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Antifungal potential of fenugreek coriander, mint, spinach herbs extracts against *Aspergillus niger* and *Pseudomonas aeruginosa* phyto-pathogenic fungi

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ABSTRACT

We investigated the antimicrobial properties of leaf extracts of fenugreek (*Trigonella foenum-graecum*), coriander (*Coriandrum sativum*), mint (Mentha Piperita), and spinach (*Spinacia oleracea)* against the phytopathogenic fungi and bacteria i.e. *Aspergillus niger* and *Pseudomonas aeruginosa* respectively. The phytochemical analysis of these leaf extracts showed presence of total phenolics and flavonoids. GCMS analysis identified the antimicrobial molecules in leaf extracts as phenol, 2,4-Bis (1,1-dimethyl), phenol, gallic acid, cinnamic acid, kaempferol-3-o-rutinoside and other compounds. The main phenolics (Phenol, 2,4-Bis(1,1-dimethyl), Ovidin A, 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester, Cinnamic acid etc) showed antifungal activity at two concentrations (10mg/ml & 50mg/ml) and MIC was determined. More research is required to develop these phenolics as ecofreindly fungicides to control plants disease.

Key Words: *Aspergillus niger,* 1,2-Benzenedicarboxylic acid, 2,4-Bis(1,1-dimethyl), bis(2-ethylhexyl) ester, Cinnamic acid, *Coriandrum sativum*, GCMS, Mentha Piperita, Ovidin A, Phenol, *Pseudomonas aeruginosa, Spinacia oleracea*, *Trigonella foenum-graecum*.

INTRODUCTION

The applied aspect of allelopathy research are in agriculture and forestry to reduce environment pollution and increase crop production in sustainable agriculture. Bacteria and fungus ranked second only to insects to reduce crop yields and they cause 20% reduction in yields of major crops (1). Fungicides are used to control plant diseases but plant pathogens are developing resistance to them due to their continued use. Hence, there is persistent search for new eco-friendly and effective fungicides (14). Green plants are valuable sources of natural pesticides (3). Plant metabolites and plant based pesticides are better alternatives as they have minimal environmental impact than synthetic pesticides (19). This study aimed to evaluate the potential of fenugreek (*Trigonella foenum-graecum*), coriander (*Coriandrum sativum*), mint (*Mentha piperita*) and spinach (*Spinacia oleracea*), to control the pathogenic fungi *Aspergillus niger* and *Pseudomonas aeruginosa*.

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MATERIALS AND METHODS

Trigonella foenum-graecum, *Coriandrum sativum*, *Mentha piperita* and *Spinacia oleracea*, were grown in garden of Bioscience Department without fertilizers and pesticides. Their leaves of were collected 30 days after sowing, washed with tap water; surface sterilized with 2% sodium hypochlorite for 5 min and washed 2-3 times with sterile distilled water then shade dried at room temperature. Dried leaves were pulverized in electric blander to obtain fine powder. The powdered leaves were extracted with sterile distilled water at room temperature by soaking (10mg in 10ml of distilled water) Then extracts were filtrated through double layered muslin cloth and finally by Whatman filter paper No. 1. With distilled water 4 concentrations (5, 10, 15 and 20% W/V) of extracts were prepared. Extracts were stored at 4°C in pre-sterilized flasks until used. Pure cultures of both fungi *Aspergillus niger* and *Pseudomonas aeruginosa* were obtained from Biosciences Department, of our University. *Aspergillus niger* was maintained on PDA plate (Potato Dextrose Agar) at 28 ± 2°C for 48-72 h and *Pseudomonas aeruginosa* on NFb (Nitrogen Free Broth) agar plate at 28 ± 2°C for 24 h.

Antimicrobial activity assay:

We used Agar well diffusion method. A drop of culture suspension was placed in centre of nutrient agar plate and spread all over plate with sterile spreader. Three wells were made on the Nutrient agar medium containing plate with sterile cork borer and wells were filled with 100 µl of plant extract. Plates were placed in refrigerator for 30 min for diffusion of extract in the nutrient agar and then transferred to incubator at 37 °C for 24 h, plates were observed for zone of inhibition by measuring the colony diameter. A 100 % DMSO was used as control.

