



## HPLC Phenolic Compounds Analysis and Antifungal Activity of extract's from *Cymbopogon citratus* (DC) Stapf against *Fusarium graminearum* and *Fusarium oxysporum sp tulipae*

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### Authors' contributions

This work was carried out in collaboration between all authors. Author EKK carried out laboratory work, contributed to the experimental design and the protocol (writing the first draft of the manuscript). Authors IC, PR and AP contributed to the protocol and managed the analyses of the study. Author SO performed the statistical analysis and managed the literature searches. Author AO supervised the work and contributed to the protocol. All authors read and approved the final manuscript.

### Article Information

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### ABSTRACT

**Aims:** To analyze phenolic compounds and evaluate antifungal activity of methanolic and ethanolic extract's from *Cymbopogon citratus* against *Fusarium graminearum* and *Fusarium oxysporum sp tulipae*.

**Study Design:** Activity directed antifungal activity, total phenolic compounds determination, total antioxidant determination and High Performance Liquid Chromatography (HPLC) phenolic compounds analysis of extract's from *Cymbopogon citratus* using *in vitro* methods.

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**Place and Duration of Study:** 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:** Growth inhibitory effect of ethanolic and methanolic extracts of *Cymbopogon citratus* were tested *in vitro* by applying agar slant double dilution method, analyze the profile of phenolic compounds in the two extracts by HPLC and to measure antioxidant activity of the tested extracts through 2, 2' diphenyl-1-picrylhydrazyl (DPPH) scavenging method.

**Results:** Methanolic and ethanolic extracts of *Cymbopogon citratus* inhibited the *in vitro* growth of *Fusarium graminearum* and *Fusarium oxysporum* sp *tulipae*, but the activity of methanolic extract was better. This study revealed the presence of phenolic compounds such as gentisic acid, chlorogenic acid, sinapic acid, protocatechuic acid, caffeic acid, rutin, p-couramic acid, kaempferol and quercetin in the tested extracts. Methanolic extract which was more concentrated in total phenolic, exhibited higher antioxidant activity than ethanolic extract.

**Conclusion:** *Cymbopogon citratus* is a powerful antioxidant. This plant is not only a natural antifungal but also a potential source of antioxidant and nutraceuticals. Further work in this direction can allow to isolate a novel natural bioactive compound against *Fusarium* contamination.

*Keywords:* *Cymbopogon citratus*; antifungal activity; phenolic compounds.

## 1. INTRODUCTION

Fungal species producing *Fusarium* mycotoxins (Trichothecenes, Zearalenone and Fumonisin) are regularly implicated in agricultural and horticultural contamination, both during production in the fields and storage [1]. Beyond the considerable economic losses from *Fusaria*, the presence of mycotoxins they produce and found in food or in the production of tulip bulbs is a real public health problem. Indeed, according to Food and Agriculture Organization of the United Nations (FAO), up to 25% of production in agriculture was contaminated with mycotoxins, and ingestion of mycotoxins is the origin of several disorders including degradation of the nervous system, liver disorders and cancer [2]. To limit such contamination, chemical control using synthetic fungicides is most commonly used with its corollary of adverse effects on the environment [3]. In addition, according to the literature, the filamentous fungi *Fusarium spp.* are 100% resistant to some synthetic antifungals such as Itraconazole [4]. The use of natural compound, such as phenolic compounds, essential oils and extracts from natural products in the formulation of fungicides in order to reduce the risk of *Fusarium* contamination and to solve the problem of *Fusarium* resistance to synthetic antifungals, is an essential component of alternative methods to contribute in the reduction of the use of synthetic antifungals. The

