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Original Article

SYNERGISTIC EFFECT OF *CURCUMA LONGA* AND *GLYCYRRHIZA GLABRA* EXTRACTS WITH COPPER IONS ON FOOD SPOILAGE BACTERIA

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ABSTRACT

Objective: To evaluate the synergistic antibacterial activity of ethanolic and aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra* in combination with copper metal ions.

Methods: The phytochemical analysis of *Curcuma longa* and *Glycyrrhiza glabra*'s extracts were observed by standard procedures. Aqueous and ethanolic extracts of *Curcuma longa* and *Glycyrrhiza glabra* were evaluated for their antibacterial activity against *Paenibacillus popilliae* by an agar well diffusion method. Antibacterial activity of copper ions and their synergistic effect was also evaluated.

Results: The phytochemical analysis of the aqueous and ethanolic extracts was carried out for the detection of flavonoids, tannins, saponins, terpenoids, alkaloids and coumarins. The results of combinatorial effects of copper metal ions with aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra* showed maximum antibacterial activity (30±0.33 mm and 30±0.00 mm) when 25 µl of plant extract combined with 25 µl of copper ions, whereas minimum antibacterial activity (23±0.33 mm and 22±0.33 mm) was shown when 45 µl of plant extract combined with 5 µl of copper ions, when evaluated against *Paenibacillus popilliae*. An increase of 187.5% and 12.55% (least) was observed in aqueous and ethanolic extracts of *Curcuma longa* on supplementation of copper metal ions.

Conclusion: The results suggest that crude extracts from these plants can be used for therapeutic purposes as they possess potent antibacterial properties due to the presence of various phyto chemicals in them. The aqueous plant extracts showed enhanced activity in conjugation with copper metal ions against food spoilage bacteria as compared to ethanolic plant extracts.

Keywords: *Curcuma longa*, *Glycyrrhiza glabra*, Copper metal ions, Food spoilage bacteria, *Paenibacillus popilliae,* Zone of inhibition.

INTRODUCTION

A medicinal plant consists of natural compounds which have been known to exhibit many therapeutic activities. Microbiological experiments and techniques have shown that medicinal plants exhibit antimicrobial properties against food spoilage bacteria [1]. Medicinal plants contain the richest resources of drugs used in traditional system of medicine, food supplements, pharmaceutical compounds and chemical entities for synthetic drugs [2]. Moreover, phyto medicines have shown great promise in the treatment of bacterial infections and disease like chronic infections [3]. Phyto chemicals also possess antioxidant and anti inflammatory properties. Increasing failure of chemotherapeutics and development of antibiotic resistance in pathogenic infections agents, has led to an increased interest in the screening of plants having medicinal properties. The beneficial products of medicinal plants are believed to result from the combination of antibacterial properties of elicitors. Exploitation of these natural compounds produced by medicinal plants can serve as the solution to the problem of ever increasing resistance to bacterial infections and diseases.

Spices are plant materials widely used in the food industry to improve the flavor and texture of food products. *Curcuma longa* (Turmeric) which belongs to the Zingiberaceae family is used as colouring agent and exhibits antimicrobial activity against food spoilage bacteria [4, 5]. Rhizome of Turmeric is cleaned, boiled, dried and the powder formed is used in spices [6, 7]. Curcumin and oil extract from *Curcuma longa* control the growth of several microorganisms like, *Streptococcus sp.*, *Staphylococcus aureus* etc [8]. Traditionally, root parts of *Glycyrrhiza glabra* have been used in the clinical purposes [9]. *G. glabra* is used in medicinal purposes for cough, colds and painful swellings [10]. Its roots are widely used for the treatment of anaemia, gout, sore throat, tonsillitis, fever, cough, skin diseases, swelling, acidity, bleeding and jaundice [11]. Copper is an essential metal for most living organisms as it serves as a redox-active cofactor important for terminal respiratory oxidases and superoxide dismutase enzymes. However, high concentration of copper can be extremely toxic for microorganism, since copper ions can produce deleterious reactive oxygen species and also form extremely stable complexes with cellular components [12]. This work comes up with an interesting finding of an increase in antimicrobial activity of *Curcuma longa* and *Glycyrrhiza glabra* extracts when used along with copper metal ions against food spoilage bacteria.

MATERIALS AND METHODS

Plant collection

The rhizomes of *Curcuma longa* and roots of *Glycyrrhiza glabra* were collected from Herbal Health Research Consortium, Punjab, India.

