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Effect of Aqueous, Ethanolic and N-hexane Extracts of *Annona senegalensis* on Germinability of Okra Seeds

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Authors' contributions

This work was carried out in collaboration between both authors. Author OIA designed the study, wrote the protocol and wrote the first draft of the manuscript. He also identified the plant species, carried out the extraction of the plants using various solvents. Author NDT managed the experimental processes, carried out data analysis, reported and did the discussion and conclusion of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Annona senegalensis, generally known as African custard-apple has been indicated to inhibit the growth of pathogenic strains of *Shigella flexneri*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* while flavonoids isolated from the stem bark of *Annona senegalensis* have been shown to inhibit the growth of *Salmonella typhi*, *Escherichia coli*, and *Shigella* sp. The aim of this study was to develop a safe, cheap and cost effective treatment against selected plants pathogens and also at evaluating the protective potential of the extracts of *Annona senegalensis* against selected fungal pathogens of okra seeds. In this study, the effect of Aqueous, Ethanolic and N-hexane Extracts of *Annona senegalensis* on germinability of *Abelmoschus esculentus* was observed. A total of ten different concentrations (1000 mg/L to 10000 mg/L) of the respective crude extracts were used for the study. To each universal bottle containing 20 mL of the known concentrations of the respective extracts, approximately 20 surface sterilized seeds were added and allowed to stand for 30 min. At the termination of the planting period, the seedling height, % germination,

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germination rate, germination time, germination capacity, germination index and seeds vigor of each of the slots were estimated. In conclusion, it can be seen from the results of the study that aqueous, ethanol and N-hexane *A. senegalensis* extracts solution tested in this study have priming and antifungal potency.

Keywords: Annona senegalensis; germinability; aqueous; ethanolic and N-hexane extracts.

1. INTRODUCTION

Seeds being the source of plant may play a vital role in the total biological yield per unit time and per unit plant surface. It is indicated that the longer duration of seed treatment with plant extracts was effective in controlling the growth of all the surface borne seed organisms. Application of plant extracts for the control of seed borne diseases is a method devoid of any health hazard problem. Plant extract has least hazardous effects on the seeds as well as on soil. Seed treatment of plant extract will not create any problem of pollution and the chemicals of plant extracts are easily degraded in the soil [1].

Medicinal plants contain various compounds which may serve as potential antibacterial agents and they serve as an alternative, cheap, effective, and safe antibacterial for treatment of common bacterial infections. The use of natural compounds as plant extracts is one which can be characterized by lack of toxicity for humans and environment, selectivity, biodegradable activity and a great variety of chemical composition, with a large variety of secondary metabolites, most of them not yet studied in correlation with their fungicidal action [2].

Annona senegalensis, generally known as African custard-apple belongs to the family Annonaceae. It is a constant shrub that is widely grown in Nigeria and known as abo ewe-aso in Yoruba land, Gwandandaajii in Hausa land and Nungbera in Nupe land. It is a species of seed plant, which grows in dry and rainy seasons and a savannah plant that is broadly spread from Nigeria to Senegal, as well as Central African Republic. The wild fruit trees of the plant are found in sub humid to semi-arid regions in Africa [3,4,5]. *Annona senegalensis* is indicated to inhibit the growth of pathogenic strains of *Shigella flexneri*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Flavonoids isolated from the stem bark of *Annona senegalensis* have been shown to inhibit the growth of *Salmonella typhi*, *Escherichiacoli*, and *Shigella* sp. [6]. Diseases play a vital role in the decrease of the yield of cereal crops. Agents causing diseases comprise fungi, bacteria, nematodes, viruses, weeds and nutrient deficiencies. There are a lot of fungal, bacterial, viral diseases of sorghum [7]. The initial remedy of combating plant diseases is prevention and it involves combination of strategies including cultural practices, sanitation and spray applications. Sanitation is to remove the source of disease infection that may occur in the future using a clean-up program [8]. The aim of this study was to develop a safe, cheap and cost effective treatment against selected plants pathogens and also at evaluating the protective potential of the extracts of *Annona senegalensis* against selected fungal pathogens of okra seeds.

