

Allelopathic effects of *Alchornea laxiflora* (Benth.) Pax & K Hoffm. leaves on seed germination and seedling growth of tomato

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ABSTRACT

We investigated in petri plates bioassay and pot culture the allelopathic effects of *Alchornea laxiflora* (Benth.) Pax & K Hoffm. on seed germination and seedling growth of tomato (*Solanum lycopersicum* L.). Tomato seeds were grown in soil in pots watered with 0.02 %, 0.04 % and 0.08 % of aqueous leaf extracts of *Alchornea laxiflora* and atrazine. For the control, only water was used. Subsequently, effects of *n*-butanol and ethyl acetate fractions of extract were tested on germination and growth of tomato seeds in laboratory assays. Increasing concentration of extracts significantly reduced the seed germination and growth of tomato seedlings. The effects of applied extracts significantly ($p < 0.05$) reduced the activities of antioxidant enzymes but increased lipid peroxidation in tomato plants. The effects of ethyl acetate fraction were similar to control (water). These results showed that *Alchornea laxiflora* ethyl acetate fraction eluents C and D were identified as rutin and quercetin, these compounds inhibited the germination and seedling growth of tomato. Hence, these compounds may be developed as herbicides to control weeds.

Keywords: *Alchornea laxiflora*, allelochemicals, aqueous, bio-herbicides, enzymes, extract, leaves, seeds germination, seedlings growth, quercetin, rutin, *Solanum lycopersicum*, solvent, tomato, toxicity

INTRODUCTION

Weeds interfere with crop growth and reduce crop yield and quality depending on their extent of infestation. Herbicides are used to control weeds and Atrazine is common herbicide which interferes with photosynthetic electron transport and thus induces the oxidative stress (1). Indiscriminate use of herbicides causes many problems including presence of their residues in human food and water reserves. Hence scientists are exploring alternative methods to control weeds in crop fields and allelopathy may be employed for this purpose (29).

Allelopathy refers to the ability of certain plant species to dominate the environments in which they grow (1). Allelopathic plants have the ability to inhibit or stimulate the growth

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and survival of surrounding plants via the release of chemical substances known as allelochemicals through their leachates, root exudates, volatilization and biomass decomposition. Allelopathy hinders the growth of neighbouring weeds, thereby, allowing the allelopathic plants to dominate (3). Phytotoxic compounds in the roots and leaves of crop plants may be used to manage weeds, if the technology is developed (11,16,19). Some plants viz. *Citrullus colocynthis* and *Xanthium italicum* are used for weeds control (21).

Solanum lycopersicum L. (tomato) is most popular vegetable crop after potato and tops the list of canned vegetables (28). Its products are rich in antioxidants and good sources of vitamins C, E and carotenoids particularly lycopene and β -carotene (10,22). In human body, consumption of tomato enhances the production of endogenous defence mechanisms including oxidative enzymes such as peroxidase and polyphenol oxidase (4).

Alchornea laxiflora (Benth.) Pax & K Hoffm. (Figure 1) is a common shrub in Nigeria and Cameroon where it exhibits dominance on other plants, a characteristic of allelopathic plants (3). It is rich in phytochemicals, hence used in ethno-medicinal practice in many African countries (5,12). Its leaves are used as preservatives and branchlets are used as chewing sticks to care for the teeth. Farmers use it for mulching.



Figure 1. *Alchornea laxiflora* plant

The plant usually dominates the areas of its growth; hence, we suspected that its allelopathic potential may adversely affect susceptible species. Therefore, this study aimed (i) to evaluate the allelopathic effects of *Alchornea laxiflora* leaf extract on seeds germination and seedling growth of tomato, a common plant used for allelopathic study and (ii) discover the bioactive compounds which may be developed as herbicide.

MATERIALS AND METHODS

The study was conducted in Fountain University, Osogbo, Nigeria (Latitude 7°46'15.7" N and Longitude 4°33'25.1" E, mean height above sea level: 320 m, annual rainfall: 1241 mm and minimum and maximum temperature of 24° C and 30 °C, respectively). The experimental soil was air dried, pulverized and passed through wire mesh

sieve of 2 mm. The soil was sandy loam (11.46 % clay, 26.56 % silt and 61.98 % sand) with pH 5.98. Silica gel (230–400 mesh 60 Å, 150 Å; Sorbent Technologies, USA), column, TLC plate, tomato seeds were purchased from JK Agric. Genetics Ltd., Hyderabad, India and Atrazine from Ningbo Agrostar China. All other chemicals and reagents used were of analytical grade.

