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Laboratory evaluation of freshly prepared juice from garlic (*Allium sativum L.*) Liliaceae as protectants against the maize weevil, *Sitophilus zeamais* (Motsch.) [Coleoptera: Curculionidae]

Ifeanyi D. Nwachukwu[1,3] and Elechi F. Asawalam[2]*

[1]Department of Plant Physiology, Institute for Biology, RWTH Aachen University, Worringerweg 1, D-52056 Aachen, Germany.
[2]Department of Plant Health Management, Michael Okpara University of Agriculture, Umudike P.M.B 7267 Umuahia, Abia state Nigeria.
[3]Department of Human Nutritional Sciences, University of Manitoba, W542-190 Dysart Road, Winnipeg, Canada MB R3T 2N2.

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Fresh prepared garlic (*Allium sativum* L.) juice, containing the antimicrobial allicin, was evaluated as a possible grain protectant against the maize weevil, *Sitophilus zeamais* (Motsch.). Each experiment was set out in completely randomized design (CRD) with four replications and a control treatment. Adult mortality and percentage weight loss were investigated. There was an observed increase in adult mortality following days of exposure in all treatments. Statistically significant (P<0.05) reduced grain loss was also observed in all the treatments when compared with the control. The juice prepared from an indigenous Nigerian garlic cultivar (GUN) was more lethal (causing 93% adult mortality), when applied topically on the freshly emerged *S. zeamais* adults, compared to the juice prepared from a clove of garlic purchased at a supermarket in Germany (GAG). High performance liquid chromatography (HPLC) analysis indicated that the amount of allicin in GUN was 1883.2 µg/ml while that in GAG was 3500.93 µg/ml. This study highlights the potential of *A. sativum* containing allicin for biorational control of maize grains against *S. zeamais* infestation and damage.

Key words: Allicin, *Allium sativum*, biopesticide, biorational control, crop protectant, *Sitophilus zeamais,* stored product.

INTRODUCTION

Maize (*Zea mays L.*) or corn is a major source of dietary carbohydrate as well as the most important cereal in Sub-Saharan Africa (IITA, 2009), while the maize weevil, *Sitophilus zeamais* (L.) (Coleoptera: Curculionidae), is a major pest of stored maize grain in many regions of the world including Nigeria (Adedire, 2001). Although

***Corresponding author. E-mail: elechiasw@yahoo.com**

Abbreviations: GUN, Umuahia main market, Nigeria; **GAG,** supermarket in Aachen, Germany; **HPLC,** high performance liquid chromatography; **DADS,** diallyl disulfide; ***TRPA1*,** transient receptor potential ankyrin-1.

synthetic insecticides have long been widely used in the control of insect pests, the indiscriminate application of synthetic products has led to various problems including toxic residues in the treated products, environmental pollution and growing resistance against insecticides by insects and pests (Huang et al., 1997). There is therefore an urgent need to continue the search for eco-friendly, cheap, sustainable and safe plant protection agents that will not contaminate food products in their use as grain protectants in storage systems for small holder farmers. Moreover, because they are often viewed as “mild” on the environment, compounds of biogenic origin are generally more positively regarded compared to substances partially or completely chemically synthesized in laboratories (Slusarenko et al., 2008), and are therefore more likely to gain wider acceptance among farmers in the long run. Crop protection agents of natural origin also have the advantage of possessing novel modes of action against insects and thus have the potential to reduce the risk of cross-resistance while offering new leads for the design of target-specific molecules (Zhou et al., 2012).

