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The bioactivities of the neem (seeds, leaves and their extracts) against *Callosobruchus maculatus* on *Vigna Subterranean L.*

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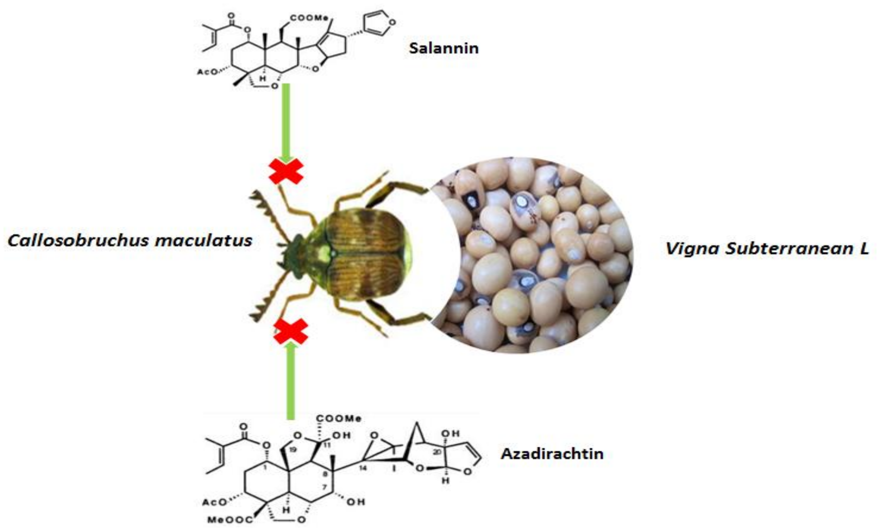
GRAPHICAL ABSTRACT



KEYWORDS

Biopesticides
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ABSTRACT

This research reports the novel bioactivities of neem leaves powder, neem leaves concentrate, neem seeds powder and neem seeds oil against the weevil (*Callosobruchus maculatus*) on bambara groundnut. The leaves were ground, crushed and subjected to ethanol to get the concentrate. While the seeds were dried under shade for one week, ground into fine particles. Cold bulk extraction was then used to obtain the neem seeds oil. The mortality count of *Callosobruchus maculatus (C. maculatus)* was studied by exposing the weevil to neem treated bambara groundnut at different days under laboratory condition. The neem dosage were 0.0, 0.1, 0.5 1.0 g/10 g of bambara groundnut. The percentage weight loss and seed damage were also determined at the end. The results indicated that neem seeds oil and neem leaves concentrate provided effective and comparable mortality counts against the *C. maculatus*. Again the neem powder (seeds and leaves) mortality counts were also comparable, but were less effective against the weevils as compared to the neem oil and concentrate at least for the first two days. Therefore, these neem materials could be used as biopesticides at the dose of 0.1 per 10 g to prevent *C. maculatus* attack on bambara groundnut during storage. This will reduce the usage of synthetic pesticides, thereby preventing their adverse residual effects on human and the environment.

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1. Introduction

In sub saharan Africa, a popular but neglected nutritional legume is the bambara groundnut (*Vigna subterranean (L) Verdc.*) [1-3]. It is also known as ground peas. The bambara groundnut can adapt to various climatic and ecological conditions. Its seeds are rich in lysine and methionine, but bambara groundnut is available in very low amount to consumers [1]. It is also highly caloric plant with 387 kcal/ 100 g, rich in vitamins, minerals and very balanced protein components [1, 2]. Report has it that the seed contains about 63% carbohydrate, 19% protein and 6.5% oil [3]. The ground peas can be used to prepare soup, porridge and various fried or steamed food such as 'akara', 'moi-moi' and 'okpa'. The fresh pods are also boiled with salt and pepper and eaten as snack [3]. Dry seeds are equally roasted with salt and eaten. Furthermore, bambara groundnut cultivation offers soil fertilization through the fixation of symbiotic nitrogen associated with rhizobium bacteria. In spite of the obvious and numerous advantages, bambara groundnut is still a neglected crop and rarely used in plant breeding programs [1]. There is need to enhance the production of this crop and raise it as a strategic crop to fight hunger. On the other hand, stored insect pests are nuisance worldwide because they reduce the quantity and quality of grain. Their damage to stored grains may amount to 40% in favorable conditions [4]. Two bruchids (Coleoptera: Chrysomelidae): *Callosobruchus subinnotatus* (Bridwell) and *Callobruchus maculatus* (Fabricius) are major pests that attack the common morphotypes of bambara groundnuts [1] in sub saharan Africa. Therefore, there is need for improvement in the production and preservation of bambara groundnut so as to facilitate food sustainability and overcome chronic proteomic deficiency [5]. Otherwise, is known that the production in agriculture is reduced by losses as high as 40 - 45% on average before or after harvesting due to attack of variety of pests including insects, nematodes, virus and bacteria, induced diseases and competition by weeds [5]. In addition, about one third of global agricultural production valued at several billion dollars is destroyed annually by over 20,000 species of insect pests in field and storage [6].

