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Research Paper

Insecticidal activity of the main flavonoids from the leaves of *Kalanchoe beharensis* and *Kalanchoe longiflora*

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ABSTRACT

The flavonoids quercetin**,** quercetin-3-O-β-L-galactopyranoside, quercetin-3-O-β-L-glucopyranoside, kampferol-3-O-α-L-rhamnopyranoside and kampferol-3,7-di-O-α-L-rhamnopyranoside were isolated from the methanolic extracts of the leaves of *Kalanchoe beharensis* and *Kalanchoe longiflora* family *Crassulaceae* using different chromatographic techniques. Identification and structure elucidation of the isolated compounds were done using different spectroscopic data and/or by comparing these data with those reported in literature. Methanolic extracts and isolates were tested for their insecticidal activity against cotton leaf worm, *Spodoptera littoralis*.

Keywords: Kalanchoe; Carssulaceae; Flavonoids; Insecticidal**.**

INTRODUCTION

kalanchoea is a genus belonging to family *Crassulaceae*, it comprises about hundred species that are native to tropical areas, Africa and Brazil (Boules, 1999). Several traditional uses for *Kalanchoe* juice were reported including local treatment of periodontal disease, cheilitis, cracking lips in children, bruises, wounds, boils (Mourao, et al., 1999). An extensive phytochemical study of several *kalanchoe* species was done leading to separation and identification of different classes of compounds including flavonoids and flavonoid glycosides (Nielsen, et al., 2005; Singab, et al., 2011; Tatsimo, et al., 2012; Megawati, et al., 2013), anthocyanins from *K. blossfeldiana* (Nielsen, et al., 2005). Bufadienolides isolated from leaves and whole aerial parts of different Kalanchoe species (Supratman, et al., 2000; 2001), sterols and triterpenes from the leaves of *K. thrysiflora* (Singab, et al., 2012). All these phytoconstituents proved to possess different biological activities as antimicrobial activity of *K. petitiana* (Tadeg, et al., 2005), analgesic, antihyperglycemic and anticonvulsant effect of *K. crenata* (Nguelefack, et al., 2004; 2006; Kamgang, et al., 2008) respectively, anti-inflammatory and antiviral effect of several Kalanchoe species (Shirobokov, et al., 1981), hepatoprotective effect (Yadav, et al., 2003), significant cardiovascular effects shown by the n-butanol extract of *K. crenata* leaves (Nguelefack, et al., 2008). Previous studies have shown that *K. pinnata*

and *K. daigremontiana* exhibited strong insecticidal activities against third instar larvae of silkworm (Supratman, et al., 2000; Maharani, et al., 2008).

Based on our interest to help in confronting one of the most destructive agricultural pests affecting the economy in our country and all over the world as well as studying two species belonging to the wide spread *Crassulaceae* family well known for its variable both phytochemical content and biological activities. We describe the isolation of five different flavonoids for the first time from the methanolic extracts of *K. beharensis* and *K. longiflora* leaves in addition to testing of the methanolic extracts and isolates for their insecticidal activity against cotton leaf worm, *Spodoptera littoralis* family *Noctuidae* which is one of the most destructive agricultural lepidopterous pests within its subtropical and tropical range. It can attack numerous economically important crops throughout year (EPPO, 2013).

MATERIALS AND METHODS

***Instruments and material*:** Column chromatography was run using silica gel G60 (Merck), Polyamide 6S and sephadex LH-20 (Sigma). TLC was carried out on silica gel plates silica gel 60 F_{254} with $CHCl_3$-MeOH- H_2O, 6:3:0.5 (S_1) and $CHCl_3$-MeOH, 5:1 (S_2). PPC was carried out using Whatman paper No 3 using H_2O (S_3). The chromatograms were first visualized under UV light and then spraying with $FeCl_3$ spray reagent. The UV spectra were obtained on Shimadzu UV 240 spectrophotometer. NMR spectra were recorded on a JEOL α 400 MHz spectrometer. Chemical shifts were given on δ-scale with TMS as internal standard.

