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Quantification of selected polyphenols in hydroethanolic garlic extract using High Performance Liquid Chromatography and their effects on Drosophila neuropeptides: A molecular docking study

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Abstract
Garlic (*Allium sativum*) is a bulbous plant, which has been established to possess diverse medicinal properties. The neuropeptides of *Drosophila melanogaster* function to control major metabolic activities of the fruit flies. Thus, this study aimed to identify polyphenols that can distort the expression of some selected neuropeptides that are responsible for satiety in *Drosophila melanogaster*. High Performance Liquid Chromatography (HPLC) analysis was performed on hydroethanolic garlic extract, after which an *in-silico* pharmacokinetics screening and molecular docking studies were performed using 10 selected polyphenols in garlic as ligands, and UDP2, Hugin and dNPF as neuropeptide targets responsible for satiety. The hydroethanolic garlic extract had gallic acid to be most abundant, followed by isovallinic acid and protocatechuic acid (7.736, 3.024 and 2.235 ppm respectively). The least present polyphenols are quercetin, rutin, orientin and isoorientin (0.561, 0.907, 0.948 and 0.913 ppm respectively). However, after docking analysis, isoorientin showed the best binding affinity with all the neuropeptide targets that were analysed at their respective binding site with docking score of upto -7.8 kcal/mol. This study predicted that isoorientin could serve as therapeutic agents to control satiety.

Keywords: garlic; isoorientin; satiety, neuropeptide, drosophila
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Introduction

Garlic is a bulbous plant of central Asia origin of the Amaryllidaceae family [1] which is widely used as a spice and for dressing in cooking [2]. The bulb has been used as a medicinal herb in traditional medicine across the world [3,4]. The stalk of garlic originates from the bulb and gives out white spherically arranged flowers, the bulb consist of bulblet [cloves] covered by white membranous scales [2]. Many studies have investigated the medicinal benefits of garlic such as antifungal, antidote (for heavy metal poisoning), hepatoprotective, anti-ageing and antimicrobial potentials [5,6]. Several studies have also reported that garlic has the potential to reduce the risk of developing cardiovascular diseases and cancer [7-10]. Garlic preparations, used as ethnomedicine, comprise garlic powder, garlic oil, extracts (aqueous and solvent) of raw or dried garlic and aged garlic extracts. The medicinal potentials of garlic depend on the unique profile of its phytoconstituents [11,12], acting synergistically.

A significant number of distinct neuropeptides have been discovered in a wide range of neuron types and cells throughout the Drosophila nervous system[13]. Forty-two (42) genes in *Drosophila melanogaster* encode precursors of neuropeptides and protein hormones [14-16]. Generally, in insects, peptides regulate a wide range of functions including development, growth, longevity, control of carbohydrate and lipid metabolism, modulation of heart, skeletal muscle contractions, regulation of water and ion transport, activation of pheromone biosynthesis, and ovary and egg maturation. Peptides also operate as output factors and modulators of biological clock circuits, triggering and orchestrating ecdysis behavior as well as regulating foraging and feeding activities. Within the *Drosophila melanogaster* lamina, direct neurotransmission and neuromodulation appear to be mediated solely by classical transmitters such as acetylcholine, glutamate, and γ-amino butyric acid (GABA)[17].

The study was aimed at quantifying selected polyphenols present in hydroethanolic garlic extract as well as molecular docking of the polyphenols against drosophila neuropeptides that are responsible for energy homeostasis.

Materials and Methods

Sample preparation

The garlic bulb (purchased at Oje-Olobi Market in Ede, Osun state, Nigeria) was peeled, washed, chopped, air dried, and pulverized. The sample of garlic was identified and confirmed at the Herbarium, Department of Botany, Obafemi Awolowo University, Ile – Ife, Osun State, Nigeria (Voucher number: 21/17735). The powdered sample (200 g) was extracted with 1000 mL ethanol (50%, v/v) for 72 h and filtered. The filtrate was concentrated in vacuo to produce the crude extract.

Phytochemical Quantification using High Performance Liquid Chromatography (HPLC)

The phytochemical quantification was carried out using Agilent 1100 series HPLC machine (Thermo Electron Corporation, USA) with HyPurity Advance chromatographic column (250 × 4.6 mm, i.d, and 5 µm particle size). The separation was achieved isocratically, using a mobile phase of water and methanol as organic modifier (60:40 v/v) pumped at a flow rate of 0.8 mL/min at 25°C. The injection volume was 10 µL and the detection was made at 280 nm.

