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Chemical analysis of Alliin-Rich *Allium sativum* (Garlic) extract and its safety evaluation in *Drosophila melanogaster*

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ABSTRACT

Garlic (*Allium sativum*) has been traditionally valued for its medicinal properties attributed to the presence of organosulfur compounds. Despite its benefits, concerns about herbal extract toxicity have arisen, necessitating safety assessment. This study was designed to evaluate the chemical analysis and safety profile of Alliin-Rich Garlic Extract (ARGE) using *Drosophila melanogaster* as a model organism. The ARGE was extracted from garlic cloves (*Allium sativum* Linn: UIH-23262) using a microwave-assisted method and characterized using UPLC-ESI-MS, ^{1}H NMR, HPLC and IR. Its safety evaluation was determined using *D. melanogaster* (Harwich strain), and various assays were conducted on 1–3-day-old flies. Toxicological markers and oxidative stress were assessed to understand the impact of ARGE on the flies. Chemical profiling of ARGE using UPLC-ESI-MS, confirmed the presence of alliin (S-ally-L-cysteine-S-oxide), L-arginine, γ-glutamylmethionine, S-(2-carboxypropyl) glutathione, N-γ-glutamyl-S-(1-propenyl) cysteine, N-γ-glutamyl-S-(2-propenyl) cysteine, N-γ-glutamylphenylalanine, S-(allylthio) cysteine, γ-glutamyl-S-allylthiocysteine and eruboside B. HPLC confirmed an alliin content of 0.073 mg/g. Toxicological assessment in *D. melanogaster* revealed that ARGE enhanced antioxidant defenses by increasing total thiol levels and GST activity, while reducing acetylcholinesterase activity. No significant alteration was observed in catalase activity and cellular metabolic rate. Histological examination revealed no alterations in the histoarchitecture of the brain, fat body or gut of *D. melanogaster*. The study demonstrated the safety of ARGE in *D. melanogaster*, supporting its potential as a safe herbal remedy.

1. Introduction

Garlic (*Allium sativum* Linn), a member of the family of Alliaceae, is one of the most used *Allium* species worldwide, second only to onions. It is often referred to as the "golden onion" due to its prominent use in herbal concoctions as well as its antiseptic properties [1,2]. Garlic has long been valued for both culinary and medicinal purposes. Traditionally, it has been employed for treating cardiovascular diseases, diabetes, and infections [3]. Garlic contains a high concentration of organosulfur compounds, making it more potent compared to other *Allium* species like onions, shallots and leeks. These compounds in garlic are believed to have evolved as a defense mechanism against animals such as birds,

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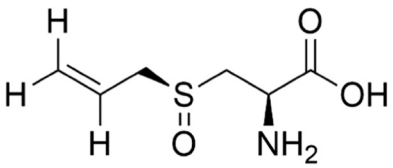


Fig. 1. Structure of Alliin.

insects and worms ensuring its survival through several generations of evolution [4].

Several studies have shown that the antioxidant property as well as the pungent smell of garlic is due the presence of organosulfur compound allicin [1]. However, little is known of its percussor molecule, alliin because it is readily lost once garlic tissue is chopped. Chopping of the garlic bulb leads to conversion of alliin to allicin by the enzyme alliinase, thus, alliin is not readily available. Alliin ($C_6H_{11}NO_3S$, Fig. 1) is a sulfoxide derived from cysteine [5,6]. In our current study, the garlic was extracted through a specific extraction method to preserve its alliin content. This necessitated the need to carry out a safety study to ensure the alliin-rich garlic extract (ARGE) is safe for consumption. Although, herbal remedies are generally assumed to be risk-free, however, there have been several cases of toxicity associated with herbal extracts. This can result from factors such as modifications during extraction, plant misidentification, contaminations, or inherent toxicity of certain plants [7]. Thus, drug regulating agencies emphasize the need to carry out safety profile and identify constituents of herbal extract regardless of their efficacy [8,9].

Drosophila melanogaster has emerged over the past century as a versatile model organism to study the safety assessment of drugs. This is due to the physiological and genetic similarity of flies with humans. About 75 % of disease-causing genes in humans are conserved in the flies [10,11]. Drug testing in flies can be conducted via micro-injection, inhalation or through diet. The introduction of drugs through diet is preferred because it makes the flies less susceptible to tissue damage compared with the injection route of drug administration [12,13]. Indeed, several toxicological markers have been established to understand the extent of tissue damage in *D. melanogaster*. Toxicological markers include survival rate, life span, as well as oxidative stress. The oxidative stress enzymes are similar in *Drosophila* and humans [14]. In this study, we evaluated the safety profile of ARGE using *D. melanogaster* as a model organism.

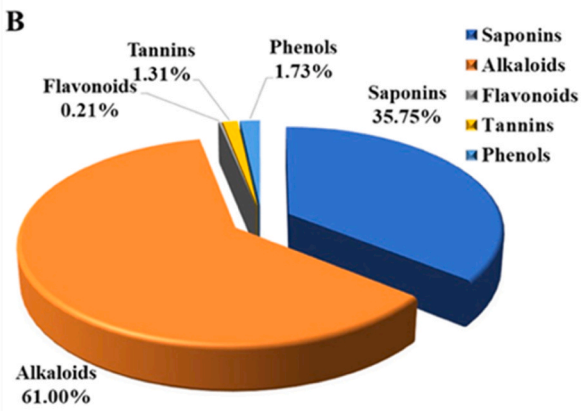
2. Materials and methods

2.1. Chemicals

Ethylacetate, acetylthiocholine iodide and sodium dodecyl sulphate (SDS) were purchased from Sigma Aldrich (St Louis, MO) and were of analytical grade.

A

S/N	Parameters	ARGE
1	Saponin (Froth's Test)	+
2	Alkaloid (Hager's Test)	+
3	Flavonoid (Lead acetate Test)	+
4	Tannin (Braymer's Test)	+
5	Phenol (Braymer's Test)	+
6	Steroid (Salkowaski's Test)	+
7	Terpenoid (Salkowaski's test)	+
8	Cardiac Glycosides (Keller Killani Test)	+
9	Glycosides (Lieberman Test)	+
10	Quinones	+
11	Anthraquinone (Bontrager Test)	-
12	Anthocyanin	-



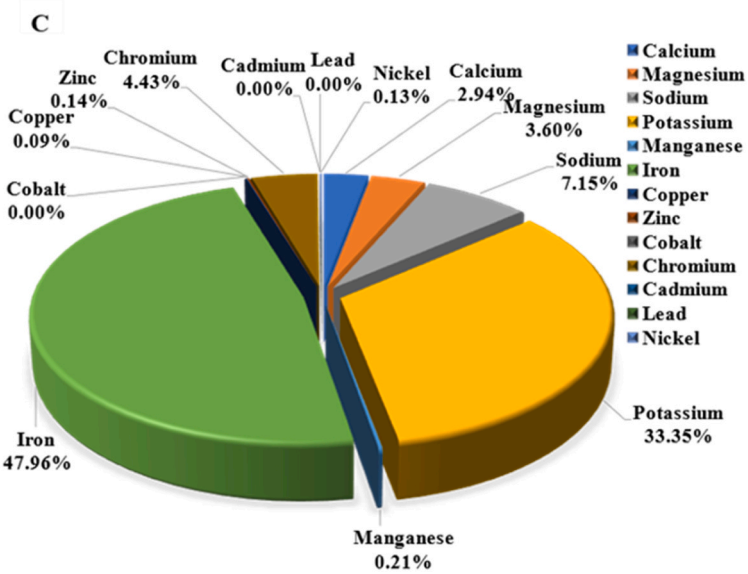


Fig. 2. Phytochemical analysis of Alliin Rich Garlic extract (ARGE). A: Qualitative phytochemical analysis, B: Percentage composition of major phytochemicals, C: Percentage composition of minerals and heavy metals.

2.2. Plant materials

Garlic bulbs were purchased from a local market in Ibadan, Nigeria. The plant was identified and validated at the University of Ibadan Herbarium with herbarium number UIH: 23262. The plant name was checked through the website: https://wfoplantlist.org/taxon/wfo-0000757248–2023-12?page=1.

2.3. Fly strain

D. melanogaster (Harwich strain) was cultured in the Drosophila Research Laboratory, Department of Biochemistry, University of Ibadan, Nigeria. They were maintained at constant temperature and humidity (23±2 °C; 60 % relative humidity, respectively) with a 12-hour dark/light cycle. Standard *Drosophila* medium was used for culturing the flies: cornmeal (1 % w/v), brewer's yeast (2 % w/v), agar and nipagin (0.08 %). All experiments were performed on 1–3-day old flies.

