Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(2): 1740-1744
Received: 10-01-2020
Accepted: 13-02-2020

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Commonly available spices to be used as feed additive to improve health of fish

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Abstract
Since antibiotics are banned to be used as growth promoters and therapeutic measures in aquaculture. The present study was conducted to evaluate the antimicrobial and antioxidant activities of solvent extracts of four Indian spices viz., Turmeric, Cumin, Ginger and Garlic. The antioxidant capacity of the spice extracts were found in descending order: Cumin>Garlic>Turmeric>Ginger by DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) method, Garlic>Cumin>Turmeric>Ginger by Ferric Reducing Antioxidant Power Assay (FRAP) method and Turmeric>Garlic>Cumin>Ginger by Total Phenolic Content (TPC) method. Cumin had the highest antimicrobial effect (9mm) at maximum concentration on the growth of bacterial strains *Vibrio vulnificus* and *Micrococcus leutieus* followed by Garlic (8mm), Ginger (8mm) and Turmeric (7mm). These results indicated that the spice extract supplement is a promising prophylactic measure for the fish health improvement.

Keywords: spices, solvent extraction, DPPH, FRAP, TPC, antimicrobial activity

Introduction
Many beneficial effects of the common food spices on the health have been understood. There are also new concerns about food safety due to increasing occurrence of new food-borne disease outbreaks caused by pathogenic micro-organisms. This raises considerable challenges, particularly since there is increasing unease regarding the use of chemical preservatives and artificial antimicrobials to inactivate or inhibit growth of spoilage and pathogenic micro-organisms (Arques *et al.* 2008; Aslim and Yucel, 2007; Brandi *et al.* 2006) [6, 7, 11]. Spices can be added to foods in several forms: as whole spices, as ground spices, or as isolates from their extracts.

Spices are aromatic and pungent food ingredients, like herbs, spices can have significant antioxidative effects (Suhaj 2006). Wojdyło *et al.* (2007) [34, 40] measured total equivalent antioxidant capacities and phenolic contents (Folin–Ciocalteu) of 32 spices. Spices can also have antibacterial effects. Shan *et al.* (2005, 2007) [29, 30] found that, of 46 spice extracts evaluated, many exhibited antibacterial activity against foodborne pathogens. Gram-positive bacteria were generally more sensitive than Gram-negative bacteria. *Staphylococcus aureus* was the most sensitive, while *Echerichia coli* were the most resistant. The antibacterial activity of the extracts was closely associated with their phenolic content.

Antioxidant can be defined as substances whose presence in relatively low concentrations significantly inhibits the rate of oxidation. These are the substances that may protect cells from the damage caused by unstable molecules known as free radicals. Scientific research now confirms that free radicals play a major role in the development of cancer, heart disease, aging, cataracts and impairment of the immune system. Antioxidants vitamins and minerals should enhance the body's natural defense mechanisms and improve the quality and length of life. Antioxidants are abundant in fruits and vegetables, spices as well as in other foods including nuts, grains, and some-meats, poultry and fish. It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine (Odugbemi, 2006) [24].

Spices are some of the most commonly used natural antimicrobial agents in foods. Addition of spices in foods not only imparts flavor and pungent stimuli but also provides antimicrobial) property (Hirasa and Takemasa, 1998, Nevas, 2004) [16, 23]. Natural antimicrobial compounds in spices were found to possess antimicrobial activity (Shelef, 1983, Kim, 1995) [31, 18]. Although some researchers have studied the antibacterial activity of spices against several species of bacteria, few serotypes of *Salmonella* have been tested. In addition, the antimicrobial property of spices may differ depending on the forms of spices added, such as fresh, dried, or extracted forms. The objective of the present study was to investigate the

antioxidant and antimicrobial properties of four Indian spices in order to rank them on the basis of their antioxidant and antimicrobial activity.

Materials and Methods

Spices: Turmeric (*Curcuma longa*), cumin (*Cuminum cyminum*), ginger (*Zingiber officinale)* and garlic (*Allium vineale)* were purchased from local market in Srinagar, Kashmir (J & K).

