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# Effect of soil amendment with *Trichoderma harzianum* and *Bacillus amyloliquefaciens* bioformulation on biochemical parameters and antioxidant activity in *Abelmoschus esculentus*

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**ABSTRACT:** Okra is a very popular vegetable fruit due to its nutritional and therapeutic potential, but its valorization is still marginalized in agricultural research centers. However, it possesses secondary metabolites not yet identified in the scientific literature and involved in the antioxidant defense system. This work aimed at assessing the effect of soil amendment on biochemical parameters of *Abelmoschus esculentus* leaf and fruit extracts, involved in the defense against ROS. It appears that the bioformulation used, highly influence some growth parameters and physicochemical parameters of soil. The screening of the different secondary metabolites was done by GC/MS performed with an Agilent 7890A GC coupled to an Agilent MSD 5975C inert mass spectrometer. The free radical scavenging activity was assessed by DPPH (2, 2-diphenyl-l-picrylhydrazyl); ABTS (2,2'-azino-bis- (3-ethylbenzothiazoline6-sulfonic acid)) reduction tests. The reducing power towards ferric ion (FRAP) was also assessed. It was determined that the leaf extract from the plot treated with the *Trichoderma harzianum* and *Bacillus amyloliquefaciens* generated more secondary metabolites than the control extract. This treated extract also showed high IPs at peak concentrations on the three assays DPPH, ABTS and FRAP which are respectively 90.37±1.21%; 91.47±1.01; 85.40 ± 0.14% against 62.55±7.28%; 67.83±4.56; 36.43 ± 0.12% for the control with a highly significant difference (p-value<0.001). The okra fruit extract where GPx, CAT and SOD were assessed, showed a highly significant difference (p-value<0.0001) among these enzymes and according to treatments. Further analysis of our study shows that these beneficial microorganisms positively influenced the agromorphological parameters and play an important role in the improvement of the biochemical parameters involved in the antioxidant activity of Abelmoschus esculentus.

**KEYWORDS:** *Abelmoschus esculentus; Trichoderma harzianum* and *Bacillus amyloliquefaciens;* antioxidant scavenging activity; secondary metabolites.

## 1 INTRODUCTION

Okra is a tropical flowering plant in the family of malvaceae and native of Africa. It has many nutritional, medicinal, artisanal, and even industrial benefits for humans. It is appreciated for its edible green colored pods [1]. In Sudan and Ghana, it represents in terms of consumption the fourth most important fruit after tomatoes, chili and eggplants; and the second in Cameroon after tomatoes [2]. It is a crop with an enormous economic potential for the poor communities because of its market potential in rural and urban areas [3]. Under optimal growing conditions, okra productivity can be expected to attain 30-40t/ha [4]. However, there is a large gap between potential and effective yield [5]. This low yield can be explained by the marginalization of okra in development and research programs [6]. It is also partly caused by inadequate soil nutrients. Thus, there is a need to minimize the difference between potential and expected yields.

It should be noted that soils are full of millions of beneficial microorganisms of indigenous origin such as growth-promoting rhizobacteria and fungi, thus playing the role of biostimulants which in the agronomic service, will take part in phytostimulation, plant defense, soil activation and play the role of agronomic additives [7]. They will degrade soil organic matter for better nutrient uptake by plants to ensure optimal growth and production. These microorganisms of indigenous origin produce both energy wealth substances such as organic acids and alcohols, sugars, amino acids and vitamins that can be easily reused by other organisms. They also produce bioactive substances and antioxidants such as vitamin E and flavonoids from organic substances [8].

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Vegetables and fruits are important sources of nutrients with nutritional and therapeutic values. Many researches showed these plants to be excellent sources of natural antioxidants for disease prevention and health care [9]. This potential is linked to their high content in secondary metabolites, including phenolic compounds. Indeed, they must on a daily basis, respond to the nutritional needs of the human body and participate in its protection against external aggressions, including diseases and premature aging [10]. The free radicals generated by the body would be the cause of several pathologies among which we can mention arthrosis, cancer, diabetes, heart disease, atherosclerosis [11]. These radicals result from oxidative stress that creates a state of imbalance between their production and antioxidant defense. This results in the variation of the oxidative stress of enzymes activity indirectly supporting the antioxidant defense system by catalyzing the dismutation of hydrogen peroxide. This potential is linked to the large amount of secondary metabolites that they have and that cannot be synthesized by humans. The ingestion of antioxidants such as phenolic compounds, through these fruits and vegetables, could allow our body to strengthen its defenses against the oxidation processes that threaten our cells daily [12]. Okra is a vegetable that provides an interesting contribution in antioxidants; but despite this, very limited data have been reported in the literature regarding its chemical composition, its content of secondary metabolites and on the biological activities of its extracts. It remains poorly known to the scientific community and marginalized in development and research programs [6]. This is why our study aimed at assessing the physicochemical parameters of soils and their influence on plant growth and biochemical parameters through the screening of secondary metabolites in leaf extracts and the antioxidant activity and oxidative stress enzymes in fruit extracts of *Abelmoschus esculentus* cultivated with some benefit microorganisms.

## 2 Material And Methods

### 2.1 Location

The study was conducted between August 2020 and February 2021 at Mbele II, a locality in the OBALA Subdivision, LEKIE Division, in the Centre Region of Cameroon.

The area is located at the altitude of 528 meters with 4°10'0'' N and 11°31'60'' E geographical coordinates. Its climate is tropical humid with an annual rainfall of about 1638 mm. The temperature is approximately 24.7°C [13].

### 2.2 Analysis Of Soil Sample Of The Study Area

Soil samples were taken with a hand auger on the first 20 cm of soil on the control plot before bioformulation application and at week five on the treated plot. The variables to be analyzed were pHwater content, organic matter (OM), cation exchange capacity (CEC), total nitrogen (Ntot), assimilable phosphorus (Pass), potassium (K), total carbon (C) according to the international methods recommended by [14] in Laboratory of Soil Analyses and Chemical Environment.

### 2.3 Preparation And Application Of The Bioformulation

The bioformulation was prepared according to the modified method described by [15] using the microbial strains *Trichoderma Harzianum* and *Bacillus amyloliquefaciens* acquired at the Dora AGRI-TECH laboratory associated with rice bran after their activation in molasses. A concentration of 20 g per hole was applied when seeding and three weeks later on the treatment except on the control. Harvesting was done 45 days after sowing.

### 2.4 Agromorphological Parameters

Agronomic parameters included plant emergence (%), plant vigor (%), average capsule weight (g) per treatment, leaf area ($cm^2$), fruit length (cm) and yield per treatment. This capsule production was estimated following a progressive harvest every seven days.

### 2.5 Vegetal Material

Young okra fruits and leaves were material used. The study focused on the Clemson Spineless variety grown in an experimental field in the bimodal rainfall agroecological zone of Cameroon in September 2020, on the T0 control and the T1 treatment based on bioformulation with T. *Harzianum* and B. *Amyloliquefaciens*. Harvesting was done 45 days after sowing in November 2020. The collected plant parts were previously washed and dried at the National Laboratory of Diagnostic Analysis of Agricultural Products and Intrants of the Ministry of Agriculture and Rural Development.

