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The Effects of Different Concentrations of Crude Aqueous Extract of *Artemisia annua* on Soil Microbes

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Authors' contributions

This work was carried out in collaboration between all authors. Author VFE designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author OBI managed the analyses of the study. Author MGS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Artemisia annua (L) is known to inhibit the growth of seedlings leading us to hypothesize that it may also adversely affect soil microbial community. The effect of crude, aqueous leaf extracts of *A. annua* on soil microorganisms was therefore studied at (w/v) 0 (control), 1.0, 2.5, 5.0 and 7.5 percent (%) concentrations. The frequency of occurrence of bacterial isolates across treatments varied in the order *Agromyces spp* < *Klebsiella spp* < *Micrococcus spp* < *Bacillus spp* < *Athrobacter spp* < *Pseudomonas spp*. After 12 weeks of treatment application, the bacterial mean counts (cfu/g) were 173 x 10^5 for the control, 57.5 x 10^5 for the 1.0 %, 52.16 x 10^5 for the 2.5 %, 47.8 x 10^5 for the 5 %, and 44.3 x 10^5 for the 7.50 % crude extract concentrations; the fungal mean counts were 6.78 x 10^4, 30.5 x 10^4, 34.3 x 10^4, 35 x 10^4 and 39.5 x 10^4 cfu/g respectively. The fungal population varied in the order *Aspergillus spp* > *Penicillium spp* > *Rhizopus spp* > *Corynespora Spp* > *Mucor spp* > *Microsporum spp* > *Fusarium spp*. Thus, the extracts of *Artemisia annua* significantly reduced the bacterial population but increased the fungal population.

Keywords: Artemisia annua; crude extract; concentrations; soil microbial properties.

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1. INTRODUCTION

Inhibition of plant growth and production of phytotoxic compounds by certain plants and their exudates is a well-established phenomenon. In searching for potential herbicides from plants, screening of compounds known to function in plant-plant interactions is a logical strategy. Phytotoxic exudates exert an effect that may be inhibitory or stimulatory on microorganisms but cannot be quantified precisely and are not reproducible [1]. Toxic exudates can upset the process of bacteria turning nitrogen into plant-friendly nitrates. Loss of beneficial bacteria lowers soil quality in several ways since bacteria help soil store nutrients and water, regulate water flow throughout the soil and assist in filtering pollutants. *Artemisia annua* is reported to produce large quantities of essential oil from glands located on its leaf surfaces [2,3]. The foliage and aqueous extracts of *Artemisia annua* has been reported to inhibit the growth of seedlings [2,4,5,6]. The sesquiterpenoid lactone, artemisinin from *Artemisia annua* (L), 1, 8-cineole, arteannuic acid and arteanniuim B are likely to be developed as herbicides because they are strongly phytotoxic [7,8]. Herbicides can harm microorganisms as well, most notably if used in high concentrations. However, simply because a compound is a natural product, does not ensure that it is safe; the toxicological and environmental consequence of these compounds from *Artemisia annua* on soil microbial properties must be studied and understood through research.

2. MATERIALS AND METHODS

2.1 Study Area

The experimental plots were cited along MCC Road, Calabar (04º55" and 0 05 N and 08º15" and 08º25' E). The area has a mean annual rainfall of 2360 mm (range 2290 to 2680 mm) with a bimodal distribution pattern and a distinct dry season of 4 months. The mean daily minimum temperature is 21-24ºC and the mean maximum temperature is 27-31ºC. The mean relative humility ranges from 82 to 89% (Federal Airport Authority of Nigeria (FAN) Meteorological data, Calabar, 2015).

