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Evaluation of antioxidant, anti-inflammatory, and antinociceptive properties of aerial parts of *Vicia sativa* and its flavonoids

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Abstract

Objective: In this study, we aimed to identify the minor flavonoids of the areal parts of *Vicia sativa,* a biologically active plant growing in El-Hassana, North Sinai, Egypt, and to monitor the antioxidant, anti-inflammatory, and antinociceptive activities of the plant extract and its purified flavonoids. Methods: antioxiant activity of *V. sativa* was assessed by four different assays including DPPH assay, quenching of ROO radicals, respiratory burst in rat neutrophils, and lipid peroxidation. On the other hand, anti-inflammatory and antinociceptive effects of *V. sativa* and its flavonoids were assessed by carrageenan-induced inflammation in mice, acetic acid-induced writhing and formalin-licking test. Results: Nine flavonoids were isolated from the ethanol extract of the aerial parts of *V. sativa* and identified as active components: apigenin 4`-O-β-D-glucopyranoside [1], apigenin 6,8-di-C-β-D-glucopyranoside [2], apigenin 6-C-α-L-arabinopyranoside-8-C-β-D-glucopyranoside [3], kaempferol-3-O- β-L-dirhamnopyranosyl (1→2, 1→6)- β -D-glucopyranoside [4], kaempferol 3-O-(4-β-D-xylopyranosyl)-α-L-rhamnopyranoside-7-O-L-rhamnopyranoside [5], kaempferol-3-O- α-L-rhamnopyranosyl (1→6)- β-D-glucopyranoside [6], Luteolin-7-O- β-D-glucopyranoside [7] quercetin 3-O-β-D-glucopyranoside [8], and quercetin 3,7 di-O- glucopyranoside [9]. Our findings indicated a potent antioxidant activity of *V. sativa* extract against different radicals (DPPH, superoxide anion, and peroxyl) and an inhibitory activity against carbon tetrachloride-induced lipid peroxidation. compound 6 showed effective inhibitory activity of both peroxyl radicals; compounds 1, 2, 3, 6, 8 and 9 decreased both superoxide anion radical production in PMA-stimulated neutrophil, and compounds 2, 3, 5, and 7 were dramatically effective in inhibition of CCl_4 -induced lipid peroxidation. Additionally, our results revealed that *V. sativa* extract exhibited inhibitory activity against different inflammatory and nociceptive chemical agents. The purified flavonoids generally showed low anti-inflammatory and antinociceptive activities in a range of 0.9-18.3 % of inhibition, whereas compounds 1, 3, 4, and 8 were the most effective flavonoids. Structure-activity correlation was also discussed. Conclusion: *V. sativa* extract possessed antioxidant, anti-inflammatory, and antinociceptive activities to variable extents, which may be attributed to the radical scavenging and anti-inflammatory activity of some of its flavonoid constituents. These activities may help in prevention and/or treatment of the pathological disorders in which free radical oxidation, inflammation, and cellular activation are suggested to play a fundamental pathogenic role.

Key words: Superoxide, lipid peroxidation, carrageenan, formalin test, acetic acid test.

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1. Introduction

Genus *Vicia* compries 140 species, distributed in the North temprate extending to south America, Hawaii, and tropical East Africa [1, 2]. Boulos [3] reported 14 species in Egypt, which are occuring in different phyto-geographical territories. Vicia species were placed into three main groups on the basis of the major protein amino acids: Canavanine, b-cyanoalanine, to which *Vicia sativa* is belonging, and arginine [4].

The common vetch (*Vicia sativa*) is a legume that grows in Egypt. It is consumed by the rural populations in many countries, as lentil, and it is frequently utilized in animal feeding [1]. Many investigations reported that *V. sativa* forage has a high protein content [1, 5, 6], and that *V. sativa* could be used as protein sources for animals [7].

Subsequently, Araya *et al.* [1] estimated that the *V. sativa* protein concentration and total dietary fiber were 23.5% and 14.2% higher than in the legumes commonly consumed, respectively. The authors suggested that these findings proved thus common vetch has no termolabile toxics, which are normally present in legumes and that it is a promisory food resource.

However, it is also known that *V. sativa* contains a neurotoxic, the B-ciano-L-alanine [8-10]. In a recent feeding trial, donkeys fed the oats-vetch mixture were heavier than other group fed traditional forage. The oats-vetch association was evaluated positively due to the higher yields and good condition of the equids [11].

Examination of the other constituents of *V. sativa* revealed that lecithin, phosphatidyl ethanolamine and phosphatidyl inositol were the major polar lipids in the seeds [5]. A lectin with a mitogenic activity on human and mice peripheral blood lymphocytes was also isolated from *V. sativa.* It agglutinates horse, rabbit and human erythrocytes, but does not agglutinate calf and sheep erythrocytes [12, 13].

Moreover *V. sativa* is an estrogenic legume due to its high estrogen concentration [14]. Recourt *et al.,* [15] demonstrated that roots extracts of *V. sativa* contain also four major non-inducing glycosylated flavonoids. Furthermore, the same laboratory found that roots contain four 3-O-glycosides of the flavonol kaempferol [16].

Superoxide anion radical ($02^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and peroxyl radical ($ROO^{\bullet\circ}$) are products of the biological reduction of 0_2. They are very reactive and poorly tolerated within living systems [17]. Oxidative stress is associated with normal aging process, various toxic reactions, and pathophysiological tissue damages such as inflammation, ischemia and cancer [18].

Biological antioxidants, that catalytically scavenge these products, act as a defense mechanism, but unbalance between oxidants and biological antioxidants evoked the oxidative stress status. Diet contains various antioxidant and anti-inflammatory compounds that are able either to inhibit the production of these oxygen radicals or to scavenge them, thus they can help the biological antioxidants in prevention of oxidative stress.

Investigation of antioxidant capacity of the traditional folk plants and of their constituents is one of the successful strategies that may help in preventing of many oxidative stress-related disorders, especially those spread in the same society.

The objective of the present work was to perform more analyzing investigation of the aerial parts of *V. sativa* to isolate and identify its unreported flavonoid constituents and to monitor the possible antioxidant, anti-inflammatory and antinociceptive activity of the plant extract and its flavonoids that may help in the prevention of

oxidative stress-related complications and inflammation-based disorders.

2. Material and methods

Fresh aerial parts of *V. sativa* were collected in 1999 from El-Hassana, North Sinai and identified by the Herbarium of National Research Centre, Cairo (NRC), Egypt. A voucher specimen has been deposited in the Herbarium of NRC. All the chemicals were analytical grade and purchased from Sigma-Aldrich, Steinheim, Germany.

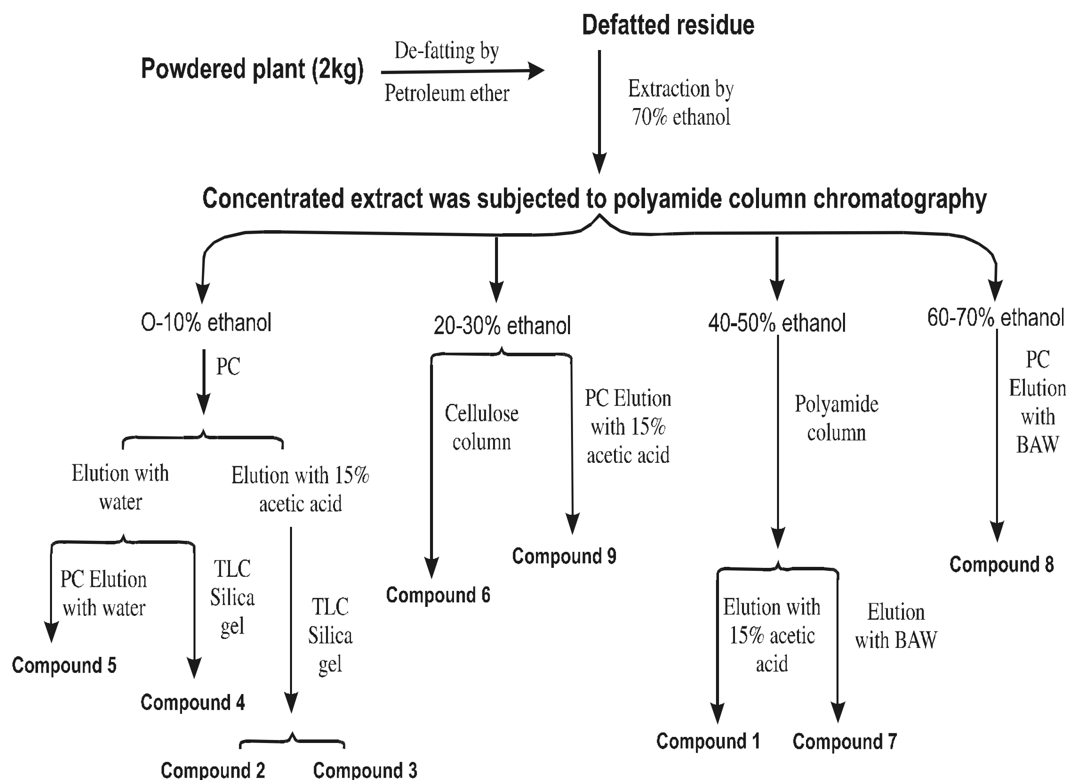
2.1 Plant extraction and flavonoid isolation