2. MATERIALS AND METHODS

2.1 Preparation of Extracts

The plant used for the study was *Annona senegalensis* leaves. The leaves of the plant were obtained from the environment of Landmark University, OmuAran, Kwara State. The leaves were first washed with clean tap water to eliminate sand and other debris and air-dried, before pulverizing using a laboratory grinder.

The pulverized leaves were soaked for 24 h in respective beakers containing the extraction solvents to be used (1:2 w/v). The extraction solvents used for the study were water, ethanol and N-hexane, which were referred to as aqueous, ethanolic and N-hexane extracts, respectively. At the end of the 24 h extraction period in the respective solvents, the extracts were filtered using Whatman no. 1 filter paper. While the supernatants were discarded, the respective filtrates were evaporated using a water bath at 37℃ and later concentrated in a rotary evaporator (MRC-ROVA 100) and freeze dried in a free-drier (LYOTRAP).

2.2 Selection of Test Seeds

Okra, with botanical name *Abelmoschus esculentus*, family Malvaceae were used for the study. The seeds were purchased from the local market in Omu-Aran, Kwara State, Nigeria. The

seeds were stored in cellophane bags and kept in a freezer, until when needed.

Prior to use, seeds soaked in distilled water to remove floating ones, which were perceived to be non-viable. This was followed by surface-sterilization of the seeds with 5% sodium hypochlorite (v/v) for 5 min. The surface sterilized seeds were afterwards rinsed several times with distilled water to remove the residual sodium hypochlorite. The viable seeds were kept in a plastic bag in the refrigerator till use. Prior to use, all seeds were confirmed to be viable.

2.3 Determination of Optimum Concentration

A total of ten different concentrations (1000 mg/L to 10000 mg/L) of the respective crude extracts were used for the study. To each universal bottle containing 20 mL of the known concentrations of the respective extracts, approximately 20 surface sterilized seeds were added and allowed to stand for 30 min. At the end of the 30 min soaking time of the seeds in the extracts, seven seeds were withdrawn at each concentration and planted on blotters that were placed in transparent plates with dimensions of 9 cm diameters and 4 cm heights. The respective blotters were pre-soaked in 50 mL of distilled water. In this study, the blotters used were absorbent cotton wools.

The plates containing the planted seeds were arranged on trays in the presence of fluorescent light and observed for daily germination of seeds for eight days. On a daily basis, 10 mL of distilled water were added to each plate containing the blotters and planted seeds to keep the environment moistened. At the termination of the planting period, the seedling height, % germination, germination rate, germination time, germination capacity, germination index and seeds vigor of each of the slots were estimated. All experimental sets were in duplicate.

2.4 Determination of Optimum Soaking Time

Using the optimum concentration for each of the respective extracts in respective beakers, the surface sterilized seeds were added. Every 30 min, for duration of 180 min, seven seeds were removed from each extract and planted in transparent plates containing the blotters. The plates were then incubated under fluorescent light for germination. On a daily basis germination profile of the seeds were recorded. Approximately 10 mL of distilled water was added to each plate containing the planted seeds on a daily basis. This was to help to keep the environment moist. Daily germination values were recorded while at the expiration of incubation, seedling height, % germination, germination rate, germination time, germination capacity, germination index and seeds vigor of each of the slots were estimated.

2.5 Assessment of Protective Potential of the Extracts

Using the optimum concentration and soaking time in each extracts, the protective potential of the extracts against selected fungal pathogens was investigated. The fungal pathogens used for the experiment were *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*. The isolates were already sub cultured on sabouraud dextrose broth after confirming them as pure cultures on sabouraud dextrose agar plates after incubation at 25℃ for 72 h.

The experimental groups were as follows:

- Seeds soaked in the respective extracts only.
- Seeds soaked in broth cultures of the respective fungal pathogens.
- Seeds that were soaked in the broth cultures of the fungal pathogens before soaking in the extracts.
- Seeds soaked in distilled water only.

In all experimental groups, apart from the groups that were first soaked in broth cultures of the pathogens before treatment with the extracts, the soaking time before planting was 60 min. In the group that was first infected before treatment, the soaking time was 60 min each in the broth cultures of the pathogens and in the extracts.

