# Antimicrobial activity, antioxidant capacity and attempt to identify Flavonoids Analyses of *Crocus sativus* leaves and petals by ultra-performance LC-DAD-MS.

**Jadouali si mohamed** ( jadoualisimohamed@gmail.com )
EST Khenifra, Université Sultan Moulay Sliman, Khénifra, Morocco.

**Hajar Atifi**
Equipe de Matériaux Catalyse et Valorisation des Ressources Naturelles, Faculté des Sciences, Université Ibn Zohr, Agadir, Morocco; INRA-CRRA Agadir – Natural Resources and Ground Products Research Unit, Agrophysiology Laboratory, B.P. 124, Inezgane, Morocco

**Elbir Mustapha**
Equipe Systèmes Chimiques Complexes, University Sultan Moulay Slimane Faculty of Science and Technology.

**Rachid Mamouni**
Equipe de Matériaux Catalyse et Valorisation des Ressources Naturelles, Faculté des Sciences, Université Ibn Zohr, Agadir, Morocco

**Khalid Majourhat**
Laboratoires des Biotechnologies, Valorisation et Environnement, Faculté Polydisciplinaire de Taroudant

**Zakia Bouzoubaa**
INRA-CRRA Agadir – Natural Resources and Ground Products Research Unit, Agrophysiology Laboratory, B.P. 124, Inezgane, Morocco

**Neffa Mounsef**
Laboratory of Biochemistry and Biotechnology, Department of Biology, Faculty of Sciences, University Mohamed Premier, Oujda 60000, Morocco

**Alilou Hakim**
Equipe de recherche sciences de 'environnement et matériaux appliqué facultés polydisciplinaires de khouribga, Morocco

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# Abstract

Saffron leaves and petals are the main by-products of saffron processing which is producing in large amounts annually. The aim of this work is to identified polyphenols in leaves and petals from *Saffron*. Ultra-performance liquid chromatography coupled to diode array detection (UPLC-DAD) and ion trap mass spectrometry with either electrospray ionization (ESI-MS). Crude extracts from leaf and petal of *Crocus sativus* were screened for their in vitro antioxidant and antimicrobial properties. A significant content of polyphenols, essentially glycosides of kaempferol, quercetin, Isorhamnetin, Malvidin have identified in petals and leaves of *Crocus sativus*. The leaf extract revealed higher activity (IC50= 190,43 µg $ml^{-1}$) for DPPH inhibition in comparison with the petals ethanolic extract (IC50= 504.26 µg $ml^{-1}$). The leaf extracts gave higher antioxidant capacity (1,090 nm) measured with the FRAP assay. Our tested extracts petals displayed a strong antimicrobial activity with MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values as equal as 6,25 µg/µl against *Aspergillus niger* and *Escherichia coli*. The quantitative analysis data obtained thus far have shown that *Crocus sativus* leaves and petals are a rich source of flavonoids. The results were showed that the by-products of *C. sativus* can be exploited to create a different conception of *C. sativus L*. The identified compounds arouse growing interest in their antioxidant and biological activity as a food supplement, in functional foods, beverages, pharmaceutical preparations, and cosmetic formulations. Farmers and local scientists may no longer view *C. sativus* flowers as waste but as an important resource for health-enhancing products.


# Introduction

Saffron derives its names from Zaafarn, an Arab word which means yellow flowers (Ramadan, Soliman, Mahmud, Nofal, and Abdel-Rahman, 2010, Tayebeh Shahi et al, 2016). Filaments of saffron are made from the dried and dark red stigmas of *Crocus sativus L.* flowers (an autumn-flowering geophyte) belonging to the Iridaceae family. Picking saffron flowers are done daily by hand, lasting only 2 to 3 weeks Molina et al. (2005); Tayebeh Shahi et al. (2016). After separating stigmas from flowers, large quantities of saffron floral bio-residues consisting of petals and remaining stigmas are completely discarded (by-products account for about 86% of each crop in Morocco) Jadouali et al. (2018). during processing, a large quantity of leaves, approximately 1500kg to get one kilogram of saffron is required Tajik et al. (2019). This biomass is, however, a potentially significant source of bioactive compounds whose exploitation would greatly increase the profitability and sustainability of saffron production Santana-Méridas et al. (2012; Sánchez-Vioque et al. (2016). Nevertheless, high-quality byproducts of saffron such as petals have been previously reported to be rich in crocin and kaempferol, thus representing a significant source of bioactive compounds for the development of potential functional foods and cosmetic formulations Luigi Menghini et al. (2018); Tirillini et al. (2006); Zeka et al. (2015). Multiple studies Lahmass et al. (2017); Tuberoso et al. (2016); Jadouali et al. (2018a) also revealed the potential application of high-quality byproducts such as spaths, leaves, corms and floral-derived juices as cheap sources of bioactive compounds endowed with antioxidant activity. Although saffron leaves have not received much attention as a source of bioactive components, the presence of many phenolic compounds that could be used as natural antioxidants has been described Sánchez-Vioque et al. (2012). In This study we aimed to further characterize saffron byproducts from a phytochemical point of view, using performance liquid chromatography with diode array detection coupled to a mass spectrometer. Furthermore, we determined the efficacy of *Crocus sativus L* leaf and petal extract against selected microbial strain and antioxidant activity. To better understand their potential value, make the crop highly profitable in terms of biomass, minimize losses and ensure better management of these *Crocus sativus* by-products.

# Materials And Methods

### 2.1 Plant material

The *C. sativus L.* petals and leaves were harvested in autumn in Taliouine-Morocco. The stigmas were carefully removed to produce saffron. The leaves were harvested in the winter. By-products of the Crocus have been ground using a grinder and the resulting powder is stored in the dark until use.

### 2.2 Total phenolic content

Total phenolic content of leaf and petal extracts were performed according to the method of Hamood Al Saeedi and Amzad Hossain (2015). Extract solution (1mg) was taken in a volumetric flask, 2,5 ml of méthanol and 2,5 ml Folin-Ciocalteu reagent were added. after 5 min in a dark place for 5 min, 1,5 ml of solution 6% $Na_2CO_3$ was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The concentration of total phenol in the samples was calculated using a gallic acid (0, 12,5, 25, 50, 100 et 200 µg / ml) calibration curve.

### 2.3 Total flavonoid content

The total flavonoid content of the extract was mesured using methode established by (Hamood Al-Saeedi and Amzad Hossain, 2015). a 250µl of the extract was mixed whith 125µl of water and 75 µl of $NaNO_2$. after standing for 6 min at room temperature, 150µl of AlCl3 and kept for another 2 h in a dark place, and 500µl of NaOH and water (500µl). The absorbance was mesured at 515nm and the content of flavonoid in the extract catechin was used as a reference standard and total flavonoid content was expressed in catechin equivalent (CE mg/g dw).

