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Evaluation of phytochemicals from lemon and curry leaves as potential protectants against the beans weevil, *Callosobruchus maculatus* (Fabs) (Coleoptera: Bruchidae)

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Abstract
Three phytochemicals; saponins, flavonoids and alkaloids from two medicinal plants, Cymbopogon citratus (DC.) Stapf, and Murraya koeinigi (L.) Spreng, were evaluated as grain protectant against the beans weevil, Callosobruchus maculatus (Fabs), in the laboratory at 0.2g, 0.4g, and 0.6g/ml concentration per 100 grains of cowpea. Parameters assessed were adult mortality, adult emergence, grain damage effect and weevil perforation index (WPI). There was increase in adult mortality with increase in concentrations of phytochemicals irrespective of plant type. Phytochemicals from the test plants showed similar mortality pattern with more effectiveness in the first 3 days of treatment. Saponins from both test plants were most potent in adult mortality whereas, flavonoids and alkaloids showed better potency in adult emergence. The results of the study reveal saponin as a potent botanical against adult C. maculatus, and flavonoid and alkaloid as more potent botanicals against the immature stages of the stored product pest. Hence, flavonoid and alkaloid from both test plants are better bioinsecticide for protecting cowpea grains from C. maculatus infestation and damage.

Keywords: mortality, *Callosobruchus maculatus,* weevil, perforation index, phytochemicals, grain

Introduction
Cowpea (*Vigna unguiculata*) (L.) Walp is a very important and cheap source of dietary protein for many countries in the tropics (1). It is a major food crop in tropical countries and popularly used as protein supplement for meat and fish; it contains digestible carbohydrates and lysine (1). The seeds of this crop is however vulnerable to insect pests of which the cowpea beetle, *Callosobruhus maculatus* (Coleoptera: Bruchidae) is the most important. Postharvest losses of cowpea grain are serious problems and in Africa, as much as 20 - 50% of grain is lost because of infestation due to this pest. It has also been estimated that about 40% or 30,000 tonnes valued at over 30 million dollars is lost annually to *Callosobruchus maculatus* (F) (2). Owing to the problems of synthetic organic chemicals, there is renewed interest on plants as alternative materials for use as stored grain protectant because they have been found to have broad spectrum insecticidal properties with reduced persistence compared to the organochlorines and organophosphates, carbamates and pyrethroids (3). The use of synthetic chemical insecticides is an old age practice to control pests in general and stored product pests in particular. However, the indiscriminate use of many synthetic insecticides has been critically linked to many fold human problems including; technical, environmental, impart on non-target organisms and other insect pest management problems. Some of such problems include resistance of insect pests, food and food product contamination with toxic residues, increased cost of application, handling hazards, environmental contamination, biodiversity, erosion and other negative impacts to human health (4, 5). In addition, the increased public awareness and concern for environmental safety, increased regulatory constraints, unavailability of insecticides to countryside farmers and ventilation restrictions in storage are some of the negative effects of synthetic chemicals (6).

Thus, the search for eco-friendly, cost effective, easily available insect pest management options is intensified worldwide to reduce the use of synthetic chemicals in pest management. Among the benign alternative insect pest management options is the use of plant based products, with broad spectrum of action, to curtail the menace caused by insect pests in storage (7).

The groups of insect species associated with post-harvest products are commonly called stored product pests. Approximately 1,660 insect species may be found in agricultural products during storage, processing, transportation, and marketing (8). Stored products pests are cosmopolitan pests which have been distributed throughout the world by international trade. In developed countries, the losses are up to 9%. In developing countries the losses can be more than 50% (9). Mankind has used plant parts or extracts to control insects since ancient times. Plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (10, 11, 12).

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However, insecticides of plant origin have been extensively used on agricultural pests and to a very limited extent, against insect vectors of public health importance (13). They are easily available and can be produced within the farmers' vicinity, thus providing a more sustainable approach to pest control (14). Oil extracts from various aromatic plants have been widely investigated and their effect on stored insect pest has been of special interest in recent years. Despite the numerous investigations of the effects of botanicals against stored product pests, not much have been achieve in the area of purification and characterization of the specific phytochemicals. Nawrot and Harmatha (15) while reviewing phytochemical feeding deterrents for stored product insect pests submitted that, more than 200 compounds (mostly sesquiterpenes) and over 160 plant extracts have been tested to date. Also, feeding inhibitors may be used along with food or sex attractants in biorational control of the stored food pests.

