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Microbiological, Heavy Metal and Antioxidant Analysis of Some Commonly used Spices in Pakistani Cuisine

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A B S T R A C T

Background: Spices have been an integral part of global cultures for centuries not only in the culinary world, but also in medicine, beauty products, and traditional healing. In Pakistani cooking, they're indispensable not only for their deep flavors and tantalizing aromas, but also for their role in preserving food.

Methodology: In this study we investigated the microbiological quality, presence of heavy metals, and antioxidant potential of some selected spices from local market namely, S and N. Fourteen samples of spice, such as chili, turmeric, chicken tikka masala, garam masala, korma masala, biryani masala, and chat masala were analyzed for microbial contamination.

Results: Microbiological analysis revealed varying colony-forming units (CFU/ml) across samples, with chicken tikka masala from S exhibiting the highest CFU/ml (6×10^5). Gram staining and spore staining indicated dominance of gram-positive and spore producing bacteria, while no Gram-negative bacteria were detected. Fourteen bacterial strains showed resistance to chromium and lead salts, with strain N6 displaying the maximum resistance (MIC for chromium: 3500µg/ml; lead: 3900µg/ml). Atomic Absorption Spectroscopy revealed elevated lead and chromium levels in the spice (NT) sample (average concentration: 2.40 ppm; 0.78 ppm). Antibacterial activity was observed (9 mm), highest in isolate N6. Exopolysaccharides (EPS) production analysis indicated biofilm-forming potential, confirmed by the ring test. SEM and FTIR analysis of N6 strain showed EPS presence and functional group diversity. 16S rRNA analysis identified the strain as Bacillus sp. Phytochemical analysis revealed the presence of beneficial plant compounds, and antioxidant assays indicated varying levels among spices, with N3 (N biryani masala) exhibiting the highest 77.19% DPPH value, N1 (N korma masala) showing the highest 88.22% FRAP value, and S5 (S biryani masala) displaying the highest total 83.95% phenolic content.

Conclusion: In conclusion, Turmeric Powder (NT) exhibited the highest antibacterial, antioxidative, and antimitotic potential among all the tested extracts of selected spices. Future research could focus on isolating and modifying the phytochemicals responsible for these activities for potential pharmacological applications.

Keywords: spice contamination, atomic absorption spectroscopy, antimitotic activity, Fourier transformed infrared spectroscopy.

Key words: Respiratory tract infection, pathogenic bacteria, antimicrobial susceptibility.

Introduction

Spices have been an integral part of global cultures for centuries not only in the culinary world, but also in medicine, beauty products, and traditional healing arts.[1] In Pakistani cooking, they're indispensable not only for their deep flavors and tantalizing aromas, but also for their role in preserving food. Their influence extends far beyond shores, too. Ancient trade routes such as the Silk Route and the Incense Route were not mere commerce, they were channels for the exchange of spices and the cultural heritage associated with them. Aside from giving flavor to our food, spices provide a surprising array of health benefits. They are said to possess anti-inflammatory[2], anticancer, and antioxidant properties due to phytochemicals such as flavonoids and saponins.[3] Other phytochemicals, including steroids, tannins, cardiac glycosides, and anthraquinones, also make them therapeutic.[4,5] It's not all good, though — there are dangers involved. Spices can at times contain toxic heavy metals such as lead and chromium picked up during cultivation, processing, or storage. Consumed in excess, they can have severe health consequences, so it's crucial that there's monitoring and public awareness.[6]

Spices are now receiving increased attention not only because of their taste and medicinal value, but also as natural substitutes for chemical preservatives. Clove, turmeric, cinnamon, and oregano spices have exhibited significant antibacterial action and are good contenders for safer food preservation and herbal medication.[7,8] However, the presence of heavy metals like lead and chromium in common spices remains a concern for food safety.[9] Lead exposure can result in neurological damage, especially in children, affecting cognitive function. Chromium exposure has been reported to with an increased danger of respiratory and gastrointestinal problems, and it may pose carcinogenic risks especially hexavalent chromium. This study in Lahore investigates the antioxidant capacity, microbiological safety, and heavy metal content of popular spice brands, S and N in Pakistani cuisine.

