Pharmacological Research - Modern Chinese Medicine 11 (2024) 100420

Contents lists available at ScienceDirect

Pharmacological Research - Modern Chinese Medicine

journal homepage: www.elsevier.com/locate/prmcm

Anti-obesity, phytochemical profiling and acute toxicity study of ethanolic extract of saffron (*Crocus sativus* L.)

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ARTICLE INFO

Keywords:
Crocus sativus
High-performance liquid chromatography
Mass spectrometry
Toxicity
Zebrafish
Body mass index

ABSTRACT

Introduction: *Crocus sativus* (*C. sativus*), known as saffron, has been recognized for its medicinal benefits since ancient times. The plant was first introduced to the Chongming Island region of Shanghai, China, in 1980. Chinese Saffron, derived from this plant, is one of China's most valuable traditional herbs, yielding about 1 ton in 2013. Traditional Chinese Medicine (TCM) has used this plant as an antianginal for a long time. The plant has been extensively studied for its pharmacological benefits in treating obesity and gastrointestinal problems. This study aimed to conduct a comprehensive phytochemical analysis of *C. sativus* stigma (saffron) ethanolic extract using LCMS/MS, quantify its major compound, i.e., trans-4-GG-crocin, and study its acute toxicity. In addition, this study aimed to investigate the effect of the extract on anti-obesity in a high-fat diet-induced zebrafish model.
Methods: Before the anti-obesity study, we conducted a comprehensive profiling of saffron ethanolic extract using UHPLC-ESI-MS/MS in both positive and negative modes. Then, the principal constituent, i.e., trans-4-GG-crocin, was quantified using HPLC with a UV detector. The study was followed by an acute toxicity study for 72 h to evaluate the safety profile of this extract using five concentrations of extract, from 93.75–1500 mg/L. Six groups of wild-type zebrafish were then segregated according to food regimen, i.e., (1) control (5 mg artemia/fish/day), (2) overfeeding (60 mg of artemia/fish/day), (3–6) high-fat diet (5 mg artemia+30 mg egg yolk/fish/day), then starting week 9–16, groups 4–6 were received (4) 50 mg/L, (5) 100 mg/L and (6) 200 mg/L of saffron extract.
Results: Negative mode analysis revealed twenty phytoconstituents, while twenty-two were found in positive mode LCMS/MS analysis. The extract contained 536.91 μg/g of trans-4-GG-crocin. The highest concentration tested in the acute toxicity killed 100% of the testing animals within 24 h, and probit analysis showed that the LC_{50} of the extract was 1021 mg/L. Eight weeks of treatment with 100 and 200 mg/L of saffron significantly reduced the body mass index of the high-fat diet-induced zebrafish model ($p<0.0001$).
Conclusion: Saffron ethanolic extract contains mainly flavonoid, carotenoid, and terpenoid compounds. This extract is highly toxic in high concentrations (1500 mg/L), which kills 100% of the tested zebrafish. Eight weeks of treatment with 100 and 200 mg/L of saffron extract via immersion significantly reduced body mass index in the high-fat diet zebrafish model. The findings suggest that Saffron extract could be a promising natural anti-obesity agent that warrants further investigation. The study provides valuable insights into the potential of natural compounds as a source of anti-obesity agents and highlights the need for further research in this area. Even though this plant was studied for anti-obesity in rodent models, its mode of action is still unknown and warrants further investigation, which could be explored more in a cheaper and easier way by using another translational animal model, such as zebrafish.

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https://doi.org/10.1016/j.prmcm.2024.100420
Received 30 November 2023; Received in revised form 18 February 2024; Accepted 23 March 2024
Available online 30 March 2024

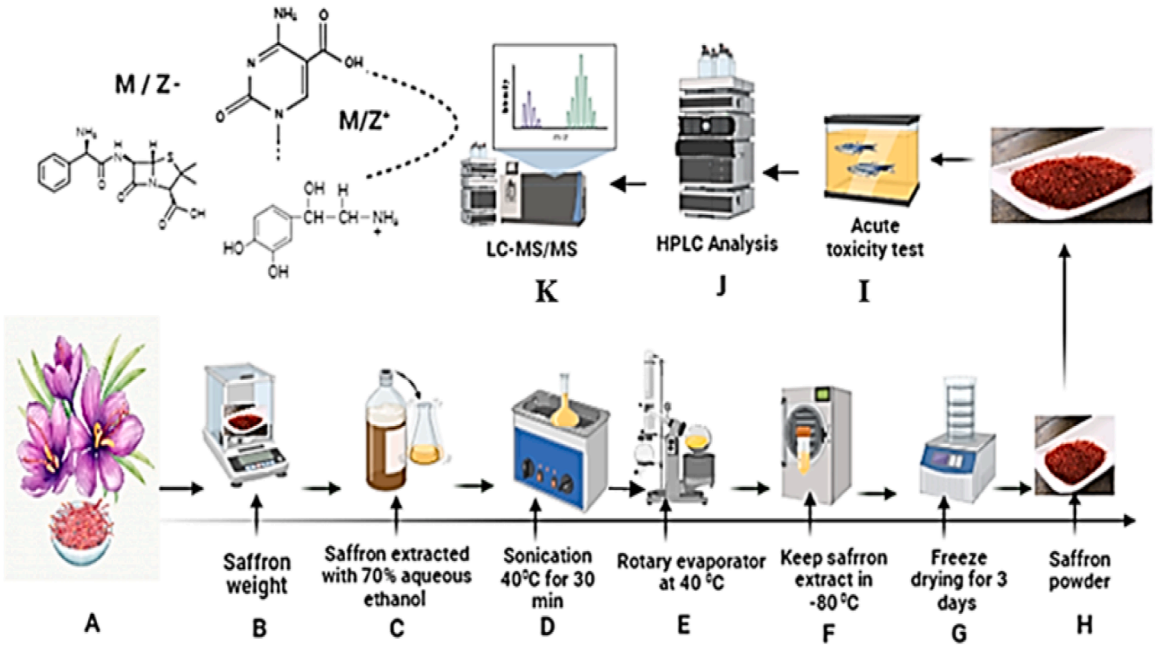


Fig. 1. Overview of experimental design.

Introduction

Obesity is a serious health concern as it can lead to various severe conditions such as diabetes, cardiovascular diseases, and cancers. Individuals who are obese often have lipid abnormalities, including high levels of cholesterol, low-density lipoprotein (LDL), and triglycerides. Although anti-obesity medications are available, they are often associated with undesirable side effects that can harm the kidneys, lungs, and liver. As an alternative, medicinal plants are used for their lower cost and perceived safety, as they are often consumed as food. Among the medicinal plants, *C. sativus* has been widely studied for its anti-obesity effects.

Saffron is a valuable herbaceous geophyte plant widely known for its culinary, dyeing, and medicinal properties [1]. The stigmas of flowers have been used in traditional medicine for centuries due to their therapeutic potential, such as antispasmodic, eupeptic, gingival sedative, nerve sedative, and many more [2]. This plant has been used as an antianginal in Traditional Chinese Medicine (TCM) for a long time [3]. In terms of preparation, there is no standard for drying processing. Most saffron products were dried by a water heating system based on the operator's experience. The rich bioactive profile of *C. sativus*, overwhelmed with crocin, safranal, and picrocrocin, underscores its significance in therapeutic research.

Phytochemicals from the *C. sativus* plant extract are widely studied for authentication evaluations, focusing on quantifying their main components, such as crocin and crocetin [4]. Studies have been done on improving the isolation of crocin and other constituents in saffron using current technologies such as molecular imprinting polymers embedded with quantum dots and others [5,6]. The complex composition of compounds in plant extract demands a comprehensive analytical approach for characterization. Liquid chromatography-tandem mass spectrometry (LCMS/MS) has emerged as an essential technique in phytochemical profiling due to its accurate identification and quantification of compounds, even at trace levels. High-performance liquid chromatography (HPLC) aids in separating complex mixtures, thereby streamlining method development for accurate profiling.

Although the benefits of *C. sativus* have been reported, it is crucial to evaluate its safety profile. A recent comprehensive review of *C. sativus* toxicity was reported [7]. A high dose of *C. sativus* extract could be developmental toxic as it caused reduced tail length, biparietal diameters, placental diameter and fetal weight during gestation in BALB/c mice after administration of 0.8, 0.4 and 0.2% of *C. sativus* aqueous extract. Zebrafish (*Danio rerio*) has emerged as a robust model organism in toxicity studies. It can offer several advantages, such as the concordance between zebrafish and mammals for developmental toxicity, which is 55–100%, and it is widely used for developmental origins of health and disease (DOHaD) [8]. Toxicity studies employing zebrafish can provide valuable insights into the potential adverse effects of *C. sativus* extracts and strengthen its therapeutic credentials. The anti-obesity potential of saffron is widely studied in clinical and preclinical settings, but the mechanism of action is still unclear [8]. Zebrafish were selected for anti-obesity because this animal model is simple and economical, with several anatomical characteristics comparable to humans, such as the skeletal muscle, adipose tissue, and digestive system [9].

Thus, the purpose of our study was to thoroughly analyze the phytochemicals present in the ethanolic extract of *C. sativus* using HPLC-ESI-MS/MS. We also developed and validated the HPLC method and assessed the acute toxicity of the extract using zebrafish. We aimed to help fill the existing knowledge gap and strengthen the scientific understanding of this herb.

