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Development of Extraction Methods and Quantification of Safranal by High Performance Liquid Chromatography from Cuminum cyminum L. and Studying its Antimicrobial Properties

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Abstract. Safranal is a monoterpene aldehyde in the essential oil of saffron affecting its characteristic aroma. Different solvents like water, methanol, ethanol, glacial acetic acid, isopropanol, ethyl acetate, acetone, butanol, chloroform, benzene and toluene were used for extraction of safranal from cumin seeds. Methanol showed better yield of safranal as compared to other solvents. Safranal concentration in cumin (*Cuminum cyminum* L.) was detected by HPLC method with photodiode-array detector using 100% acetonitrile at 308nm wavelength. Various extraction methods were selected to increase yield of safranal. Maximum yield of safranal was obtained through microwave pretreatment with ultrasound assisted extraction (44.8679 µg/g of cumin) using 80% methanol considering the economic benefits. The crude extract and standard safranal were tested against *S .aureus* (Gram positive), *E. coli* (Gram negative) and *C. albicans* (fungus) strains. Both samples showed good inhibition for all the microbial strains.

Keywords: Safranal, Extraction methods, Cumin, HPLC, Antimicrobial activity, Well diffusion method

1. Introduction

Saffron (Crocus sativus L.) is the most expensive spice [1]. Saffron contains more than 150 volatile aroma-yielding compounds. Crocin, picrocrocin and safranal are main chemical compounds of saffron responsible for saffron's exclusive color, taste, odour respectively [2,3]. The amount of these compounds in dried stigma tissues is the most important indicator of quality of this spice [4]. Picrocrocin is converted to safranal by enzymatic or thermal degradation during the drying process of saffron stigmas [5,6,7,8,9].

Safranal (2, 6, 6-trimethyl-1, 3-cyclohexadien -1-carboxaldehyde) or $C_{10}H_{14}O$ is a cyclic monoterpene aldehyde of saffron which constitutes about 60-70% of volatile fraction of saffron's essential oils. Safranal is insoluble in water but soluble in methanol, ethanol, petroleum ether, glacial acetic acid and acetonitile. Its molecular weight is 150KDa with density 0.9734g/c and boiling point 70˚C. In order to evaluate safranal content in aqueous extract, ISO proposes spectrometric method at 330 nm. Due to the interference caused by other compounds use of this method for safranal analysis becomes questionable. *Centaurea sibthorpii, Centaurea consanguinea, Erodium cicutarium, Chinese green tea, Calycopteris floribunda, Crocus heuffelianus, Sambucus nigra, Citrus limon, Cuminum cyminum* L., *Achillea distans* are the reported sources of safranal other than saffron [2,10]. Safranal shows various pharmacological effects like antidepressant, anticonvulsant, antioxidant, antihypertensive, anti-ischemia, anti inflammatory, antimicrobial, anticancer, genoprotective activities and also affect sexual behavior negatively [2]. A variety of techniques including HPLC have been used by investigators for qualitative and quantitative analysis of safranal in plant extract [4,6,7,11,12,13].

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Hydro distillation, solvent extraction, ultrasound assisted extraction, supercritical fluid extractions are the reported methods for extraction of volatile components of saffron [2,14]. Extraction of safranal from cumin, to the best of our knowledge, is first time reported. The aqueous extract of cumin inhibits growth of many pathogens [15,16]. Therefore, overall purpose of this paper is to develop a better method of extraction and quantification of safranal from cumin and to study its antimicrobial activity. The crude and standard safranal were tested against Gram positive, Gram negative and on fungal stains, namely *S. aureus, E. Coli* and *C. albicans* respectively.

2. Materials And Methods

Cumin seeds were collected from local market. All solvents/chemicals used were of AR/HPLC grade and used as purchased. The standard safranal purchased from SIGMA-ALDRICH (Germany).

For all the extraction methods below cumin to solvent ratio was kept as 1:10(w/v) initially. Water, methanol, ethanol, glacial acetic acid, isopropanol, ethyl acetate, acetone, butanol, chloroform, benzene and toluene were used as a solvent for ultrasonic assisted extraction with microwave pre-treatment (All solvents used were 100%).

