Cogent Food & Agriculture

ISSN: (Print) 2331-1932 (Online) Journal homepage: https://www.tandfonline.com/loi/oafa20

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To cite this article: Syed Muzaffar, Sajad A. Rather & Khaliquz Zaman Khan | (2016) In vitro bactericidal and fungicidal activities of various extracts of saffron (*Crocus sativus* L.) stigmas from Jammu & Kashmir, India, Cogent Food & Agriculture, 2:1, 1158999, DOI: 10.1080/23311932.2016.1158999

To link to this article: https://doi.org/10.1080/23311932.2016.1158999

Muzaffar et al., *Cogent Food & Agriculture* (2016), 2: 1158999
http://dx.doi.org/10.1080/23311932.2016.1158999

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FOOD SCIENCE & TECHNOLOGY | RESEARCH ARTICLE

In vitro bactericidal and fungicidal activities of various extracts of saffron (*Crocus sativus* L.) stigmas from Jammu & Kashmir, India

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Received: 09 December 2015
Accepted: 19 January 2016
Published: 21 April 2016

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Reviewing editor:
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Abstract: Antimicrobial activities of methanolic and petroleum ether extracts of *Croccus sativus* L. (saffron) stigmas, were tested against various bacterial strains (*Proteus vulgaris*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*) and fungi (*Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus*) by agar well diffusion method. Minimal inhibitory concentration and minimum bactericidal and fungicidal concentration values of each active extract were also determined. The results showed a strong activity of the petroleum ether and methanolic extracts of saffron stigmas against bacteria and fungi used as test organisms. The results of different antimicrobial assays also indicate that the extracts had significantly higher bactericidal than fungicidal activities ($p < 0.05$). The results suggest that these extracts can be used in pharmaceutical and food formulations for inhibiting pathogenic bacterial and fungal species.

Subjects: Bioscience; Engineering & Technology; Food Science & Technology

Keywords: antimicrobial; methanolic; petroleum ether; bacteria; fungi

1. Introduction

Croccus sativus L. (saffron) (Fam. Iridaceae) is a widely used plant, especially as a food additive and colouring agent. Saffron is cultivated almost exclusively for its stigma, which once dried forms saffron spice, the most expensive in the world (Muzaffar, Rather, Khan, & Akhter, 2015; Sánchez-Vioque et al., 2012). This spice has been used in seasoning, medicine, cosmetics, perfume and dye for over three millennia (Ulbricht et al., 2011). These properties are basically related to its contents of picrocrocin,

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PUBLIC INTEREST STATEMENT

India is the one of key centres of saffron production that has painted the face of this "golden" spice throughout history has been in the plateaus of Pampore Pulwama, Kashmir (India), where saffron has been cultivated since AD. Introduction of antimicrobial study of Indian saffron will enhance its economical value and simultaneously wide application in different field such as in pharmaceutical, food and medical fields.

safranal and crocins (Carmona, Zalacain, & Alonso, 2006; Carmona, Zalacain, Salinas, & Alonso, 2007). Picrocrocin is the glycoside precursor of safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), which is in turn the most abundant of the volatile compounds responsible for the aroma of saffron (Maggi et al., 2011). Crocins are crocetin esters with glucose, gentiobiose, neapolitanose or triglucose sugar moieties. Spices have been used since ancient times to hide spoilage in foods.

Antibiotics have been used for the treatment of infectious diseases for a long time. But, antimicrobial resistance, among pathogenic bacteria, against drugs used in the treatment of human infection is increasing. This situation has forced scientists to search for new antimicrobial substances from various plants which are the good sources of novel antimicrobial chemotherapeutic agents (Karaman et al., 2003). For a long past, plants have been used as a valuable source of natural products for maintaining human health, with more intensive studies for natural therapies. The antimicrobial compounds from plants may inhibit bacteria by different mechanisms than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains. Because of the side effects and microbial resistance against the antibiotics, the scientists developed new drugs from natural sources such as plants, which have been extensively used as alternative treatment for diseases (Manoj, Kailas, Balaji, & Sajid, 2010; Sumitra & Yogesh, 2010).