struggle based on the use of plant extracts could be an appropriate response, economically and culturally viable for farmers and horticulturists in developing countries. Indeed, many plants have shown antibacterial, antioxidant and antifungal potentialities. And among these plants, figure *Cymbopogon citratus* (DC) stapf (lemongrass). This plant of gramineae (Poaceae) family is a perennial grass which grows spontaneously in western Africa [5]. Infusions of the leaves are used in traditional medicine as antimicrobial, anti-inflammatory, febrifuge, antiseptic, in skin disorders, rheumatism and cough, headache, malaria [6, 7] and sedative [8-10]. It was reported that this plant has no toxic effect in human [11]. The essential oil of this plant is used in the food, perfumery, soap, cosmetic, pharmaceutical and insecticidal industries [12,13]. Lemongrass has been used over many years to make caffeine free tea and natural herb drink and this confers its advantage over caffeinated tea products [14]. The essential oil of this plant has been widely studied for its antimicrobial and antioxidant properties whereas the crude extract has been little studied. The aim of this study is to evaluate the antifungal effect of crude extracts obtained from the fine powder of *Cymbopogon citratus* (DC) Stapf leaves on the *in vitro* growth of *Fusarium graminearum* and *Fusarium oxysporum* sp. *tulipae* and to analyse their phenolic compounds profile using HPLC.

## 2. MATERIALS AND METHODS

### 2.1 Collection, Identification and Preservation of the Plant Sample

*Cymbopogon citratus* (DC) stapf (lemongrass) was obtained from a small garden in Daloa, Region of Haut Sassandra, Côte d'Ivoire in January 2014. Leaves of plant material sample was taken to the National Floristic Center (NFC), Felix Houphouët Boigny University-Abidjan (Côte d'Ivoire), for proper identification and authentication. The identity of the plant to the specimen CNF 11068, was confirmed. The leaves were removed; air dried for a week, powdered, labeled and stored in the air tight container before extraction.

### 2.2 Fungus

Strains of *Fusarium graminearum* and *Fusarium oxysporum* sp *tulipae* plant pathogenic fungi were provided by the Laboratory of Microbiology and plant Biotechnology, University of Agricultural Sciences and Veterinary Medicine (USAMV) Cluj-Napoca. These strains were isolated from corn seeds and *Tulipea* infected according to recommendations contained in "the *Fusarium* Laboratory manual" [15].

### 2.3 Preparation of Plant Extracts

Plant extracts were obtained by extraction with SER 148 Velp scientific. A mass of 3 x 10 g of fine powder of the dried leaves of *C. citratus* (DC) Stapf were introduced into 3 cartridges of the extraction apparatus. Subsequently, these cartridges were introduced separately for 30 minutes into 3 x 100 ml of the boiling extraction solvent contained in 3 extraction beakers according their boiling temperature (ethanol and methanol). After 45 min of refluxing, organic solvent was completely evaporated and the dry extract contained in each extraction beaker was weighed [16]. Extraction yield was determined. The different extracts of *C. citratus* were coded following: Ethanol extract: CCEt and Methanol extract: CCMet. They were stored in a refrigerator pending further investigations.

### 2.4 Antimicrobial Susceptibility Testing, Determination of Minimum Inhibitory Concentration (MIC) and MFC (Minimum Fungicidal Concentration)

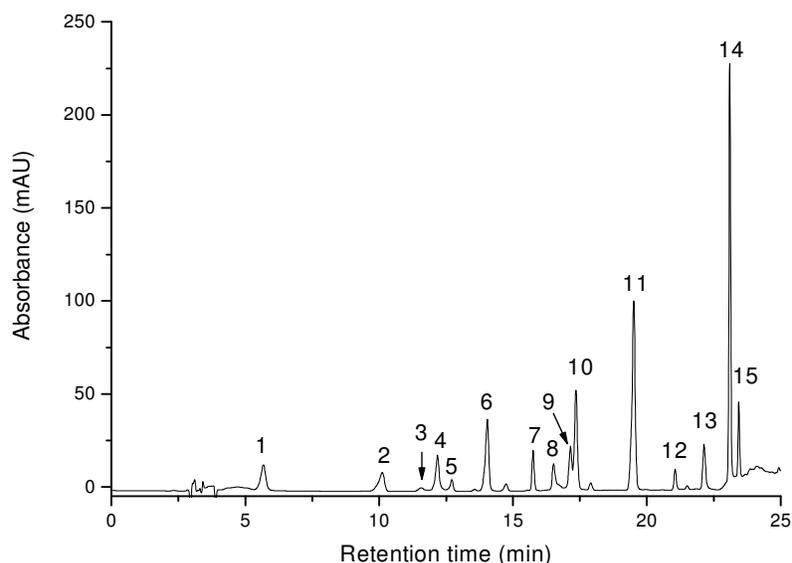
Antifungal susceptibility testing was carried out on PDA (Potato Dextrose Agar) prepared

