Pharmacogn. Mag.
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ORIGINAL ARTICLE

Flavonoid and Phenolic Compounds from *Carissa macrocarpa*: Molecular Docking and Cytotoxicity Studies

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Submitted: 12-03-2018 Revised: 11-04-2018 Published: 10-09-2018

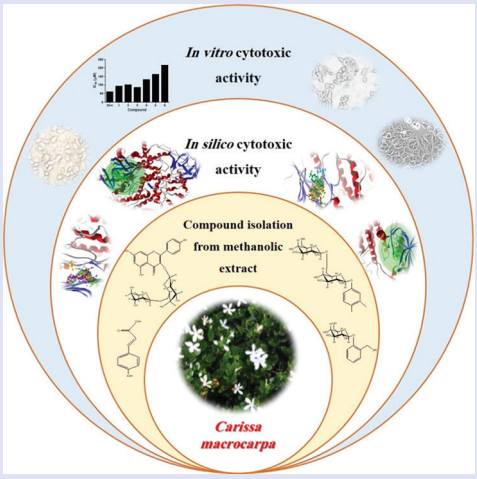
ABSTRACT

Objective: The objective of the study is to investigate the phytochemical contents of the methanol extract of leaves of *Carissa macrocarpa*, the possible anticancer activities of the isolated compounds through molecular docking approaches as well as the potential cytotoxic activity. **Materials and Methods:** The methanol extract of the plant was subjected to several chromatographic procedures. *In silico* studies of the isolated compounds against four anticancer target kinases, namely, protein kinase B (PKB/AKT), phosphatidylinositol 3-kinase, protein kinase C, and rapidly accelerated fibrosarcoma kinase were performed. Potential cytotoxic activity of the isolated compounds was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay against A549 cells. **Results:** Phytochemical investigation led to the isolation of three known flavonoid compounds: kaempferol 3-O-robinobioside (1), Kaempferol-3-O-α-L-rhamnopyranosyl (1-6)(4``-p-coumaroyl)β-D-galac topyranoside7-O-α-L-rhamnopyranoside (2), and variabiloside E (3) as well as three phenolic compounds: p-coumaric acid (4), salicin (5), and 3,4-dimethylphenol β-gentiobioside (6). *In silico* studies revealed that three out of the six compounds were strongly bound with one or more of the targets enzymes. Compound 3 showed broad-spectrum binding with the four targets with high docking score. Compounds 1–3 showed IC_{50} comparable to that of positive control, doxorubicin. The rest of the compounds 4–6 showed relatively discrete IC_{50}. **Conclusion:** The isolated compounds were reported for the first time from this plant. Compounds 1–3 could serve as lead compounds for development of new anticancer drugs.

Key words: *Carissa macrocarpa,* cytotoxicity, docking, flavonoids, phenolic

SUMMARY

- The methanol extract of *Carissa macrocarpa* was phytochemically analyzed
- Three flavonoid and three phenolic compounds were isolated from the methanol extract
- The isolated compounds were *in silico* docked against four known anticancer target enzymes
- The cytotoxic activity of the isolated compounds was investigated *in vitro*.



Abbreviations used: PKB/AKT: Protein kinase B; PI3K: Phosphatidylinositol 3-kinase; PKC: Protein kinase C; RAFK: Rapidly accelerated fibrosarcoma kinase; ^{1}H- and ^{13}C-NMR: Proton and carbon-13 nuclear magnetic resonance; SCC: Silica gel column chromatography; RPCC: Reversed-phase silica gel column chromatography; TLC: Thin-layer chromatography; MVD: Molegro Virtual Docker; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; DMSO: Dimethyl sulfoxide.

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DOI: 10.4103/pm.pm_104_18

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INTRODUCTION

Globally, cancer is considered one of the most common life-threatening diseases. Cancer affects both genders leading to severe health and socioeconomic negative impacts.[1] The treatment of cancer is designed by different major approach targeting to discover potent antitumor bioactive metabolites, based on that more than 75% of anticancer drugs are directly or indirectly derived from medicinal plants.[2]

Saudi Arabia has one of the most diverse floras in the Middle East, which contains about 2282 species in 855 genera.[3] *Carissa* (*C.*) is one of genera belonging to family *Apocynaceae* and consists of about 35 species distributed in tropics and subtropics of Africa, Asia, and Australia.[4] *Carissa macrocarpa* (family: *Apocynaceae*) grows in various habitats worldwide including Saudi Arabia. Despite the worldwide

Cite this article as: Khalil HE, Mohamed ME, Morsy MA, Kandeel M. Flavonoid and phenolic compounds from *Carissa macrocarpa*: Molecular docking and cytotoxicity studies. Phcog Mag 2018;14:S304-10.