Interest in the antimicrobial properties of active compounds is strengthened by the findings that they affect the behaviour of pathogenic bacteria or fungi of agro-food or medical field. Indeed, their use as natural additives in food industry is increased in recent years (Nazzaro et al., 2009). Antimicrobial agents, including food preservatives and organic acids, have been used to inhibit foodborne microbes and extend shelf life of processed foods. Many naturally occurring compounds found in edible and medicinal plants, herbs and spices have been shown to possess antimicrobial function and could serve as a source of antimicrobial agents against food pathogens (Lai & Roy, 2004). The antifungal activity of saffron has been investigated by earlier workers (Kamble & Patil, 2007; Sekine, Sugano, Majid, & Fujii, 2007). In addition, the inhibition of *Helicobacter pylori* by methanol extracts, as well as by safranal and crocin, has also been reported (Nakhaei, Khaje-Karamoddin, & Ramezani, 2008). Therefore, in this work we examine the *in vitro* antimicrobial activities of petroleum ether and methanolic extracts of *C. sativus* stigmas obtained from Jammu and Kashmir, India.

2. Materials and methods

2.1. Sample collection

The samples (*Crocus sativus* L.) were collected from Pampore Pulwama, Kashmir, India during October–November, 2014. Fresh stigmas were separated manually from the whole flowers of saffron by traditional procedure. The samples were vacuum dried and kept at 4°C in absence of light until their analysis. All solvents used were purchased from Hi Media, Pvt. Ltd. Mumbai, India.

2.2. Preparation of the crude extract

Ten gram of fresh *C. sativus* stigmas of plant material was extracted with 200 ml of methanol in a shaking incubator (100 rpm) overnight at room temperature. The methanolic extracts were filtered using Whatman No. 1 filter paper. Similarly, 10 g of *C. sativus* stigmas was used for petroleum ether extraction in a soxhlet apparatus (6 h for each solvent). The extracts were evaporated under reduced pressure and dried using rotary evaporator. Dried extracts were stored in labelled sterile screw capped bottles at −20°C.

2.3. Test organisms for evaluation of antimicrobial activity

The test micro-organisms used in this study were bacterial ssp. (*Proteus vulgaris*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*) and fungal spp. (*Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus*) obtained from Bacteriological and Mycological section of Department of Microbiology, SKIMS, Soura, Srinagar and Veterinary Microbiology and Immunology Division, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-Kashmir, Shuhama.

2.4. Antimicrobial activity

The *in vitro* antimicrobial activity test was carried out by agar well diffusion method. Standard cork borer of 5-mm diameter was used to make wells. Chloramphenicol and Amphotericin-B (Sigma–Aldrich) were used as positive control for bacteria and fungi, respectively, and DMSO alone as negative control. Every Petri dish was sealed with parafilm to avoid contamination. The plates were then incubated at 37 ± 1°C for 16–20 h in case of bacterial strains and 35 ± 2°C for 36–48 h for fungal strains. Finally, zone of inhibition was measured to the nearest size in mm with the help of standard scale (Norrel & Messley, 1997).

2.5. Minimal inhibitory concentration

The micro-dilution broth susceptibility assay was used for the evaluation of minimal inhibitory concentration (MIC) as recommended by (Clinical & Laboratory Standards Institute, 2012). After incubation, the first well without turbidity was determined as the MIC.

2.6. Determination of minimum bactericidal/fungicidal concentration

Equal volume of the various concentrations of each extract and Mueller Hinton broth (Hi-Media, Pvt. Ltd. Mumbai, India) were mixed in micro-tubes to make up 0.5 ml of solution. Then 0.5 ml of the organism suspension was added to each tube (Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37 and 25°C for 24 h for bacterial and fungal strains. Two control tubes were maintained for each test batch. These include tube containing extract without inoculums and the tube containing the growth medium and inoculums. The MBC and MFC was determined by subculturing the test dilution on Nutrient Agar medium/Potato Dextrose Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration (Akinyemi, Oladapo, Okwara, Ibe, & Fasure, 2005).

3. Statistical analysis

All the experiments were carried out in triplicates. Mean values, standard deviation and analysis of variance were computed using a commercial statistical package SPSS 16 (USA). The data were then compared using Duncan's multiple range tests at 5% significance level.