As was carried out in the optimum concentration and soaking times, after planting, daily germination values were recorded while at the expiration of incubation, seedling height, % germination, germination rate, germination time, germination capacity, germination index and seeds vigor of each of the slots were estimated.

2.6 Assessment of Antibacterial Potential of the Extracts

The bacterial isolates used were *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* and *Proteus*. They were all subcultured in nutrient broth after

culturing on nutrient agar plates to ensure they are pure colonies by incubating at 37℃ for 24 h.

To assess the antimicrobial potential of the extracts, sterile nutrient agar in 250 mL capacity flask was allowed to cool to 45℃, after which aliquots of broth cultures of a bacterial isolate was added. The flasks were swirled to allow homogenization of the organisms with the agar. Each of the agar was poured into labeled plates and allowed to set. Different concentrations of the extracts (10000 mg/L, 8000 mg/L, 6000 mg/L, 4000 mg/L and 2000 mg/L) were prepared into universal bottles. Four holes each were bored into the plates containing the organisms according to the concentrations of extracts. To the bored holes, 0.2 mL of the respective concentrations of the extracts were added using a micropipette. The plates were allowed to stay for a while and incubated at 37℃ for 24 h. The zone of inhibition was measured using a ruler and recorded in millimeters.

2.7 Statistical Analysis

Statistical analysis was carried out using the SPSS Statistical Software. Comparison of means was carried out using the One-Way Analysis of Variance (ANOVA) at 95% confidence interval.

3. RESULTS

3.1 Effects of Different Concentrations

As shown in Table 1. At the end of the planting period, at different concentrations of the aqueous extracts of *Annona senegalensis*, the percentage germination ranged from 30.95%-78.57%, with concentration of 4000 mg/l being the least at 30.95% and 6000 mg/l being the highest at 78.57%. The germination index ranged from 1.89-4.05 with 4000 mg/l being the least and 7000 mg/l being the highest. Germination rate ranged from 0.16-0.24d^{-1}. 3000 mg/l, 6000 mg/l, 8000 mg/l and 9000 mg/l were the least with a value of 0.16d^{-1}. 1000 mg/l had the highest germination rate of 0.24d^{-1}. Germination time ranged from 4.22 – 6.39d. 10000 mg/l had the least germination time and 6000 mg/l had had the highest germination time. Seed vigor ranged from 0.00-919.28. 1000 mg/l had the highest seed vigor while 3000 mg/l, 4000 mg/l, 5000 mg/l, 6000 mg/l and 7000 mg/l had the least seed vigor of 0.00 (Table 1).

In presence of the ethanolic extract, the concentration with the highest percentage germination 78.57% is 5000 mg/l while the concentration with the least percentage germination 42.86% is 8000 mg/l. the least germination index 2.31 is at a concentration of 8000 mg/l while the highest germination index 5.61, is at a concentration of 5000mg/l. the least germination rate was 0.16d^{-1} at concentrations of 4000 mg/l and 9000 mgh/l while the other concentrations had a germination time of 0.17d^{-1}. 3000 mg/l and 4000 mg/l have the least seed vigor of 200.01 and 1000 mg/l had the highest seed vigor of 642.55 (Table 2).

When soaked in the N-hexane extract, percentage germination ranged from 38.57-78.57% with 10000 mg/l having the least percentage germination and 6000 mg/l having the highest percentage germination. Germination index ranged from 2.84-5.48 with 10000 mg/l

Table 1. Effect of the different concentrations of the aqueous extract on the germination and vigor parameters of Okra seeds

Concentration (mg/L)	%	GI	GR	GT	SV
1000	64.29	3.86	0.24	4.22	919.28
2000	66.67	3.36	0.17	6.03	566.70
3000	42.86	2.04	0.16	6.25	0
4000	30.95	1.89	0.17	5.90	0
5000	60.00	2.90	0.17	5.90	0
6000	78.57	3.32	0.16	6.39	0
7000	64.29	4.05	0.17	5.93	0
8000	57.15	3.21	0.16	6.11	571.45
9000	35.72	2.09	0.16	6.08	410.72
10000	42.86	2.91	0.18	5.71	235.73