2.1. Methods of extraction

2.1.1. Soxhlet extraction

Ten gram of cumin powder was packed in filter paper and extraction was done with 300ml of 80% methanol (B.P=68˚C) for sixteen hours using Soxhlet apparatus.

2.1.2. Ultrasound assisted extraction (UAE)

Extraction was carried out for four hours using 80% methanol at room temperature in ultrasonicator.

2.1.3. Ultrasound assisted extraction with microwave pre-treatment (UAEMP)

Cumin powder was treated with disrupted microwave treatment for 10 minutes at medium-low temperature and ultrasonic assisted extraction was carried out as above.

2.1.4. Ultrasound assisted extraction with heat drying pre-treatment (UAEHDP)

Cumin powder was dried in drier at 55˚C for 45minutes and ultrasonic assisted extraction was carried out using methanol.

2.1.5. Ultrasound assisted extraction with enzyme pre-treatment (UAEEP)

Cellulase and diastase (20mg each) were dispersed separately in 10ml of sodium acetate buffer solution at their optimum pH 4.5 and 5.5 respectively. Solutions were kept at room temperature with continuous stirring for four hours after addition of 1gm of cumin in it. Ultrasound assisted extraction was carried out by adding absolute methanol to make final concentration of methanol equal to 80%.

All the extracts were centrifuged at 3000 rpm for 15minutes and the supernatants were collected for each.

To compare the results of all the extraction processes, extraction using 80% methanol without ultrasonic treatment, was carried out as a control at room temperature.

2.1.6. Optimization of extraction process

For optimization of ultrasound assisted extraction procedures extractions were carried out as:

- Increasing the methanol concentration (20%, 40%, 50%, 60%, 80%, 100%)
- Increasing the amount of cumin (0.5g,1g,1.5g,2g,2.5g,3g) in 10ml of solvent
- Increasing volume of solvent (20ml, 40ml, 60ml, 80ml, 100ml) keeping fixed amount (1g) of cumin

2.2. Methods for analysis

2.2.1. Spectrophotometric analysis

Estimation of safranal content in the extract was done at 316nm (λmax of standard safranal detected for spectrophotometer used) by UV- spectrophotometry.

2.2.2. HPLC analysis

A Acclaim C18 column (25cm length, 4.6mm internal diameter, 5µm particle size with a pore diameter of 120Å) was used for entire analysis. 100% acetonitrile was used as a mobile phase with a flow rate of 1.0ml/min for a maximum retention time of 3.9 min at room temperature and total run time used was 10 minutes for each sample. The sample size was 20µl of the test solution. The analysis of safranal was done with photodiode-array detector at 210nm, 308nm and 330nm.

2.3. Antimicrobial activity

The size of inoculum was adjusted to 108 CFU/ml for each of the test bacterium (Escherichia coli, Staphylococcus aureus, Candida albicans). The inoculum suspension was spread over a nutrient agar plate, to achieve a confluent growth, and allowed to dry before 10 mm diameter wells were bored in the agar using a sterile cork-borer. A 10µl-50µl of crude extract and standard safranal samples were placed into a well and the plates were held for 1 hour at room temperature for diffusion of sample into the agar. Subsequently, the plates were incubated for 24 hours at 370C. After incubation, the diameters of the zones of inhibition were measured to the nearest millimeter (mm).

3. Results and Discussion

3.1. Comparative study of extraction methods

Maximum yield was obtained using methanol as a solvent so methanol was selected for further extraction process. For the extraction methods mentioned above, the yield of safranal per gram of cumin was calculated (Table 1) using the standard curve of safranal plotted from HPLC analysis. Maximum yield was obtained using methanol as a solvent by Soxhlet method but considering the economical benefits as time and volume of solvent required for extraction, ultrasonic assisted extraction with microwave pretreatment (UAEMP) was found to be the most suitable method (Fig. 1). Saffron which is collected from local market showed less yield of safranal than cumin. That could be due to improper drying, ageing processes or poor quality of saffron. It was observed that UV-spectrometric analysis method was not appropriate due to interference caused by other compound. Comparing with the control (as mentioned above), yield of safranal was not appreciably increased by ultrsonication.