The dried aerial parts of *V. sativa* (2 Kg) were defatted using petroleum ether before extracted with 70% ethanol. The ethanol extract was evaporated under reduced pressure and low temperature affording 500g of crude extract. The residue was fractionated using polyamide column and ethanol-H_2O as eluent.

Purification process was carried out using primitive paper chromatography (PC) Whatmann No. 3 mm paper with BAW (butanol: acetic acid: water, 4:1:5, respectively, upper phase), 15% acetic acid and water for elution, and preparative thin layer chromatography (TLC) on Silica gel GF254 eluted with chloroform, methanol, and water (65:45:12, respectively).

Final purification was performed using sephadex LH-20 column with methanol as eluent. Mild acid hydrolysis was carried out using 0.1 N HCl at 100º C on 2 mg of each compound. Aliquots were removed after 5, 10, 15, 20, 30, 45, and 60 min. and immediately examined by paper

Fig. 1: Schematic representation of the flavonoid isolation procedure from *V. sativa.*



chromatography using BAW, 50% HoAc, and phenol as developing solvents.

The final aqueous acidic mixture was treated with acetic acid to isolate aglycone and sugar moieties. Complete acid hydrolysis was performed using 2N HCl at 100ºC for 2h. The final hydrolysate was treated as in the mild hydrolysis procedure. The isolation procedure of flavonoids is represented in Fig.1.

2.2. Spectral data

^{1}H and ^{13}C spectra were recorded on a 500 MHz Bruker AMX and/or a varian unity Inova with TMS as an internal standard and DMSO-d_6 as solvent. FAB mass spectra were obtained using a Finigan MAT TSQ 700 spectrometer, while UV was recorded using a Shimadzu UV 240 spectrophotometer.

2.3 Antioxidant activity assays

Before applying the probes (extract and flavonoids) to the screening antioxidant assays, freeze-dried *V. sativa* ethanol extract was standardized to 4 mg/ml in DMSO, except when different solvent is mentioned, and different dilutions of probes were prepared as convenient to each assay.

2.3.1 Scavenging of 1, 1 diphenyl-2-pycryl-hydrazyl (DPPH) radicals

Scavenging activities of the extract and the purified flavonoids against DPPH radicals were examined according to the method of Ratty *et al.* [19] In brief, 200 µL of DPPH (50 µM in ethanol), a stable free radical, were added to 5 µL of different concentrations of *V. sativa* extract, flavonoid, or DMSO (control) in a 96-well microplate and incubated at 37°C for 30 min.

The absorbance was measured at 520 nm. The percentage of DPPH bleaching utilized for IC_{50} (half maximal inhibition concentration) were calculated as being 0% is the absorbance of DPPH with DMSO and 100% is the absorbance of DPPH with an efficient scavenger (10mM ascorbic acid in DMSO). The calculation was performed using the following expression: % inhibition = $[(A_{control} - A_{probe}) / A_{control}] \times 100$, where A is the absorbance, and each IC_{50} was obtained from two curves (ascorbic acid and tested probe) in triplicate (eight concentrations).

2.3.2 Scavenging of peroxyl radicals

Scavenging of peroxyl radicals ($ROO^{\bullet}$) was monitored by ferrous ion oxidation-xylenol orange assay (FOX) according to Nourooz-Zadedeh [20], with modifications. The assay is based on the oxidation of ferrous to ferric ions under acidic conditions by ($ROO^{\bullet}$) generated by thermo-decomposition of 2,2'-azobis(2-amidopropane) hydrochloride (AAPH), and the change in absorbance was detected spectrophoto-meterically.

The working solution of FOX reagent was prepared by mixing solution A with solution B 1:9 (v/v), [Solution A: 98.03 mg ammonium ferrous sulfate/100 ml 250 mM H_2SO_4, 76.06 mg xylenol orange, kept under stirring for 10 min, Solution B: 969.76 mg butylated hydroxytoluene (BHT) 900 ml methanol].

Briefly, the assay was initiated by mixing of 25 µl of plant extract (1.0 mg/ml DMSO), test compound (1.0 mM in DMSO), trolox (1.0 mM in DMSO, positive control), or DMSO (blank), with 10 µl of 21.70 mg/ml AAPH for 5 min at 37ºC. Then, 210 µl FOX working solution were added and incubated for 30 min. The oxidation level was monitored at 560 nm against blank in comparison to trolox activity.

2.3.3 Inhibition of respiratory burst

Polymorphonuclear leukocytes (PMN) were obtained from whole blood samples of Sprague-Dawley rats by separation on a Ficoll-Hypaque density gradient. The reduction of superoxide anion radicals by tested probes was estimated

using previously reported procedure [21]. Briefly, PMN suspension (5x 10^9PMN/ml) were incubated at 37°C in flat-bottom polystyrene microplate with 5 µL of different concentrations of the extract, flavonoid or DMSO for 15 min.

Then, 45 µl of phosphate buffered-saline and 50 µl of phorbol myristate acetate (PMA) (1.625 µmol/L) were added to initiate the respiratory burst. After 10 min of mixing, 50 µl of nitro blue tetrazolium (NBT, 2.4 mmol/L) were added and the inhibition of superoxide production was followed by the NBT reduction rate at 490 nm.

Each test was carried out three times and NADPH oxidase activity was expressed as the mean of absorbance values in the 30 min period. IC_{50} were calculated as the concentration that lead to 50% inhibition in superoxide production, whereas 0% is the absorbance in presence of unstimulated PMN (without PMA addition) and 100% is the absorbance PMA-stimulated PMN. The specificity of the assay was tested by a parallel running measurements in presence of excess superoxide dismutase.

In addition, the viability of neutrophil after treatment with different concentration of extract, flavonoids or DMSO was examined using trypan blue and the treatments showed no toxic effect.

2.3.4 Inhibition of lipid peroxidation

Microsomes were prepared from liver of male Sprague-Dawley rats (250-300 g) by differential centrifugation as described previously [22]. Lipid peroxidation in rat liver microsomes was induced by carbon tetrachloride (CCl_4) and measured by thiobarbituric acid-reactive substances test (TBARS) [23]. Serial dilutions of 4mg/ml extract and 10mM flavonoids were added in 10µl of ethanol. Each IC_{50} was calculated by average values of three curves compared with catechin curve as a reference inhibitor (average of triplicate series of concentrations).

2.4 Anti-inflammatory and antinociceptive activity assays

2.4.1 Animals

All experiments were performed on male Swiss mice (30±5 g) obtained from the Animal House of NRC. The animals were fed a certified diet with free access to tap water and were housed on a 12 h-light:12 h-dark cycle at 50% humidity and a temperature of 24±1°C. All experiments were done in the morning.

2.4.2 Carrageenan-induced paw edema

Injection of carrageenan in the mouse hindpaw produces an acute, restricted inflammation associated with thermal and mechanical hyperalgesia and allodynia that peak 3 h after carrageenan injection [24].

