ISOLATION AND IDENTIFICATION OF DEFENSIVE FLAVONOIDS FROM *BRASSICA NAPUS* LEAVES EXTRACT WITH PROMISING BIOLOGICAL ACTIVITY TO CONTROL THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.)

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Abstract

Brassica napus plant has defensive secondary metabolites against phytophagous insects and pathogens. In extraction process, the end product contains traces of residual solvent, so, the solvent should not be non-toxic and should not interfere with the bioassay. The preliminary biological activity of ethanol, petroleum ether, methylene chloride, ethyl acetate and butanol fractions of *B. napus* leaves was investigated against the 4th instar larvae of *Spodoptera littoralis* under laboratory conditions. The ethanolic fraction showed the most toxic effect against the 4th instar larvae of *S. littoralis*. By phytochemical screening, *B. napus* leaves contain flavonoids as a major constituent. Four flavonoid compounds were isolated as crystals by column chromatographic technique using silica gel and purified on Sephadex LH-20. Their structures were elucidated as *Quercetin* (1), *Kaempferol* (2), *Kaempferol-3-O-glucoside* (3) and *Quercetin-7-O-glucoside* (4) by using UV and ^{1}H-NMR techniques. On toxicological studies, LC_{50} values (0.455 and 0.091 gm/100 ml) at 7 and 14 days post treatment, respectively, showed that the toxicity of *B. napus* extract increased with increasing concentration and time post treatment. This indicated that *B. napus* extract can be used as green insecticide to control the cotton leafworm, *S. littoralis*.

Keywords: *Brassica napus*, defensive secondary metabolites, flavonoids, column chromatograhy UV, ^{1}H-NMR, cotton leafworm, *Spdoptera littoralis*.

INTRODUCTION

The environmental problems caused by overuse of pesticides have been the matter of concern for both scientists and public in recent years due to the high toxicity and non-biodegradable properties of pesticides, the residues in soil, water resources and crops that affect human health.

The move toward green chemistry processes need for developing new crop protection tools with novel modes of action makes discovery and commercialization of natural products as green pesticides. Natural products are excellent alternative to synthetic pesticides as a mean to reduce negative impacts to human health and the environment.

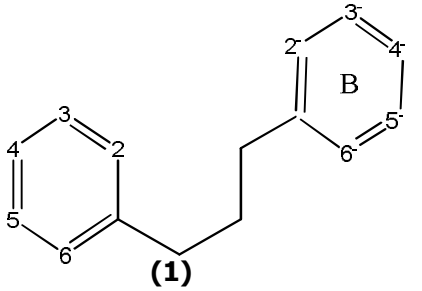
The concept of "Green pesticides" refers to all types of nature-oriented and beneficial pest control materials that can contribute to reduce the pest population and increase food production. They are more compatible with the environment components than synthetic pesticides (Isman and Machial, 2006).

Plants produce a great variety of secondary metabolites including terpenoids, alkaloids, steroids, flavonoids, tannins and saponnins are often important for mediating interactions between plants and their biotic environment (Kessler and Baldwin, 2002). They can be models of active defense against phytophagous insects and pathogens.

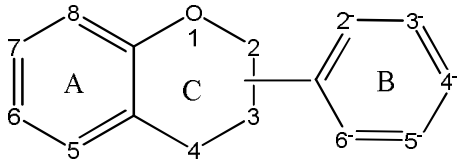
Phenolic compounds are a large group of secondary metabolites widespread in the plant kingdom. They can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids, depending on their structure.

Flavonoids are a ubiquitous and widely distributed group of natural products found in plants, they have been classified into different types of structures according to differences in aglycone, sugar moieties, inter-saccharide linkage and glycosylation position.

The most flavonoids that possessing fifteen carbon atoms with two aromatic rings joined by a linear three carbon chain. It can be described as a series of $C_6 - C_3 - C_6$ (1). The chemical structure of flavonoids is based on C_{15} skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4 (2). In a few cases, the six-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a five-membered ring (3). The oxygen bridge involving the central carbon atom (C_2) of the 3C-chain occurs in a rather limited numbers of cases, where the resulting heterocyclic is of the furan type.



(1)



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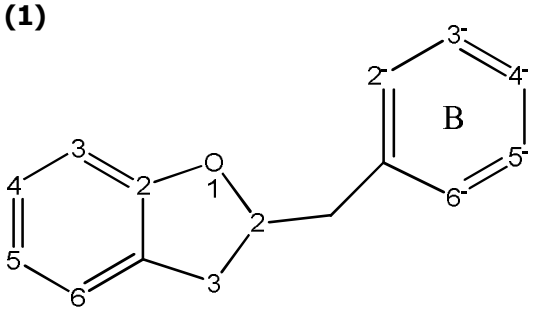


(3)

Flavonoids play important roles in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors and precursors to toxic substances, besides having long been recognized as possessing anti-allergic, anti-inflammatory, anti-viral, anti-proliferative and anti-carcinogenic activities in animals (Nijveldt *et al.,* 2001)

In Egyptian fields we observed that, canola (*Brassica napus*) plant has defensive secondary metabolites against insects; especially lepidopteran cotton leafworm, *Spodoptera littoralis,* which has its importance as one of the most destructive phytophagous lepidopterous pests in Egypt.

Brassica napus is a member *Brassicaceae* family which consists of 350 genera and about 3.500 species, it is known that seeds of winter rapeseed (*B. napus*) contain high amount of phenolic compounds. In fact, canola seeds are much richer than in phenolic compounds compared to other oilseeds (Padillia *et al.,* 2007).