2.4. Preparation of Alliin-Rich garlic extract

The ARGE was extracted using the microwave-assisted method [15,16]. The garlic bulbs were peeled to remove the scales and subsequently microwaved at 750 W for 2 min. This allows for permanent inactivation of the enzyme Allinase. The garlic bulbs were then blended with ethyl acetate, in a solid-liquid ratio of 1:2. This allowed the removal of fat-soluble components. The homogenate was then allowed to stand for an hour and centrifuged at 8000 rpm for 5 min, to remove garlic oil. The precipitate containing the alliin was then extracted with water. The solid-liquid ratio was 1:5 at 35 °C for 60 min, while stirring the mixture. The final product was thereafter obtained using vacuum filtration and freeze-dried Fig. 2.

2.5. Chemical profiling of Alliin-Rich garlic extract

2.5.1. UPLC-ESI-MS analysis

2.5.1.1. UPLC analysis. Waters UPLC coupled in tandem with a Waters SYNAPT G1 HDMS mass spectrometer was used to produce accurate mass data. Chromatographic separation Optimization was carried out with a Waters HSS T3 C18 column (150 mm × 2.1 mm, 1.8 μm) at controlled column temperature of 60 °C. A binary solvent mixture composed of water (eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (eluent B) containing 10 mM formic acid was used. The initial conditions were 100 % of A at a flow rate of 0.4 mL/min, maintained for 1 min, followed by a linear gradient to 1 % A at 25 min. These conditions were kept constant for 2 min and then changed to the initial conditions. The runtime was 30 min and the injection volume was 1 μL. Samples were kept cool at 6 °C in the Waters Sample Manager during the analysis.

2.5.1.2. TOF mass spec analysis. The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to aid in detection of all ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant (Lock Mass) to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in both ESI positive and negative modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 4.0 V. The scan time was 0.2 seconds covering 50–1200 Dalton mass range with an interscan time of 0.02 seconds. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. The nebulization gas used was nitrogen gas at a flow rate of 550 L/h and cone gas was added at 50 L/h. The described UPLC-ESI-MS procedure was adopted from a previous study [17], though with slight modifications. The software used on the hyphenated system and with which data manipulation was carried out was MassLynx 4.1 (SCN 872). This provided exact masses, possible elemental formulae, fragment masses and other features that aided compound identification while comparing with those available in the Dictionary of Natural Products (DNP) database.

2.5.1.3. ^1H NMR fingerprinting. The ARGE was subjected to ^1H NMR analysis for additional characterization of the constituents. The extract (15 mg) was dissolved in 0.5 mL deuterium oxide (D_2O) (Sigma Aldrich, St Louis, MO, USA), transferred into a 5 mm NMR tube at room temperature. NMR data was acquired on a 600 MHz Varian NMR spectrometer (Varian Inc., Palo Alto, CA, USA). The ^1H NMR spectrum was processed using Mnova Software (vers 10.1, Mestrelab Research), prior to annotation of compounds [18]. Compounds were annotated by comparison of NMR data with those available from the Human Metabolome Database (HMDB) and literature values.

2.5.1.4. Quantitative determination of Alliin by HPLC. The HPLC set up component includes an autosampler, a quaternary pump, a UV detector set at 254 nm, a data aggregation system (waters HPLC with Empower 3 software, USA) and a stationary phase column (4.6 mm × 150 mm, 5 μm, Zorbax Eclipse XDB, U.S.A) with guard column. The eluent A consist of water containing 0.008 %$^{v}/_{v}$ triethylamine adjusted to pH 6.5 with 0.06 % acetic acid and the solvent B is methanol. The solvent mixture was programmed as 70 % A for 30 min with flow rate of 1 mL/min for separation of ARGE. Then the gradient was changed to 100 % A and kept for 5 min for flushing the rest of the amino acids. Five milligrams of L- (+)-alliin were purchased from AKT Scientific (Lot#: LC64431) were dissolved in distilled water to make up a solution of 500 ppm was used as the external standard [19].

2.5.1.5. Infra-Red Spectroscopy. Infra-Red spectrum was obtained by using a Perkin ELMER FTIR spectrometer (PIKE MIRacle™ ATR). Infrared spectra were recorded from 4000 to 450 cm^{-1} [20].

2.5.1.6. Mineral and heavy metal analysis. Analysis of ARGE for its mineral and heavy metal contents was done as follows [21]. One gram of each ARGE was accurately weighed into separate conical flasks (100 mL). 10 mL of a freshly prepared mixture of concentrated HCl-HNO_3 (3:1, v/v) were placed on each flask and kept for 10 min at room temperature. The content of flasks was heated on an electric hot plate at 800 °C, until clear solutions were obtained. The flasks contents evaporated, and the semi dried mass was dissolved in 5 mL of distilled water, filtered through Whatman No. 42 filter paper, and made up to final volume of 50 mL in volumetric flasks with distilled water. It was kept as a stock sample solution. The blank solution was prepared by digesting the HCl-HNO_3 (3:1) reagent mixture. The digest was analyzed for minerals and heavy metals, using Perkin Elmer Analyst 200 at. absorption spectrometer.

2.6. Phytochemical screening

2.6.1. Alkaloid determination

Five grams of the ARGE were weighed into a 250 mL beaker and 200 mL of 10 % acetic acid in ethanol was then added. The mixture was covered and allowed to stand for 4 hrs. This mixture was then filtered, and the extract concentrated to one-quarter of the original volume on a water bath. Concentrated ammonium hydroxide was then added in drops on the extract until precipitation was complete. The whole solution was allowed to settle, and the precipitate was collected and washed with dilute ammonium hydroxide and filtered. The residue is the alkaloid which was dried and weighed [22].

2.6.2. Saponin determination

The method used was that of Obadoni and Ochuko (2001) [23]. As many as 20 g of ARGE were placed into a conical flask and 100 mL of

20 % aqueous ethanol were added. The samples were heated on a hot water bath for 4 hrs under continuous stirring at about 55 °C. The mixture was filtered, and the residue re-extracted with another 200 mL of 20 % ethanol. The combined extract volume was reduced to 40 mL over a water bath at about 90 °C. The concentrate was then transferred into a 250 mL separatory funnel and 20 mL of diethyl ether was also added. The content was then shaken vigorously. The aqueous layer was recovered and the ether layer discarded. The purification process was repeated followed by an addition of 60 mL of n-butanol. The combined n-butanol extract was rinsed twice with 10 mL of 5 % aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in an oven to a constant weight; the saponin content was then calculated in percentage.

2.6.3. Determination of total phenolic content

Phenolics content of ARGE was determined spectrophotometrically using the Folin-Ciocalteu method [24]. The reaction mixture consisted of 1 mL of extract and 9 mL of distilled water, which were then shaken together in a 25 mL volumetric flask. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken well. After 5 min, 10 mL of a 7 % sodium carbonate (Na_2CO_3) solution was added to the mixture. The volume was adjusted to 25 mL. A set of standard solution of gallic acid (20, 40, 40, 60, 80 and 100 μg/mL) were prepared in a similar manner as described earlier. This was followed by incubation for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an ultraviolet (UV/visible) spectrophotometer. Total phenol content was expressed as mg of GAE/gm of extract.

2.6.4. Determination of tannin content

The tannin content was determined with the aid of the Folin-Ciocalteu method. 0.1 mL ARGE was added to a volumetric flask (10 mL) containing 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu Phenol reagent and 1 mL of 35 % Na_2CO_3 solution. The mixture was then diluted to 10 mL with distilled water. The mixture was well shaken and then kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100 μg/mL) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV/visible spectrophotometer. The tannin content was expressed as mg of GAE/g of extract.

2.6.5. Determination of total flavonoid content

Total flavonoid content was determined with the aluminum chloride colorimetric assay [25]. For the reaction mixture, 1 mL of extract and 4 mL of distilled water were added to a 10 mL volumetric flask. Then, 0.30 mL of 5 % sodium nitrite was added and mixed with 0.3 mL of 10 % aluminum chloride. After 5 min, 2 mL of 1 M sodium hydroxide was added and the solution was diluted to 10 mL with distilled water. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 μg/mL) were prepared in a similar manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract.

2.6.6. HPLC determination of water-soluble vitamin (vitamin C and B1, B2, B3, B6, B12) and Fat-soluble Vitamin (A and E)

Water-soluble vitamin standards of VB1, VB2, VB3, VB6, VB12 and VC were prepared by weighing 10–20 mg of the vitamin powder and adding 10–20 mL of distilled water to make stock solutions of 1.0 mg/mL for each vitamin. VC was freshly prepared because of its limited stability. The solution was then filtered through a 0.45 μm membrane filter. Retinyl palmitate (vitamin A) and α-tocopherol (vitamin E) standards were prepared by accurately weighing 5 mg of the standard into a 10 mL volumetric flask followed by the addition of 0.5 mL of acetone. Methanol was added to make up the solution to the 10 mL mark and stock solution of 0.5 mg/mL. the final concentration of the vitamin standards solution ranged from 32.25 – 250 ppm. HPLC conditions were: column = ACE 5 C18, 150 × 4.6 mm, column temperature = ambient, mobile phase = (97:3) 25 Mm KH_2PO_3, pH 3.0: acetonitrile, flow rate = 1 mL/min, run time = 10 min and detection = UV at 254 nm [26,27].