Plant extract preparation

Aqueous extract

Plant parts of the rhizome and roots were washed thoroughly in tap water 4-5 times. For extraction with distilled water, 100 g of dried powder of *Curcuma longa* and *Glycyrrhiza glabra* was added into 200 ml of distilled water and shaken well in a flask for 24 h. The aqueous extract solution was filtered with the help of filter paper. After filtration, extract was evaporated in the water bath at 40-50 °C in order to obtain a thick paste of aqueous extract as described by Mukhtar & Ghori [13].

Ethanolic extract

Freshly rhizome of *Curcuma longa* and roots of *Glycyrrhiza glabra* were washed with sterilized distilled water and dried in an oven at 50 °C for 48 h. Extraction with ethanol, 100 g dried powder of *Curcuma longa* and *Glycyrrhiza glabra* was carried out by using soxhlet apparatus for 6 to 8 h. Finally, the extract was dried in the rotary evaporator to eliminate the extra solvent from extract and to obtain a thick paste as described by Chopra *et al.* [9].

Isolation of bacteria from spoiled food product

Spoiled pasta was inoculated in nutrient broth and incubated at 37 °C for 24 h in order to isolate the bacteria causing spoilage [13].

Phytochemical analysis

Rhizome and root of *Curcuma longa* and *Glycyrrhiza glabra*, respectively were subjected to preliminary phytochemical screening for the absence or presence of both primary and secondary metalbolities [14]. Various phytochemicals viz: alkaloids [15], saponins [15], tannins [16], terpenoids [17], coumarins [18], anthocyanines [18], flavonoids [15] and steroids [19].

Anti-bacterial activity

Isolated bacterial culture was maintained on Muller Hinton agar both aqueous and ethanolic extracts of *Curcuma longa* and *Glycyrrhiza glabra* were subjected to screening for antimicrobial activity by an agar well diffusion method using Muller Hinton agar plates. 50 µl of freshly prepared pure culture of bacteria was uniformly spread on agar plates with the help of the spreader and kept for 5 min. Three wells approximately 6 mm were bored with the help of metal borer. 100 µl of concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* was poured into wells. Ethanol and distilled water were used as negative control, while ciprofloxacin served as positive control. Zone of inhibition was measured after incubation time of 24 h at 37 °C. Zone of inhibition diameter was used as a measure of potency of antimicrobial activity of the plant extract [9, 20].

Determination of minimum inhibitory concentration (MIC) of plant extracts and Copper ions

The minimum inhibitory concentration is defined as the minimum concentration of antimicrobial agents that will inhibit the visible growth of microorganism after the time of incubation [21]. This was carried out by agar well plate method. For MIC, plant extracts @ (1, 0.9, 0.8 and 0.7 mg/ml) and copper metal ions @ (0.97, 0.96, 0.95, 0.94 and 0.93 µl/ml) were transferred into wells made in agar petriplates. These agar plates were incubated at 37 °C for 24 h. Inhibition diameter was measured and minimum concentration of the compound which gave visible reduction in growth was calculated as MIC [20].

Effect of copper ions on antibacterial properties of plant extracts

Synergistic antibacterial activity of plant extracts of *Curcuma longa* and *Glycyrrhiza glabra* and copper ions was determined at different concentration. A stock solution of 1% of copper metal ions was prepared. 50 µl inoculums of bacterial isolate was uniformly spread on Muller Hinton agar plates with the help of the glass spreader approximately 5 mm diameter wells were bored. Ethanolic and aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra* were added to punched wells along with copper metal ions (50 µl) with different concentration in the following manner: first well 10% (45 µl plant extract+5 µl copper metal ion), second well 20% (40 µl plant extract+10 µl copper metal ion), third well for 30% (35 µl plant extract+15 µl copper metal ion), forth well for 40% (30 µl plant extract+20 µl copper metal ions and fifth well for 50% (25 µl plant extract+25 µl copper metal ions). The plates were incubated at 37 °C and inhibition diameter was measured after 24 h [22].

Molecular characterization of bacteria

Molecular characterization of isolated bacteria was carried out by 16s rRNA sequencing. 16S rRNA universal primers gene fragment was amplified by using 27F and 1492R PCR primers; 518F and 800R were used as sequencing primer for isolated bacteria. Sequence data were aligned and analyzed for identifying the bacteria sample. 16s rRNA sequence was blast using NCBI blast similarity search tool and phylogeny analysis of sample sequence with the closely related sequence of blast results was performed by multiple sequence alignment.

Estimation of Curcumin content in ethanolic extract of *Curcuma longa*

The maximum antibacterial activity was obtained in the case of ethanolic extract of *Curcuma longa*. So, estimation of Curcumin content in this sample was carried out by high performance thin layer chromatography (HPTLC) technique. HPTLC parameters are described as spray gas: inert gas; sample solvent type: ethanol; dosage speed: 150 nl/s and pre-dosage volume: 0.2 µl.