In extraction process, successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. As the end product in extraction will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay Ncube *et al*., (2008). Initial screening of plants for possible insecticidal and antimicrobial activities typically begins by using the crude or alcohol extractions and can be followed by various organic solvent extraction methods.

This study was directed to investigate the biological activity of *B. napus* leaves extract against the cotton leafworm, *S. littoralis*. Isolation and purification of bio-active flavonoids using Column Chromatography (CC), Thin Layer Chromatography (TLC) and Sephadex LH-20. Structure identification of isolated flavonoids using different spectral methods as UV and ^{1}H-NMR techniques.

MATERIALS AND METHODS

Materials

a. Plant material

Brassica napus leaves were collected from a field of experimental farm in agricultural research institute, Ismailia governorate, washed to clean and left to dry under room temperature. The dried leaves were homogenized to fine powder by using electric mill.

b. Solvents

Ethanol, ethyl acetate, methylene chloride, petroleum ether and n-butanol were obtained from Edwic Company.

c. Insect

S. littoralis strain used in this study is a laboratory susceptible strain reared in the plant protection research institute, Dokki, Giza, Egypt according to EL-Defrawi *et al.*, (1964). The culture was maintained in climatic chamber under optimum conditions 25±2ºC, 75±5% R.H and (16L:8D) light: dark photoperiod, where reared on fresh castor bean leaves. The newly molted 4th instar larvae were selected for this study.

Instrumentations

^{1}H-NMR spectra were measured by 300 MHz-NMR spectrometer (at Cairo University, Nuclear Magnetic Resonance Laboratory), chemical shifts are given in δ (ppm) relative to TMS as internal standard compound. UV spectra were recorded on a Shimadzu 240-PC spectrometer (at the National Research Center). CC Column Chromatography was performed on silica gel Merck grain size 0.2-0.063 nm and on Sephadex LH-20. TLC Thin Layer Chromatography was carried out with Silica gel GF_{254} pre-coated plates 20 x 20 cm on aluminum sheets. The isolated compounds were sprayed with 10% sulfuric acid and were visualized on plates under UV lamp at 254 and 365 nm lamp.

Extraction

Dried leaves powder (400 g) was extracted by soaking at room temperature in ethanol 60% (v/v). The resulting extract was filtrated and evaporated under vacuum at 40ºC by using rotary evaporator to give ethanol extract (65.8 g) as a dark green residue. Part of the extract (30 g) was dissolved in least amount of ethanol (~ 10 ml) and suspended in distilled water and subjected to sequential liquid-liquid extraction with petroleum ether, methylene chloride, ethyl acetate, and n-butanol.

We used ethanol solvent in the extraction process, the choice will also depend on the targeted compounds to be extracted, the flavonoid and phenolic acid compounds were affected by the concentration of ethanol solvent. The single-factor experiment showed that 60% ethanol was suitable to extract the phenolic constituents from the plant. The levels of phenolic contents were decreased as the concentration of ethanol increased Harborne (1984); Harmala *et al.*, (1992).

Preparation of tested concentrations

The residues were diluted in distilled water containing one drop of an emulsifier Tween® 20 (Tween® 20; Sigma-Aldrich), to ensure complete solubility of the material in water. The concentrations used were selected after preliminary bioassays with a wide range of concentrations to determine the range needed. After

the proper range was obtained, at least five serially diluted concentrations covering the range 10-90% mortality were prepared.

Two negative control treatments were used in each experiment. The one was one drop of emulsifier (Tween® 20) in water, since the Tween® 20 solutions showed no significant mortality effect against the *S. littoralis* larvae (data not shown) compared with water.

Biological studies

Leaf dipping technique was applied; Castor bean leaves were dipped for 30 seconds in each concentration of ethanol extract and fractions then left to dry. The treated leaves were offered to newly molted 4th instar larvae of *S. littoralis* for 48 hrs then replaced by untreated ones. Accumulative mortality percentages were recorded till 7 and 14 days post treatment, then corrected according Abbott's formula (1925). From the corrected mortality percentages the corresponding toxicity lines (LC-P lines) were estimated in addition to determine LC_{25} and LC_{50} values and their confidence limits, slope values of tested extract were also estimated.

Abbott's formula

$$\text{Corrected mortality percentage} = T - C/100 - C$$

Where; T: Larval mortality percentage in treatment

C: Larval mortality percentage in control.

Preliminary phytochemical screening of *B. napus* extract

Phytochemical screening of *B. napus* leaves extract was performed by following standard procedures (Sofowara, 1993; Harborne, 1998); In brief, 0.5 ml of extract was added with a drop or two of Mayer's regent by the side of test tube and the formation of white to yellowish precipitate indicates presence of alkaloids. Appearance of faint pink to dense pink color within three minutes after addition of 1 ml of extract with conc. Hydrochloric acid and magnesium powder indicates presence of flavonoids. Change of color from violet to blue or green (lower layer) after the addition of 2 ml of acetic anhydride and sulphuric acid gives positive result for steroids.

Addition of 2 ml of chloroform and 3 ml of conc. sulphuric acid to the extract and formation of reddish brown layer at the junction of two solutions confirms presence of terpenoids. Existence of froth formation during warming and vigorous shaking indicates presence of saponins. Appearance of brownish green or blue black coloration after adding 0.1% ferric chloride to the cooled extract indicates tannins.