There has been a heightened interest in the last few decades in plants like garlic, which have been equipped by evolution to defend themselves against invading pathogens and pests, not only because of environmental concerns trailing the use of chemically synthesized plant protection products, but also because of farmers’ and consumers’ preference for organic farming strategies and produce, respectively (Nwachukwu et al., 2012; Slusarenko et al., 2008). For many of such plants, protection against pathogens and pests often comes in the form of sulphur-containing secondary metabolites synthesized following external attacks on them (Nwachukwu et al., 2012). Allicin (diallylthiosulfinate), the major antimicrobial substance in garlic, has attracted the attention of investigators because of its widely acclaimed potency. Garlic is known for its positive effect on health particularly the prevention of cardiovascular diseases and certain digestive cancers (Lalla et al., 2013). Previous studies have shown that Garlic also possess some insecticidal, fungicidal, acaricidal, nematicidal and bactericidal properties (Lalla et al., 2013). With a widespread antimicrobial activity comparable to those of common antibiotics like ampicillin (Curtis et al., 2004) and penicillin (Cavallito et al., 1944), it is hardly surprising that this compound has shown activity against some of the world’s most notable plant pathogens including *Phytophthora infestans* and *Pseudoperonospora cubensis* (Portz et al*.,* 2008)*.*

Allicin is a phytoanticipin which means that its synthesis, from preformed precursors already present in garlic, occurs prior to any external attack or irritation, and so does not involve any expenditure of energy (Nwachukwu et al., 2012; van Etten et al., 1994). Allicin is formed as a volatile organosulfur compound following the disruption of garlic tissues either by crushing, piercing, or wounding. The substrate alliin (S-allyl-L-cysteine sulfoxide) held in the cytosol prior to tissue disruption reacts with the now liberated vacuolar enzyme, alliinase to give allicin with pyruvate and ammonia as by-products.

Although the search for and use of plant materials with grain protectant ability is not new (Parugrug and Roxas, 2008; Asawalam and Emosairue, 2006; Asawalam et al., 2006; Rajapakse, 2006; Udo, 2005; Arannilewa et al., 2002; Adedire and Ajayi, 1996; Odeyemi, 1993; Lale, 1992), given the growing role of allicin from garlic in crop protection, we decided to evaluate the efficacy of freshly prepared *Allium sativum* juice as protectants of maize grains against infestation by *S. zeamais*. While significant progress has been recorded in the use of such active agents from natural products, it is hoped that concerted efforts by stored product entomologists will lead to greater success in the biorational control of insects.

MATERIALS AND METHODS

Sitophilus zeamais culture

Adult *S. zeamais* was cultured in the laboratory at 27±2°C, 60-65% r.h and 12 h: 12 h light: dark regime. *S. zeamais* was obtained from stocks maintained at the Crop Science Laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria. The food media used was whole maize grains, purchased from Umuahia main market, Abia State Nigeria. Fifty (50) pairs of *S. zeamais* were introduced into 1 l glass jars containing 400 g weevil-susceptible maize grains. The jars were then covered with nylon mesh held in place with rubber bands. Freshly emerged adults of *S. zeamais* were subsequently used for the experiments.

Preparation and application of *Allium sativum*

The *A. sativum* (Garlic) bulbs used for the study were locally purchased from Umuahia main market, Nigeria (GUN) and from a supermarket in Aachen, Germany (GAG). The garlic juice was prepared by blending axillary buds from composite garlic bulbs using a NAKAI Japan Model 1706 Extractor. Prior to high performance liquid chromatography (HPLC) determination of allicin, the juice was introduced into a sterile 50-ml Falcon tube and centrifuged (Megafuge 1.0R; Heraeus Instruments, Osterode, Germany) at 5000 rpm (3000 g) for 10 min to separate the majority of the pulp from the liquid. Remnants of the pulp were then carefully removed from the top of the liquid with a clean spatula. A diaphragm vacuum pump (Vacuubrand GmbH, Wertheim, Germany) was used to separate the remaining pulp from the pure liquid juice under pressure. The pure filtrate was then transferred to a second sterile Falcon tube and sealed preparatory to HPLC analysis.

