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Short Communication: Identification of phytochemical constituents of *Syzygium aromaticum* L. using gas chromatography coupled with mass spectrometry and evaluation of antimicrobial activity

ZOUBIDA BENMAKHLOUF[1,2,♥], OUAFA BENSERRADJ[1,2] , RABAH KELLAB[1,2]

[1]Department of Science of Nature and Life Science, University Center of Abd Alhafid Boussouf, Mila, Algeria
[2]Laboratory of Natural Sciences and Materials, University Center of Abd Alhafid Boussouf, Mila, 43000, Algeria. Tel: +213-657582522,
[♥]email: z.benmakhlouf@centre-univ-mila.dz

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Abstract. *Benmakhlouf Z, Benserradj O, Kellab R. 2022. Short Communication: Identification of phytochemical constituents of* Syzygium aromaticum *L. using gas chromatography coupled with mass spectrometry and evaluation of antimicrobial activity. Biodiversitas 23: 2586-2593. Syzygium aromaticum* L. is a traditional spice that has various pharmacological activities attributed to its content of bioactive molecules. The purpose of this work is to evaluate phytochemical constituents of *S. aromaticum* L. extract using gas chromatography coupled spectrometry mass (CG/MS). The number detected of phenolic compounds was 45, which the most abundant being eugenol (54.63%), followed by Phenol,2-metoxy-4-(2-propenyl)-, acetate (eugenol acetate) (21.57%) and caryophyllene (16.71%). In addition, the antimicrobial activity was screened by the paper disc diffusion method for three bacterial strains, (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella* sp. and *Staphylococcus aureus* 6538) and by the direct contact method on three fungal strains (*Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*). The results showed that extract presents an inhibitory activity with all the strains tested. *Salmonella* sp. is very sensitive (++) to the stock solution (100 mg/mL), sensitive (+) to dilutions (50 and 25) mg/mL with inhibition diameters of (16, 12, 10) mm respectively. The other bacterial strains are sensitive (+) only to the stock solution. The results of our experiment confirm that fungi strains: *A. niger*, *A. flavus* and *C. albicans* show a remarkable antifungal activity at 100 mg/mL. The result indicated that the extract obtained from dried buds of *S. aromaticum* could be considered an agent for antibacterial and antifungal in the pharmaceutical field.

Keywords: Antimicrobial, antifungal, CG/MS, phenolic compounds, *Syzygium aromaticum*

INTRODUCTION

Throughout the world, infectious diseases constitute a real public health problem. Most of the time, bacteria and fungi have the potential to cause several infectious human diseases. As antibiotics and antifungal drugs are increasingly used and misused, microbial strains become resistant, and difficult to deal with many infection cases. (David et al. 2019; Hiwandika et al. 2021).

Therefore, it has become extremely important to develop an alternative from extracts of medicinal plants to strengthen the immune system or inhibit the growth of unwanted bacteria. Traditionally, man has widely used the crude extracts of different plant organs such as root, stem, flower, fruit and twigs to treat some diseases (Khan et al. 2013). Unfortunately, so far only 5% of the 750,000 plants that exist on earth have been tested for their biological activity (Raman 2017). Spices, herbs, plant extracts and their phytoconstituents: vitamins C, E, carotenoids and phenolic compounds, flavonoids, tannins, and flavone (Kardong et al. 2013; Sokamte et al. 2019) have been reported for their anti-inflammatory, antidiarrheal, antimicrobial, antioxidant, and insecticidal activities (Li et al. 2019). Depending on their nature, whether they are dry, fresh, or extracted forms, different types of spices exhibit antimicrobial activity (Saeed et al. 2016). Thus, the antimicrobial activity of certain plant species has been widely sought after.