4. Results and discussion

4.1. Antimicrobial activity of C. sativus extracts

The test for antimicrobial effect of petroleum ether and methanolic extracts of *C. sativus* stigmas represents an important source of substances with antimicrobial activity. The results of the study provide evidence that *C. sativus* stigmas can be a potential source of new antimicrobial agents. The antimicrobial activity of extracts at different concentrations (500, 750 and 1,000 µg/disc) were assessed by the presence or absence of inhibition zone and zone diameter (Table 1). The petroleum ether extract showed maximum zone inhibition against *P. vulgaris*, *Bacillus subtilis* and *Pseudomonas aeuroginosa*, respectively. Whereas, methanolic extract showed maximum zone inhibition against *S. aureus* and *E. coli*, respectively. The extracts were found to differ significantly in their activity against different test micro-organisms ($p < 0.05$). In addition as the concentration of different extracts increased, the antimicrobial spectrum of extracts also increased significantly ($p < 0.05$). Similar results were reported by earlier workers (Soureshjan & Heidari, 2014), who found an increase in antimicrobial activity of *Glaucium elegans* and *Crocus stavius* extracts with increasing concentrations. The results also indicated that no antimicrobial activity was observed against *A. niger* and *A. fumigatus* by petroleum ether and against *C. albicans* and *A. niger* by methanolic extracts at 500 µg/disc dosage level. The standard antimicrobial compounds showed significantly highest zone of inhibition against tested micro-organism ($p < 0.05$). In other studies, the methanolic extracts of various *Crocus* spp. were found to have significant antimicrobial effect against different bacteria (Acar, Dogan, Duru, & Kıvrak, 2010). The antimicrobial activities of saffron extracts have been reported by safranal and crocin compounds (Carmona et al., 2007). These compounds can easily reach the contaminant micro-organism because of their volatility and/or water solubility and contribute to microbial killing

Table 1. Antimicrobial activities (inhibition areas diameter) of petroleum ether and methanol extracts of *C. sativus* stigmas using agar well diffusion method

Compounds and extracts tested	Concentration (µg/disc)	Inhibition zone (mm)								
		Bacterial strains						Fungal strains		
		P.v.	K.p.	B.s.	P.a.	S.a.	E.c.	C.a.	A.n.	A.f.
Petroleum ether extract	500	3 ± 0.11^{aB}	2 ± 0.07^{aA}	3 ± 0.00^{aB}	3 ± 0.11^{aB}	2 ± 0.22^{aA}	2 ± 0.12^{aA}	1 ± 0.41^{aA}	ND	ND
	750	8 ± 0.12^{bC}	6 ± 0.00^{bB}	9.2 ± 0.13^{bC}	9 ± 0.22^{bC}	7 ± 0.17^{bC}	6 ± 0.00^{bB}	4 ± 0.31^{bB}	2 ± 0.17^{aA}	3 ± 0.24^{aA}
	1,000	14 ± 0.11^{cC}	13 ± 0.12^{cB}	13.96 ± 0.32^{cC}	14 ± 0.07^{cC}	12 ± 0.11^{cB}	13 ± 0.24^{cB}	6 ± 0.22^{cA}	5 ± 0.11^{bA}	4 ± 0.11^{aA}
Methanolic extract	500	2 ± 0.20^{aA}	1 ± 0.12^{aA}	2 ± 0.23^{aA}	2 ± 0.22^{aA}	4 ± 0.10^{aB}	3 ± 0.09^{aB}	ND	ND	1.23 ± 0.10^{aA}
	750	7 ± 0.20^{bC}	6 ± 0.21^{bC}	6 ± 0.14^{bC}	7 ± 0.14^{bC}	9 ± 0.22^{bD}	7.84 ± 0.22^{bCD}	3 ± 0.12^{aA}	2 ± 0.13^{aA}	4 ± 0.22^{bB}
	1,000	12 ± 0.03^{cCD}	10 ± 0.70^{cC}	11 ± 0.33^{cC}	12 ± 0.24^{cC}	15 ± 0.14^{cD}	14 ± 0.23^{cD}	5 ± 0.11^{bA}	4 ± 0.07^{bA}	6 ± 0.13^{cB}
Amphotericin-B	10 µL	ND	ND	ND	ND	ND	ND	38 ± 0.12^{cA}	33 ± 0.11^{cA}	31 ± 0.14^{dA}
Chloramphenicol	10 µL	28 ± 0.11^{dA}	34 ± 0.00^{dB}	31 ± 0.22^{dB}	25 ± 0.03^{dA}	27 ± 0.11^{dA}	33 ± 0.22^{dB}	ND	ND	ND

All values are mean ± standard deviation of three replicates.