Fifty gram of clean and uninfested weevil-susceptible Bende white maize variety used for the study were weighed, using an MP Citizen Electronic weighing balance, and subsequently introduced into four sterilized plastic vials. To each plastic vial, 1 ml of each garlic juice type was added and mixed thoroughly by manual agitation of the vials. A control experiment containing no garlic juice was also set up. Five pairs of adult *S. zeamais* were introduced into treated and untreated maize grains. The lids of the plastic vials were perforated in order to maintain aerobic conditions in the vials. Muslin textile materials were used to secure the top of the plastic vials and served to ensure aeration while preventing entry or exit of insects. The contents of the plastic vials were then shaken gently for proper and uniform mixing. Each treatment was replicated four times. The samples were arranged in a completely randomized design on a laboratory table.

Mortality and damage assessment assays

The number of dead insects in each vial was counted at 7, 14, 21, 28 and 35 days after treatment to estimate mortality. Maize weevil mortality was assessed as

Maize weevil mortality = Number of dead insects/Total number of insects x 100.

Data on percentage adult weevil mortality were corrected using Abbott's formula (Abbott, 1925) thus:

$$P_T = \frac{P_o - P_c}{100 - P_c}$$

Where, P_T, is the Corrected mortality (%); P_O, is the observed mortality (%); P_C, is the control mortality (%).

Weight loss was assessed by re-weighing the grains to determine percentage weight loss. Percentage weight loss was calculated following the method of the FAO (FAO, 1985) as follows

$$\text{Percentage weight loss} = \frac{[UaN - (U + D)]}{UaN} \times \frac{100}{1}$$

Where, U is the Weight of undamaged fraction in the sample; N is the total number of grains in the sample; Ua, is the average weight of undamaged grains and D, is the weight of damaged fraction in the sample.

Contact toxicity test by topical application

The fresh garlic juice samples at 1 ml dosage were applied uniformly to the bottom of the plastic vials and the control was set up in which there was no garlic juice. Five male and five female adult weevils of about 5 days old were introduced separately into each vial. Each treatment was replicated four times and weevil mortality was recorded after 12, 24, 36 and 48 h of exposure. Insects were presumed dead if they remained immobile and did not respond to five jabs with a blunt dissecting probe after an arbitrary 5-min recovery period.

High-performance liquid chromatography (HPLC) determination of allicin in garlic juice

Determination of the amount of allicin in the garlic juice preparations was performed using a JASCO HPLC. The method used was taken from Krest and Keusgen (2002). HPLC-grade water was used to dilute freshly prepared garlic juice in the ratio of 1:10. Thereafter, 1 ml of the diluted sample was introduced into a sterile vial with the injection volume set at 20 µl.

In order to protect the column, the diluted garlic juice was passed through a polyether sulfon membrane (0.2 µm pore size, Steriflip, Millipore), before introduction into the vial and subsequent injection into the HPLC (JASCO Chromatography Data System, with intelligent UV detector, Jasco Labor-u. Datentechnik GmbH, Groß-Umstadt, Germany). 1.5 ml of a 0.05 mg ml^{-1} solution (in methanol) of butyl-4-hydroxybenzoate was used as internal standard. Using the HPLC software ChromPass (version 1.8.6.1), a mixed gradient elution [solvent A, 30% (v/v) HPLC grade methanol adjusted to pH 2.0 with 85% (v/v) orthophosphoric acid; solvent B, 100% HPLC grade methanol] was performed. Elution spectra were recorded between 200-600 nm with detection at 254 nm for the chromatogram.

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and significant difference (P>0.05) means were separated by using Student Newmans Keul (SNK) test.

RESULTS

Mortality

The effect of fresh garlic juice on the mortality of *S. zeamais* is presented in Figure 1. The results obtained show that fresh GAG juice with a mortality rate of 73% and GUN at 87% mortality rate were significantly more effective in causing *S. zeamais* mortality at 28 DAT compared to the control.