### 2.6 Extraction

The extraction was carried out on leaves previously delipidated with soxhlet (with methanol as solvent) according to the method of [16] by decoction under reflux of 50 g of powder in 500 ml of a hydro-methanolic mixture (20V/80V respectively) for 30 min with the aim

of extracting large quantities of polar compounds such as polyphenols. After filtration on Whatman paper, the filter residues were evaporated with a rotary evaporator at 60°C. The residues of this extrudate were dried in an oven for 48 h at 45 °C to obtain the dry extracts.

### 2.7 PHYTOCHEMICAL SCREENING

The methanolic extract for the screening of the different secondary metabolites analyzed by GC/MS was performed with an Agilent 7890A GC coupled to an Agilent MSD 5975C inert mass spectrometer. The gas chromatography is equipped with a VF-1MS capillary column (100% dimethylsiloxane, 20 m x 150 µm (inner diameter) x 0.15 µm (film thickness)) from Varian, 4 mm inner diameter liner. Carrier gas: helium (constant flow rate of 1 mL/min); oven programming: from 37 °C (1 min) to 250 °C at 5 °C/min, then 11 min step at 250 °C; source temperature: 230 °C; transfer line temperature: 260 °C; ionization energy: 70 eV. Electron ionization (EI) mass spectra were recorded between 40-400 u.

Constituent identification was based on comparison of the mass spectra with commercial, NIST 2012, Wiley, and in laboratory internal databases [17].

### 2.8 EVALUATION OF ANTIOXIDANT ACTIVITY

#### 2.8.1 DPPH TEST

The DPPH antiradical activity of the polysaccharide fraction was assessed according to the protocol of [18]. It is based on the reduction of the purple DPPH (1,1-diphenyl-2-picrilhydrazyl) radical to a yellow compound by hydrogen atom transfer to form the stable DPPH-H (1,1-diphenyl-2-picrylhydrazine). The intensity of the coloration, measured with a spectrophotometer at 517 nm, is inversely proportional to the antiradical activity of the compounds contained in the extract to be analyzed. In different test tubes were introduced 500 µL of extract at concentrations 50, 100, 150 and 200 µg/mL; 500 µL of a freshly prepared 400 µM DPPH methanolic solution.

Gallic acid at concentrations of 0.1; 1; 10; 100 and 1000 µg/mL was used as the standard. The control solution consisted of 500 µL of distilled water and 500 µL of DPPH solution. After 30 min of incubation at RT and in the dark against a blank consisting of 500 µL of methanol and 500 µL of distilled water, the optical density reading was taken at 517 nm on a Shimadzu UV-1605 spectrophotometer.

The free radical scavenging activity was expressed as percentage of DPPH- radical reduced and calculated as follows:

$$\text{Antiradical activity (\%)} = [1- (\text{DO sample } 517/\text{DO control } 517)] \times 100$$

#### 2.8.2 ABTS TEST

It was done according to the protocol described by [19]. In the presence of antioxidant proton donator, the green ABTS. + radical pulls out a proton leading to ABTS+. This leads to a decoloration of the solution with a maximum absorption at 734 nm. In different tubes, 500 µL of polysaccharide fraction at concentrations 50; 100; 150 and 200 µg/mL; 500 µL of freshly prepared ABTS reagent were introduced.

The OD was determined at 734 nm on a Shimadzu UV-1605 spectrophotometer after 30 min of incubation at RT and in the dark against distilled water (White). Gallic acid used as standards, was treated the same way at concentrations of 0.1; 1; 10 and 100 µg/mL. The polysaccharide fraction was replaced by distilled water in the control solution.

$$\text{Antiradical activity (\%)} = [1- (\text{DO sample } 734/\text{DO control } 734)] \times 100$$

The free radical scavenging activity was expressed as the percentage of reduced ABTS- radical

#### 2.8.3 FRAP TEST

The Ferric Reducing Antioxidant Power assay was performed according to the modified protocol of [20]. First, a 300 mM acetic acid/sodium acetate buffer solution at pH = 3.6 was prepared. The 10mM TPTZ reagent, diluted in 40mM HCl, is prepared extemporaneously. The second reagent FeCl3 at 20 mM is also prepared extemporaneously. Finally, the FRAP work solution is made by mixing 2.5 ml of TPTZ solution, 2.5 ml of FeCl3 solution and 25 ml of buffer solution. This solution must be kept in a bath at 37°C.

The test consists in mixing 100 µl of diluted extract (or methanol) in glass hemolysis tubes with 300 µl of distilled water and 3000 µl of FRAP working solution maintained at 37°C. The absorbance is measured at 595 nm after incubating the reaction in a thermostatic bath at 37°C in the dark for exactly 30 minutes.

The standard curve is established from the absorbances read for the range of ferrous sulfate heptahydrate solutions used as reference compound. It is of the form: **Abs= a× [Fe (II)] + b.** Each extract is tested, and the absorbance that results allows to determine its concentration in µM of Fe (II) equivalent.

Then considering the concentration of the extract under study, the AO is then expressed either directly in µM of Fe (II/) equivalent or in µmol of Fe (II) equivalent/g of dry extract and dry matter. Gradient gallic acid was used as a standard. Optical density was determined at 593 nm on a Shimadzu UV-1605.spectrophotometer after 30 min of incubation at room temperature and in the dark against distilled water. Iron reducing activity was expressed as µg gallic acid equivalent (GAE) /mg dry extract.

### 2.9 Determination Of The Enzyme Markers Activity Of The Oxidative Stress

#### 2.9.1 Superoxide Dismutase

Its activity was determined according to the method described by [21]. The principle is based on the capacity of inhibition of the autoxidation of pyrogallol by SOD at 420nm.

The measurement of the activity is performed in a final volume of 3ml. To 2.8ml of Tris HCl buffer (0.1mol, pH 8.2) are added 0.1ml of enzyme extract, 25µl of catalase (30µM in 0.1M phosphate buffer, pH 9) and 25µl of pyrogallol (24mM prepared in 10mM HCl). After incubation for 30s, the change in absorbance is measured at 420nm every minute, in the interval time of 3minutes.

The SOD activity is expressed as Unit/mg of protein. One unit of SOD activity is defined as the enzyme that would cause 50% inhibition of pyrogallol autoxidation.

#### 2.9.2 Catalase

Its activity is measured according to the method described by [22]. The breakdown of hydrogen peroxide is determined by the diminution of the absorbance at 240nm. In the presence of catalase, the breakdown of hydrogen peroxide leads to a decrease in the absorbance of the $H_2O_2$ solution as a function of time. The reaction medium contains 2.9ml of 0.1M phosphate buffer, pH 7 and 0.1ml of 20mM $H_2O_2$, and the enzyme extract. After incubation for 5 min, Titanium oxide sulfate (TiOSO4) reagent is added. The assay is performed at 240 nm every minute for a maximum of two to three minutes. The concentrations of the remaining $H_2O_2$ are determined from a standard range of $H_2O_2$ at concentrations of 0.5 to 2 mmol/l.