ARGE was prepared for the determination of VB1, VB2, VB3, VB6, VB12 and VC by weighing 0.500 g into 10 mL volumetric flasks. 8 mL of distilled water was then added to the flask. After 15 min of ultrasonic extraction, distilled water was then added to make up the 10 mL mark. The solution was then filtered through a 0.45 μm membrane filter. ARGE was then injected into the HPLC system through the sample syringe. A similar procedure was used for retinyl palmitate (vitamin A) and α-tocopherol (vitamin E) determination of ARGE, however solvent used was methanol/dichloromethane (1:1, v/v). column = ACE 5 C18, 150 × 4.6 mm, column temperature = 30 °C, mobile Phase = methanol, flow rate = 1 mL/min, run time = 10 min and detection = UV at 280 nm.

2.7. Experimental design

2.7.1. Survival assays

Both sexes of *D. melanogaster* (50 1- to 3-day old flies in 5 replicates per vial) were fed ARGE (25, 50, 100, 200 mg/g diet) for 14 days. The number of live and dead flies was recorded daily till the end of the experiment. Data were expressed as percentage of live flies [28].

2.7.2. Behaviour (negative geotaxis) assay

The ability of treated flies to climb to 6 cm mark in 6 s in a vertical glass column (length, 15 cm) was compared with that of control flies. The flies were previously immobilized using CO_2. The assay was carried out after recovery from anesthesia. Data were expressed as percentages of flies that climbed beyond the 6 cm mark in 6 s. The experiment was repeated twice and the average recorded [29,30].

2.7.3. Whole-fly tissue Homogenate preparation

Flies were allowed to mate for 24 hrs in ARGE and control diets. Adult flies were removed and the resulting emergent flies 1 – 3 days old were used for the experiment. Fifty flies per vial were weighed and then homogenized in 0.1 M buffer, pH 7.4 and then centrifuged at 4000 g for 10 min. The supernatant was maintained in ice for determination of protein, total thiol, acetylcholinesterase, GST, hydrogen peroxide, catalase and nitric oxide levels [31,32].

2.8. Ex vivo assays

2.8.1. Protein determination

Protein content of the whole fly homogenate was determined as described by Abolaji *et al.,* (2013) [13] with the aid of bovine serum albumin as the standard.

2.8.2. Total thiol determination

Total thiol was determined spectrophotometrically as described by Ellman [33] with the aid of DTNB reagent (5,5-dithio-bis-(2-nitrobenzoic acid)). DTNB reacts with SH groups to yield a pigmented substrate. Colorimetric analysis was carried out at 412 nm and the content of -SH group was calculated using GSH as standard. Thiol levels were expressed in μmol/mg protein.

2.8.3. Acetylcholinesterase activity

The AChE activity was determined by Ellman and Ecker [31,33]. Briefly, aliquots of supernatant (20 mL) were incubated at 30 °C for 2 min with 0.1 M phosphate buffer, pH 7.4 and 10 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acethylthiocholine (8 mM) as a substrate. Absorbance was determined at 412 nm after 2 mins. Enzyme activity was expressed in μmol/min/mg protein.

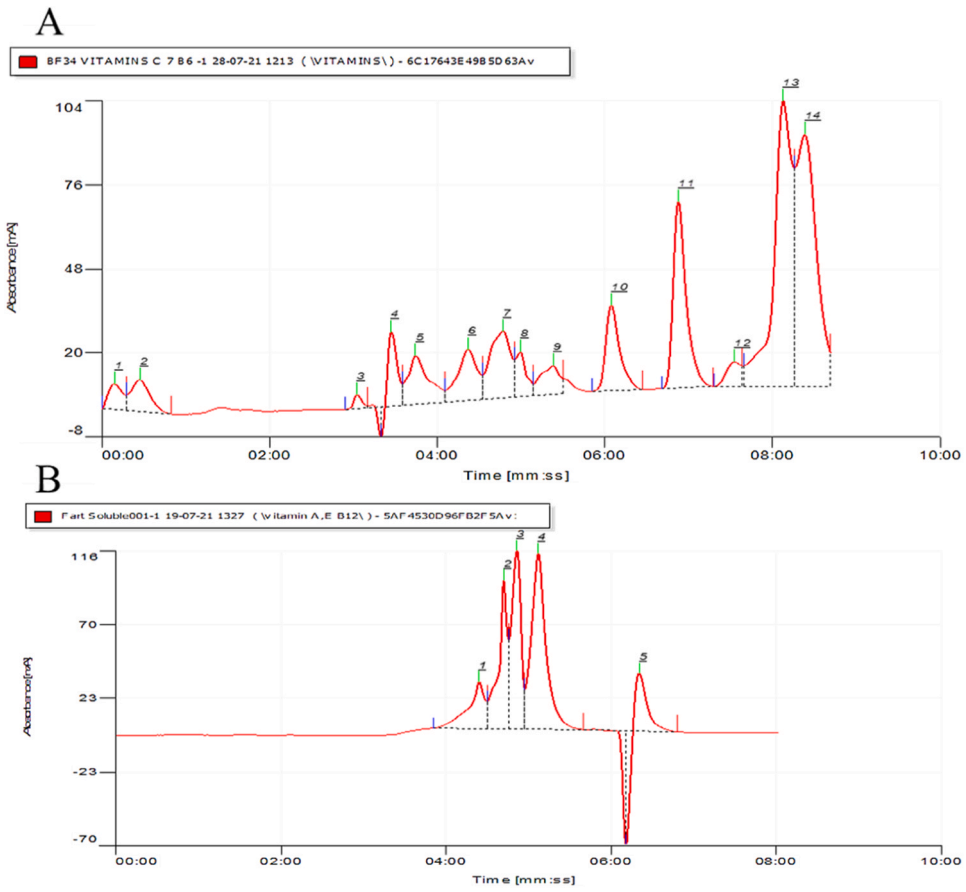


Fig. 3. HPLC analysis of vitamin content of ARGE. (A) water soluble vitamin C and B6 and (B) Fat soluble vitamin E and A.

2.8.4. GST activity

GST activity was determined following the method described by Habig and Jakoby *et al.*, (1981) and Oboh *et al.,* (2018) [34,35]. This method is based on the principle that GST enzyme catalyzes the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (GSH), resulting in a thioether (S-2,4-dinitrophenyl glutathione) which can then be monitored by the increase in absorbance at 340 nm. The molar extinction coefficient used for CDNB was 9.6 $mM^{/}cm$. The results were expressed as mmol/min/mg protein.

2.8.5. Catalase activity

Catalase (CAT) activity was determined spectrophotometrically following the method described by Aebi *et al.*, (1984) [36]. This depends on the ability of CAT to degrade H_2O_2. Kinetic analysis of CAT activity started from the addition of H_2O_2 to reaction medium. The decrease in optical density at 240 nm was measured over 2 min at 25 °C and the results were linear with regard to time. The activity of CAT was expressed as mmol of H_2O_2 consumed/min/mg of protein.

2.8.6. Nitric oxide levels

The amounts of nitrite in supernatants were measured following the Griess reaction [37,38]. It involves incubation of equal volume of Griess reagent (0.1 % N-(1-naphthyl)ethylenediamine dihydrochloride; 1 % sulfanilamide in 5 % phosphoric acid; (1:1)) and the supernatant at room temperature for 20 min. Absorbance was then read at 550 nm.

2.8.7. Hydrogen peroxide levels

Total hydroperoxide such as Hydrogen peroxide levels were determined following the method of Wolf *et al.,* (1994) [39]. This involves incubation of 590 μL of FOX1 reagent with 10 μL of the supernatant at room temperature. The absorbance was then read at 560 nm.

2.8.8. MTT assay

Cellular metabolic activity was evaluated by the ability of NADH or other reducing molecules to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolim bromide), a yellow tetrazolium salt to purple formazan by metabolic active cells [40,41]. The resulting purple formazan formed is then solubilized in DMSO and then measured at 570 nm and 630 nm. Values are expressed as % of control.