Usually synthetic pesticides are used to reduce post-harvest losses. Hence, pesticides are substances or mixture of substances used to prevent, destroy, repel, sterilize, or mitigate pests. The use of synthetic pesticides to prevent insect infestations is currently popular due to their simplicity and cost-effectiveness [4, 7]. Unfortunately, these chemicals contaminate both food and environment [1]. The organochlorine, organophosphate, carbamate, and pyrethroid insecticides are of a particular concern because of their toxicity and persistence in the environment [5]. Another literature has it that, the drawbacks of synthetic pesticides are pest resurgence and resistance, lethal effects on non-target organisms, the risk of users self-contamination, food residues, and environmental pollution [4, 6]. It was also found that synthetic pesticides can play a role in the cancer process by either non-genotoxic mechanisms such as promotion, peroxisome proliferation, and hormone imbalance, or by affecting the carcinogenic process in a variety of ways, of which both can alter the genome and provide a growth advantage for neoplastic cells [5]. Pesticides may induce oxidative stress leading to the generation of free radicals and alteration in antioxidant or oxygen free radical scavenging enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione transferase [5]. Human population is exposed to these chemicals primarily through the consumption of pesticide contaminated farm products, leading to long-term health hazards.

In view of the above, there is an urgent need to develop safe alternatives to conventional pesticides for the protection of grain products against insect infestations. Biopesticides are type of pesticides derived from natural materials like animals, plants, bacteria, and certain minerals [6]. Higher plants are rich source of novel pesticides and have been used traditionally for time in memoria [4, 5]. Biopesticides are becoming more popular with food manufacturers and consumers. Many experts forecast huge growth in the sales of biopesticides over the next decade. Biopesticides could grow from 4–5% of the global pesticide market to as much as 20% by 2025. The United States, China, India and Europe have been the leaders in the commercialization of botanical pesticides in the last few years, mainly due to regulatory schemes that encourage and facilitate the movement of 'reduced-risk' products so as to get rid of older conventional pesticides which have less desirable properties [5]. Most of the plant secondary metabolites are reported as insecticidal agents that could be an effective alternative for insect pest management. Moreover, the plants secondary metabolites (such as flavanoids, terpenes, phenols, alkaloids, sterols, waxes, fats, tannins, sugars, gums, suberins, resin acids carotenoids etc.) protect them against microbial pathogens and invertebrate pests [6]. Biopesticides are safer, less expensive and easily aplied by farmers and small industries than synthetic ones [4]. They affect only target pest and closely related organisms, are effective in very small quantities, decomposed quickly and provide residue free food and a safe environment to live [6, 8]. Well known example of botanical insecticides is the natural pyrethrin, found in *Chrysanthemum* sp., which led to development of a class of synthetic insecticides: pyrethroids. Neem (*Azadirachta indica)* is the most important botanical insecticide presently in use through the world [4, 6]. Neem was 'discovered' in the western world in 1959 when a German entomologist noticed that it was the only green standing after a swarm of locusts swept through the Sudan. Among the recognized 'pesticidal' plants, *Azadirachta indica* provides a unique source for numerous active ingredients having insecticidal properties [9]. Neem is native to India and naturalized in most of tropical and subtropical countries. Neem seed oil cream has proven to be very valuable as insect repellent particularly at higher concentrations of 7.5% and 10.0% w/w. This can be considered as an alternative to conventional pesticides that have been associated with certain adverse effects as earlier mentioned [10]. Again, *Azadirachta indica*, *Schinnus molle*, and *Phytolacca odecandra* extracts were found to produce high percentage larval mortality (>95%) against Fall Armyworm (FAW) after 72 h application [11]. Lemon grass (*Cymbopogoncitratus*) leaf extracts also protected bambara ground nut better than Jatropha (*Jatropha curcas)* against field pest [2]. Azad and Sarker [12] reported that *Nicotiana tabacum and Ficus hispida* extracts have effective performance against pest attack in eggplant field [12]. Similarly, essential oil products have recently emerged as the most important botanical insecticides [13]. Therefore, when incorporated into integrated pest management programs, botanical pesticides can greatly decrease the use of conventional pesticides or can be used in rotation or in combination with other insecticides, potentially lessening the overall quantities of conventional pesticides applied and possibly mitigating or delaying the development of resistance in pest populations [6]. One wonders, in spite of the effectiveness, advantages and potentials of the biopesticides, they are still grossly underutilized in most part of the world. This has left much to be desired of and exploited in order to achieve food safety and sustainability without sabotaging future generation from doing same. Hence, this paper reports the novel and facile bioactivities of the neem (seeds, leaves and their extracts) against *Callosobruchus maculatus* on *Vigna Subterranean L.* under laboratory condition.

