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PHYSICO-BIOCHEMICAL AND MICROBIOLOGICAL STATUS OF THE DRY SPICES FROM JAN-BAZAAR, KOLKATA

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ABSTRACT
Seventeen different dry spices including herbs were used to study their physico-biochemical features and the associated microorganisms. The moisture content and pH of the dry spice were determined. The amount of total carbohydrate was found low in most of the cases (2 to 18%). The free amino acids like glycine, alanine, arginine, lysine, phenylalanine *etc*. were common in spices and the available lipids present were similar to di-, tri-acyl glycerols and other free fatty acids when determined following TLC of chloroform soluble fraction of spice. The diluted suspension of spice was plated on nutrient agar, eosine methylene blue agar, blood agar, Rose Bengal agar and potato dextrose agar media to take the viable count as well as to determine the variety of microbes. A total of 240 microbial strains were isolated, 84.17% were fungi and 15.83% bacteria including a few *Actinomycetes*. The predominating bacteria and fungi were genus *Bacillus and Penicillium*, respectively.

KEY WORDS: nutrients in spice, microbes related to spice.

INTRODUCTION
The role of plants in human health has extensively revealed due to the emergence of numerous advancements in the medicine and nutrition disciplines. The awareness of the benefits of plants in food as wealthy additives poses researchers to pursue for discovering the influence of such ingredients to the human health. Spices and herbs are well known food ingredients, which enhances the flavour and aroma of the food items. Spices are mainly present in the tropical provinces. These could be either as seeds, flowers, bark or leaves. On the other hand, herbs are fragrant and non woody plants in which they are used in flavouring food dishes; Nutritionally, the spices and herbs are significant in reducing the peroxidation of lipids (Goswami *et al.,* 2013; Zhang *et al*. 2015), which are the changes (off flavour) in the nature and the chemical composition of lipids during the processing, preservation and the final preparation of foods. Generally, spices and herbs prevent the lipid oxidation process due to the presence of natural antioxidants. They have also antimicrobial properties that can help in the preservation of foods (Gottardi *et al.,* 2016) and efficient in replacing synthetic preservatives to increase the shelf life by reducing the growth of microorganisms or by reducing their viability. More recently a variety of spices have been used in animal feed as antimicrobials digestive aids and to reduce methane emission (Wenk, 2002).
The chemical composition of herbs and spices, responsible for their properties, have been reported to be greatly influenced by many factors such as part of the plant used, its vegetative state, environmental conditions, harvesting technique *etc.* (Al-Jasass and Al-Jasser, 2012). Spices are generally contaminated with xerophilic storage moulds and bacteria. The most frequent fungal contaminants of spices are species from the genera *Aspergillus* and *Penicillium.* They are known as potential producers of different toxic substances such as aflatoxins, ochratoxins and sterigmatocystine, that exhibit toxic, mutagenic, teratogenic and carcinogenic effects in humans and animals (Toma and Abdulla, 2013; Kumar *et al.,* 2016). Apart from the spore-forming *Bacillus* and *Clostridium* spp. *Salmonella* has occasionally been found in spices like pepper and dried herbs (Van Doren *et al*., 2013). However, most of the foodborne bacterial pathogens cannot survive under the condition of low moisture like of dry spices.
Spices commonly contain carbohydrates as their constituents since they are originated from plants. Free amino acids in almost all spices (Parthasarathy *et al.,* 2008), free phytosterols and various sterol esters in some spices are also found (Fernandes and Cabral, 2007). Hence, the type of microbes mainly depends on the characteristic chemical components present in the dried spices. The aim of this study is to isolate the microflora present on the dry spices collected from the local market Jan Baazar, Kolkata, their biochemical studies and to correlate those with chemical nature and contents of spices.

MATERIALS AND METHODS

Samples

Dried spices like bay leaf, caraway seed, cardamom, carom seed, celery, cinnamon, clove, coriander, cumin, fennel, fenugreek leaf, mace, mustard, nutmeg, pepper, red chili, turmeric collected from the open market of Jan-Bazar, Kolkata were considered as the source of microorganisms. One gram of the dried spices were properly crushed into powdery form and suspended in water. The mixtures were properly vortexed to create homogenized solutions. The filtrate was taken for further assays.