Materials and methods

Plant materials

The *C. sativus* stigmas were obtained from Hannah Saffron Agriculture Company, Afghanistan. The overview of plant material preparation

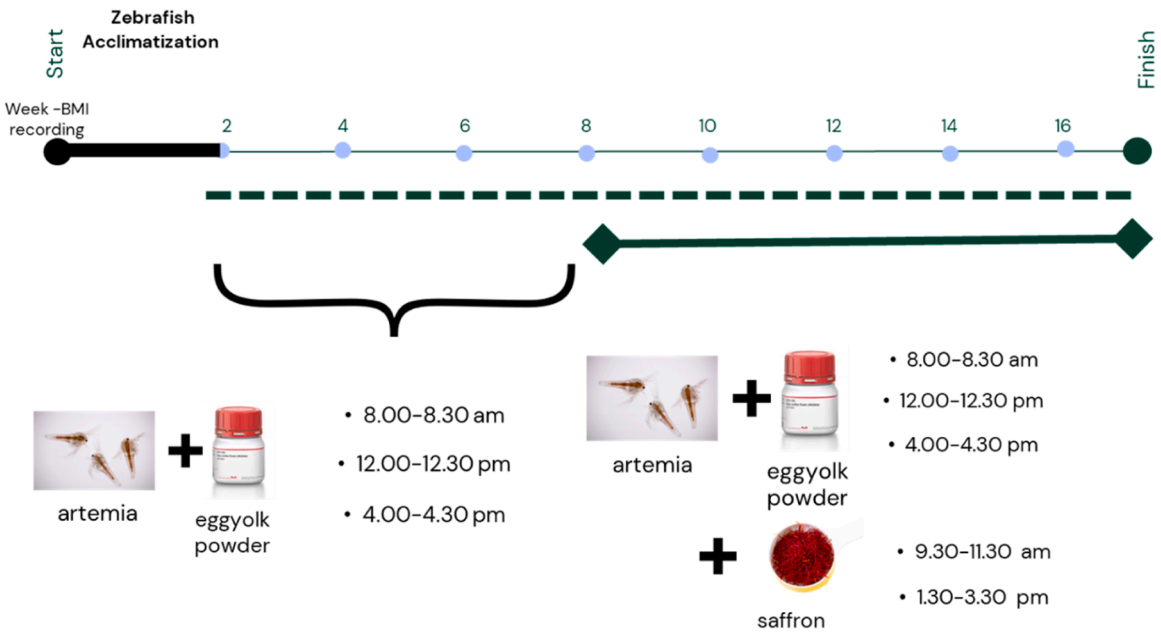


Fig. 2. Overview of anti-obesity study.

is presented in Fig. 1.

Chemicals

The chemicals utilized were of analytical grade, while the solvents (ethanol and acetonitrile) were of HPLC grade and procured from Fisher Scientific. For HPLC and LCMS/MS analysis, ultrapure water with a resistivity exceeding 18 m, obtained from a certified Milli-Q system (Millipore, Bedford, MA, US), was utilized. *Trans*-4-GG-crocin (Crocin I) (purity: 98.71%) was purchased from MedChem Express (USA).

Animals

The study used adult zebrafish (*Daino rerio*) 3-month-old, weighing (0.3±0.1 g) mixed-sex, (50:50 male: female) shortfin wild-type that was obtained from a local supplier (3B Aquatics, Bandar Baru Bangi, Selangor, Malaysia). The fish acclimated to their new environment for 14 days. The fish were kept in dechlorinated water with a pH of 7.0 ± 0.1 and a temperature of 26 ± 0.2 ∘C with a 14 h light and 10 h dark cycle. The fish were held in plastic tanks with dimensions 20 cm (length) x 8 cm (width) x 5 cm (height) filled with 2.5 L holding water. An aerator maintained the water's dissolved oxygen (DO) level at greater than 6.0

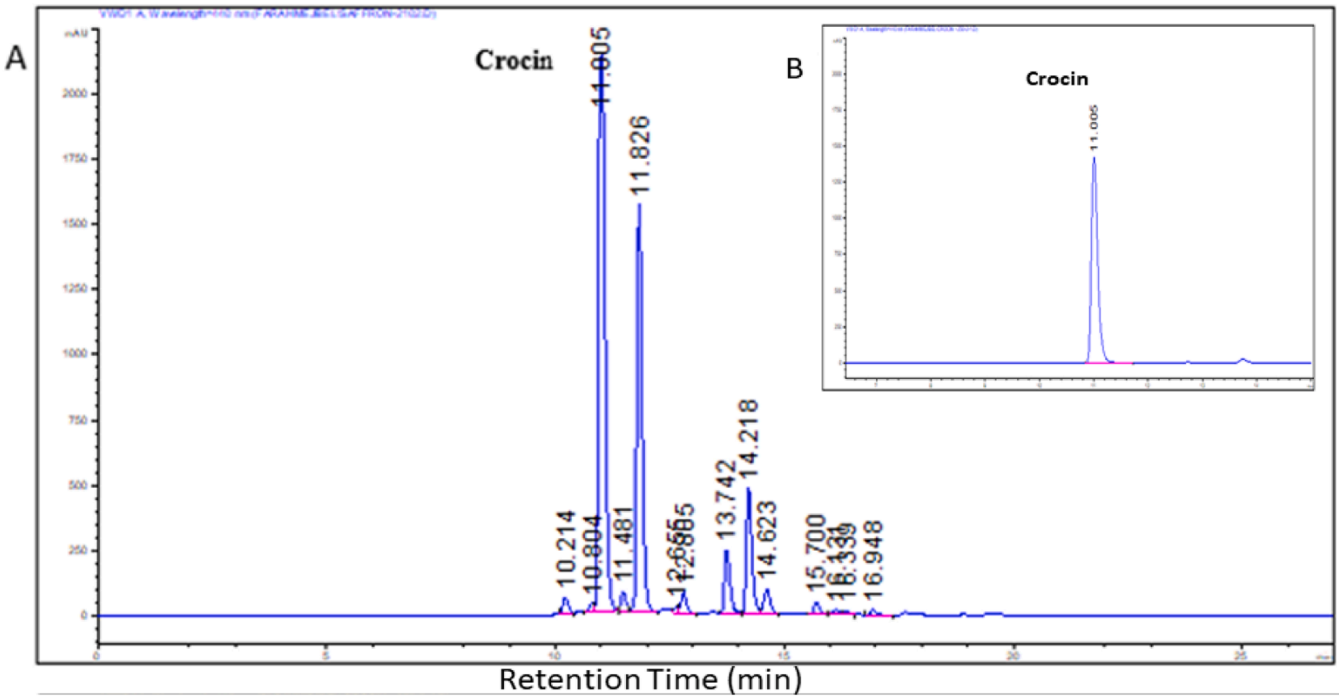


Fig. 3. HPLC chromatogram (UV detector, wavelength: 440 nm) of (A): Ethanolic extract of *C. sativus* and (B): crocin I standard. Crocin I was detected at $t_R = 11.005$ min in the standard and 11.005 in the extract.

Table 1
Linearity and sensitivity data for crocin I in ethanolic *C. sativus* extract.

No	Linear range (μg/ mL)	Regression equation	Slope (a)	R^2
1	15.62–500	y = 37.568x + 1763.4	429.12	0.9907
2	15.62–500	y = 37.626x + 1744.2	426.41	0.9908
3	15.62–500	y = 37.621x + 1754.5	428.80	0.9907

mg/L. During acclimatization, the fish were fed twice daily with tropical tetramine flakes (calories approximately 2000/fish/day). After acclimatization, the zebrafish were used for the acute toxicity test, induction of obesity, and anti-obesity experiment. All animal experiments were conducted following the approved protocols of the Animal Care USM Animal Ethics Committee (IACUC) at Universiti Sains Malaysia (No: (130) (1155)/2021). The experiments involving animals comply with the WMA statement on animal use in biomedical research.

Sample and standard solutions preparation

The dried stigmas were ground with a laboratory grinder and sifted through a 0.5 mm sieve. Then, 100 g of the dried stigmas powder was extracted three times using ultrasonic-assisted extraction in aqueous ethanol (70:30) for 30 min at 40 °C. Each time, the extract was filtered through No. 101 filter paper and then lyophilized in a freeze-dryer (Scanvac, Europe). Finally, the extract was stored at -4 °C until further analysis. To prepare a standard crocin I solution, 2 mg was accurately weighed and dissolved in 2 ml of ethanol. This resulted in a concentration of 1 mg/ml (1000 ppm). From there, serial dilutions were made to produce four additional concentrations of crocin (500, 250, 125, and 62.5 ppm). To prepare the extract, 2 mg of extract powder was added to 2 ml of ethanol to produce a 1000 ppm extract solution. Before HPLC and LCMS analysis, all solutions were filtered through a 0.22 μm filter membrane. All solutions were prepared fresh for the study.

Quantitative analysis of crocin by RP-HPLC

Chromatographic conditions

High-Performance Liquid Chromatography (HPLC) method development and validation were conducted at the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The chromatographic separation of *C. sativus* extract was performed on a UV detector Agilent HPLC 1260 series by using an Agilent column (RP-18, 4.6 mm x 250 mm, 5 μm) by gradient elution composition made up of ultrapure water+0.5% acetic acid (solvent A) and acetonitrile+0.5% acetic acid (solvent B). The mobile phase composition, represented as a ratio of A to B, was maintained at 95:5 for the initial 25 min of the analysis, following which it was transitioned to a ratio of 5:95 between the 25th and 26th minute and held constant for the ensuing 5 min at 5:95. The injection value was 20 μL. The flow rate was set at 0.8 mL/min. The injection value was 20 μL, and the analysis was performed at ambient temperature. The wavelength for the peak detection was set to 440 and 250 nm. The total analysis time was 30 min. The wavelengths used to quantify picrocrocin, safranal, and crocin following ISO 3632–2 2010 were 250 nm, 330 nm, and 440 nm, respectively [10].

