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Insects' Deterrent Flavonoids from *Cynara cardunculus* for Controlling Cotton Leafworm; *Spodoptera littoralis.*

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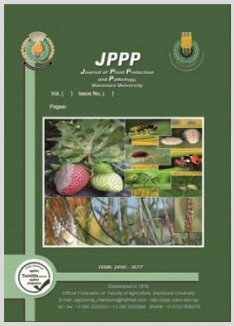

ABSTRACT

Globally, striving to find eco-friendly pest control agents increased day after day toward plant metabolites. Through current work, three flavonoids were isolated from Cynara cardunculus and identified as Apigenin, Luteolin, and Luteolin-7-O-glucoside using UV, IR, and MS spectrometry analyses. The three compounds were evaluated for their anti-feeding and deterrence activity against 4th instar larvae of cotton leafworm *Spodoptera littoralis* using both dual and no-choice leaf disk tests. results revealed that Luteolin-7-O-glucoside recorded the strongest insect antifeedant activities reached (74.50, 91.40%) followed by Apigenin (53.45% & 71.61%) in both choice tests respectively, while the antifeedant index of Luteolin did not exceed 22% in the concentration of 1000 ppm. Moreover, the activity of digestive enzymes; amylase, and invertase decreased significantly according to the deterrence activity level of the tested compound while protease activity showed non-significant changes in treated larvae. Noteworthy, the structure -feed deterrence activity relationships of flavonoids was observed and considered. Thus flavonoids can protect crop plants from insect infestation according their chemical structure.

Keywords: *Cynara cardunculus* ; Flavonoids; *Spodoptera littorals* ;Antifeedants ;Digestive enzymes.

INTRODUCTION

Indeed, One of the most complex aspects of plant-insect interactions involves the, phagostimulant and phagodeterrent. Flavonoids play an important role in plants interactions with the environment partially with insects. Since some insects, especially bee, can discriminate UV light flavones and flavonols acts as visual attractants where Flavone and flavonol glycosides were detected in flowers of many plants, as visible pigments. (Harborne & Baxter, 1999).

On other hand many flavonoids are used as feeding deterrents against harmful insects by some plants, together with terpenoids, alkaloids, hydrocarbons (Harborne, 2014). Some flavonoid classes;,flavonols, flavones, proanthocyanidins, flavan 3-ols, flavanones, flavans, and isoflavonoids mainly detected from the legume plants as feeding deterrents (Iwashina, 2000, and 2003). Moreover, flavanones compounds; 5- hydroxyisoderricin, 7-methoxy-8-(3-methylbutadienyl) - flavanone and 5-methoxyisoronchocarpin, which were isolated from three Tephrosia species, were proved to be feeding deterrents against *Spodoptera exempta* and *S. littoralis*. (Simmonds *et al*., 1990 and Simmonds, 2001).

Flavonoids activities on insects not limited to antifeedance or deterrence effects but also It has been reported that some types of flavonoids have had an effect on agricultural pests with ovicidal effect, oviposition, fecundity, mortality, weight reduction, and emergence of adults (Salunke *et al* 2005) beside toxicological impacts that suggested by some researches. For instance (Mesbah *et al.*, 2007 a&b) reported the quercetin activity and also its synergistic effect when combined with four insecticides; profenofos, deltamethrin, and tebufenozide as insect growth regulators against the studied insect-pest *Spodoptera littoralis*. Likewise, (Goławska *et al*., 2014) revolved the effects of two polyphenolic flavonoids; naringenin and quercetin on development, fecundity, and mortality of the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), were determined in vitro, on an artificial diets. Larvicidal effect of quercetin against *Spodoptera litura* Fab. (Noctuidae: Lepidoptera) was also reported by Stevenson *et al.* (1993).

Egyptian leafworm*; S. littoralis.* a polyphagous insect and can attack various economical crops throughout the year (EPPO, 2014). Mainly, it results in economic losses to a broad range of ornamental, industrial and vegetable crops in Egypt (Daddi *et al*; 2013). When synthetic insecticides have been used continuously over the year; insect resistance was the hatful result (Abo-El-Ghar *et al.*, 1986; El-Baramawy *et al* 1991). In spite of the compulsory limitation of the application of synthetic pesticides to exhibit pest resistance, the environmental pollution and human health still the main hazard of this approach (Zielhuis *et al* 2012; Zhu *et al*., 2016 and Awad, 2017). That's what persuaded the global public to search for alternative save methods to control agriculture pests. Over the last two to three decades, greater attention has been focused on exploring the bioactivity of phytochemicals for their potential as pesticides against insect- pests (Padmaja and Rao, 2000). Because of the phagostimulant and phagodeterrent effects of plant metabolites, the current study aimed to reveal the axial role

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of flavonoids as plant metabolites in protecting crops against insects' infestation.