Means in the same row (A–D) and column (a–d) with different superscripts differ significantly: $^{*}p < 0.05$.

Diameter in mm of the zone of inhibition; ND: No zone of inhibition, P.v.: *Proteus vulgaris*, K.p.: *Klebsiella pneumonia*, B.s.: *Bacillus subtilis*, P.a.: *Pseudomonas aeuroginosa*, S.a.: *Staphylococcus aureus*, E.c.: *Escherichia coli*, C.a.: *Candida albicans*, A.n.: *Aspergillus niger*, A.f.: *Aspergillus fumigatus*.

(Pintado et al., 2011). Therefore, the antimicrobial activity of *C. sativus* stigma extracts described here represents an added value for saffron as a means of use in pharmaceutical and food industry.

4.2. Minimal inhibitory concentration

Some of the uses of saffron in traditional medicine have been related to its antimicrobial activity due to the presence of components such as safranal and crocin (Pintado et al., 2011; Soureshjan & Heidari, 2014). In the present study, the MIC of *C. sativus* stigma extracts was tested against six species of bacteria and three species of fungus. The MIC of the crude petroleum ether and methanolic extracts is shown in Table 2. The MIC of petroleum ether extract ranged from 0.4 to 0.66 mg/ml for bacterial strains and from 2.13 to 3.2 mg/ml for fungal strains, respectively. Similarly, the MIC of methanolic extracts ranged from 0.40 to 0.80 mg/ml for bacterial strains and 3.13–3.2 mg/ml for fungal strains, respectively. The results indicate that petroleum ether extracts showed most effective MIC values for *P. vulgaris* and *Pseudomonas aeurogenosa* and methanolic extracts showed for *S. aureus* and *E. coli* bacterial strains. However, both the extracts showed significantly higher MIC values than the standard antibacterial chloramphenicol ($p < 0.05$). In case of MIC values of *C. sativus* extracts for fungal strains, the petroleum ether extract showed the most effective MIC value for *C. albicans* and methanolic extract showed most effective value for *Aspergillus fumigates* ($p < 0.05$). The MIC values were significantly higher than standard antifungal amphotericin-B ($p < 0.05$). In our study, the petroleum ether and methanolic extracts showed the most effective MIC values than the earlier workers (Vahidi, Kamalinejad, & Sedaghati, 2002) who used ethyl acetate extracts of different *C. sativus* parts against bacterial and fungal strains

4.3. Determination of MBC and MFC

The minimum bactericidal and fungicidal concentrations (MBC and MFC) of petroleum ether extract ranged between 3.2–6.4 mg/ml and 10.67–12.8 mg/ml, respectively, for bacterial and fungal strains (Table 3). Similarly, MBC and MFCs of methanolic extract ranged between 1.6–6.4 mg/ml and 8.53–12.8 mg/ml, respectively, for bacterial and fungal strains (Table 3). The results indicate that both petroleum ether and methanolic extracts from the *C. sativus* stigma extracts were active against tested micro-organisms. The methanolic extract showed significantly lower antimicrobial activities against *S. aureus, E. coli* and *C. albicans* ($p < 0.05$). However, both the extracts were less active against

Table 2. Determination of minimum inhibitory concentration (MIC) of petroleum ether and methanol extracts of *C. sativus* stigmas