Spice extraction

The extract preparation was done according to the method previously described by Virdi *et al.* (2003) [37] with some modification. The spices were ground into powder in a laboratory grinder and sieved into fine powder to be used for extraction. The spice materials were extracted by ethyl acetate solvent. About 10g of finely powdered spices was weighed separately and extracted with solvent ethyl acetate in a soxhlet apparatus for at least 24 hours at 70 ^{0}C. The solvent with extract was filtered with Wathman no.1 filter paper and centrifuged for 5 minutes at 5000rpm to obtain particle free supernatant. In order to obtain pure extract, the extraction solvent was removed by using rotary evaporator (IKA HB10 basic) at 70 ^{o}C. Then, solvent fee extract was finally stored at 4 ^{0}C until use.

Determination of Antioxidant Activity 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

The antioxidant activity of extracts was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, Brand-Williams *et al.* (1995) [12]. The ethyl acetate stock solution (20µL) of the extracts (concentration of stock solutions were 25, 20, 15, 5, 2 and 1mg/ml) was put into an appendroff tubes and 2mL of $6x10^{-5}$ mol/L ethyl acetate solution of DPPH was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517nm was determined in spectrophotometer after 1 h for all samples. Ethyl acetate was used to zero the spectrophotometer. Percent inhibition of the DPPH radical by the samples was calculated according to the formula of Yen & Duh (1994).

$$\% \text{ inhibition} = ((A_{C(o)} - A_{A(t)} / AC_{(o)}) \times 100$$

Where $A_{C(o)}$ is the absorbance of the control at t=0 min and $A_{A(t)}$ is the absorbance of the antioxidant at =1 h.

Determination of Ferric Reducing Antioxidant Power (FRAP Assay)

The FRAP assay was done according to the method of Benzie and Strain (1996) [9] with some modifications. The stock solutions included 300mM acetate buffer (3.1g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), Ph 3.6, 10mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40mM HCL, and 20mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution followed by warming at 37 ^{0}C before use. Spice extracts (150µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593nm. The standard curve was linear between 25 and 800 µM Trolox. Results were expressed in umol(FeII)/gDW.

Determination of Total Phenolic Contents

The total phenolic contents were estimated according to the Folin-Ciocalteu method of Singleton *et al.* (1999) [33]. To 50 µL sample were added 250 µL of undiluted Folin-Ciocalteu-reagent. After I min, 750 µL of 20 % (w/v) aqueous Na_2CO_3 were added, and the volume was made up to 5.0ml with distill water. The controls contained all the reaction reagents except the extract. After 2 h of incubation at 25 ^{0}C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg/100g extract) and the values are presented as means of triplicate analysis.

Microbial strain and growth media

A loopful of 24 h surface growth on a Nutrient Agar (NA) and Tryptic Saya Agar (TSA) slope of each bacterial strain was transferred individually to 5 ml of Brain heart Infusion (BHI) broth (pH 7.6, Difco). After incubation at 37 ^{0}C for 24 h, bacterial cells were collected by centrifugation at 3000 rpm for 15 min, washed twice and resuspended in PBS. Turbidity was adjusted to match that of a 0.5 McFarland standard (10^6 CFU/ml). Then, a 1:10 dilution of the cell suspension was performed to give an inoculum concentration of 10^7 CFU/ml.

Screening of spice extracts using disk diffusion technique

The disk diffusion test was performed using the standard procedure as described by Bauer *et al*. (1966) [13]. The bacteria, *Vibria vulnificus* was incubated in Nutrient Broth (NB) and *Micrococcus lutieus* was incubated in Tryptone Soya Broth (TSB) (Difco) at 37± ^{0}C for 24 hr, The inoculums suspension of bacterial stains, *Vibrio vulnificus* (MTCC) and *Micrococcus leuteus* (MTCC) was swabbed on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 ± 0.1, Difco). Sterile 6 mm filter paper discs (Schleicher & Schuell) were aseptically placed on MHA surfaces by pressing slightly, and solvent extracts of spices were immediately added to discs in volume of 15 µL respectively. The plates were left at ambient temperature for 15 min to allow excess rediffusion of extracts prior to incubation at 37 ^{0}C for 24 h. Diameters of inhibition zones were measured. Each experiment was done in duplicate. All of the extracts individually were injected into sterile paper discs having a diameter of 6 mm in the amount of 15 µL. Discs injected with pure ethyl acetate and distilled water served as negative control whereas as disc containing 10 µg amoxicillin was placed in the plate as a positive control.

