Original Research Article

Total phenolic compounds extraction in leaves of *Ocimum gratissimum* L. and their potential activity against some agricultural contaminants

ABSTRACT

Aims: Phenolic compounds are secondary metabolites that are important in the plant due to their role in plant defense and their antioxidant activity with other biological properties such as antipyretic, analgesic and antimicrobial activities. This study focused on the biological potential activity of total phenolic compounds extracted by soxhlet method from *Ocimum gratissimum* leaves (Lamiaceae), a popular medicinal plant harvested at Daloa (Côte d'Ivoire).
Study design: Activities were directed on the *in vitro* antifungal and antioxidant activities combined to phenolic compounds analysis.
Place and Duration of Study: The study was carried out at the Department of Environment and Plant Protection, and Laboratory of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine (USAMV) between March to July 2014, Laboratory of Biochemistry and Microbiology (Bioactives Natural Substances Unit), Jean Lorougnon Guédé University between September 2014 to January 2015.
Methodology: The extract obtained named TPCOG was tested against *Fusarium* species for its antifungal activity by applying agar slant double dilution method and for its antioxidant activity by DPPH radical scavenging assay. Qualitative and quantitative evaluation of phenolic compounds were carried out by HPLC analysis method with sigma chemical standards.
Results: Tests showed that TPCOG was a powerful antifungal extract with MIC and MFC ranging between 3.125 µg/mL to 12.5 µg/mL. This extract was fungicidal and its antioxidant activity reached F= 541.25± .25 mM Trolox/mL (I= 70 ±1.85%) with a total phenolic content equal 195.70±1.33 mgGAE/g. Radical scavenge and antifungal activities correlated very well with total phenolic compounds. Phenolic content analysis showed presence of phenolic acids and flavonoids with a higher concentration for Quercetin (393.475 mg/100 g sample).
Conclusion: All this results demonstrated the biological potential activity of total phenolic compounds extracted from leaves of *O. gratissimum* and the possibility to use it in replace to essential oil for the formulation of biofungicides and nutraceuticals by industries

Keywords: Antioxidant, Phenolic compounds, Ocimum gratissimum, HPLC, Soxhlet

1. INTRODUCTION

The genus *Ocimum* is a member of the Lamiaceae family, comprising more than 150 species. It grows widely and is distributed throughout temperate regions of the world with the greatest number of species in Africa. The best known species are the strongly aromatic herb

Ocimum basilicum L. (Thai basil) and *Ocimum gratissimum* L. (African basil) as well as the medicinal herb *Ocimum sanctum* (holy basil or tulsi in Hindi) [1; 2]. Different parts of these plants including the leaves, stems, flowers, roots, seeds, and even the whole plant are useful. *O. gratissimum* is widely used in traditional medicine. In African traditional medicine, this plant is used for treating whooping cough and various types of fever [1; 3]. In West Africa, *O. gratissimum* is used for treating coughs, fever and malaria. Many researchers have investigated the antimicrobial, antiinflammatory, antinociceptive potential of *O. gratissimum* and most of the reported studies focus on the activity of essential oils [4; 5; 6]. Therefore, previous studies showed that some crude extracts of *O. gratissimum* obtained by maceration in ethanol and methanol have good antimicrobial and antioxidant properties [3; 7]. However, literature doesn't give more information about crude extracts of this plant obtained by soxhlet extraction. Does the heat change the properties and composition of crude extract of this plant? To answer this question, this study tries to evaluate the antioxidant activity, the antimicrobial activity against agricultural contaminants of total phenolic compounds obtained by soxhlet of this plant and analyze their polyphenol profile.