This study is therefore aimed at assessing the mortality and grain protectant potential of three phytochemicals (alkaloids, flavonoids and saponins) from lemon (*Cymbopogon citratus)* and curry (*Murraya koeinigi)* leaves against the cowpea bruchid, *Callosobruchus maculatus* (Fabricus), the notorious stored product pest of cowpea.

Materials and Methods

Collection, Culturing of Callosobruchus maculatus and grain used

The adults *Callosobruchus maculatus* used for the study were collected from infested stored local brown beans purchased from Oba market in Benin metropolis. Culturing was carried out on 4kg of previously sterilized local brown beans by freezing for seven days after a period of sieving and hand picking to remove debris, unwholesome and broken ones. The 4kg of disinfested beans were then air dried for three hours to prevent moldiness and later divided in 500g lots into kilner jars. More than 2000 adult *Callosobruchus maculatus* isolated from the infested beans were introduced into the sterilized beans grains in each jar. The jars were then covered with muslin cloth to ensure ventilation and prevent moldiness. These served as stock cultures for the experiment. The adults were allowed to lay eggs and develop. The cultures were maintained at an ambient temperature of 28±2ºC and a relative humidity ranging from 70% to 80%. The freshly emerging adult from the cultures were used for the study. Similarly, healthier brown beans were also purchased from the same market, handpicked and sieved to ensure that only healthier and uninfested whole beans were used for the experiment. These were disinfested according to the method described in Iloba and Ekrakene (2).

Preparation of Whole Extract

Fresh leaves of lemon (*Cymbopogon citratus)* and curry leaves (*Murraya koeinigi)* were collected from a private garden in Evbareke Quarters in Uselu, Benin City. The leaves were rinsed with water and air dried in an open shade. The dried leaves were then ground into powder. 100g of each leaf were soaked in 300ml of ethanol for 48hrs and then filtered. The filtrate was concentrated using a rotatory evaporator at 40ºC and dried in a water bath at 50ºC for storage.

Phytochemical Screening

Phytochemical screening tests were carried out on the ethanol extracts using standard methods described by Kumar *et al*., (16).

Screening for flavonoids

1cm^3 of 10% NaOH was added to 3cm^3 of the extract. A yellow colouration indicates the presence of flavonoid.

Screening for saponins:

Emulsion test: 5 drops of olive oil was added to 3cm^3 of the extract in a test tube, the mixture was vigorously shaken. A stable emulsion indicates the presence of saponin.

Screening for alkaloids

To 1cm^3 of the extract, 2 drops of Mayer's reagent was added. A creamy precipitate indicates the presence of alkaloids in the extract.

Estimation of flavonoids content

Ten grams of dried plant sample was repeatedly extracted with 200ml of water and then100 ml of 80% aqueous ethanol at room temperature. The mixture was then filtered through a Whatman filter paper No. 1 into a pre-weighted 250 ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The yield was calculated by difference (17).

Determination of Saponins content

The dry leaves (5.0 g) were defatted with petroleum ether (2 50 mL). The defatted phases were removed, and ethanol (75%, 150 mL) was added. Reflux (soxhlet) was done at 70^{o}C for four hours, the extract filtered and evaporated at 40 C in rotary evaporator. The dry residue was extracted with n-butanol saturated with water (3 x 40mL). The combined extracts were evaporated to dryness. The dry plant extract was weighed and yield determine by difference (18).

Determination of alkaloids content

Five grams of the dried plant sample was placed in a 250 ml soxlet extraction apparatus and 250ml of 95% ethanol which has been made alkaline with ammonia was used for the extraction. The mixture was extracted for 4 hours. The ethanol extract was concentrated in a rotatory evaporator at 60^{o}C. The crude alkaloid extract was further treated with 1.0 N hydrochloric acid. This was filtered and the filtrate was collected. The filtrate was made alkaline with ammonia and placed in a separatory funnel. Measured quantities of chloroform was added into the separatory funnel, mixed and

shaken for about five times and allowed to separate into two layers. The lower layer of chloroform contained the alkaloids and the upper layer the aqueous portion. The upper layer was extracted severally. The combined chloroform extract was concentrated in a rotatory evaporator at 60^{o}C and evaporated in water bath maintained at 60^{o}C temperature until semi-dry. The residue was weighed (19).

Preparation of Phytochemicals

20g each of the extracted phytochemicals (i.e. alkaloids, flavonoids and saponins) before administration was reconstituted in beakers by dissolving in a final volume of 100ml 95% ethanol. From these solutions 0ml (control group with only ethanol), 1ml (0.2g equivalent), 2ml (0.4g equivalent), and 3ml (0.6g equivalent) for each phytochemical types were used as the standard concentration for the treatment.