***Plant material*:** The leaves of *Kalanchoe beharensis* and *Kalanchoe longiflora* were collected from Orman Public Garden, Giza, Egypt in March 2011. Identification of the plant was confirmed by Dr. Therese Labib senior head of specialists for plant identification. Two voucher specimens (no. KB-31 *Kalanchoe beharensis* & KL-32 Kalanchoe *longiflora*) have been deposited in the Herbarium of Pharmacognosy department, Faculty of Pharmacy, Helwan University.

Biological material: A laboratory strain of *Spodoptera littoralis* was reared on castor leaves under controlled conditions (25±2˚C and 65±5% R.H.), away from any insecticidal contaminations. Fourth instar larvae were used. All materials were provided by Plant Protection Department, National Research Center, Dokki, Giza.

General method of acid hydrolysis: Each glycoside (2mg) dissolved in dioxan (50µl) and 2N HCL (1:1) was heated at 95°C for 30 minutes. Dioxan was evaporated and the residue was diluted with water and extracted with ethyl acetate in which the aglycone was detected by TLC. The remaining aqueous layer was repeatedly diluted with methanol and evaporated to dryness. The residue was investigated to detect the sugar portion using PC eluted with solvent system n-BuOH-AcOH-H2O system (4:1:5 v/v, upper layer). Sugar components were identified by comparison with standard samples after spraying with aniline phthalate.

Extraction and isolation: Air dried and powdered leaves of both *K. beharensis* (750g) and *K. longiflora* (820g) were extracted with n-hexane (1Lx3) followed by extraction with MeOH (2Lx3) at room temperature. The total alcoholic extract of *K. beharensis* was concentrated under reduced pressure. The residue obtained of of *K. beharensis* (7.7g) was subjected to VLC technique using polyamide, elution was started with water and continued with water containing from 10% to 100% increment of methanol. 20 fractions, 150ml each, were collected. The fractions were monitored on TLC plates using S_1 and S_2 and visualized under UV light followed by spraying with $FeCl_3$ reagent. The fractions eluted at 70% MeOH were combined after TLC analysis. The combined fractions were repeatedly chromatographed on preparative PC

eluted with water. Repeated purifications of the components on sephadex LH-20 column eluted with MeOH, afforded compound **1**. The fractions which eluted with 30-50% MeOH were combined and subjected to PPC. Elution was performed with water followed by repeated purifications on sephadex LH-20 column using MeOH as eluting system to yield inseparable mixture of **2** and **3** (37mg).

The total alcoholic extract of *K. longiflora* was concentrated under reduced pressure (9.5g) and subjected to silica gel column where elution started with 100% methylene chloride and continued by 1% increment of MeOH. The effluent was collected in fraction (100ml each). Fractions eluted at 7% MeOH were combined and further purification on preparative TLC plates using S_2 was done followed by repeated purification on sephadex LH-20 column eluted with methanol to yield compound **4**. Fractions eluted at 8% MeOH from the silica gel column were also subjected to preparative TLC technique using S_2 followed by successive purification on sephadex LH-20 column, elution done with methanol to yield compound **5**.

Testing insecticidal activity: A (5%) Concentration of each extracts was prepared separately in acetone using few drops of Tween 80 as emulsifier. Methanolic extract of *Kalanchoe beharensis* and its isolates **2** & **3** as well as *Kalanchoe longiflora* and its isolates **4** & **5** were tested for their insecticidal activity against the Egyptian cotton leaf worm *Spodoptera littoralis*. Strips of castor leaves were immersed in the required concentration of the extracts and left to dry. Newly molted 4th instar larvae were allowed to feed for 48 hrs on treated leaves. Three replicates, each of ten larvae, were used for each tested material and the control group was fed on untreated leaves. Mortality counts were recorded daily till adult emergence and corrected according to (Abbott's formula, 1925).