Agricultural crops contaminations with fungal diseases are becoming more and more common, causing a food safety risk for the consumers. Indeed, fungal species like *Fusarium* species are regularly implicated in crops contamination (maize, Tomatoes, wheat, horticulture…), both during production in the fields and during storage [8]. Among *Fusarium* species that may infect crops, *F. graminearum*, *F. oxysporum* subsp, *F. verticillioides* and *F. proliferatum* are implicated in 70 % of cases [8; 9]. Beyond the considerable economic losses due to *Fusarium*, the presence of fusariotoxins in cereal food is a real public health problem, especially since these substances are highly carcinogenic (e.g. cancerous liver tumors) [9]. The common way of limiting these types of fungal contaminations in modern agriculture relies mainly on the use of synthetic chemicals, but misuse and overuse of these compounds can lead to adverse effects both on human and environmental health [10, 11]. The increasing restrictions on the use of synthetic fungicides in the environment has given an impetus to the search natural products that could interfere with the fungal pathogenicity factors and without harm effects on human. Besides being harmless and non-phytotoxic, it has been proved that some plant extracts with high antifungal properties are also richness in polyphenols [12; 13]. In addition, analyzing polyphenolic content and potential antioxidant of this extract could improve their use in the management of several chronic health problems such as cancers, inflammation, aging and atherosclerosis [14; 15] due the increasing of free radicals.

2. MATERIAL AND METHODS

2.1 Plant Material

The leaves of *Ocimum gratissimum* were collected in a medicinal plant garden at Daloa (Côte d'Ivoire). The specie was authenticated at National Floristic Center (NFC) of Felix Houphouet-Boigny University (Côte d'Ivoire, Abidjan) to be identified to specimen NCF 304. The leaves of *O. gratissimum* were dried in a dark ventilated room for 7 days. These parts were milled, sifted in sieve 0.75 mm size and kept in polythene bag in absence of sunlight and oxygen until required.

2.2 Microbial Agent

Strains were composed by crops fungi: *Fusarium oxysporum* subsp *tulipae* PR 102/2014, *Fusarium graminearum* PR 124/2014 *and Fusarium oxysporum* subsp *radicis-lycopersici* NM 033/2014 provided by the Laboratory of Microbiology and Plant Biotechnology, University of Agricultural Sciences and Veterinary Medicine (USAMV) Cluj-Napoca, Romania. These strains were isolated on Potato Dextrose Agar (PDA) from corn seeds, Tulip and tomato infected according to recommendations contained in "the *Fusarium* Laboratory manual" [16].

2.3 Extraction of total phenolic compounds of *Ocimum gratissimum* (TPCOG)

This extraction was made according to the method described by authors [17] with slight modification. The extraction was performed by extraction with SER 148 Velp scientific according to the principle of soxhlet extraction. A mass of 30 g of fine powder of the plant were introduced into 3 cartridges (10 g/cartridge) of the extraction apparatus. Subsequently, these cartridges were introduced separately into boiling methanol contained in 3 extraction beakers. After refluxing, methanol was completely evaporated and the content weighted [11; 18]. In order to extract total phenolic compouds, the dried extract was dissolved in 4% formic acid solution/acetonitrile (90:10 v/v) [19]. The residue left after evaporation of the solvent was pre-purified with CHCl3 under reflux. After evaporation of CHCl3 under vacuum, dry chroroform extract was obtained. This extract was treated with hexane to remove lipids, concentrated under reduced pressure, and dried to obtain total phenolic compounds of *O. gratissimum* leaf extract [20]. Then, this extract obtained was the total phenolic compounds of *O. gratissimum* named (TPCOG). This extract was stored in a refrigerator pending further investigations (antioxidant and antifusarium activities, profile of phenolic compounds).

