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EFFECT OF FERTILIZATION AND HARVESTING TIME ON ANTIOXIDANT ACTIVITY OF THREE LEAFY VEGETABLES COMMONLY USED IN BENIN

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ABSTRACT

Leafy vegetables are an excellent source of bioactive factors, traditionally used as important medical ingredients. Recently, some leafy vegetables are domesticated without the use of fertilizer, as well as the assessment of the effect of fertilizer on their nutritional value. This study aims at testing the effect of three mineral and organic fertilizers (Cowpat, NPK and NPK + cowpat) on three traditional leafy vegetables: *Ceratotheca sesamoïdes*, *Sesamum radiatum* and *Justicia tenella*. Their antioxidant activities were assessed at different harvesting times varying from six (6) to fourteen (14) Weeks After Transplantation (WAT) using three *in vitro* methods: Ferric reducing antioxidant power (FRAP), 2,2'-Azinobis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picryl hydrazil (DPPH). The FRAP, DPPH, ABTS assays were consistent and positively correlated ($p < 0.001$). Total antioxidant activities of leafy vegetables depended on species, harvesting time and methods. They ranged from 32.0 to 45.7 µmol Fe/g DW (for FRAP), with non fertilized *J. tenella* cut at 12 WAT giving the highest antioxidant activity. Percentage of inhibition using DPPH assay ranged from 11.4 to 87.2 % and showed that *J. tenella* fertilized with NPK and cowpat, and harvested at 9 WAT had the highest antioxidant activity. Regarding ABTS, the range of 17.6 to 28.9 µmol TE/g DW was recorded, and the leaves of *C. sesamoïdes* harvested at 10 WAT and fertilized with cowpat showed the highest level. Compared to other species, those studied here may best contribute to improve human health related to degenerative diseases. Moreover, significant and positive correlations were observed between the total phenolic compounds content and antioxidant activities of leaves regardless the methods used. The positive and significant correlations between the three assays (FRAP, DPPH, ABTS) allow to suggest the use of only anyone of them to check factors in the study. This paper highlights the potential of antioxidant capability of the leafy vegetables even fertilized.

Key words: Fertilizer, antioxidant, *Sesamum, Ceratotheca*, *Justicia*

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INTRODUCTION

Sesamum radiatum Schumach. & Thonn., *Ceratotheca sesamoïdes* Endl. and *Justicia tenella* Nees T. Anderson are common leafy vegetables used in many foods preparation in the Central and Northern parts of Benin [1]. Besides their use as traditional medicinal ingredients against many diseases, they constitute excellent sources of bioactive factors such as β-carotene, ascorbic acid and phenolic compounds [1, 2, 3, 4]. These compounds act as antioxidants and play important roles in the prevention of cell damage and degenerative diseases. Moreover, antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [5]. Leafy vegetables are rich sources of antioxidants. It was demonstrated that much of the total antioxidant activity of fruits and vegetables depends on their total phenol content [6]. The role of polyphenols in the prevention of degenerative diseases, particularly cardiovascular, cancers osteoporosis and neurodegenerative diseases and diabetes was acknowledged [2]. As antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress [7].

The recent domestication of some important leafy vegetables widely used by local communities in their daily diets seems a logical endogenous strategy that will strengthen regional capacity for agro biodiversity conservation. This strategy is rooted in the understanding of the variability within materials being traditionally domesticated across broad geographical ranges in order to increase the global production of leafy vegetables in vulnerable areas. Recent studies have mentioned contradictory theses of the effect of environmental factors and agricultural techniques on antioxidant activity. It was found that the use of NPK for fertilization influenced antioxidant capacity of Campbell and Export II varieties of tomatoes and bio-fertilizers enhanced the antioxidant activity of basil plant extract [8, 9]. Reversely, other studies concluded that nitrogen fertilization did not affect significantly antioxidant activity of pepper [10].

In view of this situation and due to the lack of information on antioxidant activity of fertilized local leafy vegetables consumed in Benin, it is necessary to establish the trends of fertilizers' response in producing these leaves.