Phytochemical study of ethanol extract

From preliminary biological studies, the most toxic fraction of *B. napus* leaves against the 4th instar of *S. littoralis* was studied phytochemically in details.

1. Isolation of flavonoids from *B. napus* leaves extract

The extract was chromatographed using column chromatography on a Silica gel eluted with chloroform-methanol starting from 10:0 to 4:1 gradually; eluted fractions were combined on their TLC pattern to yield nine fractions. The chloroform-methanol sub-fraction (9:1) was purified on a Sephadex LH-20 column eluted with chloroform-methanol (1:1) to yield pure compounds (1) and (2). The chloroform-methanol sub fraction (6:4) chromatographed on a Sephadex LH-20 column eluted with methanol and further purified using preparative TLC developed in a mixture of $CHCl_3$/ MeOH (6:4, v/v) yielded compounds (3) and (4) (Harborne and Mabry, 1982).

2. Investigation of the sugar moiety of isolated flavonoids

The aqueous acidic mother-liquor remained after extraction with ethyl acetate was neutralized with excess barium carbonate, then filtered. The filtrate after removal of the precipitated barium sulphate was evaporated under vacuum, the residue contained the sugar moiety (glycone) was then dissolved in 10% isopropanol. The alcoholic solution was subjected to paper-chromatography using various monosaccharides samples for comparison as authentic (glucose, galactose, xylose, rhamanose and arabinose). The paper chromatogram was developed twice with n-butanol-acetic acid-water (40:10:50) upper layer. Detection was carried out by spraying with aniline / phthalic acid reagent and heating at 110 ºC in an oven, a brown to reddish-brown colored spots were developed.

3. UV-measurements of isolated flavonoids

The UV absorption of isolated flavonoids was measured according to (Horowitz and Jurd 1961; Mabry *et al.,* 1970; Mears and Mabry 1972). Flavonoids contain conjugated aromatic system and show intense absorption bands in UV and visible regions of spectrum. The absorption spectra of isolated flavonoids were measured using Shimadzu 2401-PC spectrometer. A stock solution of the flavonoid substance was prepared by dissolving a small amount of the compound (about 0.1 mg) in about 10 ml of spectroscopic methanol. The concentration was adjusted so that the optical density of the major absorption peak between 240 and 400 nm gave an optical density (OD) reading in the region 0.6 to 0.8.

Stock Solutions and Solids reagents for UV- analysis

- Sodium Methoxide (NaOMe): Freshly cut metallic sodium (2.5 g) was added in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a glass container with tightly fitting plastic stopper.
- Aluminium Chloride ($AlCl_3$): Five grams of fresh anhydrous reagent grade $AlCl_3$ were added cautiously to spectroscopic methanol (100 ml).

- Hydrochloric Acid (HCl): Concentrated reagent grade hydrochloric acid (50 ml) was mixed with distilled water (100 ml); the solution was stored in a glass stopped bottle.
- Sodium Acetate (NaOAc): Anhydrous powdered reagent grade sodium acetate was used.
- Boric Acid (H_3BO_3): Anhydrous powdered reagent grade boric acid was used.

Procedure of UV- measurements

1. The methanol spectrum was measured at normal scan speed (about 50 nm / min.) using 2-3 ml of the stock solution of the flavonoid.
2. The sodium methoxide was measured immediately after the addition of three drops of sodium methoxide stock solution to the solution used for step 1, and after 5 min. the spectrum was rerun to check for flavonoid decomposition.
3. The aluminum chloride spectrum was measured immediately after the addition of three drops of the aluminum chloride stock solution to 2-3 ml of fresh stock solution of the flavonoid.
4. The aluminum chloride / hydrochloric acid spectrum was recorded immediately after the addition of three drops of the stock hydrochloric acid solution to the solution used for step 3.
5. The NaOAc spectrum was determined by the addition of excess coarsely powdered anhydrous sodium acetate to 2-3 ml fresh stock solution of the flavonoid and shaking the cuvette, (about 2 mm layer of sodium acetate remained at the bottom of the cuvette), and the spectrum was recorded within 2 min. then it was rerun after 5-10 min. to check the flavonoid decomposition.
6. The sodium acetate / boric acid spectrum was determined by the addition of sufficient powdered anhydrous boric acid to give a saturated solution to the cuvette from step 5 containing the sodium acetate.

RESULTS AND DISCUSSION

Leaves of canola (*Brassica napus*) were used in this study due to the non-economically importance of the plant´s leaves, we observed that *B. napus* plant has self defense property in field against insects especially lepidopteran cotton leafworm, *Spodoptera littoralis. B. napus* may be containing defensive secondary metabolites against insects, so its leaves extract could be used as botanical insecticide against *S. littoralis*.

Flavonoids have multiple additional roles in plants, including attracting insects for seed dispersion and pollination. They are also part of the natural defense system against insects, fungi, viruses and bacteria and they act as plant hormone controller. Also they have been reported to possess many useful properties for human health, including anti-inflammatory, enzyme inhibition, antimicrobial, vascular and cytotoxic

antitumor activity, but the most action of flavonoids their antioxidant activity (Chu *et al*., 2000).

Preliminary biological activity

The *B. napus* leaves ethanolic extract and its fractions; petroleum ether, methylene chloride, ethyl acetate, and *n*-butanol were investigated for their biological activity as insecticides against the 4th instar larvae of *S. littoralis* under laboratory conditions.

Table 1. The preliminary insecticidal activity of *B. napus* leaves extracts at conc. (1gm / 100 ml) against 4th instar larvae of *S. littoralis* at 7 and 14 days post treatment.