IC_{50} and MIC determination

To compare the allelopathic potential of leaf extracts, the half maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values of total leaf extracts obtained from the herbs were calculated by dose-response relationship.

Phytochemicals constituents

Total phenolics content : The total phenolics content of leaf extracts was determined using colorimetric Folin-Ciocalteu method (18). 1 ml leaf extract was mixed with 5 ml distilled water and 250 µl 1N folin-ciocalteau reagent. The mixture was covered and allowed to stand for 3 min at 25°C. In this mixture, 1 ml saturated Na_2CO_3 and 1 ml of distilled water were added. The mixture was incubated for 1 h. at 25°C for colour development and measured at 725 nm using a spectrophotometer. Standard graph was prepared by using different concentration of phenol crystals.

Total flavonoids content: Total flavonoid content was determined by aluminum chloride colorimetric assay (13). One ml leaf extract was added to 10 ml volumetric flask containing 4 ml distilled water, then 0.3 ml of 5% $NaNO_2$ was added, followed by 0.3 ml of 10% $AlCl_3$ in the above mixture. Just after 5 min incubation, 2 ml 1M NaOH was added

and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured at 510 nm against (prepared reagent) blank. Standard graph was prepared by using different concentration of gallic acid.

Gas Chromatography and Mass Spectroscopy Analysis

Analysis by GC-MS was performed using a Thermo Gc–Trace Ultra Ver: 5.0, Pyrolysis auto sampler interfaced to a Perkin Elmer Turbomass Gold equipped with a fused silica capillary column (J & W; DBI; 30m length x 0.25 mm id. film thickness 0.25µm). The fraction was pyrolysed at 610 °C and then introduced to the GC column. The transfer line was held at 280 °C and the source temperature was maintained at 180 °C and ionization energy was set at 70eV. Helium was employed as carrier gas (1 mL/m). The GC oven temperature was programmed: The column was held initially at 70 °C/ m (isothermal) and then increased by at 8 °C/m to 260°C/ m min-1 (isothermal). Qualitative identification of the different constituents was performed by composition of the relative retention times and mass spectra with those of authentic reference compounds by retention indices (RI) and mass spectra. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns.

Antimicrobial activity of main phenolic compounds identified in GCMS

The agar plate containing the appropriate medium was spread with the inoculums previously adjusted to the microbial densities cited above. Wells were made on the Nutrient agar medium containing plate with the help of sterile cork borer. Leaf extracts were dissolved in sterile DMSO at 10 mg mL^{-1} and 50 mg mL^{-1} of phenolic compounds identified in GCMS analysis. Wells were filled with 100 µl of above solution and placed in refrigerator for 30 min and then transferred to incubator at 37 °C for 24 h. Antimicrobial activity was calculated by measuring the diameter of inhibition zone. The experiment was done by measuring the diameter of inhibition zone and 100% DMSO was used as control.

Statistical analysis

All experiments were performed three times independently, with measurements taken from three (for all other measurements) different plants for each treatment in each three independent experiments. One-way analysis of variance (ANOVA) test was used to analyze all data and mean (of three independent experiments) were compared at 5 % level of significance.

RESULTS AND DISCUSSION

The plants synthesize the aromatic secondary metabolites [phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins (7). Such compounds were found present in our test plants. Phytochemical constituents of the herbs were qualitative analyzed and result showed presence of different phytochemical constituents as alkaloid, saponins, cardiac glycosides, steroids, phenols and flavonoides (Table 1). Quantitative analysis of phytochemicals constitutes as total phenolic and total

Table 1. Qualitative analysis of phytochemicals in fenugreek, coriander, mint, spinach

Phytochemicals	*Trigonella foenum-graecum*	*Coriandrum sativum*	*Mentha Piperita*	*Spinacia oleracea*
Alkaloid	+	+	+	+
Saponins	+	+	+	+
cardiac glycosides	-	-	-	-
Steroids	-	-	-	+
Phenols	+	+	+	+
Flavonoides	+	+	+	+

+: Present and - : Absent (It was only analyzed for its presence and absence , so concentration is not known)

flavonoid contents was done in all donor plants. Total phenolics content was highest in *Mentha piperita* (1.87 mg/g), while total flavonoids content was highest in *Spinacia oleracea* (10.23 mg/g) (Table 2).