3. Results

3.1. Chemical analysis of alliin-rich garlic extract

3.1.1. Phytochemical screening and mineral analysis

Qualitative analysis reveals that the extract consists of cardiac glycosides, quinones, steroids and terpenoids. Quantitative analysis revealed phenol (61 μg/kg), tannin (46 μg/kg), flavonoids (7.3 μg/kg), saponin (1.26 %), and alkaloid (2.15 %). Mineral analysis revealed the extract rich in iron = 24.22 mg/g, potassium =16.84 mg/g, chromium = 2239 μg/g, sodium = 1819 μg/g, calcium = 1485 μg/g, manganese = 105.5 μg/g, zinc = 70.5 μg/g, nickel = 65.0 μg/g, copper = 43.5 μg/g. Toxic heavy metals such as lead and cadmium were not be detected in the extract. HPLC analysis for vitamin content show the presence of vitamin C (168.62 mg/L), vitamin B6 (11.78 mg/L), vitamin E (1570 μg/kg) and vitamin A (50 μg/kg).

3.1.2. HPLC analysis of vitamin content of ARGE

HPLC analysis was performed to determine the presence of water- and fat-soluble vitamins as depicted in Fig. 3A and Fig. 3B respectively.

3.1.3. UPLC-ESI-MS profile of the aqueous extract of Allium sativum

Ten compounds were identified from ARGE based on the UPLC-ESI-

Table 1
A UPLC-ESI-MS profile of the aqueous extract of *Allium sativum*.

Peak No	Rt (min)	Compound name	Molecular formula	Calculated mass	Detected mass	Mass accuracy (mDa)	MS/MS fragmentation ions
1	0.78	L-Arginine	$C_6H_{14}N_4O_2$	174.1117	175.1212	1.7	158.1, 130.1, 116.1, 70.1
2	1.97	γ-Glutamylmethionine	$C_{10}H_{18}N_2O_5S$	278.0936	279.1019	0.4	237.1, 181.1, 144.1, 127.1
3	2.06	S-(2-Carboxypropyl)glutathione	$C_{14}H_{23}N_3O_8S$	393.1206	394.1257	2.3	265.1, 248.1, 230.1, 162.1
4	2.79	Alliin	$C_6H_{11}NO_3S$	177.0460	178.0551	1.2	116.1, 70.1
5	3.35	N-γ-Glutamyl-S-(1-propenyl)cysteine	$C_{11}H_{18}N_2O_5S$	290.0936	291.0974	2.5	274.1, 162.1, 145.1, 73.0
6	4.76	N-γ-Glutamyl-S-(2-propenyl)cysteine	$C_{11}H_{18}N_2O_5S$	290.0936	291.0970	2.1	274.1, 162.1, 145.1, 73.0
7	5.89	N-γ-Glutamylphenylalanine	$C_{14}H_{18}N_2O_5$	294.1216	293.1121	1.6	275.1, 257.1, 164.1 128.0
8	8.23	S-(Allylthio)cysteine	$C_6H_{11}NO_2S_2$	193.0231	194.0336	2.7	N/A
9	8.23	γ-Glutamyl-S-allylthiocysteine	$C_{11}H_{18}N_2O_5S_2$	322.0657	323.0733	0.2	229.1, 194.1 183.1, 152.0
10	14.86	Eruboside B	$C_{51}H_{84}O_{24}$	1080.5353	1081.5414	1.7	757.4, 595.4, 433.3, 289.2

All detected compound adducts were $[M+H]^+$ except for N-γ-glutamylphenylalanine which was $[M-H]^-$ adduct.

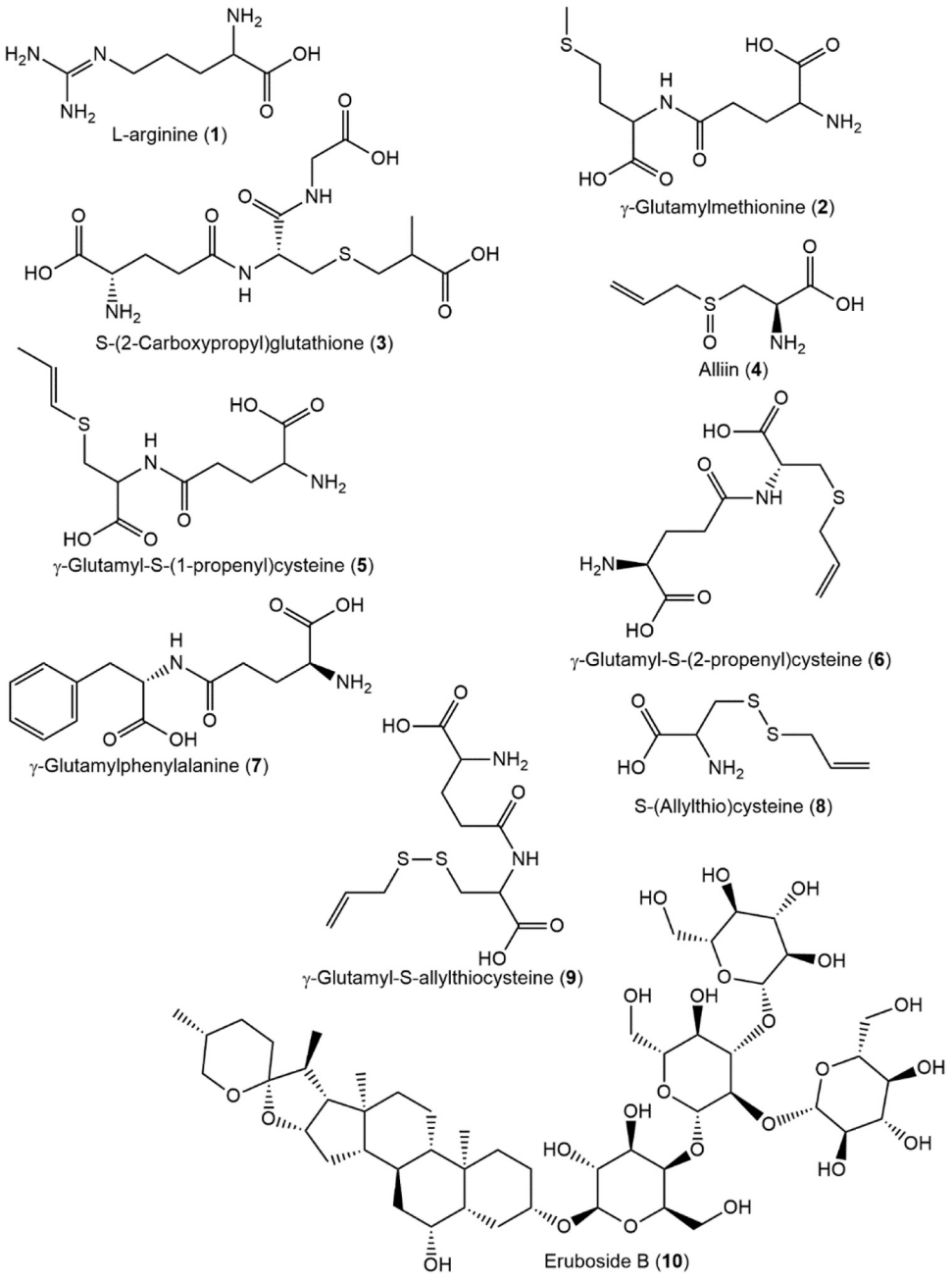


Fig. 4. Chemical structures of compounds identified in alliin-rich garlic extract.