Extract	Mortality percentages %	
	7-Days post treatment	14- Days post treatment
Ethanol	60 ± 4.3	86.0 ± 5.9
Petroleum ether	42.0 ± 9.0	67.67 ± 8.1
Methylene chloride	33.33 ± 5.9	40.0 ± 7.5
Ethyl acetate	40.0 ± 4.7	55.7 ± 4.3
n-butanol	10.0 ± 2.3	20.0 ± 5.9

The preliminary insecticidal activity of concentration 1gm/100 ml for each extract at 7 and 14 days post treatment revealed that ethanol extract is the most toxic extract followed by petroleum ether, ethyl acetate, methylene chloride and finally butanol extract against the 4th instar larvae of *S. littoralis* under laboratory conditions. So, the ethanolic extract of *B. napus* leaves is the promising extract therefore, it was selected to be studied in details.

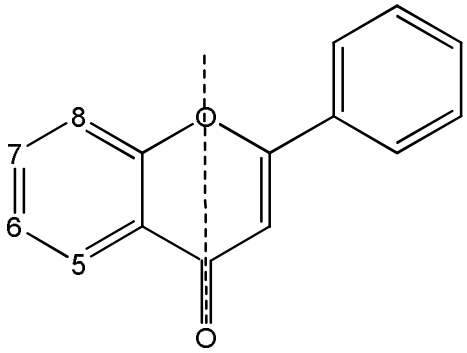
Preliminary phytochemical screening

Phytochemical screening of *B. napus* extract revealed the presence of flavonoids as a major constituent; steroids and terpenoids.

Isolation and identification of flavonoids

The isolated compounds were identified using UV and ^{1}H-NMR spectral data, and determined as *Quercetin* (1), *Kaempferol* (2), *Kaempferol-3-O-glucoside* (3) and *Quercetin-7-O-glucoside* (4) as follow;

The UV spectra of most flavonoids consists of two major absorption maxima; one of which occurs in the range 240-285 nm (Band-II) and the other range 300-400 nm (Band-I). In general terms Band-II absorption may be considered as having originated from A- ring (benzoyl system) and Band-I from B-ring (cinnamoxyl system) (Jangaard, 1970).



II **I**

Benzoyl **Cinnamoxyl**

1. Structure elucidation of compound (1)

Compound (1) was isolated and purified as yellow crystals. The UV spectrum in methanol showed two major absorption bands at λ_{max} (256 and 370 nm) as shown in Table (2), indicator for this compound is flavonol, moreover, Band-II at λ_{max} (256 nm) indicated the presence of two oxygen atoms in B-ring.

Table 2. UV-absorption spectrum of compound (1)

Band	MeOH	NaOH	NaOAc	NaOAc/H_3BO_3	$AlCl_3$	$AlCl_3$/HCl
B-II	256	267	267	277	281	280
B-I	370	426	425	391	340-441 sh	340-428 sh

In addition of sodium hydroxide (NaOH) caused a bathochromic shift which leads to absorption Band-I (λ_{max} 426 nm), this proved the presence of free 4\-OH group, also a bathochromic shift which leads to absorption Band-II (267 nm) indicator for free 7-OH group, where NaOH is strong base that ionizes to some extend all hydroxyl groups on the flavonoid nucleus. Hence, for most hydroxylated flavonoids, shifts to longer wavelength are observed in both bands (Horowitz and Jurd, 1961).

Addition of sodium acetate (NaOAc) caused a bathochromic shift in Band-II (267 nm) indicated presence of free 7-OH group. Addition of boric acid caused a bathochromic shift in Band-I (391 nm) which proved the presence of catecholic OH-groups. This is compatible with Mabry *et al.,* (1970), where sodium acetate is a weaker base than sodium hydroxide and tend to ionize significantly only the more acidic phenolic hydroxyl groups (i.e. the 3,7- and 4\ - hydroxyl groups).

After addition of aluminum chloride ($AlCl_3$), high bathochromic shift leading absorption Band-I (340, 441 sh) indicated the presence of free 5 and 7-OH groups. The hypsochromic shift of $AlCl_3$ spectrum in Band-I (340, 428 sh) after addition of HCl, this indicated the presence of free ortho 3\, 4\-OH groups in ring B. Where aluminium chloride chelates with functional groups such as 5-hydroxyl-4-keto, 3-hydroxyl-4-keto and o-dihydroxy-systems and this reaction is evidenced by bathochromic shift of one

or both bands in the spectrum. In presence of small amounts of aqueous acid, the complexes formed with o-dihydroxyl groups and the 3-hydroxyl-4-keto system in dihydroflavonols decomposes. When o-dihydroxyl groups are present in the flavonoid together with 5- or 3-hydroxyl, a double complex is formed. Traces of water in ethanol (but not in methanol) prevent the formation of Al-o-dihydroxyl complexes. Methanol is thus the solvent of choice for spectral detection of o-di-hydroxyl groups (Mears and Mabry, 1972).