### 2.6 Mass spectrometry analysis conditions

The leaves and the petals are analyzed in the Moroccan Foundation for Advanced Sciences, Innovation and Research (Mascir-RABAT) using the LC-MS technique (ESI) in waters acquitory synapt G2 equipped with a PDA detector: UPLC-LG 500nm, a pump: waters Acquity SDS and a helium cell 7.98 e-5. The extracts (10 mg/ml of methanol) were injected into an Acquity UPLC-BEH-C18 column. 1.7µm, using a solvent gradient A (water/acetonitrile) and solvent B (methanol) were used for the separation of polyphenols. The mobile phase flow rate was 1 ml/min and the solvent B was raised from 10 to 100% in 24 min. The MS parameters were as follows: 450C gas temperature, 0.5 kV Cappilar, collision energy 4, ES + ionization mode, mass range 50 to 1500, and transfer wave speed 247m / s. The data was acquired by Software Version: 1.50.1121. A PDA detector detector detector: LG 500 nm UPLC was used to monitor UV spectra at 250, 320, and 440 nm. The compounds present in the samples were provisionally identified according to their UV spectra, mass spectra and retention times and compared with data reported in the literature and/or with compounds identified in other reference plant samples.

### 2.4 DPPH radical scavenging assay.

The DPPH radical scavenging of the Crocus sativus leaves and petals extracts was mesured according to Hossain et al., (2013). briefly, each by product sample (2mg) at different concentration ( 12,5 µg / ml, 25 µg / ml, 50 µg / ml, 100 µg / ml et 200 µg / ml) were dissolved in 10 ml of methanol. 3,3 mg of DPPH was dissolved whid 100ml of methanol. Test tub of 1,5 ml of extract sample ware mixed with 2,5 ml of DPPH. The mixture were left for 90 min at room temperature in the dark. Then the absorbance was measured at 517 nm. finally, the percent of DPPH decoloration of the extracts was calculated according to the formula:

antiradical activity %= % Inhibition = 100* ((ADPPH- Acrude extract)/ADPPH )

### 2.5 Determination of reducing power

the total antioxidant capacity of sample was determined using o ferric reducing antioxidant power (FRAP) assay of Oyaizu et al. (1986). diffetent concentration of extrat (12,5 µg / ml, 25 µg / ml, 50 µg / ml, 100 µg / ml et 200 µg / ml) were mixed whith 0.5 ml of 1% potassium ferricyanide (K3Fe(CN)6) and 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6). the

mixture was incubated at 50 C° for 20 min. 2ml of Trichloroacetic acid (10%) was added to the mélange, which was then centrifuged at 650 tr / min for 10 min. The upper layer (0,5 ml) was mixed with 0,5 ml of deionized water and 0.5 ml of FeCl3 (0.1%) and the absorbance was mesured at 700 nm.

### 2.6 Antimicrobial Activity

#### 2.6.1 Detection of antibacterial activity

The *in vitro* antimicrobial activity test was carried out by agar well diffusion test (AWDA) (Tagg and Mc Given, 1971). MHA plates (Agar Mueller Hinton, Biokar Diagnostic) were covered with 5 ml of fused TSB agar (0.75% agar) inoculated with 100 µl of a culture of the indicator microorganism overnight. Wells (10 mm in diameter) were cut from the plates. Methanolic extracts of petals and leaves of *Crocus sativus* were filtered and placed in wells with the appropriate amounts. Methanol was used as a negative control. The plates were refrigerated (4 ° C) for 1-2 h to allow the radial diffusion of the compounds contained in the wells, then incubated at 37 °C for 10-16 h. Antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

#### 2.6.2 Measurement of the MIC and MBC

The antimicrobial activity tests were carried out on 11 strains of bacteria and 3 strains of fungi. The strains were obtained from the bio-industry quality control laboratory and bioactive molecules from El Jadida (Chouaïb Doukkali University of El Jadida). The microbial activity was assessed on the PDA culture medium (Potato Dextrose Agar) in the case of fungi and the HD culture medium (Muller and Hinton) in the case of bacteria. The culture media are prepared, brought to a boil and with stirring using a hot plate until complete dissolution and then sterilized at a temperature of 120 ° C for 20 min. The culture media are then distributed in sterile Petri dishes. A rejuvenation of the strains was necessary for the conduct of the test and the evaluation of the antibacterial and antifungal activity of the extracts. Indeed, the bacterial strains were seeded on HD medium and then incubated at 37 °C for 24 hours. While, the mushrooms were deposited, in the form of 1.2 cm discs, in the centers of the Petri dishes containing the PDA medium and then incubated at 28 °C for 7 days. For the launch of the antimicrobial test, discs of filter paper 0.6 cm in diameter were sterilized in an autoclave at 120 °C for 15 minutes. The microbial suspension (bacterial and fungal) was prepared from the rejuvenated strains for 24 hours on the HD agar medium in the case of bacteria and for seven days on the PDA medium for fungi. The suspensions were obtained by sampling the bacteria / fungus strains using a handle, which was subsequently suspended in distilled water. The microbial suspension was homogenized until a suspension with an optical density of 0.1 was obtained. A series of dilutions were made to obtain a concentration of 10-8 CFU / ml. The antibacterial activity was evaluated using the disc diffusion method and the well method.

The evaluation of the antibacterial activities of extracts of whole leaves, petals and flowers of *Crocus sativus* is made by the disc diffusion method. Thus, on sterile Petri dishes containing HD medium, 2 mL of the bacterial suspension was spread. Afterwards, four sterile discs with a diameter of 0.6 cm of filter paper were deposited and successively soaked with different volumes (5, 10 and 15 µl) of the extracts obtained which are diluted with DMSO (Dimethylsulfoxide) (Plant extracts were dissolved in DMSO at a concentration of 0.2 mg /ml). Two negative controls were prepared, one with DMSO and the other with distilled water. The Petri dishes were incubated in the oven for 24 hours at 37 °C. After 24 hours of incubation at 37 °C, the results were read by measuring the three diameters of the zones of inhibition appearing around the disks containing the extract. The average of the diameters was calculated and expressed in mm. The dilution methods are the most suitable for determining the minimum MIC concentration values of an extract (Rios *et al.,* 1988). Because they offer the possibility of estimating the concentration of the antimicrobial agent tested in the Muller Hinton broth. Minimum inhibitory concentrations (MIC) were determined using the 96-well microtiter plates. 100 µl of MH broth was placed in all the wells of the plate. The wells in the first vertical row are filled with 100 µl of the bacterial suspension at $10^8$ CFU / ml (OD = 0.1), as a control for bacterial growth, and in the wells in the second vertical row add 15 µl of DMSO as a

negative control. From the third vertical row, 100 µl of each product *Crocus sativus* extract - were tested in order to obtain a successive serial dilution by geometric progression of reason 2 (concentrations of the extracts ranging from 0.19 to 100 µg / µl), and 100 µl of the bacterial suspension at $10^8$ CFU / ml was added. Each test is carried out in two repetitions. After one hour of inoculation, the microplate was incubated at 37 °C for 24 hours. After incubation of the plates at 37 °C for 18-24 h the MICs are determined as the lowest concentration for which microbial growth is not observed with the naked eye (Wilkinson, 2006). The minimum bactericidal concentration (MCB) is defined as being the lowest concentration leading to a notable destruction of bacteria, estimated at 0.01% of the surviving germs. The contents of the wells, in which no clouding was observed, were taken and inoculated using a calibrated loop (10 µl) on a sterile Petri dish filled with HD agar. After incubation at 37 °C for 24 hours, the presence or absence of the colonies of the bacteria is mentioned. The MBC corresponds to the first concentration where no colony is observed.