distribution of *C. macrocarpa*, only few investigations were conducted on it, specifically those from South Africa.[5-7] There are no studies on *C. macrocarpa* from Saudi Arabia. In addition, careful examination of the available literature data revealed only few studies examining the phytochemical content or the medicinal activities of the plant's leaves. As well as, a preliminary pilot study revealed presence of the saponins and phenolic compounds including flavonoids and alkaloids.[8]

Phenolic and flavonoid derivatives are important derivatives with significant anticancer properties.[9,10] The molecular mechanisms associated with these compounds include apoptosis by inhibition of protein kinase B (PKB/AKT),[11] phosphatidylinositol 3-kinase (PI3K),[12] protein kinase C (PKC), and rapidly accelerated fibrosarcoma kinase (RAFK).[13]

Because of the studies on the methanol extract of leaves of *C. macrocarpa*, the current work reported the isolation and structural elucidation of six isolated compounds (phenolic and flavonoid derivatives) for the first time from *C. macrocarpa*. In addition, we examined the cytotoxic activities of the isolated compounds and their possible *in silico* mechanisms of cytotoxic activity. The molecular basis of interaction of a set of phenolic and flavonoid derivatives was investigated. The binding interactions with four anticancer targets: PKB/AKT, PI3K, PKC, and RAFK and their forces contributing to compound recognition were investigated.

MATERIALS AND METHODS

General

The following instruments and chemicals were used to get results: ^{1}H- and ^{13}C-NMR spectra, Bruker's Avance III spectrometer at 400 MHz and 125 MHz, respectively. Diaion HP-20 was purchased from Mitsubishi Chemical Co., Ltd. (Tokyo and Japan). Silica gel column chromatography (SCC) was performed on silica gel 60 (E. Merck, Darmstadt, Germany; 70–230 mesh). Reversed-phase silica gel column chromatography (RPCC) was performed on a Cosmosil 75C18-OPN column (Nacalai Tesque, Kyoto, Japan; Φ=50 mm, L = 25 cm, linear gradient: MeOH-H2O). Precoated silica gel 60 F_{254} plates (E. Merck; 0.25 mm in thickness) were used for thin-layer chromatography monitoring visualized by spraying with a 10% solution of H_2SO_4 in ethanol and heated to around 150°C on a hotplate.

Plant material

Leaves of *C. macrocarpa* were collected from gardens of King Faisal University, Al-Ahsa region (September 2013). The plant under investigation was identified by Dr. Mamdouh Shokry, director of El-Zohria botanical garden, Giza, Egypt. A voucher specimen of the plant was deposited at the Herbarium of the Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia (01-13-Sept-CM).

Extraction and isolation of the plant major compounds

Air-dried leaves of *C. macrocarpa* (3.0 kg) were exhaustively extracted using cold maceration three times with methanol (MeOH) (for 7 days using 10.0 L of 70% MeOH) at room temperature and then concentrated under reduced pressure. The concentrated MeOH extract (195.0 g) was partitioned with n-hexane to give hexane fraction (70.0 g), and remaining mother liquor was concentrated to give (120.0 g) defatted extract.[14] The defatted extract (115.0 g) was subjected to Diaion HP-20 CC (2.0 kg, H_2O [15 L] → MeOH [15 L] → acetone [5 L]) to give H_2O-(40.0 g), MeOH-(55.0 g), and acetone-eluted fractions (20.0 g). The MeOH-soluble fraction (55.0 g) was subjected to SCC (1.0 kg, $CHCl_3$ [3.0 L] → $CHCl_3$-MeOH [[9:1 [3.0 L] → 7:3[3.0 L] → 1:1 [3.0 L] →3:7 [3.0 L]] → $CHCl_3$-MeOH:H_2O [15:6:1] [3.0 L] → MeOH [3.0 L]]) to yield 9 fractions (Fr. 1 [4.2 g], Fr. 2 [1.1 g], Fr. 3 [2.1 g], Fr. 4 [2.0 g], Fr. 5 [1.7 g], Fr. 6 [2.3 g], Fr. 7 [3.4 g], Fr. 8 [1.5 g], and Fr. 9 [2.6 g]). Fraction 4 (2.0 g) was separated by RPCC (100.0 g, MeOH-H_2O [2:3 → 1:1 → 3:2 → 7:3 → 4:1 → 9:1] → MeOH) to yield 5 fractions (Fr. 4-1 [300.1 mg], Fr. 4-2 [30.7 mg], Fr. 4-3 [100.6 mg], Fr. 4-4 [70.2 mg], and Fr. 4-5 [260.0 mg]). Fr. 4-3 (100.4 mg) was purified by repeated RPCC [15 g, MeOH-H_2O (3:7 → 2:3 → 1:1 → 3:2 → 7:3 → 4:1 → 9:1) to give kaempferol 3-O-robinobioside (1, 8.6 mg)]. Fr. 4-5 (260.0 mg) was purified by RPCC (15.0 g, MeOH-H_2O [3:7 → 2:3 → 1:1 → 3:2 → 7:3 → 4:1 → 9:1]) to give Kaempferol-3-O-α-L-rhamnopyranosyl (1-6)(4``-p-coumaroyl) β-D-galactopyranoside7-O-α-L-rhamnopyranoside (2, 11.3 mg) and variabiloside E (3, 14.6 mg). Fraction 6 (2.3 g) was separated by RPCC [120 g, MeOH-H_2O (1:9 → 1:4 → 3:7 → 2:3 → 1:1 → 3:2 → 7:3 → 4:1)→ MeOH] to yield 6 fractions (Fr. 6-1 [210.0 mg], Fr. 6-2 [70.6 mg], Fr. 6-3 [40.2 mg], Fr. 6-4 [60.5 mg], Fr. 6-5 [80.9 mg], and Fr. 6-6 [342.0 mg]). Fr. 6-6 (342.0 mg) was separated by RPCC (20.0 g, MeOH-H_2O [3:7 → 2:3 → 1:1 → 3:2 → 7:3 → 4:1 → 9:1] → MeOH) to yield p-coumaric acid (4, 9.3 mg) and salicin (5, 7.6 mg). Fraction 8 (1.5 g) was separated by RPCC (100.0 g, MeOH-H_2O [3:7 → 2:3 → 1:1 → 3:2 → 7:3 → 4:1 → 9:1] → MeOH) to yield 5 fractions (Fr. 8-1 [100.3 mg], Fr. 8-2 [80.4 mg], Fr. 8-3 [10.5 mg], Fr. 8-4 [60.4 mg], and Fr. 8-5 [30.2 mg]). Fr. 8-1 (150.4 mg) was purified by RPCC (10.0 g, MeOH-H_2O [3:7 → 2:3 → 1:1] to give 3,4-dimethylphenol β-gentiobioside [6, 7.1 mg]).