MATERIALS AND METHODS

Plant material

Cynara cardunculus L. was collected "March 2015" in the flowering stage from the medicinal plants garden, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt. The plant identification was verified by Prof. Dr. Husain Abdel Basset, professor of Plant Taxonomy, Faculty of Science, Zagazig University, Zagazig, Egypt. A voucher specimen is deposited to the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Extraction and fractionation of the aerial parts of *Cynara cardunculus* L.

Arround (1 kg) of the air dried aerial parts of *C. cardunculus*. was milled and extracted by cold maceration with 80 % ethanol at room temperature till complete exhausion. The resulting crude hydroalcoholic extract was evaporated under vacuum. The concentrated extract (400 g) was suspended in water/methanol (9:1) then partitioned successively with petroleum ether, chloroform and ethyl acetate to afford 35.0 g, 27.0 g, and 15.0 g of petroleum ether, chloroform, and ethyl acetate fractions respectively.

Isolation of apigenin and luteolin from chloroform fraction of *Cynara cardunculus* L.

About 20 g of the chloroform soluble fraction was subjected to silica gel column, elution was started with benzene and the polarity was increased gradually with chloroform and ends with methanol. Fractions of 500 ml each were collected, screened by TLC.

Apigenin: Fractions 16-30 eluted with 4% methanol in chloroform gave 80 mg of yellow sandy crystals (MeOH). Rf 0.79 (system ethyl acetate: formic acid: H2O (12:0.5:0.5)) and m. p. 350-352 ºC. The compound gave yellow color with NH4OH, AlCl3 (T.S.) and 50% aqueous H2SO4 and a blue color with FeCl3 (T.S.).

Luteolin: Fractions 40-68 eluted with 10% methanol in chloroform gave 100 mg of yellow sandy crystals (MeOH). Rf 0.70 (system ethyl acetate: formic acid: H2O (12:0.5:0.5)) and m. p. 328-330 ºC. The compound gave yellow color with NH4OH, AlCl3 (T.S.) and 50% aqueous H2SO4 and a blue color with FeCl3 (T.S.).

Isolation of luteolin-7-*O*-glucoside from ethyl acetate fraction of of *Cynara cardunculus* L.:

About 15 g of the ethyl acetate soluble fraction of the air dried aerial parts of *C. cardunculus*. was placed on the top of silica gel column and gradually eluted with chloroform then methanol. Fractions of 250 ml each were collected and monitored with by TLC.

Luteolin-7-*O*-glucoside: Fractions 57- 81 eluted with 25% methanol in chloroform gave 300 mg of yellow sandy crystals (MeOH). Rf 0.59 (system ethyl acetate: formic acid: H2O (12:0.5:0.5)) and m. p. 240- 242 ºC. This compound gave positive results with Molisch's and Fehling's tests after hydrolysis yellow color with NH4OH, AlCl3 (T.S.) and 50% aqueous H2SO4 and a blue color with FeCl3 (T.S.).

Acid hydrolysis of luteolin-7-*O*-glucoside.

About 20 mg of the glycoside in aqueous H2SO4 (25 ml, 7 % reflux for 2 hrs) and the resulting sugar was rescreened by TLC (system, Butanol: acetic acid: water, 4:1:5). The aglycone gave a spot corresponding to luteolin.

Structure of isolate flavonoids were confirmed by Ultraviolet (UV), Infra-Red (IR) and Mass spectroscopy analyses.

Antifeedant Activity of isolated flavonoid on cotton leaf worm *Spodoperta littoralis*:

A laboratory strain of *S. littoralis* was reared under controlled conditions on clean fresh leaves of castor bean, *Ricinus communis* L. according to El-Defrawi *et al.* (1964) at 26±1°C and 70±5% relative humidity in e Plant Protection Research Institute Zagazig, Sharqia Governorate, Egypt.