### 2.7 Statistical analysis

the statistical data analyses were performed by one-way analysis of variance (ANOVA) using Minitab 17 software. Differences between means at the 95% (P 0,05) confidence level were considered statistically significant.

## Results And Discussion

### 3.1 Determination of Total phenolic content and total flavonoid content

The Figure 1 presents the total phenolic content (TPC) of *Crocus sativus* leaves and petals. Concerning leaves, TPC for *Crocus sativus* leaves were higher than petals. *Sengul* (2009) shows that the stigma of crocus sativus contains 42.29 mgGAE / g dry weight basis. another study by *Hossein Goli* (2012) showed that the saffron petal had 3.42 ± 0.2 mg of phenolic content equivalent to gallic acid per g of dry weight. our values were greater than the quantity of phenolic acids (1.38 mg caffeic acid / g dry weight) reported by *Termentzi* and *Kokkalou* (2008) for the saffron petals harvested in Greece. while other authors such as *Lahmas et al.,* (2018) have shown that the crocus sativus spath has a total polyphenol content 21.36 ± 0.025 µg GAE / mg extract, while in the stigma this content is 16 , 63 ± 0.100 µg GAE / mg.

Flavonoids are very important constituents of plants because of the scavenging ability conferred by their hydroxyl groups. The flavonoids may contribute directly to anti-oxidative and antimicrobial action *Sun et al. (2011*).

- **Polyphenol composition**
    - **Major polyphenols identified by liquid chromatography-mass spectrometry in leaf of C. sativus.**

Table 1 show the polyphenol composition of the leaf extracts as determined by HPLC-DAD-MS and figure 2 shows UPLC-DAD chromatograms of methanolic extract of *Crocus sativus* leaves at 440 nm, 320 nm and 250 nm. Peak **F4** (Rt= 4, 16, m/z 775,04) could be corresponding to a Malvidin 3-O-glucoside (6-O-malonyl) 7-glucoside with a $MS^2$ fragment ion at m/z 332,09 $[M+H-324-86]^+$. This anthocyanin it is the first time it is found in leaves. The malvidin 3,5-di-O- glucoside was identified in the Floral Bioresidues *crocus* (*Lahmas et al, 2017*). This malonated anthocyanin was identified in perianth segments *Crocus Nørbæk et al. (2002*). Peak **F5** (Rt= 4,75; m/z 639,11) (figure 3) could be corresponding to Isorhamnetin 3,4-di-O-b-glucoside with a $MS^2$ fragment ion at m/z 317,03 [M+H-162-162]. This flavonol was identified in perianth segments *Crocus Nørbæk et al. (2002*) and in pollen of *Crocus sativusBazylak et al. (2011*). Another flavonoid the quercetin derivative was observed (compound F8; Rt=21,27 ; m/z 1085) was identified with fragment ion at m/z 301,17. All flavonoid compounds found in leave by-products have demonstrated a strong antioxidant capacity in-vitro as in-vivo *Lahmas et al. (2017)*; *Miguel (2011*). In addition, it was demonstrated that antioxidant capacity can be related to the increased presence of the OH and methoxyl groups and their position but also to the presence of sugar moiety rather than the amount of polyphenol content *Lahmas et al. (2017*).

**Table 1.** Main polyphenols provisionally identified by liquid chromatography-mass spectrometry in leaves of *C. Sativus*.

| Peak | Rt (min) | UV $\lambda_{max}$ (nm) | $[M+H]^+$ (m/z) | Fragments (m/z in MS1 or MS2 | Tentative identification | Reference |
|---|---|---|---|---|---|---|
| F1 | 1,52 | 440; 320, 250 | 468,95 | 124,01(100); 165,02(15); 184,02(15); 266 (25); 366,68(10) | n.i | |
| F2 | 2,79 | 440; 320; 250 | 311,09 | 310,09(100); 292,08(20); 264,09(10) | n.i | |
| F3 | 3,49 | 440; 320; 250 | 311,09 | 310,09(100); 292,08(26); 264,09(15) | n.i | |
| F4 | 4,16 | 440; 320; 250 | 775,04 $[M+35]^+$ | 199,03(100); 383,05(48); 181,03(28); 332,09 (5) $[M+H-324-86]^+$ | Malvidin 3-O-glucoside (6-O-malonyl) 7-glucoside-chloride | Nørbæk et al. (2002) |
| F5 | 4,75 | 440; 320; 250 | 639,11 | 199,03(100); 317,03(10) [M+H-162-162]; 469,04(25) | Isorhamnetin 3,4-di-O-b-glucoside | Nørbæk et al. (2002) |
| F6 | 6,50 | 440; 320; 250 | 309,04 | 281,06(12);263,04(10);155,95(17); 139,98(15) | n.i | |
| F7 | 17,87 | 420 | 473,29 | 420,16(100);398,18(98); 326,33(14); | n.i | |
| F8 | 21,27 | 420 | 1085 | 301,17(100); 240,2(20); 365,23(85); 420,16(75);429,26(50); 473,13(42);561,18(35); 605,2(22); 780,84(17); 890,39(20); 978,44 (18) | Quercetin derivative | - |

n.i: not identified

### 3.2.2 Major polyphenols detected and tentatively identified by liquid chromatography-mass spectrometry in petal of C. sativus.

Table 2 shows the polyphenol composition of the petal extracts determined by HPLC-DAD-MS. The UPLC DAD chromatogram typical of the methanolic extract at 440, 320 and 250 nm (Figure 4.) Showed a major peak (P2), three other peaks (P4 and P6) at 440 nm and twelve minor peaks (P1, P5, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16) at 250, 320 and 440 nm. All peaks (except P2, P3 and P7) had a characteristic absorption band of flavonols. All the substances present in the chromatograms are indicated in the table by their retention time sequence and their UV and MS characteristics. The petal extract was rich in flavonols, particularly derivatives of kaempferol and quercetin. Specifically, 4 kaempferol, 2 quercetin and 7 unidentified compounds. Quercetin 3,7-O-diglucoside (Rt = 6.73, $[M + H]^+$ at m / z 649.06), the identified fragment (m / z 303) corresponds to a loss of 324, $[M + H -162-162]^+$ (Figure 5-A), were found in the ethyl acetate and butanol fractions by *Termentzi (2008)* in petal extract. The kaempferol 3,7 di-O-glucoside (P6, Rt = 8, 08 min) with a molecular ion at m / z 633.07, in their MS2 spectrum, a common ionic fragment at m / z 287.02 was found

