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Chemical composition, antibacterial and antioxidant activities of Tunisian garlic (*Allium sativum*) essential oil and ethanol extract

Raja Zouari Chekki [1,2,3], Ahmed Snoussi [1,2], Imen Hamrouni [1] and Nabiha Bouzouita [1,2,*]

[1] Ecole Supérieure des Industries Alimentaires, 58 Avenue Alain Savary, 1003 Tunis, Tunisia.
[2] Université de Tunis El Manar, Laboratoire de chimie organique structurale, Faculté des Sciences de Tunis, 2092 El Manar, Tunisia
[3]Centre Technique de la Chimie, Departement Transfert Technologique, Rue de l'Usine 12, ZI Charguia II, Tunis Carthage 2035, Tunisia

Abstract: The aim of the study is to establish some nutritional properties of garlic cultivated in Tunisia and to evaluate the antioxidant and the antimicrobial activites of its essential oil and ethanol extract. Tunisian garlic (*Allium sativum*) was characterized for moisture, ash and protein contents which were determined as 66%, 1.4% and 5.2% respectively. In addition, Fe (5.90 mg/kg), Cu (1.61 mg/kg), Mg (15 mg/kg) and P (140 mg/kg) were reported such as the major minerals in garlic. The fat profile of tunisian garlic was conducted, the main fatty acids identified were lauric acid (49.3%) and linoleic acid (20.4%). Essential oil obtained from *A. sativum* was analysed by capillary GCMS. Diallyl disulfide (49.1%) and diallyl trisulfide (30.38%) were the main components of the five identified components. The phenolic content of the ethanol extract are analysed for its phenolic profiles, colorimetric analysis revealed that the total phenols, flavonoids and proanthocyanidins contents were respectively 43.63 mg GA/g, 13.18 mg quercetin/g and 24.24 mg of catechin/g. Antioxidant activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, essential oil presented the highest antioxidant activity compared to its ethanol extract. IC_{50} values observed for the essential oil and ethanol extract were 300 µg/ml and 600 µg/ml respectively. The essential oil and ethanol extract from raw garlic were tested for antimicrobial activity against seven microorganisms. The results showed that ethanol extract was active against all tested strains: *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes, Yersinia enterocolitica* and *Bacillus cereus.*

Keywords: *Allium sativum*; chemical composition; essential oil; ethanol extract antioxidant activity; antibacterial activity.

Introduction

Garlic (*Allim sativum*), one of the oldest plants used in medicine ranks the highest of all the herbal remedies consumed for its health benefits. Scientific and clinical studies have shown that garlic can enhance immunity, protect against infection and inflammation and help lower the risk of cancer, heart disease and dementia[1]. Evidence supports the fact that regular consumption of garlic can reduce factors associated with cardiovascular disease[1].

The unique flavor and functions promoted the health are usually attributed to sulfur compounds of garlic, namely alliin, γ-glutamyl and their derivatives[2]. It has been estimated

**Corresponding author: Bouzouita Nabiha*
E-mail address: bouzouita.nabiha@gmail.com
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that the cysteine sulfoxides and γ-glutamylcysteine peptides are non-volatile over 82% of the total sulfur content of garlic[3].

Allicin is the most predominant thiosulphate in garlic that is responsible for the characteristic odor and has an antibacterial effect and toxic to insects[4]. One milligram of alliin is considered equivalent to 0.45 mg of allicin[3].

The organosulfur compounds from *A. sativum* such as alliin, allicin and diallyl sulfide, provide the most powerful of its biological activity in protection against oxidative damage[5].

Organic-soluble allyl sulfur compounds are formed from the parent compound to give the alliin, ajoene, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), while the water-soluble sulfur compounds of garlic may occur especially after alcoholic fermentation and the parent compound was alliin and gamma-glutamyl S-allylcysteine which is converted to S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC) and others.