The calculation of a unit of enzyme activity is:

**A = log A1-log A2**

A1 is the starting $H_2O_2$ concentration

A2 is the concentration of $H_2O_2$ after incubation (after 5 min)

The specific activity is expressed in U/min/ml of protein.

#### 2.9.3 Glutathione Peroxidase

The GPx assay was based on the method used by [23]. The activity of this enzyme is determined via the formation of oxidized glutathione (GSSG) from reduced glutathione (GSH). The system requires the presence of an oxidant (cumene-$H_2O_2$) and glutathione reductase (GR) which reduces GSSG by oxidizing NADPH to NADP. The measurement consists therefore in following by spectrophotometry the disappearance of NADPH at 340 nm at 25°C in a mixture formed by the addition of: sodium-phosphate buffer (100 mM, pH 7.5) containing EDTA (1 mM), GSH (2mM), NADPH (0. 12 mM), GR (1Unit/ml), DL-dithiothreitol (1 mM) to stabilize enzymes containing sulphydryl groups, cumene hydroperoxide (0.8 mM) and a volume of the sample or blank buffer that completes the total volume (1 ml). A GPx assay setup was performed first to determine the appropriate dilution of the samples. This setup consisted in detecting a significantly different absorbance decay than the blanks by testing different dilutions. In the case of this assay, the molar extinction coefficient is 6.22 mM-1. cm-1. The GPx activity was thus calculated by applying the Beer-Lambert law:

**((ΔA sample-Δ blank) / ε l)**

Then it is multiplied by the dilution factor and finally normalized to the concentration of total protein in the corresponding S100 fraction and consequently expressed in µmoles of NADPH consumed per minute and per mg of total protein (mmoles/L Prot).

### 2.10 DATA ANALYSIS

The data were previously encoded on EXCELL and then analyzed using the Rcmd package of the Rversion 3.6.3 software where the normality and homogeneity of the different data were tested using the Shapiro-Wilk and Bartlett's K-squared tests respectively. The Tukey test was used to compare the different means through one-factor analyses of variance in order to determine the significant differences between them.

## 3 RESULTS

### 3.1 ANALYSIS OF THE SOIL SAMPLE

Table 1 presents the results of the different parameters assessed. It appears from these analyses that the difference is significant between these parameters and with treatments T0 and T1 ($p<0.05$), reporting thus the treated soil rich in organic matter (3.21%) with an almost neutral pH water (6.70).

***Table 1. Effect of bioformulation on soil physicochemical parameters before and after treatment***

| treatments | PHwater | OM (%) | Carbon (%) | N (%) | K (méq/100g) | Na (méq/100g) | CEC (méq/100g) |
|---|---|---|---|---|---|---|---|
| T0 | 5,50±0,00a | 0,97±0,02a | 0,54±0,02a | 0,07±0,01a | 1,11±0,01a | 0,02±0,01a | 24,33±2,08b |
| T1 | 6,70±0,00b | 3,21±0,03b | 1,50±0,10b | 0,14±0,01b | 1,90±0,09b | 0,02±0,00a | 18,40±0,10a |

**Means with the same letters on the same column are not significantly different according to Tukey's test P<0.05*
*OM=organic matter; N=nitrogen; K=potassium; Na=sodium; CEC=cation exchange capacity*

### 3.2 AGROMORPHOLOGICAL PARAMETERS

Table 2 shows the different agronomic parameters of the plants assessed on the control and the treatment. The analysis of variance shows that there was a significant difference between these agronomic parameters according to the treatments ($p<0.05$) except for the number of leaves and plant emergence. Treatment T1 showed the best parameters compared to T0 in leaf area (878.33 vs. 620 $cm^2$); plant height (32.56 vs. 22.46cm); plant vigor (86.48 vs. 71.77%); fruit length (11.53 vs. 7.30cm) and average weight of capsules per treatment (1868 vs. 960.66g).

***Table 2. Effect of bioformulation on agromorphological parameters of okra after five weeks***

| Treatments | Plants emergence (%) | Plant height (cm) | Number of leaves | Leaf area ($cm^2$) | Plant vigor (%) | Fruits length (cm) | Poids moyen des fruits par traitement (g) |
|---|---|---|---|---|---|---|---|
| T0 | 15,33±1,52a | 22,46±1,13a | 8,33±0,57a | 620,00±68,63a | 71,77±1,4a | 7,30±0,36a | 960,66±167,28a |
| T1 | 17,33±0,57a | 32,56±1,45b | 9,33±0,57a | 878,33±48,22b | 86,48±3,59b | 11,53±0,49b | 1868,00±279,45b |

**Means with the same letters on the same column are not significantly different according to Tukey's test P<0.05*

### 3.3 PHYTOCHEMICAL SCREENING

Phytochemical screening of T0 and T1 methanolic extracts through GS/MS, revealed several peaks (Figure 1&2). The retention times ranged from 3.788min to 7.141min for T0 and 3.587min to 11.312min for T1, respectively. This revealed the overexpression of secondary metabolites in okra leaves in the treated plot than in the control plot (Table3&4).