Docking study

Retrieval of structure complexes

The Protein Data Bank (PDB, URL: www.rcsb.org) was browsed to retrieve the 3D crystal structure files containing the target enzymes bound with standard inhibitors. PDB IDs, 3CQW, 5UBR, 4RAR, and 5fD2 were retrieved for PKB/AKT, PI3K, PKC, and RAFK, respectively.

Structure preparation

The crystal structures were imported to Molegro Virtual Docker (MVD version 5.5). The proteins were optimized by assigning charges, correction of missed bonds, correction of bond orders, and ligand torsion. Energy minimization was performed by MVD (2000 maximum steps per residue).

Preparation of compounds

The structures of compounds were drawn by ChemDraw (CambridgeSoft, USA) and 3D optimized by Chem3D version 15. Compounds were saved as MOL files.

Docking

The saved optimized and energy minimized structures were used in docking studies. At first, a docking template was generated by Molegro docking template wizard by assigning a template from the original inhibitors bound with every protein. Docking was performed by energy grid resolution of 0.3 Å and a grid radius of 10–15 Å. MolDock scoring function was used after 10 rounds of runs of 1500 maximum iterations. The obtained docking results were compared to standard known inhibitors or modulators (reference compounds) specific for every protein [Table 1].

Cytotoxicity assay

This assay was performed using human lung cancer cell line (A549 was obtained from the RIKEN Cell Bank, Japan), which is adenocarcinoma human alveolar basal epithelial cells, and the viability was determined by the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The used cell culture medium was Dulbecco's

Modified Eagle's Medium (Sigma-Aldrich Co., USA) supplemented with fetal bovine serum (Invitrogen Co., USA), kanamycin (100μg/ml) (Wako, Japan), and amphotericin B (5.6μg/ml) (Sigma-Aldrich Co., USA). The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and added to each well of the 96-well microtitration plates at 1% as the final concentration. A549 cells (5×10^3 cells/well) were cultured in a 5% CO_2 incubator (Asahi, 4020, CO_2 incubator, Japan) at 37°C for 72 h, and then, MTT solution (MTT, Nacalai, Japan) was added to each well and the plates were incubated for 1.5 h; then, the formed formazan precipitates were dissolved in DMSO, and the optical density values for each well were determined at 540 nm with a microplate reader (VersaMax Tunable UV Microplate Reader [Molecular Devices, USA]). Doxorubicin (Wako, Japan) was used as a positive control. The cell growth inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = 1 - \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100$$

where $A_{control}$ is the absorbance of the control reaction mixture (containing DMSO and all reagents except for the test compounds). IC_{50} was determined as the concentration of sample required to inhibit the formation of MTT formazan by 50%.[15]