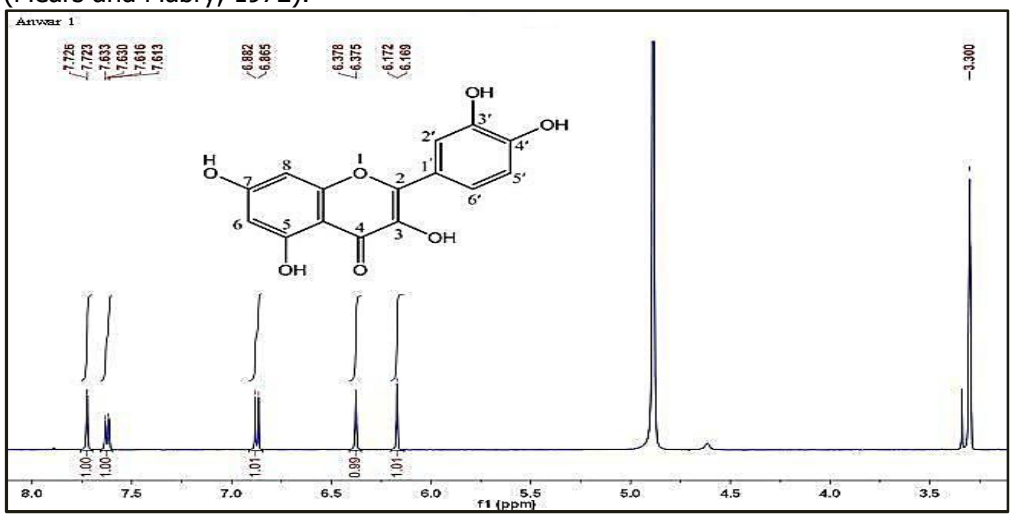


Fig. 1. ^{1}H-NMR spectrum of compound 1 (*Quercetin*).

The ^{1}H-NMR spectrum of compound (1) was characteristic for favonol as shown in Table (6) and Fig. (1), it showed two signals at δ 6.17 (1H, *d*) and 6.37 ppm (1H, *d*) consistent with the meta protons H-6 and H-8 on A-ring, On the other hand, the observation of ABX system at 7.72 (1H, *d*, H-2\), 7.61 (1H, *dd*, H-6\), and 6.88 (1H, *d*, H-5\) corresponding to the catechol protons on B-ring. All spectral data of compound (1) showed that the compound is *Quercetin* were compatible with those of Dhasan *et el.*, (2008).

2. Structure elucidation of compound (2)

Compound (2) was isolated and purified as yellow crystals. The UV spectrum in methanol showed two absorption Bands at λ_{max} (275 and 366 nm) as shown in Table (3), indicator for this compound is flavonol.

Table 3. UV-absorption spectrum of compound (2)

Band	MeOH	NaOH	NaOAc	NaOAc/H_3BO_3	$AlCl_3$	$AlCl_3$/HCl
B-II	275	284	285	285	280	280
B-I	366	420	380	380	340	340

The addition of sodium hydroxide (NaOH) caused a bathochromic shift which leads to absorption Band-I at λ_{max} (420 nm), this proved the presence of free 4\-OH group,

also a bathochromic shift which leads to absorption Band-II (284 nm) indicator for free 7-OH group.

After addition of sodium acetate, a bathochromic shift was observed in the spectrum of Band-II (285 nm) indicated presence of free 7-OH group. Addition of boric acid caused no shift in Band-II (285 nm) which proved the absence of any catecholic OH- groups.

In addition of aluminum chloride ($AlCl_3$), a bathochromic shift leading absorption Band-II (280 nm) indicated the presence of free 5 and 3-OH groups. No change was observed in Band-I (380 nm) of $AlCl_3$ spectrum after addition of HCl indicating the absence of any catecholic OH- groups.

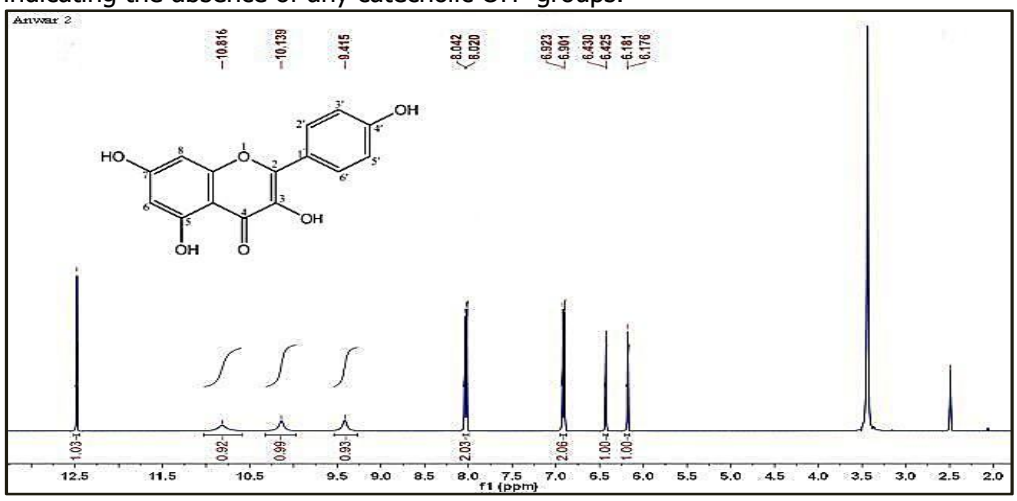


Fig. 2. ^{1}H-NMR spectrum of compound 2 (*Kaempferol*).

The ^{1}H-NMR spectrum of compound (2) is characteristic for flavonol as shown in Table (6) and Fig. (2), it showed two signals at δ 6.17 (1H, *d*) and 6.42 ppm (1H, *d*) consistent with the meta protons H-6 and H-8 on A-ring and an AA\ BB\ system at 8.04 (2H, *d*, H-2\ and H-6\) and 6.93 (2H, *d*, H-3\ and H- 5\) corresponding to the protons on B-ring. All mentioned spectral data showed that the compound (2) is *Kaempferol* which was isolated and identified previously from *Cissus quadrangularis* by Thakur *et al.,* (2009).