HPLC method validation

The HPLC method was then validated for its specificity, linearity, accuracy, and sensitivity, according to the International Conference of Harmonisation (2009) [11].

a) Linearity

To test for linearity, 10 μL crocin I standard solutions with varying concentrations (15.65, 30.25, 62.50, 125, 250, and 500 μg/mL) were injected in triplicate. The peak area was plotted against the concentration to create a calibration curve, and the least-square regression method (R^2) was used to determine the linearity of the calibration graph.

b) Specificity

Specificity was tested to determine if the analytical method could distinguish crocin I peak from other components in the *C. sativus* extract. Separate injections were made of a blank solution, crocin I standard, and *C. sativus* extract samples, and the retention time of crocin I was compared in the extract to the peak of the standard crocin. The compound's identity was confirmed by spiking the crocin I standard compound (1000 ppm) into the *C. sativus* extract solution. A significant increase in crocin I peak was observed in the spiked *C. sativus* extract compared to the un-spiked sample, confirming the presence of crocin I in the extract.

c) Accuracy

Accuracy was evaluated as a relative standard deviation of the percentage recovery of the known concentration of spiked crocin I standard added to 1 mg/mL of the *C. sativus* extract. The un-spiked sample was prepared by mixing 0.7 mL of *C. sativus* extract stock solution (1 mg/ 1 mL) with 0.3 mL HPLC grade ethanol. Three spiked samples were prepared by mixing 0.3 mL of one of three concentrations of 400, 500, or 600 μg/mL of crocin I standard, added to 0.7 mL of 1 mg/mL of *C. sativus* extract. All prepared samples were injected in triplicate, and results are presented as average ± SD (n = 3). The percentage recovery was then calculated using the following formula:

$$\% \text{ Recovery} = ((A\text{-}B)/C) \times 100$$

where A is the concentration of the spiked sample calculated from the calibration curve equation. B is the un-spiked sample concentration, and C is the added amount calculated theoretically.

d) Precision

To assess precision, three different concentrations (62.5, 125, and 500 μg/mL) of crocin I were tested on the same day in triplicates (3 concentrations X 3 times = 9 injections/day); then, it was repeated for three consecutive days. Inter-day and intra-day precision were measured by calculating each concentration's relative standard deviation (%RSD) of the average recovery value.

e) Limit of detection (LOD) and limit of quantification (LOQ)

The LOD refers to the minimum concentration (expressed as a percentage or parts per million) that can be accurately determined with a precision of ±5%. The LOD can be calculated using the formula LOD = 3.3 δ/S, where δ represents the standard deviation of the y-intercepts of

Table 2
Inter-day and intra-day precision validation of crocin I in Saffron extract.

Day	Conc. (ppm)	Peak area [mAU*s]	Average	SD	% RSD
Intra-day	250	11,761.8	11,773.23	10.20	0.09
	250	11,776.5			
	250	11,781.4			
	62.5	4447.72	4447.90	2.79	0.06
	62.5	4445.20			
	62.5	4450.78			
	15.625	1546.33	1551.22	10.14	0.65
	15.625	1544.44			
	15.625	1562.88			
Inter-day					
1	250	11,755.90	11,750.60	5.57	0.05
2	250	11,744.80			
3	250	11,751.10			
1	62.5	4458.30	4458.37	12.50	0.28
2	62.5	4445.91			
3	62.5	4470.90			
1	15.625	1525.33	1517.23	7.46	0.49
2	15.625	1515.71			
3	15.625	1510.65			
Tested compound	**Retention time (n = 10)**	**Retention factor (k')**	**LOD (ppm)**	**LOQ (ppm)**	
Crocin I	11.005 ± 0.01	2.32	5.80	17.85	

Table 3
Positive mode LCMS/MS analysis of *C. sativus* ethanolic extract.

No.	Rt (min)	$(M+H)^+$	Exact Mass	Mass Error	Molecular Formula	LC-MS/MS fragmentation	Class of compound	Predicted compounds	Refs.
1	1.25	268.1038	267.09675	-4.95	$C_{10}H_{13}N_5O_4$	136.0612 (100%), 154.0467,115.0387	Amino acid	Adenosine	[17]
2	2.21	447.1860	446.17881	-2.64	$C_{20}H_{30}O_{11}$	439.2502 (100%), 307.1112,89.0597	Sugar	Crosatoside B	[17]
3	4.14	155.1071	154.09937	-4.20	$C_9H_{14}O_2$	137.0955 (100%)159. 0210,109.1010	Terpenoid	Crocusatin A	[20] [17]
4	4.70	611.1606	610.15338	-1.89	$C_{27}H_{30}O_{16}$	287.0537 (100%), 303.1400,213.1918	Flavonoid	Rutin	[20] [18],
5	4.93	151.1124	150.10446	-2.90	$C_{10}H_{14}O$	151.1110 (100%), 123.1164 (100%), 81.0699, 133.1006, 109.1009	Terpenoid	Safranal	[12] [20]
6	5.49	331.1753	330.16785	-2.79	$C_{16}H_{26}O_7$	123.1184, 169.1215 (100%), 348.1614	Terpenoid	Picrocrocin	[19]
7	5.78	449.1068	448.10056	-4.75	$C_{21}H_{20}O_{11}$	287.0536 (100%), 177.0658, 145.0173	Flavonoid	kaempferol-3-o-glucoside (Astragalin)	[17] [20]
8	5.92	611.1611	610.15338	-1.07	$C_{27}H_{30}O_{16}$	287.0537 (100%), 347.0674, 329.0640	Flavonoid	kaempferol-3,7-di-O-β-D-glucoside	[17]
9	6.84	287.0549	286.04773	-4.22	$C_{15}H_{10}O_6$	238.0519 (100%), 285.0524, 241.0476	Flavonoid	Kaempferol	[17]
10	7.51	977.3832	976.37875	-4.01	$C_{44}H_{64}O_{24}$	329.1729 (100%), 675.8837, 999.3835	Carotenoid	Cis-4-GG-Crocin	[7]
11	8.00	491.2259	490.22028	-5.61	$C_{26}H_{34}O_9$	329.1730 (100%), 675.2587, 473.2148	Carotenoid	Crocetin glucosyl ester	[17]
12	8.45	653.2822	652.27310	1.10	$C_{23}H_{44}O_{14}$	329.1729 (100%), 635.3459,473.2166	Carotenoid	Cis-2-G-Crocin	[7]
13	8.79	653.2828	652.27310	2.02	$C_{23}H_{44}O_{14}$	329.1727 (100%), 311.1627,513.0129,	Carotenoid	trans-2-G-Crocin	[7]
14	9.62	1139.4388	1138.43157	-1.00	$C_{50}H_{74}O_{29}$	329.1732 (100%), 675.2592, 837.3107	Carotenoid	Cis-5-tg-crocin	[7]
15	10.01	329.1746	328.16745	-3.74	$C_{20}H_{24}O_4$	128.1630 (100%), 131.0954, 293.1524	Carotenoid	Crocetin	[17]
16	10.28	977.3828	976.37875	-4.42	$C_{44}H_{64}O_{24}$	329.1732 (100%), 674.8837,837.3107	Carotenoid	Trans-4-ng-Crocin	[7]
17	10.9	977.3890	976.37875	1.91	$C_{44}H_{64}O_{24}$	675.0285 (100%), 329.1653,491.1572	Carotenoid	Trans-4-GG-Crocin	[7]
18	11.4	815.3365	814.32592	2.69	$C_{38}H_{54}O_{19}$	329.1654 (100%), 491.1112, 145.0491	Carotenoid	Trans-3-Gg Crocin	[7]
19	12.5	977.3837	976.37875	-3.50	$C_{44}H_{64}O_{24}$	959.3198 (100%), 637.3107,491.1637	Carotenoid	Cis-4-ng-Crocin	[7]
20	13.6	653.2831	652.27310	2.47	$C_{23}H_{44}O_{14}$	329.1727 (100%), 655.3459,473.2149,	Carotenoid	Cis-2-gg-Crocin	[7]
21	17.1	343.1830	342.18310	-1.92	$C_{21}H_{26}O_4$	118.0852(100%),293.1523,211.1107	Carotenoid	Methyl Crocetin	[20]
22	21.2	815.3367	814.32592	2.93	$C_{38}H_{54}O_{19}$	329.1727 (100%), 311.1627,473.2149,	Carotenoid	Cis-3-gg-Crocin	[7]

the regression lines and S is the slope of the calibration curve. The LOQ is the lowest quantity of the analyte that can be measured with both precision and accuracy in a sample, and it can be determined by calculating 10δ/S.

Tandem LCMS analysis of C. sativus extract

C. sativus ethanolic extract profiling was done at the Institute of Bioscience, Universiti Putra Malaysia (UPM), using a Q Exactive Focus Orbitrap LC-MS/MS Mass Spectrometer, equipped with Thermo Scientific Dionex model Ultimate 3000 UHPLC system (Thermo Scientific, USA). The UHPLC column was used (100 × 2.1 mm, 1.9 um, Hypersil Gold Aq) (Thermo Fisher Scientific, USA). Gradient elution was performed at a flow rate of 0.3 mL/min at a 30 °C column temperature using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a 40 min total run time. The injection volume of the sample was 2 μL. The gradient started at 5% B (0–25 min), 95% B (25–26 min), and 5% B (26–30 min). The accurate mass measurements were acquired using a Q Exactive Focus, Thermo Scientific (USA) mass spectrometer in positive and negative ESI mode. The data-dependent acquisition (DDA) experiment was performed for MS/MS acquisition. The five most abundant ions per MS scan were selected and fragmented with three collision energies (15, 30, and 45). This mode was used for structure elucidation. The raw data obtained were converted from the workflow to identify plausible candidates. Raw (Thermo data format) to a .mzML file (open data format) using MSConvertGUI software (https://proteowizard.sourceforge.io/download.html), freely assessable for public use [9]. Then, the data were submitted to MZmine 3.0 for spectral visualization, MS pre-processing, and metabolite annotations [12]. The LC-MS peaks were annotated by evaluating the MS/MS fragment via open access MS/MS library (https://mona.fiehnlab.ucdavis.edu/), followed by confirmation of the identification using in silico fragmentation using MetFrag (https://msbi.ipb-halle.de/MetFrag/) and CFM-ID (https://cfmid.wishartlab.com/), and the identified precursor ions, neutral loss, accurate mass and fragment were again searched using Metline (https://metlin.scripps.edu/). These results were then cross-checked with the published articles [2,13–17]. The compound's classification and exact mass were retrieved from the https://pubchem.ncbi.nlm.nih.gov/ website.

Acute toxicity study of C. sativus ethanolic extract in zebrafish model

According to the Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals number 203, an acute toxicity test was carried out. Fifty adult zebrafish wild-type shortfin were acclimatized to laboratory conditions for 14 days before the acute toxicity test. The conditions of zebrafish at LC_{50} are room temperature was set at 26 ± 2C, pH was monitored daily and maintained at 7.0 ± 0.2, and the light-dark cycle is 14 h:10 h. The conditions set are the same for the whole animal study.

For the acute toxicity study, zebrafish were segregated into five groups (n = 10/group), and each group received a single concentration of the extract by immersion. The extract concentrations of *C. sativus* were prepared in a geometric series with a factor of 2, ranging from 93.75 to 1500 g/mL. The dose selection is based on our previous acute toxicity studies using zebrafish animal models on other plant extracts. The mortality rate of each group was monitored and recorded at intervals of 3, 8, 24, 48, 72 and 96 h. LC_{50} value was determined at the end of the testing period using Probit regression analysis on SPSS v.27 software. The toxicity of *C. sativus* extract was evaluated under static conditions; the test solution was added once and left undisturbed throughout the experiment. Mortalities and visible abnormalities related to appearance and behaviour were recorded. The pH and water temperature in the test chambers were monitored daily. All animal experiments were conducted following the approved protocols of the Animal Care USM Animal Ethics Committee (IACUC) at Universiti Sains Malaysia.