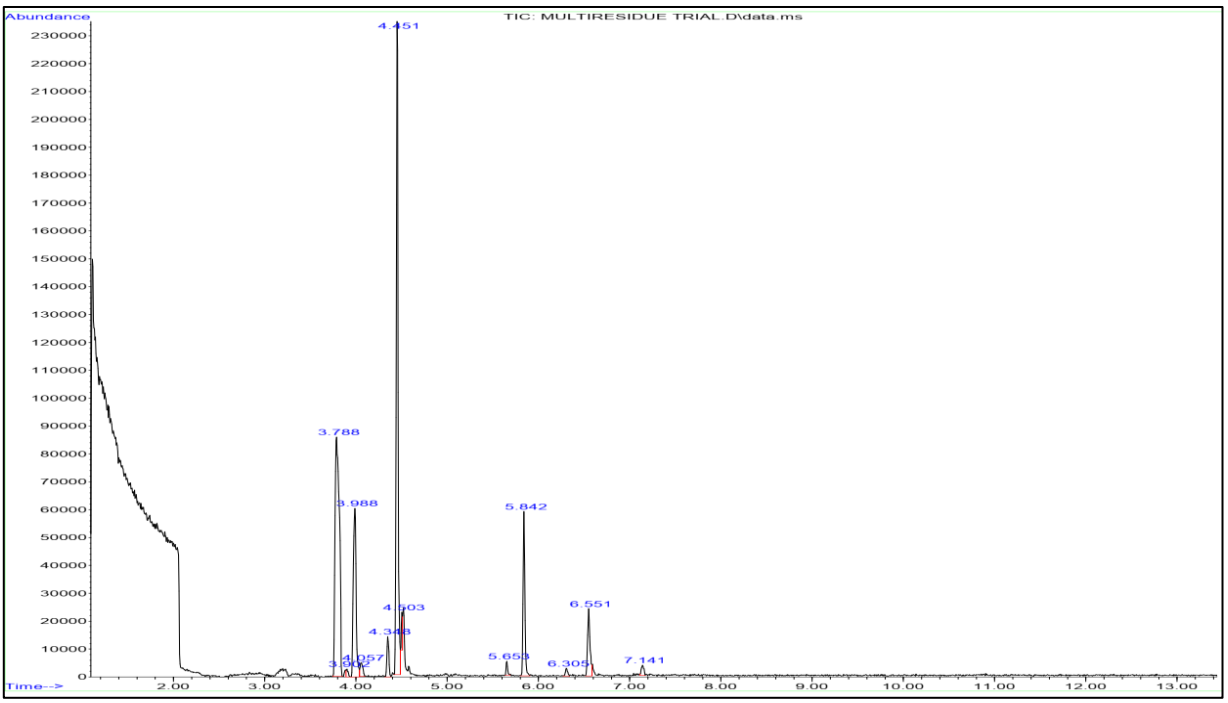


*Fig. 1. GC-MS chromatographic profile of methanolic extract of the control*

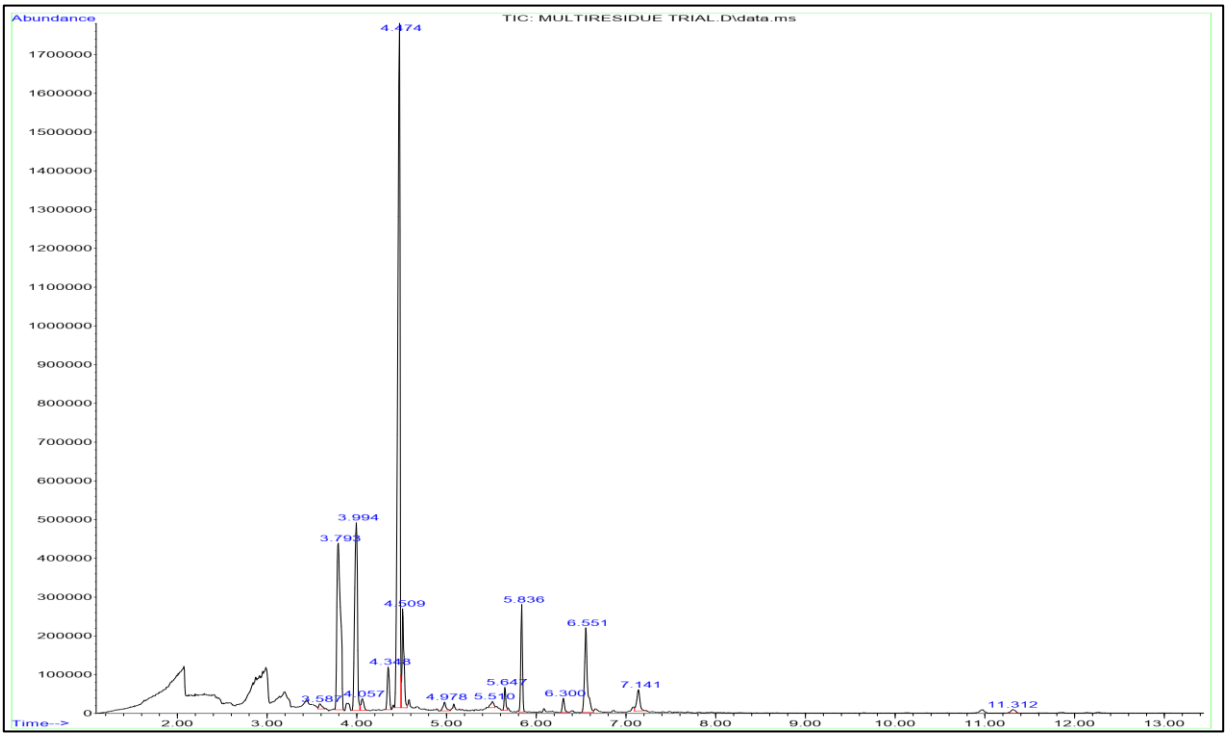


*Fig. 2. GC-MS chromatographic profile of the methanolic extract from the treated plot*

Several compounds of different molecular weight were obtained at each peak (Table 3&4). Acidic compounds such as decanoic, hexadecanoic and tetradecanoic acids were revealed. The sugar 2-O-Methyl-D-mannopyranose was also obtained as well as unidentified compounds (Table 4). Most of the compounds are acids.

***Table 3. Secondary metabolites expressed in the treatment***

| Hypothetic name | RT | Formula | Molecular weight (g/mol) | Abundance (%) |
|---|---|---|---|---|
| 3H-1,3,4-Benzotriazepin-2-one,1,2-dihydro-3,5-dimethyl- | 3,58 | $C_{10}H_{11}N_3O$ | 189,2 | 100 |
| 1-Phthalazinecarboxamide,3,4-dihydro-4- oxo- | | $C_{12}H_{13}N_3O_2$ | 279,29 | 27,06 |
| 2,4-Dimethyl-3-phenyl-isoxazol-5 (2H) -one | | $C_{11}H_{11}NO_2$ | 189,2 | 23 |
| unknown | | | 89 | 18 |
| unknown | | | 102 | 17,95 |
| 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester | 3,79 | $C_{16}H_{24}O_4$ | 278,3 | 100 |
| Phthalic acid butyl hept-2-yl ester | | $C_{19}H_{28}O_4$ | 320,4 | 13,57 |
| Phthalic acid, hexyl propyl ester | | $C_{17}H_{24}O_4$ | 292,4 | 12,76 |
| unknown | | | 57,10 | 11,82 |
| unknown | | | 150 | 9,70 |
| n-Hexadecanoic acid | 3,99 | $C_{16}H_{32}O_2$ | 256,4 | 100 |
| Tetradecanoic acid | | $C_{14}H_{28}O_2$ | 228,37 | 95,76 |
| unknown | | | 57,10 | 59,64 |
| unknown | | | 6910 | 46,77 |
| Hexadecanoic acid, ethyl ester | 4,05 | $C_{18}H_{36}O_2$ | 284,47 | 100 |
| Ethyl 13-methyl-tetradecanoate | | $C_{17}H_{34}O_2$ | 270,5 | 62,62 |
| unknown | | | 73 | 46,32 |
| unknown | | | 57 | 45,16 |
| 9,17-Octadecadienal, (Z) - | 4,34 | $C_{18}H_{32}O$ | 264,44 | 100 |
| 9,12-Octadecadienoic acid (Z, Z) -, methyl ester | | $C_{19}H_{32}O_2$ | 294,5 | 64,85 |
| 11-Octadecynoic acid, methyl ester | | $C_{19}H_{34}O_2$ | 294,5 | 50,54 |
| unknown | | | 82 | 49,25 |
| unknown | | | 60,10 | 34,93 |
| cis-13-Octadecenoic acid | 4,47 | $C_{18}H_{34}O_2$ | 282,46 | 100 |
| Oleic Acid | | $C_{18}H_{34}O_2$ | 282,46 | 63,33 |
| trans-13-Octadecenoic acid | | $C_{18}H_{24}O_2$ | 282,46 | 60,05 |
| unknown | | | 82,10 | 49,66 |
| unknown | | | 84,10 | 41,93 |
| Octadecanoic acid | 4,50 | $C_{18}H_{36}O_2$ | 284,47 | 100 |
| unknown | | | 58,10 | 51,70 |
| unknown | | | 70 | 50,62 |
| 4-Piperidinemethanol,.alpha.,.alpha.-diphenyl-1-methyl- | 4,97 | $C_{19}H_{23}NO$ | 281,4 | 100 |
| 1H-Imidazole-2-methanol | | $C_4H_6N_2O$ | 98,1g/mol | 72,44 |
| 1H-Inden-1-one, octahydro-7a-hydroxy- | | $C_9H_{14}O_2$ | 154,21 | 70,94 |
| unknown | | | 68,10 | 45,96 |
| unknown | | | 80,10 | 29,35 |
| 7-Hydroxy-3- (1,1-dimethylprop-2-enyl) coumarin | 5,51 | $C_{14}H_{14}O_3$ | 230,26 | 100 |
| Octanoic acid, 7-oxo-, ethyl ester | | $C_{10}H_{18}O_3$ | 186,25 | 66,14 |
| trans-2-Methyl-.beta.-methyl-.beta.-nitrostyrene | | $C_{10}H_{11}NO_2$ | 177,2 | 62,82 |
| unknown | | | 80 | 58,87 |
| unknown | | | 78 | 48,71 |
| Glycerol 1-palmitate | 5,64 | $C_{19}H_{38}O_4$ | 330,50 | 100 |
| Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester | | $C_{19}H_{38}O_4$ | 330,50 | 85,73 |
| Palmitoyl chloride | | $C_{16}H_{31}ClO$ | 274,9 | 78,49 |
| unknown | | | 75 | 54,94 |
| unknown | | | 70,10 | 53,82 |
| Bis (2-ethylhexyl) phthalate | 5,84 | $C_{24}H_{38}O_4$ | 390,54 | 100 |
| Phthalic acid, 2-ethylhexyl isohexyl ester | | $C_{22}H_{34}O_4$ | 362,5 | 31,32 |