The experimental setting was based on Both Dual choice and No choice leaf-disks methods described by Morimoto *et al*. (2000) and (Kannan, *et al*., 2013). Disks of 6 cm diameter, were prepared with a cork borer from fresh castor (*Ricinus communis*) leaves. Disks were dipped in the previous prepared concentrations of the three tested compounds in acetone separately for 5 sec. some were dipped in the acetone solvent only that used as control. All left to dry then weighted before provided to tested larvae.

In dual choice test; one treated disk and another control disk were set in alternating positions in the same petri dish (15 cm diameter) containing wet filter paper. Ten 4th instar larvae (starved in room at 25 ºC for 3 hr) were replaced in and left to feed for 48 hr. Three replicate were maintained for each concentration of each compounds. The partially consumed leaf-disks were weighted and from the weight of the complete disks before feeding with consideration of moisture loses to reference disk. The percent antifeedant index was calculated using the formula of Jannet *et al*. (2000).

Antifeedant Index (AFI) = (C - T)/(C + T) × 100

Where C is the consumption of control leaf disks and T is consumption of treated leaf disks.

For no- choice test, the treated disks were set in separate Petri dishes (15 cm diameter) containing wet filter paper; one per one while the control contain one untreated disk. Then, 10 4th instar larvae (starved in room at 25 ºC for 3 hr) were placed in and left to feed for 48 hr. 3 replicate were maintained.

The percent antifeedant index was calculated using the formula suggested by Gebbinck *et al*, (2002)

Antifeedant Index (AFI) = (C - T/ C) × 100

Where C is the consumption of control leaf disks and T is consumption of treated leaf disks.

Biochemical assays of digestive enzymes.

Sampling for biochemical assays were performed in both untreated and starved larvae as control. Larvae treated with 1000 ppm of the three tested flavonoid that collected from the no choice test. Samples were homogenized in cold distilled water with rate (50 mg /1 ml). Homogenates were centrifuged at 4000 rpm for 10 min. The supernatants, were stored at 5ºC.

Amylase and invertase activities were assayed by the dinitrosalicilic acid (DNS) according to Ishaaya and Swiriski (1976). The proteolytic activity of Digestive protease was determined using Azocasein as a substrate according to (Olga *et al.*, 2002; Mohen and Gujar, 2003)

Statistical analysis.

One-way analysis of variance (ANOVA) and Significant differences between treatments were determined by Tukey's multiple range tests ($P < 0.05$).

The maiden deterrent concentration DC_{50} value of each tested compounds was calculated using Probit Analysis (Finney, 1971).

RESULTS AND DISCUSSION

The MS of apigenin showed a parent ion peak at m/z 270 (for C15H10O5) with a fragment at m/z 152 of ring B suggesting the presence of two hydroxyl groups. Also, fragment t m/z 118 indicated the presence of only one hydroxyl group on ring A. The crucial replacement of two hydroxyl groups on ring A at C-5 and C-7 as well as the hydroxyl group on ring B at C-4" was indicated by UV shift reagents as indicated by (Mabry *et al*., 1970) .

IR νKBr max cm-1: 3500 – 3300 (broad OH-stretching), 2924 (CH- stretching), 1652 (C=O), 1607 (C=C), 1400, 1354(CH3- bending), 1297, 1244, 1181, 1163, 1116 (C-O), 908, 829, 692, 670(out of plane bending). EI MS m/z (% relative abundance): 272 (2), 271 (13), 270 (M+,100), 253 (1), 242 (17), (185) (0.1), 153 (12), 152 (8), 139 (1), 124 (12), 121 (10), 120 (1), 118 (11), 112 (0.4), 97 (1), 83 (1), 69 (10) and 49 (11). UV λmax max (MeOH) nm: 267, 335; (+ NaOMe): 275, 325(sh), 340; (+ AlCl3): 275, 301 (sh) 340; (+ AlCl3 + HCl): 276, 299, 343, 381 (+ NaOAC): 274, 300 (sh), 342, 375 (+ NaOAC + Boric acid): 268, 337.

The UV of luteolin showed two absorption bands at 260 and 348 nm indicating its flavone nature (Mabry *et al.*, 1970). The MS with a parent ion peak at m/z 286 (for C15H10O6) in accordance with a flavone containing four hydroxyl groups. The fragments at m/z 152 and 134 confirming the presence of two hydroxyl groups in each ring, (A and B). The placement of these hydroxyl groups was established by UV shift reagents (Mabry *et al*., 1970).