RESULTS

Isolation and identification of major compounds

The concentrated methanol extract was subjected to several chromatographic techniques to give three known flavonoid compounds: kaempferol-3-O-robinobioside (1),[16] Kaempferol-3-O-α-L-rhamn

Table 1: The docking scores of the six isolated compounds (1-6) with protein kinase B, phosphatidylinositol 3-kinase, protein kinase C, and rapidly accelerated fibrosarcoma kinase

Tested compounds	PKB/AKT	PI3K	PKC	RAFK	Color scale
	MK-2206	Wortmannin	Brostatin	Sorafenib	
Compound 1	−97.3[a]	−48.8	−50.1	−126.5	−160
Compound 2	−65.3	−128.1	−59.1	−139.9	−140
Compound 3	−134.3	−132.5	−121.1	−101.9	−120
Compound 4	−50.7	−53.3	−35.5	−61.6	−100
Compound 5	−51.4	−46.6	−31.2	−57.5	−80
Compound 6	−50.7	−46.7	−31.6	−73.7	−60
Reference compound	−95.4	−80.6	−131.1	−102.5	−40

[a]The score was calculated based on MolDock algorithm calculated by MVD. For every enzyme, a standard inhibitor was undertaken as a reference compound. A color scale was applied to identify the potency of compounds. The color range from red to blue implies lower to higher docking score, respectively. PKB/AKT: Protein kinase B; PI3K: Phosphatidylinositol 3-kinase; PKC: Protein kinase C; RAFK: Rapidly accelerated fibrosarcoma kinase

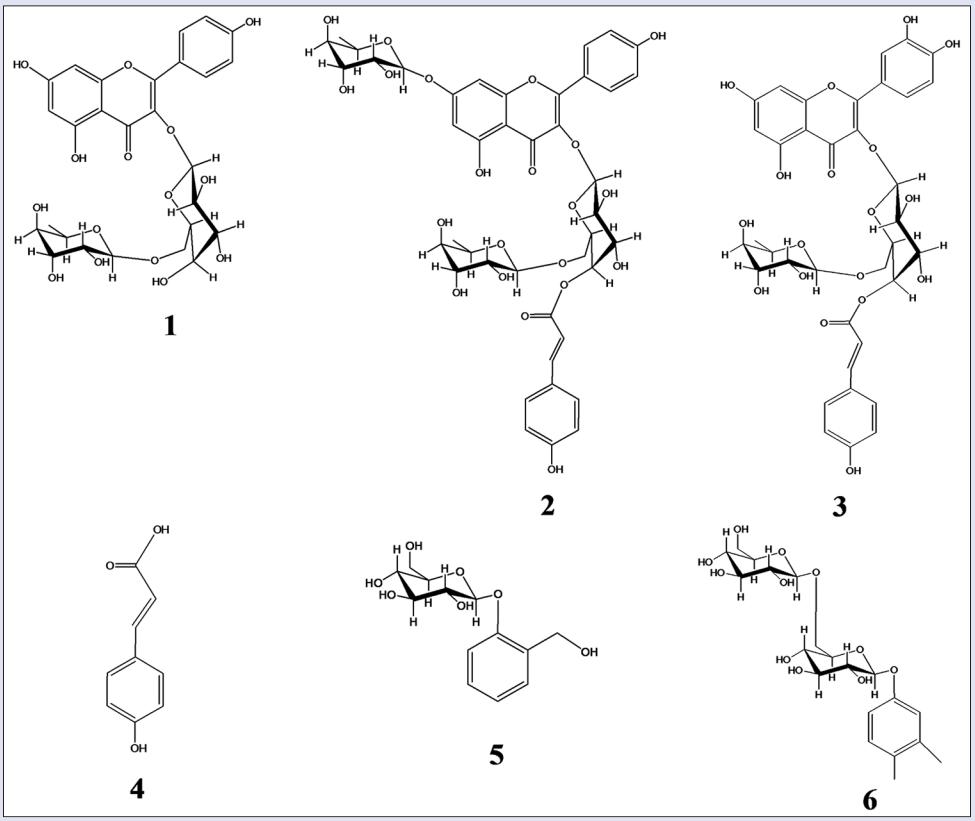

Figure 1: Isolated compounds from *Carissa macrocarpa*

opyranosyl (1-6)(4``-p-coumaroyl)β-D-galactopyranoside7-O-α-L-rhamnopyranoside (2),[17] and variabiloside E (3)[18] as well as three phenolic compounds: p-coumaric acid (4),[19] salicin (5),[20] and 3,4-dimethylphenol β-gentiobioside (6)[21] [Figure 1]. These structures were elucidated by extensive inspection of 1D and 2D NMR spectroscopic data and comparison with reported values.