Cloves, aromatic dried flower buds of *S. aromaticum* L. of a tree in the family Myrtaceae native to the small islands of Maluku in Eastern Indonesia (Kamatou et al. 2012) have been used as a traditional spice in many foods. Also, clove essential oil is used in traditional Chinese medicine and Western herbalism to treat many diseases such as gastric disorders (stomach pain), soothe dental pain due to its antiseptic properties (Nassan et al. 2015) and treat human dermatoses (Han and Parker 2017). It is also able to relieve different microorganisms such as cholera, malaria and tuberculosis and increase blood circulation (Bhowmik et al. 2017). In addition, clove is known for its many pharmacological activities such as antioxidant, anticancer, antiviral, anti-inflammatory, antifungal, antibacterial and immunomodulatory activity (Batiha 2020). On account of the presence of many phytochemicals as follows: sesquiterpenes, monoterpenes, hydrocarbon and other phenolic compounds (Radünz et al. 2018), monoterpenes are the dominant components of clove extracts of which eugenol ($C_{10}H_{12}O_2$) is the main bioactive molecular compound (Mohamed et al. 2018). Note that this molecule has been extracted from various other plants such as

lemongrass, tulsi, and cinnamon (Mak et al. 2019). The term eugenol is derived from the species name *Eugenia caryophyllata* L. (*Syzygium aromaticum* L.) which contains too high a level of eugenol (45-90%) (Kamatou et al. 2012).

Pharmacologically, eugenol possesses an antiviral, antifungal, and antibacterial effect against several pathogenic microorganisms (Banerjee et al. 2020; Sugihartini et al. 2019).

Considering the importance of clove as an antimicrobial agent, the present study was designed to identify the phytochemical constituents of *S. aromaticum* L extract using gas chromatography coupled spectrometry mass (CG/MS) with the elucidation of its main components attributed to its recognized antibacterial and antifungal properties.

MATERIALS AND METHODS

Plant material

Dried clove buds (*S. aromaticum* L.) were purchased from a local market.

Preparation of extracts

Samples are cleaned and then left to dry at room temperature away from light and ground using a grinder (Moulinex) until powders are obtained. To obtain the plant extract, we used the soxhlet. The cartridge containing 30g of clove powder is inserted into the extractor and placed on a boiler containing 300 ml of organic solvent (Hexane). above the extractor, a condenser is placed, serving to liquefy the vapors of Hexane (solvent). Hexane drips back down to the extractor containing the sample. The temperature of the apparatus was applied at 70-80° C., 2 days later, the separation of the solvent from the extract was carried out using Rotavapor.

Phytochemical analysis (CG/SM determination of phenolic compounds)

Phytochemical compounds were identified using gas chromatography coupled with mass spectrometry (CG/MS) of type Shimadzu QP2010 type EL 70 ev quadrupole equipped with column: OV 1701 (25m), at the Educational Laboratory of the SNV faculty of the University of Jijel Algeria. The GC specifications were as follows: column oven temperature: 70 °C, injector temperature: 250 °C, injection mode: Split, Split Ratio: 20, Flow control mode: Linear velocity 35.9 cm/sec, Total Flow 18 ml/min, Column Flow: 0. 80 mL/min, Purge flow 1.2 ml/min, Pressure 31.7 KPa, Carrier Gas: Helium 99.99 % purity, volume injected: 1µl. Oven temperature program: rate temperature (°C) hold time (min) (-, 70°C, 3 min), (3, 130°C, 5min), (5, 240°C, 3min), column: OV 1701 (25m). The MS specifications were as follows: Ion source temp: 200°C, interface temp: 250°C, solvent cut time: 2 min, Detector Gain Mode: Relative, Detector Gain: 0.00 KV, Threshold: 1000, ACQ mode: scan, Interval: 0.5 sec, Scan Speed: 666, Start m/z: 40, End m/z: 350, start time: 2 min, End time: 53 min (Hemalatha et al. 2016).

Study of antimicrobial activity

Tests for antibacterial activity

Bacterial strains

This study is carried out on reference strains belonging to the American Type Culture Collection (ATCC). Three bacterial strains, 2 Gram-negative (*P. aeruginosa* ATCC 27853, *Salmonella* sp.) and 1 Gram-positive (*S. aureus* ATCC 6538).