IR KBr νmax cm-1: 3418 – 3294 (broad OH-stretching), 2926, 2837, 1660 (C=O), 1614 (C=C) 1455, 1361(CH3- bending), 1264, 1168, 1118, 1031(C-O) and 837, 754, 685, 642, 600(out of plane bending C=C). EI MS m/z (% relative abundance): 286 (M+, 100) for C15H10O6 285 (11), 258 (17), 153 (27), 152 (1), 137 (4), 134 (13), 129 (17), 124 (9) and 105 (8). UV λmax max (MeOH) nm: 251, 265 (sh), 290 (sh), 348; (+ NaOMe): 270, 333(sh), 406; (+ AlCl3): 273, 300 (sh) 330, 421; (+ AlCl3 + HCl): 265, 273, 295, 353, 382; (+ NaOAC): 269, 324 (sh), 358; (+NaOAC + Boric acid): 261, 374, 430 (sh).

Luteolin-7-*O*-glucoside gave positive Molischs test suggesting its glycosidic nature. Acid hydrolysis yielded an aglycon identical to luteolin (MS, UV, IR, co- TLC). The sugar part was glucose (Rf, 0.25, System, Butanol: acetic acid: water, 4:1:5) against authentic samples. The sugar position was established by UV shift reagents and through comparison with the reported data (Mabry *et al.*, 1970).

IR KBr νmax cm-1: 3500-3400 (broad OH-stretching), 2930, 2868 (CH- stretching) 1635(C-C), 1457, 1379(CH3 bending), 1165, 1075, 1024(C-O) and 800(out of plane bending).C=C).EI MS m/z (% relative abundance): 286 (M+, 43) for C15H10O6, 256 (10), 153 (20), 152 (10), 137 (4), 134 (12), 124 (13), 105 (3) and 60 (100). UV λmax (MeOH) nm: 255, 267 (sh), 344; (+ NaOMe): 265, 300(sh), 382,394; (+ AlCl3): 274, 299 (sh) 339, 429; (+ AlCl3 + HCl): 274, 294 (sh), 351, 388; (+ NaOAC): 257, 266 (sh), 361, 410; (+NaOAC + Boric acid): 259, 371.

Based on the antifeedant index percentage and the DC50 of the three tested flavonoids (Table 1); Luteolin-7-O-glucoside were shown to have the strongest insect antifeedant activities in both dual and no-choice tests antifeedant index 74.50, 91.40 % respectively at a concentration of 1000 ppm while its DC_{50} values were 458.2 and 303.5 ppm.in both leaf-disks choice tests. In contrast, Luteolin aglycon revealed the lowest antifeedant activity reflected in the high DC_{50} values (7916.6 ppm. and 1224.9 ppm). Where the AFI at all tested concentrations didn't exceed 22.57% in the dual choice test and 45.40 % in the no-choice test respectively .

Furthermore, Apigenin cleared moderated antifeedant effects compared to the two other flavonoids with DC_{50} values of 850.30 ppm and 449.1 ppm while recorded 53.45% and 71.61% as antifeedant index percent via both dual and no choice testes respectively with 1000ppm.

Table 1. Antifeedant activity of Apeginin, Luteolin and Luteolin-7-*O*-glucoside against n 4th instar larvae of *Spodoptera littoralis*.

Tested compounds	Concentrations	Dual - choice test		No choice test	
	ppm	AFI %	DC_{50} (ppm)	AFI %	DC_{50} (ppm)
Apigenin	250	$20.37\pm0.076_{g}$	850.30	$36.34\pm0.048_{g}$	449.1
	500	$38.49\pm0.077_{d}$		$50.34\pm0.002_{d}$	
	1000	$53.45\pm0.109_{c}$		$71.61\pm0.052_{c}$	
Luteolin	250	$11.24\pm0.013_{i}$	7916.6	$19.59\pm0.090_{i}$	1224.9
	500	$19.27\pm0.025_{h}$		$33.47\pm0.143_{h}$	
	1000	$22.54\pm0.056_{f}$		$45.40\pm0.089_{f}$	
Luteolin-7-*O*-glucoside	250	$24.57\pm0.127_{e}$	458.2	$40.39\pm0.069_{g}$	303.5
	500	$59.40\pm0.103_{b}$		$74.56\pm0.018_{b}$	
	1000	$74.50\pm0.067_{a}$		$91.40\pm0.036_{a}$	