The soaking time of the seeds in the extract was 30 min. Values are averages of duplicate samples. %, GI, GR, GT and SV represent % germination, germination index, germination rate, germination time and seed vigor, respectively

having the least value and 8000 mg/l having the highest value. Germination rate range from 0.16-0.19d^{-1}; 4000 mg/l has the least germination rate of 0.16d^{-1}, 800 mg/l has a germination rate of 0.18d^{-1}, 10, 000 mg/l has the highest germination rate of 0.19d^{-1} while the other concentrations have a germination rate of 0.17d^{-1} each. Germination time ranged from 5.33-6.18d, 10,000 mg/l has the least germination time of 5.33d while 4000 mg/l has the highest germination time of 6.18d. Seed vigor ranged from 0.00 – 2100. 1000 mg/l and 2000 mg/l have zero seed vigor while 5000 mg/l has the highest seed vigor (Table 3).

Generally, at the different concentrations of the extracts, although a comparison of % germination, germinate rate, germination time and seed vigor index of the seeds treated with the respective extracts were observed to be different, these differences were not observed to be significant. However, in the case of germination index, values were observed to be significantly higher in seeds treated with the N-hexane extracts than in those treated with the aqueous extract ($p \leq 0.01$).

3.2 Effect of Soaking Time

In presence of the aqueous extract, highest % germination and germination index of 42.86% and 2.34 were observed at soaking time of 150 min. The highest germination rate 0.81d^{-1} was at 120 mins while 60 mins showed the highest seed vigor of 142.88. 30 mins, 90 mins, 120 mins and 180 mins had the least % germination of 21.43% each. Germination index 1.79 and germination time 1.23d was least at 120 mins; 150 mins had the least value of germination rate 0.43d^{-1}. 90 mins had the least seed vigor 21.43 (Table 4).

Table 2. Effect of the different concentrations of the Ethanolic extract on the germination and vigor parameters of the okra seeds

Concentration mg/L	%	GI	GR	GT	SV
1000	64.29	3.86	0.17	6.05	642.85
2000	50.00	3.41	0.17	5.79	475.00
3000	57.15	3.97	0.17	5.88	200.01
4000	57.15	3.05	0.16	6.12	200.01
5000	78.57	5.61	0.17	5.79	196.43
6000	71.43	4.06	0.17	6.04	405. 01
7000	64.29	3.67	0.17	6.07	385.71
8000	42.86	2.31	0.17	6.04	235.73
9000	57.14	2.67	0.16	6.31	571.40
10000	71.43	4.24	0.17	6.02	249.99

The soaking time of the seeds in the extract was 30 min. Values are averages of duplicate samples. %, GI, GR, GT and SV represent % germination, germination index, germination rate, germination time and seed vigor, respectively

Table 3. Effect of the different concentrations of the N-hexane extract on the germination and vigor parameters of the okra seeds

Concentration mg/L	%	GI	GR	GT	SV
1000	64.29	4.23	0.17	5.91	0.00
2000	57.15	3.67	0.17	5.84	0.00
3000	57.15	4.04	0.17	5.81	342.87
4000	64.29	3.42	0.16	6.18	289.28
5000	75.00	5.18	0.17	5.87	2100.00
6000	78.57	4.99	0.17	5.96	408.56
7000	57.15	3.80	0.17	6.02	369.64
8000	64.29	5.48	0.18	5.53	406.92
9000	56.25	4.61	0.17	5.90	365.63
10000	38.57	2.84	0.19	5.33	269.99

The soaking time of the seeds in the extract was 30 min. Values are averages of duplicate samples. %, GI, GR, GT and SV represent % germination, germination index, germination rate, germination time and seed vigor, respectively

Table 4. Effect of soaking time on germinability of the okra seeds at different soaking periods in the respective extracts