Evaluation of the antibacterial activity expressed by diameter inhibition in millimeter (mm)

Antimicrobial activity of clove extract was evaluated by the paper disc diffusion method (Bachiri 2016). In this method, sterilized paper discs were used Whatman n°3, 6 mm in diameter, impregnated with 5 µl for each concentration (100, 75, 50 and 25) mg/mL of the clove extract and deposited on the surface of a medium agar (Muller Hinton "AMH" agar), previously inoculated with a surface swab with the bacterial suspension. Sterile dimethyl sulfoxide (DMSO) served as a negative control. After incubation for 24 h at 37°C. Sensitivity to the extract and its different dilutions were classified according to the diameter of the inhibition halos as follows: non-sensitive ( ) for diameter less than 8 mm; sensitive (+) for diameter 9 - 14 mm; very sensitive (++) for diameter 15 -19 mm and extremely sensitive (+++) for diameter greater than 20 mm.

Tests for antifungal activity

Fungal strains. For this study, two fungal strains from the soil (*A. niger* and *A. flavus*) and a yeast: *C. albicans* were used.

Evaluation of the antifungal activity. Inoculation consists of taking agar cylinders 6mm in diameter from a young mushroom culture (7 days) on PDA agar and placing them on a sabouraud medium mixed with the different concentrations of extract (100, 75, 50 and 25) mg/mL. The whole was incubated at 28°C for 7 days.

RESULTS AND DISCUSSION

Organoleptic properties of extract

The organoleptic properties of the extract are shown in Table 1.

Phytochemical analysis

Phytochemical analysis of clove extract

Analysis showed the richness of clove extract in phenolic compounds. The obtained chromatogram profile is shown in Figure 1.

Based on the retention time, the sample peak area and the standard peak area, several compounds were identified. 45 phenolic compounds were identified in *S. aromaticum* L. extract of which the most abundant is Eugenol (54.63%) followed by Phenol,2-metoxy-4-(2-propenyl)-, acetate (Eugenol acetate) (21.57%) and caryophyllene (16.71%) (Table 2).

Antimicrobial activity

Antibacterial activity

After 24 hours of incubation at 37°C, the zones of inhibition observed around the discs impregnated with the extract for 100 mg/mL and their different dilutions studied were measured (Figure 2, Table 3).

Figure 2 shows that the stock solution of *S. aromaticum* L. extract at 100 mg/mL records an antibacterial effect with all the strains tested, especially against *Salmonella* sp. compared to other bacterial strains and the negative control (DMSO). Noting that with the dilutions of the extract, the activity against *salmonella* sp is present up to dilution (D3).

According to the scale given by Panda et al. (2016) *Salmonella* sp is very sensitive (++) to the stock solution, sensitive (+) to dilutions (1 and 2) and non-sensitive (-) to dilutions (3 and 4) with inhibition diameters of (16, 12, 10, 8 and 7) mm respectively. The other bacterial strains are sensitive (+) to the stock solution but not sensitive (-) to all dilutions of the extract (Table 3).

Antifungal activity

After 5 days of incubation of *A. niger* and *A. flavus* and 24 hours of *C. albicans* at room temperature, the results of the antifungal activity show that the two strains *A. niger* and *A. flavus* have the same sensitivity to the extract of *S. aromaticum* L. They are inhibited at the same concentration (stock solution = 100 mg/mL). On the other hand, good growth is observed with the different dilutions in the two strains. The same extract with the same dilutions shows an antifungal effect against *C. albicans* species (Figure 3, Table 4).