Mei-chin Yin and al. conducted a study on antioxidant and antimicrobial protection of diallyl sulfide (DAS), diallyl disulfide (DADS), S-ethyl cysteine (SEC), n-acetyl cysteine (NAC) for five inoculated pathogenic bacteria, *Salmonella typhimurium, Escherichia coli, Listeria monocytogenes, Staphyllococcus aureus and Campylobacter jejuni*. They showed that DAS and DADS exhibited both antioxidant and antimicrobial protection contrarily to both SEC and NAC that might directly stabilize the redox status or protein structure[6].

The aim of this investigation is to establish some nutritional properties of garlic cultivated in Tunisia, evaluate the antioxidant activity of garlic essential oil and its ethanol extract by using the DPPH radical assay and study their effects on seven bacterial pathogens.

Results and Discussion

Chemical composition of Tunisian garlic

Tunisian garlic was analyzed for moisture, ash and protein contents. The obtained values were respectively 66%, 1.4% and 5.2%.

Table 1. Chemical properties of Tunisian garlic

	Tunisian	**Turkish garlic[7]**	**Indian garlic[8]**
Moisture %	66	66.3	62
protein content %	5.2	9.26	6.3
Ash content %	1.4	2.3	1.0

Protein content for Tunisian garlic was found to be considerably higher than concentrations in other vegetables but moisture was lower compared with the vegetables[7]. Turkish garlic present higher protein content than Tunisian garlic.

The major minerals in Tunisian garlic were established as Fe (5.90 mg/kg), Cu (1.61 mg/kg), Mg (15 mg/kg) and P (140 mg/kg). For Turkish garlic[7] higher values were observed for Mg (1056 mg/kg) and P (6009 mg/kg). For indian garlic[8], higher value are observed for P (4600 mg/kg) but lower one was observed for Mg (0.77 mg/kg). These results showed that environment influences the mineral content of garlic. The use of mineral profiles constitutes an adequate tool for determining the geographic origin of garlic[9].

Table 2. Mineral content of Tunisian garlic (*A. sativum)*

Minerals	**Tunisian**	**Indian Garlic[10]**	**Turkish garlic[7]**
Iron	5.90 mg/kg	0.39 mg/kg	52.91 mg/kg
Copper	1.61 mg/kg	0.3 mg/kg	9.12 mg/kg
Magnesium	25 mg/kg	0.77 mg/kg	1056 mg/kg
Phosphorus	140 mg/kg	4600 mg/kg	6009 mg/kg

Fatty acids

Fatty acid composition including total saturated fatty acids, polyunsaturated fatty acids, n-3 and n-6 fatty acids is presented in table 3:

Table 3. Fatty acids composition of the *A. sativum*

Fatty acids		**Content (%)**		
Saturated fatty acids		**Tunisian Garlic**	**Indian Garlic[11]**	**Greek Garlic[12]**
Caproic acid	C6:0	1.25	-	-
Capric acid	C10:0	0.17	0.5	0.6
Lauric acid	C12:0	**49.32**	**0.5**	**-**
Myristic acid	C14:0	0.59	-	-
Palmitic acid	C16:0 23.2	**6**	**24.6**	**20**
Heptadecanoic acid	C17:0	3.72	-	0.42
Stearic acid	C18:0	0.75	-	0.4
Heneicosanoic acid	C21:0	1.30	-	-
Tricosanoic acid	C23:0	0.60	-	0.4
Lignoceric acid	C24:0	0.32	-	0.4
Unsaturated fatty acids				
Palmitoleic acid	C16:1	1.48		0.32
Oleic acid	C 18 :1	**3.1**	**3.1**	**3.7**
Linoleic acid	C18:2 52	**20.42**	**64.8**	**53.6**
Linolenic Acid (GLA)	C18:3 7.6	**3.76**	**5.7**	**4.5**
Arachidonic acid	C20:4	0.94	-	-

22 fatty acids were detected in Tunisian garlic lipids; lauric acid (49.3%) and linoleic acid (20.4%) were the major components.