2.4 Evaluation of antifungal activity

Antifungal assays were performed on culture medium PDA. Incorporating TPCOG extract in agar was made by agar slant double dilution method. For the test, the series included 9 test tubes with 7 tubes tests containing TPCOG extract and 2 control tubes used as growth control and control of sterility of the culture medium. The concentrations of the extracts of these tubes ranged from 1.562 µg/mL to 100 µg/mL binding by a geometrical progression of 9. All 9 tubes were autoclaved at 121°C, 1 bar for 15 minutes and then the tubes were inclined to room temperature so that the agar formed an oblique slope and a 3 cm base. The fungi cultures in media previously prepared was made by inoculation of 1000 cells of the *Fusarium* strains corresponding to 10 µL inoculum 10^{-1} suspension containing 10^5 cells/mL. To prepare the inoculum, a well isolated colony from a six-day culture of the fungal strain was taken with a calibrated loop, transferred in 10 mL of sterile distilled water, homogenized by the vortex and gave a suspension 10° containing 10^6 cells/mL, then 1 mL of this suspension was transferred to 9 mL of sterile distilled water giving a suspension 10^{-1} containing 10^5 Cells/mL. For each test, the load of the inoculum was verified by a series of secondary dilutions and confirmed by a spectrometer. Thereafter, the cultures produced were incubated at 25°C for 6 days. Minimum Inhibitory Concentration (MIC) was determined by the lowest concentration of extract that inhibited visual growth of fungi in an agar dilution susceptibility testing. In addition, for each test, new sterile PDA medium was used for reseeding content of tubes with concentrations superior or equal to MIC values. Then, they were incubated at 25°C for 6 days in the goal to determine Minimum Fungicidal Concentration (MFC). MFC was determined by the tube with lowest concentration of extract with the growth of one colony [11; 13]. Three replicates were carried out for each test

2.5 Qualitative and quantitative evaluation of phenolic compounds of TPCOG

2.5.1 Determination of total antioxidant activity of TPCOG

This determination was made by DPPH (2, 2' diphenyl-1-picrylhydrazyl) radical scavenging Activity. DPPH (80 µM) was dissolved in pure ethanol (98%). The radical stock solution was prepared fresh daily. The mixture was shaken vigorously and allowed to stand at room temperature (25°C) in the dark for 10 min. A volume of 250 µL of TPCOG with 1.75 mL radical solution were added to each microplate well. The decrease in absorbance of the resulting solution was monitored at 515 nm for 30 min. The results were corrected for dilution and expressed in mM (S)-(-)-6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid, also known as Trolox (T). All determinations were performed in triplicate. TPCOG extract (0.1 g sample/1 mL solvent) was used [21; 22; 23]. Inhibition percent (I %) was calculated using the formula:

I% = [(AB –AA)/ AB] X 100
AB = Absorbance of blank solution; AA =Absorbance of standard solution (t = 30 min).

2.5.2 Determination of total Phenolic compounds

Total phenolics compounds of crude extract of *O. gratissimum* leaves were measured by the Folin & Ciocalteu method and the concentration was calculated using the calibration curve (Fig. 1). The results were expressed as mg Gallic acid Equivalents (mgGAE)/g. Exactly, 200 µL of TPCOG (1 mg/ml) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin-Ciocalteu phenol reagent for 3 min followed by the addition of 2 mL of 7% Sodium carbonate (Na2CO3) solution. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 765 nm on a UV-visible against the reagent blank. To obtain a calibration curve, various concentration of gallic acid solutions were prepared [24; 25].

Table 1. HPLC solvent gradient

Time (min)	% Solvent A	% Solvent B
0	95	5
2	95	5
18	60	40
20	10	90
24	10	90
25	95	5
30	95	5

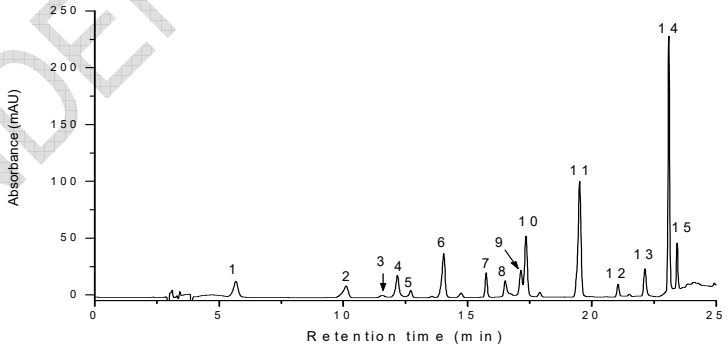

Fig. 1. Chromatogram of standards (0.1mg/mL

2.5.3 HPLC Phenolic compounds analysis method

The High Performance Liquid Chromatography (HPLC) analysis was performed with Eclipse XDB C18 column reversed phase (150x4.6 mm) 5 µm particle size from Agilent 1200 system