Polyphenols (gallic acid, protocatechuic acid, isovallinic acid, vitexin, kampferol, catechin, orientin, isoorientin, rutin and quercetin) (0.01 gm) were accurately weighed in a 10 mL volumetric flask separately, dissolved by sonication, and made up with methanol to obtain 1000 µg/mL as the stock solutions. The working standard solution was prepared with 5 mL of stock solutions diluted in a 50 mL flask with the same solvent to give a 100 µg/mL solution. A volume of 0.1 mL of sample was diluted in a 10 mL volumetric flask, and this solution was further diluted (1:10 v/v) in order to obtain a final concentration of each compound.

Molecular docking and scoring studies

The 3-dimensional X-ray crystallographic structure of drosophila neuropeptides were

retrieved from flybase protein data bank [18]. The peptides were prepared for docking by the following steps: (a) metal ions, water, and cofactors were removed, (b) bounded ligands was removed, (c) polar hydrogen bond was added, and (d) finally, the proteins were minimized using the relevant tools PyRx.

The 3D structures of all the HPLC polyphenols were obtained from the PubChem database [19] in simple document format (SDF). The polyphenols were optimized using open babel in PyRx which was then minimized and further converted to the most stable structures in pdbqt format.

Molecular docking was accomplished via a flexible docking protocol[20], using an autodock suite housed in the PyRx module. The specific target site for the receptor was set using the grid box with dimensions and the center was adjusted based on the binding site of the peptide. The molecular interaction was viewed with the aid of Discovery Studio 2016©.

ADMET Properties

Physicochemical and absorption, distribution, metabolism, excretion, and the toxicity properties of the studied molecular compounds were examined using SwissADME [21]. In this study, several factors such as human intestinal absorption, blood-brain barrier and CYP450 inhibition were considered.

Results

HPLC quantificantion of hydroethanolic garlic extract

The phytochemical quantification in Table 1 showed that gallic acid was most abundant in the hydroethanolic garlic extract, followed by isovallinic acid and proto-catechuic acid (7.736, 3.024 and 2.235 ppm respectively). The least present polyphenols were quercetin, rutin, orientin and isoorientin (0.561, 0.907, 0.948 and 0.913 ppm respectively).

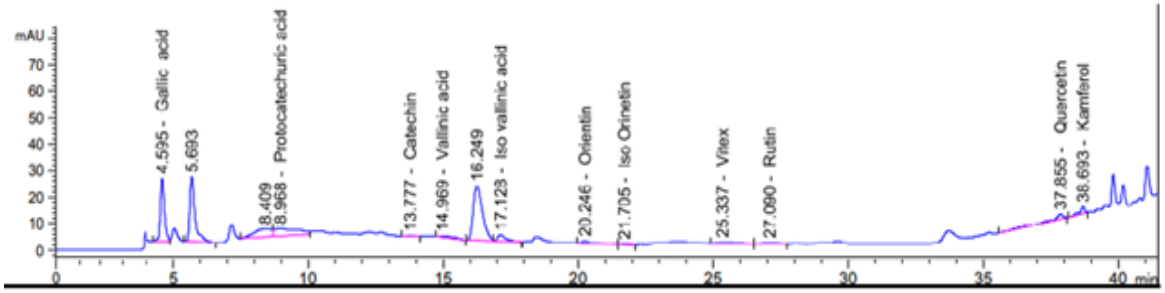
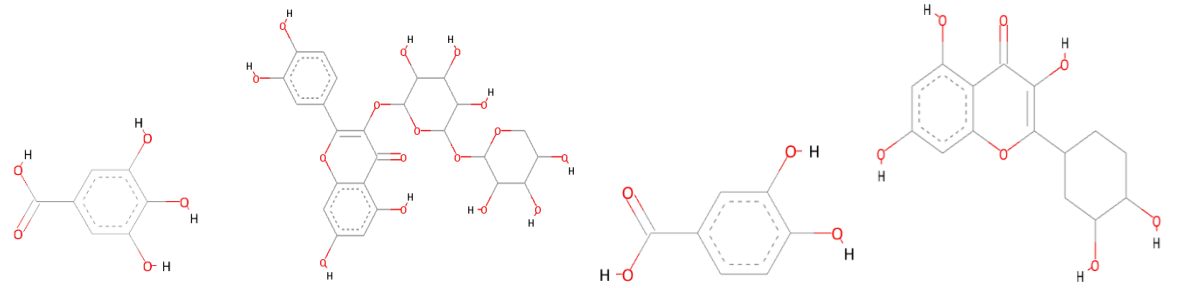