RESULTS

Phytochemical analysis

Phytochemical analysis of aqueous and ethanolic extracts of *Curcuma longa* and *Glycyrrhiza glabra* were studied and compared (table 1). The crude extracts were screened for the presences of eight phytochemicals viz. tannins, flavonoids, terpenoids, saponins, steroids, coumarins, anthocyanin and alkaloids. Aqueous and ethanolic extract of *Curcuma longa* and *Glycyrhiza glabra* showed the presence of steroids, while tannins and terpenoids were absent in aqueous and ethanolic extract of *Curcuma longa* and *Glycyrhiza glabra.* Anthocyanin was abundant in the ethanolic extract of *Curcuma longa*.

Table 1: Phytochemical results of ethanolic and aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra*

S. No.	Plant constituents	Test/reagents	*Curcuma longa*		*Glycyrrhiza glabra*	
			Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract
1.	Saponins	Foam test	-	-	+	+
2.	Alkaloids	Dragendroff's test	-	+	-	+
3.	Flavonoids	Alkaline reagent test	+	-	+	-
4.	Tannins	Lead acetate test	-	-	-	-
5.	Anthocyanins	Sulphuric acid test	++	-	-	+
6.	Steroids	Salkowshi test	+	+	+	+
7.	Terpenoids	Salkowshi test	-	-	-	-
8.	Coumarins	Sodium hydroxide test	-	+	-	-

Key:+-present; --absent; ++-abundant present

Anti-bacterial activity

The *in vitro* antibacterial activity was determined for the aqueous and ethanolic plant extracts (table 2) and copper ions (table 3). The results clearly indicate that ethanolic extract of *Curcuma longa* showed more inhibitory effect as compared to the aqueous extract. Aqueous extract of *Glycyrrhiza glabra* showed the greater inhibitory effect as compared to ethanolic extract.

In comparison, combinatorial antibacterial activity of copper metal ions with ethanolic and aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra* increased with increase in concentration of copper metal ions at 10%, 20%, 30%, 40% and 50%. Aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra* demonstrated maximum zone of inhibition of 18±0.33 mm and 21±0.33 mm, respectively. But synergistic effect of copper metal ions (50%) and plant extracts demonstrated maximum zone of inhibition of 30±0.33 mm and 30±0.0 mm, respectively. However, this effect was more pronounced when aqueous plant extracts were used in combination with copper ions as compared to ethanolic extract (table 4). The maximum percentage (%) increase in antibacterial activity of aqueous extract of *Curcuma longa* when used in conjunction with copper ions were found as 187.5%, 100%, 73.33%, 55.55% and 45.25% as compared to other extracts (table 5).

Table 2: Antibacterial activity of ethanolic and aqueous extracts of *Curcuma longa* and *Glycyrrhiza glabra*

Curcuma longa	Concentration (mg/ml)	Zone of inhibition (mm)		CD@5%
		Aqueous extract	Ethanolic extract	
	1	8±0.33	9±0.33	1.31
	2	12±0.33	13±0.33	NS
	3	15±0.33	15±0.33	NS
	4	18±0.33	22±0.00	0.93
	1	10±0.33	8±0.00	0.93
Glycyrrhiza glabra	2	13±0.00	12±0.33	NS
	3	17±0.00	15±0.33	0.93
	4	21±0.33	17±0.33	1.31

NS: Not significant; Extracts of *Curcuma longa*: 0.66; Concentration of *Curcuma longa*: 0.47; Extract-x-concentration of *Curcuma longa*: 0.94; Extracts of *Glycyrrhiza glabra*: 0.56; Concentration of *Glycyrrhiza glabra*: 0.39; Extract-x-concentration of *Glycyrrhiza glabra*: 0.79. All values are expressed as mean±standard deviation of triplicates.

Table 3: Antibacterial activity of copper metal ions against food spoilage bacteria

Concentration of copper ions (µ/ml)	Zone of inhibition (mm)	
	Copper ions	Distilled water
0.125	18±0.33	-
0.135	19±0.00	-
0.140	22±0.33	-
0.153	25±0.00	-

(-): No activity; All values are expressed as mean±standard deviation of triplicates.