corresponding to a kaempferol fraction, $[M + H-324-23]^+$ (Fig.5-B). This flavonol has already been detected in tepals by *Goupy (2013),* in *Crocus* leaves by *Lahmaset al. (2017)*. Kaempferol-3-glucoside (P7, Rt = 8.5 min) with a molecular ion at m / z 471.03, in their spectrum MS2, a ionic fragment common to m / z 287 corresponding to a kaempferol, $[M + H-162-23]^+$ (Fig. 5-C), these flavonol monoglucosides have already been found in petals of Crocus *Goupy et al. (2013*). The identification of compound P8 as Kaempferol-3-trimethyl ether-7 (6-gluconyl-malonyl) was also confirmed after comparison of their $[M + H]^+$ m / z (577.29) and MS2 ions m / z 451, 1 had a common loss of 128 (= 86 + 42), [M + H-86-42] corresponding to a malonyl-trimethyl ether and m / z 287.02 had a common loss of (= 162 + 86 + 42), [M + H-162-86-42] corresponding to a malonyl-trimethylglycosyl unit Crupi et al. (2012). The kaempferol caffeoyl-p_coumaroyl biester (compound P10, Rt = 8.81, $[M + H]^+$ at m / z 596.22) was identified on the basis of MS2 and by comparison with previously published data *Termentzi et al. (2008*). The identification of compound P12 (Rt = 15,16) as quercetin was also confirmed after comparison of their $[M + H + Na]^+$. The configuration, substitution and total number of –OH groups substantially influence several mechanisms of antioxidant activity such as radical scavenging, activation of antioxidant enzymes (*Nijveldt et al. (2001*). These compounds were supposed to play an important role in the antioxidant activity of Crocus petals. The conjugated double bonds allow electron delocalisation across the molecule thus stabilizing the phenoxyl radical. Flavanols are also potent antioxidants in lipid systems where they reduce oxidative modifications of membranes by restricting the access of oxidants to the bilayer and the propagation of lipid oxidation in the hydrophobic membrane matrix *Verstraeten et al. (2003*). The free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH. *Ishige et al. (2001)* reported that three structural characteristics of flavonol were crucial for the neuroprotective effect: the presence of the hydroxyl group in position C3, an unsaturated C cycle and hydrophobicity. The different study shows the flavonoid-rich plant extracts from different plants possess antibacterial activity *Karak (2019*).Apigenin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been proved to possess potent antimicrobial activity *Cushnie and Lamp (2005*). The mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt bacterial membranes. That flavonoid glycosides petals and leaves of *Crocus sativus* damage the cytoplasmic membrane and cause loss of intracellular components. Very little is known about the structure–function relationships of natural antimicrobials, but it seems that different substituent groups within the compounds had a great influence on their biophysical and biological properties *Mandalari et al. (2007*). Structural features such as the presence of an aromatic ring, the sugar moiety or the numbers of hydroxyl and methoxyl groups can significantly change membrane permeability and subsequent affinity to external and internal binding sites in the bacteria, thus influencing the compound's antimicrobial properties *Fitzgerald et al. (2004*). *Cushnie and Lamp* (2005) reported that kaempferol and quercetin have antimicrobial activity against human pathogenic microorganisms with some mechanisms of action such as inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolisms. Antimicrobial activity from petals and leaves of *Crocus sativus* might be due to the presence of kaempferol, quercetin, Malvidin and isorhamnetin. The results showed that all identified kaempferols could inhibit growth rate of *Staphylococcus aureus*, *Enterococcus faecalis*, *E. coli*, *Bacillus cereus* and *Klebsiella pneumonia.*

**Table 2.** Major polyphenols tentatively identificated by liquid chromatography-mass spectrometry in petals of *C. Sativus.*

| Peak | Rt (min) | UV $\lambda_{max}$ (nm) | $[M+H]^+$ (m/z) | Fragments (m/z in MS1 or MS2 | Tentative identification | Reference |
|---|---|---|---|---|---|---|
| **P1** | 1,46 | 440 | 474,17 | 262,09(100);276,11(95);244,08(28) | n.i | |
| **P2** | 4,66 | 440 ; 320 ; 250 | 633,07 [M+H+Na] | 287,02(100);[M+H-324] | Kaempferol 3,7 di-O-glucoside | Goupy et al (2013); Lahmas et al (2017) |
| **P3** | 5,55 | 440 ; 320 ; 250 | 471,03 [M+H+Na] | 287,02(100); [M+H-162] | Kaempferol-3-glucoside | Goupy et al (2013) |
| **P4** | 6,11 | 440 | 577,29 | 155,95(100) ; 451,1(65) ; [M+H-86-42] 287,02(10)[M+H-162-86-42] | Kaempferol-3-triméthyl ether-7(6-malonyl glucoside) | ------ |
| **P5** | 6,73 | 440 | 649,06 | 303,01(100);465,04(5) | Quercetine 3,7-O-diglucoside | Termentzi and Kokkalou (2008) |
| **P6** | 8,08 | 440 | 566,24 | 155,95(100) ; 309,04(70) ; 272,9(20) ; 341,2(33) | n.i | |
| **P7** | 8,50 | 440 ; 320 ; 250 | 356,31 | 200,17(100) ; 157,95(5) ; 139,98(8) | n.i | |
| **P8** | 9,06 | 440 | 277,11 | 276,11(100); 258,1(20); 248,12 (5) | n.i | |
| **P9** | 12,25 | 440 | 283,1 | 264,09(100);272,9(18);256,93 | n.i | |
| **P10** | 2,75 | 320 ; 250 | 596,22 | 155,95(100) ; 369,14(40) ; 287,02(10) )[M+H-146-162] | Kaempferol caffeoyl-p-coumaroyl biester | Termentzi and Kokkalou (2008) |
| **P11** | 4,26 | 320 ; 250 | 600 | 450 (100) ; 370 (30) | n.i | |
| **P12** | 15,16 | 250 | 326,33[M+H+Na] | 326,33(100) ; 157,95(5) ; 139,98(8) | Quercetine | ---- |
| **P13** | 1,64 | 250 | 360,93 | 157,95(100) ; 139,97(90) ; 326,33(10) | n.i | |
| **P14** | 3,75 | 250 | 420,16 | 398,18(100) ; 296,09 (8) ; 233,09(5) ; 149(45) | n.i | |
| **P15** | 12,16 | 250 | 326 (100) | n.i | | |
| **P16** | 16,16 | 250 | 360 | 157 (100) ; 139 (90) ; 326 (10) | n.i | |

### 3.3 DPPH radical-scavenging activity

The DPPH method is a simple, rapid, sensitive and reproducible test used to assess the antioxidant activity of plant extracts *Ebrahimzadeh et al. (2008*). In the DPPH test, an antioxidant eliminates free radicals and is used to measure the ability of extracts to recover the stable DPPH radical formed in solution by donation of hydrogen atom or an electron (Tepe et al.( 2011). Crude extracts of different polarities such as water, 80% methanol and 80% ethanol obtained from different

parts of the *Crocus sativus* plant were used to determine the antioxidant activity by the DPPH method established by *Hossain (2014)*. The study has shown that the ethanolic extracts from the leaves have a fairly high antioxidant power. This power is confirmed by low IC50 values and high inhibition percentages (Table 3). Thus, for the extracts of the petals, the IC50 values vary between 268.02 ± 5.6 µg/ml for the methanolic extract and 528.3 ± 29.4 µg/ml for the aqueous extract. The CI50 obtained for the different fractions are shown in Table 3. The phenolic compounds contained in the extracts of *Crocus sativus* are probably responsible for the antioxidant activity of these extracts. In addition, of the two types of tissue tested, the leaf showed the highest antioxidant activity in the aqueous fraction. The activity of all extracts was found to be concentration dependent and increased with increasing concentration of the extract. The IC50 values for the methanolic petal and leaf extracts were 268.02 ± 5.6 and 337.66 ± 9.37, compared to ascorbic acid (2.5 ± 0.34) used as positive control and α-tocopherol with 182.02 ± 2.09. *Baba (2015)* showed that the ethanolic extracts of the stigmas, leaves and corms of Crocus sativus have an CI50 only of 345.21 ± 5.2, 362.32 ± 3.32 and 412.42 ± 3.7 respectively. Lahmas et al. (2018) have shown that Free radical scavenging activity of spath extracts was similar to that of stigma extract and does not exceed 12 of inhibition % at an extract concentration of 500 µg/ml. Hossein Gholi (2012) determined that the petal extract at a concentration of 500 ppm showed an antioxidant activity of 74.23 ± 1.82%.