3. Structure elucidation of compound (3)

Compound (3) was isolated and purified as yellow crystals. The UV spectrum in methanol showed two absorption bands at λ_{max} (275 and 331 nm) indicator for this compound is flavonol.

Table 4. UV-absorption spectrum of compound (3)

Band	MeOH	NaOH	NaOAc	NaOAc/H_3BO_3	$AlCl_3$	$AlCl_3$/HCl
B-II	275	285	280	280	285	285
B-I	331	390	341	340	330, 340 sh	330, 340 sh

In addition of sodium hydroxide caused a bathochromic shift of Band-I at λ_{max}(390 nm), proved the presence of free 4\ -OH group. Also a bathochromic shift in

Band-II at λ_{max} (285 nm) indicated the presence of free 7- OH group. In addition of sodium acetate a bathochromic shift which leads to absorption Band II at λ_{max} (280 nm) and Band II (341 nm) indicating of free 7- hydroxyl group. Addition of boric acid caused no shift, which proved the absence of any catecholic OH groups.

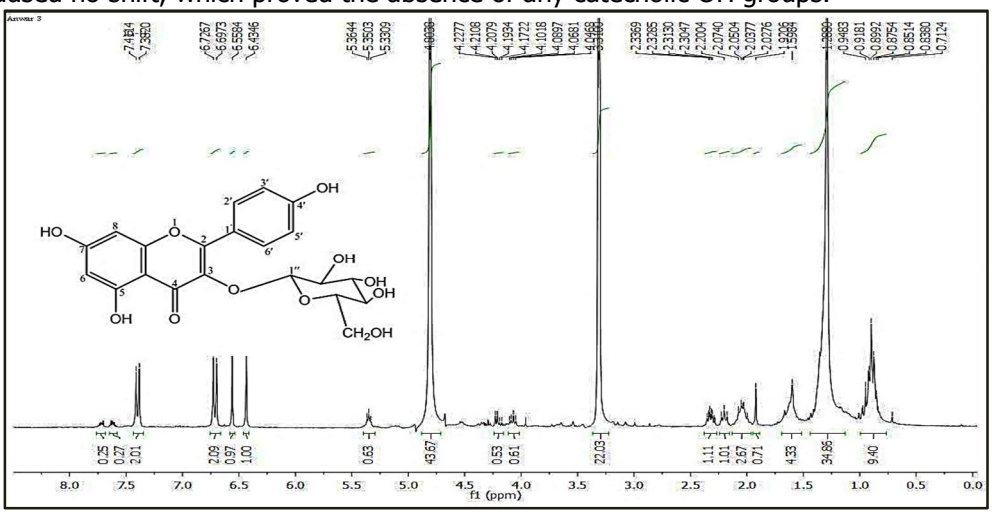


Fig. 3. ^{1}H-NMR spectrum of compound 3 (*kaempferol-3-O-glucoside*).

The ^{1}H-NMR spectrum is characteristic for flavonoid glycoside as shown in Table (6) and Fig. (3), it showed two signals at δ 6.42 (1H, *d*) and 6.55 ppm (1H, *d*) consistent with the meta protons H-6 and H-8 on A-ring. AA\ BB\ system at 7.38 (2H, *d*, H-2\ and H-6\) and 6.69 (2H, *d*, H-3\ and H- 5\) corresponding to the protons on B-ring. Compound 3 presented the same aglycone signal patterns of compound (2) but the signal at δ 4.803 ppm (1H, *d*) followed by other characteristic additional signals indicates the presence of a sugar moiety in compound (3). It showed signal at δ (3.3 ppm, *m*), where the sugar moiety was determined to be glucose bound to the C-3 position of the aglycone by comparison of proton and carbon upfield shift values with the literature data of Awaad *et al*., (2004), the compound (3) was identified as *kaempferol-3-O-glucoside.*

4. Structure elucidation of compound (4)

Compound (4) was isolated and purified as yellow crystals. The UV spectrum in methanol showed two absorption bands at λ_{max} (255 and 372 nm) indicator for this compound is flavonol, as shown in Table (2). The Band II at λ_{max} (255 nm) indicated the presence of two oxygen in B-ring.

Table 5. Ultra-Violet absorption spectrum of compound (4)

Band	MeOH	NaOH	NaOAc	NaOAc/H_3BO_3	$AlCl_3$	$AlCl_3$/HCl
B-II	255	243	255	262	273	273
B-I	372	432	380,433 sh	390	340, 434 sh	340, 420 sh

In addition of sodium hydroxide caused a hypsohochromic shift which leads to absorption Band-II at λ_{max} (243 nm) indicated the 7-OH substituent. Moreover, caused a bathochromic shift of Band-I at λ_{max} (432 nm), which proved the presence of free 4\- OH group.

After addition of sodium acetate, no bathochromic shift of spectrum in Band-II (255 nm) indicated presence of 7-OH substituent. After addition of boric acid caused a bathochromic shift in Band-I (390 nm), which proved the presence of catecholic OH-groups.

In addition of aluminum chloride, a bathochromic shift of Band-I (434 nm) indicated the presence of free 5,3-OH groups. The hypsochromic shift of $AlCl_3$ spectrum in Band-I (420 nm) after addition of HCl, this indicated, this indicated presence of free ortho 3\, 4\ hydroxyl groups in B-ring.

