Indian Journal of Natural Products and Resources
Vol. 7(1), March 2016, pp. 32-39

Effect of extraction methods on yield, phytochemical constituents, antibacterial and antifungal activity of *Capsicum frutescens* L.

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Received 23 April 2015; Accepted 8 January 2016

Capsicum frutescens L. (Family Solanaceae) commonly used to make one of the most common spice, red peppers is also associated with multiple health benefits. In the present study, extract yield and phytochemical constituents of *n*-hexane, chloroform, ethyl acetate, acetone and methanol extracts of dried seeds of *C. frutescens* L. prepared by microwave assisted solvent extraction technique, also known as "Green extraction" were compared with the solvent extracts prepared by two common conventional extraction methods. *In vitro* antibacterial and antifungal activity of all extracts were determined by using agar well diffusion method against three gram positive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *S. aureus MRSA*), five gram negative bacteria (*Escherechia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris*) and two fungi (*Candida albicans* and *C. krusei*). Results were also compared with positive (Cotrimoxazole and Nystatin) and negative controls (Dimethyl sulphoxide). Solvent extracts prepared by microwave assisted method showed significant activity and *n*-hexane extract formed inhibition zone of 14.4 mm against *P. aeruginosa*, 20.0 mm against *C. albicans*, while ethyl acetate extract formed largest inhibition zone (15.0 mm) against *C. krusei*. Minimum inhibitory concentration evaluated by two fold serial broth tube dilution method ranged between 0.312 to 5 mg/mL.

Keywords: Antibacterial, Antifungal, *Capsicum frutescens* L., Microwave, Minimum inhibitory concentration

IPC code; Int. cl. (2015.01)− A61K 36/00

Introduction

Asia is well known as "Land of spices" and tropical climate can be considered as the reason for development of hot, spicy cuisine in many of Asian countries like India, Pakistan, Bangladesh, Malaysia and Thailand; as climate conditions are favorable for easy growth of food spoilage pathogens. According to food microbiologists, many of the spices that appear most often and most abundantly in recipes from hot climates especially garlic, onion and hot peppers can inhibit 75 to 100 % of the bacteria present in food[1]. Even millenniums ago, traditional Indian medicine system "Ayurveda" explained the role of spices in digestion and assimilation of food and thereby contributing in maintaining a balanced and healthy Agni or digestive fire. The use of spices also helps in cleansing *ama* (toxins) from body formed by food[2]. Spices are important sources of bioactive compounds such as alkaloids, terpenoids, steroids, flavonoids and phenolics, known for their health promoting effects against degenerative diseases[3]. In present scenario also, therapeutic efficacy of spices for specific pharmacological action is being established by experimental and clinical studies and efforts are being made to identify and isolate active compounds from them[4]. Profound antimicrobial effects of commonly used spices against most common bacteria and fungi that contaminate food (*Bacillus cereus*, *Listeria* spp., *Staphylococcus* spp., *Salmonella* spp., *Escherichia* spp., *Pseudomonas* spp., *Aspergillus* spp. and *Cladosporium* spp.) have been confirmed[5-7].

Capsicum frutescens L. (Family Solanaceae) is commonly known as *Mirch* or *Lalmirch* in Hindi, *Marichiphala* in Sanskrit, *Jolokia* in Assamese, *Lanka marich* in Bengali and Chili in English. It is a short-lived evergreen shrub usually 1 to 1.5 m in height. The ovate to ovate lanceolate leaves vary in size, while greenish-white to yellowish-white flowers with blue, violet or yellow anthers occur in groups of two or more at the nodes. The berries are red or red-orange at maturity, elongated with a pointed or rounded tip, normally 1.5 to 3.5 cm long and 0.5 to 1.2 cm thick. The fruit contains cream to yellow lenticular seeds