Table 1. Organoleptic properties of the extract

Extract	Color	Odor	Appearance
S. aromaticum	Yellowish	Very intense and strong	Slightly liquid, viscous

Table 3. Diameters of the inhibition zone of *Syzygium aromaticum* L. extract (100 mg/mL) and its different dilutions

Extract	Bacterium		
	***Salmonella* sp.**	***P. aerginosa***	***S. aureus***
SS	16	9.5	9
D1	12	7	7.5
D2	10	6	6.5
D3	8	6	6
D4	7	6	6

Note: SS: stock solution: 100 mg/mL; D1: 50 mg/mL; D2: 25 mg/mL; D3: 12.5 mg/mL; D4: 6.25 mg/mL

Table 4. Sensitivity of fungal strains to *S. aromaticum* extract at different concentrations

Fungal strains	Extract concentrations			
	SS	**D1**	**D2**	**D3**
A. flavus	-	+	++	+++
C. albicans	-	-	-	-

Note: (-) lack of growth; (+) presence of growth. SS: stock solution: 100 mg/mL; D1: 50 mg/mL; D2: 25 mg/mL; D3: 12.5 mg/mL

Discussion

In modern scientific studies, cloves extracted with different methods are studied for their medicinal properties. Extraction with soxhlet allows us to have a yellowish extract, with a very intense and strong odor with a slightly liquid, viscous aspect. According to Sugihartini et al. (2019), these organoleptic properties are attributed to the presence of eugenol. Our results agree with those of Banerjee et al. (2020), who report that eugenol is a volatile bioactive constituent that is characterized by a strong odor, intense flavor, poorly soluble in water and its color changes from colorless to light yellow.

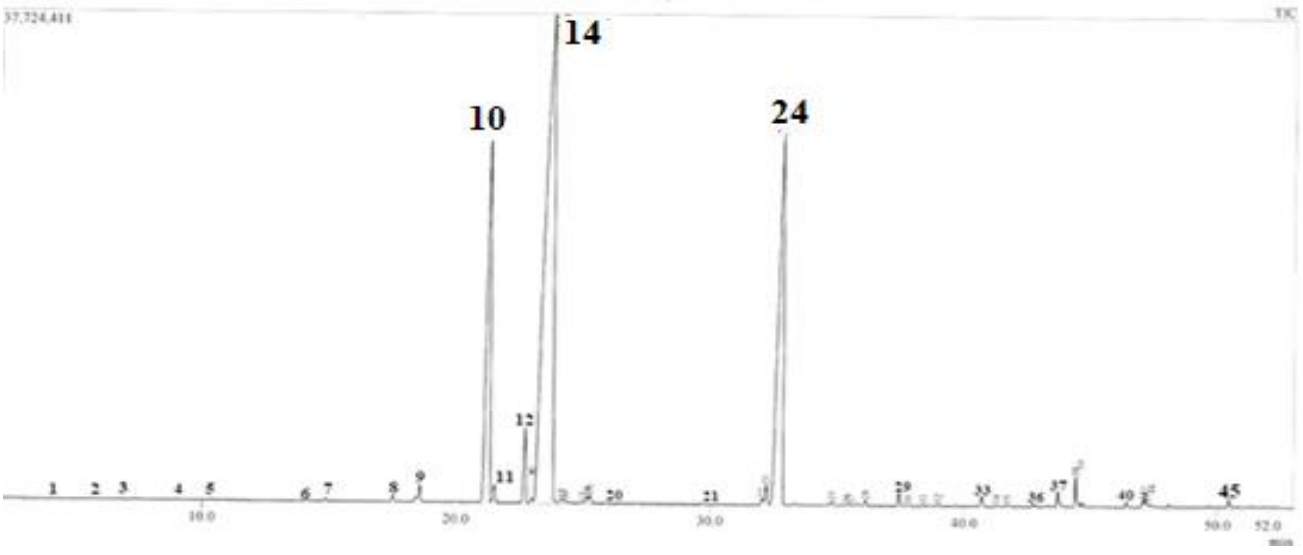

Figure 1. GC/MS chromatogram profile of clove extract

Table 2. Phenolic compounds of clove extract using CG/MS at the Educational Laboratory of the SNV faculty of the University of Jijel, Algeria