Plant collection, authentication and study period

Alchornea laxiflora (Benth.) Pax & K Hoffm. leaves were collected from our University Campus, in December 2015. The plant was identified and authenticated at the Herbarium of University of Ilorin, Nigeria and was deposited with voucher number UILH/002/666. The study was conducted from December 2015 to June 2016. The collected leaves were thoroughly washed in tap water followed by distilled water to remove dirt, dust and debris. They were air dried in shade in the laboratory for 2-weeks to constant weights. The dried leaves were powdered using an electric blender and stored in dry air-tight glass bottle until used. Fifty g powdered leaf sample was soaked in 500 mL distilled water for 24 h at room temperature (26 °C). This was followed by 2 h orbital shaking at 200 rpm for maximum extraction and then filtered through Whatman No. 1 filter paper. The resulting filtrate was concentrated on a water bath at 40 °C and stored in a refrigerator at 4 °C until used.

Pot culture

The tomato seeds were surface sterilized with 0.1% mercuric chloride solution for 20 min and rinsed with water 2-3 times to remove residues from seeds (27). The seeds were immersed in 1 L distilled water to remove the non-viable seeds floating on water surface. The viable seeds that settled at the bottom of beaker were collected, air dried and used in the experiment (8). Ten tomato seeds were sown per plastic pot (5 L) with 250 g soil for 3 weeks. Each pot was irrigated with 450 ml distilled water, 0.02 %, 0.04 %, 0.08 % of leaf extract of *Alchornea laxiflora* and 0.5 µg/ml Atrazine (450 ml). After 3 weeks, the tomato plants were harvested and their germination and seedlings growth were assessed based on germination (%), root length, shoot length and dry weight (18).

Chlorophyll

Chlorophyll *a* and Chlorophyll *b* concentrations in tomato leaves were determined as per Comb *et al.* (6). Briefly fresh leaf samples (0.1 g) were homogenized and extracted with 10 ml of 80 % acetone for 24 h until the leaves turned white. The concentrations of Chlorophyll *a* and Chlorophyll *b* in extract were determined using spectrophotometer as under:

$$\begin{aligned} \text{Chlorophyll } a &= 13.19A_{664} - 2.57A_{647} \text{ } \mu\text{g/g fresh weight} \\ \text{Chlorophyll } b &= 22.10A_{647} - 5.26A_{664} \text{ } \mu\text{g/g fresh weight} \end{aligned}$$

Where, A_{664} : absorbance at wavelength 664 nm; A_{647} : absorbance at wavelength 647 nm.

Oxidative stress

Lipid peroxidation, activities of superoxide dismutase (SOD) and catalase (CAT) were determined as per Sunmonu and van Staden (27).

(i). MDA concentration: The enzyme extract (0.5 ml) was treated with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA). The mixture was incubated in a water bath for 30 min, cooled immediately in ice chips and the absorbance

was read at 532 nm. Lipid peroxidation was expressed in terms of malondialdehyde (MDA) concentration.

(ii). SOD activity: The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μ M phenazine methosulfate (PMS), 0.3 ml of 300 μ M nitroblue tetrazolium (NBT), 0.2 ml of the enzyme extract and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH (780 μ M). The mixture was left to stand for 90 s and arrested by the addition of 1.0 ml glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of *n*-butanol and allowed to stand for 10 min. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer (Cary 50 Conc, Australia). One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in 1 min.