This test was done essentially as described previously [25]. Carrageenan was dissolved in sterile physiological saline (0.9% NaCl) as 1% W/V solution, autoclaved and stored in sterile tubes at 4°C until use. *V. sativa* extract and purified compounds were dissolved in a small volume of absolute ethanol, and then diluted with 50% propylene glycol to obtain a stock solution.

Prior to use, the stock solution was diluted with physiological saline. Saline containing 0.01% ethanol and 5% propylene glycol was used as vehicle control. Indomethacin (10 mg/kg in saline, positive control), ethanol extract of *V. sativa* (100 and 200 mg/kg) or purified compounds (5 mg/kg) were administered i.p. 30 min before the injection of 25µl of carrageenan into the subplantar region of the right hindpaw under light ether anesthesia.

Paw edema was measured by water displacement plethysmography, after 3 h from carrageenan injection, by placing the paw into the chamber of the plethysmometer (Buxco, Troy, NY) up

to the ankle hairline (approximately 1.5 cm), as described by Winter *et al.,* 1962. The edema was reported as the difference between the final and the initial volumes of the paw. The displacement volume was measured in milliliters, recorded by a computer, and the percentages of edema inhibition were calculated

2.4.3 Acetic acid-induced abdominal writhing response in mice

Mice were randomly divided into several groups and used according to the method described previously [26]. Each mouse was given intraperitoneally 1% acetic acid solution (10 ml/kg body weight). The animals were pretreated i.P. with vehicle control, indomethacin (10 mg/kg in saline, positive control), morphine (2 mg/kg in saline, potential positive control), ethanol extract of *V. sativa* (100 and 200 mg/kg.), or purified compounds (5 mg/kg) at 1 h before acetic acid administration.

The mice were placed in the individual observation boxes. The total number of writhings following administration of acetic acid was recorded for 20 min, starting 5 min after the injection. Antinociceptive activity was expressed as the inhibition percentage of the number of abdominal writhes.

2.4.4 Formalin-induced licking response in mice

Formalin injection induces a biphasic stereotypical nocifensive behavior and corresponding electrophysiological responses [27, 28]. These responses are divided into an early and short-lasting first phase (0-10 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period, and then a second, prolonged phase (15-60 min) of tonic pain [29].

Each mouse was placed in the observation chamber for 5 min prior to injection. A mirror was inclined at a 45° angler to allow clear observation of animal paws. The animals were administered 25 µl of 5% formalin, pH 6.9, in saline, s.c. into the right subplantar. Then, each formalin-treated animal was observed for two distinct periods of the intensive licking response.

The first period was recorded 0–10 min after the formalin injection and the second period was recorded 10–35 min after the injection.

The time (in seconds) spent in licking responses of the injected paw was measured as an indicator of pain response. *V. sativa* extract (100 and 200 mg/kg), purified compounds (5 mg/kg), morphine (2 mg/kg in saline, positive control), or vehicle was administered 1 h before formalin injection. Nociceptive behaviors were recorded including: flinching, licking, or biting the injected paw.

2.5 Statistical analysis

The results of the inflammation test were analyzed by ANOVA followed by the Tukey test. For the nociceptive model analysis we used the non-parametrical analogous test, the Kruskal-Wallis followed by Nemmenyi test. Inhibition percents were calculated by the formula: inhibition percent =(1-Vt / Vcx100, where Vt and Vc represent the average paw volume, the number of writhes, or licking paw time of the treated and control groups, respectively.

3. Results

After several chromatographic separations, the aqueous ethanol extract of the aerial parts of *V. sativa* afforded nine identified flavonoids as active components: apigenin 4`-O-β-D-glucopyranoside [1], apigenin 6, 8-di-C-β-D-glucopyranoside [2], apigenin 6-C-α-L-arabinopyranoside-8-C-β-D-glucopyranoside [3], kaempferol-3-O- β-L-dirhamnopyranosyl (1→2, 1→6)-β-D-glucopyranoside [4], kaempferol 3-O-(4-β-D-xylopyranosyl)-α-L-rhamnopyranoside-7-O-L-rhamnoside [5],

kaempferol-3-O- α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside [6], Luteolin-7-O- β-D-glucopyranoside [7] quercetin 3-O- ββ-D-glucopyranoside [8], and quercetin 3,7 di-O-glucopyranoside [9], (.1).

The identities of compounds were confirmed by ^{1}H-NMR as well as by ^{13}C-NMR spectroscopy and comparing with literature data of Agrawal [30], the molecular structure of the compounds is represented in Fig.2.

3.1 Apigenin 4`-O-β-D-glucopyranoside [1]

UV λmax (MeOH) 271, 321 (NaOMe) 280, 322, 396 ($AlCl_3$) 274, 303, 342 ($AlCl_3$/HCl) 274, 303, 340 (NaOAc) 277, 380 (NaOAc/H_3BO_3) 272, 318; ^{1}H-NMR (DMSO-d_6): δ 8.0 (d, J = 9.0 Hz, H-2', 6', 2H), 7.1 (d, J = 9.0 Hz, H-3', 5', 2H), 6.65 (s, H-3, 1H), 6.75 (d, J = 2.0 Hz, H-8, 1H), 6.4 (d, J = 2.0 Hz, H-6, 1H), 5.25(d, J = 8.0 Hz, H-1'').

3.2 Apigenin 6,8-di-C-β-D-glucopyranoside (vicenin-2) [2]

UV λmax (MeOH) 273, 333 (NaOMe) 282, 330, 400 ($AlCl_3$) 280, 304, 343, 385 ($AlCl_3$/HCl) 280, 303, 345, 381, (NaOAc) 282, 392 (NaOAc/H_3BO_3) 276, 322, 346; ^{1}H-NMR (DMSO-d_6): δ 8.0 (d, J = 8.0 Hz, H-2', 6', 2H), 6.90 (d, J = 8.0 Hz, H-3', 5', 2H), 6.85 (s, H-3, 1H), 4.8 (d, J = 7.5 Hz, H-1''), 4.6 (d, J = 8.0 Hz, H-1'''); ^{13}C-NMR; δ164 (C-2), 102.6 (C-3), 182.3 (C-4), 158.5 (C-5), 107.5 (C-6), 161.2 (C-7), 105.3 (C-8), 155.1 (C-9), 103.8 (C-10), 121.5 (C-1'), 129 (C-2', 6'), 115.8 (C-3', 5'), 160.7 (C-4'), 61.3 (C-6''), 81.1 (C-5''), 70.6 (C-4''), 77.5 (C-3''), 73.2 (C-2''), 75.3 (C-1''), 60.7 (C-6'''), 80.8 (C-5'''),70.6 (C-6'''),78.9 (C-3'''), 72.0 (C-2'''), 74.0 (C-1''').

3.3 Apigenin 6-C-α-L-arabinopyranoside-8-C-β-D-glucopyranoside [3]

UV λmax (MeOH) 272, 334 (NaOMe) 282, 335, 400 ($AlCl_3$) 279, 304, 345 ($AlCl_3$/HCl) 280, 304, 344 (NaOAc) 282, 392 (NaOAc/H3BO3) 276, 322,346; ^{1}H-NMR (DMSO-d_6): δ 8.0 (d, J = 8.0 Hz, H-2', 6', 2H), 6.90 (d, J = 8.0 Hz, H-3', 5', 2H), 6.85 (s, H-3, 1H), 4.8 (d, J = 8.0 Hz, H-1''), 4.6 (d, J = 8.0 Hz, H-1'''); 13C-NMR; d 164.5 (C-2), 103 (C-3), 182.6 (C-4), 161.6 (C-5), 108.4 (C-6), 161.6 (C-7), 105.3 (C-8), 155.6 (C-9), 103.9 (C-10), 121.9 (C-1'), 129.4 (C-2'), 116.4 (C-3'), 158.7 (C-4'), 116.4 (C-5'), 129.4 (C-6'), 74.6 (C-1''), 74 (C-2''), 71 (C-3''), 69.6 (C-4''), 69 (C-5''), 73.7 (C-1'''), 71.5 (C-2'''→), 79 (C-3'''), 70.6 (C-4'''), 82.8 (C-5'''), 61.4 (C-6''').