Test dose selection

The test doses were chosen based on our acute toxicity evaluations of the saffron extract, with LD_{50}=1021 mg/L. We selected the concentrations to be tested, which are 200 mg/L, 100 mg/L, and 50 mg/L.

Anti-obesity study of saffron in high-fat diet zebrafish model

The experimental overview of the anti-obesity study is presented in Fig. 2. The method for inducing obesity in zebrafish followed two established procedures by Oka et al. [18] and Landgraf et al. [19], with some modifications. Male and female (50:50) adult zebrafish were used for this study. During the experimental period, zebrafish were divided into six groups (n = 10/group), i.e., (1) control (regular diet, 5 mg artemia/fish/day), (2) negative control (high-fat diet, no treatment), (3) high-fat diet + treatment with 200 mg/L saffron, (4) high-fat diet + treatment with 100 mg/L saffron, (5) high-fat diet + treatment with 50 mg/L saffron and (6) overfeeding group (60 mg artemia/fish/day). The high-fat diet was given thrice daily from week one until week 16, while treatment with saffron via immersion was done from week 8 to week 16. The high-fat diet consisting of freshly hatched artemia (22% fat, 16% carbohydrate, and 44% protein, total calorie: 150 cal) and egg yolk powder (59% fat, 32% protein, 2% carbohydrates) (E-6025, Sigma Aldrich) while overfeeding (OF) group received 60 mg of artemia/-fish/day divided into 20 mg/feeding/fish (20 cal), fed thrice a day. The first feeding started from 8.00–8.30 am; the second was from 12.00–12.30 pm. The last feeding was from 4.00–4.30 pm. The fish tanks were clean after each feeding and saffron treatment procedure. Body mass index (BMI) was measured every two weeks. After it was confirmed that the fish's BMI in the high-fat diet group showed a significant increase, the treatment with saffron was initiated from week eight onwards. The fish immersion in the saffron solution was done after 60 min of the first and second feedings for two hours each. The pH of holding water was recorded before and after the treatment to ensure the conditions followed the conditions suggested in the zebrafish book (https://zfin.org/zf_info/zfbook/zfbk.html). The food regimen and treatment are summarized below:

Group 1: The control group received 5 mg of freshly hatched artemia/fish/day.

Group 2: The overfeeding group received 60 mg of freshly hatched artemia /fish/day.

Group 3: The high-fat diet group received 5 mg of freshly hatched artemia and 30 mg of egg yolk /fish/day.

Group 4: The high-fat diet group received 5 mg of freshly hatched artemia and 30 mg egg yolk + 50 mg saffron extract /fish/day.

Group 5: The high-fat diet group received 5 mg of freshly hatched artemia and 30 mg egg yolk + 100 mg saffron extract /fish/day.

Group 6: The high-fat diet group received 5 mg of freshly hatched artemia and 30 mg egg yolk + 200 mg saffron extract /fish/day.

Measurement length and weight (BMI)

The weight and length of the fish were measured every two weeks, starting from a day before the obesity induction. The fish were anaesthetized using ice water (about 8℃) before length measurement until only gills movement was observed. The length of the fish was measured

Table 4
Negative mode LCMS/MS analysis of ethanolic *C. sativus* extract.

No.	Rt (min)	*m/z* $(M\text{-}H)^-$	Exact Mass	Mass Error	Molecular Formula	LC-MS/MS Fragmentation	Class of Compound	Predicted Compound	Refs.
1	3.83	771.1960	772.20620	-3.80	$C_{33}H_{40}O_{21}$	285.0308 (100%), 609.1442, 255.0286, 446.0825	Flavonoid	kaempferol-3,7,4-triglucoside	[2]
2	4.71	329.1621	330.16785	4.62	$C_{16}H_{26}O_7$	167.0697 (100%), 152.0464, 283.5397, 303.0561	Terpenoid	Picrocrocin	[14] [11]
3	4.86	609.1461	610.15338	-2.63	$C_{27}H_{30}O_{16}$	285.0315 (100%), 255.0289, 151.0022, 446.0910	Flavonoid	Kaempferol-3-O-Sophoroside	[15]
4	5.91	329.0666	330.07395	-1.75	$C_{17}H_{14}O_7$	301.0674 (100%), 151.5125, 284.3628	Flavonoid	Dimethyl quercetin	[15]
5	7.1	593.1497	594.15847	-2.51	$C_{27}H_{30}O_{15}$	285.0314 (100%), 255.0280, 277.0338,159.9771	Flavonoid	Kaempferol-3-O-rutinoside	[15]
6	7.64	623.1612	624.16903	-3.14	$C_{28}H_{32}O_{16}$	315.0421 (100%), 271.0237, 151.2417	Flavonoid	Isorhamnetin-rutinoside	[15]
7	8.3	447.0404	448.10056	-3.09	$C_{21}H_{20}O_{11}$	285.0316 (100%), 255.0287, 159.0020, 229.0336	Flavonoid	Kaempferol-hexoside	[15]
8	9.4	1137.4243	1138.43157	-3.78	$C_{50}H_{74}O_{29}$	813.3160 (100%), 283.1692,327.1591	Carotenoid	Crocetin gentiobiosyl neapolitanosyl ester (Crocin F)	[11] [14]
9	9.84	651.2637	652.27310	-3.27	$C_{32}H_{44}O_{14}$	327.1588 (100%), 283.1693, 239.1794	Carotenoid	(Trans-2-gg Crocin) (Crocin C)	[12] [13]
10	10.61	149.0971	150.10446	2.08	$C_{10}H_{14}O$	113.0545 (100%), 105.0697, 81.0700	Terpenoid	Safranal	[14]
11	11.38	813.3157	814.32592	-3.63	$C_{38}H_{54}O_{19}$	283.1692 (100%), 327.1591, 651.1795	Carotenoid	Trans-3Gg-crocin (Crocin B)	[12] [14]
12	11.99	430.0905	431.09782	1.29	$C_{21}H_{19}O_{10}$	285.0316 (100%), 133.0287, 159.0020,	Flavonoid	Kaempferol-rhamnoside	[15]
13	13.08	975.3698	976.37875	-2.74	$C_{44}H_{64}O_{24}$	651.2638 (100%), 327.1590, 283.1693	Carotenoid	Trans-crocetin di(β-D-gentiobiosyl) ester	[13]
14	13.86	975.3698	976.37875	-3.46	$C_{44}H_{64}O_{24}$	651.1848 (100%), 327.1745, 153.0905	Carotenoid	Cis-4GG-Crocin	[12]
15	14.87	975.3698	976.37875	-2.84	$C_{44}H_{64}O_{24}$	651.2638 (100%), 327.1590, 283.1693, 329.1793	Carotenoid	trans-4-gg- Crocin (Crocin A)	[12] [13] [14]
16	15.5	813.3157	814.32592	-3.63	$C_{38}H_{54}O_{19}$	327.1590 (100%), 651.2629, 489.2109, 833.4432	Carotenoid	Trans -Crocin-3(gen-glu)	[19]
17	16.93	489.2115	490.22028	-3.07	$C_{26}H_{34}O_9$	327.1591 (100%), 283.1693, 323.1793	Carotenoid	(Crocin E) Crocetin -D glucosyl ester	[14] [11]
18	18.25	651.2637	652.27310	-2.65	$C_{32}H_{44}O_{14}$	239.1794 (100%), 327.1588, 283.1693, 119.0850	Carotenoid	Crocetin β D gentobiosyl ester	[11]
19	12.73	535.4207	536.43820	1.90	$C_{40}H_{56}$	327.1590 (100%), 283.1692, 489.2114	Carotenoid	β-Carotene	[14]
20	19.52	327.1611	328.16745	2.80	$C_{20}H_{24}O_4$	165.1832 (100%), 239.1805, 283.7473	Carotenoid	Crocetin	[11]

from the anterior most point of the mouth to the most posterior region of the caudal peduncle using an electronic measuring ruler. The weight of the fish was measured by subtracting the weight of water in a beaker with the fish from the weight of the water in a beaker without the fish. BMI was calculated using the following equation:

$$BMI = [\text{Weight (g)} / (\text{length (cm)})^2].$$

Statistical analysis

Data analysis for the study was carried out using GraphPad Prism9 software. Data were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was carried out to compare all groups with the Duncan test used as the post hoc test. The Tukey test offers improved statistical control and more reliable multiple comparisons among groups. All the analyses defined values with $P < 0.05$ at a 95% confidence interval as statistically significant.