Physico-biochemical tests of spices

To determine pH of spices individual stock suspension was tested with fractional pH paper, Qualigens and moisture content was determined following the standard method of subtractions of weights of fresh and oven dried sample in each case. The lipid fraction of the filtrate was identified using chloroform followed by thin layer chromatography (TLC) where the solvents used were petroleum ether, diethyl ether, and acetic acid in the ratio of 90:10:1. Same filtrates of the respective samples were used to detect the amino acids present it them and the TLC was performed using solvents butanol, acetic acid and water in the ratio of 80:20:10. The total carbohydrate was estimated after digestion with concentrated HCl following standard Anthrone method.

Media and isolation of microorganisms

The total count of microorganisms was determined following haemocytometer count from the original suspension. For isolation of microorganism, varieties of microbiological media were used. These were nutrient agar (NA) as universal medium for viable count, potato dextrose agar (PDA) for molds, Rose Bengal Agar (RBA) for yeast as well as molds, Blood agar (BA) as enriched medium and Eosin Methylene Blue agar (EMBA) as selective medium for pathogenic microorganisms. For microbiological assays the stock mixture (filtrate) of individual sample were serially diluted and 100µl of the diluted solutions was taken and spread evenly on the aforementioned media on respective medium plate. After incubation of plates for affixed duration colonies were enumerated and characterized. The viable cells present in the suspension of samples were determined by counting the colony forming units (cfu) grown on the media.

Characterization of microorganisms

The microorganisms were characterized by their gram nature, sporulation properties and presence of capsule of bacterial strains and the asexual structure of the fungal strains following standard differential staining procedures.

RESULTS AND DISCUSSION

Biochemical assay of spices

Attempt has been made to detect lipid and free amino acid from the chloroform extract and aquatic extract of spices, respectively. The TLC result showed that lipid in the form of sterols, sterol ester, DAG and free fatty acids were present in most of the spices as depicted from the thin layer chromatography and the Rf values of those lipid components were represented in Table1.

TABLE 1. Presence of probable lipids present in spices as per their Rf values in TLC

Sample	RF1	RF2	RF3	RF4	RF5	RF6	RF7
Mustard	ND						
Turmeric	DAG (0.127)	ND (0.237)	Free FA (0.382)	Sterol ester (0.927)			
Cardamom	ND						
Nutmeg	ND (0.073)	DAG (0.109)	ND (0.209)	ND (0.245)	ND (0.4272)	TAG (0.773)	Sterol esters (0.936)
Cumin	ND						
Bay-leaf	DAG (0.091)	Sterols (0.182)	ND (0.445)	Cho lesterol(0.6)			
Pepper	DAG (0.091)	Sterols (0.164)					
Fenugreek	Fee FA (0.309)						
Celery	ND						
Cinnamon	ND (0.064)	DAG(0.118)					
Mace	DAG (0.109)	ND (0.218)	ND (0.273)	Free FA (0.355)	Free FA (0.4)	Free FA (0.373)	
Carawaseed	ND						
Clove	DAG (0.1)	Sterol (0.173)	ND (0.428)	Cho (0.609)			
Red chilli	Sterol esters (0.927)						
Fennel	ND						
Carom seed	ND						
Coriander	ND						

ND- not determined.

*10µl of the chloroform fraction of the spice extract was spotted: Petroleum ether, diethyl ether, and acetic acid in the ratio 90:10:1 were used as solvents and for detection, Iodine vapour was used.

Free lipids were not determined in mustard, cardamom, cumin, celery, caraway seed, fennel, carom seed and coriander.

The TLC experiment for detection of free amino acids present in spices were also showed most of the spices were with aromatic (tyrosine in carom, phenylalanine in clove, cumin, fenugreek, tryptophan in mustard) and non aromatic amino acids like cysteine, lysine, asparagine, glutamine, valine *etc*. However, from bay leaf, cinnamon, mace (jaitri) and fennel (mouri) no as such free amino acids were detected. A few documentation regarding the fatty acids and free amino acids detection from dry spices has been made so far (Nithya and Ramachandramurty, 2007; Bouba *et al*., 2016). Most of the spices, originated from plants materials showed very high content of carbohydrates (26% - 68%, w/w) as stated in Fig.1 and the similar character was described by Parthasarathy *et al*., 2008. The fatty acid containing spices like mustard or clove showed low carbohydrate compare to the contents of leafy spices like fenugreek or bay leaves.