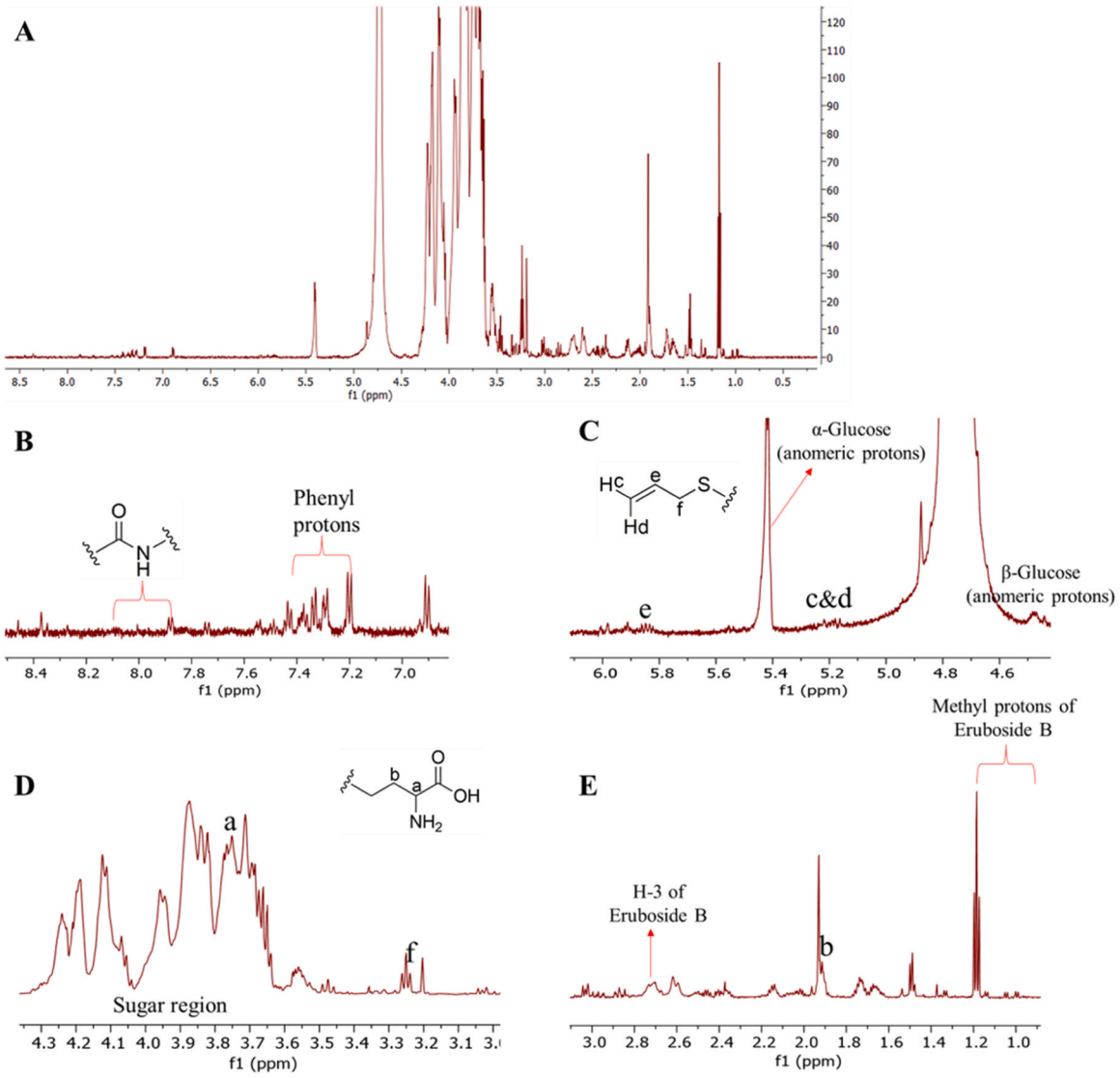


Fig. 5. Full and expanded ^{1}H NMR spectra of Alliin Rich Garlic extract (ARGE). A: Full spectrum, B: 6.90 – 8.50 ppm, C: 4.50 – 6.10 ppm, D: 3.00 – 4.35 ppm, E: 1.00 – 3.05 ppm.

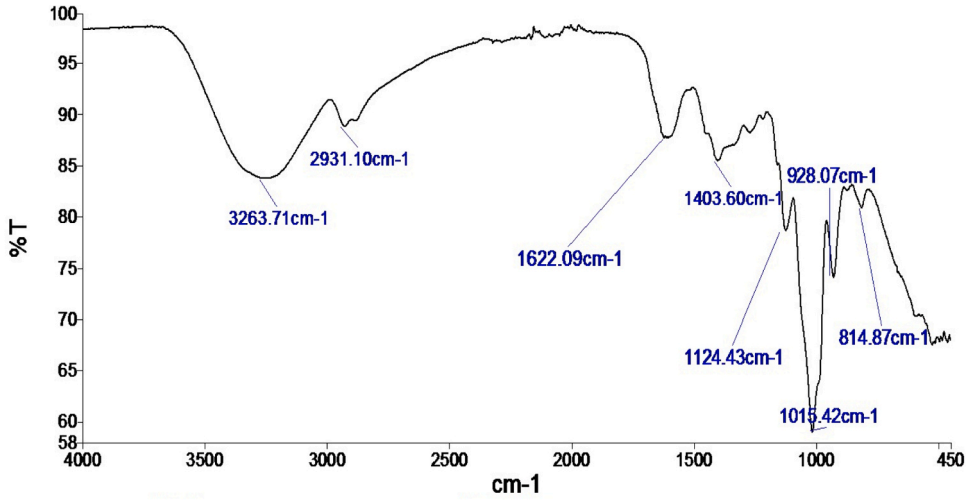
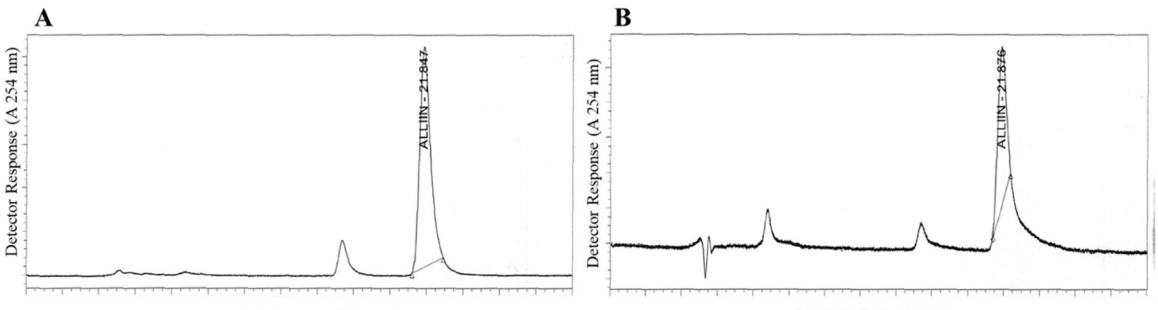


Fig. 6. The infra-red spectra of Alliin Rich Garlic Extract.



	Processed Channel Desc	Peak Name	RT	Area	%Area	Height	Amount	Units
1	W2489 ChA 254nm	Alliin	21.847	204753	100.00	5050	479.866	ppm

	Processed Channel Desc	Peak Name	RT	Area	%Area	Height	Amount	Units
1	W2489 ChA 254nm	Alliin	21.876	28262	100.00	947	73.513	ppm

Fig. 7. HPLC chromatogram of (A) L- (+)-Alliin standard (B) ARGE.

MS analysis. These include seven organosulfur compounds among which is alliin (that eluted at 2.79 mins), γ-glutamylmethionine, S-(2-carboxypropyl)glutathione, N-γ-glutamyl-S-(1-propenyl)cysteine, N-γ-glutamyl-S-(2-propenyl)cysteine, S-(allylthio)cysteine and γ-glutamyl-S-allylthiocysteine. Others include two non-sulfur amino acids, L-arginine and N-γ-glutamylphenylalanine and the steroidal saponin, eruboside B. All the compounds eluted between 0.78 and 8.23 min with the exception of eruboside which eluted at 14.86 mins. A comprehensive report of the UPLC-ESI-MS analysis is presented in Table 1 while the chemical structures of the identified compounds are shown in Fig. 4.

3.1.4. ^{1}H NMR fingerprint

Further verification of the compounds identified using UPLC-ESI -MS were achieved based on ^{1}H NMR fingerprinting. Full and expanded ^{1}H NMR spectra of the garlic extract are provided in Fig. 4. The highly deshielded amide protons were observed between δ_H 7.85 and 8.11. Phenolic protons of γ-glutamylphenylalanine and other related metabolites were observed from δ_H 7.19–7.50. Presence of alliin in the extract was registered with characteristic chemical shifts at δ_H 5.84 (a multiplet assigned to the more deshielded -C═C-H- of the allyl unit), δ_H 5.25 and 5.34 (assignable to the terminal allylic CH_2 protons). The alpha proton to the NH_2 group resonated around δ_H 3.72 which overlaps significantly with sugar protons in addition to other amino acids, thus, contributing to the very high intensities of the peaks between δ_H 3.60 and 4.40. Other alliin peaks include δ_H 3.47 and 3.65, assignable to the bridging methylene group between the allyl and sulfoxide groups. Chemical shift values adopted for the annotation of alliin are consistent with those that were previously reported by Hakamata *et al.,* (2015). The functionally annotated ^{1}H NMR spectrum of ARGE is shown in Fig. 5. Peaks that correspond to various chemical environments and characteristic of the identified compounds are denoted with lowercase letters.

3.1.5. Infra-Red Spectroscopy

The FTIR spectrum (Fig. 6) revealed the various functional groups associated with ARGE compounds. First peak (3263.71 cm^{-1}) can be assigned to COOH group, the second peak (2931.10 cm^{-1}) can be assigned to the NH_2 group, the third peak (1622.09 cm^{-1}) can be assigned to the group C═C and the sixth peak (1015.42 cm^{-1}) assigned to S═O and the 7th peak (928.07 cm^{-1}) assigned to C-S. This is in accordance with the standard infrared spectra of alliin.