The carvacrol, eugenol, and thymol, were highly active against the pathogen. These compounds are antimicrobial and serves as plant defense mechanisms against pathogenic microorganisms (8). Water extract of *Coriandrum sativum* showed the highest antibacterial activity (6 mm) against *P. aeruginose* and *Spinacia oleracea* (3 mm) against *Aspergilus niger* than others. No activity was observed in *Trigonella foenum-graecum* and *Mentha piperita*, while methanol extract of *Trigonella foenum-graecum* (4 mm) showed highest antimicrobial activity against *Aspergilus niger* and (6 mm) against *P. aeruginose* compared to other herbs.

Ethyl extract of *Mentha piperita* showed maximum inhibition (9 mm) against *Aspergilus niger* followed by *Trigonella foenum-graecum* (8 mm) against *P. aeruginosa* than others. Petroleum extract of *Coriandrum sativum* showed largest zone of inhibition (9 mm) against *Aspergilus niger*, while *Spinacia oleracea* showed (5 mm) against *P. aeruginosa* than others (Table 3). The antibacterial activities in the aqueous extracts of donor plants may be due to the presence of phenolic compounds, the antimicrobial capacity of phenolic compounds is well-known (15,16). They act by causing the leakage of cytoplasmic constituents such as protein, glutamate or potassium and phosphate from bacteria and fungi due to disruption of cell wall peptidoglycan layer or damage of cell membrane. Also, extracts may be more active than isolated constituents, because bioactivity of individual component is changed in presence of other compounds present in extract (4).

The use of various solvents (methanol, ethanol, chloroform, ethyl acetate, acetone and petroleum ether) of different polarities in extraction methods greatly help in extraction of allelochemicals from the plants (6). The plants extracts showing highest zone of inhibition in agar well diffusion method were selected further for minimum inhibitory concentration (MIC) method. In this method plant extract with different concentrations ranging from 100 mg/ml-0.1953 mg/ml was prepared by two folds double dilution method, depending on the appearance of red color of 2,3,5-triphenyl tetrazolium dye in appendroff tube. The MFO of petroleum ether extract of *Aspergilus niger* of all four test donor plants were 1.43-2.67 mg/ml and MIC of ethanol extract of *Pseudomonas aeruginosa* of all four donor plants were 1.78- 3.12 mg/ml. While Half maximal inhibitory concentration (IC_{50})

Table 2. Quantitative analysis of phytochemicals in fenugreek, coriander, mint, spinach

Phytochemicals	*Trigonella foenum-graecum*	*Coriandrum sativum*	*Mentha Piperita*	*Spinacia oleracea*
Total Phenolic content (mg/g of extract)	0.83 ± 0.01^{d}	1.12 ± 0.12^{c}	1.87 ± 0.11^{a}	1.47 ± 0.12^{b}
Total Flavonoid content (mg/g of extract)	7.89 ± 0.02^{c}	3.11 ± 0.02^{d}	8.76 ± 0.01^{b}	10.23 ± 0.11^{a}

For each Parameter, values in columns followed by the same letter are not significantly different at ($P\leq0.05$).