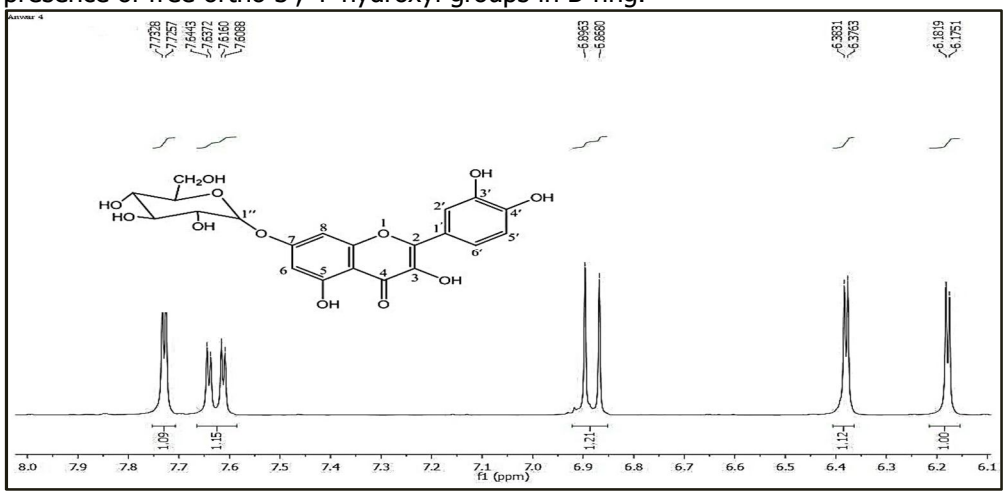


Fig. 4. ^{1}H-NMR spectrum of compound 4 (*Quercetin-7-O-glucoside*).

The ^{1}H-NMR spectrum of compound (4) was characteristic for favonol as shown in Table (6) and Fig. (4), it showed two signals at δ 6.18 (1H, *d*) and 6.37 ppm (1H, *d*) consistent with the meta protons H-6 and H-8 on A-ring, On the other hand, the observation of ABX system at 7.72 (1H, *d*, H-2\), 7.64 (1H, *dd*, H-6\), and 6.86 (1H, *d*, H-5\) corresponding to the catechol protons on B-ring. 7-O-substituted flavonol was indicated for corresponding anomeric proton at δ 4.83 ppm (1H-, *d*, H-1\\) characteristic for glucosyl moiety, upfield shift signal at δ 3.29 ppm (1H, *m*, glucosyl protons). All the previous spectral data of compound (4) showed that the compound is *Quercetin-7-O-glucoside* which was previously isolated and identified by Ying (2000).

Table 6. ^{1}H-NMR spectra of isolated flavonoids recorded in MeOH

H - atom	δ value (ppm) multiplicity	δ value (ppm) multiplicity	δ value (ppm) multiplicity	δ value (ppm) multiplicity
	1	2	3	4
6	6.17 (1H, *d*)	6.17 (1H, *d*)	6.42 (1H, *d*)	6.18 (1H, *d*)
8	6.37 (1H, *d*)	6.42 (1H, *d*)	6.55 (1H, *d*)	6.37 (1H, *d*)
2\	7.72 (1H, *d*)	8.04 (1H, *d*)	7.38 (1H, *d*)	7.72 (1H, *d*)
3\	-	6.93 (1H, *d*)	6.69 (1H, *d*)	-
5\	6.88 (1H, *d*)	6.93 (1H, *d*)	6.69 (1H, *d*)	6.86 (1H, *d*)
6\	7.61 (1H, *dd*)	8.04 (1H, *d*)	7.38 (1H, *d*)	7.64 (1H, *dd*)
1\\	-	-	4.803 (1H, *d*)	4.83 (1H, *d*)
Glucosyl protons	-	-	3.3 (1H, *m*)	3.29 1H, *m*)

Biological studies

In our screening program which is designed to evaluate the biological activity of ethanolic extract of *B. napus* leaves, it was found that the extract showed toxicity against the 4th instar larvae of *S. littoralis* under lab-conditions using leaf dipping technique, where the larvae fed on treated castor bean leaves for 48 hrs then transferred to untreated ones.

Table 7. Insecticidal activity of ethanolic extract of *B. napus* leaves against the 4th instar larvae of *S. littoralis*.

parameter	LC_{25} (gm/100 ml)	LC_{50} (gm/100 ml)	Slope ± SE
7-Days post treatment	0.064 (0.013 – 0.120)	0.455 (0.315 – 0.751)	0.794 ± 0.19
14-Days post treatment	0.016 (0.002 – 0.042)	0.091 (0.031 – 0.146)	0.91 ± 0.20