Results

Quantitative analysis of crocin I by RP-HPLC

Fig. 3 presents the HPLC chromatograms of saffron ethanolic extract

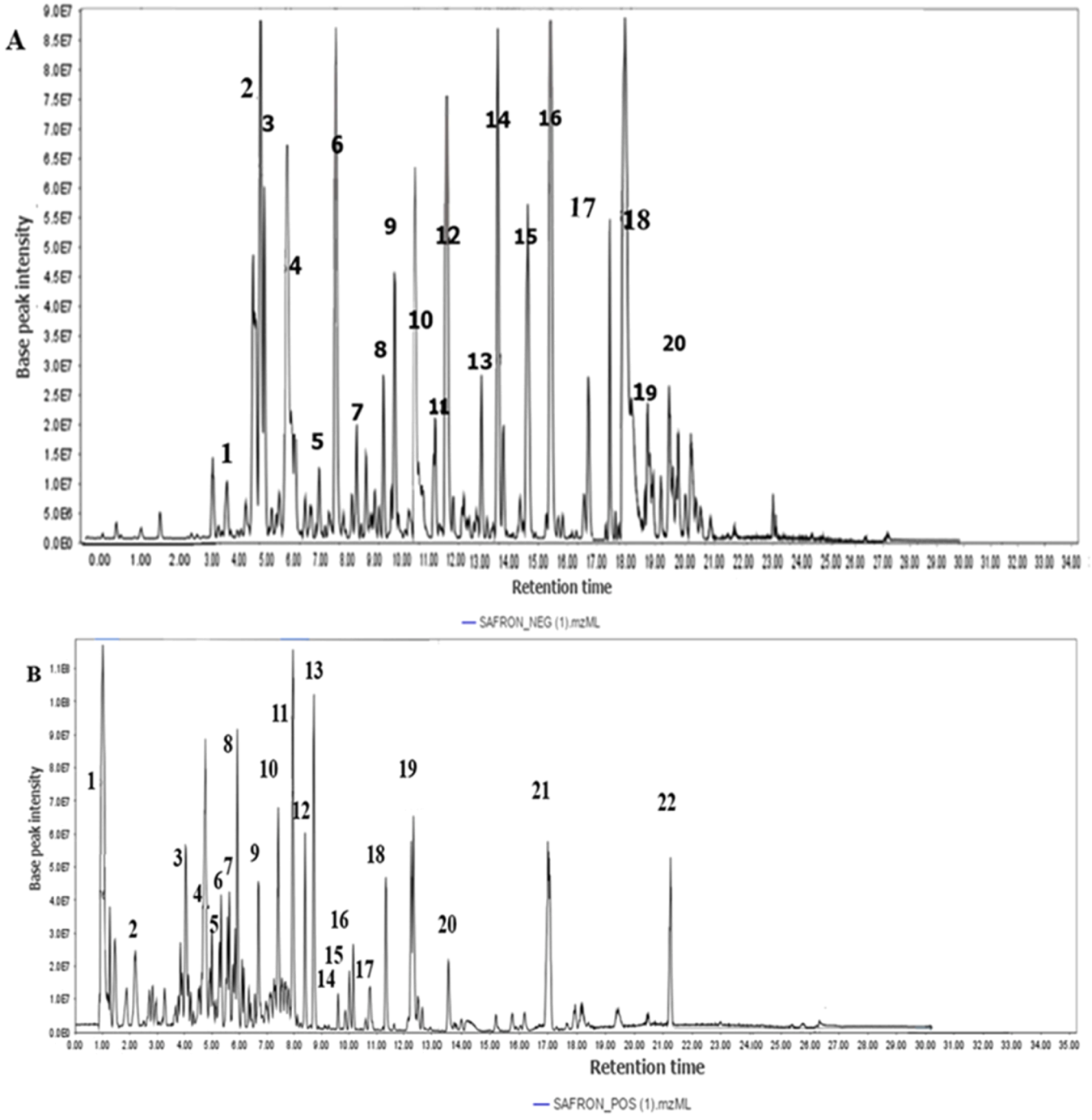
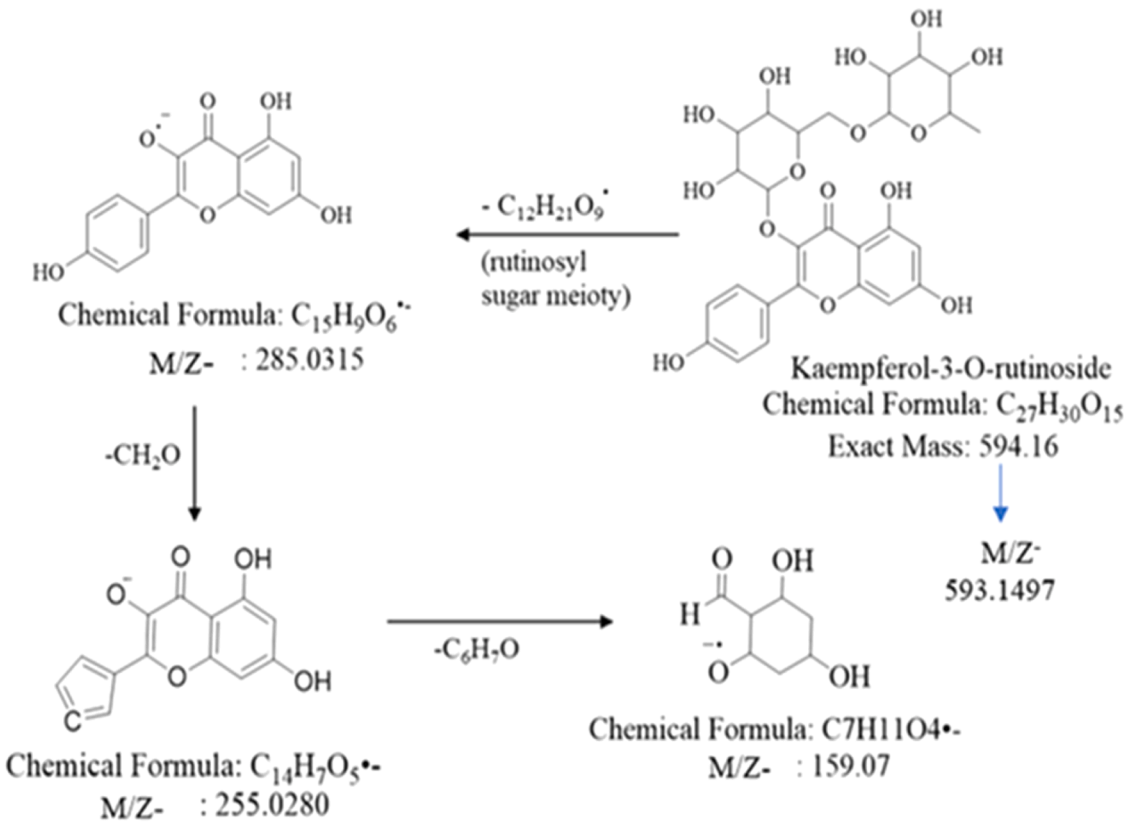


Fig. 4. Base peak chromatogram (BPC) of *C. sativus* ethanolic extract; A: Negative mode, B: Positive mode. Numberings refer to the constituents presented in Tables 3 and 4.



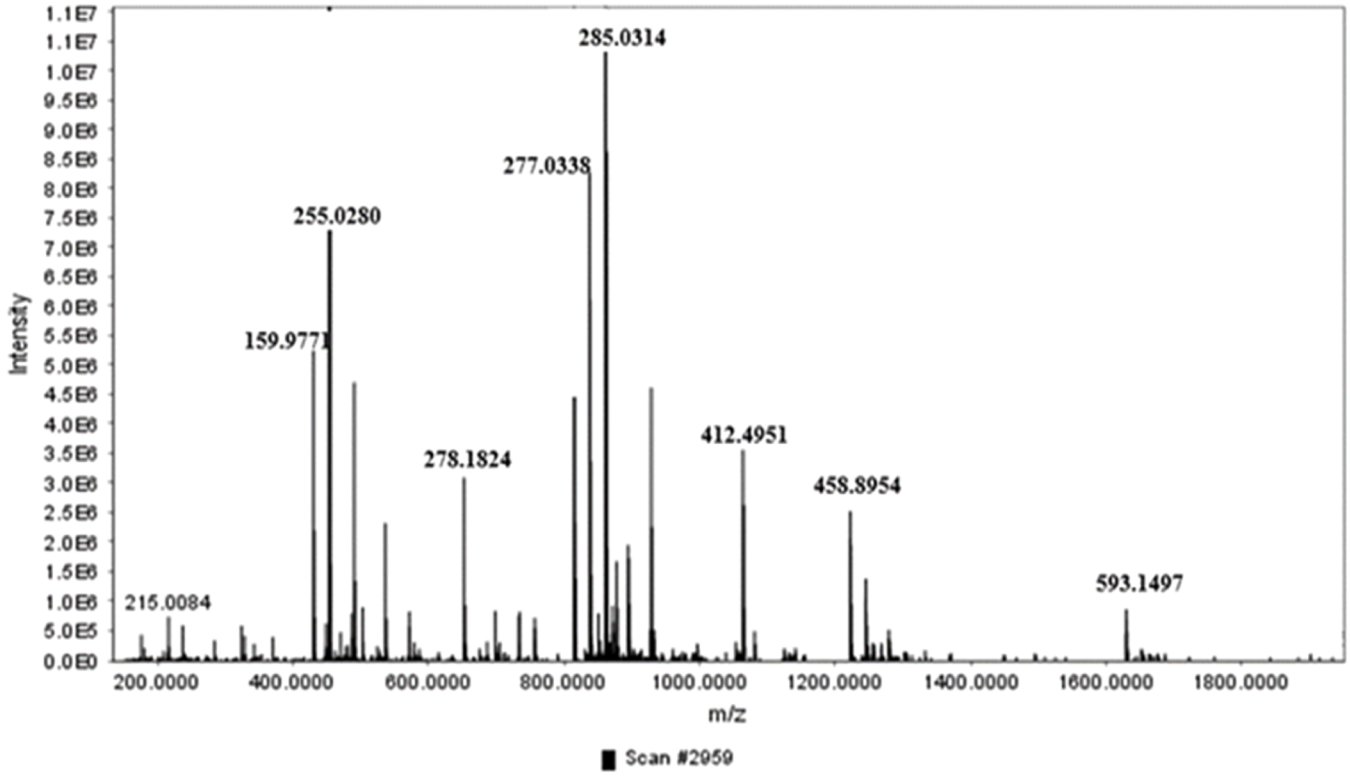


Fig. 5. Fragmentation of kaempferol-retonoside with its respective mass spectrum.

and crocin standard to confirm the peak of crocin I (trans-4-GG-crocin) in the extract based on retention time. The quantitative analysis determined that the ethanolic extract contained 536.91 μg/gram of crocin I, which was validated using external calibration to ensure accurate, precise, and reproducible data. The validation procedure followed the International Conference on Harmonization (ICH) guidelines. It included the following validation characteristics: linearity, specificity, accuracy, precision, LOD and LOQ.

(a) Linearity

The linearity of the calibration curve was assessed via the linear regression equation (y = mx + c). The obtained standard calibration curves depicted linearity in the tested range with good correlation coefficients for the compound with all concentrations, with R^2 ranging between 0.9907 and 0.9908. As shown in Table 1, the HPLC method has a good linearity.