Table 3. Effects of various extracts of herbs on Inhibition Zone (mm) of phyto-pathogenic microorganisms

Test organisms	*T. foenumgraecum*	*C. sativum*	*M. Piperita*	*S. oleracea*
		Aqueous extract		
Aspergilus niger	00	02 ± 0.01^{d}	00	03 ± 0.04^{bc}
Pseudomonas aeruginosa	05 ± 0.02^{bc}	06 ± 0.03^{b}	05 ± 0.03^{c}	04 ± 0.01^{ab}
		Methanol extract		
Aspergilus niger	04 ± 0.02^{cd}	02 ± 0.01^{d}	01 ± 0.02^{de}	03 ± 0.02^{bc}
Pseudomonas aeruginosa	06 ± 0.02^{b}	04 ± 0.03^{c}	10 ± 0.04^{a}	05 ± 0.01^{a}
		Ethyl acetate extract		
Aspergilus niger	02 ± 0.01^{e}	00	09 ± 0.01^{ab}	05 ± 0.02^{a}
Pseudomonas aeruginosa	08 ± 0.02^{a}	06 ± 0.03^{b}	02 ± 0.04^{cd}	05 ± 0.02^{a}
		Petroleum ether extract		
Aspergilus niger	01 ± 0.01^{ef}	09 ± 0.01^{a}	05 ± 0.02^{c}	03 ± 0.03^{bc}
Pseudomonas aeruginosa	04 ± 0.02^{cd}	04 ± 0.03^{c}	01 ± 0.01^{de}	05 ± 0.01^{a}

Note: Each value is expressed as mean (n = 3). For each Parameter, values in columns followed by the same letter are not significantly different at ($P\leq0.05$).

Table 4. Half maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values of total extracts obtained from extract of fenugreek, coriander, mint, and spinach against the plant pathogenic microorganisms

Test organism	Minimum Inhibition Concentration (MIC) (mg/ml)			
	T. foenum-graecum	*C. sativum*	*M. Piperita*	*S. oleracea*
Aspergilus niger	1.43 ± 0.01^{d}	2.34 ± 0.01^{b}	2.67 ± 0.02^{a}	1.87 ± 0.02^{c}
Pseudomonas aeruginosa	1.78 ± 0.02^{d}	2.66 ± 0.01^{b}	3.12 ± 0.01^{a}	1.99 ± 0.01^{c}
	Half maximal inhibitory concentration (IC_{50}) (mg/ml)			
Aspergilus niger	0.56 ± 0.01^{b}	0.39 ± 0.02^{c}	0.72 ± 0.01^{a}	0.24 ± 0.01^{d}
Pseudomonas aeruginosa	0.62 ± 0.02^{b}	0.41 ± 0.01^{c}	0.83 ± 0.01^{a}	0.31 ± 0.01^{d}

For each Parameter, values in columns followed by the same letter are not significantly different at ($P\leq0.05$).

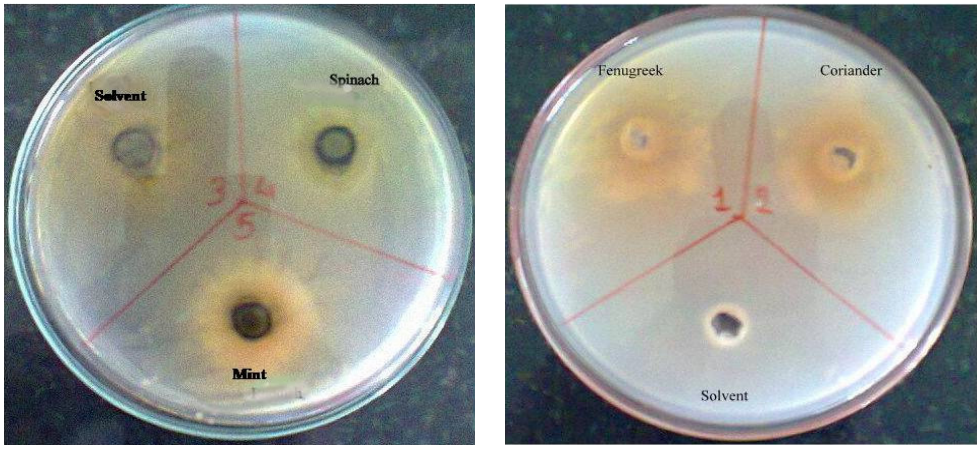

Figure 1. Antibacterial activities of ethanol extract of fenugreek, coriander, mint, spinach against bacteria *Pseudomonas aeruginosa.*