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about 3 mm in diameter. Dried fruit of *C. frutescens* L. has been used as a flavouring agent for centuries and is also associated with different traditional medicinal remedies. Fruit of *C. frutescens* can reduce the risk of heart attack, stroke and pulmonary embolism as it stimulates the blood circulation aiding in removal of blood cholesterol, triglycerides and platelets aggregation. The fruit also has analgesic, anti-inflammatory, antidiabetic, antiobesity, antirhinitic, anticancer and antipsoriatic properties[8,9]. These pungent fruited peppers are important in the tropics as gastrointestinal detoxifiers and used in treatment of diarrhoea and dysentery[10]. It also possesses insecticidal properties[11]. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the active component of chilli pepper has a wide range of medicinal applications and can be used as blood sugar level regulator[12,13], anticancer agent[14,15] and pain reliever[16]. It is also helpful in treatment of gastric and duodenum ulcers[17].

The polarity of solvent used for extraction and the method of extraction play a vital role in efficiency (yield) and efficacy (magnitude of bioactivity) of prepared extracts[18]. With the key principle of solubility "like dissolves like", phyto constituents of varied polarity present in plant matrix can be extracted by suitable solvents. There is continuous research going on to develop and optimize the non-conventional extraction techniques by using ultrasound waves, microwaves, supercritical fluids, etc. to extract bioactive ingredients from plant cells in less time and solvent consumption with minimum sample degradation in comparison to conventional extraction methods like infusion, maceration, digestion, percolation and hot continuous method (soxhlet). Hence, with the objective to compare both the efficiency and efficacy of non-conventional microwave assisted extraction method with conventional methods (maceration and soxhlet) and to study the effect of polarity of solvent used for extraction on phytochemical constituents, antibacterial and antifungal potential of *C. frutescens* seeds (most pungent and antimicrobial active part of fruit), present study was conducted. In addition, minimum inhibitory concentration (MIC) was also determined against microorganism with significant results.

Materials and Methods

Plant material

Air dried pods of *C. frutescens* were collected from the local agricultural field of District Ratlam, Madhya Pradesh, India and identified by Dr Vrinda Gupta, Head, Department of Botany, Government Arts & Science College, Ratlam. Seeds were separated from the pods and washed thoroughly to remove adhering material and shade dried at room temperature. Seeds were further ground by means of an electrical blender to fine powder.

Preparation of plant extracts

Plant extracts were prepared in five different organic solvents (*n*-hexane, chloroform, ethyl acetate, acetone and methanol) using three methods (Maceration, Soxhlet and Microwave-assisted Solvent Extraction)[19].

Maceration Solvent Extraction (MSE)

Seeds powder (5 g) was suspended in 150 mL of each solvent separately at room temperature (32±2 ^{o}C) for 16 h in a closed container and was stirred at regular intervals after every 2 h.

Soxhlet Solvent Extraction (SSE)

Seeds powder (5 g) was extracted in 150 mL of each solvent separately using Soxhlet extractor at boiling temperature + 2 ^{o}C over water bath for 16 h.

Microwave-assisted Solvent Extraction (MASE)

Seeds powder (5 g) was extracted in 50 mL of each solvent separately for 10 min with intermittent cooling of 1 min after every 1 min, at 180 Watt (20 % power) in an LG domestic unmodified microwave oven model MG-555 F.

All Liquid extracts obtained were separated from the solid residue by filtration through whatman No. 1 filter paper and concentrated using a rotary evaporator. Dried extracts were kept in sterile sample tubes and stored at 4 ^{o}C till further analysis.

Preliminary phytochemical screening

Phytochemical screening of all the fifteen extracts for presence of steroids, alkaloids, reducing sugars, phenolic compounds, flavonoids, saponins, flavones and tannins was carried out according to standard method[20].