3.4 kaempferol-3-O-β-L-dirhamnopyranosyl (1→2,1→6)-β-D-glucopyranoside (nicotiflorin) [4]

UV λmax (MeOH) 266, 346 (NaOMe) 277, 323, 405 ($AlCl_3$) 273, 346 ($AlCl_3$/HCl) 273, 344 (NaOAc) 273, 365 (NaOAc/H_3BO_3) 267, 353; ^{1}H-NMR (DMSO-d_6): δ 8.0 (d, J = 8.0 Hz, H-2, 6', 2H), 6.85 (d, J = 8.0 Hz, H-3`, 5', 2H), 6.34 (d, J = 2.0 Hz, H-8, 1H), 6.15 (d, J = 2.0 Hz, H-6, 1H), 5.30 (d, J = 7.0 Hz, H-1''), 4.40 (d, J = 1.1 Hz, H-1'''). FAB-MS m/z 739 $[M-H]^-$, ^{13}C-NMR; δ 159.7 (C-2), 132.4 (C-3), 177.0 (C-4), 156.5 (C-5), 93.8 (C-6), 164.0 (C-7), 98.6 (C-8), 161.1 (C-9), 101.4 (C-10), 121.0 (C-1'), 130.6 (C-2'), 115.0 (C-3'), 156.9 (C-4'), 115.0 (C-5'), 130.6 (C-6'), 98.4 (C-1''), 77.0 (C-2''), 77.3 (C-3''), 70.2 (C-4''), 75.5 (C-5''), 66.85 (C-6''), 100.7 (C-1'''), 70.4 (C-2'''), 70.59 (C-3'''), 71.7 (C-4'''), 68.27 (C-5'''), 17.2 (C-6'''), 100.7 (C-1''''), 70.3 (C-2''''), 70.52 (C-3''''), 71.8 (C-4''''), 68.25 (C-5''''), 17.6 (C-6'''').

Kaempferol 3-O (4-β-D-xylopyranosyl)-α-L-rhamnopyranoside-7-O-L-rhamnopyranoside [5]:

FAB-MS m/z 709 $[M-H]^-$, UV λmax (MeOH) 265, 344 (NaOMe) 265, 388 ($AlCl_3$) 394, 347, 299, 274 ($AlCl_3$/HCl) 394, 340, 274, (NaOAc) 265, 352 (NaOAc/H_3BO_3) 265, 346; ^{1}H-NMR

(DMSO-d_6): δ7.8 (d, J = 8.5 Hz, H-2', 6', 2H), 6.95 (d, J = 8.5 Hz, H-3', 5', 2H), 6.75 (d, J = 2.0 Hz, H-8, 1H), 6.45 (d, J = 2.0 Hz, H-6, 1H), 5.55 (d, J = 1.1 Hz, rham-7, H-1'''), 5.39 (d, J = 1.1 Hz, rham-3,H-1''), 4.19 (d, J = 8.0 Hz, xyl-terminal, H-1'''); ^{13}C-NMR; δ157.9 (C-2), 134.5 (C-3), 179.0 (C-4), 162.9 (C-5), 100.5 (C-6), 163.0 (C-7), 96.1 (C-8), 159.5 (C-9), 106.1 (C-10), 119.2 (C-1'), 131.9 (C-2'), 116.1 (C-3'), 161.8 (C-4'), 116.1 (C-5'), 131.9 (C-6'), 101.0 (C-1''), 73.9 (C-2''), 71.8 (C-3''), 80.9 (C-4''), 71.6 (C-5''), 17.7 (C-6''), 99.8 (C-1'''), 72 (C-2'''), 71.5 (C-3'''), 73.5 (C-4'''), 71.2 (C-5'''), 18.1 (C-6'''), 106.6 (C-1''''), 76.2 (C-2''''), 77.2 (C-3''''), 70.4 (C-4''''), 67.5 (C-5'''').

kaempferol-3-α-L-rhamnopyranosyl (1®6)-β-D-glucopyranoside [6]:

UV λmax (MeOH) 346, 266 (NaOMe) 405, 323, 277; ($AlCl_3$) 346, 273, ($AlCl_3$/HCl) 344, 273 (NaOAc) 365, 273 (NaOAc/H_3BO_3) 350, 267; ^{1}H-NMR (DMSO-d_6): δ8.0 (d, J = 8.0 Hz, H-2', 6', 2H), 6.85 (d, J = 8.0 Hz, H-3`, 5', 2H), 6.34 (d, J = 2.0 Hz, H-8, 1H), 6.15 (d, J = 2.0 Hz, H-6, 1H), 5.30 (d, J = 7.0 Hz, H-1'', 1H), 4.40 (1H, S, H-1'''), 0.95 (3H, d, J = 5.0 Hz, CH3-rhamnose).

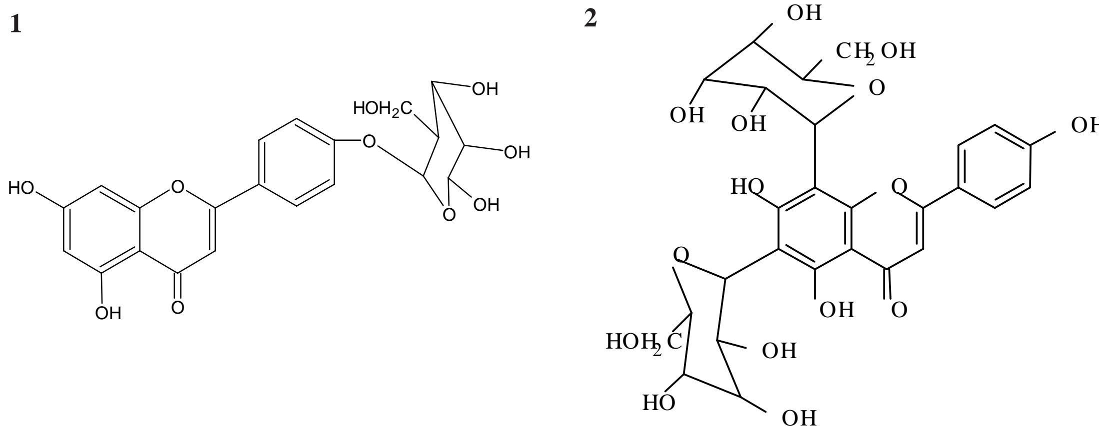
Luteolin-7-O-β-D- glucopyranoside [7]:

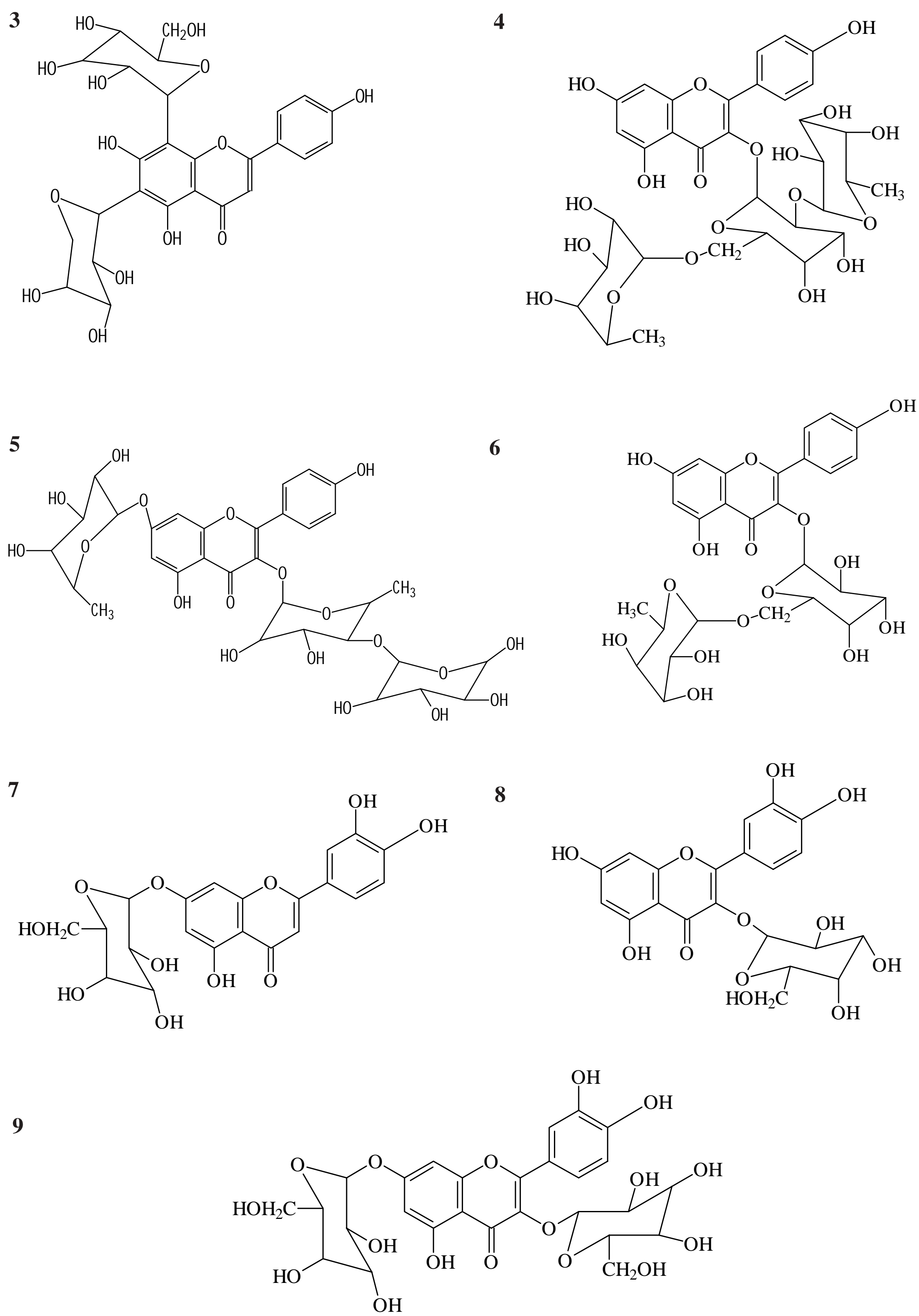
UV λmax (MeOH) 264, 345 (NaOMe) 273, 398 ($AlCl_3$) 273, 344, 400 ($AlCl_3$/HCl) 273, 341, 393 (NaOAc) 264, 360 (NaOAc/H_3BO_3) 265, 348; ^{1}H-NMR (DMSO-d_6): δ 8.0 (d, J = 7.5 Hz, H-6', 1H), 7.6 (d, J = 2.5 Hz, H-2', 1H), 7.3 (d, J = 7.5Hz, H-5', 1H), 6.9 (d, J = 2.5Hz, H-8, 1H), 6.7 (d, J = 2.5Hz, H-6, 1H), 6.1 (s, H-3, 1H), 5.03 (d, J = 7.5 Hz, H-1'').

Quercetin 3-O-β-D-glucopyranoside (isoquercetin) [8]:

FAB-MS m/z 464 [M-H]-, UV λmax (MeOH) 257, 266sh, 358 (NaOMe) 272, 330, 411 ($AlCl_3$) 272, 324, 385 ($AlCl_3$/HCl) 269, 297, 363, 398, (NaOAc) 272, 327, 375 (NaOAc/H_3BO_3) 261, 379; ^{1}H-NMR (DMSO-d_6): 7.58 (dd, J =7.5 Hz, H-6', 1H), 7.53 (d, J = 2.5 Hz, H-2', 1H), 6.85 (d, J = 8.5 Hz, H-5', 1H), 6.25 (d, J = 2.5 Hz, H-6, 1H), 6.4 (d, J = 2.0 Hz, H-8, 1H), 5.45 (d, J = 7.5 Hz, H-5''). ^{13}C-NMR; δ 156.3 (C-2), 133.3 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.4 (C-7), 93.6 (C-8), 156 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.3 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 116.2 (C-5'), 121.6 (C-6'), 100.9 (C-1''), 74.1 (C-2''), 76.5 (C-3''), 69.9 (C-4''), 77.5 (C-5''), 60.9 (C-6'').

Fig. 2: Molecular structures of compounds (1-9) isolated from *V. sativa.*

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Quercetin 3,7 di-O- glucopyranoside [9]

UV λmax (MeOH) 265, 345 (NaOMe) 268, 401 ($AlCl_3$) 272, 406 ($AlCl_3$/HCl) 267, 355, 397, (NaOAc) 265, 391 (NaOAc/H_3BO_3) 261, 373; 1H-NMR (DMSO-d_6): δ 7.58 (dd, J= 7.5 Hz, H-2', 6', 2H), 7.05 (d, J=8.0 Hz, H-5', 1H), 6.8 (d, J=2.0 Hz, H-8, 1H), 6.4 (d, J=2.0 Hz, H-6, 1H). 5.40 (d, J=7.0 Hz, H-1''), 4.85 (d, J=7.0 Hz, H-1'''); ^{13}C-NMR; δ 156.8 (C-2), 133.5 (C-3), 177.5 (C-4), 164.6 (C-5), 98.7 (C-6), 162.8 (C-7), 94.5 (C-8), 157.1 (C-9), 104.7 (C-10), 121.01 (C-1'), 115.5 (C-2'), 145.0 (C-3'), 148.8 (C-4'), 116.6 (C-5'), 121.6 (C-6'), 100.6 (C-1''), 74.0 (C-2''), 76.5 (C-3''), 69.8 (C-4''), 77.8 (C-5''), 61.6 (C-6''), 99.8 (C-1'''), 73.1 (C-2'''), 75.5 (C-3'''), 69.8 (C-4'''), 77.5 (C-5'''), 60.8 (C-6''').

3.1 Antioxidant activity

3.1.1 Quenching of DPPH radicals (Fig. 3, panel A)

Ethanol extract of *V. sativa* act as active free radical scavenger as indicated by DPPH assay. Most of the purified flavonoids from *V. sativa* exhibited potential scavenging activity against DPPH radicals in the following order 9>5>8>7>4>2, where quercetin 3,7 di-O-glucopyranoside (compound 9) was the most active antioxidant with the lowest IC_{50} value of 2.50±0.25 μM compared with the IC_{50} value of 3.2±0.19 μM of ascorbic acid, a known physiological antioxidant. Compounds 1, 3, and 6 showed inactive reactivity against DPPH.

3.1.2 Scavenging of ROO$^•$ radicals (Fig. 3, panel B)

Ethanol extract of V. sativa inhibited the AAPH-induced ROO$^•$ radical production as indicated by FOX assay. Most of the purified flavonoids from *V. sativa* (compounds 4, 5, 7, 8, 9) are inactive against ROO$^•$ radicals, while Kaempferol 3-O (4-β-D-xylopyranosyl)-α-L-rhamnopyranoside-7-O-L-rhamnoside (compound 6) was the most active ROO$^•$ scavenger with an inhibition percentage of 24.8±2.2 compared with that of Trolox 76.1±6.40, a known ROO$^•$ scavenger.