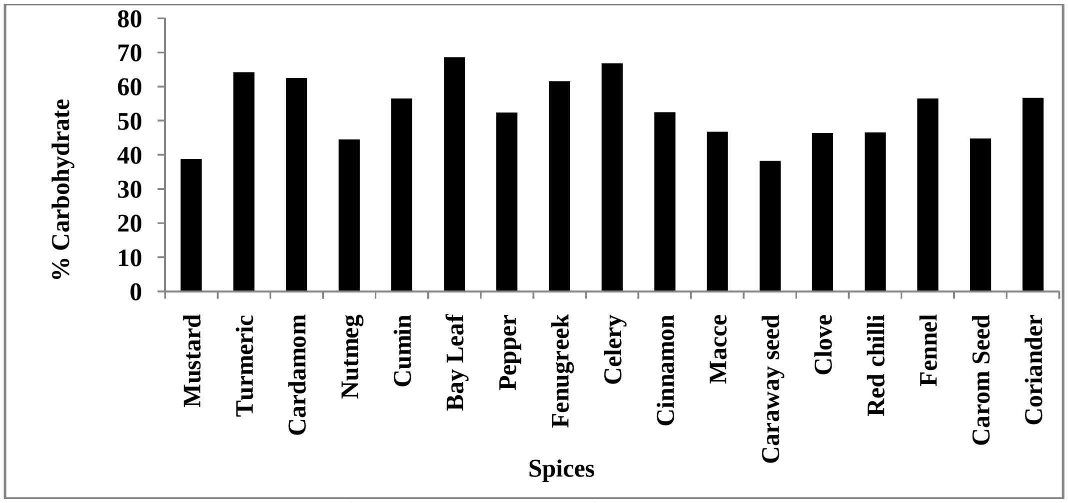

FIGURE 1 Determination of carbohydrate content of the collected spices. (Anthrone Method)

PLATE 1. Isolation of microbes from spices. 1. From turmeric on NA **2.** From caraway seed on RBA 3**.** From Red chili on PDA **4.** From Clove on BA **5**. From pepper on EMBA; Plates after 48h of incubation at 37°C for NA, BA and EMBA and at 28 °C for PDA and RBA

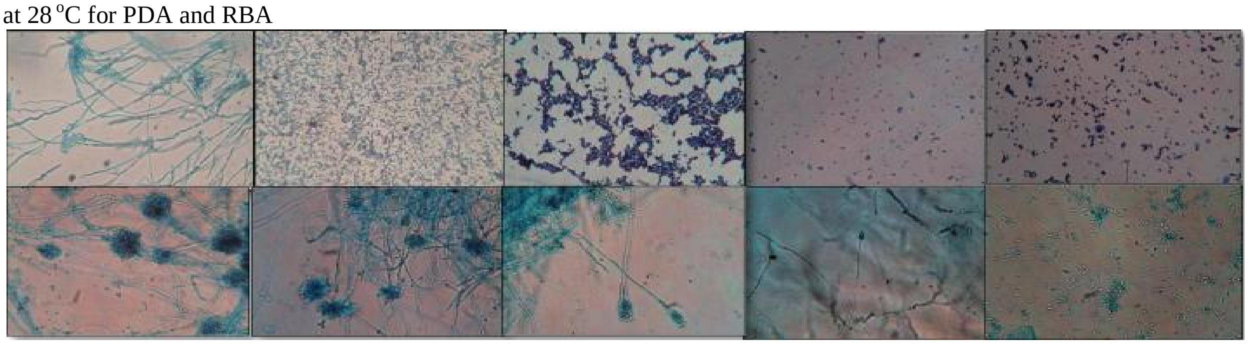

PLATE 2. Bacteria under Microscope (from left to right, in first row): Actinomycetes from clove, Bacilli from nutmeg, Staphylococci from Cardamom, Coliforms from pepper, Streptococci from mace. **Fungi under microscope** (left to right in second row): *Penicillium* from fenugreek leaf, fenugreek, fennel, *Rhizopus* from red chilli, yeast from cumin (tentatively identied). Photos not in scale.

Physical and microbiological status of spices

All the spices appeared slightly acidic to neutral pH (5.5-7.2) except clove (pH 3.0) and the moisture content varied from 3% -12% (except mustard and mace, with 0.3% and 39% moisture, respectively) as represented in the Table 3. The total microbial count, evaluated following haemo cytometer counting and viable count on nutrient agar showed least from celery and maximum from cardamom compare to other spices. Superficial growth of few bacterial isolates on blood agar medium was noted, though not as such haemolytic action (surrounding halo zone) by them had been observed. Coliform was very rare except from pepper. Apart from celery all the spices were contaminated with either molds or yeast or by both types of fungal isolates and their colony forming units per gram of spice varied 2 X 10^4 to 29 X 10^4.