(b) Specificity

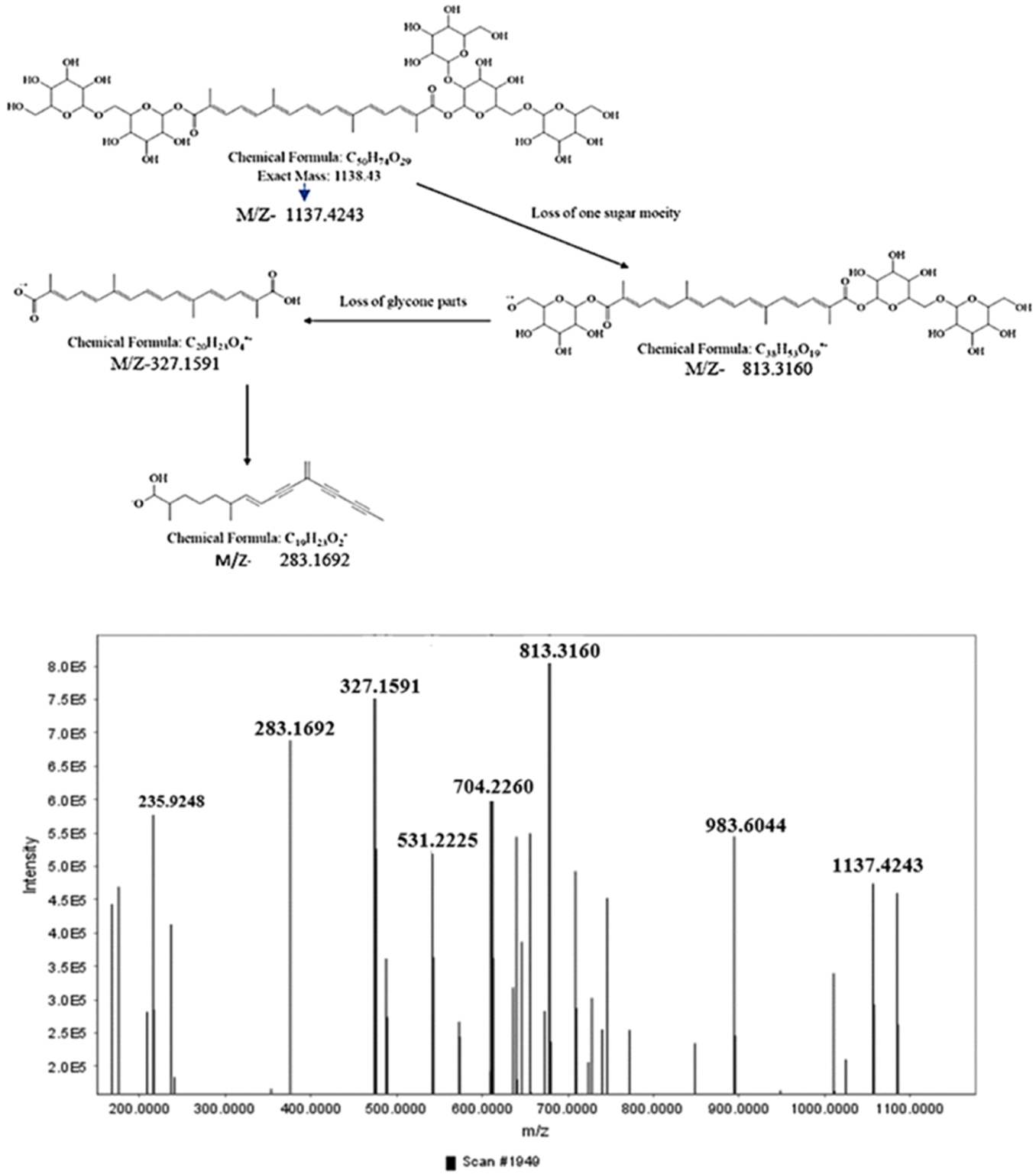
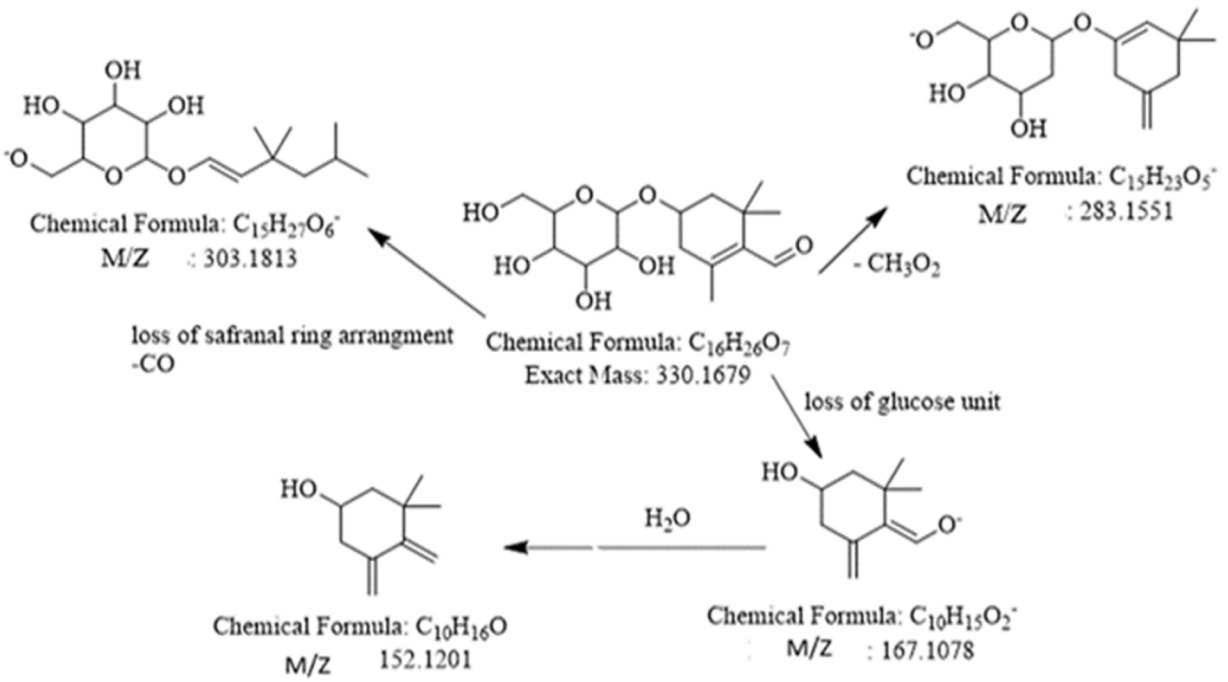


Fig. 6. Crocetin fragmentations with respective mass spectrum.



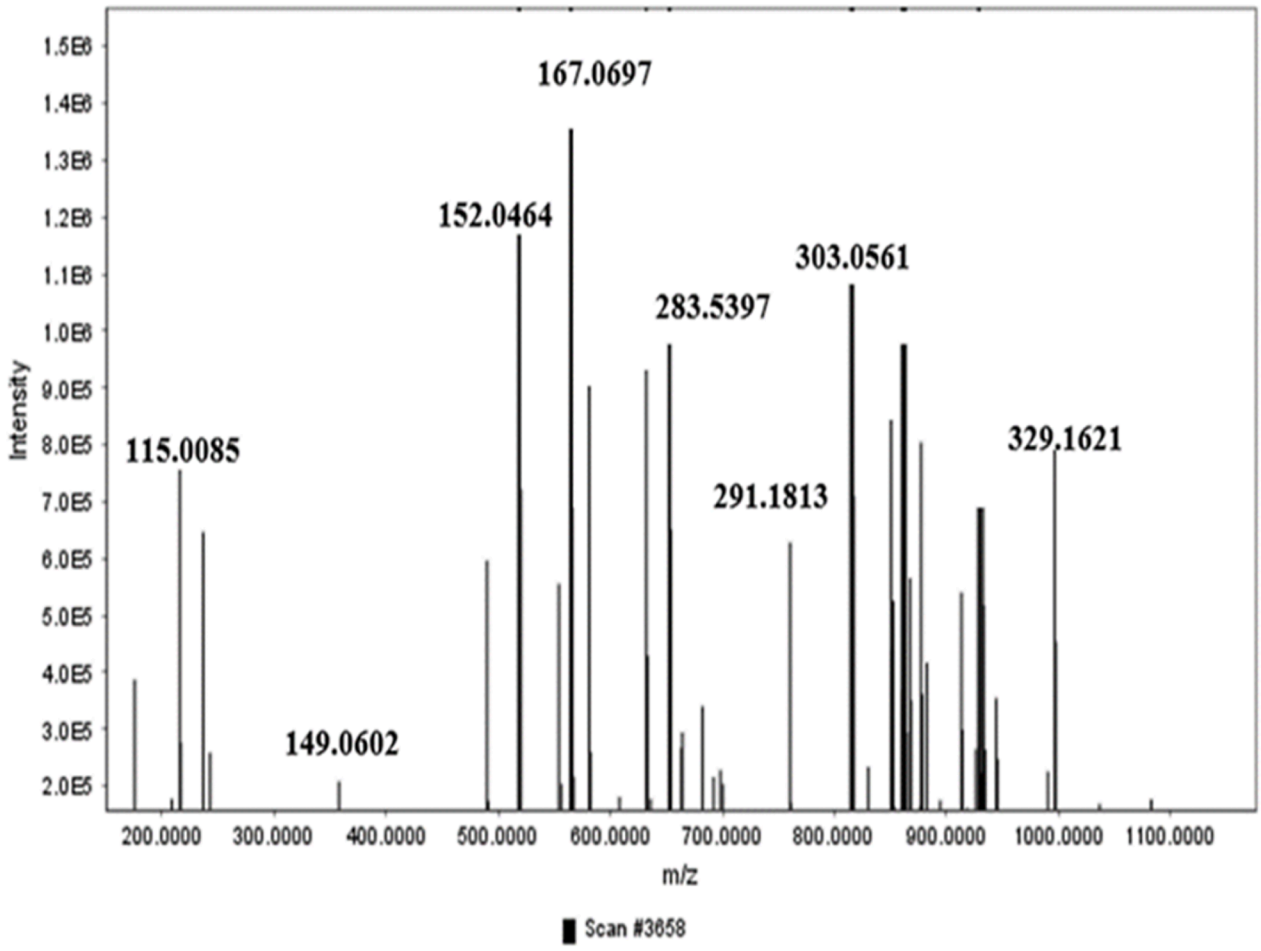


Fig. 7. Picrocrocin fragmentations with respective mass spectrum.