equipped with a quaternary solvent delivery system, coupled to a UV visible detector. A binary gradient composed of solvent A (water/acetic acid/ acetonitrile 99/0.1/1 v/v/v) and solvent B (acetonitrile/ acetic acid 100/0.1 v/v) was used. Injection volume was adjusted to 20 µL and chromatogram was recorded at 280 nm. The column flow rate was adjusted to 0.5 mL/min at 30°C with a HPLC solvent gradient as shown in Table 1. Standards were obtained from Sigma Chemical Company (USA). Phenolic acids and flavonoids standards were dissolved in 95% MeOH to make a concentration of 0.10 mg/mL (Fig. 1 and Table 2). For the preparation of TPCOG extract, a mass of 0.2 g sample was extracted using 2 mL methanol with 1% HCl and sonicated for 30 min. The mixture was centrifuged at 3000 rpm for 10 min and filtered through a 0.45 µm nylon filter [19; 24].

Table 2. Retention time of phenolic compounds standards

Peak	Retention time t_R (min)	UV λ_{max} (nm)	Area$_{280nm}$ (mAU)	Phenolic compound
1	5,6	280	177,4	Gallic acid
2	10,1	270,300	154,7	Protocatechuic acid
3	11,5	330	30,8	Gentisic acid
4	12,1	250,340	198,9	Chlorogenic acid
5	12,7	280	53,3	Catechin
6	14,1	250,340	358,1	Caffeic acid
7	15,7	250,360	125,4	Rutin
8	16,5	280,340	148,7	p-Coumaric acid
9	17,1	240,340	191,8	Sinapic acid
10	17,3	240,330	431,9	Ferulic acid
11	19,5	250,360	865,6	Myricetin
12	21,0	280,330	68,9	Tilirosid
13	22,1	260,370	183,2	Quercetin
14	23,1	280	1093,1	trans-Cinnamic acid
15	23,4	260,370	256,4	Kaempherol

2.6 Statistical Analysis

Statistical analysis of total phenol and antioxidant activity was evaluated using student's *t-test* analysis [26]. The mean was considered statistically significant at $p<0.05$. The results of all experiments performed were expressed as Mean ± SD of three determinations, the test of significance was applied wherever necessary and values obtained as $p<0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antifungal activity

Total phenolic compounds extracted from *O. gratissimum* (TPCOG) at different concentrations (100 µg/mL to 1.562 µg/mL) exhibited antifungal activity against the test *Fusarium*. This was also observed in a dose dependent manner. *F. oxysporum tulipae* and *F. oxysporum* sp *lycopersici* had the lest MIC and MFC values (3.125 µg/mL) following the results of this study (Fig 2). *F. graminearum* had the highest MIC and MFC values (12.5 µg/mL). The inhibitory role of TPCOG was more active on *F. oxysporum* species. The findings from this study explain the use of this plant in folklore medicine for the treatment of infections and in fumigation of granaries for storing agricultural products [7; 10; 27]. In addition, the equality between MIC and MFC values on each strain showed that this extract is probably fungicidal and it could be used to develop an eco-friendly fungicidal. In comparison with previous studies conducted on the same strains by an author [11], it appears that TPCOG extract was more active than Methanolic and Ethanolic extracts of *Cymbopogon citratus* for which values of MIC and MFC ranging between 12.5 and 50 µg/mL. Similar studies directed by Odamiro and Jimoh (2015) showed the antimicrobial activity of ethanolic leaf extract of *O. gratissimum*. All these results confirmed the antimicrobial potential activity of *O. gratissimum* crude extracts. TPCOG obtained by soxhlet extraction didn't lose their activity so the heat at 80°C didn't influence the nature of bioactive compounds present in TPCOG extract. This natural herb is therefore effective in controlling microbial growth by selectively inhibiting *Fusarium* growth, protein synthesis, cell wall and membrane synthesis, nucleic acid synthesis and the essential metabolic pathways that exist in the *Fusarium* [10]. Moreover, results showed that antifungal activity can be obtained from crude's extract of *O. gratissimum* not only with essential oil of this plant. For this reason, industrial exploitation of this plant in the production of bio-fungicides could be carried out from the crude extract of this plant in place of the essential oil which presents a low yield. The basis for the antifungal activity can be credited to phenolic compounds concentrated in TPCOG like phenolic acids and flavonoids which had been reported to have antimicrobial and medicinal activity [18; 20; 28].