| | | | | |
|---|---|---|---|---|
| Phthalic acid, di (oct-3-yl) ester | | $C_{24}H_{38}O_4$ | 390,6 | 25,64 |
| unknown | | | 56,10 | 14,26 |
| unknown | | | 71,10 | 13,82 |
| Butanedioic acid 2,3-dimethyl-, dibutyl ester | | $C_{14}H_{26}O_4$ | 258,35 | 100 |
| Succinic acid, ethyl 3- (2-methoxyethyl) nonyl ester | | | 130 | 71,02 |
| 5-Chloro-2-pyridinol | 6,3 | $C_5H_4ClNO$ | 129,54 | 69,97 |
| unknown | | | 102 | 50,82 |
| unknown | | | 82 | 48,78 |
| 9,17-Octadecadienal, (Z) - | | $C_{18}H_{32}O$ | 264,44 | 100 |
| 9,12-Octadecadienal | | $C_{18}H_{32}O$ | 264,44 | 74,03 |
| 9-Octadecenal, (Z) - | 6,55 | $C_{18}H_{34}O$ | 266,46 | 60,18 |
| unknown | | | 70,10 | 48,25 |
| unknown | | | 96,10 | 43,03 |
| E, Z-1,3,12-Nonadecatriene | | $C_{19}H_{34}$ | 262,5 | 100 |
| 1,3,12-Nonadecatriene | | $C_{19}H_{34}$ | 262,5 | 87,52 |
| 9,12-Octadecadienoyl chloride, (Z, Z) - | 7,14 | $C_{18}H_{31}ClO$ | 298,9 | 78,14 |
| unknown | | | 82 | 59,30 |
| unknown | | | 80,10 | 48,43 |
| 1- (2 Adamantylidene) semicarbazide | | $CH_5NO_3$ | 92,1 | 100 |
| 1,3-Bis- (2-cyclopropyl,2-methylcyclopropyl) -but-2-en-1-one | | $C_{16}H_{26}O$ | 258,4 | 73,20 |
| 2,8-Decadiyne | 11,31 | $C_{10}H_{14}$ | 134, 21 | 70,72 |
| unknown | | | 208 | 67,64 |
| unknown | | | 77,90 | 61,28 |

***Table 4. Secondary metabolites expressed in the treatment***

| Hypothetic name | RT (min) | Formula | Molecular weight (g/mol) | Abundance (%) |
|---|---|---|---|---|
| Phthalic acid, 8-chlorooctyl isobutyl ester | | $C_{20}H_{29}ClO_4$ | 368,9 | 100 |
| 2- ((prop-2-ynyloxy) carbonyl) benzoic acid | | $C_{11}H_8O_4$ | 204,17 | 15,40 |
| Phthalic acid, butyl isohexyl ester | 3,78 | $C_{18}H_{26}O_4$ | 306,39 | 14; 42 |
| unknown | | | 58,10 | 10,97 |
| unknown | | | 151 | 8,7 |
| Nonanoic acid, methyl ester | | $C_{10}H_{20}O_2$ | 172,26 | 100 |
| 2-O-Methyl-D-mannopyranosa | | $C_7H_{14}O_6$ | 194,18 | 76,22 |
| Octanoic acid, methyl ester | 3,90 | $C_9H_{18}O_2$ | 158,24 | 54,35 |
| unknown | | | 149,90 | 38,18 |
| unknown | | | 75,90 | 28,13 |
| n-Hexadecanoic acid | | $C_{16}H_{32}O_2$ | 256,42 | 100 |
| Tridecanoic acid | 3,98 | $C_{13}H_{23}O_2$ | 214,34 | 90,91 |
| unknown | | | 58,10 | 57,66 |
| unknown | | | 70,10 | 43,38 |
| Pentanoic acid, 4-methyl-, ethyl ester | | $C_8H_{16}O_2$ | 144,21 | 100 |
| Hexanoic acid, ethyl ester | 4,05 | $C_8H_{16}O_2$ | 144,21 | 77,13 |
| unknown | | | 73,90 | 64,75 |
| unknown | | | 58,10 | 54,63 |
| 11,14-Eicosadienoic acid, methyl ester | | $C_{21}H_{38}O_2$ | 322,5 | 100 |
| 9,12-Octadecadienoic acid, methyl ester, (E, E) - | | $C_{19}H_{34}O_2$ | 294,5 | 60,30 |
| 8-Hexadecyne | 4,34 | $C_{16}H_{30}$ | 22,41 | 49,72 |
| unknown | | | 70 | 43,61 |
| unknown | | | 75 | 37,11 |
| 9,12-Octadecadienoic acid (Z, Z) - | 4,45 | $C_{19}H_{34}O_2$ | 294,5 | 100 |
| 9,17-Octadecadienal, (Z) - | | $C_{18}H_{32}O$ | 280,44 | 75,62 |