Crocin I standard retention time was 11.005 min, like the ethanolic saffron extract's (t_R = 11.005) (as shown in Fig. 3A and 3B). Adding a crocin standard to the extract enhanced the compound's peak area without affecting the retention time. This confirmed that the compound was indeed crocin and that the HPLC method was specific.

c) Accuracy

The results show that the % recovery data of spiked samples with crocin I standard at the three accuracy levels (400, 500, and 600 μg/mL) ranged from 99.46% to 103.28%. The average % recovery is within the acceptable range of 100% ± 5%, which indicates the method's accuracy. The %RSD (relative standard deviation) measures the method's precision. The data in Table 2 shows that the %RSD values at the three accuracy levels range from 1.26% to 1.94%, which indicates good precision of the method.

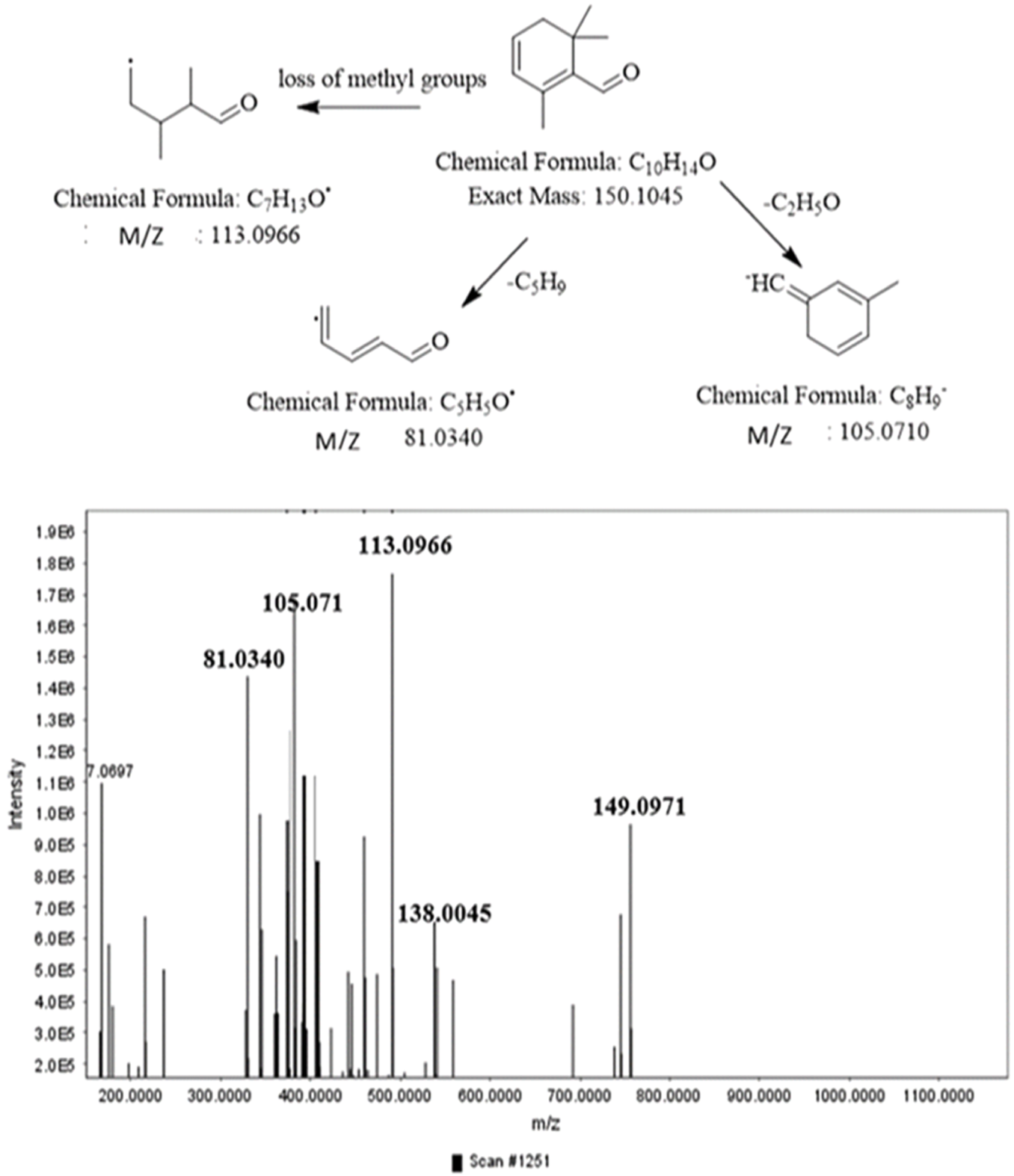


Fig. 8. Safranal's *m/z* fragmentation (bottom) and predicted fragmentation mechanism (top).

d) Precision

The precision of the developed analytical method was examined using the intra-day precision (repeatability) and the inter-day precision (intermediate precision) analysis conducted on three concentrations of crocin standard. The results were expressed as %RSD = (SD/mean) ×100%. The % relative standard deviations (RSD) for inter- and intra-day precision was below 2%, indicating an excellent analytical method precision. The results are summarized in Table 2.

e) LOD and LOQ

The LOD and LOQ were calculated to be 5.80 ppm and 17.85 ppm, respectively, indicating the developed method's acceptability.

Phytochemical characterization of C. sativus by LCMS/MS analysis

C. sativus's constituents were identified and presented in Tables 3 and 4. The base peak chromatogram is shown in Fig. 4A and 4B. Fragmentation patterns of main constituents, i.e., safranal, picrocrocin, kaempferol-retonoside, and crocetin were presented in Figs. 5–8.

Acute toxicity study of C. sativus extract

According to the findings, all the fish in the group exposed to the highest concentration (1500 mg/L) died within 24 hours of exposure. However, no mortality was observed in the other test concentrations. Based on our probit regression analysis, the IC50 for this extract was calculated to be 1021.374 mg/L.

Anti-obesity effect of saffron in high-fat diet-induced zebrafish

During the first two weeks after starting the high-fat diet, there were no significant changes in the BMI of the testing fish. However, starting from week 4, a significant increase in BMI was observed in both the OF and HFD groups. After conducting a one-way ANOVA followed by Tukey's post hoc analysis, it was found that the group treated with saffron 100 mg/L ($p<0.05$) and saffron 200 mg/L ($p<0.001$) had a significant decrease in BMI in week 12 compared to the HFD group. This decrease in BMI was sustained until week 16, specifically in the group treated with saffron 100 mg/L ($p<0.001$) and saffron 200 mg/L ($p<0.00001$) (Figs. 9, 10).

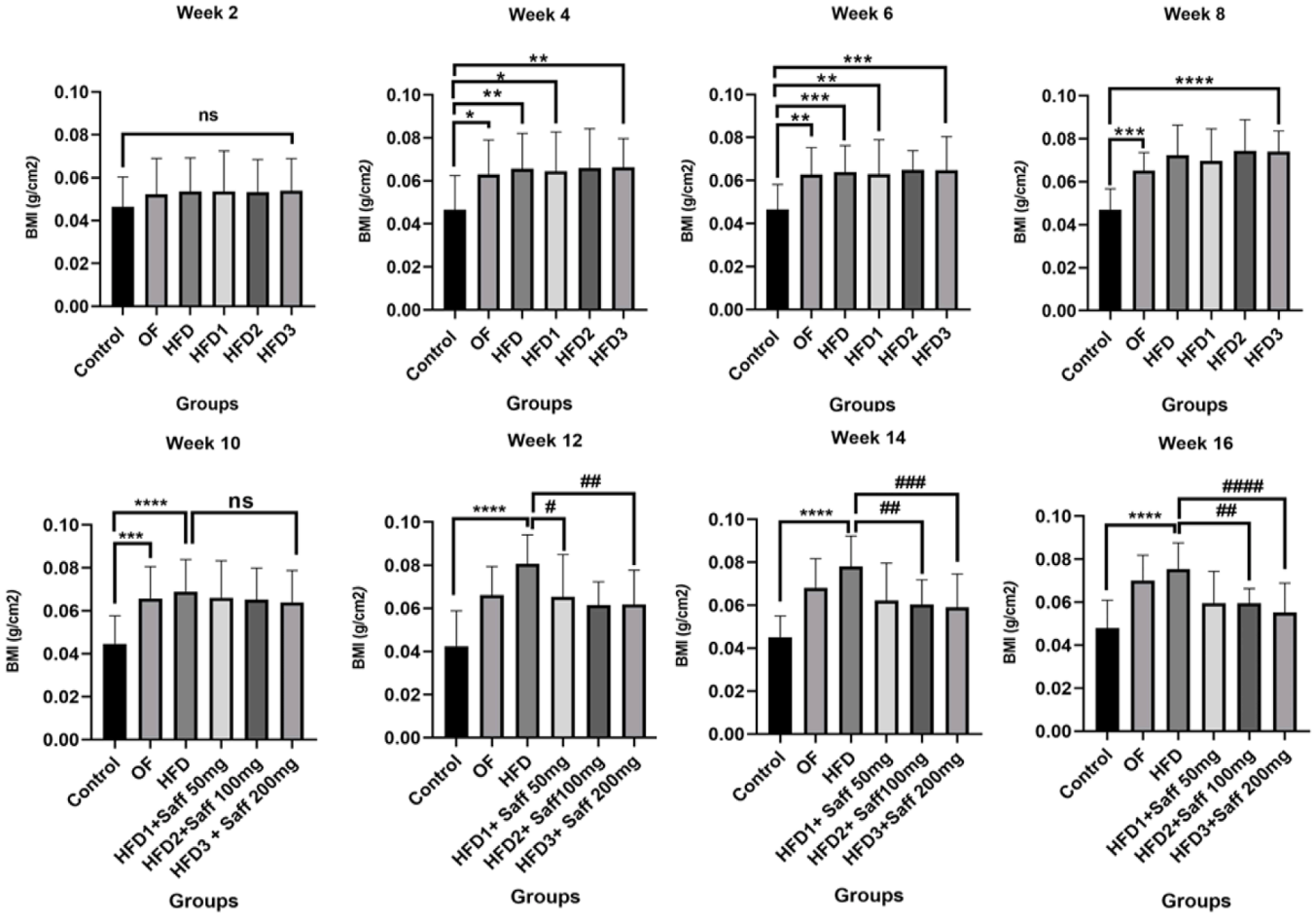


Fig. 9. Changes in body mass index (BMI) before and after treatment with saffron ethanolic extract (50, 100 and 200 mg/L, immersion) in 6 groups (n=10/group). Week 2–8 represented results before treatment, and Week 10–16, after saffron's treatment. Level of significance: * is comparing control and high-fat diet (HFD) groups with *p<0.05, **p<0.001,***p<0.0001,****p<0.00001, while # is comparing HFD with treatment groups, with #p<0.05, ##p<0.001, ###p<0.0001, ####p<0.00001., ns = not significant. Control = normal fish (normal diet, no treatment), HFD = high-fat diet, OF = overfeeding, saff 50 = treated with 50 mg/L saffron, saff 100 = treated with saffron 100 mg/L saffron, Saff 200 = treated with 200 mg/L saffron. Saffron treatments were via immersion.