(iii). CAT activity: The hydrogen peroxide phosphate buffer (3 ml, 0.067 M, pH 7.0) was measured in a test tube followed by the addition of an aliquot of 40 μ l of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm using a spectrophotometer (Cary 50 Conc, Australia). The enzyme solution containing hydrogen peroxide-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

Solvent partitioning of *Alchornea laxiflora* extract

The aqueous extract of *Alchornea laxiflora* was subjected to solvent partitioning as per Kupchan and Tsou (14). Briefly, 15 g extract was dissolved in 100 mL distilled water in beaker and transferred into 5 L burette. The resulting solution was partitioned using 1 L each of two different partitioning solvents (*n*-butanol and ethyl acetate) to increase their solubility gradients. The fractions collected and the residue were concentrated *in vacuo* using a rotary evaporator and thereafter stored in a refrigerator at 4°C until used.

Petri-dish germination assay using the fractions

The 5-experimental treatments were: aqueous extracts (0.04 %), ethyl acetate fraction, *n*-butanol fraction, residue and distilled water as control. Ten tomato seeds were evenly placed in 9 cm diameter Petri dishes lined with double layers of Whatman No. 1 filter paper. Each Petri plate was moistened with 5 mL aqueous extracts (0.04 %), ethyl acetate fraction, *n*-butanol fraction, residue and distilled water. The Petri dishes were sealed with parafilm and incubated for 7 days at $27 \pm 2^\circ\text{C}$ in dark. These treatments were replicated 4 times in completely randomized design. After the 7-day experimental period, seeds growth was assessed based on lengths of plumule and radicle relative to the control (27).

Thin layer chromatography (TLC), petri-dish bioassay with eluents and high-performance liquid chromatography (HPLC)

Based on phytotoxic activity, the ethyl acetate fraction (EAF) was fractionated on silica gel (230-400 mesh 60 Å) using direct column chromatography (21) and the column was isocratically eluted with ethyl acetate: methanol: water (10:1.3:0.7). The eluents obtained were pooled into four (A to D) based on similarity of R_f values using Thin Layer Chromatography (TLC) profile (K₅ Silica gel, 150 Å; Sorbent Technologies, USA) obtained

with Ethyl acetate: Methanol: Water (10:1.3:0.7) as the mobile phase. Another experiment was done with 5-Treatments: 4 Eluents (A, B, C, D) and distilled water as control.

The germination (%), shoot length and root length of tomato were recorded after 7 days. The active eluents C and D were subjected to High Performance Liquid Chromatography (HPLC) analysis using acetonitrile: water (75:25) as carrier to identify the compounds present.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test for multiple comparison using the Statistical Package for Social Sciences (SPSS) Version 17.0 for windows software package. Results were expressed as Mean \pm SEM and they were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Seed germination

Exposure of tomato seeds to *Alchornea laxiflora* leaf extract adversely affected germination and growth based on the parameters measured. Specifically, the extract significantly ($p < 0.05$) inhibited percentage germination, root length, shoot length and dry weight of leaves in the tomato plant by about 40.6 %, 50.2 %, 41.0 % and 56.0 % respectively (at 0.08% concentration) compared to the control. Atrazine had similar effect inhibiting the parameters by about 46.0 %, 64.3 %, 40.7 % and 60.4 % respectively compared to the control (Figure 2). Phytotoxic compounds inhibit seed germination by blocking enzymatic activities which are responsible for mobilization of stored nutrients in such seeds (8). In this study, *Alchornea laxiflora* extract may have affected the activities of hydrolytic enzymes in tomato seeds, and this probably accounted for the significant inhibition of seed germination (8) and subsequent significant reductions in the number of leaves, shoot and root lengths of the seedlings (26) Similar observations were recorded in the atrazine-treated seeds, confirming the phytotoxicity of the chemical. Thus, there are indications that *Alchornea laxiflora* aqueous leaf extract contains allelochemicals which hinder the survival of seedlings.

Photosynthetic pigments

Chlorophylls *a* and *b* contents in the tomato plant were significantly ($p < 0.05$) reduced following treatment with the extract by about 50.60 % and 66.02 % respectively (at 0.08 % concentration) compared to the control. Atrazine on the other hand reduced chlorophylls *a* and *b* by about 59.84 % 23.27 % respectively compared to the control (Table 1). Photosynthetic ability is usually hampered when there is a reduction in the levels of photosynthetic pigments thereby causing oxidative stress in affected plants (9,26). The observed reduction in chlorophyll content of tomato plants following treatment with *Alchornea laxiflora* aqueous leaf extract and atrazine suggests a disruption of photosynthetic pathway. Our findings agree with previous studies where exposure of seedlings to plant extracts caused inhibition of photosystems and consequently a reduction in the levels of photosynthetic pigments (8,14). Bai *et al.* (1) also reported reduction of photosynthesis in *Phaeodactylum tricornutum* at doses $> 100 \mu\text{g/ml}$ after 48 h.