Furthermore, antioxidant capacities involved many mechanisms evidenced by different assays. The 2, 2'- Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay is frequently used for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidant in many plant extracts [11]. Like ABTS, 2,2-diphenyl-1-picryl hydrazil (DPPH) assay is one of the compounds that possess a proton free radical; so it measures radical scavenging activity. Ferric reducing power (FRAP) in leaf tissue is related to their electrons transfer ability and may, therefore, serve as a significant indicator of its potential activity [12]. It is a simple assay that gives fast and reproducible results [13]. In this respect, it was

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suggested to perform more than one assay in order to take into account the different mechanisms of antioxidant activity.

This research aims at investigating the effect of three fertilizers and different harvesting times on the antioxidant potential of three leafy vegetables, using DPPH, FRAP and ABTS assays [14].

MATERIAL AND METHODS

Study area

The experiment was carried out from May to November 2008 in two contrasting ecological zones of Benin: the experimental site of the International Institute of Tropical Agriculture (IITA, Benin Sub-station in the Guineo-Congolese region) and the local farmlands in Savè, a village located in Central Benin in the Soudano-Guinean region (Table 1).

Field experiment

The experiment was set up in a random complete block design with five replications and two (2) main factors. The first factor (fertilizer) was constituted of four (4) treatments: control (no fertilizer), chemical fertilizer (NPK), organic fertilizer (Cowpat) and mixture of chemical and organic fertilizers. The NPK used in this study is a combination of urea, tri-super phosphate (P_2O_5) and potassium sulfate (K_2SO_4), with a formulation ratio of 10-10-20. The second factor was the harvest time (Table 2). The seeds of *S. radiatum* and *J. tenella* were collected at Savè in central Benin and Natitingou in Northern Benin, and germinated during 30 days in the experimental areas. In the case of *C. sesamoïdes*, the seeds were not germinated, but young plants, which grow naturally in the area, were just dug up. The experimental units were rectangular plots of 4.5 m x 1.5 m. The plots were prepared one week before transplanting because of burring of cowpat, potassium sulfate and tri-super phosphate. Thirty (30) days after nursery, young plants of *S. radiatum*, *J. tenella and C. sesamoïdes* were transplanted. Recommended cultural practices such as watering and eradication of weeds were adopted uniformly according to standard crop requirements. Moreover, weeds were manually controlled with hoe each week.

Sampling

For all leafy vegetables, harvests began six weeks after transplanting (WAT) and took place three times (Table 2) from randomly selected rows per plot. For *S. radiatum* and *J. tenella,* harvest took place at 6, 9 and 12 WAT while the harvest of *C. sesamoïdes* took place at 6, 10 and 14 WAT (because of low quantity of leaves of *C. sesamoïdes* observed at 9 WAT).The cutting was realized at 10 cm of collar of plants. The plants which were located in the border lines and in the end of harvest lines were not considered. After harvesting, edible parts were separated from the branches. Edible leaves from *S. radiatum* and *C. sesamoïdes* were wiped with a kitchen towel because of their slimy characteristic and *J. tenella* leaves were soaked in tap water to remove soil, put in sieves and left to drain before analyses. Samples were oven-dried at 60 °C and reduced in powder with a Moulinex blender before analysis.

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Antioxidant activity

Preparation of the crude extract for antioxidant activity measurement

For Ferric reducing antioxidant power (FRAP) and 2, 2'- Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays, the extract was obtained [15]. 50 mg of dried leaf flour was extracted using 1.5 mL of HCl/methanol (1%v/v) for 1h under continuous stirring at room temperature. The mixture was centrifuged at 7000xg for 10 min and the supernatant was removed. The pellet was then re-extracted as described above while supernatants were pooled.

In the case of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, 25 mg of dried leaf flour was extracted with 1.125 mL of aqueous methanol (80%v/v) for 2h under continuous stirring at room temperature [16]. The mixture was treated as described above. The supernatant of the methanol extract was collected and filtered through a 90 mm Whatman paper N° 1 and diluted at 5% with aqueous methanol.

Ferric reducing power (FRAP) assay

The FRAP assay was done by measuring at 595 nm the absorbance of colored solutions as a result of reducing of Fe (II) [13].

ABTS scavenging activity

The 2,2'-Azinobis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging was determined using Benzie and Strain and Moore *et al*. modified method [17, 18]. This method is based on the capacity of antioxidant to quench the long-lived $ABTS^{+}$. Spectrophotometer was calibrated with typical Trolox standard concentrations in steps of 10, 50, 100, 200 and 300 µM. 100 µL of plant extract was incubated for 1min with 1.25 mL of $ABTS^{\cdot+}$ working solution (100 µL of $ABTS^{\cdot+}$ activated solution in 10 mL ethanol) and absorbance was measured at 734 nm with a spectrophotometer (6715 UV/Vis Spectrophotometer JENWAY). Data was expressed in µmol of Trolox equivalents (TE) per gram sample.