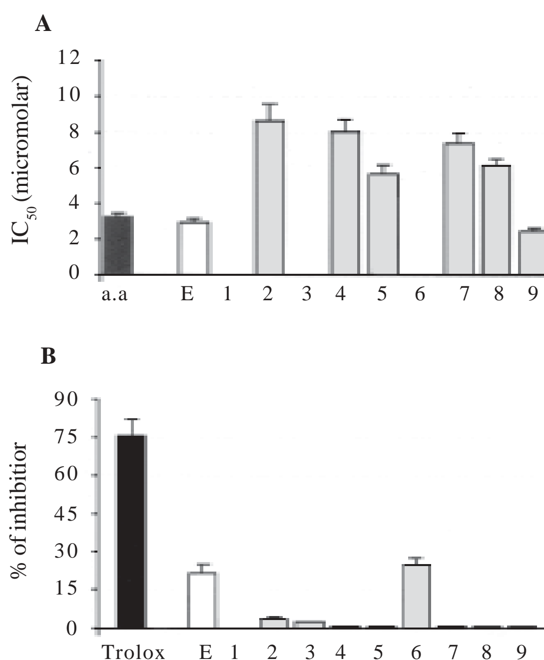

Fig. 3: Antioxidant activity (mean± S.E.) of *V. sativa* extract (E, white bar) and its purified flavonoids (1-9, respectively, gray bars) compared with suitable standard (black bar) in 3 independent experiments for each measurement.
Panel A: Scavenging activity against DPPH radicals compared with ascorbic acid (a. a.) activity. The activities are expressed as IC_{50} value of μM for flavonoids and of μg/ml for the extract
Panel B: Inhibition of peroxyl radical produced by AAPH of the *V. sativa* extract and flavonoids compared with the inhibition capacity of trolox.

3.1.3 Inhibition of respiratory burst in rat neutrophils (Fig. 4, panel A)

The respiratory burst stimulated by PMA in rat neutrophils was measured as superoxide anion radicals-sensitive reduction of NBT. The ethanol extract of *V. sativa* appeared as a mild inhibitor of neutrophil NADPH-oxidase. The purified

flavonoids exhibited variable reduction rate of PMA-induced superoxide production in neutrophil, where compounds 7, 5, and 4 exhibited the highest radical inhibitors. Compound 7, Luteolin-7-O-β-D-glucopyranoside, was the most potent superoxide radical scavenger with the lowest IC_{50} value of (15.1±1.30 μM).

3.1.4 Inhibition of lipid peroxidation (Fig. 4, panel B)

Lipid peroxidation induced in rat microsomes by CCl_4 was estimated as thiobarbituric acid-reactive substances. The potential inhibition of lipid peroxidation by *V. sativa* extract was observed at IC_{50} value of 8.20±0.9 μg/ml. The purified flavonoids revealed variable potential inhibitory effect on the CCl_4-induced lipid peroxidation, where Quercetin 3,7 di-O-glucopyranoside (compound 8) was the most effective inhibitor with IC_{50} value of 2.60±0.15 μM, compared with the inhibitory activity of catechin (IC_{50} =13.1±1.40 μM).

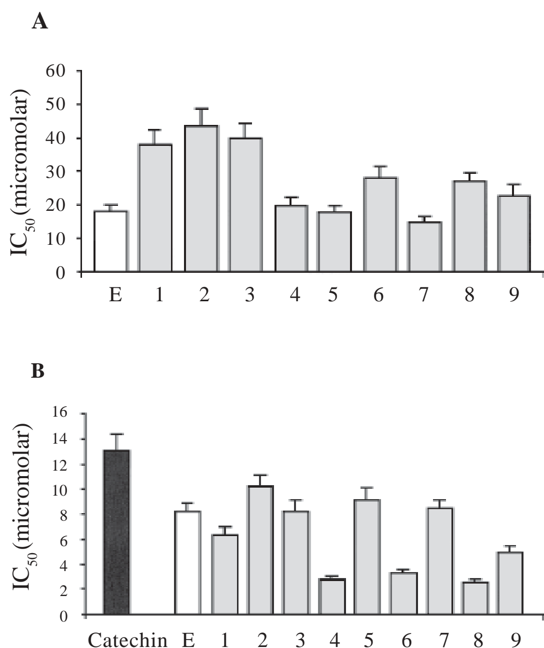

Fig. 4: Antioxidant activity (mean±S.E.) of *V. sativa* extract (E, white bar) and its purified flavonoids (1-9, gray bars respectively) compared with suitable standard (black bar) in 3 independent experiments for each measurement. The activities are expressed as IC_{50} value of μM for purified compounds and of μg/ml for the extract (n=10 for each test).
Panel A: Inhibition of superoxide radical production in PMA-stimulated rat neutrophil, compared with unstimulated neutrophil.
Panel B: Inhibition of CCl_4-induced lipid peroxidation in rat microsomes compared with catechin inhibitory activity.

3.2 Anti-inflammatory and antinociceptive activity

The anti-inflammatory and antinociceptive effects were evaluated using different chemical agents: carrageenan-induced paw edema, acetic acid-induced writhing, and formalin-induced licking.

3.2.1 Carrageenan-induced paw edema (Fig. 5, panel A)

The administration of ethanol extract from *V. sativa* showed significant inhibition ($P< 0.05$) on the carrageenan-induced paw edema at the doses of 100, and 200 mg/kg. However, among the purified flavonoids, only compounds 1, 8, 4 and 3 showed significant antiedematogenic activity ($P< 0.05$). This test is used to evaluate anti-inflammatory drugs and has been used to test the antiedematogenic effect of various substances [31].

3.2.2 Acetic acid-induced writhing (Fig. 5, panel B):

When performing the writhing test, results showed notable inhibition of the writhing frequency between the extract doses and most of the tested flavonoids ($P<0.05$). On the other hand, compounds 6, 7, 9 and 2 showed non-inhibitory effect of the writhing number. The abdominal constriction response is an unspecific nociception model used to evaluate the central and peripheral analgesic drug activity [31].

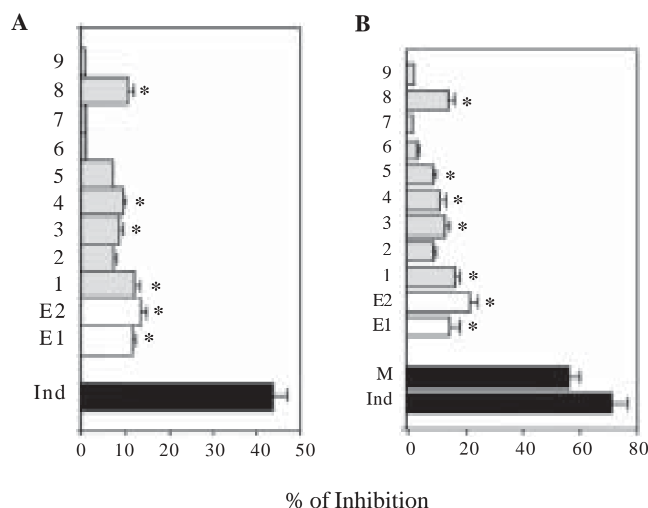

Fig. 5: **Panel A:** Anti-inflammatory activity: Inhibitory effects of indomethacin (10 mg/Kg, black bar), *V. sativa* extract (white bar, E1 and E2: 100 and 200 mg/kg, respectively) and its purified flavonoids (1-9, 5 mg/Kg, gray bars, respectively) on the mice paw edema induced by carrageenan 1% (n=10). The paw volume was measured four times after 2h from carrageenan treatment, and anti-inflammatory activity was expressed as the inhibition percentage (mean±S.E.) of the paw edema. Mean control value was 1.72 ± 0.13 mm of paw edema volume.
Panel B: Antinociceptive activity: Effects of indomethacin (10 mg/Kg, black bar), morphine (2 mg/Kg, black bar), *V. sativa* extract (white bars, E1 and E2: 100 and 200 mg/kg, respectively) and flavonoids (1-9, 2 mg/Kg, gray bars, respectively) on the writhes number (mean ±S.E.) induced in mice by acetic acid 1% were evaluated (n=10 for each test). Anti-nociceptive activity was expressed as the inhibition percentage of the writhing responses. Mean control value was 26 ± 3 of number of writhes. * represents the results significance (P<0.05).

3.2.3 Formalin-induced licking response in mice (Fig. 6, panel A, B):

Formalin administered s.c. produced a distinct biphasic behavioral response involving licking of the injured paw (Fig. 6A). This response showed an early first phase of 10 min, followed by a more exaggerated second late phase, suggesting persistent activation of nociceptive neurons. The ability of *V.sativa* extract and flavonoids to block this licking behavior in mice was tested in both phases.