Docking study

Isolated compounds were evaluated based on their MolDock score compared with reference compounds. For PKB/AKT, compounds 1 and 3 showed higher docking score compared with the reference inhibitor MK-2206 [Table 1]. For PI3K, compounds 2 and 3 showed about 59% and 64% improved binding strength compared with wortmannin, a standard inhibitor for PI3K. Compound 3 showed docking score of −121.1, which is highly comparable with the PKC inhibitor brostatin. Compounds 1 and 3 were also strong binders with RAFK by showing higher docking score compared with sorafenib [Table 1].

The docked ligands of the isolated compounds 1-6 to the active site of PKB/AKT, PI3K, PKC, and RAFK together with dissection of forces contributing to compound recognition were shown in Figures 2-5, respectively.

Cytotoxicity assay

The results of MTT cytotoxicity assay on A549 cell line are shown in Figure 6. Compounds (1-3) had comparable IC_{50} to the positive control, doxorubicin (59.7 μM). Compound 3 was the most potent of the tested compounds as it achieved the lowest (IC_{50}: 84.3 μM) followed by compounds 1 (93.6 μM) and 2 (100.4 μM). Compounds 4-6 showed relatively distinct IC_{50}.

DISCUSSION

C. macrocarpa is an evergreen spiny shrub, with crimson fruits, which is used commonly in Saudi Arabia for ornamental purposes. The shrub was verified in previous study to contain many classes of phytochemicals such as flavonoids, saponins, triterpenoids/steroids, anthraquinones, tannins, and carbohydrates.[8] The present study revealed the identification of six compounds, recorded for the first time in the plant. These compounds included three flavonoid glycosides: kaempferol-3-O-robinobioside (1), Kaempferol-3-O-α-L-rhamnopyranosyl (1-6)(4``-p-coumaroyl)β-D-galactopyranoside7-O-α-L-rhamnopyranoside (2), and variabiloside E (3) and three phenolic compounds: p-coumaric acid (4), salicin (5), and 3,4-dimethylphenol β-gentiobioside (6). Compounds 1 and 2 shared the same flavone aglycone, Kaempferol; however, compound 3 had luteolin as its aglycone. All flavonoid compounds were 3-O-glycoside, but compound 2 had an additional 7-O-glycosylation. All the three flavonoid compounds contained the sugar unit O-α-L-rhamnopyran osyl-(1-6)-β-D-galactopyranoside (i.e., robinobioside); furthermore, compounds 2 and 3 had p-coumaric acid side chain. Compound 4 was the p-coumaric acid, which seemed to be abundant in the plant to the extent that it reacted with the sugar parts in compounds 2 and 3. p-Coumaric acid is the main precursor for the synthesis of flavonoid aglycones. Salicin is a derivative of benzyl alcohol with β-glucoside sugar part. 3,4-dimethylphenol β-gentiobioside (also called 3,4-xylenol β-gentiobioside) (6) is synthesized through glycosylation of 3,4-xylenol. The identity of the isolated compounds was confirmed through comparison with the previous literature using their 1D and 2D NMR data.

Molecular modeling and docking strategies are gold standards in drug discovery and evaluation process.[22-24] The merit of accurate docking scoring functions led to rapid and accurate determination of new inhibitors against molecular targets.[21,22,25] In this work, molecular