Soaking time	%	GI	GR	GT	SV
Aqueous extract					
30 min	21.43	2.24	0.70	1.42	58.84
60 min	28.57	2.77	0.53	1.88	142.88
90 min	21.43	2.08	0.72	1.39	21.43
120 min	21.43	1.79	0.81	1.23	130.72
150 min	42.86	3.39	0.43	2.34	42.86
180 min	21.43	1.85	0.78	1.29	42.86
Ethanolic extract					
30 min	35.71	2.14	0.50	2.02	107.15
60 min	21.43	1.56	0.83	1.21	10.72
90 min	28.57	1.81	0.62	1.62	28.58
120 min	42.86	5.07	0.35	2.88	32.15
150 min	28.57	1.81	0.62	1.62	171.45
180 min	42.86	3.60	0.38	2.68	96.42
N-hexane extract					
30 min	28.57	2.52	0.57	1.76	128.57
60 min	64.29	3.96	0.29	3.50	321.43
90 min	28.57	1.56	0.66	1.52	35.71
120 min	14.29	1.16	1.11	0.90	17.86
150 min	14.29	1.00	1.15	0.87	21.43
180 min	21.43	1.69	0.80	1.26	32.15

%, GI, GR, GT and SV represent % germination, germination index, germination rate, germination time and seed vigor, respectively

The soaking time for ethanolic extract shows that 60 mins had the value of 21.43%, 1.56, 1.21d and 10.72 for percentage germination, germination index, germination time and seed vigor respectively while germination rate 0.35d^{-1} was least at 120 mins. Germination time, germination index and percentage germination were highest 2.88d, 5.07 and 42.86% at 120 mins. 180 mins also had a percentage germination of 42.86%. Seed vigor was highest 171.45 at 150 mins.

When soaked in the N-hexane extract, the soaking time 60 mins had the highest values of 64.29%, 3.86, 3.50d and 321.43 for percentage germination, germination index, germination time and seed vigor respectively while it had the least germination rate vale of 0.29d^{-1}. Percentage germination 14.29% was least at 150 min and 120 min, seed vigor 17.86 was least at 120 mins, and germination rate 1.15d^{-1} was highest at 150 min while it was least 1.00 and 0.87d for germination index and germination time respectively.

At the different soaking time of the seeds in the respective extracts, no significant differences ($p \leq 0.05$) were observed for the different parameters investigated between seeds soaked ion the respective extracts.

3.3 Protective Potential of the Extracts

When infected with the fungal species, percentage germination of the seeds was observed to be 28.57%, 35.71% and 42.86% in seeds infected with the *A. niger, A. flavus* and *A. fumigatus,* respectively. When the infected seeds were treated the extracts, germination percentage showed ranges from 28.57-35.71% and from 14.29-78.57% for seeds treated with the ethanol and the aqueous extracts, respectively. For infected seeds that were treated with the N-hexane extract, germination was observed to be 42.86%. This observation in presence of the N-hexane was irrespective of the pathogen used in infecting the seeds (Table 5).

4. DISCUSSION

In the present study, different extracts of the leaves of *Annona sengalensis* was evaluated for the investigation of their priming potential on Okra seeds and their antimicrobial activity against certain species of *Aspergillus.* In presence of the extracts, the okra seeds were

observed to show remarkable increase in germination parameters and seed vigor of the okra seeds. This observation was in agreement with similar studies by previous investigators. [9] had reported enhanced germination and seedling vigor when seeds were pretreated with cytokinins in tomato cultivars najina and pakit by dormancy breakdown. Similarly, [10,11,12] have reported improved priming and growth of tomatoes, asparagus and cucumber, respectively in similar studies. In another study, the effect of aqueous extracts of Neem (*Azadirachta indica*) on germination percentage depicted that Neem aqueous extract slightly inhibited seed germination of cow pea (90, 70 and 60%) over control, whereas it had very little or no significant effect on germination of horse gram [13]. [14] have also established increase in germination rates and an improvement in seedling development of rice seeds treated with leaf extract of *Clerodendrum viscosum*.

Table 5. Protective potential of the extracts on the infected seeds

Pathogens	%	GI	GR	GT
Infected seeds				
A. niger	28.57	1.98	0.25	4.44
A. flavus	35.71	2.04	0.21	4.67
A. fummigatus	42.86	1.89	0.20	4.94
Infected seeds treated with ethanol extract				
A. niger	35.71	2.21	0.22	4.58
A. flavus	35.71	1.71	0.21	4.88
A. fummigatus	28.57	1.98	0.23	4.44
Infected seeds treated with N-hexane extract				
A. niger	42.86	2.68	0.22	4.57
A. flavus	42.86	2.52	0.22	4.64
A. fumigatus	42.86	2.18	0.21	4.80
Infected seeds treated with the aqueous extract				
A. niger	14.29	1.03	0.23	4.38
A. flavus	42.86	3.02	0.23	4.42
A. fummigatus	78.57	3.02	0.03	4.42