2. Materials and Methods

2.1. Collection of the bambara groundnut seed

Local untreated bambara groundnut landrace (Igbogh-ahi Jato-Aka) were purchased from a local market in Adikpo, Kwande Local Government Area of Benue State, Nigeria. The weevils infected grains were separated out. Then, the uninfected and clean seeds were put into a big beaker and covered firmly with calico cloth. This was then kept for subsequent experimentation in the laboratory.

Collection and preparation of the neem biomass

Fresh neem leaves were collected from the school promises of Benue State University Makurdi - Nigeria. The collected neem leaves were dried and ground. Neem seeds were also collected within the premises of the school, Benue State University Makurdi, Benue State - Nigeria. The seeds were dried under shade for one week, ground into fine particles, sealed in thick polythene bag and kept in a dry cool place for future use.

2.2. Apparatus and reagent

The apparatus and reagents used include: test tube, thermometer, rubber band, beakers, sulphuric acid, ethanol, wire guage, tripod stand, retort stand, capillary tube, cork, acid bath, conical flask, hot plate, calico cloth, funnel, pestle, mortal, spatula, glass bottle, pippete and refractometer.

2.3. Collection of the weevils (*Callosobruchus maculatus*)

Adult *Callosobruchus maculatus* were sourced from an infected bambara seeds from Wurukum market, Makurdi, Benue State - Nigeria. About 0.50 kg clean bambara seeds were weighed out into two plastic breeding containers, respectively, and the bruchids introduced into them. The containers were them firmly covered with clean calico cloth. The introduce bruchids were discarded after 7 days. The seeds, with eggs were allowed to stay until emergence of bruchids after about 22 days. On emergence, the freshly emerged bruchids were used for the experiment.

2.4. Cold bulk extraction of the neem seed oil

Cold bulk extraction procedure was used to extract oil from the ground seeds. About 50 g of ground neem seeds was put into a big glass bottle and 1.5 L of ethanol added, agitated for proper interaction and allowed to settle. After 2 days, the liquid oil was separated from the chaff by placing a clean calico cloth at the mouth of a funnel. The filtrate was then poured into a 500 mL beaker and place on hot plate at a low temperature (70 ^{o}C) for 3 h to allow the ethanol to evaporate to obtained solvent-free oil.

2.5. Extraction of the neem leaves concentrate

About 50 g ground neem leaves was put into a big glass bottle and 1 L of ethanol added, agitated for proper mixing and it was allowed to settle. After 2 days concentrate was separated from the chaff, the filtrate was then poured into beaker and place on hot plate at a low temperature of 70 ^{o}C for 3 h to allow the ethanol to evaporate.