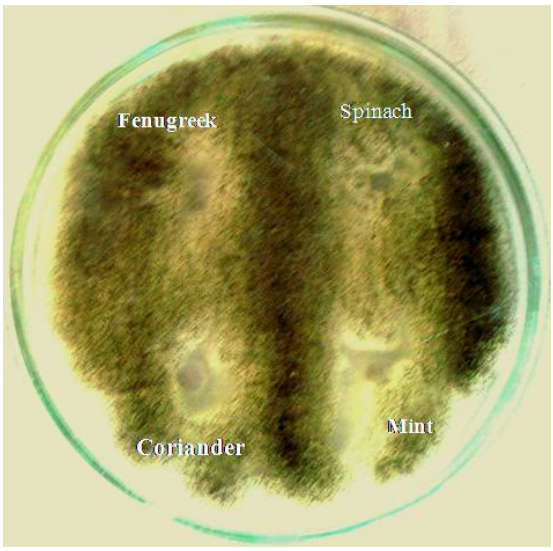

Figure 2. Antifungal activities of petroleum ether extract of fenugreek, coriander, mint, spinach against fungus *Aspergilus niger.*

of petroleum ether extract of *Aspergilus niger* of all four herbs *Trigonella foenum-graecum*, *Coriandrum sativum*, Mentha Piperita, and *Spinacia oleracea* were 0.24-0.72 mg/ml and that of *Pseudomonas aeruginosa* by ethanol extract of all four herbs were 0.31-0.83 mg/ml (Table 4). MIC of *Pseudomonas aeruginosa* by ethanol extract of all four herbs fenugreek, coriander, mint, and spinach were shown in Fig.1 and of *Aspergilus niger* by petroleum ether extract was shown in Fig. 2.

This study indicated that the ethanol and petroleum extracts were very effective against phyto-pathogenic bacteria *P. aeruginose*. Ethanol and petroleum has high dielectric constant and cohesive energy, than other solvents, which provides strong bonding between solvent molecules and polar compounds from the solutes, causing their dissolution (21). Moreover, the results revealed that ethanol caused damages to cell walls and the cell membranes of microorganisms. The presence of hydroxyl group in phenolic compound might influence their antimicrobial effectiveness by binding to the active site of enzymes, form hydrogen bonds with enzymes and alter their metabolism and lipid solubility. The degree of steric hindrance of phenolic compounds might determine their antimicrobial activity (5).

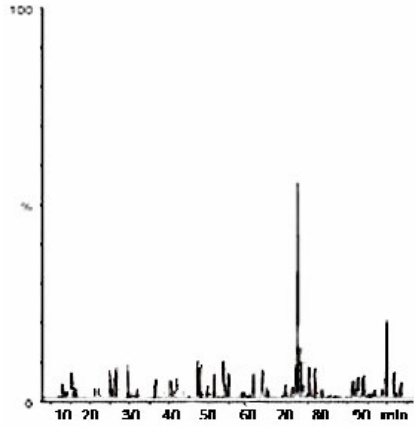

Figure 3. GC-MS analysis of bound flavonoid extract of spinach (*Spinacia oleracea*) leaves extract.

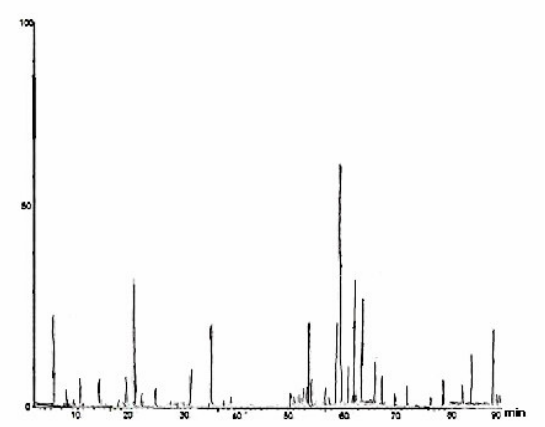

Figure 4. GC-MS analysis of phenolic extract of mint (*Mentha Piperita*) leaves extract.