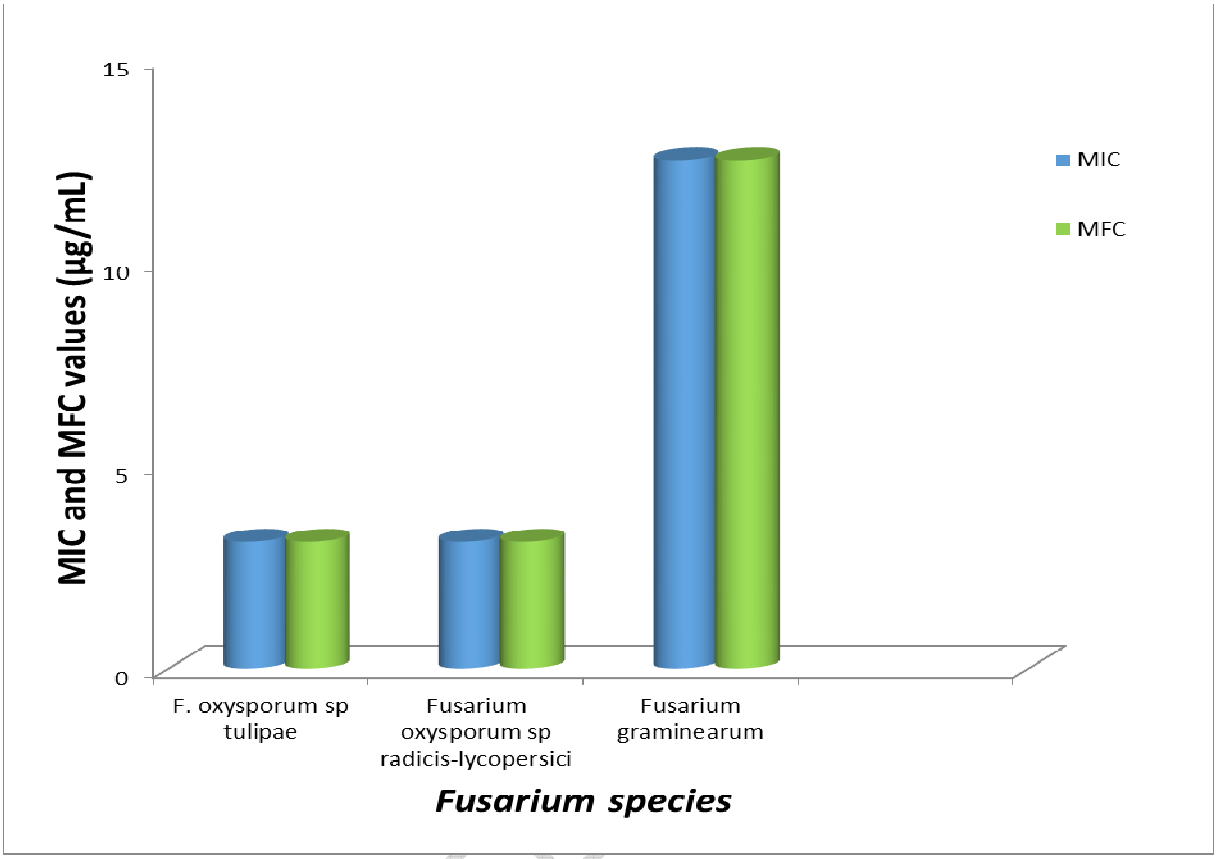

Figure 2: Histograms of MIC and MFC values of TPCOG extract

3.2 Qualitative and quantitative evaluation of TPCOG

3.2.1 Total phenolic compounds content and antioxidant activity

TPCOG extract revealed in-vitro antioxidant properties when tested with DPPH radical scavenging assay (Table 3). DPPH scavenging activity of this extract (F= 541.25± 1.25 mM Trolox/mL and I= 70 ± 1.85%) was higher than the essential oil of this plant evaluated by Elisée et al., (2018) with F= 187± 1.57 mM Trolox/mL and I= 38 ± 0.74 % [6]. The highest scavenging effect revealed by TPCOG extract was due to the high concentration of the active compound present in the plant material. The phenolic content of this extract was relatively high, suggesting that this plant has antioxidant potential. Polyphenolic compounds are well known as antioxidant and scavenging agents against free radicals associated with oxidative damage [15; 22]. The presence of these compounds in *O. gratissimum* could promote acceptance for its local usage in the management of oxidative stress induced ailment. Phenolic compounds are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis [29]. The strong antioxidant activity of *O. gratissimum* as shown in the table 3 might be related to high

contents of phenolic compounds (195.70 ± 1.33 mgGAE/g). Presence of phenol indicates that TPCOG could act as antiinflammatory, anti-clothing, anti-oxidant immune enhancers and hormone modulators. Phenols have ability to block enzymes that cause inflammation, and can also modify prostaglandin pathways leading to protection of platelets from clumping [30].

Table 3. Values of Total phenolic- and Antioxidant activity

Sample name	*Total phenolics (mgGAE/g)	Antioxidant activity	
		*F (mM Trolox/ 1 mL extrcat)	*I (%)
TGCOG	195.70 ± 1.33	545.21 ± 1.25	70 ± 1.85

*Mean±SD of three determinations