Statistical Analysis

Univariate Analysis of variance (ANOVA) was applied to the data to determine differences ($p < 0.05$). To discover where there were significant differences between the levels of the main factor, Least Significant difference was used. The statistical analyses were made using SPSS-17.

Results

The radical scavenging capacity of the spice extracts was tested using the 'stable' free radical, DPPH, FRAP and TPC. Table 1 shows the effective concentrations of each extract required to scavenge DPPH radical and the scavenging values as inhibition (%). It was observed that the extracts analyzed exhibited varying degrees of scavenging capacities. Garlic extract exhibited the highest ($p<0.05$) radical scavenging effect which was higher than the other extracts. The lowest activity was shown by ginger.

A concentration dependent ferric reducing capacity was found for all the spice extracts (Table 2). Garlic extract exhibited the strongest $p<0.05$) radical scavenging effect which was highest followed by cumin, turmeric and ginger.

Total phenolic contents of the turmeric was highest (p<0.05) among all extracts. Other extracts were in the order of garlic, cumin and ginger (Table 3).

The results of the disk diffusion test indicated that spice extracts cumin, garlic, cumin and ginger showed different degrees of growth inhibition at different concentrations depending on the bacterial stains (Table 4). Cumin showed the broadest antibacterial activity by inhibiting growth of *V.vulnifius* and *M. lutius* stains tested. Other extracts showed antibacterial activity in order of, garlic, ginger and turmeric.

Table 1: Antioxidant activity of spices using the corresponding concentrations (A=25 mg/ml, B=20mg/ml, C=15mg/ml, D=5mg/ml, E=2mg/ml and F=1mg/ml) measured by DPPH (% inhibition) method.

Spices	Concentration (mg/ml)					
	F	E	D	C	B	A
Turmeric	9.355±0.46	26.891±0.84	51.278±0.23	65.439±0.49	85.531±3.81	89.263±0.15
Cumin	9.430±.45	22.772±.30	44.458±.47	61.712±.49	75.619±.13	87.440±.27
Garlic	22.763±0.37	31.366±0.55	41.494±0.37	57.472±0.08	67.846±0.14	71.042±0.45
Ginger	3.213±0.37	23.039±0.32	46.895±0.65	63.725±0.16	68.028±0.24	84.749±0.34

Critical Difference, CD (p<0.05), Conc.: 0.39, Spices: 0.53, Spices*Conc.: 1.30

Table 2: Antioxidant activity of spices using the corresponding concentrations (A=25 mg/ml, B=20mg/ml, C=15mg/ml, D=5mg/ml, E=2mg/ml and F=1mg/ml) measured by FRAP (umol(FeII)/gDW) method.

Spices	Concentration (mg/ml)					
	F	E	D	C	B	A
Turmeric	136.291±3.98	155.375±.90	169.125±.50	178.875±1.08	184.208±1.50	237.625±10.60
Cumin	142.791±6.80	151.125±3.38	167.375±5.95	197.958±12.41	215.541±2.89	246.875±14.72
Garlic	146.375±2.81	191.041±4.62	220.291±5.39	234.625±.25	241.458±1.84	253.625±.75
Ginger	124.125±.50	125.208±.38	141.875±2.61	171.291±2.75	126.541±98.87	230.625±1.08

Critical Difference, CD (p<0.05), Conc.: 6.23, Spices: 8.44, Spices*Conc: 20.69

Total phenolic contents of the turmeric was highest (p<0.05) among all extracts. Other extracts were in the order of cinnamon, garlic, cumin and ginger (Table 3).

Table 3: Antioxidant activity of spices using the corresponding concentrations (A=25 mg/ml, B=20mg/ml, C=15mg/ml, D=5mg/ml, E=2mg/ml and F=1mg/ml) measured by TPC (mg/100g) method.