Compounds and extracts tested	MIC (mg/ml)								
	Bacterial strains						Fungal strains		
	P.v.	K.p.	B.s.	P.a.	S.a.	E.c.	C.a.	A.n.	A.f.
Petroleum ether extract	0.4 ± 0.0^{bA}	0.66 ± 0.23^{bC}	0.53 ± 0.23^{bB}	0.4 ± 0.0^{bA}	0.53 ± 0.23^{bB}	0.66 ± 0.23^{cC}	2.13 ± 0.92^{bD}	3.2 ± 0.0^{bE}	3.2 ± 0.0^{cE}
Methanolic extract	0.66 ± 0.23^{bcC}	0.8 ± 0.0^{cC}	0.8 ± 0.0^{cC}	0.53 ± 0.23^{cB}	0.4 ± 0.0^{bA}	0.4 ± 0.0^{bA}	3.2 ± 0.0^{cE}	3.2 ± 0.0^{bE}	2.13 ± 0.92^{bD}
Amphotericin-B	ND	ND	ND	ND	ND	ND	0.0003 ± 0.0001^{aA}	0.0006 ± 0.0003^{aB}	0.0006 ± 0.03^{aB}
Chloramphenicol	0.008 ± 0.0^{aD}	0.004 ± 0.0^{aA}	0.004 ± 0.0^{aB}	0.128 ± 0.0^{aE}	0.0066 ± 0.0023^{aC}	0.004 ± 0.0^{aA}	ND	ND	ND

Determined (showing no growth inhibition up to 12.8 mg/ml, the highest tested concentration).

All values are mean ± standard deviation of three replicates.

Means in the same row (A–E) and column (a–c) with different superscripts differ significantly: $^{*}p < 0.05$.

Diameter in mm of the zone of inhibition; ND: Not determined, P.v.: *Proteus vulgaris*, K.p.: *Klebsiella pneumonia*, B.s.: *Bacillus subtilis*, P.a.: *Pseudomonas aeuroginosa*, S.a.: *Staphylococcus aureus*, E.c.: *Escherichia coli*, C.a.: *Candida albicans*, A.n.: *Aspergillus niger*, A.f.: *Aspergillus fumigatus.*

Table 3. Determination of minimum bactericidal/fungicidal concentration (MBC/MFC) of petroleum ether and methanol extracts of *C. sativus* stigmas

Compounds and extracts tested	MBC and MFC (mg/ml)								
	Bacterial strains						Fungal strains		
	P.v.	K.p.	B.s.	P.a.	S.a.	E.c.	C.a.	A.n.	A.f.
Petroleum ether extract	3.200 ± 0.0	6.400 ± 0.0	4.260 ± 1.85	3.200 ± 0.0	6.400 ± 0.0	4.260 ± 1.85	10.670 ± 3.69	12.800 ± 0.0	10.670 ± 3.69
Methanolic extract	5.330 ± 1.85	4.260 ± 1.85	3.200 ± 0.0	6.400 ± 0.0	1.600 ± 0.0	1.600 ± 0.0	8.530 ± 3.69	10.670 ± 3.69	12.800 ± 0.0
Amphotericin-B	ND	ND	ND	ND	ND	ND	0.0013 ± 0.0006	0.0066 ± 0.0023	0.0066 ± 0.0023
Chloramphenicol	0.064 ± 0.0	0.008 ± 0.0	0.064 ± 0.0	0.512 ± 0.0	0.0533 ± 0.0185	0.064 ± 0.0	ND	ND	ND

ND: not determined (showing no bactericidal effect up to 12.8 mg/ml, the highest tested concentration).

P.v.: *Proteus vulgaris*, K.p.: *Klebsiella pneumonia*, B.s.: *Bacillus subtilis*, P.a.: *Pseudomonas aeuroginosa*, S.a.: *Staph Staphylococcus aureus*, E.c.: *Escherichia coli*, C.a.: *Candida albicans*, A.n.: *Aspergillus niger*, A.f.: *Aspergillus fumigatus.*

tested micro-organisms than the standard chloramphenicol and amphotericin-B the antifungal and antibacterial compounds ($p < 0.05$).

5. Conclusion

The results concluded that both petroleum ether and methanolic extracts of *C. sativus* stigmas have great potential as antimicrobial compounds against bacteria and fungi. However, the results showed that the extracts exhibited strong bactericidal than fungicidal effects. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes and can have wide applications in pharmaceutical, food and medical fields.

Funding
The authors received no direct funding for this research.

Competing Interests
The authors declare no competing interest.

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Citation information
Cite this article as: *In vitro* bactericidal and fungicidal activities of various extracts of saffron (*Crocus sativus* L.) stigmas from Jammu & Kashmir, India, Syed Muzaffar, Sajad A. Rather & Khaliquz Zaman Khan, *Cogent Food & Agriculture* (2016), 2: 1158999.

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Cogent Food & Agriculture **(ISSN: 2331-1932) is published by Cogent OA, part of Taylor & Francis Group.**