2.2 Preparation of *Artemisia annua* Crude Extract

Crude extract of *Artemisia annua* (L) upper biomass (leaves, secondary stem and florets) was prepared in four (4) concentration levels (weight/volume) namely: 1.00%, 2.50%, 5.0% and 7.5%. The various concentrations were prepared by grinding the upper biomass of fresh Artemisia annua and weighing out 15, 37.5 75 and 112.5 g respectively and extracting in 1500 ml of distilled water with shaking at 150 rmp for 24 hours at room temperature. The solution was filtered with Whatman No 4 filter paper, stored in labeled containers and kept in refrigerator till use (20 hours before use). These concentrations of pollution were deliberately chosen to mimic common occurrence in the field.

2.3 Field Studies

Fifteen experimental field plots of 2 m x 2 m were mapped out, weeded, tilled and composite samples collected for laboratory analysis. The experimental treatments consisted of the four extract concentrations and a control.

Five hundred milliliters (500 ml) of the various concentrations of *Artemisia annua* (L) crude extracts were applied to the different experimental plots using hand sprayer. The spraying was carried out between 7.00-10.30 am in the month of May. The spraying was done devoid of drift at a spray height of 10 cm, at 15 minutes for each plot. Twenty eight hours of rain-free period and 16 hours of sunshine were noted after the application. The experiment was maintained under natural environmental conditions prevalent in Calabar for twelve weeks.

2.4 Collection and Treatment of Soil Samples

Sample collection for microbial analyses was carried out forth nightly. A total of 90 samples were collected during the six (6) sampling periods. At each sampling point, soil was augered to a depth of 15 cm from the 2 m x 2 m square into sterile polythene bags and appropriately labeled.

2.5 Cultivation and Enumeration of Bacteria in the Experimental Plots

Each sample (1 g) was thoroughly shaken in 10 ml of distilled water. An aliquot (1.0 ml) was transferred aseptically into the next tube and diluted serially in one-tenth stepwise to 10^{-5} dilution [9]. From the dilution of 10^{-5} of each soil sample, 0.1 ml aliquot was transferred aseptically

onto freshly prepared nutrient Agar plates [9,10]. The dilution of 10^{-5} was used in plating for bacteria because the dilution of 10^{-4} gave a confluent count. The inoculated plates were inverted and incubated at 37ºC for 24-48 hours after which the plates were examined for growth. The discrete colonies which developed were counted and the average counts for triplicate cultures were recorded as total viable bacterial count in the sample.

2.6 Isolation, Characterization and Identification of Bacteria from the Experimental Soils

Discrete bacteria colonies which developed were aseptically transferred and sub cultured on freshly prepaid nutrient agar slopes and incubated at 30ºC for 24 hours. These served as pure stock for subsequent characterization test. Standard characterization test were performed in duplicates: Gram staining, catalase test, coagulate test, sugar fermentation test, motility test, methyl red test, voges proskauer test, indole test and citrate utilization test. The pure cultures were identified on basis of their cultural, morphological and physiological characteristics in accordance with methods by [11] and [12].

2.7 Cultivation and Enumeration of Fungi

Serial dilution as outlined by [9] was carried out. From the dilution of 10^{-3} of each soil sample, 0.1 ml aliquot was transferred aseptically onto freshly prepared sabourauds dextrose agar plate to which 0.2 ml of 0.5% of Ampicillin had been added to inhibit the growth of bacteria and allowing the growth of fungi [9,10]. The dilution of 10^{-3} was used in plating because the dilution of 10^{-4} gave fewer growths. The inoculated plates were inverted and incubated at 30ºC (room temperature) for 5 to 7 days. The colonies which developed were counted and the average count for the triplicate cultures were recorded as total viable fungi in the sample.

2.8 Isolation, Characterization and Identification of Fungi

Pure culture of fungi isolates which developed were further sub cultured onto agar slants and incubated at room temperature for 5-7 days. The pure cultures isolates which developed were stored in the refrigerator as stock cultures for subsequent characterization test. The following standard characterization tests were performed in duplicates: Macroscopic examination of fungal growth was carried out by observing the colony morphology-Diameter, colour (pigmentation), texture and surface appearance. Microscopic examination was done by needle mount method and observing sexual and asexual reproductive structures. Sugar fermentations were carried out for species identification. The complete identification of fungal isolates was done by comparing the results of their cultural, morphological and biochemical characteristics with those of known taxa [11,12,13,14].