Methods of Treatment of disinfested cowpea

The mortality effects of the phytochemicals extracted from the leaves was accomplished in small plastic dishes (6cm diameter) containing 100 grains each of disinfested local brown beans with concentrations of 0.2g, 0.4g and 0.6g/ml of extracted phytochemicals (flavonoids, alkaloids and saponins). Each phytochemical was thoroughly mixed with the aid of a glass rod at the rate of 0ml, 1.0ml, 2.0ml, and 3.0ml of the extracted phytochemicals in separate plastic dishes with lid. Each concentration was mounted in triplicate from where mean values were obtained. The set-ups were allowed to air-dry for 10-15 minutes to avoid moldiness after which 20 sexed newly emerged adult *C. maculatus* were introduced into the dishes and covered with muslin cloth and rubber band. This gives insect grain ratio of 1:5. These set-ups were observed for mortality daily for 7 days. Insects not responding to pin probe were considered dead. The set-ups with 0ml served as the control experiment and were void of the phytochemicals. The entire set-ups were maintained at room temperature of 28±2^{o}C throughout the study period.

Another level of the experimental observation was carried out on the infested and treated grains at 21days post treatment. At the end of the period of egg and larva development, observations on the number of emergence and extent of weevil damage was assessed at intervals of two days for 18-day period using the exit-holes as a measure of damage to the grains. Grains that were riddled with exit-holes were counted; the Percentage Damage (PD) and Weevil Perforation Index (WPI) of the weevils to the grains were calculated using the methods of Adedire and Ajayi (20) and Fatope *et al*., (21) respectively.

$$PD = \frac{\text{Total number of treated grains perforated}}{\text{Total number of grains}} \times 100$$

$$WPI = \frac{\text{\% of treated grains perforated}}{\text{\% control grain perforated}} \times 100$$

Results

The results of the mortalities of *C. maculatus* resulting from the administration of phytochemicals from lemon and curry leaves are presented in Table 1. The results showed that the phytochemicals from lemon leaves exerted more mortality effect when compared to the ones from curry leaves across all concentrations studied. Though the pattern of mortality of *C. maculatus* from the two test plants were similar, showing more effectiveness within the first 3 days, mortality pattern of *C. maculatus* from lemon grass increased with increase in concentration and mortality was highest within the first day (24 hours). On the basis of individual phytochemicals, saponins from both lemon and curry leaves were most effective peaking on day 1 at 0.6g/ml where average of 10 *C. maculatus* died. However, mortality effect of saponins from curry leaves peaked on days 1, 4 and 7 when an average of 4 *C. maculatus* died. Saponins from both leaf types were followed in terms of mortality of *C. maculatus* with alkaloids from lemon leaves, peaking on day 1 when about 6 *C. maculatus* died compared to alkaloid from curry leaves which peaked at days 1, 5 and 7 with an average of 3.5 *C. maculatus* dying. Hence, the order of potency of phytochemicals from lemon and curry leaves to cause death of *C. maculatus* is: Saponin > Alkaloid > Flavonoid.

The mean number of emerged *C. maculatus* at 42 days post treatment with phytochemicals from lemon and curry leaves is presented in Table 2. The results showed that, irrespective of plant type, and phytochemicals, mean number of emerged *C. maculatus* decreased with increase in concentration from 0.2g to 0.6g/ml. There was significant difference ($p < 0.05$) in the mean number of emerged *C. maculatus* compared to the control and this was independent of plant type and phytochemicals. Alkaloids from lemon leaves recorded zero emergences at 3ml concentration as was flavonoids from curry leaves at the same concentration. The order of ability to prevent or reduce emergence of *C. maculatus* in each leaf is:

Lemon leaf: Alkaloid > Flavonoid > Saponin

Curry leaf: Flavonoid > Alkaloid > Saponin

Table 1: Mean Mortality (±S.E) of *C. maculatus* resulting from the phytochemicals from Lemon and Curry leaves