TABLE 2: Presence of probable free amino acid in spices as per their R_f values in TLC

Sample	RF1	RF2	RF3	RF4	RF5	RF6	RF7
Mustard	ND (0.033)	His/Lys (0.099)	Cystine (0.143)	Ala (0.275)	Val (0.429)	Trp (0.6)	
Turmeric	ND (0.022)	Lys (0.132)	Thr/Ala (0.292)	ND (0.967)			
Cardamom	Lys/His (0.132)	Pro (0.22)					
Nutmeg	Pro (0.209)						
Cumin	ND (0.055)	Cystine (0.143)	Asn (0.229)	Gln/Gly (0.263)	Thr/Ala (0.318)	Ile (0.54)	Phe (0.758)
Bay leaf	ND						
Pepper	His/Lys (0.121)	Asn (0.209)					
Fenugreek	His/Lys (0.099)	Di /tri peptide (0.187)	Gln/Gly (0.264)	Val (0.44)	Ile (0.528)		
Celery	Arg (0.176)	Gly/Gln (0.264)					
Cinnamon	ND						
Mace	ND						
Caraway seed	His/Lys (0.077)	Asn (0.209)					
Clove	Lys (0.132)	Ala/Thr (0.341)	Val (0.462)	Phe (0.603)			
Red chilli	His/Lys(0.121)	Val (0.451)	ND (0.96)				
Fennel	ND						
Carom seed	His/Lys (0.077)	Asn (0.21)	Tyr (0.56)				
Coriander	Asn (0.198)	Cys (0.363)	Phe (0.683)				

ND- not determined. Rf=Retardation factor
Free amino acids were not determined in bay leaf, cinnamon, Mace (jaitri) and Fennel (mouri).
For TLC solvents were butanol, acetic acid and water in the ratio 80:20:10 and ninhydrin soln. was used for detection.

TABLE 3: Physical and microbiological analyses of dry whole spices

Spices	pH*	%, Moisture	Total* Count/ml	Viable count/ml#				
				NA	EMBA	BA	PDA	RBA
Mustard	6.7	0.603	12x 10^5	2 x10^5	-	-	6 x 10^4	3x 10^4
Turmeric	7.0	9.05	7.6x 10^6	8 x 10^6	-	-	16x 10^4	2x 10^4
Cardamom	6.5	12.1	15.6 x 10^6	12 x 10^6	-	1 x 10^4	1 x 10^4	-
Nutmeg	5.0	12.86	2 x 10^6	37 x 10^5	-	-	1 x 10^4	1 x 10^4
Cumin	6.5	11.73	48 x 10^6	3 x 10^6	-	2 x 10^2	2 x 10^6	11 x10^4
Bay leaf	7.2	12.36	8.8 x 10^6	6 x 10^6	-	-	22 x10^4	13 x 10^4
Pepper	7.0	9.47	7x10^6	61x 10^5	1 X 10^2	1 x 10^2	29 x 10^4	-
Fenugreek	6.6	11.98	53 x 10^5	2 x 10^5	-	-	6 x 10^4	3 x 10^4
Celery	6.8	11.35	3.4x 10^4	3x 10^4	-	1 x 10^2	-	-
Cinnamon	5.6	16.27	27 x 10^5	8.2 x10^4	-	-	-	5 x 10^3
Mace	6.5	39.47	10.4 x10^6	3 x 10^5	-	-	6 x 10^4	5 x 10^4
Caraway seed	7.2	12.36	6.1x 10^6	65x 10^5	-	-	50x 10^5	26x 10^4
Clove	3.0	3.009	2.3 x 10^6	13 x10^4	-	2 x 10^4	4 x 10^4	1 x 10^4
Red chilli	5.5	10.25	13.2X10^6	3x 10^6	-	3 x 10^4	14x 10^4	39x 10^4
Fennel	6.5	8.34	59.5x 10^5	5 x 10^5	-	-	4 x 10^4	2 x 10^4
Carom seed	5.5	14.67	7.5 x 10^6	9 x 10^5	-	-	2x 10^4	-
Coriander	6.5	7.18	15 x 10^6	7 x 10^6	-	1 x 10^2	3 x 10^4	12x 10^4

*Dry spice sample (1gm) has been grinded and suspended in double distilled water (10 ml) for determination of pH and same suspension was used for total count under microscope following haemocytometer counting.
Different standard microbiological culture media were used for specific microbial viable counting. NA-Nutrient agar; EMBA- Eosin methylene blue agar; PDA-Potato dextrose agar; BA-blood agar; RBA-Rose Bengal agar.