3. RESULTS AND DISCUSSION

The results obtained for bacterial and fungal counts and identification from field experiments with *Artemisia annua* are displayed on Table 1. From the results, the control plot had a total bacterial count of 1038 CFU with a mean of 173 x 10^{-5} cfu/g for 12 weeks while a total count of 345 CFU (57.5 x10^{5} cfu/g) 313 CFU (52.16 x 10^{5} cfu/g), 287 CFU (47.8 x 10^{5} cfu/g), 266 CFU (44.3 x 10^{5} cfu/g) were obtained for *Artemisia annua* crude extract treatments at 1.00% w/v, 2.50% w/v, 5.00% w/v and 7.5% w/v respectively. The bacterial isolates identified from the 15 experimental plots portrayed a decreasing frequency across the various treatment levels giving the trend *Agromyces spp* < *Klebsiella spp* < *Micrococcus spp* < *Bacillus spp* < *Athrobacter spp* < *Pseudomonas spp* with *Agromyces spp* being the most depressed bacterial community. The mean trend indicates that the bacterial population decreased with increase in concentrations of *Artemisia annua* crude extract (7.5% w/v > 5.00% w/v > 2.50% w/v). An initial decline in the bacterial population was observed for Weeks 2, 4 and 6, however, results for weeks 8, 10 and 12 recorded slight increase in bacterial population. The slight increase observed for weeks 8, 10 and 12, may be due to adaptation of the bacteria to the available substrate or probably due to leaching of some water soluble compounds beyond the sampling depth, owing to the high rain intensity and sandy nature of the soils noted in the study area.

Despite the slight increase observed in the bacterial population at weeks 8, 10 and 12, the mean separation indicates that the control mean significantly differed from the treatment means (Table 2). The significant difference is an indication of the suppressive (negative) effect of *Artemisia annua* compounds on the soil bacterial population. A similar depressive trend was

observed by [15] for field trials with *A. annua* dried leaves. It is most likely that coumarins, flavonoids and/or a few other constituents of *Artemisia annua* released upon water might be responsible for the inhibitory properties observed in this study. Flavonoids have been reported to be an effective antimicrobial agent against a wide array of microorganisms because of their ability to complex with extracellular and soluble proteins and with bacterial cell walls [16].

Table 1. Mean bacterial isolate count (cfu/g) from experimental plots treated with various levels of *Artemisia annua* crude extract