Contact toxicity test by topical application

Upon exposure to adult *S. zeamais*, fresh GUN juice caused 100% mortality, while GAG juice caused 90% mortality (Figure 2) 48 h after topical application. The control led to zero mortality.

Effect on grain weight

There was significant weight loss of the control (Figure 3) when compared with the maize grains treated with garlic juice indicating the effectiveness of the juice in offering protection to the stored maize grains. While the untreated control grains lost over 8% of original weight on average, the grains treated with GAG and GUN only lost a negligible < 0.5% of average weight after 60 days.

High- performance liquid chromatography (HPLC) analysis of *A. sativum*

The HPLC chromatograms depicting the amount of allicin in the freshly prepared garlic juice is shown in Figures 4 and 5. The amount of allicin in GUN and GAG was found to be 1.88 and 3.50 mg/ml, respectively.

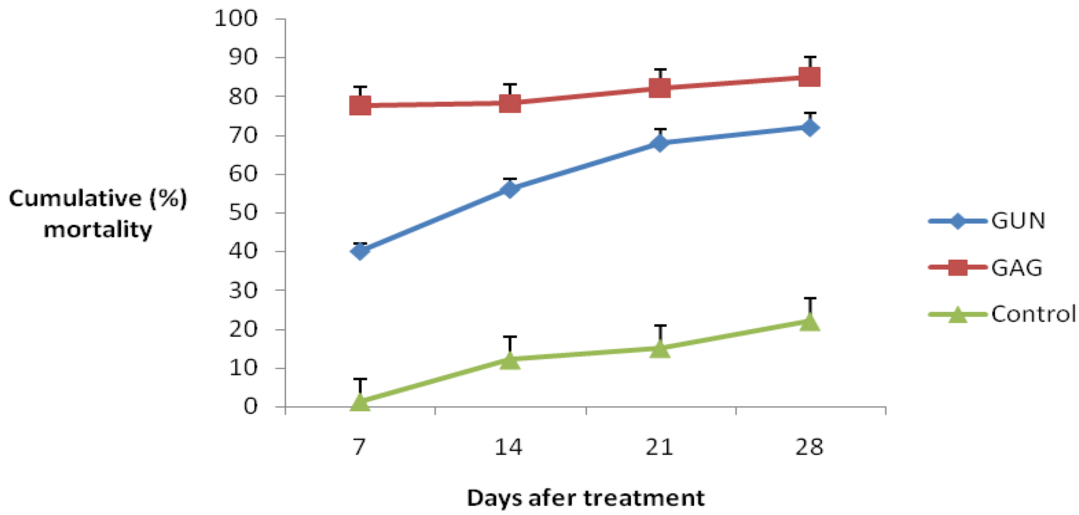


Figure 1. Cumulative mortality of freshly emerged adults of *S. zeamais* after introduction onto maize grains treated with freshly prepared *A. sativum* juice.

