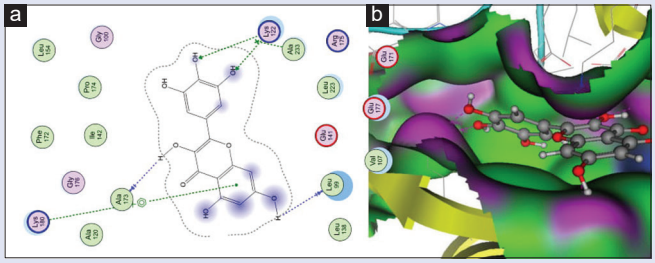

Figure 4: (a) 2D; binding modes and (b) 3D mapping surface of Myricetin with Aurora B

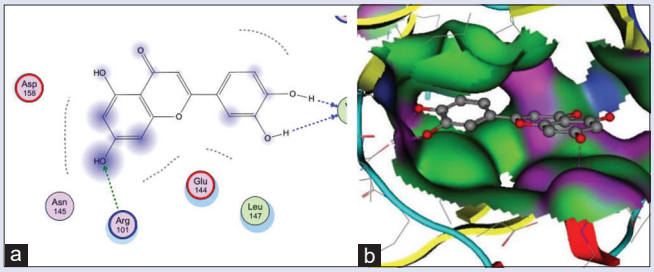

Figure 5: (a) 2D; binding modes and (b) 3D mapping surface of Luteolin with CDK4/cyclin D1

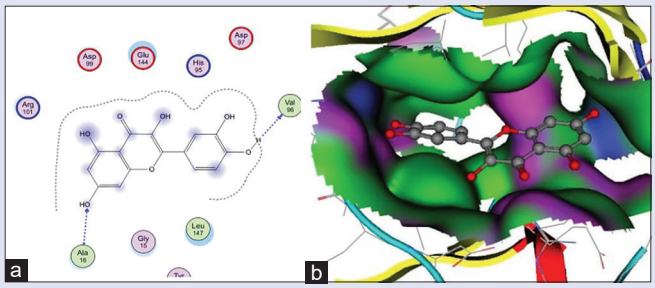

Figure 6: (a) 2D; binding modes and (b) 3D mapping surface of Quereetin with CDK4/cyclin D1

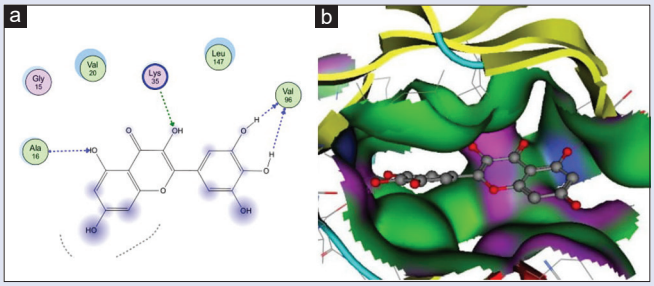

Figure 7: 2D; binding modes and (b) 3D mapping surface of Myricetin with CDK4/cyclin D1

A of the flavonoid moiety with Lys180 residue of the protein hinge region [Figure 4a] and the mapping surface showing compound 3 inhabiting the active pocket [Figure 4b]. The study also displayed a high affinity of 2 with CDK4/CycD1 with score energy = −18.4496 kcal/mol [Table 3], and its binding mode showed interactions with Val96, Lys35, and Ala16. The interactions of compounds 1 and 2 with Aurora B are showed hydrogen bonding with residues Lys122 and Lys180 [Figures 2a and 3a] and with Val96 of the hinge region of CDK4/CycD1 [Figures 5a and 6a].

CONCLUSION

Two flavones and three flavonols were isolated from *S. tortuosissima* and identified by various spectroscopic measurements; confirmation was done by comparison with literature data. *In silico* study of the highly active myricetin on Aurora B and CDK4/cyclin D1 confirmed its antiproliferative effect against MCF-7 and HepG2 (IC_{50} = 5.56 and 8.61 µM, respectively). These results reflect the potential value of those plants growing in Al-Jouf area which, in turn, attract our attention for further investigations of such plants.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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