Contrarily to our findings, Kamanna[11] and Tsiaganis[12] reported low lauric acid content (0.5%, 0%) and high linoleic acid content (64.8% and 53.6%) from Indian and Greek garlic respectively.

Fatty acids have been also reported as bioactive compounds, it has been well known for many years that α-linolenic and lauric acid to have antibacterial and antifungal activities[13]. In particular polyunsaturated free fatty acids function as the key ingredients of many antimicrobial food additives. Up to 14 carbon atoms, the bactericidal efficacy has been found to increase with increasing the chain length[13]. There is concern that dietary linoleic acid could enhance the risk of and/or exacerbate conditions associated with acute and chronic diseases (cancers, cardiovascular disease, inflammation, neurological disorders, etc.)[14].

The difference between the different varieties of garlic may be attributed to the extraction solvent (hexane-isopropanol, in our case, versus chloroform-methanol mix in the case of Indian and Greek garlic) that affects the extraction efficiency of different fatty acids based on their polarity.

Essential oil

The yield of hydrodistilled oil obtained from Tunisian garlic was 0.15 % that is similar to the yield of Turkish garlic (0.14%)[7]. The chemical composition was examined by GCMS. Three main components identified in Tunisian garlic essential oil are presented in table 4:

Table 4. Sulfur compounds in the essential oil.

	Constituent	**Percent (%)**
1	Diallyl disulfide DADS	44.6

Volatile organosulfur compounds previously reported in *Allium* species have customarily been identified using mass spectral characterization of garlic oil components.
By comparing to the literature, we found:

Table 5. Comparison of garlic oil composition.

Compounds	**Tunisian Garlic (%)**	**Seoulean Garlic (%)[15]**	**Argentinean Garlic (%)[16]**
Diallyl sulphide DAS	4.1	-	**2.2**
Allyl mehyl disulfide	6.5	0.13	-
Allyl methyl sulphide	-	-	0.9
Dimethyl trisulphide	-	0.51	2.3
DADS	**44.6**	**32.8**	**34**
Allyl methyl trisulphide	**11.8**	**7.4**	**13.1**
3-vinyl-1,2-dithiin	4.04	1.99	2.1
2-vinyl-1,3-dithiin	1.2	5.9	1.6
DATS	**27.7**	**29.1**	**24**

Composition difference of garlic oils is due to GC analytical conditions, because a study[17] explained that DADS affords DATS and thioacrolein dimers (3-vinyl-1,2-dithiin and 2-vinyl-1,3-dithiin) and these reactions were dependant on temperature.

Total phenolic and total flavonoid content

Total phenol content, expressed as g catechin equivalent/100 g garlic was effected by the extracting solvents (Table 6).

Table 6. Total polyphenols and flavonoids content of garlic

Total polyphenols	**ethanol extract (Tunisian Garlic)**	**methanol extract(1)**	**methanol extract (2)**	**methanol extract (Indian Garlic)(3)**
Total phenols content TPC mg GAE/100 g	43.6	10.6	5	64.5
Total flavonoids content TFC mg QE/100 g	13.2	59.5	0.42	-
Proanthyanidins mg cathechin/g	24.2	-	-	-

(1) extracted using a method of maceration with 70% methanol for 10 min at 70 °C [18]

(2) extracted using a method of maceration with 80% methanol for 76 h at room temperature [19]

(3) extracted using a method of maceration with 80% acidic methanol for 45 min at room temperature [20].

Total phenol content of ethanol extract for Tunisian garlic was 43.6 mg GAE/100 g. Total phenolics of methanol extracts (1) and (2) were respectively 10.6 mg GAE/100 g and 5 mgGAE/100 g which were lower than our obtained value for ethanol extract but the value and 64.5 mg GAE/100 g for the Indian extract garlic (3) was higher than our results.