%, GI, GR and GT represent % germination, germination index, germination rate and germination time, respectively

At the different soaking times, maximum germination was observed when seeds were pre-soaked before planting for 150 min, 120 min and 60 min in presence of the aqueous, ethanol and N-hexane extracts, respectively. In their study [15], reported that sorghum seed primed with 5% moringa leaf extract for 720 min was the best seed priming technique as it gave the maximum final germination percentage and the minimum time for 50% germination as well as mean emergence time. This treatment also gave significantly the highest number of roots and leaves along with root and shoots length. The result of this study indicates that seeds soaked in water only performed poorly as compared to the extracts, *Pseudomonas aeruginosa* and *Bacillus subtilis*. This finding agrees with the results of similar work carried out by [16] who reported that seeds soaked in plant extract of *Annona senegalensis* performed better as compared to when soaked in water only, but differs from the study of [17] who reported that water soaking for 720 mins effectively enhanced emergence, seedling vigor, crop establishment of the bitter gourd cultivar.

4.1 Infection and Treatment

Seed is probably the most important single input for arable cultivation that determines the potential production and thus productivity of all other inputs [18]. About 90 % of the world food crops including sorghum (*Sorghum bicolor* (L) Moench) are propagated by seed [19]. However, seeds can be passive carriers of pathogens that are transmitted when the seed hosts are sown and emerge under suitable environmental conditions. Fungi, bacteria, viruses and nematodes can be carried with, on or in the seeds resulting in tremendous yield losses [20]. Seed germination is controlled by a number of mechanisms and is necessary for the growth and development of the embryo resulting in the eventual production of a new plant [21]. The ability of plant extracts to increase seed germination could be attributed to the suppression of seed borne fungi that could have consider to kill the embryo of the seeds leading to germination failure [22]. According to [23], extracts of some higher plants exert antifungal activity against fungi. The most important of bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolics compounds [24]. Plant metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides [25]. Application of plant extracts for the control of seed borne diseases is a method devoid of any health hazard problem. Hill bunt of Wheat (*Tilletia foetida*) was effectively controlled by seed treatment with plant extracts of *Datura stramonium, Thuja*sp. and *Eucalyptus* [26].

In this study the performance of the okra seeds recorded when infected with the fungal

pathogens was not as low as anticipated. This finding deviates from the work of [27] who reported a high positive relationship between germination failure and prevalence of seed borne fungal infection on several vegetable seeds. However, the present study revealed that there were indications of antimicrobial activity when the infected seeds were treated with leaf extracts of *Annona senegalensis* prior to planting. This result is an indication that these extracts probably have some fungicidal properties that could inhibit seed infection. [22] obtained best performance in terms of reducing percentage seed infection and increasing percent seed germination through treating cotton seeds with *Azadirachta indica* and *Boswelli adalzielii* extracts. Stimulation of seed germination and suppressing seed infection was reported in rice seed treated with extracts from *A. indica*, [28]. On an all-encompassing view, aqueous extracts were more effective in controlling the *Aspergillus* spp. than other extracts, whereas ethanol extract was the least effective. This may be an indication that the active antifungal compounds in the leaves of *A. senegalensis* are more readily extracted in water as compared to the other extracts. This finding disagrees with [29] who found that plant extracts from organic solvents give more consistent antimicrobial activity compared to those from water.

5. CONCLUSION

In conclusion, it can be seen from the results of the study that aqueous, ethanol and N-hexane *A. senegalensis* extracts tested in this study have priming and antifungal potency. However further studies are needed to establish the best among the extracts. Nevertheless, the ideal concentration that will enhance germination for aqueous, ethanol and N-hexane extract is 1000 mg/L, 5000 mg/L and 4000 mg/L respectively. The best soaking time for the extracts is 150 mins for aqueous extract, 180mins for ethanol extract and 60 mins for N-hexane extracts. The pathogens did not have conspicuous effect on the germination parameters of the okra seeds.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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