according to manufacturer's instructions. Ethanol and methanol extracts were solubilized in 1% DMSO, then incorporated separately to the medium following method of double dilution tubes on sloping Agar. For each strain per test, eleven (11) tubes were used including nine (09) test tubes containing plant's extract and two (02) control tubes, one without plant's extract, as growth control of fungi and the second without fungi and plant's extract, as a control of sterility of culture medium. Extracts concentrations range in the tubes from 0.39 µg/ml to 100 µg/ml with geometrical connection in order of 1/2. All eleven (11) tubes were autoclaved at 121°C, 1 bar for 15 min and then the tubes were inclined to room temperature of the laboratory to allow their cooling and solidification of the agar. Inoculum was obtained by sampling with a loop diameter of 2 mm with a sterile single colony which was homogenized in 10 ml of distilled water giving a suspension containing 10<sup>6</sup> cells/ml. Then, 1 ml of this suspension was transferred to 9 ml of sterile distilled water giving a suspension 10<sup>-1</sup>. Fungi culture on agar slant previously prepared was made by inoculation of 1000 cells of each strain corresponding to 10 µl standardized inoculum 10<sup>-1</sup> suspension containing 10<sup>5</sup> cells/ml. For each test, the load of inoculum was verified by series of secondary dilutions. Thus, cultures produced were incubated at 25°C for six days (144 hours). After this incubation time, 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 reseeded 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) [17]. MFC was determined by the tube with lowest concentration of extract with the growth of one colony. Three replicates for each extract concentration and control against the fungi were used.

### 2.5 Determination of the Total Phenolic Compounds

Total phenolics compounds of crude extract of *C. citratus* leaves were measured by the Folin & Ciocalteu method and the concentration was calculated using the calibration curve (Fig. 1) and the results were expressed as mg Gallic acid Equivalents (mgGAE)/g.

Exactly, 200 µl of crude extracts (1 mg/ml) were made up to 3 ml with distilled water, mixed



**Fig. 1. Chromatogram of standards (0.1 mg/ml)**

thoroughly with 0.5 ml of Folin-Ciocalteu phenol reagent for 3 min followed by the addition of 2 ml of 7% Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) 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.

## 2.6 Determination of Total Antioxidant Activity by DPPH Radical Scavenging Activity

It's reported that this assay was based on the measurement of the reducing ability of antioxidants toward DPPH (2, 2'-diphenyl-1-picrylhydrazyl) [18]. Antioxidant assays were based on measurement of the loss of DPPH color at 515 nm after reaction with test compounds, and the reaction was monitored by a spectrophotometer. A measure of total antioxidant capacity would help us understand the functional properties of extracts.

An easier way to present antioxidant activity of extracts would be to reference a common reference standard. (S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, also known as Trolox (T), serves such as a common reference standard.

Inhibition percent (I%) was calculated using the formula:

$$I\% = [(A_B - A_A) / A_B] \times 100$$

where:  $A_B$  = absorbance of blank solution;  $A_A$  = absorbance of standard solution ( $t = 30$  min).

### 2.6.1 Assay protocol for microplates with 24 wells

The DPPH (80  $\mu\text{M}$ ) 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  $\mu\text{l}$  of sample 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 Trolox per 1g weight. All determinations were performed in triplicate. These analytical methods measure the radical-scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical ( $\text{O}_2^-$ ), the hydroxyl radical (OH), or the peroxy radical (ROO). The extracts (0.1 g sample/1 ml solvent) were used.

### 2.7 HPLC Phenolic Compounds Analysis Method

The High Performance Liquid Chromatography (HPLC) analyses were performed with Eclipse

XDB C18 column reversed phase (150x4.6 mm) 5  $\mu$ m 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  $\mu$ l 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).

**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

### 2.7.1 Preparation of extracts

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  $\mu$ m nylon filter.