The accumulative percent mortality was calculated for two criteria:

- **IPF, Cumulative percent inhibition till pupal formation.**
- **IAE, Cumulative percent inhibition till adult emergence.**

RESULTS

Chemistry: 5 compounds (Figure 1) were separated from the two Kalanchoe species, compounds 1, 2 and 3 separated from *K. beharensis* methanol extract of the leaves while compounds 4 and 5 separated from *K. longiflora* methanol extract of the leaves. They were identified as follow

Quercetin (1): amorphous yellow powder (20mg), UV λ_{max} nm (MeOH): 258, 267sh, 299 sh, 360; +NaOMe: 272, 326, 414, +$AlCl_3$: 275, 302, 430; +$AlCl_3$ /HCl: 271, 302, 362 sh, 400; +NaOAc: 270, 325, 390; +NaOAc/H_3BO_3: 260, 296, 387. ^{1}H NMR (400 MHz, Acetone, δ ppm) H-6', H-2'(7.28, m), H-5' (6.88, d, *J*=8.0 Hz), H-8 (6.40, d, *J*=2.5 Hz), H-6 (6.20, d, *J*=2.5 Hz), (Harborne, 1993).

Quercetin-3-O-β-D-galactopyranoside (2): amorphous yellow powder (37mg). ^{1}H NMR (400, 399.65 MHz, Acetone, δ ppm) , H-2' (7.93, d, *J*=2.0 Hz), H-6' (7.52, dd, *J*=8.5, 2.2 Hz), , Gal H-1" (5.15, d, *J*=7.2 Hz), Gal H-5" (3.9, d, *J*=3.0 Hz). ^{13}C NMR (400 MHz, Acetone, δ ppm) C-4 (178.14), C-7 (164.6), C-5 (161.26), C-2 (157.3), C-9 (156.9), C-4' (148.7), C-3' (144.5), C-3 (134.4), C-6' (121.69), C-1'(121.4), C-5' (116.6), C-2' (115.1), C-1" (104.5), C-10 (103.77), C-6 (98.9) , C-8 (94.0), C-5" (75.4) , C-3" (73,4), C-2" (71.7), C-4" (68.1), C-6" (60.9),(Gulnur et al., 2004; Markham et al., 1978).

***Quercetin-3-O-β-D-glucopyranoside (3)*:** amorphous yellow powder (37mg). ^{1}H NMR (400 MHz, Acetone, δ ppm). Glc H-1" (5.21, d, *J*=7.6 Hz), Glc H-5" (3.23, m). ^{13}C NMR (400 MHz, Acetone, δ ppm) C-1" (103.0), C-5" (76.5), C-3" (76,4), C-2" (74.3), C-4" (69.1), C-6" (61.0), (Mustafa et al., 2000).

***Kampherol 3-O-α-L-rhamnopyranosyl (4)*:** Pale yellow powder (32mg), UV λ_{max} data (MeOH) 269, 345; (NaOMe) 274, 388; (NaOAc) 276, 302, 369; ($NaOAc/H_3BO_4$) 267, 350; ($AlCl_3$) 274, 300 sh, 348, 396; ($AlCl_3$/HCl) 273, 300 sh, 362 nm. ^{1}H NMR (400 MHz, DMSO, δ ppm), OH-5 (12.67, s), H-2', 6' (7.81, d, *J*=8.4, 2H), H-3', 5' (6.89, d, *J*=8.4, 2H), H-8 (6.57, d, *J*=2.1, 1H), H-6 (6.41, d, *J*=2.1,1H), H-1'' Rha (5.51, d, *J*=1.8, 1H), Me-6'' (0.89, d, *J*=5.7, 3H), H-(2''-5'') (2.29-3.31, 4H) and ^{13}C NMR (400 MHz, DMSO, δ ppm) C-4 (178.3), C-7 (164.6), C-5 (162.2), C-4' (161.2), C-2 (158.0), C-9 (156.6), C-3 (135.1), C-6' (131.5), C-2' (131.3), C-1'(121.7), C-5' (116.3), C-3' (116.1), C-10 (105.0), C-1'' (102.1), C-6 (94.2), C-8 (94.7), C-4'' (72.13), C-2'' (70.8), C-3'' (70,6), C-5'' (70.8), C-6''(17.4).