Antimicrobial assay

Microorganisms

Nine microorganisms including gram positive bacteria [*Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25923)], gram negative bacteria [*Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 6539), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 27736) and *Proteus vulgaris* (ATCC 33420)]

and fungi [*Candida albicans* (ATCC 24433) and *C. krusei* (ATCC 14243)] along with one clinically isolated methicillin resistant strain of *S. aureus* MRSA were obtained from Department of microbiology, RD Gardi Medical College and Hospital, Ujjain, India. Screening for methicillin resistance was performed using a cefoxitin disc screen test and 6 g/mL oxacillin in Mueller-Hinton agar supplemented with NaCl (4 % w/v; 0.68 mol/L) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[21]. Multidrug-resistant isolates were defined as isolates having co-resistance to at least three antibiotic groups[22]. All microorganisms were sub-cultured on nutrient broth, incubated at 37±2 °C for 24 h and kept at 4 °C until further analysis.

Agar well diffusion method

All chemicals and antibiotic discs used in antimicrobial assay were procured from Hi-Media Laboratories Pvt Ltd, Mumbai, India. The antibacterial and antifungal activities of different solvent extracts were determined by agar well diffusion method[23]. Plant extract for activity were prepared by dissolving 100 mg of each extract in 5 mL of 10 % Dimethyl sulfoxide (DMSO). Muller Hinton Agar (38 g) was suspended in 1000 mL of distilled water and heated up to boiling point for complete mixing. To sterilize, it was autoclaved at 15 lbs pressure at 121 °C for 15 minutes. Approximately 25 mL of sterilized selective medium was poured in to each petridish and solidified at room temperature. Using a sterile cotton swab, the bacterial culture was swabbed on the surface of pre-poured nutrient agar plates. The plates were allowed to dry for 15 minutes, before being used in the test. A well of 10 mm diam. was punched off in to agar medium with sterile cup borer at previously marked petri plates and then it was filled with 100 µL of extract every time. Plates were placed for 30 minutes in refrigerator for diffusion of extracts and then incubated at 37±2 °C for 24 h. Zone of inhibition (excluding well diameter) appeared was measured as a property of antibacterial and antifungal activity of plant extracts. Standard antibiotic discs, cotrimoxazole (25 mcg/disc) and nystatin (100 units/disc) were used as positive control in case of antibacterial and antifungal activity, respectively while 10 % DMSO was used as negative control. Inhibition zones were recorded as the diameter of growth-free zones at the end of incubation period. All bioassays were performed in triplicate to get better result.

Minimum Inhibitory Concentration (MIC)

MIC of extracts was evaluated by two fold serial broth tube dilution method[24] against *E. coli*, *K. pneumoniae*, *B. cereus*, *S. aureus*, *C. albicans* and *C. krusei*. Serial two fold dilution of the extract was prepared in the test tubes containing peptone water (at 0.5 McFarland turbidity standards) as diluents from the stock solution (10 mg/mL) to give concentration ranging from 5, 2.5, 1.25, 0.625 to 0.312 mg/mL. All test tubes were inoculated with 0.1 mL of inoculums of the test organisms using sterile loop. Solvent control was prepared with DMSO (10 %), while blank control was prepared from virgin media. Tubes were incubated for 24 h at 37±2 °C for all the test microorganisms.

Statistical analysis

Samples were analyzed individually in triplicate and data were reported as mean ± standard deviation. One way analyses of variance (ANOVA) with post hoc Tukey HSD test was used to compare significant differences between extracts and control at 5 % significance level ($p < 0.05$).

Results and Discussion

Extraction yield is a measure of solvent and extraction method's efficiency to extract out specific components from plant matrix. In present study, seeds of *C. frutescens* were extracted with five solvents (*n*-hexane, chloroform, ethyl acetate, acetone and methanol) using three different extraction methods (MSE, SSE and MASE) and the extraction yields is compared in Table 1. In MSE, maximum methanol extract yield (8.5 %) was followed by chloroform, *n*-hexane, ethyl acetate and acetone. In SSE, acetone extract gave highest yield (9.5 %) followed by ethyl acetate, methanol, chloroform and *n*-hexane. In case of MASE, extraction yield increased with the increase in polarity of solvent used. Present study yielded better efficiency in MASE method over