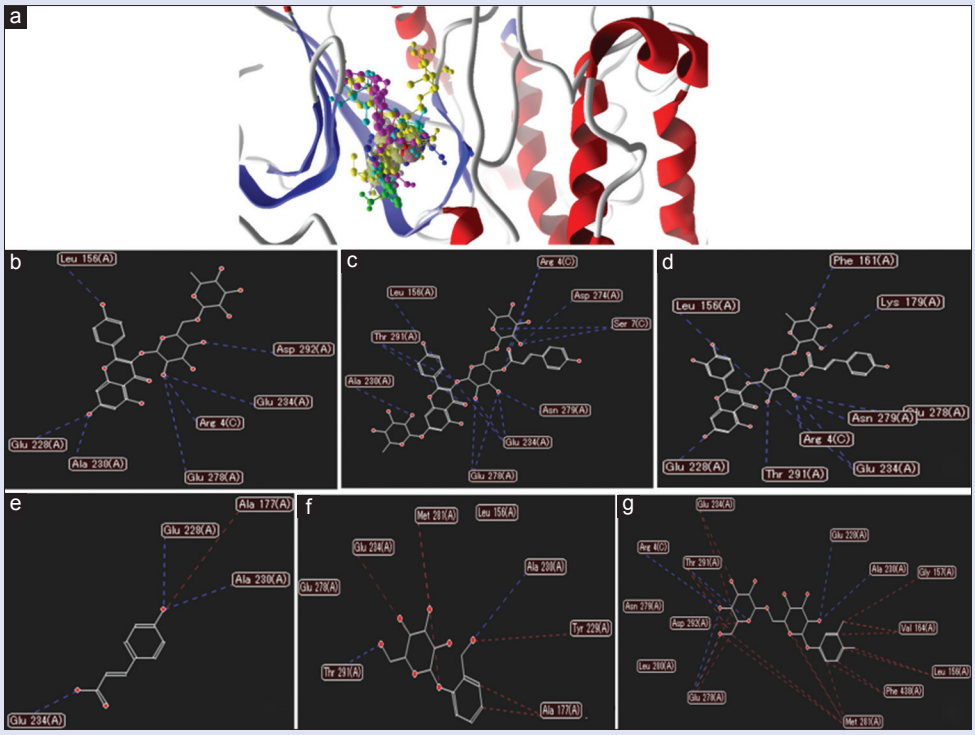

Figure 2: Molecular modeling and docking of the isolated compounds with protein kinase B. (a) The docked compounds into the active site of protein kinase B. Interactions of compound 1 (b), compound 2 (c), compound 3 (d), compound 4 (e), compound 5 (f), and compound 6 (g). Hydrogen bonds are shown as blue dashes. Steric interactions are shown as red dashes

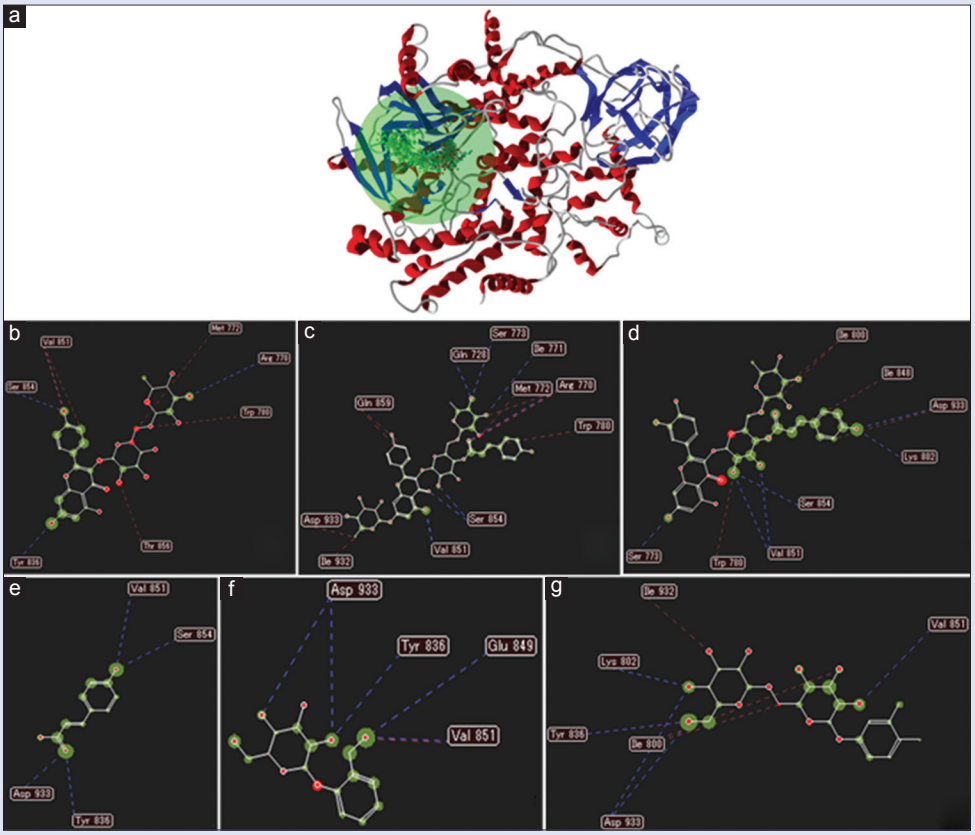

Figure 3: Molecular modeling and docking of the isolated compounds with phosphatidylinositol 3-kinase. The docked compounds into the active site of phosphatidylinositol 3-kinase (a). Interactions of compound 1 (b), compound 2 (c), compound 3 (d), compound 4 (e), compound 5 (f), and compound 6 (g). Hydrogen bonds are shown as blue dashes. Steric interactions are shown as red dashes