Early phase of paw-licking behavior was not affected by adminstration of neither extract nor flavonoids. The late phase (second phase) was blocked significantly (P<0.05) by only the lower dose of extract at 100 mg/kg (Fig. 6B). A blockade of the licking behavior (P<0.05) was also observed with the administration of some of *V. sativa* flavonoids, except compounds number 6, 7, and 9.

4. Discussion

The biochemical mechanisms of several folk and traditional remedies using plant extracts to treat inflammation-related pathological disorders are likely related to their ability to inhibit inflammatory responses, which may be linked to their antioxidant activity. In the present study, we successfully identified nine flavonoids in the aerial parts of *V. sativa*. Most of which are, to our knowledge, unreported in this plant in any previous reports.

Our strategy was aimed to evaluate the properties of *V. sativa* extract and its flavonoid constituents as antioxidant, anti-inflammatory, and anti-nociceptive agents that may help in the treatment of the oxidative stress-related diseases and inflammation-based disorders.

The reactivity of DPPH, as a stable non-physiological free radical, is useful in screening the drug antioxidant activity, but it does not permit a clear-cut definition of the antioxidant effect, while the scavenging activity against physiological radicals provide a relevant evaluation of the antioxidant activity.

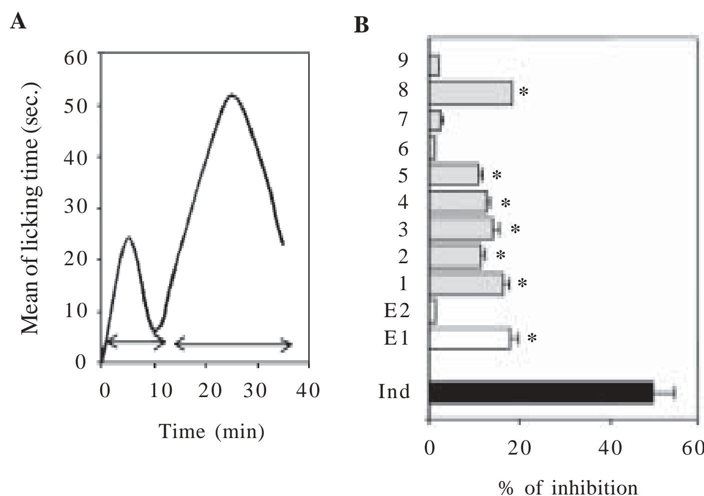

Fig. 6: Antinociceptive activity using formalin test:
Panel A: The formalin test (5% formalin, s.c. in right subplantar) involved two distinct phases of nociceptive behavior in response to formalin, an early first phase and a late second phase in which an exaggerated behavioral response (licking of the injected paw) was observed. Behavior was recorded in 5-min intervals (amount of time spent licking/5 min), each of which is represented as a data point in the graph.
Panel B: The effect of indomethacin (10 mg/Kg, black bar), *V. sativa* extract (white bars, E1 and E2: 100 and 200 mg/kg, respectively) and flavonoids (1-9, 5 mg/Kg, gray bars, respectively), which were injected 1h before formalin challenge, on the total time spent in licking (mean ±S.E.) in phase 2 was evaluated. Each point represents total licking time in the late phase. Mean control values were 152 ± 23.0 sec of total licking time in the late phase. * represents the results significance (P<0.05).

Our findings revealed the potent antioxidant activity of *V. sativa* extract assessed by four assays against different physiological and non-physiological radicals, while its purified flavonoids exhibited notable difference in antioxidant activity against different radicals. The antioxidant activity of the flavonoids is known to be associated with the number of free hydroxyl groups (OH).

From our findings, it seems that free OH group in positions 5, 7, and 4', especially position 7, may potentiate the activity against induced-lipid peroxidation with respect to the presence of glucoside groups, their types, numbers, and positions as noticed in position 8. While free OH group in positions 5 and 4' may be associated with the inhibitory activity against superoxide anion radicals,

Additionally, our results demonstrated that *V. sativa* extract exhibited inhibitory activity against different inflammatory and nociceptive chemical agents in a range of 11.5-24.6 % of inhibition.

The purified flavonoids generally showed low anti-inflammatory and antinociceptive activities in a range of 0.9-18.3 % of inhibition compared with the inhibition percentage range of known steroidal and non-steroidal inhibitors (43.8-71.5 %).

These findings may describe a link between the presence of free OH group at position 7 and anti-inflammatory and antinociceptive activities. The blocking of this position or the steiric henderance of OH group by glucoside groups may lead to dramatic decrease in both activities as shown in compounds 7, 9, and 5.

Few investigations studied the biological activities of *V. sativa.* Zielinski [32] found that compared with raw seeds, germinated *V. sativa* seeds were more effective in peroxyl radical-trapping capacity as measured using AAPH. Herein, our results add also more information about the antioxidant activity *V. sativa* against different free radicals, and that it is effective in modulation of cellular activation (respiratory burst and lipid peroxidation), moreover that *V. sativa* may possess also moderate anti-inflammatory and antinociceptive activities.

Luteolin 7-O-β-D-glucopyranoside (compound 7) was previously isolated from different plants such as *Melissa officinalis* [33], *Abutilon indicum* [34], and *Salvia officinalis* [35]. As isolated from *Buddleja officinalis,* compound 7 showed inhibitory activity on unpurified rat lens aldose reductase, enzyme involved diabetes complications [36].

Luteolin, quercetin, and their corresponding glycosides, isolated from *Carthamus tinctorius,* were reported to exhibit strong antioxidative activity against 2-deoxyribose degradation and lipid peroxidation in rat liver microsomes by hydroxyl radicals generated via a Fenton-type reaction, while apigenin-6,8-di-C-glucoside (compound 2) was relatively less active [37]. These reports are in agreement with our findings.

Kaempferol-3-O-α-L-dirhamnopyranosyl (1→2, 1→6)-β-D-glucopyranoside (compound 5) had been previouly purified from *Phytolacca americana* [38], and Solenostemma argel [39], while kaempferol 3-O-α-L-rhamnopyranosyl-(1->6)-β-D-glucopyranoside (compound 6) was isolated from *Morus alba* [40] and *Crescentia alata* [41].

quercetin 3-O-β-D-glucopyranoside (compound 8) was previously isolated from *Tachigalia paniculata* [42], *Phytolacca americana* [38], *Abutilon indicum* [34], and *Morus alba* [40]. Many invistigations studied the biological activities of compound 8.

Recently, it was reported that compound 8 purified from *Ginkgo biloba* leaves showed profound antioxidant activities in DPPH and cytochrome-c reduction assays using the HL-60 cell culture system [43]. Meng *et al.* [44] reported also that compound 8 purified from *Quercus dentata* suppressed significantly the superoxide generation induced by arachidonic acid or by PMA. Similarly, Akdemir *et al.* [45] reported the effectivness of compound 8 against free radical induced impairment of endothelium-dependent relaxation in isolated rat aorta.

In other reportrs, compound 8 was found to act as inhibitor of lipoxygenase-induced LDL oxidation more efficiently than ascorbic acid and alpha-tocopherol [46], and as effective antioxidant in solution and liposomal phospholipid suspension [47].

Additionally, compound 8 isolated from *Wedelia chinensis* revealed an inhibitory activity on both the classical and the alternative activation pathway of the complement system [48]. These reports are positively in agreement with our findings which proved the antioxidant, anti-inflammatory, and antinociceptive activities of compound 8.

Quercetin-3,7-di-O-β-D-glucopyranoside (compound 9) was previously separated from Morus alba [40]. In previous report, compound 2, 4, and 8 were isolated from leaves of *lycyrrhiza uralensis* [49]. Vicenin 2 (compound 2) isolated from *Cyperus alopecuroides,* showed moderate estrogenic activity using a strain of *Saccharomyces cerevisiae* [50], and from *Salvia officinalis* as well as *Allophyllus edulis* showed remarkable anti-hepatotoxic activities against CCl_4 and galactosamine cytotoxicity in primary cultured rat hepatocytes [35, 51].

Our findings add more information to these data, that vicenin-2 play an active role in scavenging of different radicals and in inhibiting induced inflammation and nociceptive reactions.