		Lemon Leaves			Curry Leaves		
Day	Conc. (g/ml)	Alkaloids	Flavonoids	Saponins	Alkaloids	Flavonoids	Saponins
1	0.2	2.67±0.33	2.67±0.88	3.33±0.67	2.00±1.15	0.67±0.67	1.33±0.67
	0.4	2.00±0.00	2.00±0.58	2.00±0.00	0.67±0.67	0.67±0.67	1.33±0.67
	0.6	5.67±1.76	5.33±0.88	10.00±1.53	3.33±0.67	0.00±0.00	4.00±2.00
2	0.2	3.67±0.33	2.33±0.33	2.00±0.58	1.33±0.67	4.00±0.00	0.00±0.00
	0.4	2.00±0.00	3.67±0.33	2.00±0.58	2.00±0.00	2.00±1.15	1.33±0.67
	0.6	1.33±0.67	2.00±0.58	1.67±0.33	2.67±0.67	1.33±0.67	2.67±1.33
3	0.2	4.00±1.73	3.00±0.58	2.33±0.88	1.33±1.33	2.00±0.00	3.33±0.67
	0.4	4.33±0.88	2.00±0.58	1.33±0.67	0.00±0.00	3.33±0.67	2.67±0.67
	0.6	2.33±1.86	1.33±0.88	0.33±0.33	0.67±0.67	2.00±1.15	2.00±0.00
4	0.2	2.00±1.15	2.00±0.00	1.33±1.33	2.00±1.15	0.67±0.67	2.67±0.67
	0.4	2.00±1.15	3.00±1.00	0.33±0.33	0.67±0.67	4.00±1.15	2.00±0.00
	0.6	2.50±1.34	1.00±0.58	0.33±0.33	1.33±0.67	1.33±0.67	4.00±0.00
5	0.2	1.33±0.88	0.33±0.33	2.00±0.58	0.67±0.67	2.00±0.00	1.33±1.33
	0.4	1.00±0.00	0.33±0.33	2.00±0.00	2.00±1.15	2.67±0.67	0.67±0.67
	0.6	2.00±1.15	1.00±0.58	1.67±0.88	3.33±1.33	0.00±0.00	0.67±0.67
6	0.2	1.00±0.00	0.33±0.33	1.00±0.00	2.67±1.33	1.33±0.67	2.67±0.67
	0.4	2.00±1.15	0.33±0.33	0.33±0.33	2.00±1.15	1.33±1.33	2.67±0.67
	0.6	0.67±0.33	1.33±0.67	1.33±0.67	0.67±0.67	0.00±0.00	1.33±0.67
7	0.2	0.00±0.00	0.00±0.00	0.00±0.00	2.67±1.33	3.33±0.67	4.00±0.00
	0.4	0.00±0.00	0.33±0.33	0.00±0.00	3.33±0.67	1.33±1.33	2.67±0.67
	0.6	0.00±0.00	0.00±0.00	0.67±0.33	0.00±0.00	0.00±0.00	0.67±0.67

Each value is the Mean ± SEM of three replicates.

Table 2: Mean of emerged *C. maculatus* from phytochemicals treated grains

Phytochemicals	**Conc. (g/ml)**	**Means of emerged adults (±S.D)**
	LEMON LEAVES	
Alkaloids	0.2	28.33 ± 2.33^{c}
	0.4	11.62 ± 0.67^{b}
	0.6	0.00 ± 0.00^{a}
Flavonoids	0.2	29.84 ± 4.24^{c}
	0.4	10.33 ± 2.67^{b}
	0.6	1.39 ± 0.33^{a}
Saponins	0.2	40.33 ± 3.33^{d}
	0.4	25.67 ± 6.67^{c}
	0.6	15.33 ± 4.33^{b}
	CURRY LEAVES	
Alkaloids	0.2	32.33 ± 0.67^{c}
	0.4	14.67 ± 1.33^{b}
	0.6	1.00 ± 0.33^{a}
Flavonoids	0.2	34.33 ± 1.67^{c}
	0.4	9.67 ± 2.33^{b}
	0.6	0.00 ± 0.00^{a}
Saponins	0.2	43.66 ± 6.43^{d}
	0.4	53.34 ± 8.23^{d}
	0.6	13.33 ± 2.67^{b}
Control	0.0	78.67 ± 8.33^{e}

Each value is the Mean ± SEM of three replicates. Means followed by the same letter are not significantly different ($P > 0.05$) from each other, using New Duncan's Multiple Range Test.

The percentage perforation of grains by *C. maculatus* and Weevil Perforation Indices (WPI) are presented in Table 3. The results indicate that, irrespective of plant type and phytochemical, perforation of grains decreased with increase in concentration of phytochemicals used. Highest perforation of grain was recorded from grains treated with alkaloids from lemon leaves at 0.2g/ml concentration. At concentrations of 0.4g and 0.6g/ml, all phytochemicals from lemon leaves studied (alkaloids, flavonoids and saponins) recorded no perforation on the grains thereby ensuring complete protection of the grains within the period. However, apart from alkaloids and flavonoids at concentration of 3ml from curry leaves which recorded no perforation of grains, saponins and other concentrations of alkaloids and flavonoids recorded varying percentage perforations when compared to the control which recorded 92% perforation of the grains.
The WPI followed a similar pattern, recording better protection from *C. maculatus* by the use of phytochemicals from lemon leaves compared to that of the curry leaves.