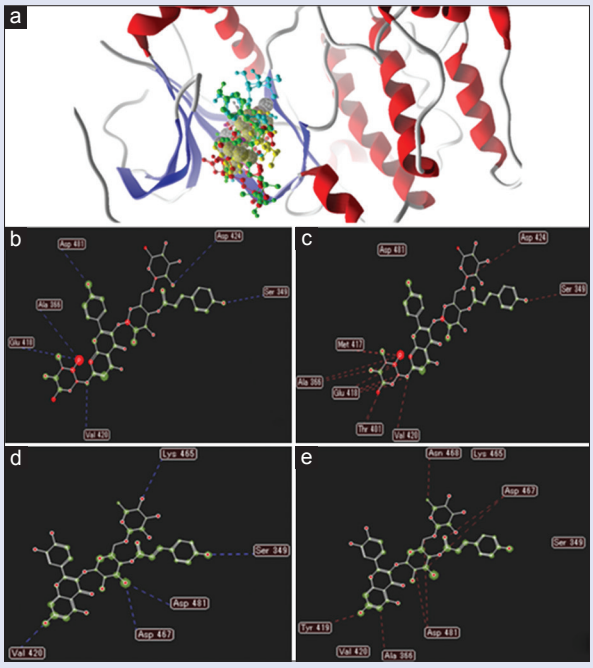

Figure 4: Molecular modeling and docking of the isolated compounds with protein kinase C. The docked compounds into the active site of protein kinase C (a). Interactions of compound 2 (b and c) and compound 3 (d and e). Hydrogen bonds are shown as blue dashes. Steric interactions are shown as red dashes

modeling tools and docking studies were carried out to investigate the binding efficiency of six flavonoid and phenolic derivatives from plant extracts with four targets in treatment of cancers, PKB/AKT, PI3K, PKC, and RAFK.

PKB/AKT is a serine/threonine protein kinase, which is activated by PI3K. PKB/AKT regulates fundamental cellular activities such as cell apoptosis, glucose metabolism, and cell cycle development. PI3K is activated by tyrosine kinase and it is an activator of PKB/AKT, and thus, it is involved in similar cellular activities such as cell proliferation, cell survival, and differentiation. PKC is groups of enzymes responsible for altering the function of other proteins through phosphorylation of OH groups of amino acid residues such as serine and threonine. Activation of such group of enzymes has an impact on various cellular events such as membrane structure modulation and receptors' deactivation. RAFKs are groups of enzymes, which induce fibrosarcoma. They are oncogene-related enzyme, which have a direct effect in the induction of cell proliferation and thus cancer initiation.

Within the six compounds, three compounds (1-3) were strongly bound with the target enzymes. Furthermore, compound 3 showed multiple expected strong binding with multiple enzyme targets.

Compounds 4-6 did not show high docking score and were expected to bind weakly with the target enzymes. Compared with reference compounds, compounds 1-3 showed higher docking score and expected to bind strongly with the target enzymes. Compound 3 was the most promising compound by showing strong binding with all the tested enzymes, PKB/AKT, PI3K, PKC, and RAFK [Table 1]. Compounds 1 and 2 showed selective binding with two of the target enzymes.