***Within the column, mean followed by the same letter do not differ significantly using Tukey's test, $P < 0.05$**

It has been noted, the effect of Luteolin aglycon is considered half of the impact of Luteolin-7-O-glucoside; the glucosyl derivative of lutoelin. This finding simply referred to that the insect antifeedant activity increased due to the glucoside substituents on A-ring of flavonoid structure (Figure 1). Thus assert the belief that the antifeedant activity of flavonoid was strongly affected by the substituent in A-ring (Morimoto *et al*, 2000) and (Morimoto and komai, 2006). In particular, the 6-substituted derivatives of flavonoids showed strong

antifeedant activity against the common cutworm. (Morimoto *et al*, (2003).Also, Bouaziz *et al*. (2005) reported similar results with the flavonoids; tricin and tricin 7-O-glucoside when tested the feed modulatory effects on the locusts *Locusta migratoria.*

By comparing the activity of the two tested flavonoids aglycons; apigenin and luteolin, it was noticeable that apigenin was stronger than luteolin as an antifeedant and a deterrent against S.littorales larvae .based on chemical structure data (Figure 1) luteolin have an extra-hydroxyl substituent at B-ring that might be affected its insect -deterrent activity. This suggests that the increase in hydroxyl substituent in the flavonoid structure could strongly decrease the antifeedant activity. (Morimoto *et al*, (2003).

It worth to mention, that apigenin and apigenin glycosides were recorded as feeding deterrents herbivores besides causing high mortality and modified the behavior of the pea aphid (Feeny *et al* 1988 and Agrell *et al*, 2003). Also, (Agrell,*et al*,2003) reported that the total concentration of flavones in *S. littoralis* infested and uninfested alfalfa plants were not significantly different. On the other hand luteolin did not influence the feeding of three species of insects. Stevenson *et al* (1993)

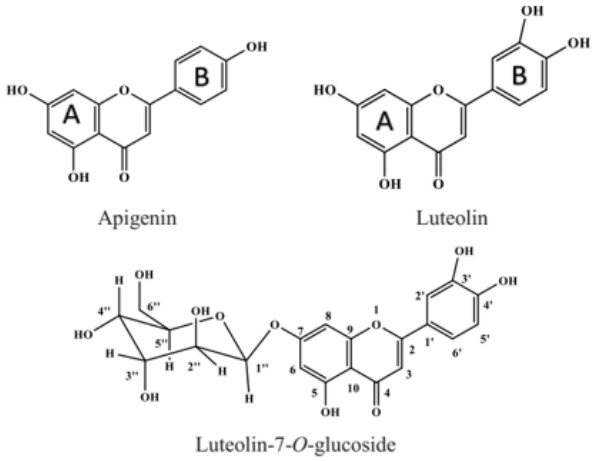

Figure 1. Chemical structure of isolated flavonoide from *Cynara cardunculus*

Mostly, if the disturbance of the digestion process was not the axial reason for in feed deterrence it will surely affected by it. This hypothesis clearly appears in recorded data of the activity of carbohydrate hydrolyzing enzyme; amylase and invertase (Figure 2). these enzymes activity decreased significantly corresponding with the increase of antifeedant activity of tested flavonoids where luteolin -7-O-glucoside recorded the lowest activity (97.22 &168.65 U/g) of amylase and invertase respectively compared to normal control (204.2 & 406.34 U/g) and starved control (165.23 & 231.45 U/g). As apigenin was moderate in deterrent activity it was also the same in inhibition action on amylase and invertase activity reading (104.59 & 178.65 U/g) respectively. The changes in amylase and invertase activities were significant using Tukey's test, $P < 0.05$.

The mechanisms of action of flavonoids at the enzymatic level are mostly unknown but, in a few recent studies, both luteolin and luteolin 7-O-glucoside recorded inhibitory effects on the pancreatic alpha-amylase in vitro (Kim *et al*., 2000). Also (Tadera *et al*., 2006) recorded that luteolin and apigenin inhibited amylase activity and evaluate their activity that predicted according to their structure. A different study by (Lo Piparo *et al*, 2008) investigated the structural requirements for inhibition of human salivary alpha-amylase by flavonoids. Results showed that the inhibitory activity of flavonols and flavones depends on hydrogen bonds between the hydroxyl groups and the catalytic residues of the binding site besides stabilizing the interaction with the active site.