**Table 3**. The antioxidant activity of extracts of petals and leaves at different concentrations and crude extracts of different polarities.

| Extracts | Concentrati-ons (µg mL-1) | Inhibition (%) | IC50% (µg mL-1) | | | | |
|---|---|---|---|---|---|---|---|
| Water | Methanol 80% | Ethanol 80% | Water | Methanol 80% | Ethanol 80% | | |
| Petals | 12,5 | 3,69±0,55 | 2,03±1,4 | 3,73±0,5 | 528,3±29.4 | 268,02±5.6 | 504,26±17.53 |
| | 25 | 9,10±1,06 | 5,19±1,27 | 6,05±0,98 | | | |
| | 50 | 11,44±1,02 | 17,61±0,92 | 11,27±0,88 | | | |
| | 100 | 16,12±1,33 | 27,24±082 | 15,38±0,96 | | | |
| | 200 | 21.36±0,61 | 33,83±1,17 | 21,39±0,66 | | | |
| Leaves | 12,5 | 4,49±0,68 | 4,28±1,05 | 7,01±1,3 | 264,49±6,23 | 337,66±9,37 | 190,43±4,59 |
| | 25 | 15,45±1,28 | 6,44±0,72 | 10,69±1,13 | | | |
| | 50 | 21,68±1,42 | 11,32±0,74 | 26,89±1,43 | | | |
| | 100 | 31,06±1,61 | 19,24±0,39 | 39,09±1,16 | | | |
| | 200 | 37,95±1,95 | 30,12±0,31 | 47,19±0,91 | | | |
| α-tocopherol | 12,5 | - | 3,69±1,2 | - | 182,02±2,09 | | |
| | 25 | | 15,6±0,7 | | | | |
| | 50 | | 25,11±0,49 | | | | |
| | 100 | | 31,92±1,06 | | | | |
| | 200 | | 52,46±1,27 | | | | |
| Ascorbic ac | 12,5 | - | 82,18±0,72 | - | 25±0,34 | | |
| | 25 | | 86,95±0,57 | | | | |
| | 50 | | 89,58±0,36 | | | | |
| | 100 | | 91,69±1,14 | | | | |
| | 200 | | 94,05±1,48 | | | | |

IC50: the concentration of antioxidant necessary to inhibit DPPH to 50% under the experimental conditions.

The values are the mean value ± standard deviation of the extracts.

### 3.4 FRAP reducing power

There were differences in antioxidant capacity determined as FRAP between the extract samples as shown in (Table 4). The principle of the FRAP method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants *Tachakittirungrod et al. (2007*). The reducing power increased in a concentration-dependent manner in all the samples, and did not reach a saturation point in the concentration range assayed. Reducing power of petals showed a low reduction of $Fe3^{+}$. The leaf extracts gave higher antioxidant capacity; it was remarkable before that leaf's extracts had exhibited more antiradical activity according to the DPPH assay. On the other hand, acid ascorbic was

the synthetic antioxidants having highest antioxidant capacity. A study has shown that the reducing activity of spath is 0.157 to 250 µg / ml, whereas that of the stigmata is of the order of 0.707 at the same concentration *Lahmas et al. (2018*). The measurement of antioxydant activity by the FRAP method carried out by Hossein Goli (2012) showed that the extract of petals has an activity of 0.3 at a concentration of 250 ppm, this value remains low compared to that found in our results. All synthetic antioxidants exhibited higher FRAP values than those of plant extracts. *Mira et al. (2002)* show that only flavones myricetin and quercetin reduced $Fe^{+}$ effectively.

**Table 4.** Antiradical activities of the methanolic extracts and synthetic antioxidants at different concentrations.

| Concentrations | Petals Abs[1] (nm) | Leaves Abs (nm) | Ac. Ascorbic Abs (nm) | A-Tocophérol Abs (nm) |
|---|---|---|---|---|
| 12,5 | 0,336±0,007 | 0,166±0,008 | 0,525±0,015 | 0,811±0,050 |
| 25 | 0,372±0,006 | 0,228±0,032 | 1,513±0,019 | 0,842±0,021 |
| 50 | 0,379±0,010 | 0,362±0,015 | 1,760±0,014 | 0,981±0,055 |
| 100 | 0,437±0,014 | 0,664±0,090 | 1,833±0,025 | 1,440±0,019 |
| 200 | 0,457±0,014 | 1,090±0,014 | 3,161±0,083 | 1,920±0,022 |

[1]Abs. Absorbance

### 3.5 Anti-Bacterial Activity.

Antimicrobial effect tests on extracts of *C. sativus* have shown that these extracts are an important source of substances with antimicrobial activity. The results of various studies show that certain herbal medicines may well be potential sources of new antibacterial agents Kone et al. (2004). The table represents the zone of inhibition in mm, found by the activity of the extracts on the microorganisms tested. Overall, each extract therefore presented a fairly well defined activity on the in vitro growth of the microorganisms tested. The results show that the extracts of the petals have antibacterial activity against *Escherichia coli* Cip54127 and *Esherichia coliTg1* with zones of inhibition respectively of 14.5 and 8, and antifungal against Cryptococcus neoformans with a zone of inhibition of 13 mm at high concentration. On the other hand, the results show that the extracts of the leaves of *C. sativus* slightly inhibit the growth of these microorganisms and that the zones of inhibition do not exceed 6 mm.

**Table 5**. Zone of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against different types of microbial strains

| Extracts | Leaves (µg/µl) | | | | | Petals (µg/µl) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentrations<br>Microorganisms | 5µl | 10µl | 15µl | MIC | MBC | 5µl | 10µl | 15µl | MIC | MBC |
| *Staphylococcus aureus* | nd | 2 | 5,80 | 25 | nd | 1 | 2 | 3 | 50 | 12,50 |
| *Esherichia coli Tg1* | nd | 2 | 6 | 100 | 50 | nd | 4 | 8 | 6,25 | 100 |
| *Esherichia coli Dg5alfa* | nd | 2 | 6 | 25 | 50 | nd | 1 | 2,50 | 100 | 12,50 |
| *Escherichia coli Cip54127* | nd | 2 | 6 | 25 | 100 | 4 | 12 | 14,50 | 6,25 | 100 |
| *Bacillus cereus* | nd | 2 | 6 | 100 | 50 | nd | 1 | 2,50 | 100 | 100 |
| *Bacillus cereus Med5* | nd | 2 | 6 | 50 | 50 | nd | 1,50 | 2,50 | 100 | 100 |
| *Klebsiella pneumoniae* | nd | 2 | 6 | 50 | 100 | nd | 1,50 | 2,50 | 100 | 25 |
| *Citrobacter freundii* | nd | 2 | 6 | 100 | 100 | 1 | 2,55 | 5 | 25 | nd |
| *Bacillus sp* | nd | 2 | 6 | 100 | 100 | 1 | 3,55 | 6 | 50 | 50 |
| *Entercoccus faecalis Atcc19433* | nd | 2 | 6 | 100 | 50 | 2 | 5 | 6 | 50 | 50 |
| *Cryptococcus neoformans Cip960* | nd | 3 | 6 | 50 | nd | 1 | 2 | 2,5 | 50 | 100 |
| *Candida albicans* | nd | 2 | 6 | 50 | 50 | 1 | 2 | 2,50 | 50 | 100 |
| *Aspergillud niger* | nd | 2 | 6 | 100 | 100 | 2 | 2,50 | 3 | 6,25 | 6,25 |
| *Cryptococcus neoformans* | nd | 2 | 6 | 50 | 50 | 2,50 | 11 | 13 | 100 | 50 |