## 2.8 Statistical Analysis

Statistical analysis of total phenol and antioxidant activity was evaluated using the *t-test* analysis. The mean was considered statistically significant at  $p < 0.05$ . The results of all experiments performed were expressed as Mean  $\pm$  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 Preparation of Extract

Percentages yield of plant extract after 9 cycle extraction by Ethanol and Methanol using SER 148 Velp scientific were calculated to respectively  $11.18 \pm 0.15\%$  and  $15.25 \pm 0.20\%$  (Table 3). Yield obtained with Methanol extract (CCMet) was more higher than Ethanol extract (CCEt) because this solvent extracted both same polar, semi polar, non polar compounds and compounds with larger molecular weights. Using SER 148 Velp scientific for extraction allowed to extract maximum of bioactive compounds and prevent the inactivation of phenolic compounds. Percentages of yield should not be considered as a criterion of efficiency but will estimate future returns to scale in production and to select the solvent depending on antifungal activity [19,20].

**Table 2. Retention time of phenolic compounds standards**

Peak	Retention time $t_R$ (min)	UV $\lambda_{max}$ (nm)	Area <sub>280nm</sub> (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

**Table 3. Percentages yields of extraction by SER 148 of crude extract from *C. citratus***

Extracts	Quantity of powdered leaves (g) extracted by SER 148	Quantity of extract (g)	Percentages (%)
CCEt	270	30.18	11.18± 0.15
CCMet	270	41.17	15.25± 0.20

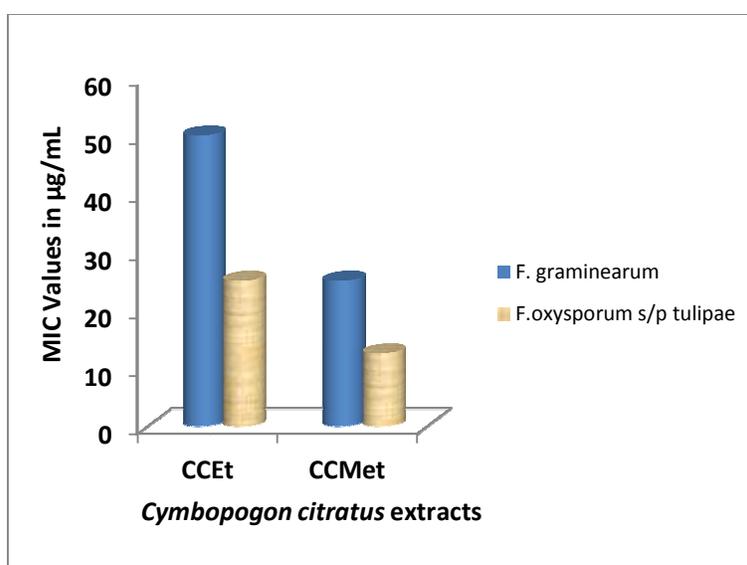
### 3.2 Antimicrobial Susceptibility Testing

Antifungal activity of crude extracts of *Cymbopogon citratus* against *Fusarium graminearum* and *Fusarium oxysporum sp tulipae* have been investigated (Fig. 2). The crude extracts exhibited after six (06) days of incubation an inhibitory activity. MIC values of CCMet and CCEt were respectively 25 µg/ml and 50 µg/ml on *F. graminearum* and were 12.50 µg/ml and 25 µg/ml on *F. oxysporum sp tulipae*. In addition, for all the tests achieved MIC values were equal to MFC values. Inhibition observed for methanol and ethanol extracts of *C. citratus* (CCMet and CCEt) on the *in vitro* growth of *F. graminearum* and *F. oxysporum sp tulipae* demonstrated that these extracts possess antifungal properties. The lowest values of MIC were obtained with CCMet, so CCMet was the most active extract. Equality between MIC and MFC proved that extracts were fungicide. In addition, results showed that antifungal activity can be obtained from crude's extract of *C. citratus* with an interesting yield, and 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 [21,22]. Various bio-fungicides were tested for biological control of *Fusarium* contamination [23,24], data obtained in this study demonstrated that *C. citratus* extracts were consistently effective in reducing *Fusarium* contamination and it is an alternative for reducing significantly environmental pollution due to chemical fungicides in agriculture and horticulture [25-27].