Taken together, compound 6 showed effective inhibitory activity of both peroxyl radicals; compounds 1, 2, 3, 6, 8 and 9 decreased both superoxide anion radical production in PMA-stimulated neutrophil, and compounds 2, 3, 5, and 7 were dramatically effective in inhibition of CCl_4 -induced lipid peroxidation.

Meanwhile, compounds 1, 3, 4, 5, and 8 were the most effective tested flavonoids in *V. sativa* against induced-inflammatory and nociceptive reactions. In conclusion, *V. sativa* extract possessed varied antioxidant, anti-inflammatory and antinociceptive activities, which may be associated with the potential activities of flavonoid constituents of *V. sativa*. These activities may open the road to use *V. sativa* as an additive food which may help in preventing some pathological disorders in which free radical oxidation, inflammation, and cellular activation are suggested to play a fundamental pathogenic role.

5. Acknowledgement

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References

1. Araya H, Alvina M, Vera G, Pak N. (1990) *Gaudin Arch. Latinoam Nutr.* 40:588-93.
2. Boulos L. (1999) *Flora of Egypt,* Al Hadara Publishing: Cairo, Egypt; 112-114.
3. Boulos L. (1995) *Flora of Egypt* Ckecklist, Al Hadara Publishing: Cairo, Egypt; 204-206.
4. Tschiersch B, Hanelt P. (1966) *Flora,* Blackwell: California USA; 157: 389-92.
5. Nierle W, el Wahab el Baya A. (1977) *Z. Lebensm. Unters. Forsch.* 164:23-7.
6. Alzueta C, Caballero R, Rebole A, Trevino J, Gil A. (2001) *J. Anim. Sci.* 79:2449-55
7. Seabra M, Carvalho S, Freire J, Ferreira R, Mourato M, Cunha L, Cabral F, Teixeira A, Aumaitre A. (2001) 89:1-16.
8. Picon SJ, Blanco Carmona JG, Garces Sotillos MD. (1991) *J. Allergy Clin. Immunol.* 88:135-6.
9. Roy DN, Sabri MI, Kayton RJ, Spencer PS. (1996) *Nat. Toxins.* 4:247-53.
10. Farran MT, Darwish AH, Uwayjan MG, Sleiman FT, Ashkarian VM. (2002) *Int. J. Toxicol.* 21:201-9
11. Velazquez-Beltran LG, Felipe-Perez YE, Arriaga-Jordan CM. (2002) *Trop. Anim. Health Prod.* 34:169-79.
12. Gebauer B, Schiltz E, Schimpl A, Rudiger H. (1979) *Hoppe Seylers Z Physiol. Chem.* 360:1727-30
13. Falasca A, Franceschi C, Rossi CA, Stirpe F. (1979) *Biochim. Biophys. Acta.* 577:71-81
14. Shore LS, Rios C, Marcus S, Bernstein M, Shemesh M. (1998) *Theriogenology* 50:101-7.
15. Recourt K, Schripsema J, Kijne JW, van Brussel AA, Lugtenberg BJ. (1991) *Plant. Mol. Biol.* 16:841-52.
16. Recourt K, Verkerke M, Schripsema J, van Brussel AA, Lugtenberg BJ, Kijne JW. (1992) *Plant. Mol. Biol.* 18:505-13.
17. Famaey JP. (1982) *Eur. J. Rheumatol. Inflamm.* 5:350-9.
18. Apaydin S, Zeybek U, Ince I, Elgin G, Karamenderes C, Ozturk B, Tuglular I. (1999) *J. Ethnopharmacol.* 67:307-12.
19. Ratty AK, Sunamoto J, Das NP. (1998) *Biochem. Pharmacol.* 37:989-995.
20. Nourooz-Zadedeh J.(1999) *Meth. Enzymol.* 300: 58-62.
21. Virella G, Thompson T, Haskill R. (1990) *J. Clin. Lab. Analysis* 4:86-9.
22. Masuda J, Murano T (1977) *Biochem. Pharmacol.* 26:2275-82.

23. Lee PY, McCay P B, Hornbrook R. (1982) *Biochem. Pharmacol.* 31: 405-409.

24. Kayser V, Chen YL, Guilbaud G. (1991) *Brain Res.* 560: 237-244.

25. Henriques MG, Silva PMR, Martins MA, Flores CA, Cunha FQ, Assreyu-Filho J Cordeiro RSB. (1987) *Braz. J. Med. Biol. Res.* 20 : 243–249.

26. Koster R, Anderson M and Debeer EJ. (1959) *Federation Proc.* 18: 412-16.

27. Dickenson AH, Sullivan AF. (1987) *Neurosci Lett.* 83: 207-21.

28. Dickenson AH, Sullivan AF. (1987) *Pain* 30: 349-360.

29. Tjolsen A, Berge O-G, Hunskaar S, Rosland JH, Hole K. (1992) *Pain* 51: 5-17.

30. Agrawal PK. (1989) *Carbon-13 NMR of Flavonoids,* Elsevier: Amsterdam, Netherlands; 6: 283.

31. Guilherme F, d. Miranda G, Vilar JC, Andria I, Alves N, Cabral S, Cavalcanti H, Roberto A. (2001) *BMC Pharmacol.* 6:1-6.

32. Zielinski H. (2002) *Nahrung* 46:100-4.

33. Patora J, Klimek B. (2002) *Acta. Pol. Pharm.* 59:139-43.

34. Matlawska I, Sikorska M. (2002) *Acta. Pol. Pharm.* 59: 227-9.

35. Lu Y, Foo LY. (2000) *Phytochemistry* 55:263-7.

36. Matsuda H, Cai H, Kubo M, Tosa H, Iinuma M. (1995) *Biol. Pharm. Bull.* 18: 463-6.

37. Lee JY, Chang EJ, Kim HJ, Park JH, Choi SW. (2002) *Arch. Pharm. Res.* 25: 313-9.

38. Bylka W, Matlawska I. (2001) *Acta. Pol. Pharm.* 58: 69-72.

39. Kamel MS, Ohtani K, Hasanain HA, Mohamed MH, Kasai R, Yamasaki K. (2000) *Phytochemistry* 53: 937-40.

40. Kim SY, Gao JJ, Lee WC, Ryu KS, Lee KR, Kim YC. (1999) *Arch. Pharm. Res.* 22: 81-5

41. Autore G, Rastrelli L, Lauro MR, Marzocco S, Sorrentino R, Sorrentino U, Pinto A, Aquino R. (2001) *Life Sci.* 70: 523-34.

42. Cioffi G, D'Auria M, Braca A, Mendez J, Castillo A, Morelli I, De Simone F, De Tommasi N. (2002) *J. Nat. Prod.* 65:1526-9.

43. Tang Y, Lou F, Wang J, Li Y, Zhuang S. (2001) *Phytochemistry* 58: 1251-6.

44. Meng Z, Zhou Y, Lu J, Sugahara K, Xu S, Kodama H. (2001) *Clin. Chim. Acta.* 306: 97-102.

45. Akdemir ZS, Tatli II, Saracoglu I, Ismailoglu UB, Sahin-Erdemli I, Calis I. (2001) *Phytochemistry* 56: 189-93

46. Luiz da Silva E, Tsushida T, Terao J. (1998) *Arch. Biochem. Biophys.* 349: 313-20.

47. Ioku K, Tsushida T, Takei Y, Nakatani N, Terao J. (1995) *Biochim. Biophys. Acta.* 1234: 99-104.

48. Apers S, Huang Y, Van Miert S, Dommisse R, Berghe DV, Pieters L, Vlietinck A. (2002) *Phytochem. Anal.* 13: 202-6

49. Jia SS, Ma CM, Li YH, Hao JH. (1992) *Yao Xue Xue Bao* 27:441-4.

50. Nassar MI, Abdel-Razik AF, El-Khrisy Eel-D, Dawidar AA, Bystrom A, Mabry TJ. (2002) *Phytochemistry* 60:385-7.

51. Hoffmann-Bohm K, Lotter H, Seligmann O, Wagner H. (1992) *Planta Med.* 58:544-8.