Table 3: Percentage perforation and weevil perforation indices of phytochemicals

Phytochemicals	**Conc. /ml (g/ml)**	**%age Perforated grain**	***WPI**
Alkaloids	0.2	82	89.13
	0.4	00	0.00
	0.6	00	0.00
Flavonoids	0.2	01	1.09
	0.4	00	0.00
	0.6	00	0.00
Saponins	0.2	06	6.52
	0.4	00	0.00
	0.6	00	0.00
Alkaloids	0.2	23	25.00
	0.4	06	6.52
	0.6	00	0.00
Flavonoids	0.2	34	36.96
	0.4	20	21.74
	0.6	0	0.00
Saponins	0.2	39	42.39
	0.4	19	20.65
	0.6	10	10.87
Control	0.0	92	92.00

WPI – Weevil Perforation Index

Discussion

The use of plant products and extracts in the control of stored products insect is an ancient practice (22, 23) and a lot of research efforts have been put into ascertaining the effectiveness of various botanicals. The presence of certain chemicals in plants prevents insects from feeding on them. This leads to starvation of the insects and, in some cases, eventual mortality (24). Several groups of insecticidal chemicals have been identified in plants, including terpenoids, alkaloids, glycosides, phenols and tannins. These compounds have been reported to elicit different behavioural and physiological effects on insects (25).
The present study investigated the mortality effects of three phytochemicals (alkaloids, flavonoids and saponins) from the leaves of *Cymbopogon citratus* (lemon) and *Murraya koeinigi* (curry). The mortality caused by these chemicals as observed in this study, could be attributed to several mechanisms, including contact toxicity causing death; disruption of normal respiratory activities resulting in asphyxiation and subsequent death (3). Others mechanisms could be by actively repelling or preventing insects from feeding thereby causing death through starvation. Many researchers in an attempt to adequately underscore the importance of exploring botanicals in tackling the insects menace in the General Integrated Pest Management System have advanced similar reasons.

Odeyemi *et al*., (24), Mahmoud *et al*., (26) and Varma and Dubey (25) among others have at varying times studied phytochemicals including saponins, alkaloids and flavonoids from different plants in which similar reasons were adduced for their mortality. The mortality potential of phytochemicals was also buttressed by Odeyemi *et al*., (24), when they surveyed desert and semi desert plants and revealed a range of chemicals such as benzopyrans and quinines serving as repellants, attractants, deterrents, anti-feedants or even modifies oviposition of most insect pests depending on the type of plant. It was also observed that the phytochemicals of each plant type had different mortality effect, which is an inherent characteristic in the plant.

Odeyemi *et al*., (24), also reported that the response of secondary metabolites may retard, accelerate development, or interfere with the life cycle of the insects in other ways. From the results obtained in this study, despite the mortality recorded, new insects emerged and the number of emergence was characteristics of plant type, individual phytochemicals and concentration. Whereas, saponins performed better as contact poison against the adult pest at increased concentration, flavonoids and alkaloids at increased concentration were better as anti-emergence and more effective against the immature stages of the pest. Hence, flavonoids and alkaloids at increased concentrations might have acted as ovicide (active against egg) and larvicide (active against larva) while saponins acted as mainly as contact and stomach poison. The pattern of performance obtained in this study regarding the phytochemicals is irrespective of plant type and concentration. This observation is in line with the findings of Wood *et al*., (27) who opined that flavonoid was more active against the immature stages of insects due to its ability to cause a higher decrease in the glutathione S-transferases (GST). This also explains why there was no emergence as concentration increased to 3ml/100 whole beans. This also supports the findings of Kotkar *et al*., (28) in the use of flavonoid on adzuki beans weevil.

The performance of the phytochemicals from the two plants (lemon and curry leaves) investigated was observed to be characteristic of the plant type. Even though saponins, flavonoids and alkaloids were extracted from these plants, their respective ability to cause the death of adult insects and other immature insect stages were characteristics inherent to individual plant type. There is also the likelihood, that botanicals from the plant types are varied in their ability to protect grains against stored product insect pests, especially *C. maculatus*. On the ground of plant type, phytochemicals from lemon plant were better either as contact/stomach poison against adult insect or actively against other immature stages of the insect.

The present study revealed that treatment of cowpea with phytochemicals at appropriate concentration have the potential to kill adult *C. maculatus* probably by contact toxicity, oviposition deterrence and/or ovicidal action resulting in reduced emergence of the stored product pest in subsequent generation.

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