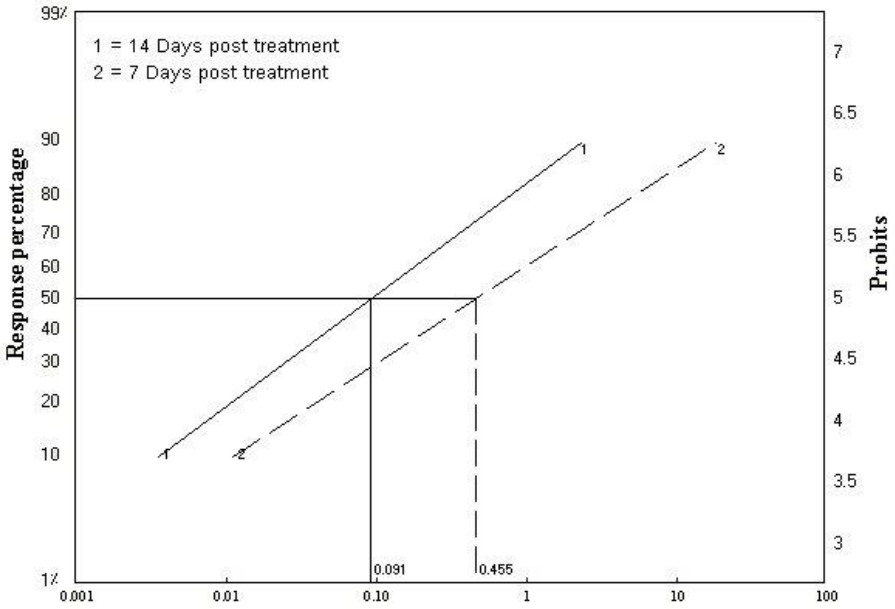


Fig. 5. comparative toxicity lines of ethanolic extract of *B. napus* leaves against the 4th instar larvae of *S. littoralis* at 7 and 14- days post treatment.

By using LC-P program; LC_{25}, LC_{50} and their confidence limits, and slope values were used as parameters in evaluation the insecticidal activity of tested extract, where mortality of larvae was recorded daily and accumulative larval mortality was recorded. It was observed that the toxicity increased with concentrations used and time post treatment according to descending order of LC_{25} and LC_{50} values, where LC_{25} values were (0.064 and 0.019 gm/100 ml) at 7 and 14 days post treatment, respectively. Also LC_{50} values were (0.455 and 0.091 gm/100ml) at 7 and 14 days post treatment, respectively. Furthermore, when the lower and upper confidence

limits were taken into consideration, it was found that all the points of LC_{25} and LC_{50} values were within these limits, as presented in Table (7) and Fig. (5).

Flavonoids are reported as a major class of phytochemicals constituting 5–10% of the known secondary metabolites in plants. They are involved, beside other things, in plant defense mechanisms by exerting toxic effects on insects, where to date, more than 5000 flavonoids are documented and their antimicrobial and insecticidal activities demonstrated (Kotkar *et al.,* 2002). The insecticidal activity of flavonoids is mainly the result of inhibition of vital enzymatic pathways, such as the actions of cytochrome-P_{450} dependent oxidases, in which affect insect ecdysone-20 monoxygenase, which is responsible for the biosynthesis of 20-hydroxyecdysone (Mitchell *et al.,* 1993), whereas the antimicrobial activity of flavonoids is attributed to changes in membrane structure (Padmavati and Reddy, 1999). Where Kuroyanagi *et al.,* (1999) found that flavonoids of *Thea sinensis* and *Sophora flavescens* have shown promising antimicrobial activity against gram-positive bacteria, *Staphylococcus aureus* Rosenbach and *Bacillus subtillis* (Ehrenberg) Cohn.

Quercetin and other pro-oxidant compounds can be metabolically activated to generate free radical species in insects upon ingestion. Their generation can result in direct cellular toxicity, or give rise to more toxic oxygen species and hydroxyl by oxygen radical cascade (Mendki *et al.,* 1999).

From all mentioned results, it can concluded that the ethanolic extract of *B. napu*s leaves and its flavonoids can be used effectively as botanical insecticide to control the cotton leafworm, *S. littoralis*, where *B. napus* leaves is by- product from the plant.

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فصل وتعريف الصيغ البنائية للفلافونيدات المسئولة عن خاصية الدفاع الذاتية من مستخلص أوراق نبات الكانولا التى لها تأثير بيولوجى لمكافحة دودة ورق القطن

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يحتوى نبات الكانولا على مركبات عضوية طبيعية لها دور فى الدفاع عنه ضد الافات و مسببات الأمراض. تم دراسة النشاط البيولوجى الأولى لمستخلصات الايثانول، الاثير البترولى، ثنائى كلور الميثان، خلات الايثيل، البيتانول لأوراق نبات الكانولا ضد يرقات العمر الرابع لدودة ورق القطن تحت الظروف المعملية، حيث أظهر مسنخلص الايثانول التأثير السام الأقوى ضد يرقات دودوة ورق القطن. تم التعرف على المكونات الموجودة فى اوراق نبات الكانولا حيث وجد أن الفلافونيدات هى المكون الرئيسى فى أوراق النبات. تم فصل و تنقية اربعة مركبات فلافونيدية عن طريق طرق الفصل الكروماتوجرافية. وتم التعرف على الصيغ البنائية لها عن طريق استخدام القياسات الطيفية مثل الأشعة الفوق بنفسجية وطيف الرنين النووى المغناطيسى للهيدروجين وهم: *Quercetin Kaempferol, Kaempferol-3-O-glucoside, Quercetin-7-O-glucoside*، أشارت نتائج دراسة سمية لمستخلص الايثانول ان سمية المستخلص تزداد بمرور الوقت بعد المعاملة حيث بلغت قيم التركيز اللازم لقتل ٥٠ % من الأفراد المعاملة (٠,٤٥٥ & ٠,٠٩١ ملجم/١٠٠ مل) بعد مرور ٧ & ١٤ يوم من المعاملة على التوالى. ربما تكون وجود هذه الفلافونيدات هى من أحد اسباب خاصية الدفاع الذاتية لأوراق نبات الكانولا، ويمكن استخدام مستخلص نبات الكانولا كمبيد من أصل طبيعى لمكافحة دودة ورق القطن.