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	4.106	93281	0.01	40279	0.03	2- Heptanone	43.00
2	5.844	116759	0.01	39850	0.03	Cyclobutane,1,2,bis(1-methylethenyl-,trans-	68.05
3	7.452	366026	0.02	109227	0.009	Acetic acid, Sec-octyl ester	43.00
4	9.046	128620	0.01	42315	0.04	Geranyl nitrile	69.05
5	10.411	305906	0.02	84776	0.07	2-Nonanone	58.00
6	14.137	460644	0.03	106184	0.09	Acetic acid, phenylmethyl ester	108.00
7	14.867	935915	0.06	201633	0.17	Benzoic acid,2-hydroxy-,methyl ester	120.00
8	17.526	2382930	0.14	504211	0.43	alpha-Cubebene	161.10
9	18.584	6591851	0.39	1267388	1.08	Copaene	161.10
10	21.323	284206042	**16.71**	**27958607**	**23.83**	**Caryophyllene**	93.05
11	21.510	7546755	0.44	1370967	1.17	3-Allyl-6-methoxyphenol	164.05
12	22.716	30649908	1.80	5695197	4.85	alpha-Caryophyllene	93.05
13	22.988	2120689	0.12	373597	0.32	3-Allyl-6-methoxyphenol	164.05
14	23.799	929295417	**54.63**	**37576655**	**32.03**	**Eugenol**	93.05
15	24.185	2562490	0.15	326604	0.28	1H-cyclopenta(1,3)cyclopropa(1,2)benzane,octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,(3as-(3a.alpha.,3b))	164.05
16	24.326	1088578	0.06	220779	0.19	1H-Cyclopropa(a)naphtalene,decahydro-1,1,3a-trimethyl-7-methylene-,(1aS-(1a.alpha.,3a.alpha.,7a.beta.,7b.alpha)	164.05
17	25.017	522185	0.03	927992	0.08	Naphthalene1,2,3,4,4a,5,6,8a octahydro-7-methyl-4-methylene-1-(1-methylethyl)-(1.alpha-,4a.beta.,8a.alpha)	161.10
18	25.171	2966725	0.17	536630	0.46	alpha-Farnesene	105.00
19	25.296	3771132	0.22	651067	0.55	Naphthalene,1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-ethylethyl)-,(1S-(1-alpha.,4a.beta.,8a.alpha)-	161.10
20	25.930	593548	0.03	99763	0.09	Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	93.05
21	29.742	573635	0.03	82575	0.07	Benzaldehyde,3-hydroxy-4-methoxy	161.10
22	32.065	3164059	0.19	539533	0.46	Phenol,2-methoxy-4-(2-propenyl)-,acetate	119.05
23	32.22	8958600	0.53	1525312	1.30	Caryophyllene oxide	151.00
24	32.823	367001507	**21.057**	**27847280**	**23.74**	**Phenol,2-methoxy-4-(2-propenyl)-,acetate**	164.05
25	34.816	1372575	0.08	303761	0.26	Longipinocarveol,trans-	136.05
26	35.382	58096	0.03	109283	0.09	1H-Indene,2,3-dihydro-1,1,3-trimethyl-3-phenyl-	121.00
27	35.524	424378	0.02	119243	0.10	1,3,6,10-Cyclotetradecatetraene,3,7,11-trimethyl-14-(1-methylethyl)-,(S-(E,Z,E,E))	41.05
28	36.130	1943056	0.11	442639	0.38	Androstan-17-one,3-ethyl-3-hydroxy-,(5.alpha)-	91.05
29	37.468	5132063	0.30	1089145	0.93	2',3',4'Trimethoxyacetophenone	194.95
30	37.814	641999	0.04	71744	0.06	Benzene,1,1'-(1,1,2,2-tetramethyl-1,2-ethanedicyl)bis-	119.05
31	38.405	695500	0.04	175243	0.15	Heptadecane	57.05
32	38.980	116912	0.07	239827	0.20	Benzyl Benzoate	105.00
33	40.703	3961010	0.23	828586	0.71	Hexadecanoic acid,methylester	74.00
34	41.292	1020559	0.06	186360	0.16	9,19-Cyclanost-23-ene-3,25-diol,3-acetate,(3.beta.,23E)-	123.05
35	41.711	483288	0.03	134042	0.11	1,2-Benzenedicarboxylic acid,bis(2-methylpropyl)ester	149.00
36	42.721	1159085	0.07	340418	0.29	Eicosane	57.05
37	43.706	6470669	0.38	1270739	1.08	Hexadecanoic acid	73.00
38	44.403	10186652	0.60	2207365	1.88	Octadecenoic acid, methylester	55.05
39	44.615	1349301	0.08	377594	0.32	Decosane	57.05
40	46.399	1518808	0.09	331500	0.28	Tetracosane	57.05
41	47.042	956313	0.06	285343	0.24	9-Octadecenoic acid (E)	55.05
42	47.137	1830013	0.11	608935	0.52	9,12-Octadecadienoic acid (Z,Z)-	67.05
43	47.237	497663	0.03	180811	0.15	Octadecanoic acid	43.05
44	47.408	875919	0.05	168508	0.14	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	79.05
45	50.427	2438553	0.14	548922	0.47	Hexanedioic acid, bis(2-ethylhexyl)ester	129.00
		1701118614	100.00	117313229	100.00		