nd: not detected

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (CMB) of extracts of leaves and petals of *C. sativus* were determined by a macro-dilution method and the results are given in Tables 5. Results reported here may be affected by factors such as the extraction method, extract yield, antifungal, and antimicrobial test methods, etc. The inhibitory effects of extracts of petals vis-à-vis *Citrobacter freundii* with a MIC value of 25 µg / µl. The inhibitory effects of extracts of leaves vis-à-vis Bacillus cereus Med5, *Bacillus sp*, *Entercoccus faecalis Atcc19433*, with MIC values of 50 µg / µl, 100 µg / µl, 100 µg / µl, respectively. The petal extracts had the maximum antibacterial effect against *Cryptococcus neoformans, Entercoccus faecalis Atcc19433*, *Bacillus sp,* and *Candida albicans* with the lowest MIC values of 6.25; 50; 50; 50µg/µl, respectively. *Okmen et al (2016)* reported that the methanol and ethanol extracts of *Crocus sativus* inhibit Staphylococcus aureus with a MIC value of 6500 µg / ml. According to Vahidi et al. (2002) that the ethanolic extract of the leaves and the extract of the stamens of Crocus sativus have no effect on *Candida Albicans.* According to *Asgarpanah et al. (2013)*, the petal extract inhibitory effect against *Staphylococcus aureus ATCC 25923* with a MIC value of 31.2 mg/ml.

The CMB of the leaf extract varies between 50 and 100 µg / µl. Regarding the extract of the petals show a strong CMB against *Aspergillud niger* with 6.25 µg / µl, and for the other microorganisms, the CMB varies between 12.5 and 100 µg / µl (table 5). A study by *Asgarpanah et al. (2013)* showed that the methanol extract of petals and stamens of *C. sativus* exhibited bactericidal effects against S. aureus with the lowest CMB of 125 mg / ml. And that the extract of the petals at a CMB of 250 mg / ml against *Bacillus cereus* PTCC 1247, on the other hand the extract of stamens the CMB is 125 mg /

ml. Because of increasing incidence of multiresistant bacterial infection caused by Gram-positive bacteria (such as *Staphylococcus*, *Enterococcus* and Bacillus species) and Gram-negative bacteria (such as *klebsiellapneumoniae*, *Enterobacter* and *Echerichia coli*) medicinal plant have been used for centuries in folk medicine as remedies for human disease because they content compounds of therapeutic value (Okwu et al. 2006). The resistance of Gram-negative bacteria could be attributed to its cell wall structure. Gram-negative bacteria have an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the extruding the plant extract. Gram positive bacteria have a mesh-like peptidoglycan layer which is more accessible to permeation by the extracts Zuhaira et al. (2018). Therefore, petals and leaves extracts possess bioactive compounds that can be used as antibacterial agent in curing bacterial infection. Phenolic contents and flavonoid contents present in the plant body mainly give the idea of its medicinal importance. These are considered the index of the antioxidant and free radical scavenging strength of the plants, as these components are involved in deleting, neutralizing or scavenging free radicals Pietta (2000); Zuhaira et al. (2018) due to the presence of conjugated ring systems and carboxylic groups.

## Conclusion

As a conclusion, the results obtained from this study showed that *Crocus sativus* leaf and petal had antibacterial effect on common microorganisms that attack human. This waste produced during the production of saffron could be an interesting source of phenolic compounds. The petal extract was rich in flavonols: Quercetin and Kaempferol. Our study confirmed the identification of malvidin in leaves *Crocus* and isorhamnetin. The presence of flavonoids could contribute to its antimicrobial activities. It is suggested that biomass of *Crocus sativus* could be considered as source of antimicrobial and antioxydant agent which might be applied in pharmaceutical and cosmetic products.

## Declaration

Competing interests: The authors declare no competing interests.