| | | | | |
|---|---|---|---|---|
| unknown | | | 70,10 | 48,38 |
| unknown | | | 80,10 | 35,29 |
| Octadecanoic acid | 4,50 | $C_{18}H_{36}O_2$ | 284,47 | 100 |
| Pentadecanoic acid | | $C_{15}H_{30}O_2$ | 242,4 | 53,98 |
| n-Hexadecanoic acid | | $C_{16}H_{32}O_2$ | 256,4 | 52,90 |
| unknown | | | 70,10 | 48,47 |
| unknown | | | 58 | 47,75 |
| Oxacyclododecan-2-one | 5,63 | $C_{11}H_{20}O_2$ | 184,27 | 100 |
| Cyclooctaneacetic acid, 2-oxo- | | $C_{10}H_{16}O_3$ | 184,23 | 84 |
| unknown | | | 85 | 57,23 |
| unknown | | | 208,10 | 54,62 |
| Phthalic acid, 2-ethylhexyl isohexyl ester | 5,84 | $C_{22}H_{34}O_4$ | 362,5 | 100 |
| Bis (2-ethylhexyl) phthalate | | $C_{24}H_{38}O_4$ | 390,55 | 29,78 |
| 2- (Hexyloxycarbonyl) benzoic acid | | $C_{14}H_{18}O_4$ | 250,29 | 19,92 |
| unknown | | | 56 | 13,39 |
| unknown | | | 105 | 13,35 |
| Butanedioic acid, 2,2-dimethyl-, bis (1-methylpropyl) ester | 6,30 | $C_{14}H_{26}O_4$ | 258,35 | 100 |
| 5.alpha.-Androstan-16-one, cyclic ethylene mercaptole | | $C_{21}H_{34}S_2$ | 350,6 | 86,82 |
| Diisopropyl adipate | | $C_{12}H_{22}O_4$ | 230,3 | 65,27 |
| unknown | | | 130 | 56,07 |
| unknown | | | 58 | 51,46 |
| 9,12-Octadecadienoyl chloride, (Z, Z) - | 6,55 | $C_{18}H_{31}ClO$ | 298,9 | 100 |
| cis, cis-7,10, -Hexadecadienal | | $C_{16}H_{28}O$ | 236,39 | 83,83 |
| 1,3,12-Nonadecatriene | | $C_{16}H_{34}$ | 262,5 | 61,43 |
| unknown | | | 82,10 | 60,48 |
| unknown | | | 96,10 | 49,60 |
| 9-Octadecenal, (Z) - | 7,14 | $C_{18}H_{34}O$ | 266,46 | 100 |
| Cyclohexylamine, N- (2-chlorocyclopentylidene) -, N-oxide | | | 56,10 | 72,65 |
| 17-Octadecynoic acid | | $C_{18}H_{32}O_2$ | 280,4 | 53,40 |
| unknown | | | 67,75 | 48,71 |
| unknown | | | 208,10 | 45,31 |

### 3.4 ANTIOXIDANT ACTIVITY

#### 3.4.1 DPPH TEST

The analysis of antioxidant activity at different concentrations analyzed, revealed a highly significant inhibitory activity between the treated and control hydro-methanolic extracts ($p<0.001$). The treated extract showed a higher inhibitory percentage at all concentrations tested compared to the control: C50 (T1=81.00±4.34% vs. T0=42.67±5.26%); C100 (T1=84.87±3.60% vs. T0=44.14±4.61%); C150 (T1=88.02±2.29% vs. T0=52.85±4.32%) and C200 (T1=90.37±1.21% vs. T0=62.55±7.28%). The treatment showed its optimal activity at the concentration of 200µg/mL with a percentage of inhibition of 90.37±1.21%. As for the control, its highest inhibition percentage 62.55±7.28% was also found at the same concentration (Figure 1). Gallic acid used as a reference inhibited 85.56±2.23% of DPPH radical at a concentration of C100 compared to 84.23±1.06% for the treated extract and 45.53±0.98% for the control extract (Figure 1).

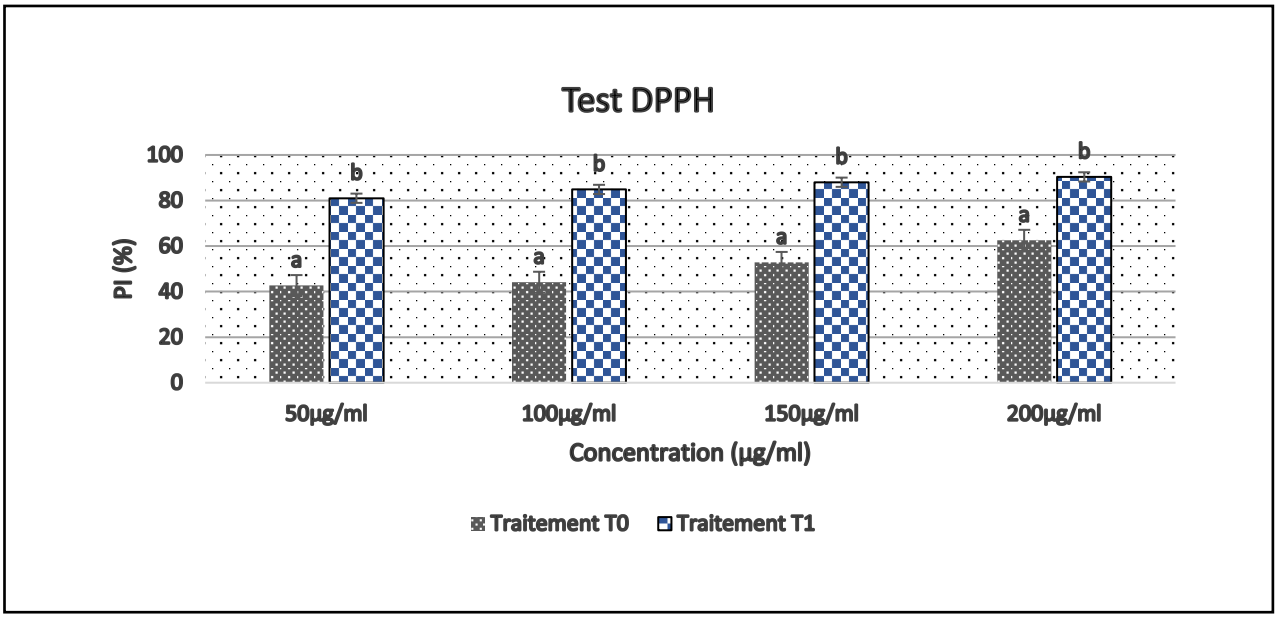


*Fig. 3. Percentage of DPPH radical inhibition by leaf extracts of Abelmoschus esculentus*

### 3.4.2 ABTS TEST

Analysis of the ABTS test results follow a normal distribution at different concentrations (pvalue>0.05). Figure 2 shows that the cation radical ABTS+- was significantly inhibited at all concentrations tested, and in a dose-dependent manner by the treated leaf extracts compared to the control extract; C50 (T1= 77.58±0.43% versus T0= 44.45±1.39%); C100 (T1=84.23±1.06% vs. T0=45.53±0.98%); C150 (T1=89.30±0.63% versus T0=48.85±1.32%) and C200 (T1=91.47±1.01% vs. T0=67.83±4.56%). Similarly, the difference was highly significant between treatments at the same concentrations (p-value<0.001). As for the DPPH assay, the extract showed better antioxidant activity in the treatment than in the control (Figure 1). At low concentration C50, the treatment showed an IP of 77.58±0.43 versus 44.45±1.39 for the control. The highest concentration C200 showed for both control and treatment, the IPs of 67.83±4.56 and 91.47±1.01. At the C100 concentration, the percentage of gallic acid inhibition taken as reference was assessed at 96.08±2.40% against 45.53±0.98% for the control and 84.23±1.06 T1.