### 3.3 Total Phenolic Compounds Content and Antioxidant Activity

The total phenolic and antioxidant activity of the crude extracts of *Cymbopogon citratus* were represented in Table 4. Antioxidant activity was recorded in terms of equivalent factor (F) and % Inhibition (I) as shown in Table 4. It was observed that this activity was  $F=178.069 \pm 1.57$  ( $I=34 \pm 0.74$ ) for CCMet, and  $F= 173.931 \pm 0.87$  ( $I=33 \pm 0.26$ ) for CCEt. For total phenolic compounds, CCMet contained  $118.14 \pm 1.05$  mgGAE/g while CCEt contained  $35.43 \pm 0.24$  mgGAE/g.



**Fig. 2. Histogram MIC\* values of crude extracts on *F. graminearum* and *F. oxysporum sp. tulipae***

\*For these tests MIC=MFC

**Table 4. Total phenolics (GAE) and antioxidant activity (Percentage inhibition (I%) and equivalent factor (F) in mM Trolox)**

Sample	*Total phenolics (mgGAE/g)	Antioxidant activity	
		*F (mM Trolox/1 ml extract)	*I %
CCMet	118.14±1.05	178.069 ± 1.57	34 ± 0.74
CCEt	35.43±0.24	173.931 ± 0.87	33 ± 0.26

\*Mean ± SD of three determinations

CCEt has minimum DPPH scavenging activity among individual *C. citratus* extracts. Total phenols content and antioxidant of CCMet extracts were significantly different from CCEt extracts. Total phenols contents in the methanolic extract of *C. citratus* leaves, using the calibration curve, was found to be 118.14 mgGAE/g dry weight of extract and 35.43 mgGAE/g in ethanol extract. The total phenolic compounds in methanolic extract was at least 3 times higher than ethanolic extract. The total antioxidant capacity of CCMet was 178.069 mM Trolox/1ml extract (I=34%) and 173.931 mM Trolox/1ml extract (I= 33%) for CCEt. These Crude's extracts of *C. citratus* are said to be powerful antioxidants, and their regular intake would allow to reduce risks of cancer, cardiovascular disease, diabetes, and other degenerative diseases associated with aging, and have the advantage of being almost devoid of harmful side effects [11]. Authors showed that the volatile oil from the leaves of *Cymbopogon citratus* has also an antioxidant property against DPPH [28]. Total phenolic compounds constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators hence it was reasonable to detect their amount in the herbal preparation. Phenolics and flavonoids possess a broad spectrum of chemical and antimicrobial activities including radical scavenging properties. The medicinal effects of plants are often attributed to the antioxidant activity of phytochemical constituents mainly phenolics and flavonoids [29]. It is claimed that phenolic compounds are powerful chain breaking antioxidants. The scavenging activity of phenolic group is due to its hydroxyl group.

The antioxidant activities of several natural polyphenol compounds present in vegetables has been reported. Moreover, the polyphenolic compounds have exhibited inhibitory effects on microbial agents and antifungal effects with respect to antioxidant properties was reported by Melyssa et al. [30]. The presence of phenolic compounds identified in the crude's extract of *Cymbopogon citratus* would probably contribute

to their efficiency against *Fusarium* strains tested.

Exploring secondary metabolites in plant, synthetic antioxidants could be replaced by naturally occurring safer antioxidants, as the synthetics have been suspected of causing or provoking unfavorable side effects, while stronger restrictions are encountered on their application. Several reports have conclusively shown a correlation between the antioxidant activity and amount of total phenolics or total flavonoids [24].