Kampherol 3,7-O-α-L-dirhamnopyranosyl 5: Pale yellow powder (28mg), UV (MeOH) 205, 265, 345; (NaOMe) 210, 246, 273, 390; (NaOAc) 222, 264, 364; (AlCl3) 203, 274, 301, 350, 400; (AlCl3/HCl) 201, 271, 339, 399nm; ^{1}H NMR (400 MHz, DMSO, δ ppm)) H-8 (6.87 d, 1H, *J*=2.1 Hz), H-6 (6.41d, 1H, *J*= 2.1Hz), H-1'''(5.62 d, 1H, *J*= 1.8 Hz), Me-6'' (1.08 d, 3H,*J*= 5.7Hz), H-6''' (1.1, d, 3H, *J*= 5.7Hz) and ^{13}C NMR (400 MHz, DMSO,δ ppm), C-7 (162.8), C-3 (136.1), C-5' (116.4), C-6 (99.9) , C-10 (107.0), C-1''' (99.99), C-8 (95), C-4''' (72.13), C-5'' (70.8), C-2''' (70.8), C-5''' (70.8), C-3''' (70.6), C-6'' (18.4), C-6''' (18.02), (Fábio de Sousa, et al., 2007).

Insecticidal activity: The insecticidal activity of the methanolic extracts was carried out against cotton leaf worm *Spodoptera littoralis* (Table 1)**.**

DISCUSSION

As described in the experimental section, from the methanolic extracts of *Kalanchoe beharensis* and *Kalanchoe longiflora* leaves, the flavonoids quercetin **1,** quercetin-3-O-β-L-galactopyranoside **2**, quercetin-3-O-β-L-glucopyranoside **3**, kampherol-3-O-α-L-rhamnopyranoside **4** and kampherol-3, 7-di-O-α-L-rhamnopyranoside **5** were isolated. The structural identification of the isolates was elucidated by acid hydrolysis, UV, ^{1}H and ^{13}C NMR spectroscopic analysis and/or comparison with published data.

Quercetin (1): The flavonoid quercetin was characterized by comparison of its spectral data with literature values (Harborne, et al., 1982).

Quercetin-3-O-β-D-galactopyranoside (2) & quercetin-3-O-β-D-glucopyranoside (3): were detected as an inseparable mixture showing UV spectra with different shift reagents characteristic to flavonol substituted in position 3 with a free 4' position (Mabry, et al., 1970). The ^{1}H and ^{13}C NMR spectra of **2** and **3** showed two sets of typical signals for quercetin nucleus (Harborne, et al., 1982).The spectra exhibited also two anomeric doublets proton signals (5.15, d, *J*=7.2 Hz) and (5.21, d, *J*=7.6 Hz) as well as two anomeric carbons at δ102.8 and 104.1 ppm that assigned to β-D-galactopyranoside and β-D-glucopyranoside respectively. They were confirmed after acid hydrolysis by comparison to standards on TLC. Comparing the obtained data with published data (Mustafa, et al., 2000; Gulnur, et al., 2004; Markham, et al., 1978) lead to suggesting compound **2** to be quercetin 3-O-β-D-galactopyranoside and compound **3** to be quercetin 3-O-β-D-glucopyranoside.

Kampferol 3-O-α-L-rhamnopyranosyl (4): was obtained as yellow crystalline substance, its UV spectrum with MeOH and different shift reagents suggested a flavonol compound with free OH groups in position 5 & 7.The ^{1}H NMR spectrum as well as ^{13}C NMR signals proved that we have kampferol nucleus substituted at C-3 when compared with the reported data (Harborne, et al., 1982). Also One doublet appearing at δ 5.51 (J=1.8) associated with a doublet at δ 0.89 ppm with a J=5.7 suggesting an α-L-rhamnopyranosyl moiety confirmed by the appearance of the anomeric carbon at δ 102.37 and acid hydrolysis with comparison with standard. All

the obtained data were in good agreement with the reported Kampferol 3-O-α-L-rhamnopyranosyl.

Kampferol 3,7-O-α-L-dirhamnopyranosyl (5): showed similar data to that of compound **4** with an additional 2 doublets in the ^{1}H NMR spectrum appearing at δ 5.26 (d, 1H, *J*= 1.8 Hz) and 1.1 (d, 3H, *J*= 5.7Hz) ppm corresponding respectively to the anomeric proton and the methyl group of another rhamnopyranosyl moiety. The structure was confirmed by comparing the obtained results with the published data (Fábio de Sousa, et al., 2007). Compound **5** was assigned the structure (Kampferol 3,7-O-α-L-dirhamnopyranosyl).