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# Figures

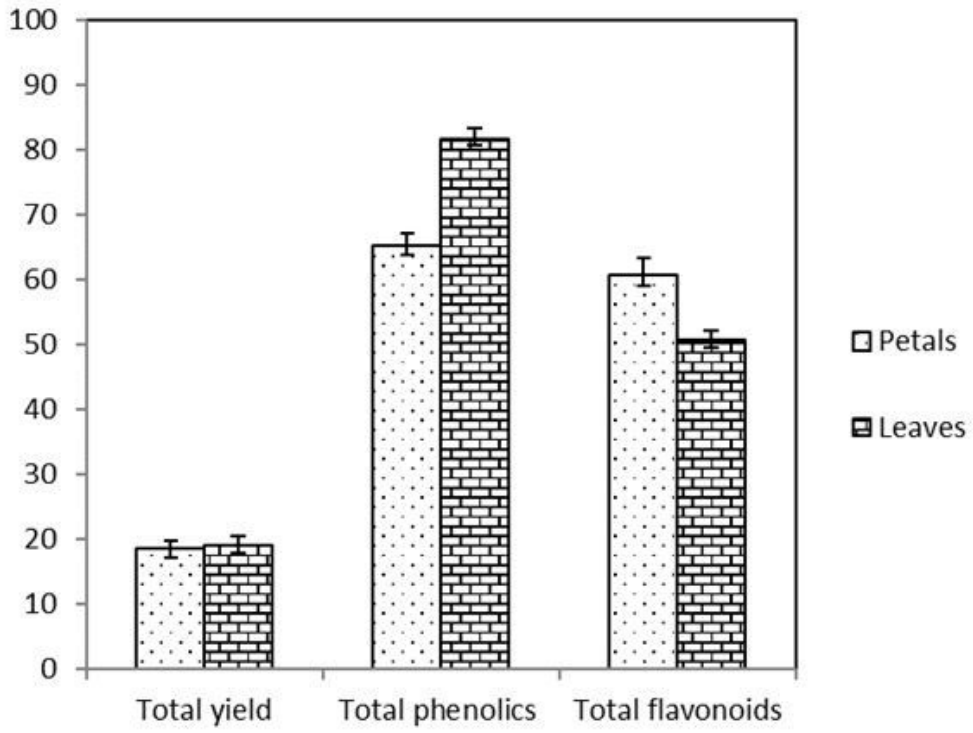
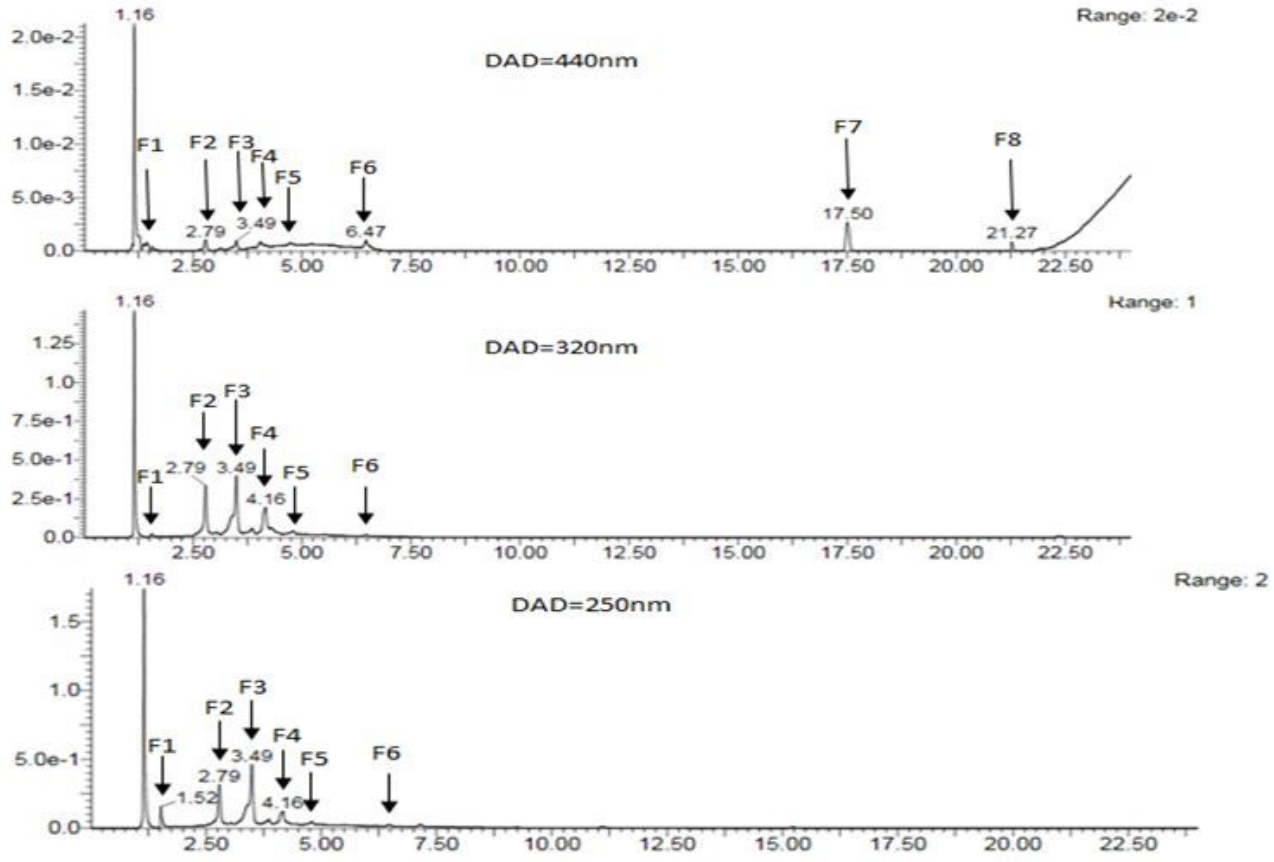


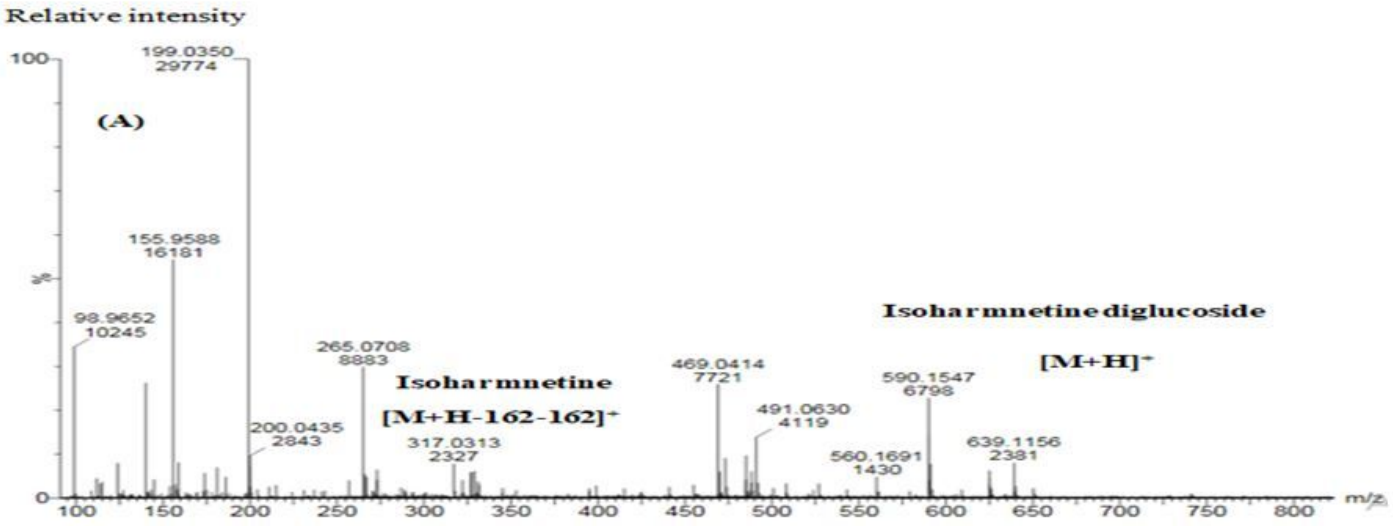

**Figure 1**

Total yield, total phenolic (mg GAE/mg), and total flavonoids contents (mg of CE/g of dry plant material) of methanolic extracts.