DPPH scavenging assay

Antioxidant activity was firstly measured using the 2,2-diphenyl-1-picryl hydrazil (DPPH) reagent according to Brand-Williams *et al*.[11] modified by Zhang and Hamauzu [16] and Turkmen *et al*. [19]. The crude extract was filtered through a 90 mm Whatman paper N°1 and diluted at 5% (6 mg/mL). A solution of 0.1 mM of DPPH in methanol was prepared and 1.5 mL of this solution was treated with 0.5 mL of the diluted extract. A control was treated with 0.2 mL of distilled water instead of the extract. The mixture was left at room temperature for 60 min before the decrease in absorbance at 517 nm was measured with a spectrophotometer (6715 UV/Vis. Spectrophotometer JENWAY). Antioxidant activity was expressed as percentage inhibition of DPPH radical.

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Statistical analysis
Analysis of variance on repeated measures [20] was performed on the data with SAS program (SAS, v 9.2) to test the effect of fertilizers and harvesting time on the antioxidant activity of the leafy vegetables. The effect of fertilizers used was compared to natural product using Dunnett test [21] at 5% significance level. Correlation between total polyphenols and antioxidant capacity assays and between methods used to evaluate antioxidant activity were calculated using Pearson's correlation coefficient (r).

RESULTS

Fertilizer and time of harvesting significantly affected ($p < 0.001$) antioxidant activity of *S. radiatum, C. sesamoïdes* and *J. tenella* leaves based on DPPH, ABTS and FRAP assays (Table 3, 4, 5). Moreover, the interaction between these factors (fertilizer type* harvesting time) was also significant.

Ferric reducing power (FRAP)
Antioxidant activity assayed by FRAP increased during the harvesting time for *S. radiatum and C. sesamoïdes* irrespective of the fertilizer while for *J. tenella,* the trends varied with the fertilizer type (Table 6, 7, 8).

In the case of *J. tenella* (Table 6), the antioxidant activity varied between 37.8 and 45.7 µmol Fe/g dry weight (DW), with the non fertilized *J. tenella* obtained at the third harvest showing the highest antioxidant activity. With the exception of cowpat fertilizer, all fertilizers used had an optimum antioxidant activity at second harvesting time.

As far as *S. radiatum* is concerned (Table 7), FRAP ranged from 32.0 to 37.2 µmol Fe/g DW. Antioxidant activity of all of leaves had an upward trend from the first to the third harvesting time, but it was particularly higher in leaves fertilized with cowpat at the third harvesting time.

With respect to *C. sesamoïdes,* leaves' antioxidant potential ranged from 34.3 to 44.5 µmol Fe/g DW (Table 8). There was no significant difference between control and cowpat fertilization at second and third times.

ABTS scavenging activity
Likewise, FRAP assay antioxidant activity had an upward trend from the first to the third harvesting time with the range of 17.6 to 26.2, and 21.7 to 28.9 µmol TE/g DW for *S. radiatum* and *C. sesamoïdes*, respectively. *J. tenella* had the lowest antioxidant activity (Table 6) for all fertilizer compared to the control at all harvesting time.

For *S. radiatum* (Table 7), cowpat gave the lowest value at the first harvesting time while the highest were obtained for NPK and for the mixture of "NPK and cowpat" compared to the control. At the second harvesting time, the highest antioxidant

activity was obtained with NPK fertilization while at the third harvesting; the cowpat gave the highest value.

Concerning *C. sesamoides* (Table 8), NPK fertilizer had significantly the highest value at the first time and the lowest at the second harvest compared to the control. Among all vegetables studied using ABTS test, non fertilized leaves of *C. sesamoïdes* at third cut had the highest total antioxidant content (28.9 µmol TE/g DW). In the case of *J. tenella*, it seemed to decrease slightly.

DPPH scavenging assay

Radical scavenging activity ranged from 11.38 to 75.02 %, 35.80 to 84.30% and 75.33 to 87.36 % for *S. radiatum, C. sesamoïdes* and *J. tenella,* respectively (Table 6, 7, 8).