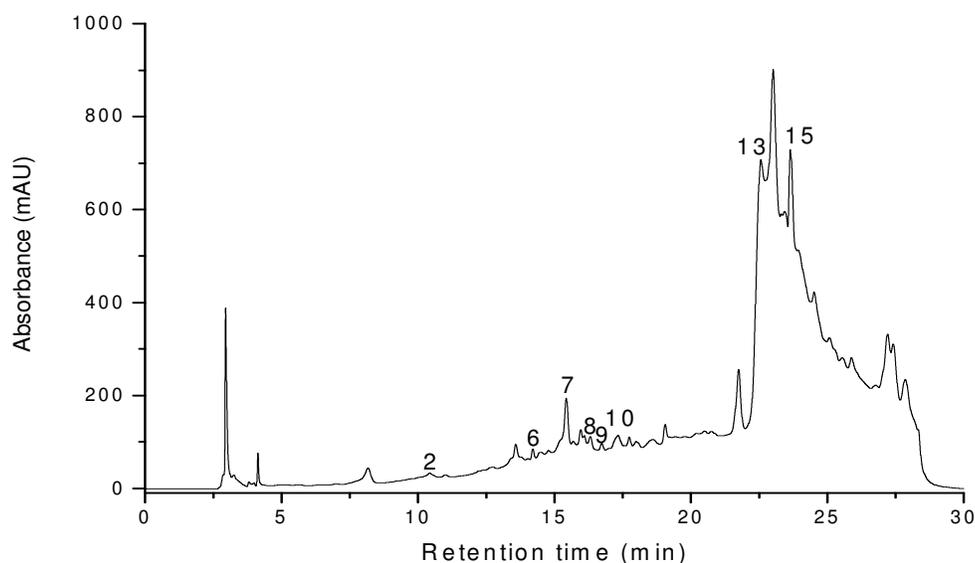
### 3.4 HPLC Phenolic Compounds Analysis Method

HPLC phenolic compounds analysis recorded in Figs. 3, 4 and 5 showed that protocatechuic acid; caffeic acid; rutin; p coumaric acid; ferulic acid; quercetin; kaempferol are common to the two extracts but they were more concentrated in CCMet (Fig. 5). Sinapic acid was specific to CCEt (Fig. 3) while gentisic acid and chlorogenic acid were specific to extract CCMet (Fig. 4).

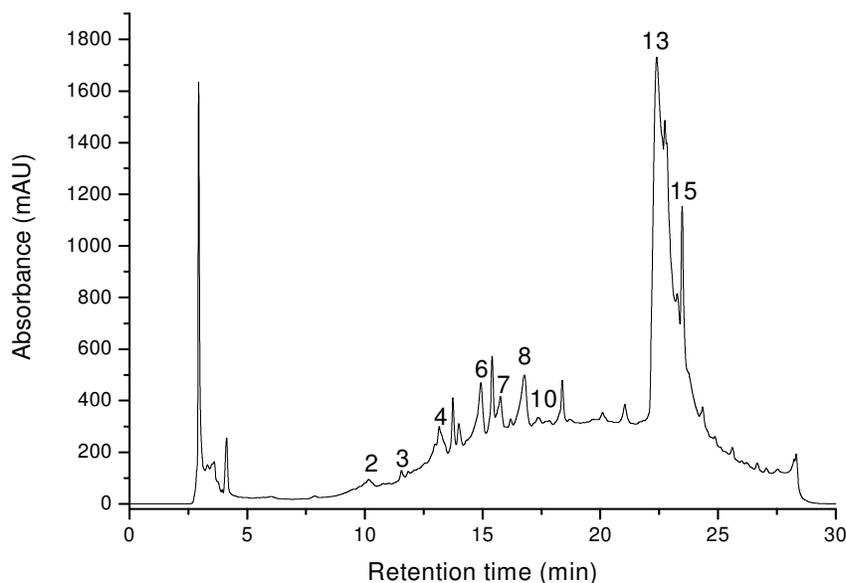
HPLC phenolics compounds analysis of CCMet and CCEt extract's showed its richness in total phenolic compounds (Phenolics acid and Flavonoids). Methanol is efficient solvent for extraction of total phenolic compounds from *Cymbopogon citratus* because in CCMet extract concentrations of phenolics acid (protocatechuic acid, gentisic acid, chlorogenic acid, caffeic acid) and flavonoids (Rutin, Kaempferol and quercetin) were higher. Bassolé et al. [31] showed that the methanol was better to concentrate total phenolic compounds. These compounds have shown to have antioxidant activity [32]. Phenolic compounds are bioactive compounds with antioxidant and anti-inflammatory properties that are found in large quantities in certain plants such as *Cymbopogon citratus*. They can be simple or complex structures containing at least one aromatic ring in which one or more hydrogens are substituted by a hydroxyl group.