3.2.2 Total phenolic compound analysis

HPLC analysis showed 10 peaks identified with Standards (Phenolic acids and flavonoids) obtained from Sigma Chemical Company (USA). The phenolic compounds identified in TPCOG extract (Fig 3) were 7 phenolic acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, caffeic acid, p-coumaric acid and ferulic acid) and 3 flavonoids (rutin, quercetin, kaempferol). Phenolic compounds are usually classified into four different groups by their number of phenol rings and by their different structural elements that bind these rings to one another. The four classes are phenolic acids, flavonoids, stilbenes and lignans [19].

Phenolic acids identified could be classified in cinnamic acid derivatives comprising Coumaric acid, caffeic acid and ferulic acid, and in Hydroxy-benzoic acids possessing C6-C1 configuration with gallic acid and protocatechuic acid [19]. Due to their enormous structural diversity, phenolic acids are associated with important functions in plants such as protein synthesis, nutrient uptake, enzyme activity, allelopathy along with other biological properties such as anti-pyretic, analgesic and anti-microbial activity [24]. The most concentrated phenolic compound in TPCOG extract was Quercetin with 393.475 mg/100 g sample (Table 4). In addition, the cumulate concentrations of flavonoids (464.84 mg/100 g sample) was more important than phenolic acids (117.53 mg/100 g sample).

Flavonoids have a C6-C3-C6 framework. The chemical structure of flavonoids is responsible for their hydrogen donating (radical scavenging) and metal chelating properties. Flavonoids

exhibit antifungal, antibacterial, antiviral, antiinflammatory and antiulcer activities [24]. So, the higher concentration of flavonoid in TPCOG extract could explain its antioxidant activity because flavonoids are well-known powerful antioxidants and have been found in other plants [5; 29].