3.1.6. Concentration of alliin in ARGE by HPLC

The concentration of alliin in ARGE was 0.073 mg/g of the extract (25 mg/g = 1.825 mg, 50 mg/g = 3.65 mg, 100 mg/g = 7.30 mg and 200 mg/g = 14.60 mg) (Fig. 6).

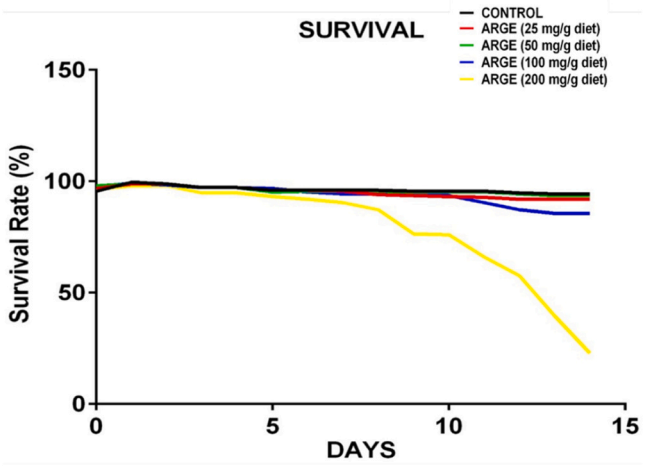


Fig. 8. Effects of ARGE on the survival rate of *Drosophila melanogaster*.

3.2. Safety Evaluation of Alliin-Rich Garlic Extract

The safety evaluation of ARGE was carried out at the following concentrations 25, 50, 100 and 200 mg/g diet.

3.2.1. Alliin-rich garlic extract maintains D. melanogaster survival rate

There was no significant change in the survival rate of flies fed with ARGE (25, 50 and 100 mg/g diet) compared with the control. However, at the highest concentration of 200 mg/g diet, there was a 54 % reduction in the fly survival rate Figs. 7 and 8.

3.2.2. Effects of Alliin-Rich Garlic on activity of acetylcholinesterase and levels of negative geotaxis, nitric oxide and hydrogen peroxide in D. melanogaster

ARGE (25, 50 and 100 mg/g diet) significantly reduced acetylcholinesterase activity by 40, 76 and 71 %, respectively compared with the control (Fig. 9A, $p < 0.05$). The negative geotaxis (locomotor activity) of the flies was not affected by ARGE at all concentrations (Fig. 9B). There was a significant increase in nitric oxide (nitrite/nitrate) level in flies fed with ARGE (25 mg/g diet) by 77 % compared with the control (Fig. 9C, $p < 0.05$). There was no significant change in the level of hydrogen peroxide in *D. melanogaster* fed with ARGE 25, 50 and 100 mg/g diet except at ARGE concentration of 200 mg/g diet (Fig. 9D).

3.2.3. Effects of ARGE on GST and Catalase Activities and Total Thiols Level and Mitochondrial Metabolic Rate

ARGE (25 and 50 mg/g diet) increased total thiol levels by 110 and

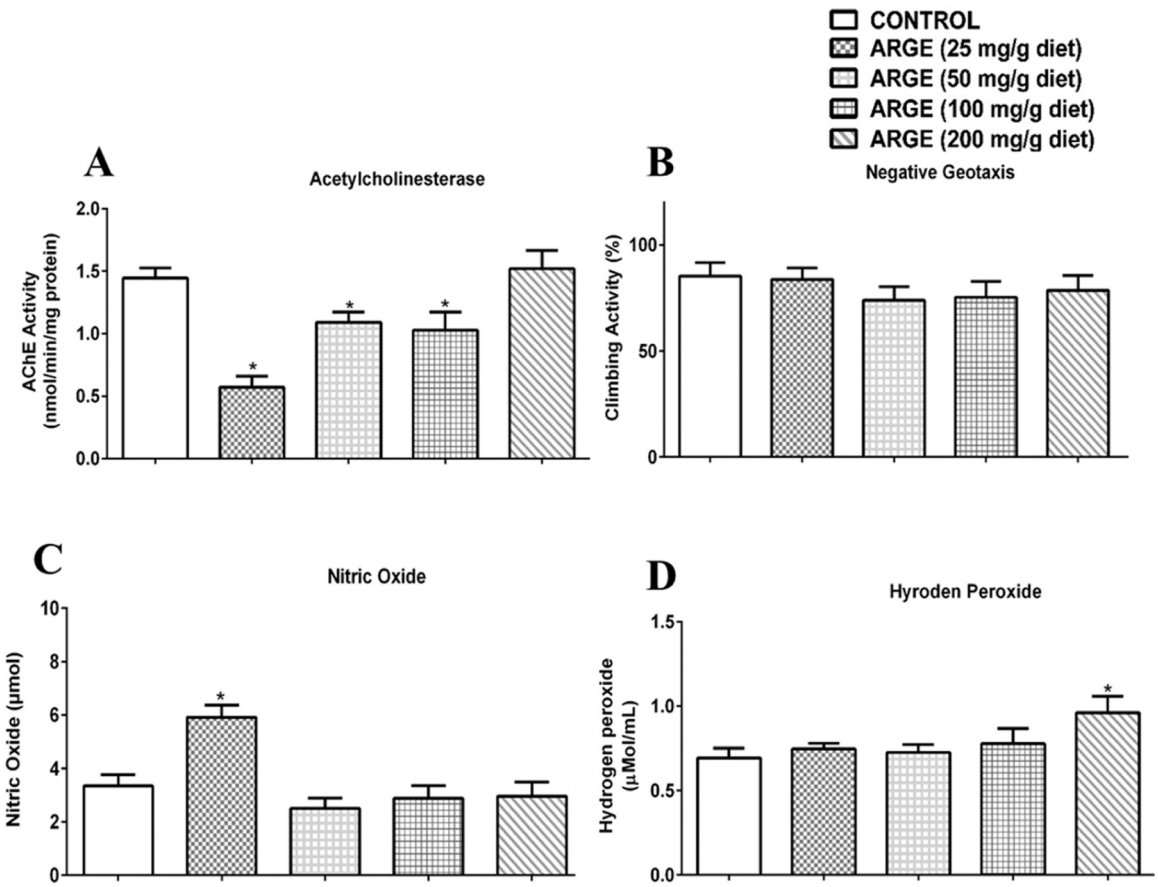


Fig. 9. Effects of ARGE on acetylcholinesterase activity (A), negative geotaxis (B), nitric oxide level (C) and hydrogen peroxide level (D) in *Drosophila melanogaster*. Values are expressed as mean ± standard error of mean with 5 replicates per treatment group. Significant differences from the control are indicated by $*p < 0.05$.

81 %, respectively (Fig. 10A, $p < 0.05$). ARGE (25 and 50 mg/g diet) did not significantly alter catalase activity of *D. melanogaster* (Fig. 10B). The GST activity of *D. melanogaster* exposed to ARGE (50, 100 and 200 mg/g diet) increased by a 2.5, 2.6 and 2.7 folds respectively, (Fig. 10C). ARGE (25 and 50 mg/g diet) did not significantly change the cellular metabolic rate (cell viability) of flies. This marker was however reduced by higher concentrations of ARGE (100 and 200 mg/g diet).

3.3. Histopathological observation of the organs

The histology of the brain, fat body and gut revealed no visible lesion on these tissues of flies treated with ARGE (Fig. 11).

Magnification: 400×

4. Discussion

Humans have always relied on herbs as medicines and most modern medicines such as ephedrine, aspirin and artemisinin originated from plants [42,43]. However, the chemical composition as well as safety profiles of many herbal extracts remain underexplored. There is a need for safety evaluation of herbal extracts due to the chemical modifications occurring during the extraction process [7]. Thus, Drug Regulatory Agencies are focusing on the safety profile and constituents of herbal extracts irrespective of their therapeutic efficacy. [44,45] Alliin is an organosulfur compound found mainly in garlic and it possesses chemoprotective properties due to its sulfur-centered stereochemistry [46]. The popularity of garlic in various herbal preparations has led to our quest to understand the bioactive components of its ARGE and its safety profile.

The use of the modern hyphenated technique, UPLC-ESI-MS and 1 H NMR spectroscopy identified several key components of ARGE. These includes: alliin (S-ally-L-cysteine-S-oxide), L-arginine, γ-glutamylmethionine, S-(2-carboxypropyl)glutathione, N-γ-glutamyl-S-(1-propenyl)cysteine, N-γ-glutamyl-S-(2-propenyl)cysteine, N-γ-glutamylphenylalanine, S-(allylthio)cysteine, γ-glutamyl-S-allylthiocysteine and eruboside B. This extract is notably rich in essential thiols of cysteine and glutathione derivatives which are important antioxidant compounds protecting tissues from oxidative stress. Thiols are used in the treatment of various diseases because they function as radical scavengers, GSH prodrugs or metal chelators [47,48]. Further analysis via HPLC confirmed that the extract is alliin-rich with an alliin concentration of 0.073 mg/g of extract (equivalent to 1.825 mg in 25 mg/g, 3.65 mg in 50 mg/g, 7.30 mg in 100 mg/g and 14.60 mg in 200 mg/g).