2.6. Bioactivity tests for the mortality count

About 0.1, 0.5 and 1.0 g each of the neem samples were weighed and added to a 10 g of undamaged and uninfected bambara groundnut in 250 mL glass beaker. The containers with their contents were gently shaken to ensure thorough mixting of the bambara groundnut seeds and neem materials. Ten (10) pairs of *C. maculatus* adults were then introduced into the beakers. The beakers were covered with calico cloth held firmly with rubber band. Untreated bambara seeds with the 10 bruchids were used as the control. These treatments were replicated three times. The mortality of *C. maculatus* was assessed after every 24 h for 4 days. *C. maculatus* was considered dead when probed with sharp objects and there was no response. On day 5 all insects, both dead and alive were removed from each container. The % mortality, % weight loss and % seed damage were determined using these equations as previously reported [14, 15].

$$\%\ \text{mortality} = \frac{Number\ of\ insects\ that\ died}{Number\ of\ initial\ insects} \times \frac{100}{1}$$

$$\%\ \text{weight loss} = \frac{Initial\ weight - final\ wight}{Initial\ weight} \times \frac{100}{1}$$

$$\%\ \text{seed damage} = \frac{Number\ of\ perforated\ grains}{Total\ number\ of\ grains\ counted} \times \frac{100}{1}$$

3. Results and Discussion

3.1. Effects of neem seeds oil and neem leaves concentrate on mortality of the C. maculatus

The mortality counts of neem seeds oil against the weevil increased with time (24 h, 48 h, 72 h and 96 h, respectively). In Table 1, at 0.1 g the result shows 5, 7, 10, 10 mortality counts at the various days, respectively. Whereas, 7, 8, 10, 10 mortality counts for 24 h, 48 h, 72 h and 96 h, respectively were found with 0.5 g neem seeds oil. Same trend was observed for the case of 1.0 g of the neem seeds oil. There was an increase in the percentage mortality count (0%, 47%, 70%, 100%) of *C. maculatus* as the concentration of the neem seed oil increased (0.0 g, 0.1 g, 0.5 g and 1.0 g, respectively) at 24 h. Similarly the % mortality counts of the pest were increased with the increasing amount of the neem seeds oil at 48 h, 72 h and 96 h. Within about 10 minutes, deaths of the pest were recorded mostly at higher amount of the neem material as similarly reported by another researcher [16]. On the neem leaves concentrate as well, the results (see Table 2) show that there is an increase in mortality of the weevils with time (24 h, 48 h, 72 h and 96 h, respectively) after their exposure to the neem leaves concentrate treated bambara groundnut. This was the general observation with time at the different amounts of the neem leaves concentrate as earlier noted with the neem seeds oil. There was a direct relationship between the amounts of the neem seeds oil and neem leaves concentrate with % mortality counts. In addition, the neem leaves concentrate and the neem seeds oil are comparatively effective in terms of the mortality counts against these weevils as previously reported [16]. More so, neem pesticides is relatively less toxic, environmentally friendly and effective substitutes of conventional pesticides for an integrated pest management [16, 17]. Previously, it is found that the neem seeds oil showed some correlations between the death rates for the insect-pests as against the time and concentration levels [16]. The higher the concentration of the neem seed oil, the faster it killed the pests [16]. Biologically active constituents of *A. indica* that makes it efficient as pesticide are Azadirachtin and Salannin [8, 18]. Especially the azadirachtin, its major modes of action are that of powerful insect growth regulator (IGR), a feeding and an oviposition deterrent. It is structurally similar to the natural insect hormone ecdysone. Azadirachtin interferes with the production and reception of this insect hormone during insect's growth and molting. Thus, azadirachtin blocks the molting cycle causing the insect to die [8]. Botanicals have been reported as antifeedant and growth inhibitors to insect, probably for their interference in hormone mechanisms [4].

3.2. Effects of neem powders (leaves and seeds) on mortality counts of the C. maculatus in bambara groundnut

The study revealed that the bambara groundnut treated with neem leaves powder (see Table 3) at the dosage of 0.1 g, 0.5 g and 1.0 g per 10 g gave promising levels of control of *C. maculatus* in because of increased in mortality of the weevils. There is an increased in the mortality of the weevil for 24 h, 48 h, 72 h, and 96 h at the different amounts of the leaves powder. In neem seeds powder, there is also an increased in the mortality counts of the weevil with time and as the amount of the neem seeds powder increased as may be seen in Table 4.