This study showed a high level of phenolic compounds in methanolic extract of *C. citratus* and activity against *F. graminearum* and *F. oxysporum sp tulipae* was better. Moreover, previous studies showed that phenolic compounds exhibited antifungal activity against a large number of pathogenic fungi [33,34]. These

compounds have a role in various functions, including the protection of plants against ultraviolet and visible light; protection against insects, fungi, viruses, and bacteria; the attraction of animals for pollination; and the action of plant hormones [35].



**Fig. 3. Chromatogram of sample CC Et 32; peak 2-protocatechuic acid; 6-caffeic acid; 7-rutin; 8-p coumaric acid; 9-sinapic acid; 10-ferulic acid; 13-quercetin; 15-kaempferol**



**Fig. 4. Chromatogram of sample CC Met 22; peak 2-protocatechuic acid; 3-gentisic ac; 4-chlorogenic acid; 6- caffeic acid; 7-rutin; 8-p coumaric acid; 10-ferulic acid; 13-quercetin; 15-kaempferol**

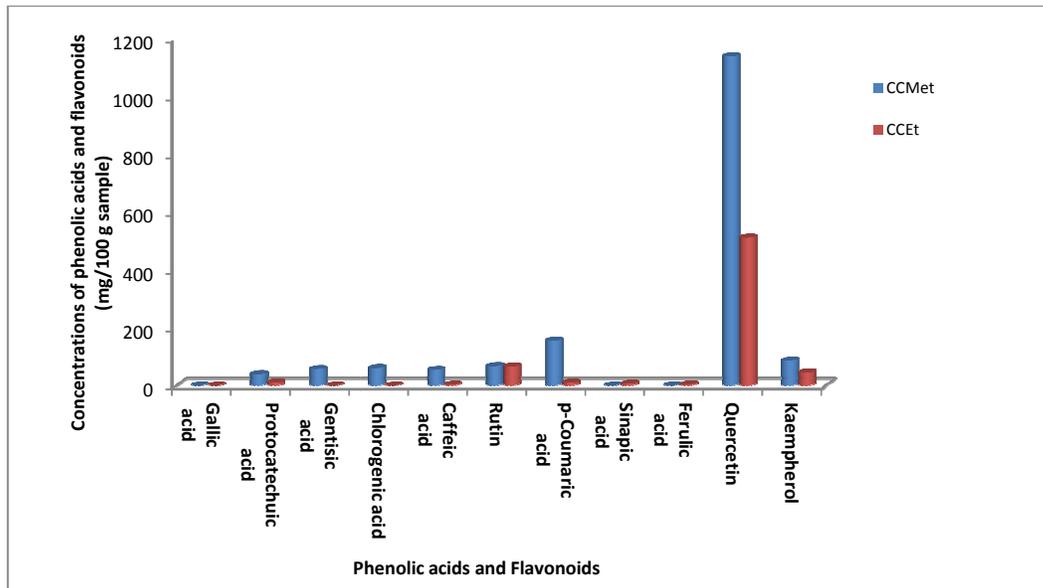


Fig. 5. Histogram showing concentrations of phenolic acids and flavonoids in CCMet and CCET extracts

#### 4. CONCLUSION

Methanolic and ethanolic extracts of the leaves of *cymbopogon citratus* possessed both antioxidant and antifungal activity against *Fusarium graminearum* and *Fusarium oxysporum* sp *tulipae*. Methanolic extract has better antifungal activity. Moreover, extracts tested contained phenolic acids such as protocatechuic acid, gentisic acid, chlorogenic acid, caffeic acid, and flavonoids such as rutin, kaempferol and quercetin. Methanolic extract was more concentrated in total phenolic compounds, and this extract presented the best antioxidant activity. This study would be able to allow the development of natural drugs directed towards *fusarium* contamination and natural antioxidants for nutraceuticals.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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