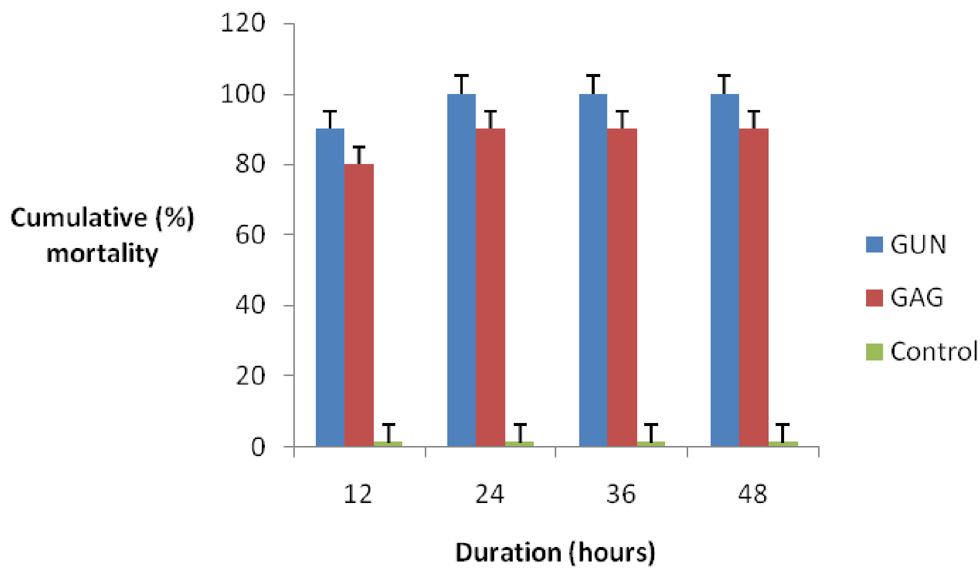


Figure 2. Contact toxicity of fresh garlic juice against *S. zeamais* adult at 48 h after treatment.

DISCUSSION

Over the years, various studies investigating the possible use of agents of biogenic origin as crop protectants have highlighted the potency of natural products as biopesticides (Gonzalez-Coloma et al., 2010; Lee et al., 2004; Isman, 2000; Qi and Burkholder, 1981). For instance, studies examining the fumigant and contact insecticidal effects of 22 plant essential oils against the bean weevil, *Acanthoscelides obtectus* (Regnault-Roger et al., 1993), and of 28 plant essential oils against four adult members of Order *Coleoptera* including the rice weevil *Sitophilus oryzae,* a close relative of *S. zeamais* (Shaaya et al., 1990), have not only added to the impressive body of evidence in literature clearly demonstrating the efficacy of using natural pesticides as biocontrol agents in the open field but also as stored product protectants against pests related to the maize weevil. While none of the two works just cited examined the use of agents from garlic but instead studied the use