Figure 1: HPLC chromatogram for polyphenols in hydroethanolic garlic extract

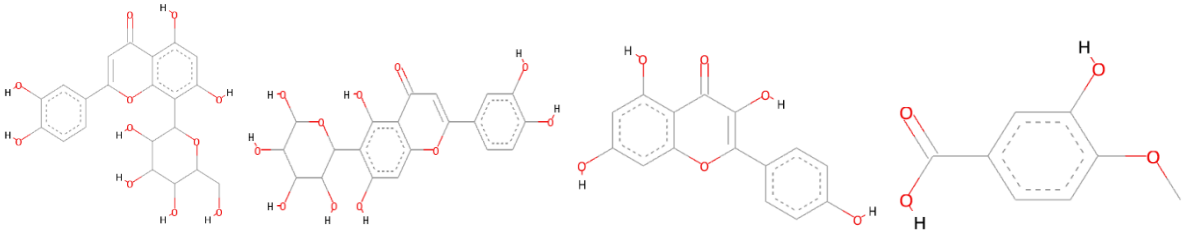
Table 1. HPLC quantification of hydroethanolic garlic extract with reported bioactivity

	Polyphenol	amount (ppm)	CAS	reported activity	references
1.	gallic acid	7.736	149-91-7	neuroprotection; antioxidant	[22,23]
2.	protocatechuic acid	2.235	99-50-3	improves brain function	[24]

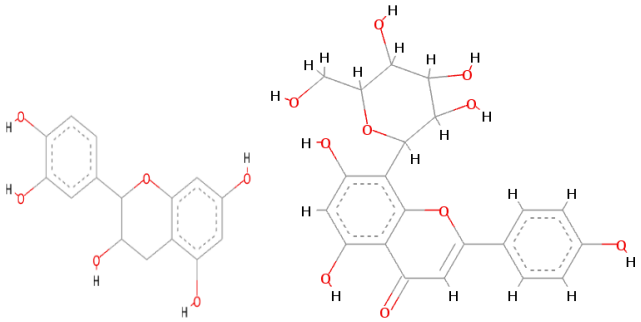
3.	isovallinic acid	3.024	211-430-5	Antioxidant	[25]
4.	Vitexin	1.975	521-33-5	antioxidant; alleviates neurotoxicity	[26, 27,]
5.	Kampferol	1.126	520-18-3	antioxidant, Improves gut microbiota, anti-obesity	[28, 29]
6.	Catechin	1.117	7295-85-4	improves brain function and cognitive impairment, antioxidant, antiadipogenic potential	[30]
7.	Orientin	0.948	28608-75-5	improves metabolic process and adiponectin	[31]
8.	Isoorientin	0.913	4261-42-1	antioxidant, and gut microbiota, regulate lipid metabolism	[32]
9.	Rutin	0.907	153-18-4	antioxidant, antihyperlipidemia, reoxygenation of hypoxia cells	[33]
10.	Quercetin	0.561	117-39-5	antioxidant, attenuates insulin resistance, and neurotoxicity	[34, 35,36]



(a) **(b)** **(c)** **(d)**



(e) **(f)** **(g)** **(h)**



(i) (j)

Figure 2. 2D structure of HPLC quantified garlic polyphenols: (a) gallic acid (b) rutin (c) protocatechuic acid (d) quercetin (e) orientin (f) isoorientin (g) kaempferol (h) isovallinic acid (i) catechin (j) vitexin

Molecular docking and scoring studies

Series of selected molecular compounds identified to be present in hydroethanolic garlic extract were docked against *Drosophila melanogaster* neuropeptides to observe the interactions that were involved. The docking scores and binding affinities obtained for the studied complexes are shown in Table 2.

Most of the studied polyphenolic compounds proved to be more effective than the fenfluramine (standard drug), as they bind more specifically with the neuropeptides as compared with the standard drug. Isoorientin showed the most binding affinity with *HUG*, *UDP2* and *dNPF* (-6.6, -7.8 and -6.7 kcal/mol respectively).