Table 1: Mortality counts of *C. maculatus* in bambara groundnut treated with neem seeds oil

Mortality counts at different times using varying amount of the oil																
	24 h				48 h				72 h				96 h			
	0.0g	0.1g	0.5g	1.0g	0.0	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g
1	0	5	7	10	0	7	8	10	0	10	10	10	0	10	10	10
2	0	4	7	10	0	7	8	9	0	10	10	10	0	10	10	10
3	0	5	7	10	0	7	9	10	0	10	10	10	0	10	10	10
Total	0	14	21	30	0	21	25	29	0	30	30	30	0	30	30	30
Mean	0	5	7	10	0	7	8	10	0	10	10	10	0	10	10	10

Table 2: Mortality counts of *C. maculatus* in Bambara groundnut treated with neem leaves extract

Mortality counts at different times using varying amount of the extract																
	24 h				48 h				72 h				96 h			
	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g
1	0	3	5	10	0	7	9	10	0	8	9	10	0	10	10	10
2	0	3	6	10	0	7	9	10	0	9	9	10	0	10	10	10
3	0	3	6	10	0	6	9	10	0	8	9	10	0	10	10	10
Total	0	9	17	30	0	20	27	30	0	25	27	30	0	30	30	30
Mean	0	3	6	10	0	7	9	10	0	8	9	10	0	10	10	10

Table 3: Mortality counts of C. maculatus in bambara groundnut treated with neem leaves powder

Mortality count at different times using varying amount of the extract																
	24 h				48 h				72 h				96 h			
Runs	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g
1	0	1	1	2	0	5	6	6	0	6	7	8	0	9	10	10
2	0	1	1	3	0	4	5	6	0	6	7	9	0	9	9	10
3	0	2	2	3	0	5	6	7	0	5	6	9	0	8	9	10
Total	0	4	4	8	0	14	17	19	0	17	20	25	0	26	28	30
Mean	0	1	1	3	0	5	6	6	0	6	7	8	0	9	9	10

Table 4: Mortality counts of C. maculatus in bambara groundnut treated with neem seeds powder

Mortality count at different times using varying amount of the extract																
	24 h				48 h				72 h				96 h			
Runs	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g
1	0	2	3	4	0	7	8	9	0	8	9	10	0	10	10	10
2	0	2	2	3	0	6	7	8	0	7	9	10	0	9	10	10
3	0	2	3	4	0	5	7	8	0	8	8	10	0	10	10	10
Total	0	6	8	11	0	18	22	25	0	23	26	30	0	29	30	30
Mean	0	2	3	4	0	6	7	8	0	8	9	10	0	10	10	10

Table 5: % weight loss and perforated Index

Samples	**% weight loss @ different conc. (g/10 g)**				**Damage @ different conc. (g/10 g)**			
	0.0g	0.1g	0.5g	1.0g	0.0g	0.1g	0.5g	1.0g
NLP	1.0	0.4	0.0	0.0	3	2	0.0	0.0
NSP	0.8	0.1	0.0	0.0	3	1	0.0	0.0
NSE	1.0	0.1	0.0	0.0	2	1	0.0	0.0

NLE	1.0	0.0	0.0	0.0	2	2	0.0	0.0

NLP = Neem leaves powder, NSP =Neem seeds powder, NLE = Neem leaves extract, NSE = Neem seeds extract

In Table 4, % mortality of 100% was observed at 1.0 g in 72 h unlike the neem leaves powder. Thus, the neem seeds powder is quicker in bioactivity against these weevils than the neem leaves powder. It was also previously and similarly reported that neem leaves insecticides are less toxic as compared to those from the synthetic origin [19]. The powdery form of neem is normally used for the preservation of stored seed-bean grains against weevil attacks or mixed with dry ground clay or sawdust and sprinkled over young plants, such as maize and sorghum, against pest infestation. The increasing demand for high quality food, free from chemical residues, makes it imperative that botanical means of protecting stored products or crops against insect damage be used [16]. Botanical pesticides (essential oils, flavonoids, alkaloids, glycosides, esters and fatty acids) have various chemical properties and modes of action and effect on insects in different ways namely; repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants. So it is preferable to use the botanical insecticides instead of synthetic insecticides. Moreso, the use of botanical insecticides is recognized by organic crop producers even in developed nations [7].