Materials and Methods

Fourteen spice samples, including red chili, garam masala powder, chaat masala, turmeric powder, tikka masala (chicken), and korma masala, were examined in the study from well-known companies one is named as N and other is named as S. Samples were procured from Iqbal town local market in Lahore, Punjab, Pakistan. Ethyl acetate was used to make the spice extract (0.02g/ml).

The content of carbohydrates, tannins, flavonoids, phlobatannins, cardiac glycosides, saponins, alkaloids, terpenoids, flavonoids, and steroids

Spices were mixed with water to make slurry. The study evaluated antibacterial properties of spices against *Escherichia coli* (GM2163) and *Bacillus sp.* (KC881030) Bacterial strains were swabbed on agar plates, wells were made, and spices samples (50µl) were placed in them. The impact of spices on bacterial growth was revealed by measuring the zone of inhibition (mm) surrounding the wells in plates that have been incubated at 37°C for 01 day.[10]

The antioxidant activity of extracts was assessed using the DPPH radical scavenging method. Ethyl acetate stock solutions of the extracts (20 mg/ml) was mixed with a DPPH solution, and absorbance at 517nm was measured after 1 hour. Percent inhibition of the DPPH radical by the samples was calculated using the formula: % inhibition = $((A_C(o) – A_A(t)) / A_C(o)) \times 100$, where $A_C(o)$ is the initial absorbance of the control and $A_A(t)$ is the absorbance of the antioxidant after 1 hour.

The FRAP assay was conducted following a modified version of the method described. Stock solutions of acetate buffer, TPTZ solution, and $FeCl_3.6H_2O$ solution were prepared, and a fresh working solution was made by combining these components. Spice extracts were incubated with the FRAP solution for 30 minutes in the dark, followed by measurement of the absorbance of the resulting ferrous tripyridyltriazine complex at 593nm. Results were expressed in µmol of FeII per gram of dry weight (DW), with a linear standard curve ranging from 25 to 800 µM Trolox.

The total phenolic contents were determined using the Folin-Ciocalteu method described by.[11] A 50 µL sample was mixed with undiluted Folin-Ciocalteu reagent and 20% aqueous Na_2CO_3. After 2 hours of incubation at 25°C, absorbance was measured at 760 nm and compared to a Gallic acid calibration curve. Total phenols were expressed as Gallic acid equivalents (mg/100g extract), with values presented as means of triplicate analyses.

A modified method based on Fiskesjo (1985) was used to assess antimitotic activity using Allium cepa roots. Bulbs (40 ± 10g) were germinated in water for 72 hours in darkness at room temperature. Uniformly developed roots were selected. These roots were exposed to water, and spices powder 20 mg/mL) for 24 hours in triplicate. After staining with acetocarmine, root tips were observed under a light microscope.[12] Dividing and non-dividing cells were counted, and the mitotic index (MI) was calculated using the formula:

Percentage mitotic index = dividing cells (number) / Total number of cells) * 100.

A comprehensive microbiological analysis was conducted involving the isolation, purification, staining, and biochemical characterization of bacterial strains. Six distinct bacterial isolates were selected based on varied morphologies. Subsequently, the isolated strains were subjected to Gram and Spore staining to assess cell shape and structure. Gram staining was used to identify Gram-positive and Gram-negative microorganisms. The Schaeffer-Fulton method of spore staining was used to differentiate bacterial spores from vegetative cells. Biochemical characterization was performed and tests included are catalase & oxidase test, starch hydrolysis test, Mannitol salt agar and Vogues Proskauer test.