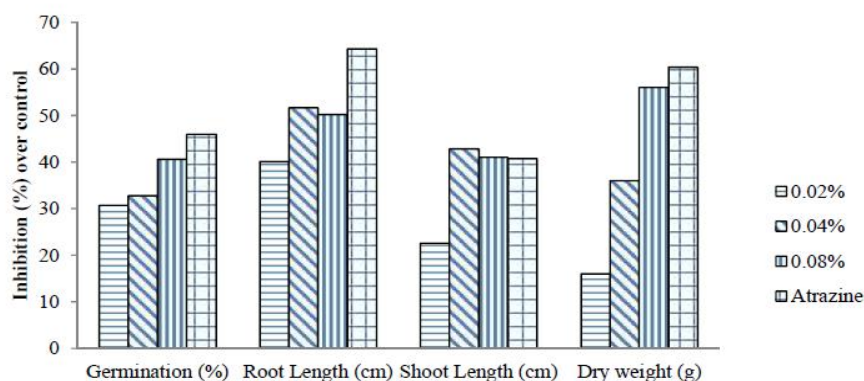


Figure 2. Inhibitory effects of aqueous leaf extract of *Alchornea laxiflora* and Atrazine on seeds germination and seedling growth of tomato plants in pot culture after 21 days

Table 1. Effects of aqueous leaf extract of *Alchornea laxiflora* and Atrazine on Chlorophyll contents in tomato plants raised in pot culture for 21 days

Treatment	Chlorophyll <i>a</i> ($\mu\text{g/g}$ of fresh weight)	Chlorophyll <i>b</i> ($\mu\text{g/g}$ of fresh weight)
Control	7.52 ± 1.05^a	2.02 ± 0.02^a
0.02 %	5.39 ± 0.94^b	2.45 ± 0.03^b
0.04 %	5.45 ± 1.40^c	2.17 ± 0.02^c
0.08 %	3.71 ± 0.04^d	1.86 ± 0.01^d
Atrazine	3.02 ± 0.14^d	1.55 ± 0.03^d

*Data are expressed as mean \pm SEM for 10 determinations. Values with different superscripts are significantly different at $P < 0.05$.

Germination of seeds and subsequent seedling growth are valuable indices in allelopathic studies (9). These indices are generally enhanced by the concentration of photosynthetic pigments including chlorophyll *a* and *b*. Consequently, the significant reduction in germination observed in the present study following treatment with *Alchornea laxiflora* aqueous leaf extract may be attributed to decrease in the photosynthetic ability of tomato plant (23). Previous studies have reported that allelochemicals have the ability to inhibit chlorophyll biosynthesis (20,31). This is most likely to be the case in this study as the compounds present in the aqueous leaf extract of *A. laxiflora* must have inhibited the development of root, shoot and leaves of tomato plant by inhibition of chlorophyll synthesis.

Antioxidant indices

Following treatment with the extract, MDA concentration was found to be significantly ($p < 0.05$) increased in the tomato plants by about 94.44 % (at 0.08 % concentration), compared to the control, while the activities of SOD and CAT were reduced by about 35.85 % and 55.00 % respectively (at 0.08 % concentration) compared to the control. Similarly, Atrazine also caused an increase in MDA concentration by about 88.89 % compared to the control while activities of SOD and CAT were reduced by about 32.58 % and 50 % respectively than control (Table 2). Toxic allelochemicals have been reported to have damaging effects on plant cells particularly the membranes (15). This

cellular toxicity may be caused by an increased production of reactive oxygen species (ROS) and a concurrent alteration in antioxidant enzyme activities (25). Therefore, MDA which is an end product of lipid peroxidation serves as an important index to determine the degree of oxidative stress in plants (32). In this study, the observed increase in MDA concentration in tomato seedlings treated with aqueous leaf extract of *Alchornea laxiflora* and atrazine may be an indication of stress induced on the plant system.