3.3. % weight loss and damage (perforated) assessment

The weight analysis of the bambara groundnut treated with neem material of 1.0 g and 0.5 g/ 10g recorded 0 % weight loss for all the different forms of the neem materials. This implied there was effective protection of the bambara nut against the weevils. Meanwhile, the % weight losses of the bambara nut were highest when 0.1 g neem material was used. In addition, the results of the seed damage showed neem leaves powder and its concentrate gave the highest seed damage of 2 at 0.1 g as given in Table 5. Generally, the % weight loss and seed damage decreased with increase in the neem materials used. Furthermore, the present research is in agreement with previous finding as given below. Nasiru *et al.*[14] reported the insecticidal activity of *Tamarindus indica*, *Azadirachta indica* and *Jatropha curcas* leaves powder against maize weevil (*Sitophilus zeamais*). The results show that T. *indica* was found to be the most effective in the seeds treatment with 97%, while A. *indica* and J. *curcas* have 66% and 40% adult mortality at 1.0, 1.5 and 2.0g/20 maize seeds respectively. The study recommended T. *indica* to be used in grain storage against insect pests especially *Sitophilus zeamais*. Also Edelduok *et al.* [20] studied insecticidal activity of cotyledon powder of melon (*Citrullus vulgaris* Schrad) against the maize weevil (Sitophilus zeamais, Motschulsky). Cotyledon powder of melon, *C. vulgaris*, was mixed with 50g maize grains as direct admixtures at seven different rates: 0g, 0.5g, 1.0g 1.5g, 2.0g, 2.5g and 3.0g, and infested with five males and five females of *S. zeamais* respectively. These were observed for twenty-eight days for natality, mortality and oviposition of *S. zeamais*. Melon cotyledon powder significantly reduced the natality and oviposition of *S. zeamais* while significantly increasing the mortality ($P < 0.05$). Cotyledon powder of melon seed could therefore be used as a grain protectant at the rate of 3.0g per 50g of maize to achieve complete mortality of *S. zeamais*, while effectively decreasing natality and oviposition of *S. zeamais* in storage pest management systems. This will reduce the usage of chemical pesticides, thereby reducing their adverse effects on stored products. Ileke and Oni [15] reported the effect of four plant powders including *Azadirachta india, Alstonia boonei, Garcina kola* and *Moringa oleifera* on the mortality adults and mergence of maize weevil (*Sitophilus zeamais*) on stored wheat grains. The powders were incorporated into 20 g of wheat grains at 0.0% (control) 2.5, 5.0, 12.5 and 25.5% (w/w). The ability of the plants powders to protect wheat grains were assessed in terms of mortality rates after 24 to 96 h of post treatment, percentage grain, weight loss and damage after the first filial generation (F1). The results indicated that *A. indica* and *A. boonei* provided the highest protection of the treated grains. Seed powders of *A. boonei* can be used as a good alternative to pesticides against *S. zeamais* in addition with that of *A. indica* which effects are well established by many former works.

Conclusion

This research has derived neem based pesticides gainst *C. maculatus* on a stored bambara nut under laboratory condition. It was found that neem seeds oil and neem leaves concentrate gave comparable mortality counts against the *C. maculatus*. Similarly, the neem powders (seeds and leaves) mortality counts were also comparable, but were less effective against the weevils as compared to the neem seeds oil and neem leaves concentrate at least for the first two days. Besides that, both the leaves and seeds and their respective extracts were quite effective as pesticides against *C. maculatus* at dose of 0.1 g/ 10 g at the end of 96 h. Therefore, these neem based materials could be used as pesticides at the dose of 0.1 g/ 10 g bambara nut to prevent *C. maculatus* during storage. This will reduce synthetic pesticide usage, remove the risks of toxic residues in foods and ensure the continued availability of insect-free bambara groundnut for food, planting and trading. Additionally, the task of looking out for botanicals to replace the synthetic pesticides as much as possible is a challenge that must be pursued wholeheartedly with the principles of Green Chemistry as our watchwords.

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