Table1—Extraction yields of solvent extracts of *C. frutescens* seeds (w/w of plant material)

Name of extract	MSE		SSE		MASE	
	g	%	g	%	g	%
n-Hexane	0.34	6.8	0.41	8.2	0.57	11.5
Chloroform	0.37	7.4	0.43	8.6	0.58	11.6
Ethyl acetate	0.18	3.7	0.46	9.2	0.66	13.2
Acetone	0.13	2.5	0.48	9.5	0.69	13.9
Methanol	0.43	8.5	0.44	8.8	0.71	14.2

MSE-Maceration solvent extraction, SSE-Soxhlet solvent extraction, MASE-Microwave assisted solvent extraction

the conventional extraction methods in terms of extraction yield. This is in accordance with earlier study supporting faster diffusion or partition of the solute from the plant cells into solvent due to direct heat generation within volume and pressure impact on cell walls[25,26].

All extracts were screened for eight phytochemicals i.e. steroids, reducing sugars, alkaloids, phenols, flavonoids, flavone, saponin and tannins (Table 2). As most abundant and pungent constituents of chilies, capsaicinoid belong to alkaloid class, hence their presence in all extracts, irrespective of polarity of solvent and extraction method was expected. Besides that, steroids and phenolic compounds were also present in most of the extracts. Similar results have been reported earlier[11,12] and presence of alkaloids, flavonoids, polyphenols, steroids and tannins was observed in methanolic extract of *C. frutescens*. Ethyl acetate extract prepared by conventional MSE showed presence of maximum number of phytochemicals, while acetone and methanol extracts prepared by MASE showed smallest phytochemical array. Many secondary metabolites like alkaloids, flavonoids, isoflavonoids, tannins, coumarins, glycosides, terpenes and phenolic compounds present in plants have been related with antimicrobial properties, while in case of spices phenols, acids, acyclic esters or lactones, alcohols, aldehydes, ketones, ethers and hydrocarbons have been recognized as major antimicrobial components[27,28].

Results of antibacterial and antifungal activity are presented in Table 3. Results revealed remarkable ability of these extracts to inhibit the growth of tested pathogens to varying magnitude, depending upon polarity of extraction solvent and method used. Nature of solvent is a deciding factor in quantitative estimation of antimicrobial activity[29]. In present study, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. cereus*, *C. albicans* and *C. krusei* were found sensitive towards all the extracts tested, while none of the extracts showed inhibitory activity against *S. aureus* MRSA. Chloroform extract prepared by MSE formed maximum inhibition zone of 15.3, 13.5 and 22.0 mm against *P. aeruginosa*, *K. Pneumoniae* and *C. albicans*, respectively whereas ethyl acetate extract prepared by MSE formed largest inhibition zone (12.0 mm) against *E. coli*. These values were larger than the zone size formed by positive controls. Activity of most of the extracts against *S. aureus* was low, but *n*-hexane extract prepared by SSE showed significant activity by forming 11.1 mm inhibition zone. Though between both conventional extraction methods, results were better with MSE, but against *B. cereus* ethyl acetate extract prepared by SSE

Table 2—Results of preliminary phytochemical screening of solvent extracts of *C. frutescens* seeds

Extracts	Steroids	Reducing sugars	Alkaloids	Phenolics	Flavonoids	Flavone	Saponins	Tannins
HE(MSE)	+	+	+	+	-	-	+	-
CH(MSE)	+	-	+	+	+	-	+	-
EA(MSE)	+	+	+	+	+	-	+	+
AC(MSE)	+	+	+	+	-	-	+	-
ME(MSE)	+	-	+	+	-	-	+	+
HE(SSE)	+	+	+	+	-	-	+	+
CH(SSE)	+	+	+	+	-	-	+	-
EA(SSE)	+	+	+	+	-	-	+	-
AC(SSE)	+	+	+	+	-	-	+	-
ME(SSE)	+	-	+	+	+	-	+	-
HE(MASE)	+	-	+	+	-	-	-	+
CH(MASE)	+	+	+	+	-	-	+	+
EA(MASE)	+	-	+	+	-	-	+	+
AC(MASE)	-	-	+	+	-	-	+	+
ME(MASE)	-	-	+	+	-	-	+	+