Infra-red spectra revealed several characteristics peaks; the first peak (3263.71 cm^{-1}) can be assigned to the COOH group, the second peak (2931.10 cm^{-1}) can be assigned to the NH_2 group, the third peak (1622.09 cm^{-1}) assigned to the group C═C and the sixth peak (1015.42 cm^{-1}) assigned to the S═O and the 7th peak (928.07 cm^{-1}) assigned to the C-S. This is in accordance with the standard infrared spectra of alliin (Tasci *et al.,* 2016).

Phytochemical analysis revealed that the extract is rich in cardiac glycosides, quinones, steroids and terpenoids. Quantitative analysis revealed phenols (61 µg/kg), tannins (46 µg/kg), flavonoids (7.3 µg/kg), saponins (1.26 %), and alkaloids (2.15 %). Mineral analysis revealed the extract rich in iron = 24.22 mg/g, potassium = 16.84 mg/g, chromium = 2239 µg/g, sodium = 1819 µg/g, calcium = 1485 µg/g, manganese = 105.5 µg/g, zinc = 70.5 µg/g, nickel = 65.0 µg/g, copper = 43.5 µg/g. There was no presence of toxic metals lead and cadmium.

HPLC analysis revealed the presence of vitamin C (168.62 mg/L), vitamin B6 (11.78 mg/L), vitamin E (1570 µg/kg) and vitamin A (50 µg/

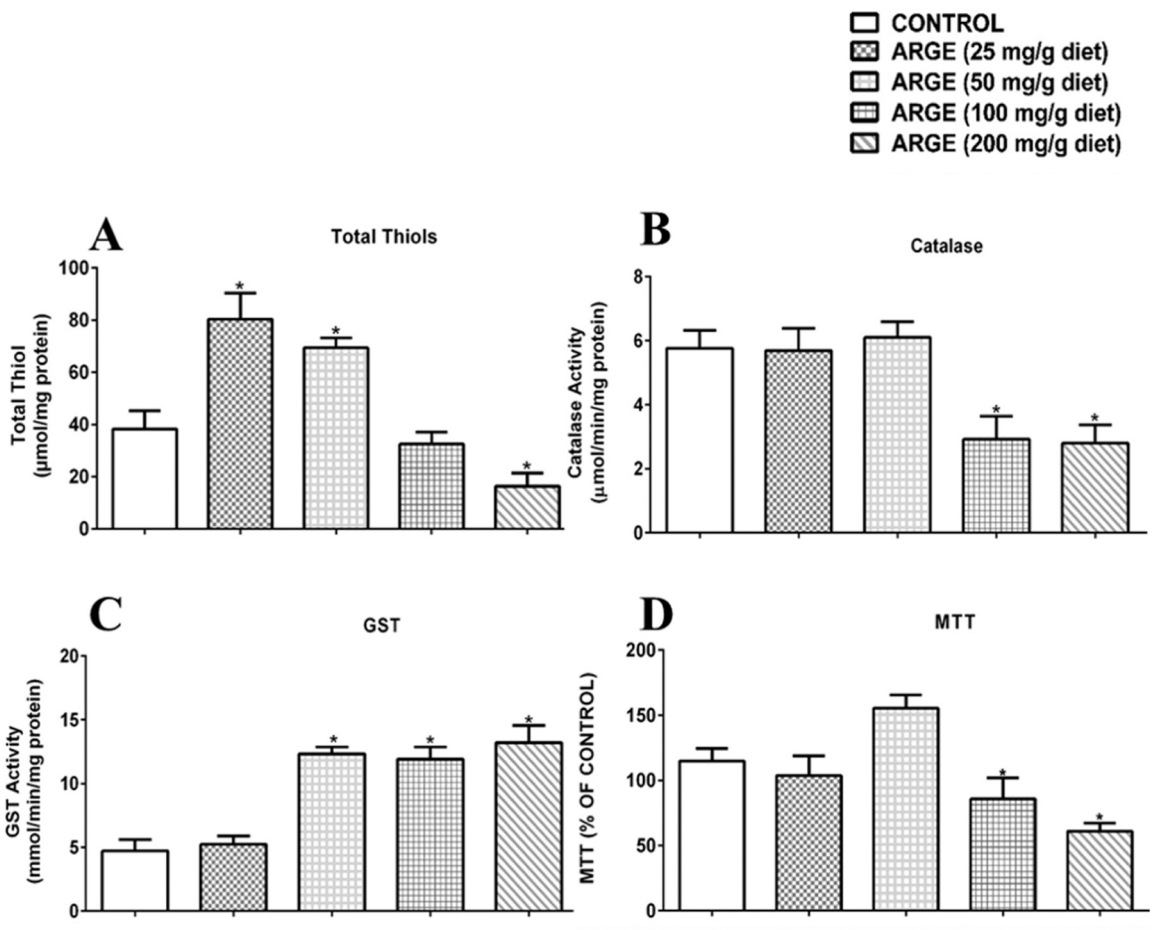


Fig. 10. : Effects of ARGE on Total Thiol (**A**), Catalase activity (**B**), GST activity (**C**) and Cellular metabolic rate (**D**) in *Drosophila melanogaster*. Values are expressed as Mean ± Standard Error of mean with 5 replicates per treatment group. Significant differences from the control are indicated by $*p < 0.05$.

kg). These results indicates that the extract is rich in essential vitamins required for normal nerve function, regulating body fluid balance and supporting overall health. ARGE is also rich in phytochemicals such as cardiac glycosides which are commonly used as a remedy for heart failures and irregular heartbeats [49,50]. Additionally, the extract contains phenols and flavonoids both of which act as antioxidants and protect against cardiovascular diseases [51]. The high concentration of vitamin C and B6 suggest that ARGE may play an important role in tissue repair as well as in supporting healthy brain function and the immune system [52].

The safety evaluation was carried out on ARGE (25, 50, 100 and 200 mg/g diet) using *D. melanogaster* (Harwich strain) as an alternative to the mammalian model. We found that ARGE is safe for oral consumption at moderate concentrations. It boosts the antioxidant status of the flies and acts as a potent acetylcholinesterase inhibitor.

The exposure of *D. melanogaster* to ARGE concentrations of 25, 50 and 100 mg/g diet for 14 days maintained the survival rate of these flies. Hence, ARGE did not induce mortality except at high concentration of 200 mg/g diet. Previous studies by *Shi et al.,* (2019) [52] among aged Chinese population (mean age = 92.9 years) showed that garlic components lowered their mortality risk. Following the survival experiment, we sought to understand the antioxidant status, cellular metabolic rate and histology of the flies.

Acetylcholinesterase is mainly located at the postsynaptic neuromuscular junction specifically in the muscles and nerves where it terminates the activity of the naturally occurring neurotransmitter acetylcholine. This it does by catalyzing the breakdown of acetylcholine into acetic acid and choline. Acetylcholinesterase terminates neuronal transmission from the brain to the muscles and between synapses, thus preventing acetylcholine dispersal and activation of nearby receptors. Alzheimer's disease patients have reduced levels of acetylcholine and their cholinergic neurons have defective projections, thus neurotransmission between the nerves in the brain and the muscles is slowed. This leads to cognitive decline or lack of coordination, reduced control of attention, thinking and processing of stimuli because these activities are organized by the cholinergic neurons. Alzheimer's patients are regularly treated with acetylcholinesterase inhibitors which serve to alleviate their symptoms by keeping the levels of acetylcholine high at the synaptic junctions, thus enhancing the potentiation of the signal. This action helps to recalibrate the neurotransmitter to appropriate levels and keeps them sensitive to cognitive functions which enhances increased attention span, thinking and procession of stimuli [53,54]. ARGE (25, 50 and 100 mg/g diet) significantly reduced the acetylcholinesterase activity by 40, 76 and 71 %, but did not alter the negative geotaxis (behavior) of the flies. This is in line with previous studies by Jing-Fang *et al.,* (2021) [55] which showed that garlic extract is effective for the treatment of cognitive impairment and Alzheimer's disease.