Insecticidal activity: The obtained results in (Table 1) revealed an apparent toxic effect against Egyptian cotton leaf worm *Spodoptera littoralis* for both plant methanolic extracts. The results indicated that the tested extracts exerted extended effects through the pupal and adult stages (Abdelaziz, 2007), the percent of inhibition of the methanolic extract of *K. beharensis* for pupae (IPF) and adult (IAE) reached up to (66.6% and 73.3%) while that of the methanolic extract of *K. longiflora* reached up to (86.6% (IPE) and 93.3% (IAE)) showing a more potent effect for *K. longiflora*. Also, the effect of the isolated flavonoids from each plant were tested separately (Table 1) exhibiting satisfactory effect but less pronounced than the total methanolic extracts of each plant. The cumulative mortalities were 60% and 73.3% for compounds **2** & **3** of *K. beharensis* leaves while for *K. longiflora* leaves, were 50% and 60% for compound **4** as well as 73.3% and 76.6 for compound **5** till pupa and adult emergence respectively. This is may be justified by a synergistic effect of all the components present in the total extract of each plant which show more powerful insecticidal effect than the single component tested individually, but it also points out the satisfactory activity of the flavonoid class in general as insecticide.

Finally, it was clear that *Kalanchoe longiflora* was the most effective plant extract against *Spodoptera littoralis* followed by its isolate compound **5**.

CONCLUSION

The present study revealed marked insecticidal activity of total MeOH extract of *K. longiflora* followed by its isolate compound 5 against the Egyptian cotton leaf worm *Spodoptera littoralis*. The structures of five isolated flavonoids were identified as quercetin (**1**)**,** quercetin-3-O-β-L-galactopyranoside (**2**), quercetin-3-O-β-L-glucopyranoside (**3**) isolated for the first time from *Kalanchoe beharensis* and kampferol-3-O-α-L-rhamnopyranoside (**4**) and kampferol-3,7-di-O-α-L-rhamnopyranoside (**5**) isolated for the first time from *Kalanchoe longiflora*.

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Table-1: Insecticidal activity of the tested plant extracts against 4th instar larvae of *Spodoptra littoralis* fed on 5 % treated leaves.

Number	Sample	IPF %	IAE %
1	Methanol extract of *K. beharensis*	66.6	73.3
2	Compounds 2 & 3 mixture	63.3	70
3	Methanol extract of *K. longiflora*	86.6	93.3
4	Compound 4	50	60
5	Compound 5	73.3	76.6

- %IPF = Cumulative percent inhibition till pupal formation.
- %IAE = Cumulative percent inhibition till adult emergence.

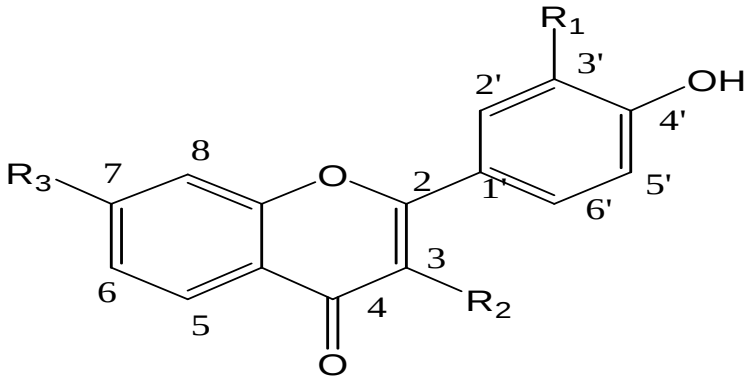

Figure- 1

Cpd No	R_1	R_2	R_3
1	OH	OH	OH
2	OH	*O-β-D-galactopyranoside*	OH
3	OH	*O-β-D-glucopyranoside*	OH
4	H	*O-α-L-rhamnopyranosyl*	OH
5	H	*O-α-L-rhamnopyranosyl*	*3-O-α-L-rhamnopyranosyl*

- Compounds: (1) quercetin, (2) quercetin-3-O-β-D-galactopyranoside, (3) quercetin-3-O-β-D-glucopyranoside, (4) kampherol 3-O-α-L-rhamnopyranosyl, (5) kampferol 3,7-O-α-L-dirhamnopyranosyl.