Table 4: Synergistic antibacterial activity of plant extracts along with copper ions against food spoilage bacteria

Curcuma longa+copper ions	Concentration (%)	Concentration of Plant extract (µl)+copper ions (µl)	Zone of inhibition (mm)		CD@5%
			Aqueous extract	Ethanolic extract	
	10	45 P. E.+5 Co.	23±0.33	21±0.00	0.93
	20	40 P. E.+10 Co.	24±0.33	23±0.00	0.93
	30	35 P. E.+15 Co.	26±0.33	25±0.00	0.93
	40	30 P. E.+20 Co.	28±0.00	26±0.33	0.93
	50	25 P. E.+25 Co.	30±0.33	28±0.33	1.31
Glycyrrhiza glabra+copper ions	10	45 P. E.+5 Co.	22±0.33	21±0.00	0.93
	20	40 P. E.+10 Co.	24±0.00	23±0.33	NS
	30	35 P. E.+15 Co.	26±0.33	25±0.33	NS
	40	30 P. E.+20 Co.	28±0.00	26±0.33	0.93
	50	25 P. E.+25 Co.	30±0.00	28±0.33	0.93

P. E. = Plant extract; Co. = Copper metal ions, NS: Not significant; Extracts of *Curcuma longa*: 0.54; Concentration of *Curcuma longa*: 0.34; Extract-x-concentration of *Curcuma longa*: 0.76; Extracts of *Glycyrrhiza glabra*: 0.54; Concentration of *Glycyrrhiza glabra*: 0.34; Extract-x-concentration of *Glycyrrhiza glabra*: 0.76. All values are expressed as mean±standard deviation of triplicates.

Table 5: Percentage (%) increase in antibacterial activity of plant extracts in combination with copper ions

S. No.	% increase in antibacterial activity of plant extracts (%)			
	Curcuma longa		*Glycyrrhiza glabra*	
	Aqueous extract+copper ions	Ethanolic extract+copper ions	Aqueous extract+copper ions	Ethanolic extract+copper ions
1.	187.5	133.33	120	162.5
2.	100	76.92	84.61	91.66
3.	73.33	66.66	52.94	66.66
4.	55.55	18.18	33.33	52.94
5.	45.25	12.55	30.18	40.85

Molecular characterization of bacteria

16s rRNA sequencing characterized the isolate as *Paenibacillus popilliae* having 33% similarity to *Paenibacillus apiaries* species fig. 1.

Estimation of Curcumin content in ethanolic extract of *Curcuma longa*

The maximum inhibition of food spoilage bacteria *Paenibacillus popilliae* was obtained in case of ethanolic extract of *Curcuma longa*. Estimation of curcumin content in ethanolic extract of *Curcuma longa* was carried out by using HPTLC techniques. The curcumin (10.37%) compound was identified as the major compound in the *Curcuma longa* rhizome extract at 425 nm as shown in the fig. 2. Curcumin as an active phytochemical is present in ethanolic extract of *Curcuma longa*.

DISCUSSION

Naturally occurring compounds are responsible for antimicrobial activity of plant extracts. In the present study steroids were found to be present in aqueous and ethanolic extracts of *Glycyrrhiza glabra* and *Curcuma longa* and the same has also been reported by many workers [15-17].

Ethanolic extract of *Curcuma longa* and copper ions showed 22 mm and 25 mm zone of inhibition at the concentration of 4 mg/ml and 0.153 µl/ml, respectively. The results are in accordance with, as obtained by essential oil extracted from *Curcuma longa* which exhibit high antibacterial activity as reported by Prinitha *et al.* [24]. The effect of copper metal ions on the antibacterial activity of various extracts was determined. Copper metal ions were found to be the best elicitors, selected in the study, to enhance the antibacterial activity of used plant extracts [25].

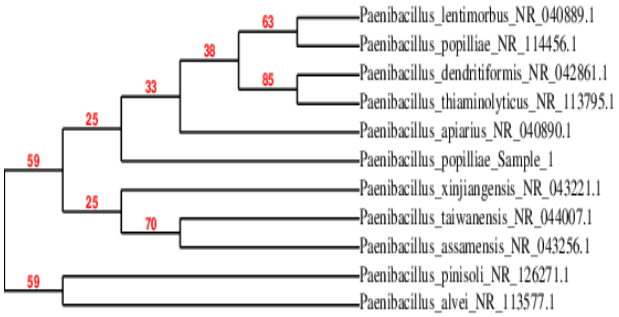

Fig. 1: Phylogenetic tree of bacterial isolate as indicated by sample 1 (*Paenibacillus Popilliae)*

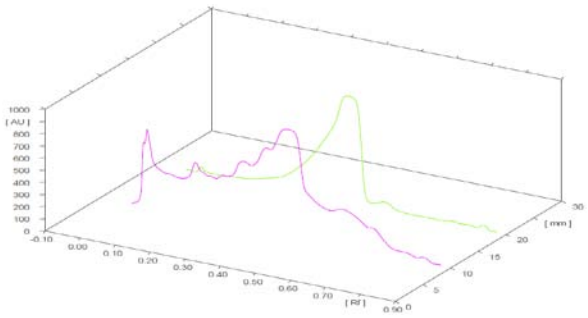

Fig. 2: Superimposed spectra of Curcumin; standard curve (in green color) and sample extract (in pink color).