The differences between our data and the findings of authors cited before can be explained by factors such as differences in experimental parameters and the natural qualitative and quantitative variability in the raw material.

Antioxidant activity determined by DPPH assay

Antioxidant activity was evaluated as free radical scavenging capacity by measuring the scavenging activity of garlic extract and garlic EO on DPPH.

Five different working solutions were used (0.5; 1,0; 1.5; 2,0 and 2.5 mg/ml).

The obtained results show that percentage inhibition of garlic essential oil (EO) and ethanol extract (EE) are in increasing order with the increase in concentration.

Investigated EO and EE reduced the DPPH radical formation, the IC_{50} values respectively for EE and EO were 600 µg/ml, 300 µg/ml.

Antioxidant activity was directly related to the contents of phenolic compounds, with the ethanol extract[19,21]. The radical scavenging activity also co-related positively with the total phenolics of the EE.

The result for the essential oil of Tunisian garlic is comparable to the essential oil of Indian garlic (IC_{50} of 500 µg/ml)[22].

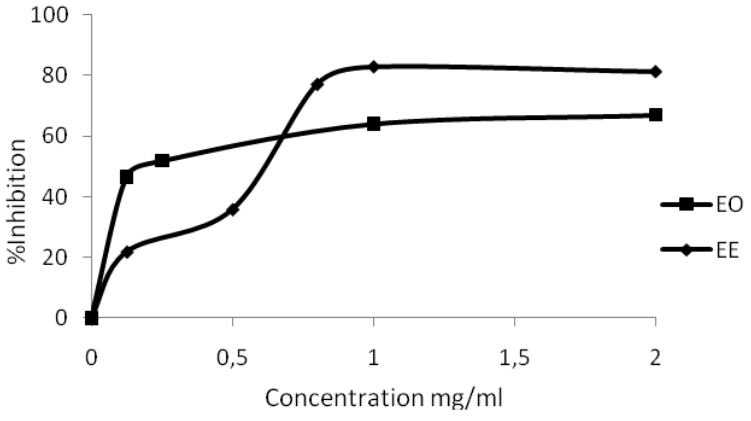
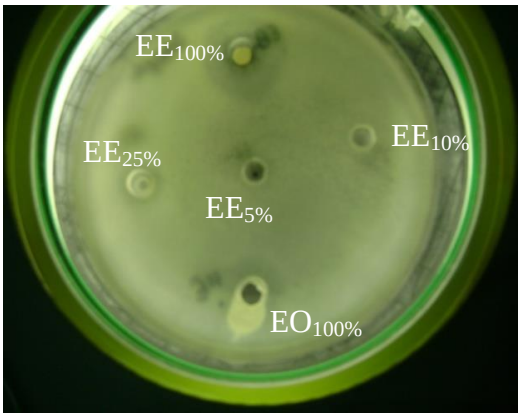


Figure 4. DPPH scavenging activities of various concentrations of *A. sativum* EE and EO

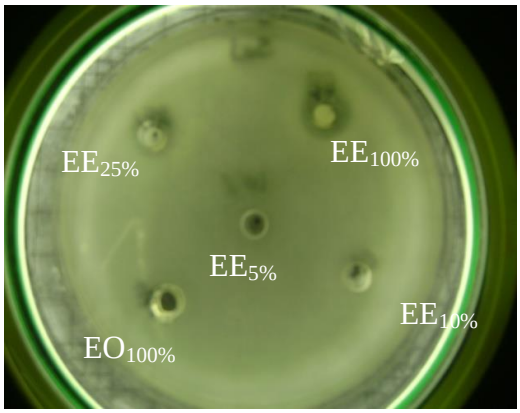
The EO and EE have concentration-dependent effects[19]. The antioxidant activity of EO is related essentially to organic-soluble sulfur compounds while for EE extract, it was due to both phenolic compounds and water-soluble sulfur compounds[21,23].