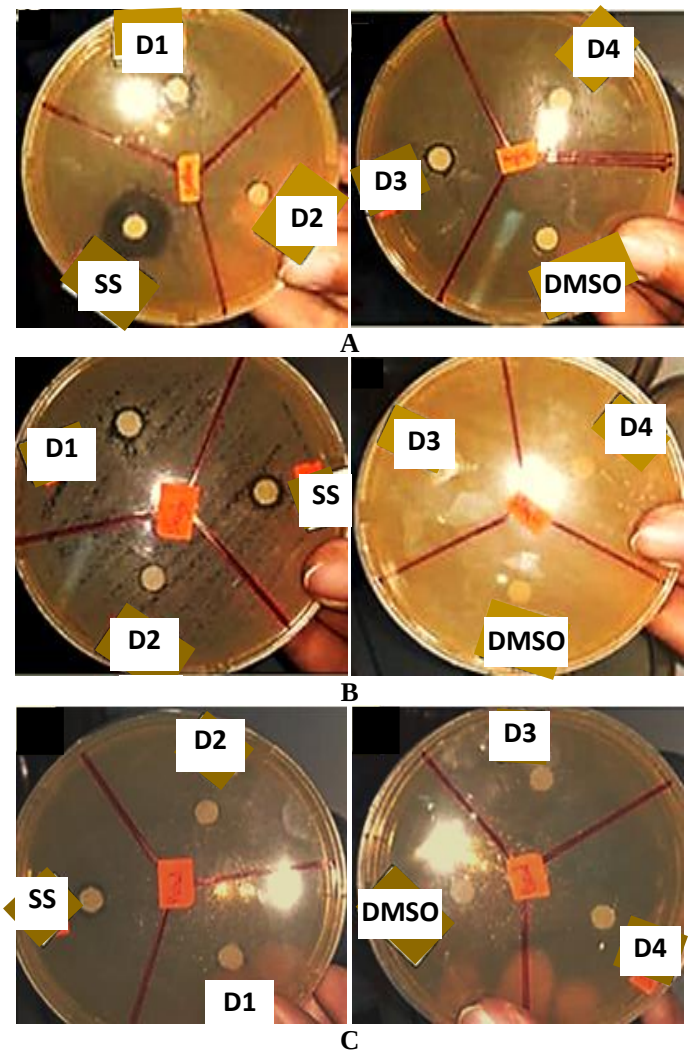

Figure 2. Antibiogram results of *S. aromaticum* L. extract for 100 mg/mL. A: *Selmonella* sp.; B: *S. aurus*; C: *P. aerginosa*; DMSO: Sterile dimethyl sulfoxide (negative control); SS: stock solution: 100 mg/mL; D1: 50 mg/mL; D2: 25 mg/mL; D3: 12.5 mg/mL; D4: 6.25 mg/mL