Regarding to digestive protease, the biochemical assays didn't reveal any significant changes in activity in current study (Figure 2). In spite, most of the antifeedant insecticides affect protein digestion by disturbing the activities of the proteolytic enzymes; proteases Smirle *et al*. (1996). Likewise, the common insect' antifeedant; azadirachtin appeared to disturb the digestive process in insects, inhibiting the activity of digestive proteases in larvae of *Spodoptera litura* Koul *et al*, (1996) and *Manduca sexta* Timmins and Reynolds. (1992). that could mean; protease was not a target in flavonoid action.

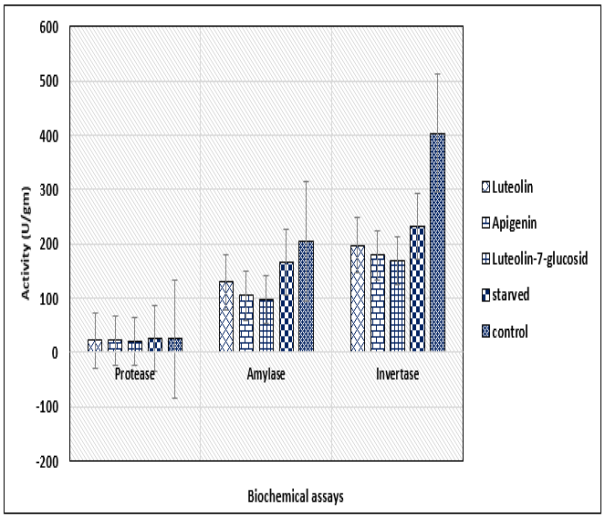

Figure 2. Changes in digestive enzymes of *Spodoptera littoralis* 4th instar larvae treated by Apigenin , Luteolin and Luteolin-7-*O*-glucoside (in 1000 ppm.)

CONCLUSION

The current research provide a new potential pesticides which developed using Flavonoids from ***Cynara cardunculus***, to control the Egyptian leaf worm; *S. littoralis*. Authors can recommended the three isolated flavonoids as an alternative to synthetic insect antifeedants and deterrents; they prevent larvae 'feeding and inhibit enzymatic activity and can protect crop plants from insect infestation according their chemical structure.

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الفلافونيدات الرادعة للحشرات من نبات الخرشوف البري ,*سينارا كارينكولارس* لمكافحة دودة ورق القطن.

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على الصعيد العالمي ، يومًا بعد يوم يزداد السعي نحو المستخلصات النباتيه للعثور على مواد مكافحة الآفات وصديقة البيئة . فمن خلال العمل الحالي ، تم فصل ثلاثة مركبات فلافونويدية من نبات الخرشوف البري *Cynara cardunculus* وتم تعريفها على أنها Apigenin و Luteolin و Luteolin-7-O-glucoside باستخدام تحليل مطياف الأشعة فوق البنفسجية والأشعة تحت الحمراء و طيف الكتلة . وتم تقييم فاعلية المركبات الثلاثة ونشاطها الرداع و المضاد للتغذية ضد يرقات العمر الرابع لدودة ورق القطن *Spodoptera littoralis* باستخدام كل من اختبارات اختيار اقرص الأوراق المزدوج والاختيار الواحد الاجباري .و أوضحت النتائج أن Luteolin-7-O-glucoside سجل أقوى نشاط مانع للتغذية و التي وصل إلى (74.50 ، 91.40٪) يليه Apigenin (53.45٪ و 71.61٪) في كلا الاختبارين على التوالي ، بينما لم يتجاوز مؤشر منع للتغذية Luteolin 22٪ بتركيز 1000 جزء في المليون. علاوة على ذلك ، فإن نشاط الإنزيمات الهضمية الاميليز والانفرتيز انخفض بشكل معنوي تبعا لمستوى نشاط ردع التغذية للمركب المختبر ، بينما أظهر نشاط البروتياز تغيرات غير معنوية في اليرقات المعاملة.وجدير بالذكر ،انه تم ملاحظة علاقة النشاط رداع للتغذية وتركيب الفلافونويد. وبالتالي فانه يمكن للفلافونيدات حماية نباتات المحاصيل من الإصابة بالحشرات وفقًا لهيكلها الكيميائي.