It is not possible for single allelochemical with low released quantity to reach the effective concentration to reduce the bacterial and fungal population. *Mentha Piperita* having maximum phenolic and *Spinacia oleracea* with maximum flavonoid were used for GCMS analysis to identify the various molecule in the extract with antibacterial and antifungal activity. GCMS analysis *Spinacia oleracea* leaf extract, identified 11 known and two unknown molecules (Phenol, Tetradecane, Naphthalene, Phenol,2,4-Bis(1,1-dimethyl) 4-hydroxybenzoicacidmethylester etc) (Table 5), whereas, in *Mentha piperita* 13 known molecules (3,4-dihydroxybenzoic acid, Vicenin-2, Gallic acid, Caffeic acid, Chlorogenic acid etc) were identified (Table 6). These molecules are well known antimicrobials. However, every plant contains numerous secondary metabolites, which jointly inhibits the target organisms (20). Mint leaf extract antimicrobial activity is due to the phenolics like rosmarinic and caffeic acid (2), which supported our results. GCMS analysis showed ellagic acid, pyrogallic acid, gallic acid and catechin like compounds released simultaneously, they exerts synergistic inhibition action on the growth of bacteria and fungus (12). The common herbs like cinnabar, garlic, ginger, basil, mint and sage containing caffeic acid (a representative of wide group of phenylpropane-derived

Table 5. Important compounds identified in the GC-MS analysis of bound flavonoid extract of *Spinacia oleracea* leaves extract

Peak	Retention Time	Area %	Compound Name	Mol. weight
1	8.12	2.42	Phenol	94
2	12.24	1.29	Tetradecane	198
3	19.33	0.37	Naphthalene	128
4	21.26	1.58	Phenol,2,4-Bis(1,1-dimethyl)	206
5	23.49	5.11	4-hydroxybenzoicacidmethylester	206
6	33.21	2.34	Kaempferol 4'-rutinoside	152
7	37.44	2.58	Kaempferol-3-o-rutinoside	594
8	46.28	1.42	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	180
9	49.23	0.87	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	180
10	68.58	4.12	Ovidin A	252
11	71.54	2.76	Tetradecane	198
12	83.34	0.87	Unidentified	NA
13.	90.23	1.63	unidentified	NA

Table 6. Important compounds identified in the GC-MS analysis of phenolic extract of *Mentha piperita* leaves extract

Peak	Retention Time	Area %	Compound Name	Mol. weight
1	5.12	4.25	3,4-dihydroxybenzoic acid	154
2	12.21	1.26	Vicenin-2	594
3	20.11	0.91	Gallic acid	170
4	23.43	0.34	Chlorogenic acid	354
5	32.54	2.27	Caffeic acid	180
6	34.49	0.53	Tridecanoic acid	214
7	55.14	1.31	Coumarins	146
8	59.31	1.44	4-(3-Benzyl-2,4-dioxo-1-phenethyl-1,2,3,4-tetrahydro-5-pyrimidinylmethyl)benzamide	435
9	63.51	2.21	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	390
10	66.48	0.73	2-(2-hydroxy-2-pchlorophenylethyl)-3,5,6-rimethylpyrazine	276
11	72.17	3.45	Diisooctyl-phthalate	390
12	79.18	1.52	Cinnamic acid	148
13.	85.37	1.16	Rosmarinic acid	360

compounds) are effective against fungi (9). The main phenolics identified in the GCMS analysis of *Mentha Piperita* and *Spinacia oleracea* were used for bioassay against the *Aspergilus niger a*nd *Pseudomonas aeruginosa*, showed that growth of phytopathogenic microorganism was inhibited by such molecule and inhibition was concentration-dependent.