The results of the analysis by gas chromatography coupled with spectrometry mass (CG-MS) of the clove extract show that the major constituents are monoterpenes (eugenol 54.63% followed by eugenol acetate 21.57%) and sesquiterpenes (caryophyllene 16.71%). Our results are similar to those of José et al. (2021) in which eugenol is the predominant compound, with a percentage that reaches at least 50%, while l'eugenyl acetate, β-caryophyllene and α-humulene make up the remaining 10-40%. Banerjee (2020) also showed that gas chromatography-mass spectrometry (GC-MS) analysis of clove oil revealed eugenol (76.11%) and eugenyl acetate (12 .41%) as major constituents. In addition, a study carried out by Xu et al. (2016) confirms that among the 22 components identified in the essential oil of clove buds, eugenol is the major component (76.23%). Fujisawa and Murakami (2016) reported that eugenol is the most abundant compound in the ethanolic extract of clove.

Nassan et al. (2015) and Liu et al. (2017) recorded antifungal and antibacterial effects against strains Gram-positive and Gram-negative, as well as yeasts and mold for extracts of oregano, cumin, cinnamon, sage and other spices like clove. For a long time, clove has been considered a powerful antiseptic against infectious diseases. Many studies have been published on the antimicrobial activities of clove compounds against different microbes.