Copper ions were found to enhance the antibacterial activity of plant extracts to a greater extent. Highest activity was obtained when a concentration of 40-50% of copper ions were used. However, higher concentrations were not found to any significant increase [22]. In the presence of copper metal ions (50%), aqueous extracts of *Curcuma longa* demonstrated maximum zone of inhibition i.e. 30±0.33 mm and 30±0.0 mm, respectively as compared to the ethanolic extracts of plant.

The degree of antibacterial property of *Curcuma longa* and *Glycyrrhiza glabra* when evaluated along with copper ions can be put in the following order: 50%>40%> 30%>20%>10%. 5 µl of copper ions increased in each concentration of plant extract showed increase in the potency of antibacterial property to inhibit the activity of food spoilage bacteria. But only slight changes were evaluated in the zone of inhibition with 60% concentration of copper ions.

The maximum percentage (%) increase in antibacterial activity in conjunction with copper ions was found as 187.5% in aqueous extract of *Curcuma longa* and least was 12.55% in ethanolic extract of *Curcuma longa*. @ a concentration of addition of metal ions with plant extracts results were obtained by Pandey and Singh [22] and its positive results against test organisms showed that at various concentrations it cannot increase the antibacterial activity of samples compare to 10%, 20% and 30%, almost zone of inhibition were equal and only slight changes were observed.

0.8 mg/ml concentration of aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* was found to be the MIC against *Paenibacillus popilliae,* whereas 0.94 µl/ml was the minimum inhibitory concentration of copper metal ion. The results are in accordance with Pandey and Singh, also reported that methanolic plant extract was subjected to get the MIC against test organisms and it was found to be 2.31 mg/ml for *E. coli*, 0.385 mg/ml for *S. aureus* and 0.010 mg/ml for *P. aeruginosa* [25].

Different active compounds of *Curcuma longa* such as curcumin, dimethoxy curcumin and bis-demethoxy curcumin were successfully detected directly from ethanolic extract of *Curcuma longa.* The quantitative determination of Curcumin content from ethanolic extract of *Curcuma longa* was found to be 10.37%. Paramasivam *et al.* also detected the presence of curcumin, dimethoxy curcumin and bis-demethoxy curcumin by HPTLC [23].

Table 6: Rf values of compounds detected by HPTLC

Peak	Start Rf	Start height	Max Rf	End Rf	Area %
1	-0.01	4.0	0.03	0.09	11.39
2	0.09	306.7	0.09	0.12	3.75
3	0.13	289.4	0.17	0.21	10.79
4	0.21	356.4	0.21	0.23	2.88
5	0.23	346.9	0.25	0.26	4.62
6	0.26	382.7	0.31	0.33	12.47
7	0.34	522.1	0.35	0.35	3.40
8	0.36	632.4	0.37	0.37	4.13
9	0.39	675.4	0.44	0.49	30.04
10	0.56	272.7	0.59	0.67	11.22
11	0.67	195.1	0.67	0.75	3.89
12	0.75	51.2	0.76	0.80	0.91
13	0.80	27.6	0.82	0.85	0.51

Rf obtained at 0.21, 0.36 and 0.39 are indicative of Bis-demethoxycurcumin, Demethoxycurcumin and Curcumin, respectively as suggested by Paramasivam *et al.* [23].

CONCLUSION

Herbal medicines are a valuable resource for health care and complementary health care systems. Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value that have to be discovered, though large numbers of plants are constantly being screened for their antimicrobial effects. These plants may prove to be a rich source of compounds with possible antimicrobial activities, but more pharmacological investigations are necessary [25].

Curcumin is an active compound present in the extract of *Curcuma longa* [4], whereas, Glycyrrhizic acid in *Glycyrrhiza glabra* is responsible for their antibacterial activity against food spoilage bacteria [26].

Addition of copper metal ion in plant extracts enhances the antibacterial properties against food spoilage bacteria. These natural compounds with copper ions are used in the food industry to overcome the problems of spoilage of food products. Shelf life of food can also be increased by adding these compounds to food products.

CONFLICT OF INTERESTS

The authors do not have any conflict of interest.

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