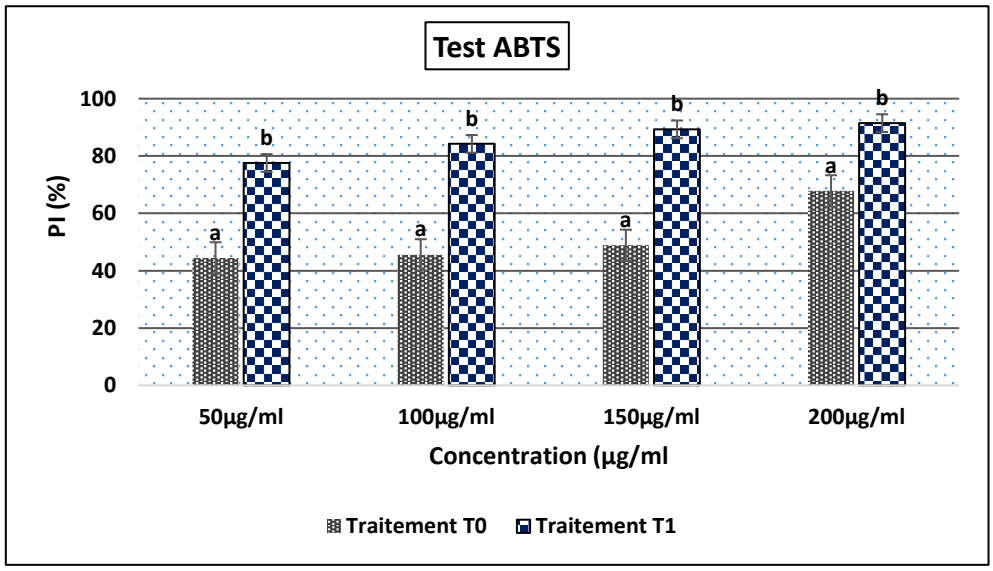


*Fig. 4. Percentage of inhibition of ABTS+ cation by extracts of leaves of Abelmoschus esculentus*

### 3.4.3 TEST FRAP

Analysis of the antioxydant data by Shapiro-Wilk test, do not follow a normal distribution at different concentrations (W = 0,68612; p-value = 0.8559). The analysis of the reduction power, revealed a highly significant inhibitory activity between the treated T1 and control T0 hydro-methanolic extracts (p<0.001). The treated extract (85,40 ± 0,14%) showed a higher inhibitory percentage compared to the control (36,43 ± 0,12%) (figure 3).

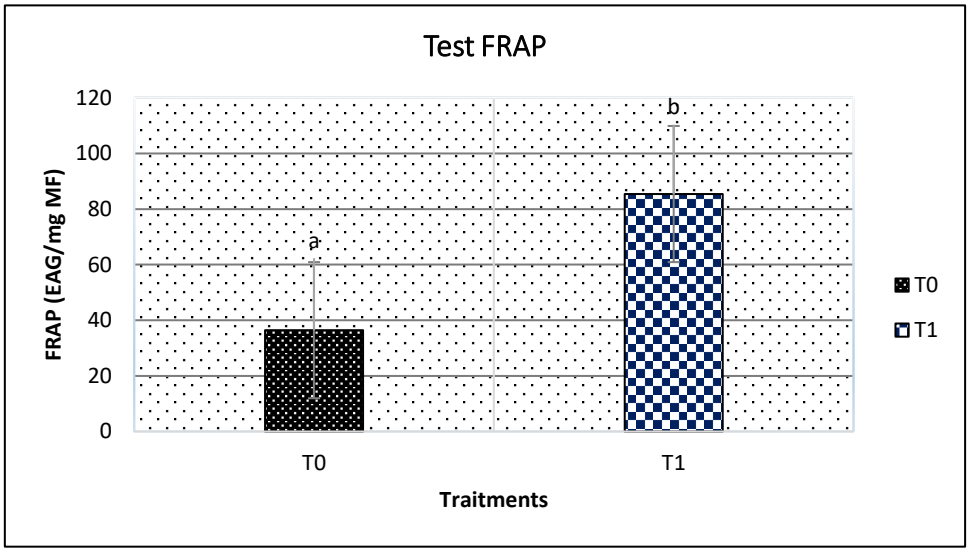


***Fig. 5. Reducing power of Abelmoschus esculentus leaf extracts***

### 3.5 OXIDATIVE STRESS ENZYMES

Analyses of variance of the oxidative power of catalase and superoxide dismutase showed a highly significant activity between enzymes according to their concentrations (p-value<0.001) and between enzymes according to the treatments (p-value<0.001). Glutathione peroxidase from its concentration also showed highly significant activity between T1 and T0 (p-value<0.001). The highest enzymatic activity was attributed to catalase whose concentration in the fruit extract of T1 treatment was 38.47 ± 1.40 U/min/ml/protein compared to that of superoxide dismutase (25.47 ± 0.021.40 U/min/ml/protein) for the same unit (Figure3). The level of glutathione peroxidase (5.70 ± 0.20 mmol/L) is higher than the control (3.21 ± 0.02 mmol/L). CAT and SOD for the control show lower activity than the treatment at the respective concentrations of 17.93 ± 0.58 U/min/ml/protein and 13.27 ± 0.02 U/min/ml/protein (Figure 4).