Table 2. Effects of aqueous leaf extract of *Alchornea laxiflora* and Atrazine on oxidative stress markers in tomato plants raised in pot culture for 21 days

Treatment	MDA Concentration (nmol/g)	SOD Activity (Unit/mg protein)	Catalase Activity (Unit/mg protein)
Control	0.54 ± 0.02 ^a	60.96 ± 0.06 ^a	0.20 ± 0.01 ^a
0.02 %	0.61 ± 0.01 ^b	51.76 ± 0.11 ^b	0.15 ± 0.01 ^b
0.04 %	0.66 ± 0.01 ^c	40.81 ± 0.22 ^c	0.11 ± 0.01 ^c
0.08 %	1.05 ± 0.02 ^d	39.10 ± 0.22 ^d	0.09 ± 0.01 ^d
Atrazine	1.02 ± 0.01 ^e	41.10 ± 0.12 ^e	0.10 ± 0.01 ^e

*Data are expressed as mean ± SEM for 10 determinations. Values with different superscripts are significantly different at $P < 0.05$.

Additionally, plants produce several antioxidant enzymes which provide secondary protection against cellular damage arising from ROS generation. Thus, the activities of antioxidant enzymes including SOD and CAT are usually taken as a measure of the degree of damage during an environmental stress condition (17,33). Reduction in the activities of these enzymes in response to extract and atrazine treatment may be attributed to high amounts of ROS generated within the seedlings. This may be attributed to the fact that ROS have the tendency to overwhelm antioxidant enzymes, resulting in depletion of their activities. These findings are consistent with previous studies which reported a reduction in antioxidant enzyme activities of seedlings exposed to phytotoxic chemicals (25,26).

Petri-plate germination of solvent-partitioned fractions and eluents

The ethyl acetate fraction and Eluents C and D significantly reduced the germination (%), shoot and root lengths in tomato seedlings (Tables 3 and 4). The inhibitory effects of ethyl acetate fraction on seeds germination and seedlings growth of tomato indicated the presence of some phytotoxic compounds in the aqueous extract. Further, the phytotoxic activity of Eluents C (rutin) and D (quercetrin) clearly showed that they may be responsible for the allelopathic activity of *Alchornea laxiflora* aqueous extract. These findings agree with earlier ones that aqueous extract from *Castanea sativa* leave and its purified constituents, quercetin and rutin, significantly inhibited both germination and growth of *Raphanus sativus* (2). This shows that isolated phytotoxic substances produce greater effects on recipient plants (7,30).

TLC Profiling of Ethyl Acetate Fraction and Identification of Bioactive Compounds

A total of 22 eluents were collected from TLC analysis of the ethyl acetate fraction, these were grouped in four zones based on TLC profiles. Each zone had compounds with similar R_f values. The active fractions C and D were found to contain rutin and quercetrin based on HPLC analysis (Figures 3 and 4).

Table 3. Effects of aqueous leaf extract of *Alchornea laxiflora* and different solvent-partitioned chromatographic fractions on germination and seedlings growth of tomato in Petri plates assay for 7 days

Treatment Group	Germination (%)	Shoot length (cm)	Root length (cm)
Control	98 ± 0.00 ^a	4.50 ± 0.03 ^a	2.74 ± 0.02 ^a
Aqueous extract	89 ± 2.15 ^b	1.90 ± 0.03 ^b	1.44 ± 0.02 ^b
Ethyl acetate	85 ± 2.85 ^b	1.58 ± 0.04 ^b	1.77 ± 0.02 ^b
<i>n</i> -butanol	97 ± 2.68 ^a	4.18 ± 0.04 ^a	2.76 ± 0.02 ^a
Residue	98 ± 2.50 ^a	4.54 ± 0.02 ^a	2.84 ± 0.02 ^a

*Data are Mean ± SEM of 10 determinations. Values with different superscripts along the same column indicate significant difference at $p < 0.05$.