Table 2. Binding affinity of the hydroethanolic extract ligand to neuropeptides

Ligands (kcal/mol)	HUG	UDP2	Dnpf
fenfluramine	-5.3	-6.0	-5.0
Catechin	-5.8	-7.3	-5.5
gallic_acid	-4.4	-5.4	-4.3
isoorientin	**-6.6**	**-7.8**	**-6.7**
isovanillic acid	-4.3	-5.5	-5.1
Kampfaerol	-5.4	-6.7	-5.4
Orientin	-6	-6.9	-6.1
procatechuric acid	-4.5	-5.6	-5.4
Quercetin	-5.9	-7.2	-5.9
Rutin	-6.3	-7.2	-6.3
Vitexin	-5.9	-7.2	-6.1

Molecular interaction studies

The 2D molecular interaction revealed the bonding between the phytochemical (isoorientin) and the neuropeptides. Isoorientin interacted with *UDP2*, *dNPF* and *Hug* with a hydrogen bonding at their amino acid residues as shown in Table 3.

Table 3. Interactions between ligands and neuropeptides

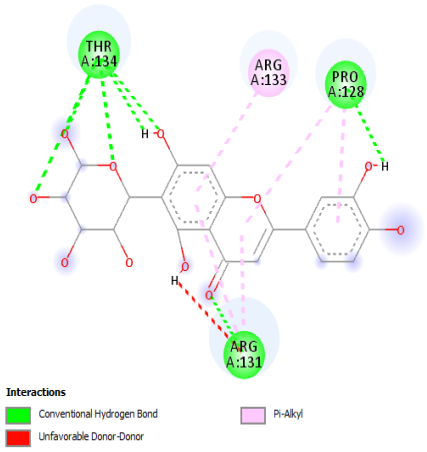
Ligands	Neuropeptides	Hydrogen bonding	Other bonds
isoorientin	Hug	PRO128. ARG131, THR134	ARG133
isoorientin	UDP2	LEU169, SER170, ARG178, ASN196	LYS181, PRO195
isoorientin	Dnpf	ASN28, THR52, ARG57	PRO31, PRO32, TYR53

ADME properties

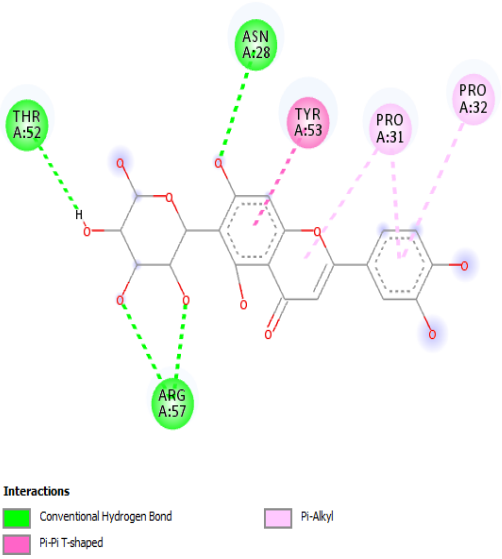
The absorption, distribution, metabolism and excretion (ADME) of the identified phytochemical (isoorientin) in hydroethanolic garlic extract are shown in Table 4. It had a low intestinal absorption and was unable to permeate the blood brain barrier as compared with the standard drug which had high gastro-intestinal absorption and could permeate blood brain barrier. Isoorientin was non-substrate and non-inhibitor to p-glycoprotein (Pgp), which was similar to the standard drug. The study also indicated that isoorientin was a non-inhibitor of cytochrome P450 (CYP 450) as compared to the standard drug which could inhibit CYP4501A2 and CYTP4593A4.

Table 4: Absorption, digestion, metabolism and Excretion predictions of the hit ligands

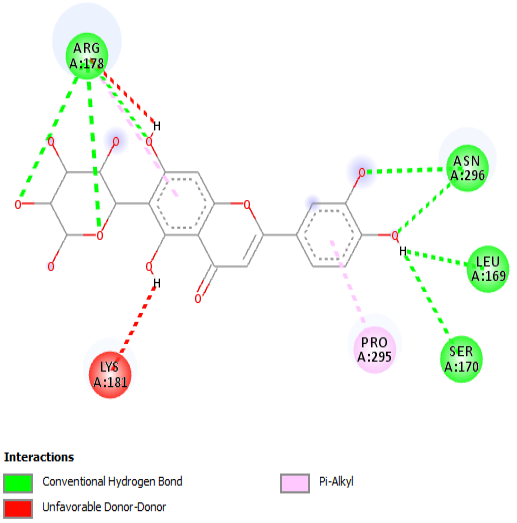
Molecules	Isoorientin	Fenfluramine
GI absorption	Low	High
BBB permeant	No	Yes
Pgp substrate	No	No
CYP1A2 inhibitor	No	Yes
CYP2C19 inhibitor	No	No
CYP2D6 inhibitor	No	Yes
CYP3A4 inhibitor	No	No
log Kp (cm/s)	-9.14	-5.3
Lipinski #violations	2	0
Bioavailability Score	0.17	0.55
Synthetic Accessibility	5.04	2.07