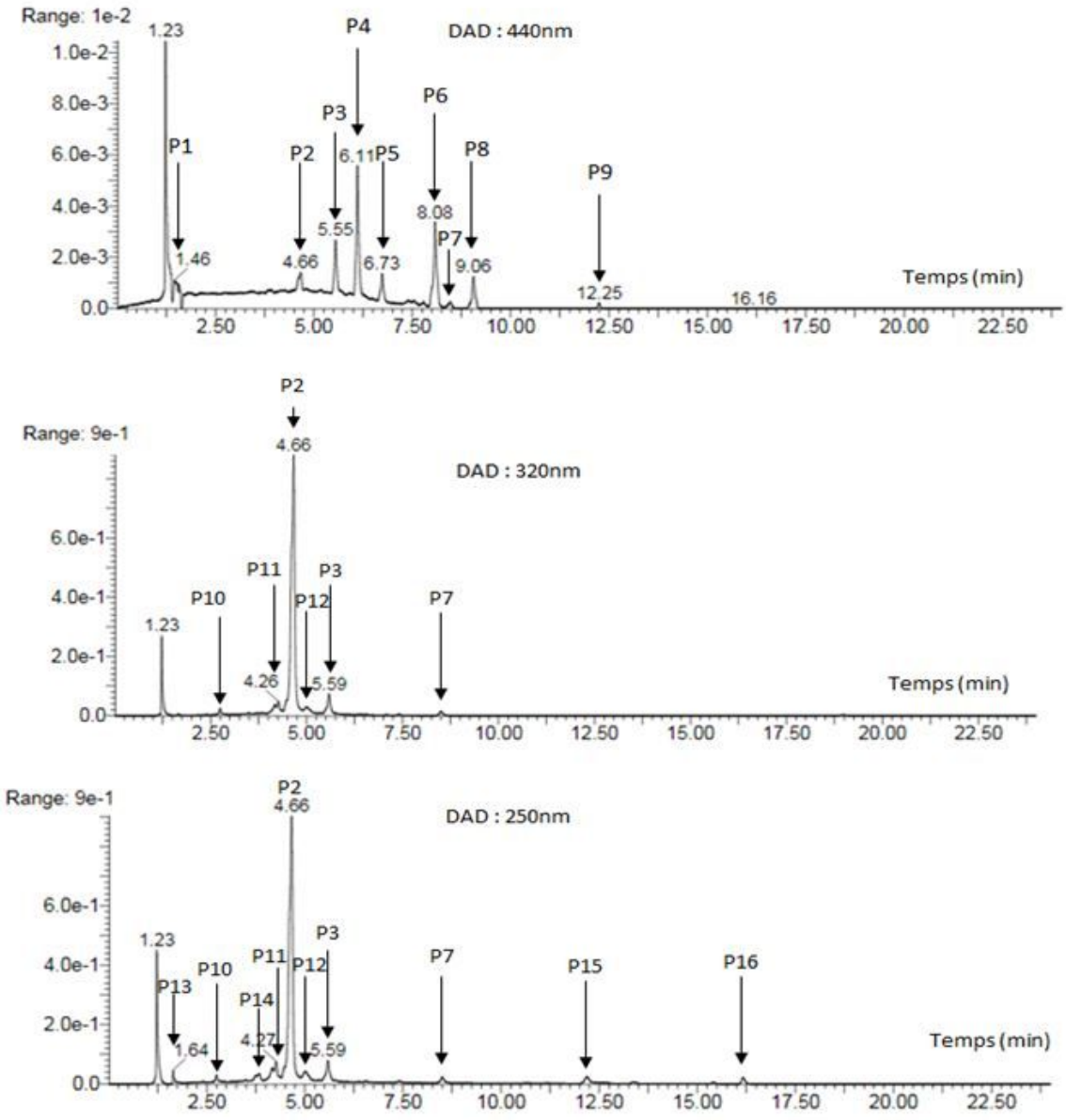

## Figure 2

UPLC DAD chromatograms of methanolic extract of Crocus sativus leaves at 440nm, 320nm and 250nm (see Table 1 for identification of numbered peaks)



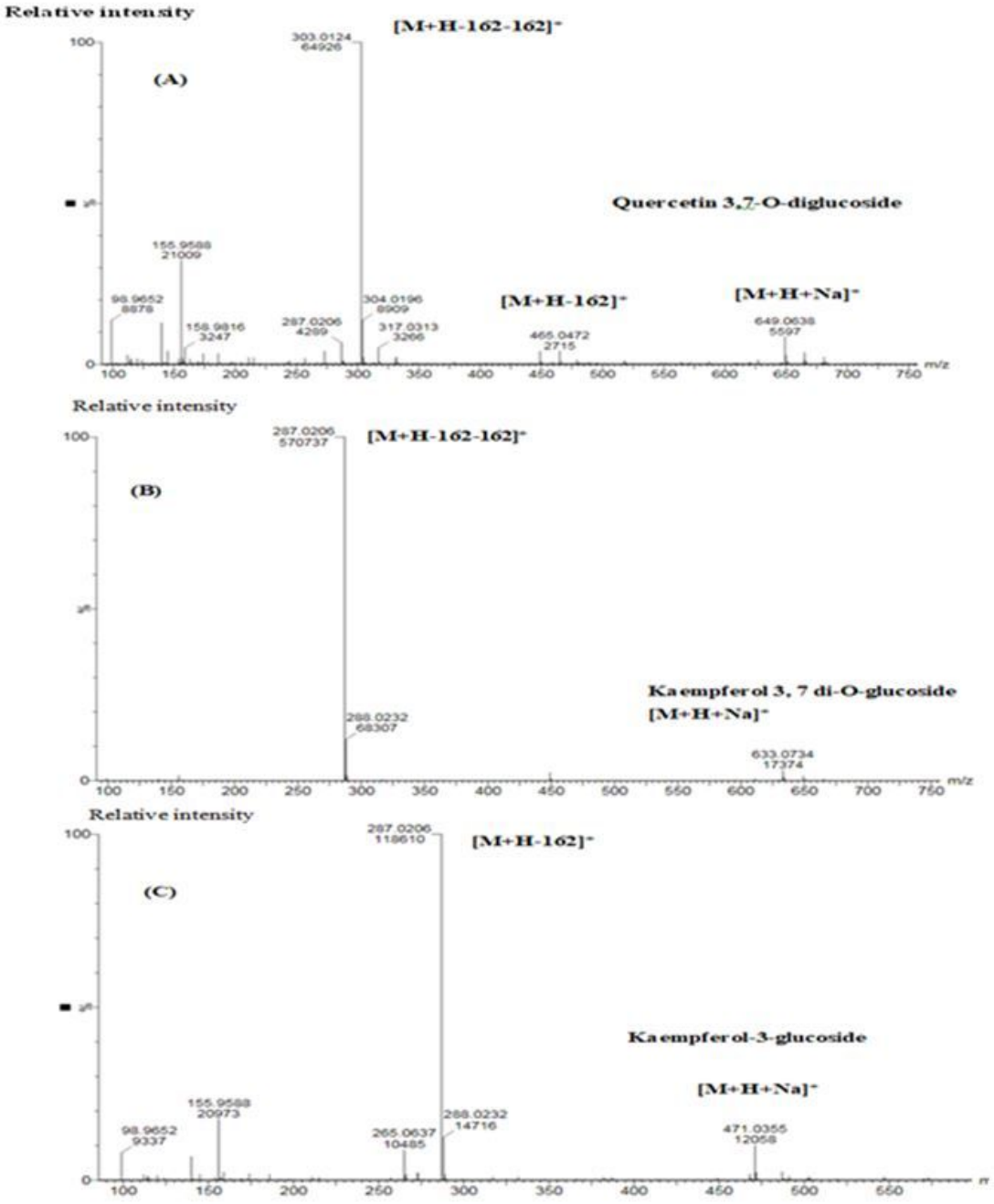

## Figure 3

Mass spectrometry fingerprints of Isoharmnetine diglucoside identified in Crocus sativus leaves.




## Figure 4

UPLC-DAD chromatograms of methanolic extract of Crocus sativus petals at 440nm, 320nm and 250nm (see Table 16 for identification of numbered peaks).




## Figure 5

Fingerprint mass spectrometry of quercetin 3, 7-O-diglucoside (A), kaempferol 3.7 di-O-glucoside (B) and kaempferol-3-glucoside (C) identified in petals Crocus sativus.