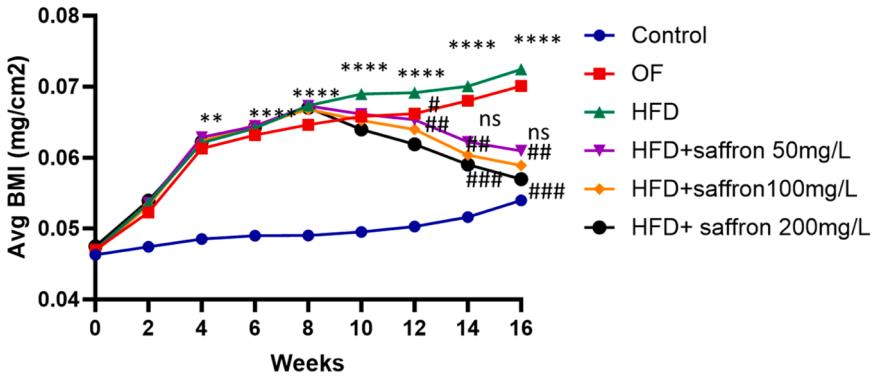


Fig. 10. Assessment of body mass index (BMI) from Week 0–16 of high-fat diet zebrafish model in 6 groups (n=10/group). Data are shown as mean ±SEM. Level of significance: * is comparing control and high-fat diet (HFD) groups with *p<0.05, **p<0.001,***p<0.0001,****p<0.00001, while # is comparing HFD with treatment groups, with #p<0.05, ##p<0.001, ###p<0.0001,####p<0.00001., ns = not significant. Control = normal fish (normal diet, no treatment), HFD = high-fat diet, OF = overfeeding, HFD+saffron 50 = high-fat diet treated with 50 mg/L saffron, HFD+saffron 100 = high-fat diet treated with saffron 100 mg/L saffron, HFD+saffron 200 = high-fat diet treated with 200 mg/L saffron. Saffron treatments were via immersion.

Discussions

The saffron ethanolic extract was found to contain 536.91 ± 7.67 μg/g (0.053%) of crocin I (trans-4-GG-crocin), which is a relatively low amount compared to other studies [10]. It has been noted in several studies that crocin is an unstable compound as it quickly forms its isomers [10]. The identification of saffron's chemical constituents was done by comparing fragmentation profiles from our LCMS/MS data with several published studies [2,4,19,21]. Our study found that most compounds identified in positive and negative ionization modes belong to carotenoids, terpenoids, and flavonoids. Among these, Crocin I (trans-4-GG-crocin) was one of the primary compounds found in *C. sativus*, along with 16 other crocin isomers [10]. Crocin comprises a crocetin molecule linked to sugars by an ester bond. It has been observed that purified crocin is less stable than crocin present in the crude saffron extract. Moreover, *cis*-crocin isomers can absorb UV–visible light at 326 and 440 nm, while *trans*-crocin isomers can only absorb at 440 nm [10]. The presence of the fragment at *m/z* 327, which corresponds to crocetin, was used to identify crocin compounds and derivatives.

Flavonoids are a group of plant metabolites that have polyphenolic molecular structures. Flavonoids possess a wide range of biological activities, including anti-inflammatory, antioxidant, antibacterial, and antitumor [22,23]. Flavonoids contain 15 carbon atoms and are soluble in water. Based on the differences in their chemical composition, flavonoids can be subclassified as flavonols, flavones, flavanones, flavan-3-ols, anthocyanins, and quercetin. Rutin, kaempferol-3-O-glucoside, kaempferol-3,7-di-O-β-D-glucoside, kaempferol-3,7,4-triglucoside, kaempferol-3-O-Sophoroside, dimethyl quercetin, kaempferol-3-O-rutinoside, isorhamnetin-rutinoside, kaempferol-hexoside, and kaempferol-rhamnoside were tentative identified in this study. A previous study discovered that saffron petals have a high kaempferol concentration of 12.6% (w/w) [24]. Fig. 5 shows kaempferol-rutinoside's MS/MS fragmentation spectrum as representative of flavonoid compounds. The distinctive feature of the fragmentation patterns for all the identified flavonoids, especially those derived from kaempferol, is the prominent ion at *m/z* 285 [25], symbolizing the kaempferol scaffold's structure after removing the rutinosyl sugar moiety. These findings align with previous studies on the analysis of flavonoids [22,24].

The LC-MS/MS analysis revealed the presence of terpenoids, another group of compounds. Picrocrocin, a bitter crystalline terpene-glucoside ($C_{16}H_{26}O_7$), is significant to the overall flavour profile of saffron, contributing to its bitterness and serving as a precursor to safranal. Picrocrocin has been widely studied for its potential health benefits, such as anti-inflammatory and antioxidant properties [22]. Although both crocins (carotenoids), picrocrocin, and safranal (terpenoids) have different structures, they derive from the same precursor molecule, zeaxanthin. Picrocrocin's fragmentation pattern is characterized by a precursor ion at *m/z* 329.1621 [M-H]-. This molecule also produces several characteristic fragments at *m/z* 167.0697, 152.0464, and 283.5397, resulting from specific bond cleavages. The ion at *m/z* 167.0697 corresponds to the detachment of a glucose moiety, while the ion at *m/z* 152.0464 signifies the release of a water molecule. Additionally, the ion at *m/z* 283.5397 results from the separation of a safranal molecule, a breakdown product stemming from picrocrocin (as shown in Fig. 7).

Fig. 8 shows the predicted mechanism of fragmentation in the safranal respective mass spectrum. The precursor ion at *m/z* 149.0971 characterizes the loss of hydrogen from the neutral safranal's chemical structure. After losing alkyl groups such as methyl, a fragment with *m/z* = 113.0545 was produced. Losing the C_5H_9 fragment produced an ion with *m/z* =81.0340 [17].

Assessing the toxicity of *C. sativus* extract is crucial to determine the potential harm the plant's constituents pose to humans and the environment. Previous studies have extensively reviewed saffron and its major components [14,26]. Clinical trials have shown that saffron is safe for consumption at doses of up to 1.5 g per day, while doses exceeding 5 mg per day can result in harmful side effects, and the lethal dose is approximately 20 g [27]. The zebrafish model was used to conduct our study, where the toxicity level of saffron ethanolic extract was mediocre, with an IC_{50} of 1021.374 mg/L. In comparison, *Centella asiatica* extract was found to be less toxic with an IC_{50} of 1250 mg/L [28], while *Polygonum minus* extract was found to be more toxic with an IC_{50} of 750 mg/L [29].

Obesity is commonly associated with developing several chronic diseases such as type 2 diabetes, dyslipidemia, hypertension, stroke, cancers and others [30]. Treatment such as bariatric surgeries and anti-obesity drugs commonly come with side-effects. Nutraceuticals and plant-based superfoods with healthy lifestyles are alternatives to reducing BMI effectively. Saffron is one of the superfoods widely studied for its anti-obesity effects. This study showed that eight-week chronic treatment significantly reduces BMI in the high-fat diet zebrafish model. Previous studies have established a direct correlation between a high-fat diet and obesity. A study showed that 40 and 80 mg/kg of saffron extract significantly decreased food consumption in obese rats, and 80 mg/kg of crocin significantly reduced plasma levels of triacylglycerol and total cholesterol [31]. A recent clinical study showed that saffron supplementation in obese man with type 2 diabetes mellitus and concurrent training showed improved inflammatory markers (TNF-α, hs-CRP, IL-6, IL-1β, IL-10), glycemic markers (FPG, Hb11c, HOMA-IR, insulin) and body composition [32]. This study opens a new perspective on the anti-obesity effect of saffron in a vertebrate model to investigate the potential of this food in reducing BMI in obese individuals.

Limitations

It is worth noting that the study on saffron extract has certain limitations that could be addressed in future research. Although the extract contains several phytoconstituents contributing to the observed anti-obesity activity, the active compound(s) and mechanism of actions are yet to be identified.

Conclusion

The constituents of the ethanolic extract of *C. sativus* were analyzed using LCMS/MS analysis in both positive and negative modes. In negative ion mode, 20 compounds were tentatively identified, and 22 were in positive mode. It should be noted that a high dose of 1500 mg/L of the extract is toxic to fish and can cause death. Therefore, caution should be exercised when using it. Quantitative analysis revealed 536.91 μg/gram of crocin I in the ethanolic extract. The study shows that the anti-obesity effect of saffron can be tested in a simple model like zebrafish. Thus, an in-depth study on how to correlate anti-obesity and other related diseases linked with saffron, such as antidepressant-like effects, can be studied more easily in less time. To the best of our knowledge, this is the only study that uses the high-fat diet zebrafish model to study the anti-obesity effects of saffron extract, which offers a new perspective on how this plant mitigates obesity in a simple animal model.

Declaration of generative AI in scientific writing

Authors used Grammarly to improve English fluency. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the *publication's content.*

CRediT authorship contribution statement

Farah Mejbel Al Jaberi: Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. **Maram B. Alhawarri:** Methodology, Validation. **Aidiahmad Dewa:** Supervision, Visualization. **Zurina Zainal:** Methodology, Visualization. **Fauziahanim Zakaria:** Conceptualization, Data curation, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Universiti Sains Malaysia for providing us with a Short-Term Research grant (304/PFARMASI/6315451) and the School of Pharmaceutical Sciences, USM staff for assistance.

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