Weeks	*Athrobacter spp*	*Agromyces spp*	*Bacillus spp*	*Klebsiella spp*	*Micrococcus spp*	*Pseudomonas spp*	Total count (CFU)
2	45 x 10^5	15 x 10^5	27 x 10^5	15 x 10^5	24 x 10^5	30 x 10^5	156
4	24 x 10^5	15 x 10^5	24 x 10^5	15 x 10^5	24 x 10^5	27 x 10^5	147
6	36 x 10^5	18 x 10^5	27 x 10^5	12 x 10^5	27 x 10^5	30 x 10^5	150
8	45 x 10^5	18 x 10^5	30 x 10^5	12 x 10^5	30 x 10^5	45 x 10^5	180
10	30 x 10^5	33 x 10^5	30 x 10^5	12 x 10^5	36 x 10^5	63 x 10^5	204
12	30 x 10^5	33 x 10^5	30 x 10^5	9 x 10^5	39 x 10^5	60 x 10^5	201
Total							**1038**
Mean							**173**
2	11 x 10^5	4 x 10^5	12 x 10^5	6 x 10^5	4 x 10^5	10 x 10^5	47
4	9 x 10^5	3 x 10^5	13 x 10^5	6 x 10^5	5 x 10^5	12 x 10^5	48
6	14 x 10^5	3 x 10^5	12 x 10^5	2 x 10^5	7 x 10^5	14 x 10^5	52
8	15 x 10^5	6 x 10^5	10 x 10^5	4 x 10^5	9 x 10^5	14 x 10^5	58
10	12 x 10^5	11 x 10^5	9 x 10^5	3 x 10^5	14 x 10^5	21x 10^5	71
12	12 x 10^5	11 x 10^5	9 x 10^5	3 x 10^5	15 x 10^5	20 x 10^5	69
Total							**345**
Mean							**57.5**
2	10 x 10^5	--	12 x 10^5	40 x 10^5	4 x 10^5	13 x 10^5	43
4	11 x 10^5	--	12 x 10^5	2 x 10^5	3 x 10^5	13 x 10^5	41
6	13 x 10^5	--	10 x 10^5	2 x 10^5	7 x 10^5	12 x 10^5	44
8	14 x 10^5	3 x 10^5	10 x 10^5	3 x 10^5	11 x 10^5	15 x 10^5	56
10	10 x 10^5	11x 10^5	10 x 10^5	2 x 10^5	13 x 10^5	22 x 10^5	67
12	10 x 10^5	9 x 10^5	9 x 10^5	2 x 10^5	12 x 10^5	20 x 10^5	62
Total							**313**
Mean							**52.16**
2	8 x 10^5	--	10 x 10^5	4 x 10^5	3 x 10^5	15 x 10^5	40
4	9 x 10^5	--	12 x 10^5	2 x 10^5	2 x 10^5	13 x 10^5	38
6	13 x 10^5	--	10 x 10^5	3 x 10^5	5 x 10^5	10 x 10^5	31
8	15 x 10^5	--	9 x 10^5	3 x 10^5	12 x 10^5	12 x 10^5	51
10	12 x 10^5	9 x 10^5	11 x 10^5	2 x 10^5	10 x 10^5	22 x 10^5	66
12	9 x 10^5	10 x 10^5	10 x 10^5	1 x 10^5	10 x 10^5	21 x 10^5	61
Total							**287**
Mean							**47.8**
2	7 x 10^5	--	10 x 10^5	--	13 x 10^5	17 x 10^5	37
4	9 x 10^5	--	10 x 10^5	1 x 10^5	1 x 10^5	15 x 10^5	36
6	12 x 10^5	--	11 x 10^5	2 x 10^5	--	12 x 10^5	37
8	13 x 10^5	--	10 x 10^5	3 x 10^5	2 x 10^5	12 x 10^5	40
10	10 x 10^5	--	10 x 10^5	1 x 10^5	10 x 10^5	23 x 10^5	54
12	10 x 10^5	--	12 x 10^5	1 x 10^5	9 x 10^5	20 x 10^5	62
Total							**266**
Mean							**44.3**

Table 2. Effects of *Artemisia annua* crude extract on soil total viable bacteria and fungi counts (cfu/g)

Treatments	Units	Total viable bacteria	Total viable fungi
Control % w/v	CFU	173.00	6.78
1.00 % w/v	CFU	57.50	31.00
2.50 % w/v	CFU	52.20	33.33
5.00 % w/v	CFU	47.80	35.00
7.50 % w/v	CFU	44.30	39.50
ESEM Mean		75.00 ± 2.96	29.12 ± 1.59
LSD (0.05)		8.74	4.68
CV (%)		18.8	17.7

ESEM- Estimated Standard Error Mean
LSD – Least Significant Difference
CV – Coefficient of Variation

The fungal species isolated from the experimental site gave a trend of *Aspergillus spp, Penicillium spp, Rhizopus spp, Corynespora Spp, Mucor spp, Microsporum spp, Fusarium spp*. The control plot had a total count of 40.68 CFU and a mean count of 6.78 x 10^5 cfu/g for the 12 weeks. A total fungal count of 186 CFU (31. 0 x 10^5 cfu/g), 200 CFU (33.3 x 10^5 cfu/g), 210 CFU (35 x 10^{-5} cfu/g) and 237 CFU (39.5 x 10^5 cfu/g) was recorded for treatments 1.00 % w/v, 2.50%w/v, 5.00% w/v and 7.5% w/v *Artemisia annua* crude extract respectively for 12 weeks.