Moreover, ARGE maintained the hydrogen peroxide levels of the flies. Increased hydrogen peroxide level has been reportedly linked to peroxidation of the lipid bilayer and tissue damage in humans [56,57]. ARGE (25 mg/g diet) increased the nitric oxide level of the flies by 77 %. Our data agrees with reports by Baik *et al.,* (2022) [58] which showed that fermented garlic extract improves vascular function through increasing vascular nitric oxide bioavailability in healthy participants. Indeed, nitric oxide is also a retrograde neurotransmitter which uses feedback mechanism to amplify its original signal source [59]. Thus,

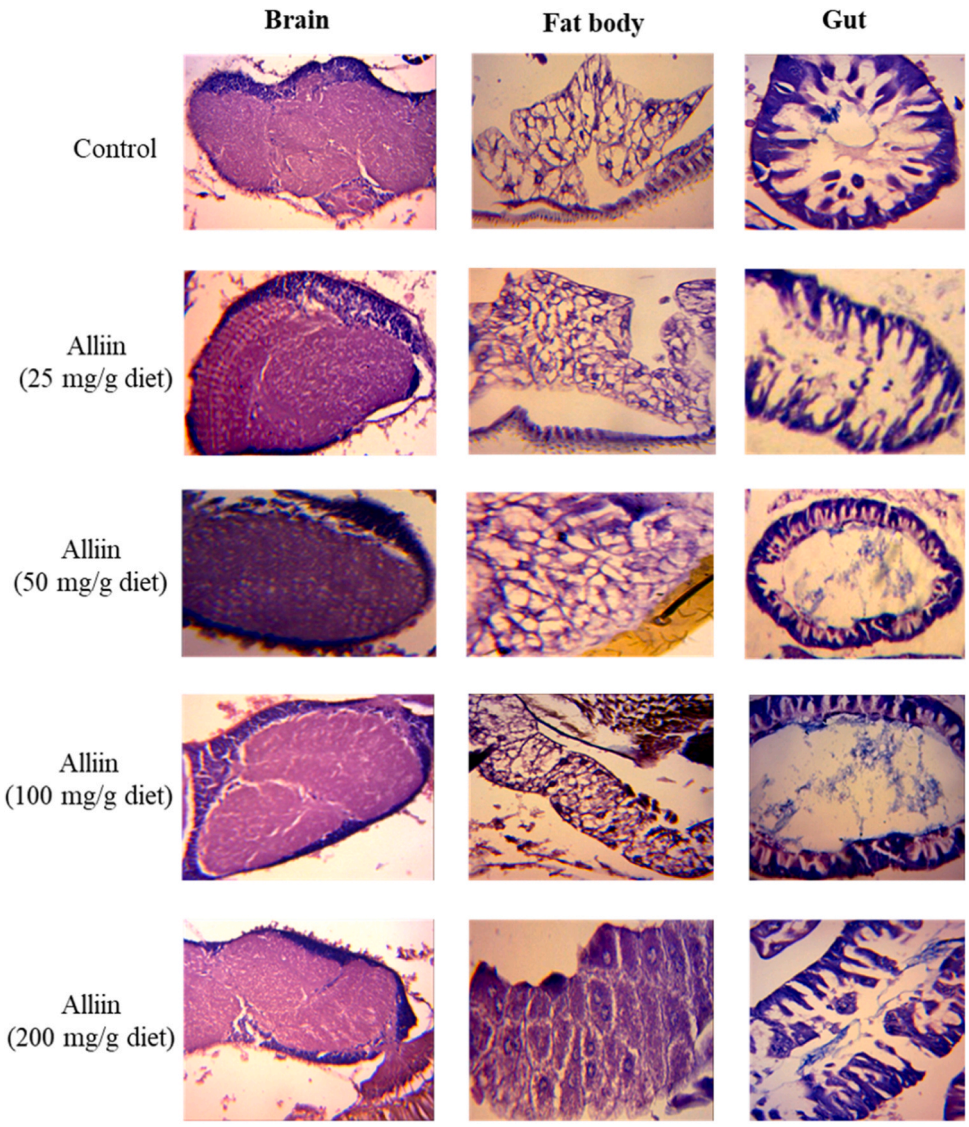


Fig. 11. Histology of the brain, fat body and gut of *D. melanogaster* fed with ARGE.

increased insulin sensitivity and improved health have been shown in diabetic patients exposed to nitrates [60].

Glutathione-S-transferase (GST), thiols and catalase protect the body against oxidation and aid the body in mopping up free radicals by converting them to less harmful substances for excretion, thus functioning as the antioxidant shield of the body [61,62]. ARGE improves the antioxidant status of the flies at medium concentrations. Glutathione-S-transferases are antioxidant enzymes which catalyze the conjugation of the reduced form of glutathione (GSH) with foreign substances mainly electrophiles for elimination from the body. These phase II metabolic isoenzymes help to protect the body against oxidative damage which is key in the survival of organisms [13]. ARGE (50, 100 and 200 mg/g diet) increased the GST levels by 2.5, 2.6 and 2.7- folds, respectively. Thus, ARGE enhanced the antioxidant status of the flies. This is in accordance with previous studies by Chia-Wen et al., (2005) [63] which have shown that garlic organosulfur compounds increase the expression of glutathione-S-transferase (π class) genes in hepatocytes of male rats.

Thiols also referred to as mercaptan, are a class of organic compounds containing a sulfhydryl group (-SH). Thiols act as reductants and therefore are easily oxidized to disulfides during oxidative stress. This thiol/disulphide homeostasis protects the body cells from oxidative damage. Thus, thiols act as an antioxidant shield, aid in regulation of antioxidant enzymes as well as maintain an oxidative balance. Most naturally occurring thiols are cysteine derivatives such as homocysteine, glutathione and c-glutamylcysteine [64,65]. ARGE (25 and 50 mg/g diet) increased the total thiol levels by 110 and 81 %, respectively, thereby increasing the antioxidant status of *D. melanogaster*. This is in accordance with studies by Lotfi *et al.,* 2021 [66] which showed that feeding diabetic rats with garlic extract restored their depleted total thiol to normal levels.

Catalase is a major antioxidant enzyme which serves to protect the body against the harmful effect of hydroxyl radical by decomposing H_2O_2 to water and molecular oxygen. Thus, catalase protects the body against oxidative stress. ARGE (25 and 50 mg/g diet) maintained the catalase activity of *D. melanogaster*. Our data correlates with a study by Pedraza-Chaverri *et al.,* (2001) [67] which showed that garlic extract maintained the catalase mRNA levels in the kidney and liver of rats and in presence of reduced H_2O_2 renal and hepatic catalase expression were diminished.

A living cell has to expend energy in the form of NADH or NADPH. Measurement of these energy currencies serves as an indicator of cell viability, proliferation and cytotoxicity. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which tend to reduce yellow tetrazolium salt of MTT (3–4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolim bromide) to purple formazan crystals which can then be detected

spectrophotometrically [68]. Consequently, cells with low metabolism such as splenocytes exhibit low MTT levels while rapidly dividing cells exhibit high levels of MTT [69]. ARGE (25 and 50 mg/g diet) did not significantly alter the cell viability (MTT levels) of *D. melanogaster*. This is in accordance with studies by Jasamai *et al.,* (2016) [70] which showed that garlic extract maintained cell viability (MTT levels) and does not induce necrosis in human leukemic cell lines (Jurkat Clone E6–1 and K-562 cells). Furthermore, the histology of the brain, gut and fat body revealed no visible damage to the flies which further indicates that ARGE is safe for oral consumption especially at lower doses.

5. Conclusion

This study shows that alliin-rich garlic extract is safe for oral consumption at moderate concentrations, rich in vitamins, minerals and essential thiol derivatives of cysteine and glutathione origins. It enhances the antioxidant status of the flies and may function as a potent acetylcholinesterase inhibitor.

List of Abbreviations

- ARGE - Alliin-Rich Garlic Extract
- UPLC - Ultra Performance Liquid Chromatography
- ESI-MS - Electrospray Ionization Mass Spectrometry
- SDS - Sodium Dodecyl Sulfate
- WHO - World Health Organization
- *D. melanogaster* - Drosophila melanogaster
- RNA - Ribonucleic Acid
- DNA - Deoxyribonucleic Acid
- HSS - High Strength Steel
- C18 - Column with Octadecyl Functional Group

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Author Statement

All the authors agree with the current state of the manuscript.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Chinedum Peace Babalola: Writing – review & editing, Supervision, Methodology, Conceptualization. **Amos Abolaji:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation. **Zeniat Emike Oyaluna:** Writing – original draft, Investigation. **Paul Steenkamp:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Gerhard Prinsloo:** Writing – review & editing, Methodology, Formal analysis, Data curation. **John Oludele Olanlokun:** Writing – review & editing, Formal analysis. **Olusola Bodede:** Writing – review & editing, Investigation, Formal analysis, Data curation.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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