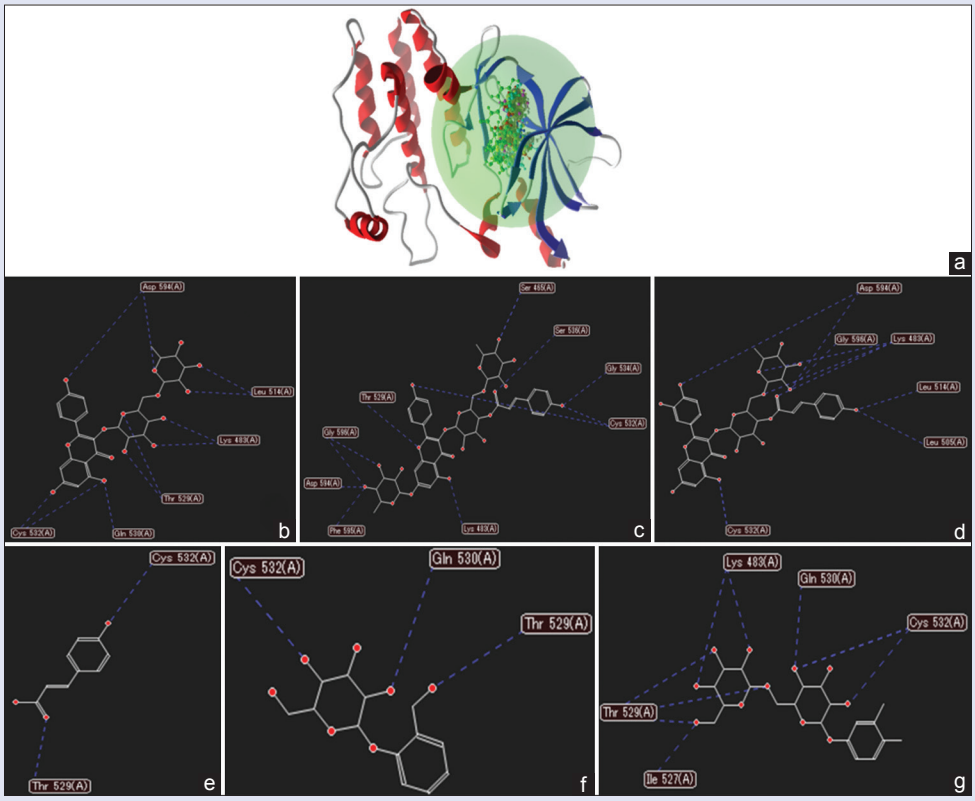

Figure 5: Molecular modeling and docking of the isolated compounds with rapidly accelerated fibrosarcoma kinase. The docked compounds into the active site of rapidly accelerated fibrosarcoma kinase (a). Interactions of compound 1 (b), compound 2 (c), compound 3 (d), compound 4 (e), compound 5 (f), and compound 6 (g). Hydrogen bonds are shown as blue dashes

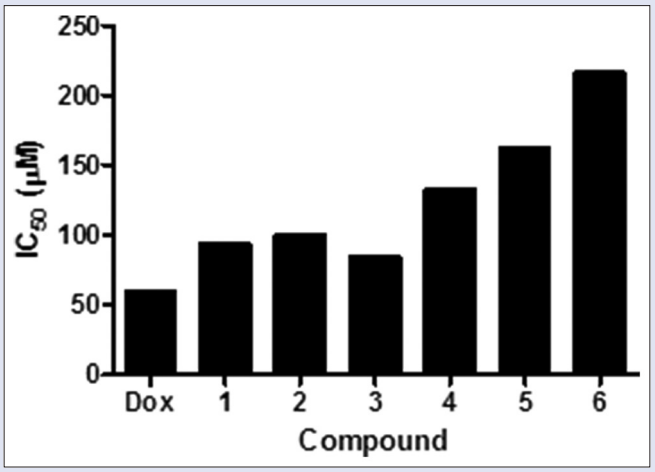

Figure 6: IC_{50} values (μM) of the isolated compounds 1–6 against the positive control (doxorubicin) using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay on A549 cell line

Analysis of the contributing forces of compound recognition by the target enzymes reveals two major forces which are attraction force guides by hydrogen bonds and favorable steric or stacking interactions. These forces are antagonized by unfavorable steric interactions with the backbone of target proteins. Figure 3b-g shows the docked ligands to the active site of PKB/AKT and dissection of forces contributing compound recognition. Despite the binding of compound 2 to PKB/AKT with strong network of nine hydrogen bonds, counteracting strong adverse steric clashes minimized the total binding energy [Figure 3c]. Compounds 1 and 3 showed 4-6 hydrogen bonds with favorable steric force contribution to yield a total stronger binding energy [Figure 3b and d]. Compounds 4-6 showed lower binding interactions with PKB/AKT with higher unfavorable contributions [Figure 3e-g]. For PI3K [Figure 4], compounds 2 and 3 showed the most favorable binding profile due to larger number of hydrogen bonds and favorable steric forces [Figure 4c and d]. A lower number of hydrogen bonds [Figure 4b] and larger negative contribution from internal compound interactions from torsions lead to loss of compound 1 interaction strength. Compounds 4-6 showed lower binding interactions with PI3K [Figure 4e-g]. The lower score of compounds 2 [Figure 5b and c] with PKC is attributed with higher degree of unfavorable steric repulsion forces compared with more favorable hydrogen bond attraction forces with compound 3 [Figure 5d and e]. The favorable interaction of compounds 1-3 with RAFK is due to formation of strong hydrogen network with the backbone of enzyme [Figure 6b-d]. Compounds 4-6 showed lower binding interactions with RAFK [Figure 6e-g].

According to the molecular docking results, compounds 1-3 could retain some cytotoxic activities. The MTT cytotoxicity assay revealed that compound 3 has the most potent cytotoxic activity. Compound 3 disclosed 70.8% activity relative to the used anticancerstandard doxorubicin. Compounds 1 and 2 showed 63.8% and 59.5% activity of the same standard, respectively. These results are very promising, taking into mind that these drugs have the preference of being natural, save and with wide therapeutic indices.

CONCLUSION

Leaves of *C. macrocarpa* were extracted and fractionated, resulting in the isolation of three flavonoid glycosides and three phenolic compounds for the first time from the plant. Molecular docking studies of the isolated compound indicated an activity of the flavonoid compounds on four enzymes, which are directly related to cancer induction, thus suggesting cytotoxic activity. *In vitro* cytotoxic assay revered the antiproliferative activity of such flavonoid glycosides.

Acknowledgement

This project was funded by the Deanship of Scientific Research, King Faisal University, Al-Ahsa, Saudi Arabia, under project number (160011). The authors, therefore, acknowledge with thanks DSR financial support.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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