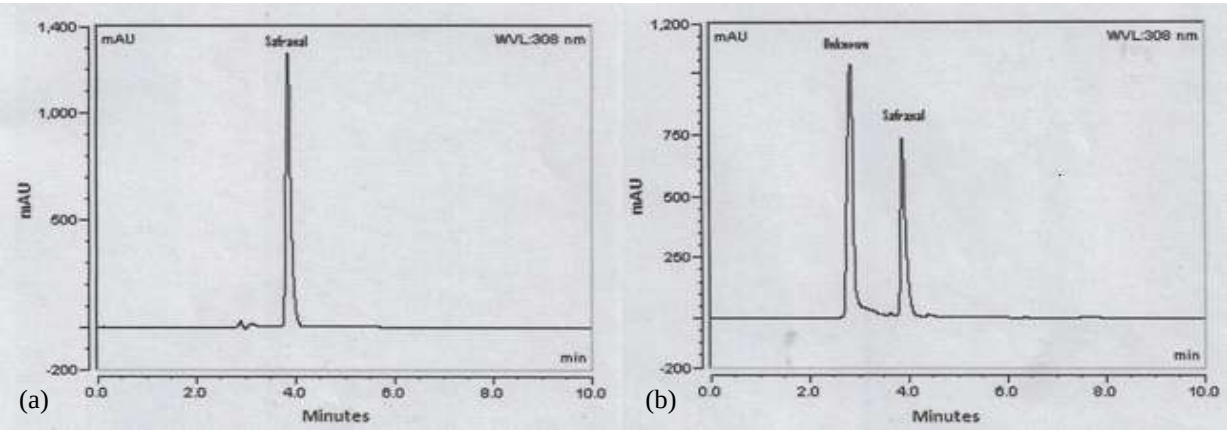

Fig. 1: (a) HPLC profile of standard safranal (b) HPLC profile of crude extract

Sr. No.	Extraction methods	Yield of safranal µg per gram of cumin
1	Soxhlet Extraction	52.4652
2	UAE	31.0912
3	UAE-Control	25.9644
4	UAEMP	44.8679
5	UAEMP-Control	38.4626
6	UAEHDP	30.6028
7	UAEHDP-Control	31.0476
8	UAEEP Cellulase	28.3156
9	UAEEP Diastase	29.9188

Table 1: Yield of safranal obtained by different extraction methods

3.2. Optimization of extraction process

It was observed that yield of safranal increased with increasing concentration of methanol. When extraction was carried out with increasing amount of cumin in fixed volume of solvent, it was observed that, concentration of safranal µg per ml increased but yield of safranal µg per gram of cumin decreased. For increasing volume of solvent and keeping amount of cumin (1g) fixed, lower increase in yield of safranal was seen.

3.3. Antimicrobial activity

Methanolic cumin extract (Fig. 2) and standard safranal exhibited antimicrobial activity against E. coli, S. aureus and C. albicans. Cumin and safranal showed more pronounced activity against C.albicans than others (Table 2).

Micro organism	80% methanol	Ampicillin (25 µg/ml)	Standard safranal (9.66mg/ml)					Cumin Extract (1gm cumin powder per 10ml of 80% methanol)				
			10 µl	20 µl	30 µl	40 µl	50 µl	10 µl	20 µl	30 µl	40 µl	50 µl
E. coli	0	34	8	10	16	20	28	5	7	7	9	15
S. aureus	0	40	0	0	0	8	8	7	8	9	12	14
C. albicans	0	33	8	12	14	19	22	10	17	17	18	20

Table 2: In vitro antimicrobial activity of cumin extract and standard safranal by agar well diffusion method
The numerical values specify corresponding diameters of zone of inhibition measured in mm

Fig. 2: Microbial growth inhibition by methanolic cumin extract
A, B, C, D, E: Wells corresponding to volume of crude cumin extract added to them as 50 µl, 40 µl, 30 µl, 20 µl, 10 µl respectively

4. Conclusions

Cumin can be used as a potential source for safranal extraction considering its various pharmaceutical applications. Ultrasonic assisted extraction with microwave pre-treatment was found to be the most suitable method for extraction of safranal due to maximum yield obtained (44.8679 µg/g of cumin). The results obtained proved that cumin extract and standard safranal has antimicrobial activity. By using natural agents like cumin which are non-toxic to humans and with no side effects, we hope that this could be used as a potential medical drug in clinical trials.

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