The lowest concentration of Cr and Pb that prevents bacterial growth is known as the minimum inhibitory concentration (MIC). A Loop full of pure bacterial cultures were streaked on nutrient agar plates that had been supplemented with variable concentrations of chromium and lead (0, 0.1, 0.5, 1, 1.5, 2, 3, 3.5 and 4 mg/ml). The plates were then incubated for 48 hours at 37°C. On the basis of growth inhibition, MIC for chromium and lead was determined.[13]

Heavy metal analysis of spices involved wet digestion of spice samples using nitric and per chloric acids. Atomic absorption spectroscopy (AAS) was used to quantify heavy metals like lead and chromium in spices.

The modified ethanol precipitation method was used to extract EPS. Centrifugation of bacterial cultures was a step in the solvent extraction process, and the supernatant was used for further processing. Through centrifugation, extracted EPS was filtered and purified. Protein estimation of exopolysaccharides was conducted using Lowry method[14] while sugars were determined via the phenol sulfuric acid assay.[15]

On Congo red agar plates, distinctive growth was used to determine the formation of slime. Using the Christensen test-tube approach, biofilm development was identified; positive results were confirmed by crystal violet staining.

Using the KBR disc approach, Fourier transform infrared spectroscopy (FTIR) was used to analyze the nature of the EPS. SEM analysis was employed to study the surface morphology of bacterial strain N_6.[16]

The selected bacterial strain underwent 16S rRNA analysis for identification, and its nucleotide sequence was used for phylogenetic analysis to develop a tree using Mega 11 software. The sequence was submitted to NCBI GenBank for accession number.

Results

On October 18, 2022, 14 commonly used spices were purchased from a well-known local store in Iqbal town, Lahore. Two brands, each with seven spice varieties, were collected in clean plastic bags and brought to the Microbiology Laboratory, University of the Punjab. A total of 14 bacterial strains were isolated and the examination of their colony morphology revealed that most of the bacterial isolate's colonies were circular in form, mostly have entire margins and convex elevation while irregular forms, undulant and serrate margins and Umbonate elevation was also found. Microscopic analysis of isolated bacterial strains showed the presence of Gram's positive rod-shaped bacteria mostly arranged in the form of chains while scattered and Diplobacilli cell arrangement was also observed. The biochemical analysis of isolated bacterial strains revealed that out of 14, 11 bacterial isolates gave negative result for oxidase and positive for catalase and Mannitol salt agar test, rest of the 3 isolates were catalase negative, oxidase positive and negative for Mannitol salt agar test. 8 bacterial isolates were positive for VP and others were negative for it, while out of 14 only 6 isolates were positive for starch hydrolysis and 8 were negative.

Phytochemical screening of spices revealed vital insights into their chemical composition, showcasing the presence of various beneficial compounds. All the spices (N_1, to N_5, N_T, N_7, S_1, to S_7) showed the presence of alkaloids, carbohydrates, phenols, saponins, cardiac glycosides with the exception of N_T, flavonoids with the exception of S_4, and all the samples showed the presence of tannins except N_7 and S_2. And all the samples showed the presence of phlobatannins and steroids except N_7, S_4, S_6 and N_5, N_7 respectively. (table I).

Table I:Analysis of phytochemicals and antibacterial activity of spice samples.

Phytochemical tests	Spices samples													
	N_1	**N_2**	**N_3**	**N_4**	**N_5**	**NT**	**N_7**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	-	+	-	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Phlobatannins	+	+	+	+	+	+	-	+	+	+	-	+	-	+
Saponins	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	-	+	-	+	+	+	+	+	+	+
Antibacterial activity against														
Bacillus Sp.	-	-	-	-	-	9mm	-	-	-	6mm	-	-	-	-
E.coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table II: Antioxidant activity of spices.