Spices	Concentration (mg/ml)					
	F	E	D	C	B	A
Turmeric	1.416±0.38	25.666±0.80	54.000±4.98	57.583±1.23	67.166±3.02	147.333±3.98
Cumin	3.666±0.38	10.083±0.38	13.416±0.14	17.833±0.62	37.916±0.62	58.000±1.32
Garlic	3.333±0.62	7.750±1.08	13.250±0.90	53.250±0.66	83.250±1.08	107.500±1.50
Ginger	3.666±0.38	7.416±0.38	2.500±1.00	8.416±0.80	17.166±2.25	35.333±0.76

Critical Difference, CD (p<0.05), Conc.: 1.06, Spices: 1.44, Spices*Conc: 3.53

Table 4: Antimicrobial activity of spices extracted by ethyl acetate against *Vibrio vulnificus* and *Micrococcus luteus* using the corresponding concentrations (A=25 mg/ml, B=20mg/ml, C=15mg/ml, D=5mg/ml, E=2mg/ml and F=1mg/ml).

	Spices	Diameter of inhibition zone (mm)											
		Vibrio vulnificus						*Micrococcus lutieus*					
		A	*B*	*C*	*D*	*E*	*F*	*A*	*B*	*C*	*D*	*E*	*F*
1	Turmeric	7	-	-	-	-	-	8	-	-	-		-
2	Cumin	9	9	7	-	-	-	10	9	8	-	-	-
3	Garlic	8	7	-	-	-	-	8	8	-	-	-	-
4	Ginger	8	7	-	-	-	-	9	8	-	-	-	-
	Amoxycillin (+ve control)	22						25					
	Ethyl acetate (-ve control)	Nil						Nil					

Discussion

The antioxidant activities of spice extracts have been widely demonstrated (Sebranek *et.al.* 2005) [32] although the mechanism of such activity is not fully understood. Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $^{\bullet}O_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentrations (Nawar 1996) [22]. Several explanations have been provided among them the following: the sequence of free radicals; hydrogen donation; metallic ion chelation; or even acting as substrate for radicals such as superoxide or hydroxyl (Al-Mamary *et al.* 2002) [8].

The DPPH assay measures the ability of the extract to donate hydrogen to the DPPH radical, resulting in bleaching of the DPPH solution. In the present study the values of antioxidant activity were in the order of: cumin>garlic>turmeric>ginger. Antioxidant activities of essential oils from aromatic plants are mainly attributed to the active compounds present in them. This can be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them (Abdalla and Roozen, 1999) [1]. In the present study, the antioxidant activities related to the contents of extracts for four spices belonging to

different extracts showed very different antioxidant capacities. Stronger activity is indicated by a higher antioxidant index determined by each of the three different methods: DPPH, FRAP and TPC. Cumin essential oil is better at reducing Fe^{3+} ions than dried or fresh ginger. The major components in cumin volatile oil are cuminal, γ-terpinene, and pinocarveol (El-Ghorab *et al.* 2010) [15]. Garlic and shallots (*Allium ascalonicum*) have antioxidant and free radical-scavenging characteristics and identifiable odors at low concentrations. It is suggested that a combination of the allyl group ($-CH_2CH=CH_2$) and the $-S(O)S-$ group is necessary for the antioxidant action of thiosulfinates in garlic extracts Okada *et al.* (2005), Amagase (2006) [26, 4]. Curcumin present in turmeric is highly effective in neutralizing free radicals (Yu *et al.* 2008) [41]. At the same concentration, curcumin has about twice the antioxidative activity of the polyphenol resveratrol (Aftab and Vieira 2009) [5]. Heating dry ginger, turmeric and their essential oils at 120 °C results in different degrees of retention of antioxidant activity (Tiwari *et al.* 2006) [35]. Of 42 commonly used essential oils, cinnamon bark, oregano, and thyme have been reported to have the strongest free radical-scavenging abilities (Wen *et al.* 2009) [39].