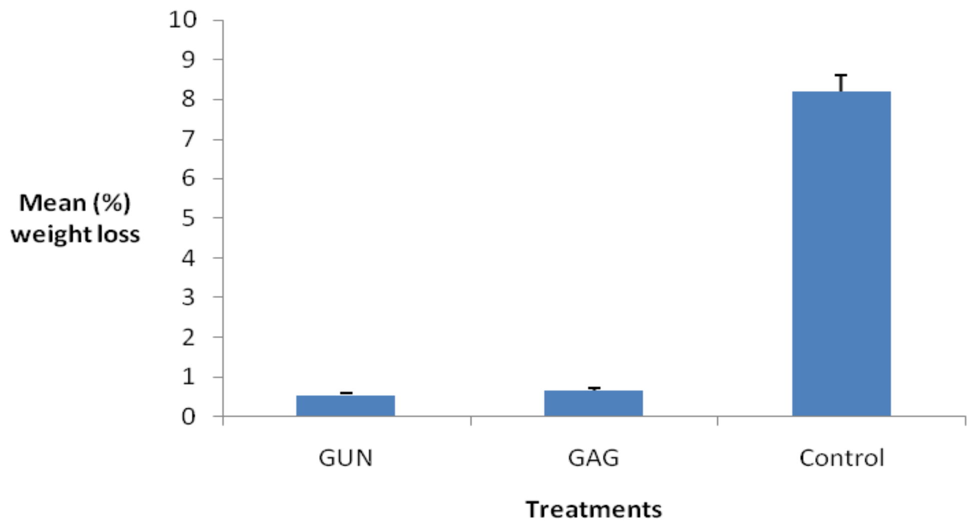
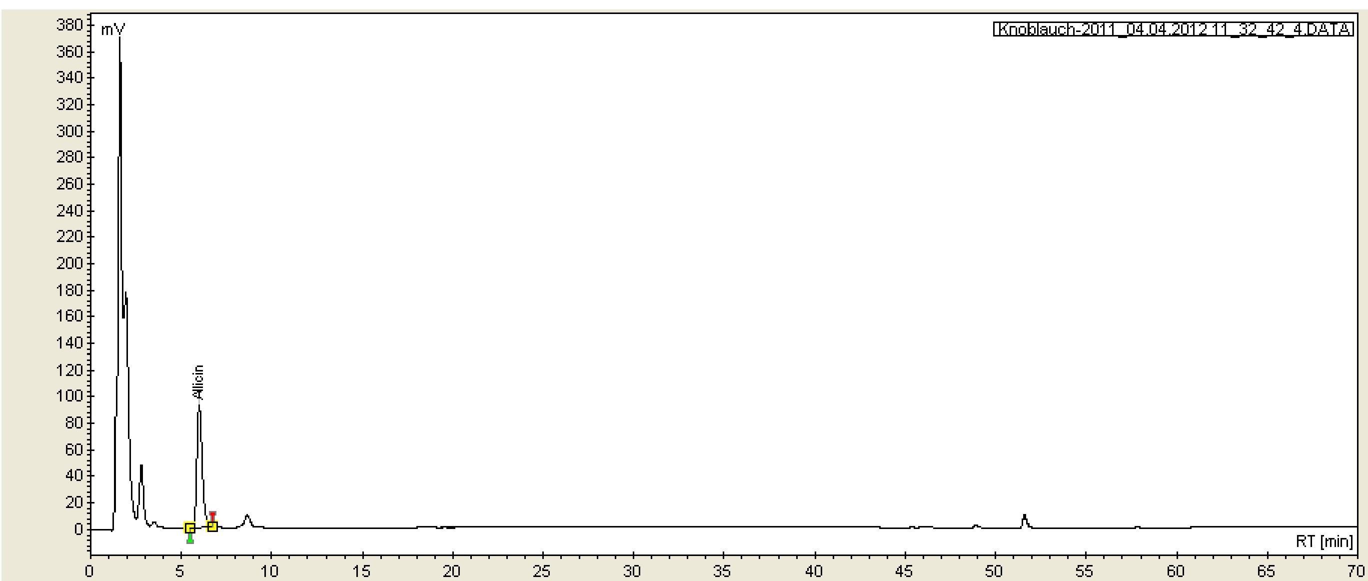


Figure 3. Effect of *A. sativum* juice on weight loss of maize grains.