Table 4. Effects of different chromatographic eluents of ethyl acetate fraction on germination and seedlings growth of tomato in Petri plates assay for 7 days

Eluents	Germination (%)	Shoot length (cm)	Root length (cm)
Control	98 ± 2.75 ^a	4.05 ± 0.01 ^a	3.14 ± 0.02 ^a
A	97 ± 2.17 ^a	3.90 ± 0.02 ^a	3.05 ± 0.02 ^a
B	95 ± 2.89 ^a	3.98 ± 0.02 ^a	2.97 ± 0.02 ^a
C	87 ± 2.85 ^b	1.34 ± 0.02 ^b	1.06 ± 0.02 ^b
D	85 ± 2.50 ^b	1.89 ± 0.01 ^b	1.75 ± 0.02 ^b

A-D represent spots with similar R_f values pulled together. Data are Mean ± SEM of 10 determinations. Values with different superscripts along the same column for each parameter indicate significant difference at $p < 0.05$.

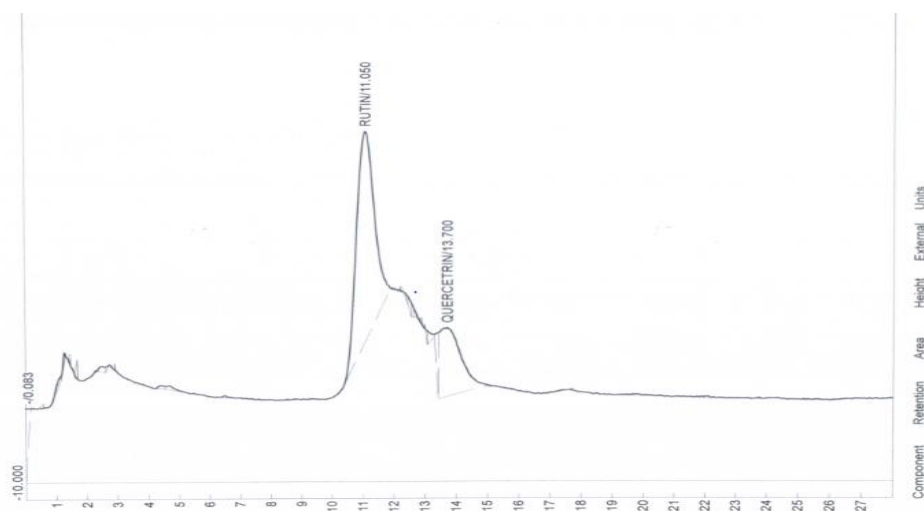


Figure 3. High-performance liquid chromatography peaks of Fraction C showing the presence of rutin and quercetrin.

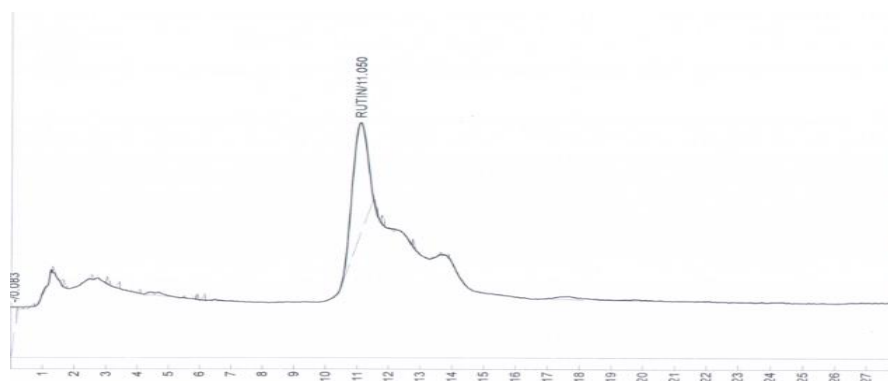


Figure 4. High-performance liquid chromatography peak of Fraction D showing the presence of rutin

CONCLUSIONS

We found that *Alchornea laxiflora* contained substances harmful to tomato plants. The ethyl acetate fraction contained the rutin and quercetin, these were most phytotoxic and may be responsible for the allelopathic activity of *Alchornea laxiflora* aqueous extract. The compounds (rutin and quercetin), found in this study may find application to develop herbicides from *Alchornea laxiflora* to control weeds.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest : The Authors declare no conflict of Interest.

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