Samples	DPPH (%age inhibition)	FRAP (%age antioxidant activity)	Phenolic content (mg/100g)
N_1	47.73	88.22	45.01
N_2	47.09	49.27	26.43
N_3	77.79	45.34	41.97
N_4	46.65	56.19	26.26
N_5	53.22	48.32	58.79
NT	54.37	6.40	37.16
N_7	46.59	40.87	75.14
S_1	53.09	48.85	30.27
S_2	55.20	44.28	40.53
S_3	46.65	9.60	25.46
S_4	48.31	30.23	14.41
S_5	47.61	79.49	83.95
S_6	51.82	56.11	63.61
S_7	46.14	34.7	68.41

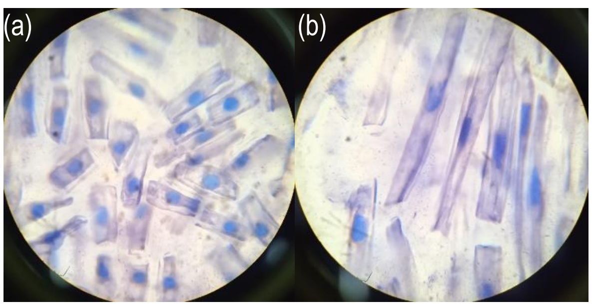

Figure 1. Antimitotic activity of spices, figure (a) showing the presence of non-dividing cells and figure (b) showing the presence of dividing (elongated) cells.

Two out of 14 tested spices (14.28%) demonstrated strong antibacterial activity against *Escherichia coli* (GM2163) and *Bacillus sp.* (KC881030). Samples (N_T) and (S_3) exhibited notable inhibition zones 9mm and 6mm against *Bacillus sp.* respectively, and did not show antibacterial activity against *E.coli*. Other samples did not show any antibacterial activity (table III). Result of time kill assay revealed that extract of national turmeric powder showed considerable bactericidal activity against test organism. After 3 hours incubation, viability of *Bacillus sp*. was reduced by 40% and of *E.coli* by 36%. The viability of *Bacillus sp.* and *E.coli.* was reduced by 78%, 75% respectively after 6 hours of incubation. Reduction continued, after 24 h incubation, viability of test organisms was reduced by 97% for *Bacillus sp.* and by 93% for *E.coli.*

Study assessing antioxidant capacity, 14 spice samples were examined using the 2, 2-diphenyl-1-picrylhydrazyl test. Results ranged from 46.14 to 77.79, with N_3 (N biryani masala) having the highest and S_3 (S turmeric powder) the lowest activity. Phenolic compounds in spices contribute to these variations. Similarly, the ferric ion reducing antioxidant power assay displayed diverse results (6.4 to 88.22), with N_1 exhibiting the highest and S_3 and N_T the lowest antioxidant capacities. Total phenolic content analysis ranged from 14.41 to 83.95 mg GAE/g, showcasing the variability in spice samples' antioxidant potential (table II).

Table III: Bacterial isolates presenting heavy metal resistance.

Bacterial strains	K2CrO4µg/ml MIC	PbCl2 µg/ml MIC
N_5L	3000	2500
N_5M	2000	1600
N_6	3500	3900
N_5B	1500	2500
N_2P	1500	2000
S_6C	1500	2000
S_5A	2100	1600
S_3A	2500	2000
S_4A	2000	2600
S_2A	2500	2000
S_3B	2500	2000
N_1B	3000	2500
N_1A	2000	1500
S_6B	2500	2000

This study aimed to investigate the antimitotic properties of 14 commonly used spices. Among them, only turmeric powder of both N and S brands exhibited significant antimitotic action, potentially obstructing cell division and deterring cancer cell formation. Spices, rich in bioactive compounds, offer a novel avenue for exploring antimitotic drugs. These findings suggest the potential for novel anticancer medications and underline the health advantages of spice integration in diets. The study examined heavy metal levels in spices through bacterial resistance analysis. Minimum Inhibitory Concentration (MIC) values were determined for chromium and lead (ranging from

1500 to 4000 µg/ml), showing bacterial MIC values between 1500-3500 µg/ml. N_6 bacterial strain exhibited the highest resistance (3500 µg/ml against chromium and 3900 µg/ml against lead).

Atomic Absorption Spectroscopy revealed the presence of lead and chromium concentrations in the spice sample (turmeric powder) 2.40ppm and 0.78ppm, respectively (Table IV).

Table IV: Concentration of detected metals in spice sample.