HE-*n*-Hexane, CH-chloroform, EA-Ethyl acetate, AC-Acetone, ME-Methanol, MSE-Maceration solvent extraction, SSE-Soxhlet solvent extraction, MASE-Microwave assisted solvent extraction, +-Present; --Absent

Table 3—Zone of inhibition (excluding well size, in mm)* formed by a specific concentration (100 µL of 20 mg/mL) of solvent extracts of *C. frutescens* seeds against pathogenic bacteria and fungi

Extracts/ Positive control	Gram negative bacteria					Gram positive bacteria			Fungi	
	E. coli	*S. typhi*	*P. vulgaris*	*P. aeruginosa*	*K. pneumoniae*	*B. cereus*	*S. aureus*	*S. aureus MRSA*	*C. albicans*	*C. krusei*
HE(MSE)	11.1±0.22	R	R	6.8±0.48	5.1±0.31	4.0±0.14	3.1±0.2	R	5.2±0.25	3.5±0.12
HE(SSE)	8.0±0.25	R	6.3±0.45	12.3±0.26	13.0±0.3	6.2±0.24	11.1±0.4	R	18.1±0.32	16.2±0.46
HE(MASE)	11.1±0.24	4.5±0.3	R	14.4±0.67	11.1±0.26	6.9±0.35	R	R	20.0±0.62	14.2±0.43
CH(MSE)	8.0±0.59	5.2±0.14	R	15.3±0.27	13.5±0.82	10.4±0.36	5.1±0.3	R	22.0±0.86	13.0±0.46
CH(SSE)	9.2±0.46	R	R	11.1±0.46	11.1±0.56	8.0±0.52	5.1±0.62	R	13.8±0.4	10.1±0.38
CH(MASE)	11.1±0.34	R	R	13.0±0.34	9.5±0.62	5.6±0.55	R	R	13.0±0.55	11.0±0.36
EA(MSE)	12.2±0.3	6.0±0.22	R	12.2±0.48	12.2±0.45	9.0±0.25	4.2±0.67	R	13.1±0.5	12.5±0.48
EA(SSE)	9.2±0.38	R	6.5±0.35	11.0±0.28	10.4±0.78	12.2±0.62	4.2±0.17	R	8.2±0.3	11.0±0.4
EA(MASE)	10.3±0.42	5.2±0.21	5.0±0.34	14.2±0.57	11.5±0.44	8.0±0.68	R	R	12.1±0.26	15.0±0.64
AC(MSE)	8.0±0.64	8.5±0.37	6.5±0.52	14.2±0.72	13.0±0.21	10.4±0.47	5.2±0.28	R	16.2±0.39	14.1±0.3
AC(SSE)	8.0±0.57	4.5±0.18	R	12.2±0.65	9.5±0.4	10.3±0.49	5.2±0.24	R	17.3±0.56	12.1±0.22
AC(MASE)	8.0±0.33	8.0±0.46	6.5±0.37	11.1±0.44	11.0±0.32	9.0±0.32	8.0±0.14	R	9.0±0.16	11.0±0.38
ME(MSE)	8.0±0.49	6.5±0.12	6.0±0.48	11.1±0.41	9.5±0.64	8.1±0.24	8.0±0.31	R	13.1±0.46	8.8±0.24
ME(SSE)	7.0±0.29	5.2±0.26	R	14.2±0.15	10.4±0.26	8.1±0.36	3.6±0.12	R	15.2±0.48	10.0±0.27
ME(MASE)	9.2±0.4	8.0±0.53	R	11.2±0.33	13.0±0.24	8.1±0.29	5.2±0.25	R	10.0±0.4	11.0±0.32
Cotrimoxazole (25 mcg/disc)	10.0±0.35	11.2± 0.47	12.2±0.18	14.0±0.54	12.2±0.46	14.0±0.32	15.0±0.48	8.1±0.30	-	-
Nystatin (100 units/disc)	-	-	-	-	-	-	-	-	19.2±0.12	16.3±0.22