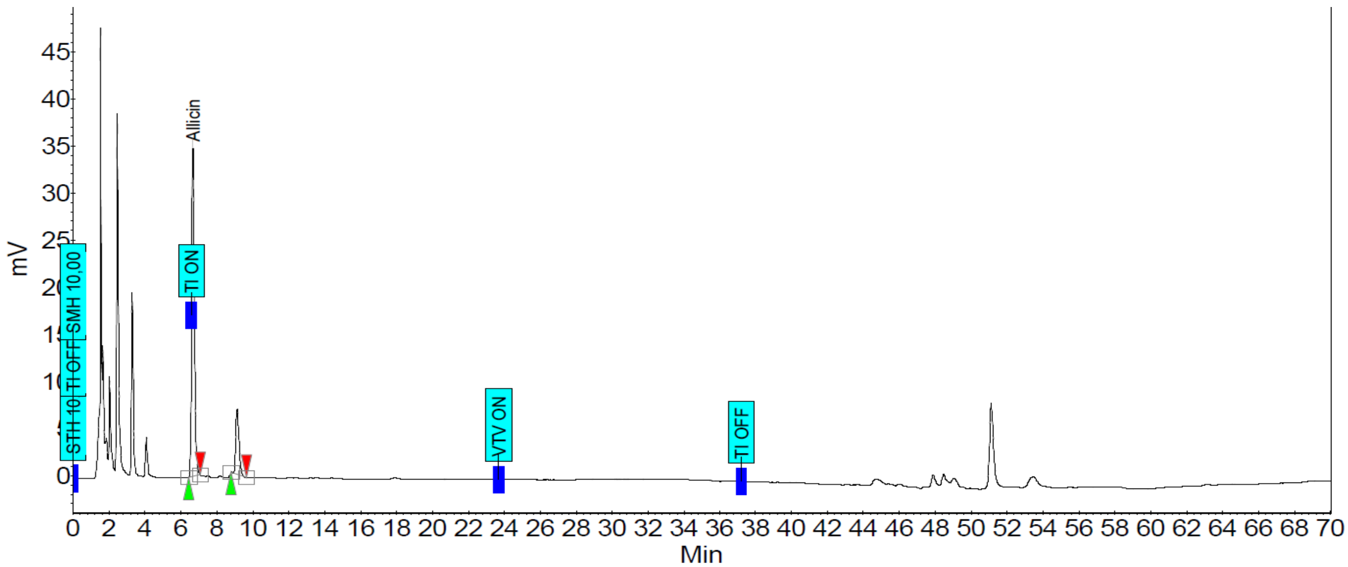


#	Name	Time [Min]	Quantity [ug/ml]	Height [mV]	Area [mV.Min]	Area % [%]
1	Allicin	5,983	188,32	92,2	31,7	100,000
Total			188,32	92,2	31,7	100,000

Figure 4. HPLC chromatogram for GUN allicin determination. The peak for allicin in diluted garlic juice (1:10) was detected at 5.98 min. Amount of allicin was determined to be 1883.2 µg/ml corresponding to an allicin concentration of 11.60 Mm.

of other natural compounds including those from lavender, coriander, lemon and celery, such as α-terpineol, α-terpinene, β-caryophyllen, and carvacrol among others, they provide materials for fascinating comparative analyses of chemical compounds in plants as crop protectants especially as allicin the major biologically active agent in fresh garlic juice is only found in garlic (Cavallito and Bailey, 1944; Cavallito et al., 1944; Jain and Apitz-Castro, 1987).

Research on the potential application of biologically active compounds from garlic abound in literature. For instance, steam-distilled garlic oil has been tested for toxicity against the eggs, larvae and adults of *Tribolium castaneum* and against the adults of *S. zeamais* (Ho et



Peak results :

Index	Name	Time [Min]	Quantity [ug/ml]	Height [mV]	Area [mV.Min]	Area % [%]
1	Allicin	6,658	35,93	35,0	6,1	80,606
Total			35,93	41,9	7,5	100,000

Figure 5. HPLC chromatogram for GAG allicin concentration determination. The peak for allicin in diluted garlic juice (1:100) was detected at 6.66 min. The amount of allicin in the freshly prepared garlic juice was determined to be 3500.93 µg/ml corresponding to an allicin concentration of 21.57 Mm.

al., 1996) while extracts from garlic (and other plants) have been vapourized and used in fumigation tests involving *T. castaneum* and *S. zeamais* (Ho, 2000). The result from this study corroborates Musa (2013) who recorded 100% mortality at 6% w/w in groundnut seeds treated with *A. sativum* clove powder. Ibrahim and Garba (2011) found garlic powder to be effective in the control of maize weevil. Zhao et al (2013) observed that the essential oil of *A. sativum* possessed contact toxicity against overwintering *Cacopsylla chinensis* (Hemiptera:Psyllidae) with an LC_{50} value of 1.42 µg per adult. The two main constituent compounds diallyl trisulfide and diallyl disulfide, exhibited strong acute toxicity against the overwintering *C. Chinensis* with LC_{50} values of 0.64 and 11.04 µg per adult respectively. Feng-Lian et al. (2011) also reported that garlic essential oil diallyl disulfide and diallyl trisulfide inhibited the development of grain moth *Sitotroga cerealella* (Lepidoptera:Gelechidae). Ofuya et al. (2010) proved that fumigation of pods with crushed bulbs of *A. sativum* and *Allium cepa*, showed a toxic effect to *Callosobruchus maculatus.*