(a)



(d)



(c)

Figure 3. 2D molecular interactions of garlic polyphenols and neuropeptides: (a) *hugin* and isoorientin; (b) *dNPF* and isoorientin and (c) *UDP2* and isoorientin.

Discussion

Phenolic compounds are phytochemicals possessing aromatic rings that bear one or more hydroxyl groups. More than 8,000 plant phenolics are naturally occurring with flavonoids in over 4,000 plants [37]. Phenolics have been established to have medicinal importance and pharmaceutical values, as they possess antioxidant, anti-cancer, anti-inflammatory, neuroprotective and cardioprotective effects [38]. In this study, the polyphenolics that were quantified include gallic acid, protocatechuic acid, isovallinic acid, vitexin, kampferol, rutin, isoorientin,

orientin, quercetin, and catechin. Liaqat *et al*.[39] also reported the presence of gallic acid and quercetin in 50% ethanolic garlic extract. These polyphenols have strong antioxidant potentials, where some acts as prooxidants in relations to their metal chelating ability [40]

Molecular docking approach can be used to model interactions between small molecules (ligands) and protein at the atomic level in order to characterize the behaviour of the ligands in the binding site of the target peptides [41]. In this study, molecular docking approach was employed to elucidate the fundamental biochemical interactions between polyphenols in hydroethanolic garlic extract with neuropeptides and transcription factors that are responsible for energy homeostasis and feeding behaviours in *Drosophila melanogaster*. The ten garlic polyphenolics docked against three (3) peptides showed good binding affinities when compared with fenfluramine (standard drug). In this study, isoorientin had the most binding affinity with the neuropeptides that are responsible for feeding behaviour; i.e. Hug, UDP2 and dNPF. This predicts that isoorientin has direct effect on the modulation of the neuropeptides that control feeding behaviour and food sensing, which affect amount of food intake and consumption. Hugin neuropeptide has been reported to modulate taste-mediated feeding behaviour and correlated with degree of food seeking response [42]. UDP is the leptin analog that suppresses stored fat breakdown during starvation, by which differing features of weight regulation is being controlled through brain circuit[43]. dNPF is the functional homolog of orexigenic neuropeptide Y, known to regulate food related appetite behaviour. Studies have reported dNPF to regulate feeding in *Drosophila melanogaster*. dNPF does not specifically influence total food intake, but may rather regulate food choice behaviour [44].

The ADMET prediction of the polyphenol (isoorientin) with strongest affinity to the studied neuropeptides revealed low intestinal absorption and impermeant through the blood brain barrier. Drugs that act in the central nervous system (CNS) need to pass over the blood brain barrier (BBB) to reach their molecular target. However, little or no BBB permeation might be required for drug molecules with a peripheral target, so as to avoid CNS side effects. The BBB permeation expresses the relative affinity of the drug for the blood or brain tissue. Isoorientin was non-substrate to p-glycoprotein and did not inhibit CYP 450. The inhibition of CYP P450 may cause pharmacokinetics related drug-drug interactions leading to toxic or other unwanted adverse effects due to the lower clearance and accumulation of the drug or its metabolites [45]. CYP P450 is important in drug elimination through metabolic biotransformation [46]. Prediction of the skin permeability of isoorientin showed that it was less permeable to the skin. The more negative the log Kp (with Kp in cm/s), the less skin permeant is the molecule [47,48].

Conclusion

This present study suggested that the hydroethanolic garlic extract has strong medicinal importance as a result of its rich polyphenolic contents. Isoorientin showed the

best binding affinity to the feeding behaviour neuropeptides. This study predicted that garlic polyphenol i.e. isoorientin may be responsible for the feeding control potential of garlic. Further *in vivo* study is recommended to confirm the outcomes of this study.

Data Availability

All data generated or analyzed during this study are included in this published article

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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