There was significant increase in the fungal population across the various experimental treatment levels. The fungal population increased with an increase in *Artemisia annua* concentration (Fig. 1), giving the trend: *Aspergillus spp* > Penicillium spp > *Rhizopus spp* > *Corynespora spp* > *Mucor spp* > *Microsporum spp* > *Fusarium spp*. The result indicates that the treatment means significantly differed from the control with treatment 7.5% w/v giving the highest fungi population.

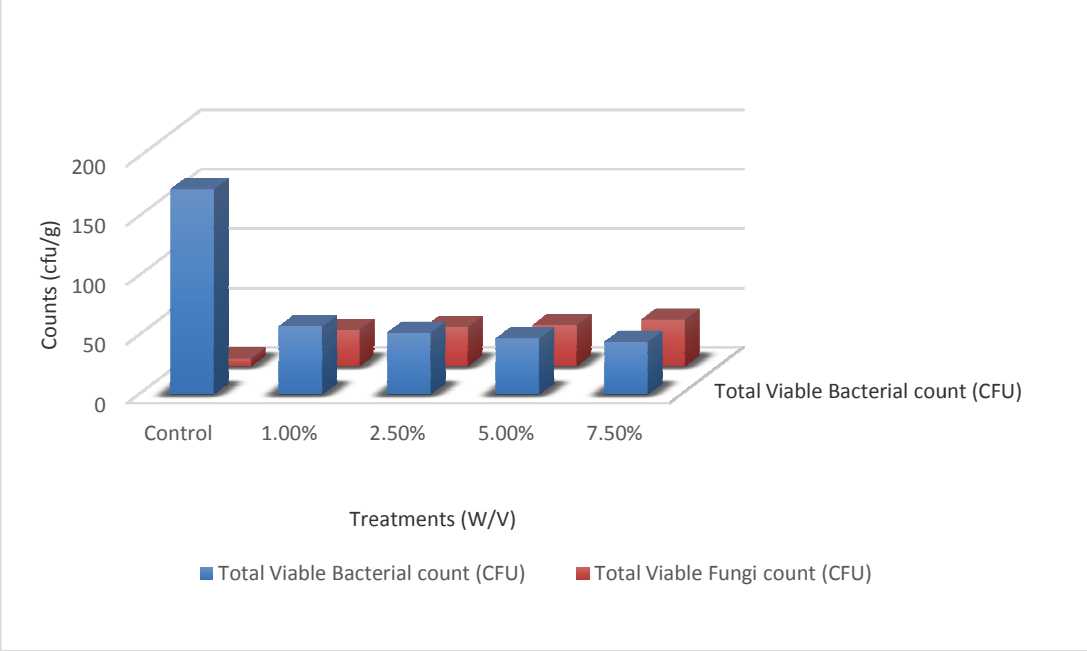

Fig. 1. Effects of different concentrations of crude aqueous extract of *Artemisia annua* on soil microbial population

Table 3. Mean fungal count from experimental plots treated with various levels of *Artemisia annua* crude extract