Other investigations include those of Hamed et al. (2012), Adedire and Ajayi (2006), and Arannilewa et al. (2006). Each of these works employed garlic essential oils obtained by steam distillation or solvent extraction. However, to the best of our knowledge, no study has been carried out on the use of fresh garlic juice as a biopesticide against *S. zeamais.* A search of the leading electronic database, *Scopus*, returned no hits for each of the search terms "garlic juice weevil", "garlic juice Sitophilus" and "garlic juice *Sitophilus zeamais*." Therefore, this work most likely represents a remarkable shift from the conventional approach to studying the use of biologically active compounds from garlic in crop protection. Importantly, given that allicin is volatile and unstable, and upon production rapidly decomposes to other breakdown products like ajoene and the vinyldithiins (Apitz-Castro et al., 1983; Block et al., 1984; Block, 1985; Voigt and Wolf, 1986; Iberl et al., 1990), we propose that allicin could not have been principally responsible for the insecticidal effects reported in previous works investigating the exposure of agricultural pests to garlic essential oil. As comprehensively discussed by Staba et al. (2001), any of a number of treatments/processing of garlic such as freeze drying, steam distillation, oil maceration, ethanolic extraction and low temperature drying, results in the production of complex mixtures including allicin, diallyl disulfides, dially trisulfides, allyl methyl trisulfides, ajoene, and vinyldithiins.

The results show that although GUN and GAG belong to the same species, they exert slightly different effects on *S. zeamais*- a difference which can be reasonably attributed to their different allicin contents. Our results are similar to the work of Hamed et al. (2012) which recorded mortality rates ranging from 78 -100% following 3-14 days of exposing *Sitophilus oryzae* adults to garlic essential oils. In addition to using garlic essential oils, other similar works such as those by Adedire and Ajayi (2006), and Arannilewa et al. (2006) also employed solvents like petroleum ether and ethanol as extraction vehicles thus making direct and accurate comparisons improbable.

Garlic's pungent smell has been attributed to the presence of organosulfur compounds such as allicin and diallyl disulfide (DADS) in the edible allium (Bautista et al., 2005). It has been suggested that garlic's pungency contributes to its toxicity in weevils by disrupting regular respiratory events (Adedire and Ajayi, 2006). Furthermore, the structures of allicin and DADS are similar to that of allyl isothiocyanate which apart from lending wasabi and other mustard plants their pungency, induces pain and inflammation by activating the transient receptor potential ankyrin-1 (*TRPA1*) ion channel in neuronal cells (Wang and Woolf, 2005; Jordt et al., 2004; Fahey et al., 2001).

Work with neuronal cell cultures have also provided molecular evidence suggesting that allicin is the main sulphur compounds in garlic that excites allyl isothiocyanate-sensitive sensory neurons as well as activate TRPA1 and the related TRPV1 ion channels (Bautista et al., 2005; Macpherson et al., 2005) which are present in pain-sensing neurons. Induction of pain could have significantly contributed to insect mortality by causing considerable stress the maize weevils. Finally, plant essential oils given their lipophilicity are able to penetrate the cuticle of insects (Richards, 1978) thus contributing to lethal effects.

Conclusion

The present findings suggest that freshly prepared garlic juice which has allicin as its main biologically active compound possesses a potentially vital insecticidal effect on *S. zeamais* when compared with the control. Thus, garlic offers significant promise for combating the threat posed by maize weevils to farmers in developing countries. The major thrust of this work is its adaptability for use by small scale farmers plagued by the challenge of not being able to afford conventional pesticides on the market. With no need for the more complex and sophisticated production of essential oils, this work's simplicity expressly lends zest to the overarching essence of providing a quick and easy solution to the problem of pest infestation in third world countries. There is need for further investigations to identify the other garlic juice constituents (apart from allicin) with toxic effects on *S. zeamais*, and to elucidate the precise mechanisms by which they exert their insecticidal effects.

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