Variation in this number might be attributed by the presence of different physical and chemical barriers in plants and direct plant originated materials like spices. The chemically active compounds have been identified in several spices, such as cinammaldehyde in cinnamon, eugenol in clove and cuminaldehyde in cumin which have proven to prevent food from spoilage and inhibit the growth of pathogenic microorganisms (Carlos and Harrison, 1999). Spice phenolic compounds have been proved as antimicrobial and antioxidant that make spices useful for medicinal and preservative uses (Bozin *et al.*, 2008; Sajilata and Singhal, 2012).

All of the spice isolates appeared aerobic, heterotrophic and microscopic observations determined that they were gram positive either sporulating bacilli (tentatively *Bacillus sp.*) or cocci (Micrococci on musturd, Streptococci on mace and Staphylococci on bay leaves). The spice-borne fungal isolates were microscopically examined and interestingly, they all were tentatively identified as strains of *Penicillium sp.* except in pepper and cumin where *Rhizopus* like mould and yeast were the contaminants, respectively. The antibacterial and antifungal activity of spices are well documented against a variety of microorganisms (Barbosa-Canovas *et al.,* 1998; Lai *et al.,* 2004; Rahaman *et al*., 2010; Marín *et al.*, 2016; Silva *et al*., 2017; Gonelimali *et al*., 2018). However, the dry spices and herbs, like other agricultural products, may be exposed to a wide range of microbial contamination during pre- and post-harvest and during marketization as well and may cause food poisoning.

TABLE 4. List of spices and the microbes dominating them

Spice	Scientific name	Edible part	Dominating Microbes*
Bay leaf	*Laurus nobilis*	Leaf	*Penicilliumsp.* Gram positive Staphylococci
Caraway seed	*Carum carvi*	Seed	Gram positive Bacilli
Cardamom	*Elettaria cardamonum*	Fruit	*Penicillium sp.*
Carom seed	*Trachyspermum ammi*	Seed	*Penicillium sp.,* Gram positive Bacilli
Celery	*Trachyspermum roxburghianum*	Seed	*Penicillium sp.*
Cinnamon	*Cinnamomum zeylanicum*	Bark	*Penicillium sp.,* Gram positive Bacilli
Clove	*Syzygium aromaticum*	Bud	*Penicillium sp.,* Actinomycetes
Coriander	*Coriandrum sativum*	Seed	*Penicillium sp.,* Gram positive Bacilli.
Cumin	*Cuminum cyminum*	Seed	*Penicillium sp.,* yeast
Fennel	*Foeniculam vulgare*	Seed	*Penicillium sp.*
Fenugreek	*Trigonella foenum-graecum*	Seed	*Penicillium sp.*
Fenugreek leaf	*Trigonella foenum-graecum*	Leaf	*Penicillium sp.*
Mace	*Myristica fragrans*	Flower	*Penicillium sp.,* Gram positive Streptococci
Mustard	*Brassica nigra*	Seed	*Penicillium sp*., Gram positive Micrococci
Nutmeg	*Myristica fragrans*	Fruit	*Penicillium sp.* , Gram positive Bacilli
Pepper	*Piper nigrum*	Seed	*Rhizopus sp.* , Coliforms
Red chili	*Capsicum annuum*	Fruit	*Penicillium sp.* , *Rhizopus sp.*
Turmeric	*Carcuma longa*	Root	*Penicillium sp.,* Gram positive Bacilli

*Tentative identification of the isolate was done as per colony morphology and the microscopic studies

CONCLUSION

Although spices are added to foods in small amounts, from the above results, microflora, both bacterial and fungal have been recognized as important source of contamination of spices collected from local market of Jan-Bazar, Kolkata. Detailed studies on the toxicity of contaminants may attribute the proper evaluation of the risk factor in future. However, to avoid that risk and hazards, specially due to mycotoxins, concerned people and consumer, both should aware about the fact and practice proper hygiene for storage of dry spices.

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Ethical Approval

The authors have declared that no ethical issues exist

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