Antibacterial activity

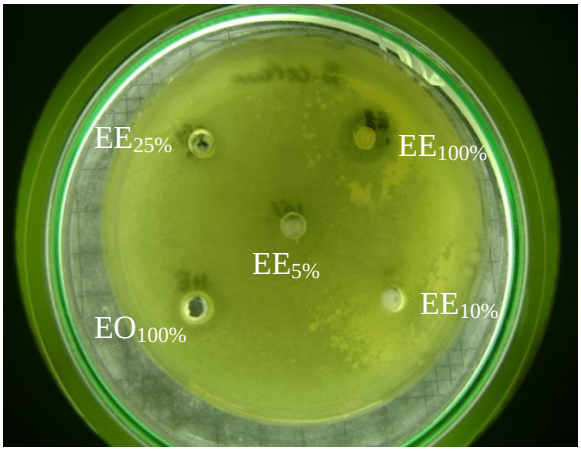
The antibacterial activities of *A. sativum* EE and EO against Gram positive (*Bacillus cereus* NCTC 7464, *Staphylococcus aureus* ATTC 6538P and *Listeria monocytogenes* NCTC 11994) and Gram negative (*Escherichia coli* ATTC25922, *Salmonella typhi* ATTC 14028, *Pseudomonas aeruginosa* ATTC 10145 and *Yersinia enterocolitic)* bacterial strains and results are shown in Figure 5 and table 8.



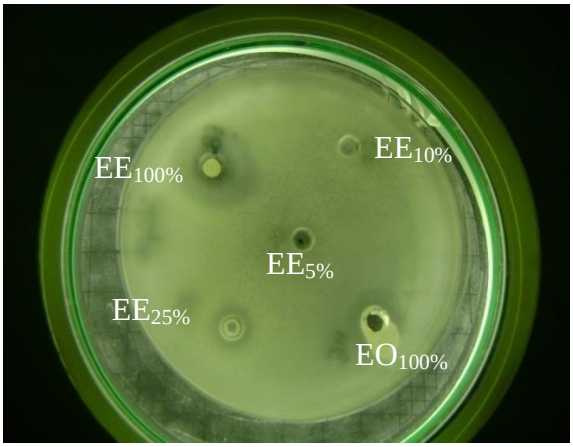
Pseudomonas aeruginosa



Listeria monocytogéne



Bacillus cereus



Staphylococcus aureus

Figure 5. Inhibition zones produced by *A. sativum* essential oil and ethanol extract on tested bacteria

(EE: Ethanolic Extract; EO: Essential Oil)

EE showed higher anti-bacterial activity than EO against all tested strains. This activity was more important against Gram-positive ones mainly towards *Staphylococcus aureus* which showed the highest Inhibition diameter (18 mm).

Following recent studies on the biology and biochemistry of Reactive Sulfur Species (RSS)[24], glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a well-characterized antioxidant in Gram-negative bacteria, where it is synthesized by the sequential action of two enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS).

Table 8. Inhibition diameters of *A. sativum* EO and EE against seven bacterial strains.

Bacteria	Concentrations				
	EE				**EO**
	100 %	**25 %**	**10 %**	**5 %**	**100 %**
Gram negative bacteria					
Escherichia. coli	7	NA	NA	NA	NA
Salmonella typhi	9	2	NA	NA	4
Yersinia enterocolitica	5	NA	NA	NA	4
Pseudomonas	7	NA	NA	NA	NA
Gram positive bacteria					
Listeria monocytogene	9	5	NA	NA	NA
Staphylococcus	18	5	NA	NA	12
Bacillus cereus	9	5	NA	NA	9

NA: No activity

Among Gram-positive bacteria only a few species contain GSH and its metabolism is poorly characterized[25]. In addition to its key role in maintaining the proper oxidation state of protein thiols, glutathione also serves as a key function in protecting the cell from the action of low pH, chlorine compounds, and oxidative and osmotic stresses. Moreover, glutathione has emerged as a posttranslational regulator of protein function under conditions of oxidative stress, by the direct modification of proteins via glutathionylation, whereas excess GSH can protect proteins from oxidation by Reactive Sulfur Species (RSS)[26]. This explains the less sensitivity of gram (-) to the extracts compared to gram (+) bacteria.