The FRAP method is a simple, very rapid, inexpensive and reproducible method, which can be applied to the assay of antioxidants in botanicals (Prior *et al.* 2006). In the present study a concentration dependent FRAP activity was found for all the extracts. A possible explanation could be that the antioxidant effect is due to several non-volatile compounds (Viuda-Martos *et al.* 2007) [36]. In an investigation Murcia *et al.* (2004) [21] found that a 5% water extract from ginger yielded nearly the same antioxidant activity toward lipid peroxidation as of BHT, both applied at a dose of 0.1%. The antioxidant capability of cinnamon essential oil is stronger than its free radical-scavenging capacity (Chen 2008) [41]. However, it is a better superoxide radical scavenger than propyl gallate, mint, anise, BHA, licorice, vanilla, ginger, nutmeg, or BHT (Murcia *et al.*, 2004) [21]. In a study (Yu *et al.*, 2008) [41] found that turmeric oil has a free radical-scavenging ability comparable to vitamin E and BHT. The results on the antioxidant activity of extracts by FRAP method were found to have highest activity in garlic followed by cumin, turmeric and ginger.

The results of the present study showed that total phenolic content of cumin extract had the highest activity belonging to family of *Apiaceae* whereas, in the spices derived from *Zingiberaceae*, only a small amount of total phenols could found. Phenolic substances have been reported for most of the examined spices. Main phenolics are quercetin and kaempferol glycosides in cumin and fennel (Kunzemann and Herrmann, 1977) [20]. Of 42 commonly used essential oils, cinnamon bark, oregano, and thyme have been reported to have the strongest free radical-scavenging abilities (Wen *et al.* 2009) [39]. Kikuzaki and Nakatani (2006) [19] reported that 12 of the 5 gingerol-related compounds and 8 diarylheptanoids isolated from ginger rhizomes exhibit higher antioxidative activity than α-tocopherol. Authors suggest that this is likely dependent upon side chain structures in addition to substitution patterns on the benzene ring. Thiosulfinates, such as allicin, give garlic its characteristic odor; however, they are not necessarily responsible for all of the various antioxidative and health benefits attributed to it (Amagase 2006) [4]. In the present study the antioxidant assays showed that all extracts can act as radical scavengers to a certain extent.

Cuminum cyminum (cumin) showed the highest antibacterial activity (09 mm inhibition zone) to the microorganisms tested as compared to other spice extracts. Agaoglu *et al.* (2006) [2] found that *C.zeylanicum* was the most effective spice against all of the test strains. Smith-Palmer *et al.* (1998) [28] found that the oils of cinnamon were the most inhibitory, each having a bacteriostatic concentration of 0.075% or less against all of five pathogens (*S. aureus*, L. *monocytogenes*, *Camphylobacter jejuni, Salmonella enteritidis, E. coli*). Con *et al.* (1998) [14] reported that cumin had an inhibitory effect against *S. aureus* and *M. luteus*. In a similiar investigation, Akgul and Kivanc (1989a) [3] reported that cumin exhibited an inhibitory effect against *S. aureus* and *K. pneumoniae* and *P. aeruginosa*. The results of the present study are in agreement with Con *et al.* (1998) [14] and Akgul and Kivanc (1989a) [3]. *Zingiber officinale* (ginger) extracts showed antibacterial activities (8-9 mm inhibition zone) to the microorganisms tested. Konning *et al.* (2004) [17] found that the methanol extracts of the plant were significantly active against the bacteria Gram (+) and Gram (-) and fungi studied. The extracts were less active against *P. aeruginosa*, which is naturally resistant to antibacterial agents (Walker and Edwards 1999) [38]. In a similar study, Bonjar *et al.* (2004) [10] reported that the methanol extracts of *Z. officinale* was active against to all of Gram (+) bacteria. These results were in accordance with the present study. Different antimicrobial activity was explained by Onyeagba *et al.* (2004) [25] that changes one country to other.

Conclusion

It is difficult to assess the antioxidant activity of spices on the basis of a single method. The results obtained using three different methods to evaluate the antioxidant activity (DPPH, FRAP and TPC) showed that the spice extracts used in the present study may be considered good sources of natural compounds with significant antioxidant activity. The degree of antibacterial property of spices tested can be put in the following order: Cumin>Ginger>garlic>turmeric. The present study indicated that the ethyl acetate extracts of the spices have got profound antibacterial and antioxidant effect and may have potential use in aquaculture.

Acknowledgement

The first author is highly thankful to the Vice-chancellor and Dean, Faculty of Fisheries, Rangil, Shere Kashmir University of Agricultural Sciences and Technology-Kashmir for providing facilities for carrying out the work.

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