Weeks	*Aspergillus spp*	*Corynespora spp*	*Fusarium spp*	*Microsporum spp*	*Mucor spp*	*Penicillium spp*	*Rhizopus spp*	Total count (CFU)
2	2 x10^3	NI	1 x10^3	1 x10^3	1 x10^3	1 x10^3	1 x10^3	7.00
4	1 x10^3	NI	1 x10^3	1 x10^3	1 x10^3	2 x10^3	1 x10^3	7.00
6	1.67 x10^3	NI	1 x10^3	1 x10^3	1 x10^3	1 x10^3	1 x10^3	6.67
8	2.67 x10^3	NI	NI	1x10^3	1 x10^3	1 x10^3	1 x10^3	6.67
10	2 x10^3	NI	NI	1 x10^3	2 x10^3	1 x10^3	1.67 x10^3	7.67
12	1 x10^3	NI	NI	1 x10^3	1 x10^3	1 x10^3	1.67 x10^3	5.67
Total								**40.68**
Mean								**6.78**
2	6 X10^3	6 x10^3	2 x10^3	3 x10^3	6 x10^3	6.x10^3	1 x10^3	36.00
4	12 x10^3	3 x10^3	NI	3 x10^3	3 x10^3	6 x10^3	3 x10^3	30.00
6	9 x10^3	3 x10^3	3 x10^3	6 x10^3	3 x10^3	3 x10^3	3 x10^3	30.00
8	12 x10^3	3 x10^3	NI	3 x10^3	3 x10^3	6 x10^3	3 x10^3	30.00
10	9 x10^3	6 x10^3	NI	3 x10^3	3 x10^3	9 x10^3	6 x10^3	33.00
12	9 x10^3	3 x10^3	NI	3 x10^3	3 x10^3	3 x10^3	6 x10^3	27.00
Total								**186.00**
Mean								**31.50**
2	9 x10^3	6 x10^3	3 x10^3	3x10^3	6 x10^3	9 x10^3	6 x10^3	42.00
4	9 x10^3	6 x10^3	NI	3x10^3	3 x10^3	6 x10^3	3 x10^3	30.00
6	9 x10^3	3 x10^3	NI	5 x10^3	3 x10^3	6 x10^3	6 x10^3	30.00
8	9 x10^3	6 x10^3	NI	3 x10^3	3 x10^3	6 X10^3	3 x10^3	30.00
10	12 x10^3	6 x10^3	NI	6 x10^3	6 x10^3	9 x10^3	6 x10^3	451.00
12	6 x10^3	3 x10^3	NI	3 x10^3	3 x10^3	5 x10^3	3 x10^3	23.00
Total								**200.00**
Mean								**33.33**
2	12 x10^3	6 x10^3	3 x10^3	6 x10^3	9 x10^3	3 x10^3	6 x10^3	45.00
4	9 x10^3	3 x10^3	NI	6 x10^3	6 x10^3	3 x10^3	6 x10^3	33.00
6	12 x10^3	3 x10^3	NI	3 x10^3	6 x10^3	6 x10^3	3 x10^3	33.00
8	11 X10^3	3 x10^3	NI	6 x10^3	3 x10^3	3 x10^3	3 x10^3	29.00
10	9 x10^3	6 x10^3	NI	3 x10^3	5 x10^3	12 x10^3	6 x10^3	41.00
12	9 x10^3	5 x10^3	NI	3 x10^3	3 x10^3	3 x10^3	6 x10^3	9.00
Total								**210.00**
Mean								**35.00**
2	15 x10^3	6 x10^3	6 x10^3	9x10^3	6 x10^3	6 x10^3	6 x10^3	54.00
4	12 x10^3	6 x10^3	NI	3 x10^3	3 x10^3	9 x10^3	3 x10^3	36.00
6	12 x10^3	6 x10^3	NI	6 x10^3	5 x10^3	6 x10^3	6 x10^3	41.00
8	11 x10^3	6 x10^3	NI	3 x10^3	3 x10^3	5 x10^3	6 x10^3	34.00
10	14 x10^3	3 x10^3	NI	3 x10^3	6 x10^3	10 x10^3	6 x10^3	42.00
12	12 x10^3	3 x10^3	NI	3 x10^3	3 x10^3	10 x10^3	6 x10^3	30.00
Total								**237.00**
Mean								39.70

4. CONCLUSION

In conclusion, this study has clearly observed that *Artemisia annua* plant temporally depressed soil bacterial population at concentrations of 7.50%, 5.00%, 2.50%, and 1. 00% as used in this study. However, this innocuous plant was also observed to boost fungal population at 7.50%, 5.00%, 2.50%, 1.00% than the control.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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