Metal detected	Concentration (ppm)
Lead	2.40
Chromium	0.78

The EPS of a bacterial strain was isolated using alcohol precipitation method, yielding 1.17g/100ml. A 0.4 mL EPS sample was analyzed for soluble protein, measuring 40µg per 0.4ml via a calibration curve. Employing the phenol-sulfuric acid and Lowry method on a 1ml and 0.4ml respectively on EPS sample, carbohydrate and protein content was estimated at 50µg/ml and 40µg/0.4ml respectively.

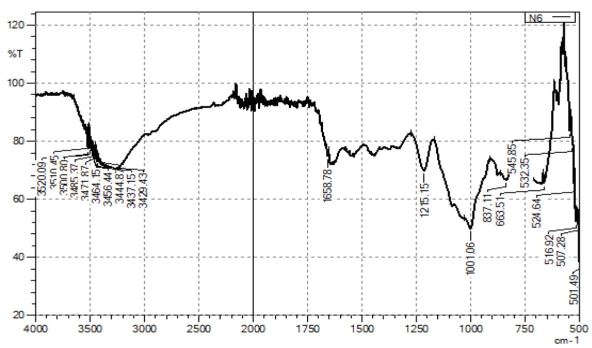

Figure 2. FTIR peaks showing the complex nature of EPS produced by strain N_6

The bacterial strain N6 displayed a positive ring test, affirming its ability to construct a biofilm. A discernible ring or pellicle was evident at the air-liquid interface within the flask, signifying successful bacterial attachment and aggregation. This discovery underscores strain N6's competence in biofilm establishment. However, no distinct black or maroon colored colony, indicative of an unsuccessful amyloid-like fiber formation, was observed.

Fourier Transform Infrared Spectroscopy (FTIR) was used to analyze EPS, revealing distinct peaks corresponding to functional groups and molecular bonds. Hydroxyl (OH) groups appear as a peak around 3200-3600 cm^{-1} in EPS constituents like proteins and carbohydrates. Carboxylic acid (COOH) groups display a strong peak between 1700 and 1750 cm^{-1}, characteristic of uronic acids and acidic amino acids. The amide I band (1600–1700 cm^{-1}) results from C=O bond stretching in EPS proteins. The amide II band (~1500–1550 cm^{-1}) indicates N-H bond bending and C-N stretching in amide groups. Aliphatic C-H bond stretching (2800-3000 cm^{-1}) and glycoside linkages (1000-1150 cm^{-1}) in carbohydrates are also characteristic FTIR features of EPS composition.

The Scanning Electron Microscopy (SEM) analysis of the isolated bacterial strain N_6 revealed distinctive morphological features. The strain exhibited a rod-shaped configuration, showcasing a well-defined and elongated cellular structure under high magnification. Notably, the SEM images illuminated the presence of Extracellular Polymeric Substances (EPS) on the bacterial surface, suggesting the secretion of biofilm-forming materials. The EPS matrix appeared to envelop the bacterial rods, indicating the potential involvement of this strain in biofilm formation.

The construction of phylogenetic tree for the sequenced bacterial isolate was achieved using the MEGA 11 programmer, employing the neighborhood-joining method coupled with bootstrap analysis. In this analysis, isolate N_6 exhibited maximum similarity (100%) to *Bacillus* sp. (MN456846.1).

Discussion

This research presents a detailed investigation into the quality and safety of commonly used spices, with a particular focus on concerns related to microbial contamination and heavy metal accumulation. Fourteen different spices were analyzed to assess both their microbial profiles and the presence of heavy metals. At the same time, their potential antibacterial, antioxidant, antimitotic, and phytochemical properties were explored.

The findings revealed the presence of gram-positive and spore-forming bacteria in several spice samples organisms known for their ability to survive under a range of storage and processing conditions Interestingly, some of these bacterial isolates exhibited strong antibacterial activity, aligning with previous studies.