The zones of inhibition of some main phenolics identified in the herbal matter were assayed against the test microorganism. The zone of inhibition obtained in the two herbal extracts indicates a higher inhibition at a concentration of 50 mg/ml than 10 mg/ml (Table 7). Among them, few molecule were more effective against bacteria *Pseudomonas aeruginosa* and few were against the fungus *Aspergilus niger*. This agrees with Furneri

Table 7. Antimicrobial activity of the main phenolic compounds identified in fenugreek, coriander, mint, spinach

Phenolic compound	Zones of inhibition (mm)			
	Aspergilus niger		*Pseudomonas aeruginosa*	
	10mg/ml	50mg/ml	10mg/ml	50mg/ml
Phenol	00	0.43±0.03^{k}	0.41±0.01^{hi}	1.12±0.01^{gh}
Naphthalene	0.45±0.01^{f}	0.67±0.02^{gh}	0.86±0.012^{a}	1.21±0.02^{g}
Phenol,2,4-Bis(1,1-dimethyl)	0.51±0.02^{e}	0.86±0.01^{f}	0.82±0.02^{ab}	1.48±0.02^{c}
Kaempferol 4'-rutinoside	00	00	0.46±0.01^{h}	1.32±0.01^{ef}
5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	0.63±0.01^{abc}	1.35±0.03^{b}	0.57±0.03^{def}	1.12±0.02^{gh}
Ovidin A	0.31±0.01^{h}	0.69±0.02^{g}	0.65±0.02^{cd}	1.76±0.03^{a}
3,4-dihydroxybenzoic acid	0.22±0.02^{i}	0.62±0.02^{i}	0.59±0.01^{de}	1.12±0.02^{gh}
Gallic acid	00	00	0.54±0.01^{efg}	1.37±0.01^{de}
4-(3-Benzyl-2,4-dioxo-1-phenethyl-1,2,3,4-tetrahydro-5-pyrimidinylmethyl) benzamide	0.54±0.03^{d}	1.02±0.02^{d}	0.35±0.02^{j}	0.89±0.01^{j}
1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester	0.64±0.01^{ab}	1.25±0.01^{bc}	0.23±0.02^{k}	1.11±0.02^{ghi}
2-(2-hydroxy-2-pchlorophenylethyl)-3,5,6-trimethylpyrazine	0.42±0.02^{g}	1.43±0.02^{a}	0.46±0.01^{h}	1.62±0.012^{b}
Diisooctyl-phthalate	00	0.45±0.01^{j}	00	0.51±0.01^{k}
Cinnamic acid	0.65±0.01^{a}	0.98±0.02^{de}	0.69±0.02^{c}	1.38±0.01^{d}

Note: Each value is expressed as mean (n = 3). For each Parameter, values in columns followed by the same letter are not significantly different at (P≤0.05).

et al. (10) that the activity of phenolic compounds is concentration dependent. The distribution of phenolic compounds in plants depends on several factors, while, the contents of individual and total phenolic compounds in plants are influenced by genetic factors, environmental conditions such as light, ripeness and post-harvest processing (11). Tropical vegetable extracts showed antimicrobial activities against *B. cereus, E. coli, Salmonella* spp, *Pseudomonas aeruginosa, S. aereus, Shigella* spp*, Enterobacter, C. sporogenes, B. subtilis, P. Vulgaris* (17).

CONCLUSIONS

The use of fenugreek, spinach, coriander and mint extracts may help in controlling the infection of phyto-pathogenic microorganisms (*Aspergillus niger* and *Pseudomonas aeruginosa*) and reduce the use of fungicides. However, before use of allelochemicals as natural fungicides, laboratory and field studies are needed to study their allelochmeicals interactions with various biological and physicochemical properties of soil, movement of allelochemicals, mode of action, selectivity etc and the impact of use of allelochemicals from agronomic and environmental point of view.

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