Thiosulfinates including allicin that are present in EE inhibit microorganisms because of their -S(O)-S- group, which reacts generally with the SH group of cellular proteins to generate mixed disulfides. The antimicrobial activity of thiosulfinates is cancelled by sulfhydryl compounds such as cysteine; adding to this, allicin and other thiosulfinates reacts with the sulfhydryl (SH) groups of cellular proteins and with non-SH amino acids[27].

The efficacy of the garlic ethanol extract as an antimicrobial has been linked to the ease by which the molecules pass through cell membranes and react biologically at the low level of thiol bonds in amino acids[28].

The antimicrobial effect of garlic is mainly attributed to organosulfur compounds such as allicin, ajoene and diallyl sulfides. This is consistent with what has been reported in previous studies which showed that the essential oil, water and ethanol extracts inhibit the in vitro growth of *Bacillus* species, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida species*[3,29].

Conclusion

The physicochemical characterization reveals the presence of minerals, fatty acids and sulphur components in Tunisian *Allium sativum*. The antioxidant and antimicrobial effects of EE and EO are attributed to organosulphur compounds. The results of this study reinforce the growing view that dietary supplementation of garlic is beneficial nutriments and food additives.

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Experimental Section

Chemicals

Folin-ciocalteu, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeCl_3$ were purchased from Sigma-Aldrich.

Plant material

Plants of *A. sativum* belonging to the variety softneck were randomly collected in October 2012, from wild population in Manouba. The plant was botanically characterized by Prof. Nadia Ben Brahim.

Chemical properties

The chemical properties of garlic bulbs were determined according to AOAC (1984). The dry matter was determined by drying in an oven maintained at 105°C until the weight becomes constant.

Preparation of ethanol extract

50 g of crushed garlic were put in 500 ml bottle. Three hundred ml of 80% ethanol were added. After three days of storage at room temperature, the supernatant and the sediment were separated by a vacuum filtration. The extract solution was dried by vacuum evaporator.

Essential oil extraction

The essential oil obtained by hydrodistillation using a Dean stark apparatus until there was no significant increase in the volume of oil collected to give the following yields (w/w). The oil was dried over anhydrous sodium sulphate and stored under N_2 at 4 °C.

GC-MS analysis of essential oil

The isolated essential oil were analyzed by GC/MS, using fused HP-5MS capillary column (30 m length, 0.25 mm i.d and 0.5 µm film thickness). The oven temperature was programmed from 45 °C (5 min) to 240 °C (5 min) at 5 °C/min. The temperature of the injector port was held at 100 °C, the temperature of the detector was set at 280 °C. The carrier gas was helium with a flow rate of 1.2 ml / min.

The mass spectrometer was operated in the electron impact (EI) positive mode (70 eV). The range of mass spectra was 35-350 m/z.

Fatty acid extraction

Hexane / Isopropanol solvent can extract neutral lipids (triglycerides), called polar lipids (partial glycerides, free fatty acids, unsaponifiables and phospholipids). The extraction was conducted according to AFNOR NF V03-030/1991: 50g garlic added to hexane/isopropanol solvent and let stand at least 2 hours, then filter through filter paper and dry the extract by using anhydrous sodium sulfate. The solution thus obtained was concentrated using a rotary evaporator in a 40°C. After concentration, the solution was used for the preparation of methyl esters of fatty acids according to ISO 5509-1978 by adding about 40 ml of methanol, 0.5 ml of methanolic potassium hydroxide solution and boiling with reflux condenser.

The esters obtained are extracted with heptane, concentrated and then analyzed by GC-FID.