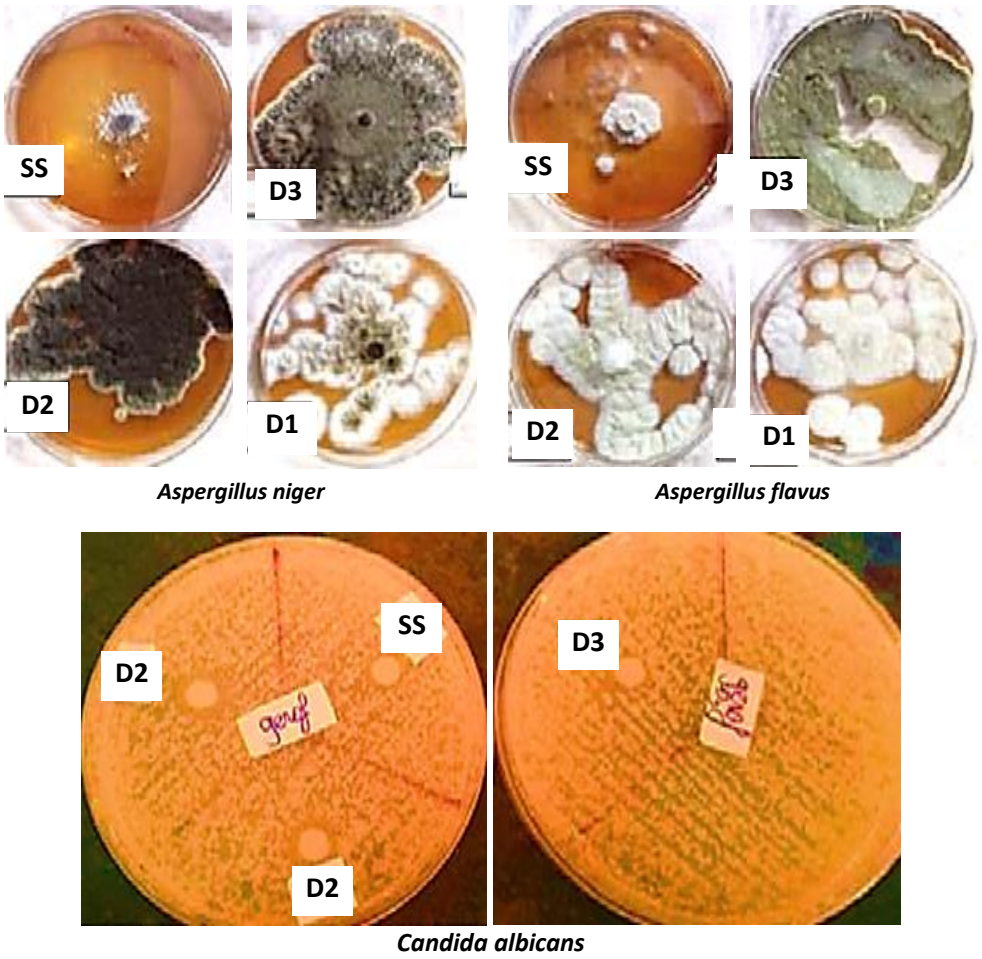

Figure 3. The results of the susceptibility test of fungal strains

Our results revealed that clove extract at 100 mg/mL registers an antibacterial effect with Gram-positive strain (*S. aureus*) and Gram-negative strains *(P. aeruginosa, Salmonella* sp.) with inhibition zones at 9, 9.5 et 16 mm respectively. Note that *Salmonella* sp. strain shows sensitivity even for dilutions of 50 mg/mL and 25 mg/mL of the same extract. These results are in agreement with those reported by Oulkheir et al. (2017) that Clove oil was found to be active against *Salmonelle* with inhibition zones at 16 mm.

Also, several studies have shown that clove has effective antifungal properties on various mycosis. The results of our experiment confirm that fungi strains: *A. niger, A. flavus* and *C. albicans* show sensitivity to *S. aromaticum* L. extract. According to Khan et al. (2012), clove oil has a broad spectrum of action which concerns not only dermatophytes, aspergillus and candidate species such as *C. albicans* but also species resistant to fluconazole such as *Candida krusei, Candida glabtra* and certain strains of isolated candidas. Additionally, Hiwandika et al. (2021) documented that bacterium strains, whether Gram-positive or Gram-negative such as *S. aureus, S. epidermidis, A. hydrophila, K. pneumoniae* show sensitivity against the clove ethanolic extract. Furthermore, Clove essential oil is able to inhibit several fungi such as *C. albicans*. The mechanisms are associated with inhibition of biofilm synthesis, virulence factor gene expression, and bacterial migration and adhesion, and destruction of the synthesized biofilm, etc. (Hu et al. 2018). Gonelimali et al. (2018) showed that clove extracts are able to induce a decrease in cytoplasmic pH and cause a hyperpolarization of the cell membrane of Gram-positive bacteria and Gram-negative bacteria. These changes indicate damage to the bacterial cell membrane, resulting in bacterial death (Vanhauteghem et al. 2013). Also, clove extracts act directly on the morphology of the envelope, which prevents colonization and causes cell cycle arrest in fungi (Kamatou et al. 2012).

Several studies have shown that the antimicrobial activity of cloves could be attributed to its main compound, which is eugenol. Recent studies proposed that eugenol is well known to present strong antimicrobial effect against Gram-positive, Gram-negative, fungi and viruses (Mak et al. 2019). Patil et al. (2013) showed that eugenol inhibits cell morphogenesis of *Candida albicans*. Batiha et al. (2020) reported that eugenol is not the only active antimicrobial in clove essential oil. Eugenol acetate and β-caryophyllene are also considered potent antibacterial and

antifungal agents. Musthafa and Voravuthikunchai (2015) have demonstrated that eugenyl acetate also exerts antibacterial activity on both Gram-negative and Gram-positive strains. In our opinion, the true properties are attributable to the synergistic action of the components, including those existing in trace amounts. These studies confirm our results concerning the richness of the ethanolic clove extract in these different molecules and also its antimicrobial properties.

In the light of our results, the biological activity of this extract marked a very important antibacterial power, especially in Gram-negative bacteria (*Salmonella*). Antifungal properties are also recorded on the strains tested. The results show that clove represents a very interesting plant rich in bioactive compounds that can serve as a basis for biological control. An additional study regarding the synergy or the antagonism between the different constituents remains necessary to better understand the mechanism of action of these molecules. This will facilitate the study of other biological properties in a more precise manner. Thus, making possible future applications in pharmaceutical industries with the aim of manufacturing drugs with fewer side effects and more effective for different diseases.

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