*All values are mean of three determinations ± standard deviation, R-Resistant, --Not tested, HE-n-Hexane, CH-chloroform, EA- Ethyl acetate, AC- Acetone, ME-Methanol, MSE-Maceration solvent extraction, SSE-Soxhlet solvent extraction, MASE-Microwave assisted solvent extraction

showed promising activity and formed 12.2 mm inhibition zone. Earlier studies have reported that extracts of *C. frutescens* possess antifungal activities against different fungi like *A. flavus*, *A. niger*, *Penicillium* sp., *Rhizopus* sp., and *C. Albicans*[30,31]. In present study also, most of the extracts showed antifungal activity against both fungi tested. Against *C. albicans*, inhibitory activity exhibited by chloroform extract prepared by MSE (22.0 mm) and *n*-hexane extract prepared by MASE (20.0 mm) was even greater than standard nystatin drug. Against *C. krusei*, largest inhibition zone was formed by *n*-hexane extract prepared by SSE (16.2 mm) followed by ethyl acetate extract prepared by MASE (15.0 mm).

The antibacterial and antifungal potency of extract showing considerable activity was quantitatively assessed by MIC determination (Fig. 1a-c). An earlier study on ethanolic extract of *C. frutescens* reported MIC values between 5-25 mg/mL against microorganisms like *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *C. albicans*[32]. In present study, MIC values ranged between 0.312-5 mg/mL and most of the cases were in correlation with the antibacterial and antifungal activity determined by inhibition zone formation.

MASE is an innovative solvent extraction technology, which offers a better alternative to several thermal extraction methods, in terms of less solvent consumption, shorter operational time, high recoveries, good reproducibility, cost effectiveness and minimal sample manipulation for extraction process[33]. In present study, ethyl acetate extract prepared by MASE showed significant activity against *E. coli* (10.3mm), *P. aeruginosa* (14.2 mm), *K. pneumoniae* (11.5 mm) and *C. krusei* (15.0 mm), while *n*-hexane extract prepared by MASE showed promising activity against *P. aeruginosa* (14.4 mm)