Compared to the control, most of fertilized leaves had significant highest scavenging activity ($p < 0.05$) for *S. radiatum* (Table 7) at the two last harvesting times, whereas samples from cowpat had the lowest value at first time. As for *C. sesamoïdes* (Table 8), all treatments had an upward trend from first to third time. In general, a significant ($p < 0.05$) lowest antioxidant activity was observed at the two last harvest times for fertilized leaves compared to control. Fertilizers had various effects on antioxidant capacity for *J. tenella* (Table 6) from one harvest to another. With the exception of NPK, all fertilizers used had an optimum scavenging activity at second harvesting time.

For DPPH assay, the highest antioxidant activity was found in *J. tenella* fertilized with "NPK and cowpat", cut at second harvest (87.61%) followed by unfertilized leaves of *J. tenella* cut at the same time. Unfertilized *C. sesamoïdes* cut at second harvest time presented also high antioxidant capacity (84.36 %). This results were similar to those obtained with FRAP assay.

DISCUSSION

Antioxidant activity

Total antioxidant in this study was measured by three methods (FRAP, DPPH and ABTS assays), which exhibited high antioxidant potential.

Compared to other vegetables, leaves studied had similar antioxidant capacity based on FRAP assay with the ones of dried okra (42 µmol Fe/g DW) but lower than those of African baobab tree dried leaves and *Moringa Stenopetela* dried leaves (480 µmol Fe/g DW) and stem (120 µmol Fe/g DW) [22].

The results obtained from FRAP and ABTS assays were probably due to various phenolic compounds present in the extracts prepared from leaves which could have different responses to various kinds of free radicals [23].

Antioxidant activity of studied leaves based on ABTS was higher than some of values of Indonesian vegetables such as *Portulaca oleracea, Centella asiatica, Talinum triangulare, Ocimum americanum* L. (0.613- 2.16 µmol TE/g DW) [24].

Some compounds, which have ABTS scavenging activity, did not show DPPH scavenging activity [25]. This is not the case in this study. All the extracts tested had the capability to scavenge different free radicals in different systems. These were in agreement with literature, which suggested that the compounds, which could scavenge DPPH radical, were also able to scavenge ABTS radical cation and reduced ferric ions [23].

All leaves studied had antioxidant activity compared to one of some tropical green leafy vegetables consumed in Nigeria (15 to 61%) and in Malaysia (15.44-83.27%) [26,27]. *C. triloba*, species from the same family with C. *sesamoïdes* and *J. flava* from the same family of *J. tenella* had high level of antioxidant activity (84% and 96%, respectively) comparable with the values obtained in the studied leaves [28]. The high antioxidant activity obtained of previous studies for *C. triloba* and *J. flava* could be explained by the high concentration of extract (100 mg/ml) used in the case of the previous study and by the difference in varieties and cultivars of the same plant.

The three *in vitro* methods used to determine antioxidant activity on leafy vegetables showed different results. The same trend was mentioned in the case of Malaysian vegetables and suggested that this observation could be due to the different antioxidant compounds detected for each assay [27].

Correlations DPPH, ABTS, FRAP assays with TPC and between methods used to evaluate antioxidant activity

The total phenolic contents (TPC) of leaves were previously investigated and the results showed the high amounts of TPC in different leaves [5]. Total phenolic contents (TPC) were highly correlated with FRAP assay ($r= 0.805$, $p<0.001$) with DPPH assay ($r=0.750$, $p<0.001$) and ABTS assay ($r=0.624$, $p<0.001$). Antioxidant measures could indirectly be estimated by using TPC since they showed high correlation with all assays [29]. This appeared to be a trend in many plant species [30]. Indeed, previous studies reported a high correlation between TPC and antioxidant activity for vegetables from Indonesia and grapes [24, 29].

These high correlations indicated that TPC in studied leaves contributed highly to their antioxidant activity.

In addition, FRAP and DPPH were highly correlated ($r=0.866$, $p<0.001$), and then DPPH and ABTS ($r=0.608$, $p<0.001$) or FRAP and ABTS showed significant correlation ($r=0.584$, $p<0.001$). These were in agreement with some works, which suggested that the compounds, which could scavenge DPPH radical were also able to scavenge ABTS radical cation and to reduce ferric ions [25]. The positive and significant correlations between the three assays (FRAP, DPPH, ABTS) allow to suggest the use of only anyone of them to check factors in study.