Finally, TPCOG extract could be the basis for formulation of nutraceuticals that regular intake in the diet should reduce the risk of coronary heart diseases, pulmonary diseases and may inhibit various forms of cancer due to the presence of phenolic compounds.

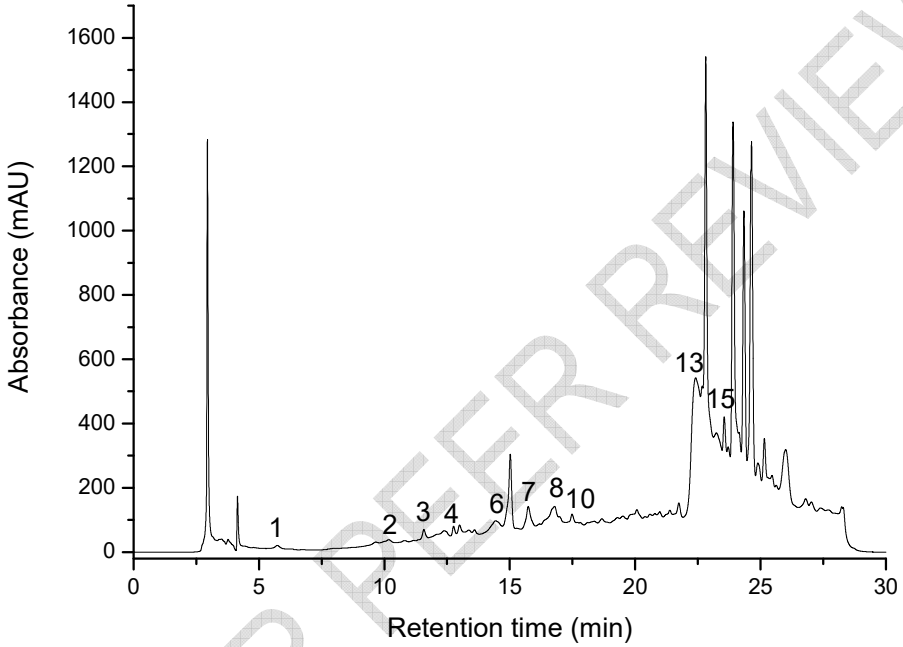

Fig. 3. Chromatogram of sample OG Met 24; peak1-gallic acid, 2-protocatechuic acid; 3-gentisic acid; 4-chlorogenic acid; 6-caffeic acid; 7-rutin; 8-p coumaric acid; 10-ferulic acid; 13-quercetin; 15-kaempherol

Table 4. Concentrations of Phenolic acids and flavonoids (mg/100g sample)

Sample name	Concentrations (mg/100 g sample)
Gallic ac	11.075
Protocatechuic ac.	11.880
Gentisic ac	79.413
Chlorogenic ac	7.562
Caffeic ac	18.510
Rutin	52.906
p-Coumaric ac	48.813
Sinapic ac	0
Ferulic ac	0
Quercetin	393.475
Kaempherol	18.459

4. CONCLUSION

Ocimum gratissimum is a popular medicinal plant used in traditional medicine for its therapeutic and biological properties due to the various active compounds. This study showed that total phenolic compounds extracted by soxhlet method from the leaves of this plant possessed antifungal activity against some post-harvest contaminants such as *Fusarium oxysporum sp tulipae, Fusarium oxysporum sp radicis lycopersici and Fusarium graminearum,* and this activity was fungicidal. In addition, the DPPH radical scavenging attested that this extract was a powerful antioxidant with a high concentration in phenolic compounds. HPLC analysis allowed to identify and quantify the phenolic compounds comprising phenolic acids (caffeic acid, ferulic acid, p-coumaryl acid, gentisic acid, gallic acid, protocatechuic acid and chlorogenic acid) and flavonoids (quercetin, rutin and kaempferol) with quercetin the major compound. These result demonstrated that phenolic compounds from leaves of this could be the basis for the development of new biofungicides for biological agriculture and nutraceuticals for human health.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and

producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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APPENDIX