Phytochemical analysis showed that the spices were rich in beneficial compounds like flavonoids and phenolic acids likewise other study.[17] Antioxidant activity varied among the samples, consistent with earlier research.[18] Notably, turmeric powders from two sources labeled 'S' and 'N' displayed unique antimitotic properties, likely due to their curcumin content, suggesting their potential for medicinal use.[19] Among them, only turmeric from source 'N' exhibited notable antibacterial effects, possibly due to higher levels of bioactive compounds.

A study was conducted and evaluated bacterial resistance to heavy metals such as lead (Pb) and chromium (Cr) and similar findings were observed.[20] One isolate, N6, showed strong tolerance to both metals, supporting prior studies on metalresistant bacterial strains. Atomic Absorption Spectroscopy (AAS) confirmed the presence of lead and chromium in several spice samples, echoing earlier reports and reinforcing concerns about ongoing heavy metal contamination in the spice supply. Additionally, the study examined extracellular polymeric substances (EPS) produced by isolate N6, which played a key role in bacterial surface adherence and biofilm formation. These findings build on existing knowledge about the ecological and medical relevance of EPS and biofilms.[21]

Using 16S rRNA gene sequencing, isolate N6 was identified as a *Bacillus* species. This supports the common occurrence of *Bacillus* in spices, attributed to their resilience and spore-forming capabilities (Smith & Hussey, 2020). These results highlight the importance of implementing strict hygiene measures during spice cultivation and processing to reduce contamination risks.

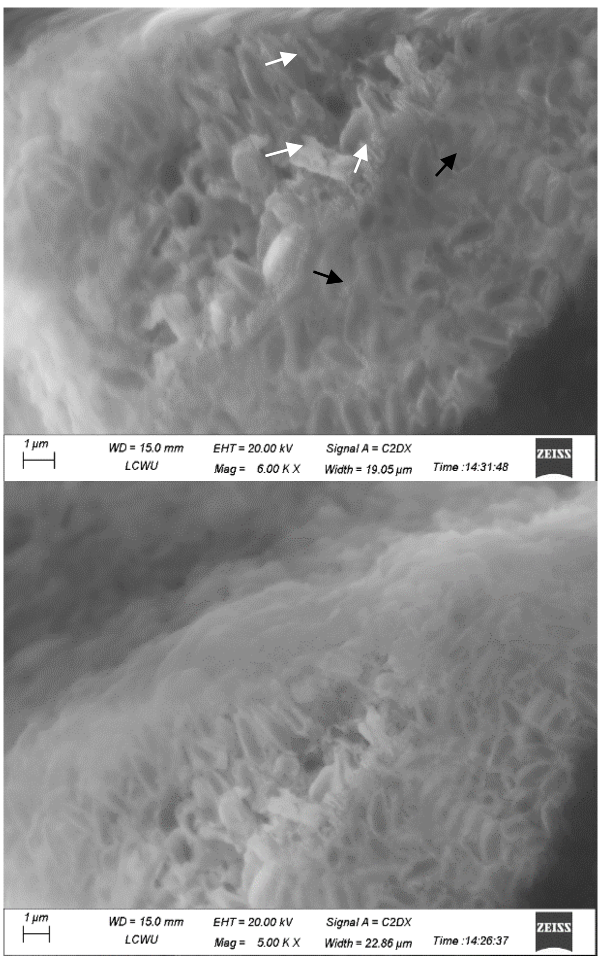

Figure 3: Scanning electron microscope images, black arrows show the presence of rod shape bacteria, white arrows show the existence of Exopolysaccharides on the cell surface of N_6

Conclusions

This study sheds light on the safety and biological value of frequently used spices. It confirms the presence of hardy gram-positive and spore-forming bacteria, alongside promising antibacterial and antimitotic properties in specific spice samples. Significant differences in antioxidant activity and phytochemical content were observed, particularly among turmeric sources. The detection of lead and chromium emphasizes the pressing issue of heavy metal contamination in spices. The study also highlighted the role of EPS in bacterial adherence and biofilm development, with strain N6 identified as a *Bacillus* species. Overall, these findings call for better hygiene and quality control practices in the spice industry, contributing to safer culinary use and expanding our understanding of their health potential.

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