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CONCLUSION

This study revealed that *C. sesamoïdes* and *J. tenella* both had a higher antioxidant activity than *S. radiatum* regardless of treatments and harvesting period. Leafy vegetables studied could contribute to improve human health related to degenerative diseases. Fertilizers had significant effect on antioxidant activity depending on species and harvesting time. Correlations between antioxidant activity and TPC and between assays revealed that TPC is a good indicator of antioxidant activity, and they allow suggesting the use of only anyone of them for determining antioxidant activity of vegetables leaves.

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Table 1: Characteristics of experimental sites in Benin: Adapted from WorldClim database (http://www.worldclim.org/current) and FAO soil database (www.fao.org/geonetwork/srv/en/main.home#). Accessed on 12th March 2010.

Place	IITA, Benin Sub-station	Local Farmland of Savè, Benin
Region of Benin	South	Centre east
Geographic location (in decimal degree)	6.36°N / 2.54°E	7.78° N / 2.28°E
Annual precipitation (mm / year)	1200	1060
Annual mean temperature (°C)	27.3	27.7
Soil types and characteristics	Clayey tropical ferralitic soil; moderately well drained, with 100 cm depth, topsoil pH:5.6, CEC (Cationic Exchange Capacity: 8cmol/kg, 0.86% topsoil carbon content	Tropical ferruginous soil, poorly to moderately drained with 100 cm depth, topsoil pH:6.3, CEC (Cationic Exchange Capacity):10.3 cmol/kg, 0.9% topsoil carbon content

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Table 2: Experimental design and harvesting time of leafy vegetables

Fertilizer	Dose (kg. ha-^{1})	Time of application	Season during experiment	Plantation density	Harvest time (Week After Transplanting: WAT)
Control	-	-	Raining season	16 plants.m^{2} (*S. radiatum* and *J. tenella*) 5plants.m^{2} (*C. sesamoïdes*)	6, 9 and 12 (*S.radiatum* and *J. tenella*) 6, 10 and 14 (*C. sesamoïdes*)
Cowpat	20.10^{3}	One week before transplanting			
NPK -tri super phosphate (TSP) with 46% of phosphorus -potassium sulfate with 50% of potassium -urea([CO $(NH_2)_2$] with 36% of nitrogen -	400	One week before transplanting One week before transplanting 1, 2 and 3 weeks after transplanting			
NPK + Cowpat	400+20.10^{3}	Each fertilizer is applied according to preceding lines			

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Table 3: F values and significance of antioxidant activity of *Sesamum radiatum* for different treatments and harvesting times

Source	df	FRAP	ABTS	DPPH
		F values		
Treat	3	8.03ns	9.55***	411.91***
Plot	2	164.42***	135.86***	3964.93***
Treat*plot	6	31.58***	6.89***	322;25***
Time	2	75.51***	1432.59***	23836***
Time*treat	6	6.63***	25.88***	604.58***
Time*treat*plot	12	20.66***	9.67***	473.85***

df: degree of freedom, *F*: Fisher, ***Significant at 0.001, *ns*: not significant, treat: treatment

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Table 4: F values and significance of antioxidant activity of *Ceratotheca sesamoïdes* for different treatments and harvesting times

Source	df	FRAP	ABTS	DPPH
		F values		
Treat	3	10.93***	19.75***	1387.87***
Plot	2	79.20***	3.44ns	38.72***
Treat*plot	6	8.52***	37.07***	50.11***
Time	2	1900.89***	3509.10***	36988.9***
Time*treat	6	32.28***	36.26***	72.92***
Time*treat*plot	12	27.67***	24.02***	36.61***

df: degree of freedom, *F*: Fisher, ***Significant at 0.001, *ns*: not significant, treat: treatment

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Table 5: F values and significance of antioxidant activity of *Justicia tenella* for different treatments and harvesting times

Source	df	FRAP	ABTS	DPPH
		F values		
Treat	3	18.02***	48.71***	173.01***
Plot	2	15.49***	44.46***	263.68***
Treat*plot	6	7.59***	14.08***	119.06***
Time	2	7.03ns	110.97***	538.52***
Time*treat	6	30.12ns	12.77***	38.45***
Time*treat*plot	12	11.28***	21.53***	53.68***

df: degree of freedom, *F*: Fisher, ***Significant at 0.001, *ns*: not significant, treat: treatment