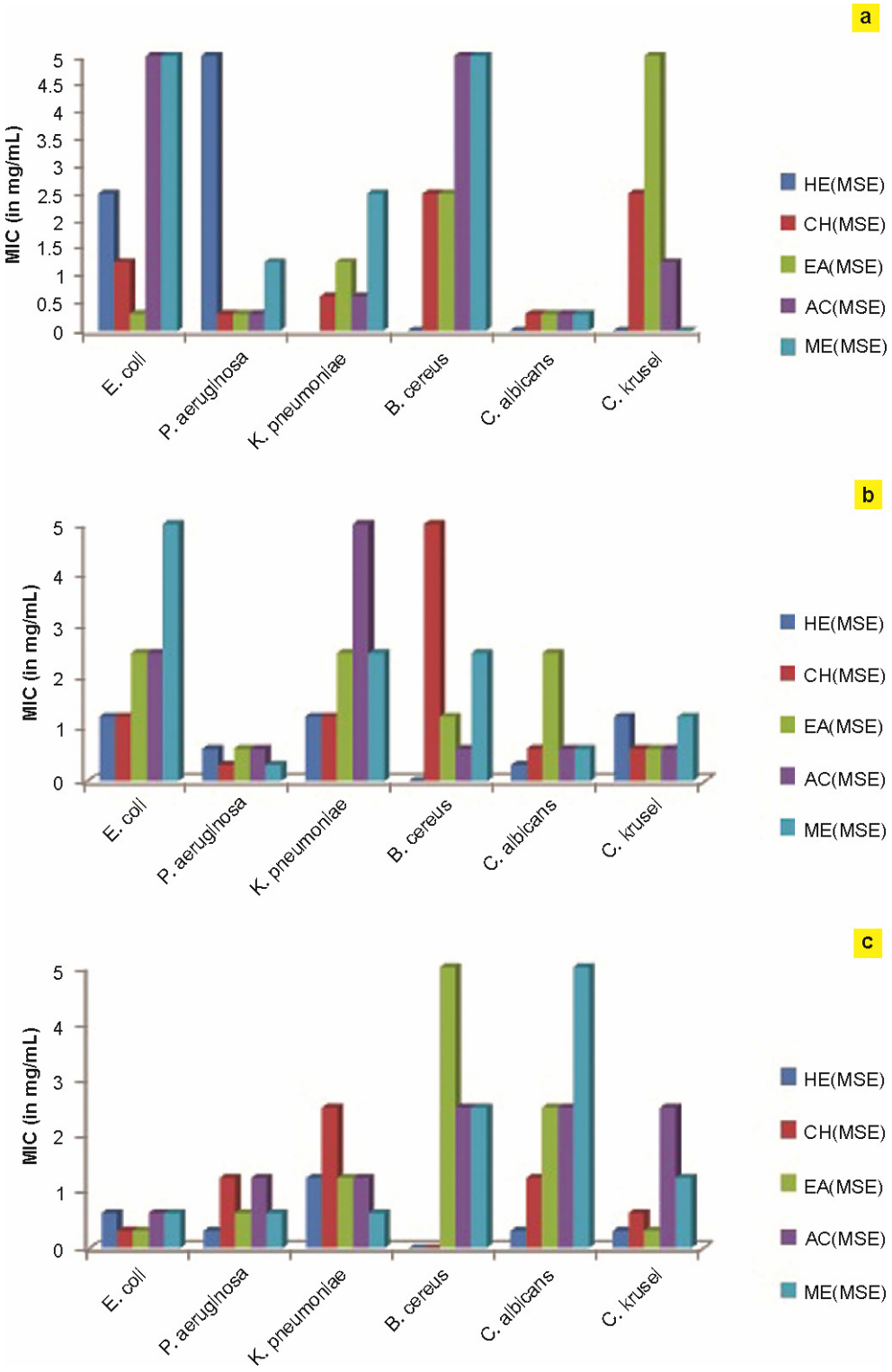

Figure 1—Minimum inhibitory concentration of solvent extracts of *C. frutescens* seeds against pathogenic bacteria and fungi prepared using a) MSE, b) SSE and c) MASE

and *C. albicans* (20.0 mm). Comparable results of antimicrobial activity of extracts prepared by MASE, with respect to extracts prepared by conventional methods may provide an improvised way to extract out antimicrobial phytoconstituents from capsicum seeds with ease.

Conclusion

With the increased resistance towards synthetic antibiotics in pathogenic microorganisms, plant products especially present in common food ingredients can provide a better alternative to cure as well as prevent the infections caused by them. The present study revealed significant antimicrobial potential of one of the most common spice, red chilies along with providing an easy, economic and less polluting way to extract out target bioactive molecules from chili seeds. However further investigations regarding the optimization of extraction parameter and isolation of individual moieties from most active extracts may help in offering the natural substitutes to treat diseases caused by investigated microbes.

Acknowledgement

We are thankful to Dr. Vrinda Gupta, Head, Department of Botany, Government Arts & Science College, Ratlam for authentication of plant material and Dr. Harshada Shah, Professor and Head, Department of Microbiology, RD Gardi Medical College, Ujjain for providing the pathogenic strains, other lab facilities and guidance to conduct bio-assay.

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