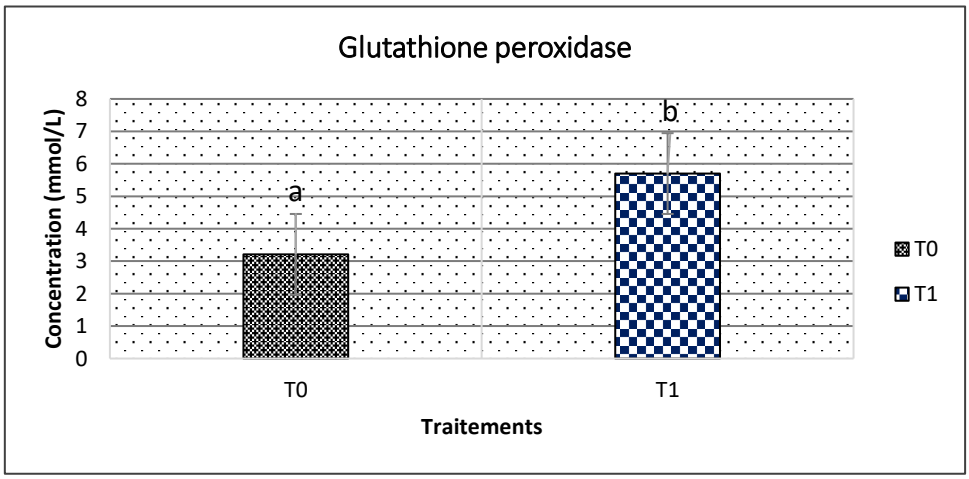


***Fig. 6. GPx content of Abelmoschus esculentus extracts***

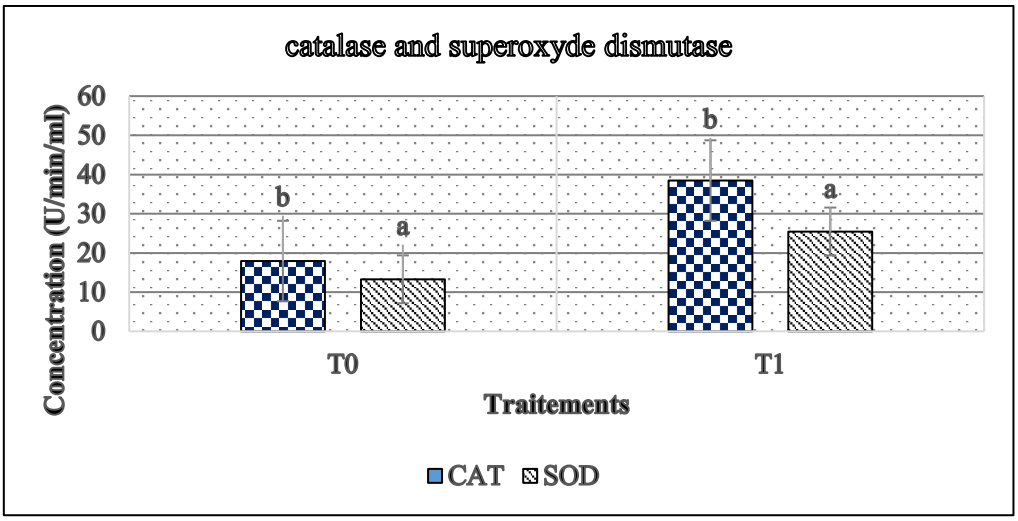


*Fig. 7. CAT and SOD content of Abelmoschus esculentus extracts*

## 4 DISCUSSION

The present study was carried out in order to evaluate the effect of soil amendment using *Trichoderma harzianum* and *Bacillus amyloliquefaciens* bioformulation on the biochemical parameters and antioxidant activity of okra extracts. It was reported that bioformulation positively influenced the organic matter content (3.21%) and pHwater of the cropland (6.70) to be nearly neutral. This contributed to the effective growth of okra, which requires an optimal pH between 6 and 7 [24]. The relative moisture of the agro-ecological zone with bimodal rainfall is about 1638 mm, which responds favorably to the water required for okra at the vegetative growth stage for an average need of 900 to 1200 mm [25]. The temperature of the environment, which is 24.5°C, also satisfies the needs of the plant for rapid growth at a temperature between 20 and 30°C [25]. Regarding the agromorphological parameters, it was found that they were favorably influenced by the bioformulation in terms of plant height, plant vigor, leaf area, fruit length, and an average weight of the treatment. This could be justified by the biostimulant activity of *Trichoderma harzianum* and *Bacillus amyloliquefaciens* that contributed to mineralize organic matter to make it available to the plant for optimal growth [26] with their ability to colonize plant roots. The increase in plant vigor could be attributed to *Trichoderma Harzianum* which has the ability to parasitize fungi belonging to a very wide range of plant pathogens producing harmful secondary metabolites to the plant according to [27].

The hydro-methanolic solution used in the present study permitted to extract secondary metabolites from okra leaves. It was found that okra leaves treated with *Trichoderma harzianum* and *Bacillus amyloliquefaciens* bioformulation have more metabolites belonging to several chemical classes compared to the control. This could be due to the capacity of these microorganisms to secrete bioactive molecules that would stimulate the expression of genes responsible for metabolic synthesis pathways in *Abelmoschs esculentus* plant, whence the overexpression of these secondary metabolites. These results confirm those of [28] that stated that the leaves of *Datura innoxia Mill*. grown out of soil and in nutritional condition overexpressed secondary metabolites of different chemical classes. Secondary metabolites such as polyphenols are not produced directly during photosynthesis but are the result of subsequent chemical reactions; suggesting that biosynthesis of these metabolites occurs in an enhanced manner in the organs at the time of their optimal growth to ensure their protection and fructification of plants. This assertion is corroborated by [29] who stated that like primary compounds, the composition of secondary metabolites in organs changes with their degree of maturity. This justifies the choice of our collection of plant material at the reproductive stage of the plant.

Regarding the antioxidant activity which corresponds to the capacity of a compound to oxidize, most of the antioxidants of synthetic or natural origin have hydroxy phenolic groups in their structure and the antioxidant properties are attributed partially to the capacity of its natural compounds to scavenge free radicals such as hydroxyl radicals ($OH^+$) and superoxide ($O_2^+$) Bartosz (2003). The results obtained showed that the hydro-methanolic extract of the fruits from the treated soil was more active than that of the untreated soil in the three tests performed. furthermore, at the same concentrations tested, the IPs obtained by the DPPH test was slightly lower than those of the ABTS method. This could be explained by the presence of substances that show absorption bands at the same wavelength as those of the $DPPH^{\cdot}$ radical leading to the increase of absorbance [11] and thus complex interpretations. The FRAP test shows a higher ferric ion reducing power of the treated leaves than the control. Thus, from these 3 tests, it was found that the antioxidant activity of the leaves extract of T1 is higher than the T0. This could be justified by the content of secondary metabolites like polyphenol class overexpressed in the plants of this treatment T1 compared to the control T0 as previously described by [12] where plant of *Piliostigma thonningii Schumach* through their secondary metabolites contain, increased their antiradical activity.

## 5 CONCLUSION

The aim of this work was to analyze the effect of bioformulation with *Trichoderma harzianum* and *Bacillus amyloliquefaciens* on the physicochemical parameters of the soil and biochemical parameters of okra plant. A phytochemical screening was carried out to identify the different chemical compounds present on okra leaves. The identification of these different compounds influenced by microorganisms would allow a better exploitation of the plant in terms of nutrition and health level. The analysis of the antioxidant activity and antioxidant enzymes stress of the fruit extract shows that the symbiosis of the beneficial microorganisms through bioformulation positively influenced these different parameters. The results also show that the symbiosis of beneficial microorganisms through bioformulation positively influenced these parameters, as well as soil physicochemical parameters and plant growth. The overall results thus show a significant influence of bioformulation on the treatment compared to the control.

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