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Table 6: Antioxidant activity in *J. tenella* leaves using FRAP, ABTS and DPPH assays[a]

Harvest times	Treatments	FRAP (µmol Fe/gDW)	ABTS (µmol TE/gDW)	DPPH (%)
First (6WAT)	Control	40.0±1.8	22.7±1.2	82.24±0.34
	Cowpat	39.7±2.8ns	20.8±0.9***	75.33±0.34***
	NPK	41.9±3.4***	21.4±0.5***	79.77±0.55ns
	NPK +cowpat	41.8±2.4***	21.5±0.4***	80.70±1.28***
Second (9WAT)	Control	40.9±1.3	23.4±1.7	87.18±0.67
	Cowpat	40.8±1.2ns	22.1±1.1***	84.69±1.07***
	NPK	42.4±2.2***	22.9±0.2***	82.78±7.08***
	NPK +cowpat	42.3±1.9***	22.8±0.5***	87.61±1.8ns
Third (12WAT)	Control	45.7±0.8	2.18±0.12	85.42±1.62
	Cowpat	37.8±5.2***	2.18±0.16ns	84.16±1.06***
	NPK	38.3±2.8***	2.07±0.12***	83.90±1.26***
	NPK +cowpat	40.3±6.2***	2.11±0.15***	84.31±1.37***

[a] Values of FRAP, ABTS and DPPH of leaves are means ± SD (n = 3).
ns= non significant and *** = significant at p <.0.05

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Table 7: Antioxidant activity in *S. radiatum* leaves using FRAP, ABTS and DPPH assays

Harvest times	Treatments	FRAP (µmol Fe/gDW)	ABTS (µmol TE/gDW)	DPPH (%)
First (6WAT)	Control	34.4±2.7	18.8±0.9	23.37±1.61
	Cowpat	34.2±3ns	17.6±0.5***	11.38±0.78***
	NPK	43±2.3ns	19.3±1.1***	24.06±1.64ns
	NPK +cowpat	32.0±1.9***	19.3±0.8***	23.29±1.75ns
Second (9WAT)	Control	35.0±1.1	20.4±1.7	24.20±3.63
	Cowpat	35.4±2.0ns	20.7±2.1ns	27.50±4.56***
	NPK	36.1±4.7***	21.8±1.3***	30.41±6.05***
	NPK +cowpat	35.7±5.4***	20.2±1.4ns	30.36±3.46***
Third (12WAT)	Control	35.7±3.2	25.8±2.0	56.00±30.05
	Cowpat	37.2±3.5***	26.2±1.8ns	75.02±21.62***
	NPK	36.3±1.4ns	25.0±2.2***	57.64±28.57***
	NPK +cowpat	36.5±1.5***	24.1±1.6***	74.59±20.65***

[a] Values of FRAP, ABTS and DPPH of leaves are means ± SD (n = 3)
ns = non significant and *** = significant at p<.0.05

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Table 8: Antioxidant activity in *C. sesamoïdes* leaves using FRAP, ABTS and DPPH assays

Harvest times	Treatments	FRAP (µmol Fe/gDW)	ABTS (µmol TE/gDW)	DPPH (%)
First (6WAT)	Control	34.3±1.4	21.8±1.5	37.01±3.36
	Cowpat	36.2±1.2***	21.7±0.6ns	35.80±4.51***
	NPK	37.0±1.9***	22.7±1.2***	42.72±6.31***
	NPK +cowpat	35.3±1.5***	21.8±0.4ns	37.95±2.60***
Second (10WAT)	Control	41.1±1.6	28.1±0.7	84.36±1.71
	Cowpat	40.8±0.7ns	28.0±0.8ns	82.12±2.27***
	NPK	40.4±0.4***	26.1±1.9***	81.65±1.48***
	NPK +cowpat	40.9±1.2ns	27.7±0.9ns	82.85±2.55***
Third (14WAT)	Control	44.2±2.3	28.9±0.8	84.15±0.78
	Cowpat	44.5±2.4ns	22.9±0.4ns	84.30±0.61ns
	NPK	42.6±1.2***	28.3±0.4***	81.70±3.95***
	NPK +cowpat	42.5±0.8***	28.3±0.4***	82.74±4.67***

[a] Values of FRAP, ABTS and DPPH of leaves are means ± SD (n = 3)
ns= non significant and *** = significant at p <.0.05

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