Analysis of the methyl esters of the fatty substance by gas chromatography

Analysis of methyl esters samples was performed using a gas chromatograph equipped with flame ionization detector. The column used in the GC was a DB-23, 60 m length x 0.25 mm i.d x 0.25 µm film thickness with a carrier gas of Helium 1.5 ml/min. The temperature program of initial temperature 150 °C raised to 200 °C at the rate of 1.3 °C/min

and maintained for 10 min, with injector temperature at 210 °C, detector temperature at 210 °C.

Determination of polyphenols content

Total phenolic content (TPC)

The total phenols content is determined by the Folin-Ciocalteu test (Lister and Wilson, 2001). 100 µl of extract were diluted with 500 µl of Folin-Ciocalteu reagent and 1 ml of distilled water. After a minute, 1.5 ml of sodium carbonate (Na_2CO_3, 20%) was added.

The absorbance is then carried out at 760 nm after incubation for 2 h in the dark using a spectrophotometer. The results are expressed as mg GA/g determined by a calibration curve.

Determination of proanthocyanidins

The dosage of proanthocyanidins is performed according to HCl/butan-1-ol method (Luximon-Ramma et al., 2005). 0.25 ml of the extract were added to 3 ml n-butanol/HCl 95% solution and 0.1 ml of ($NH_4Fe(SO_4)_2$ x12 H_2O) HCl (2N) solution. The tubes were incubated for 40 min at 95 °C.

The result performed at 500 nm is expressed as mg catechin/g and determined by a standard curve.

Total flavonoid content (TFC)

The method of aluminum trichloride is adopted for the determination of flavonoïds (Luxminon-Ramma et al., 2005) by mixing 1.5 ml of the extract with an equal volume of a 2% solution ($AlCl_3$, $6H_2O$). The resulting mixture was stirred and incubated for 10 min at room temperature.

The absorbance at 367.5 nm was carried out and the results are expressed in meq.g quercitin/g garlic and are determined by a standard curve.

Determination of antioxidant activity

In a 50 ml volumetric flask, weigh 2 mg of DPPH solution and complete with ethanol up to the mark (Sun, 2005). From the extract to be tested (essential oil and ethanolic extract), it was prepared several solutions at different concentrations in bottles protected from light, and then 2 ml were mixed with 2 ml of DPPH solution. This mixture was stirred and allowed to stand 30 minutes away from the light.

The antioxidant power of different extracts obtained from the target plant was calculated as the inhibition percent of DPPH oxidative effect by the formula:

$$IP\% = \frac{(DO_{blanc} - DO_{mixture})}{DO_{blanc}} * 100 = \frac{DPPH^{\cdot}{}_{residuel}}{DPPH_{initial}} * 100$$

with:

IP%: percent inhibition

DO Blanc: optical density of the blank at t 0

DO mixture: optical density of the mixuture

Antibacterial activity

The garlic ethanol extract and essential oil have been investigated for their antibacterial activity on seven test bacteria namely:

4 bacteria Gram (-): Escherichia.coli ATTC25922, *Salmonella typhi* ATTC 14028, *Pseudomonas aeruginosa* ATTC 10145 and *Yersinia enterocolitic.*

3 bacteria Gram (+): Bacillus cereus NCTC 7464, *Staphylococcus aureus* ATTC 6538P and *Listeria monocytogene* NCTC 11994.

In agar well diffusion method, plate count agar (PCA) plates were inoculated with each pathogenic microorganism. Wells of 8 mm size were containing the microbial inoculums and we introduced 9.1 ml TS (Tryptone Salt).

Appropriate volume of ethanol extract was added to sterile water (vol/vol) to obtain the desired concentrations to be tested.

The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar. After incubation for 24 h at 37 °C, the plates were observed. Antimicrobial activity was indicated by an inhibition zone surrounding the well containing the extract expressed in millimeters.

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