



Chemical Profiling and In Silico Docking of Phytoconstituents as Prospective Immune Modulating Agents: Investigating the Therapeutic Potential of Fermented Garlic Honey



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Abstract

Fermented Garlic Honey (FGH) is a combination of honey and garlic (*Allium sativum* L.) that undergoes extraction and fermentation with varied soaking times. The combination of these two herbs is likely to have a synergistic therapeutic effect, which can be utilized as a functional food to promote a healthy lifestyle. This method evaluates chemicals in a sample based on the length of fermentation time, allowing it to predict the compounds that contribute to its biological activity. FTIR and LC-HRMS were used to identify secondary metabolites in FGH. Principal component analysis (PCA) may easily distinguish between samples based on the extraction solvent. LC-HRMS is an advanced analytical technique that identifies 29 compounds in Raw Honey and 31, 34, and 34 compounds in the FGH at 2, 4, and 6 weeks, respectively. The bioactive substances meglutol, Gamma Glutamyl-S-Methylcysteine, Gamma Glutamyl-S-Allylcysteine, and S-Allylcysteine were found in the four samples and have been suggested to have therapeutic benefits as immunomodulating agents. Furthermore, Molegro Virtual Docker 7 has been utilized to predict the possible mode of action of the selected bioactive compounds. Among the compounds tested, Gamma-glutamyl-S-allylcysteine exhibited the most optimal interaction in three receptors (Caspase-1, iNOS, and IL-1 β). Our results suggest that the bioactive chemicals found in FGH may have potential immunomodulatory capabilities, although further verification is still required through *in vivo* or *in vitro* studies.

Keywords: Honey; Garlic; Obesity; Diabetes; Healthy lifestyle; S-Allylcysteine
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1. Introduction

For millennia, honey has been utilized for both medicinal and nutritive purposes. Honey has long been suggested as a home remedy for various medical conditions. Honey generally contains 200 different substances, including minerals, vitamins, carbohydrates, proteins, enzymes, amino acids, and polyphenols. Each honey has a unique color, flavor, viscosity, and medicinal effectiveness due to the varied ratios of these components. In this instance, the amalgamation of all these substances functions in concert across various application domains. Eighty percent of the physical characteristics and chemical makeup of most honey worldwide are similar [1]. Honey's several medicinally significant components make it a popular ingredient in manufacturing dietary supplements, medications, personal hygiene products, and other items [2]. According to Palma-Morales et al. (2023a), honey is composed of a variety of sugars (80–85%), water (15–17%), and protein (0–0.4%) [3]. It also includes enzymes, organic acids, vitamins, minerals, and phenolics. Numerous research investigations have demonstrated the multifunctional properties of honey, including its wound-healing, antioxidant, antibacterial, nematocidal, anti-inflammatory, and anticancer properties [4,5].

Plant-derived chemicals have distinct pharmacological features, including low cost, low toxicity, fewer side effects, and a lower likelihood of developing resistance. Synergism is the interaction of two or more medications that generates a decisive influence greater than either alone [6]. For many common human disorders, garlic and its secondary metabolites have shown excellent health-promoting and disease-preventing properties [7,8]. Atherosclerosis, hyperlipidemia, hypertension, diabetes, cancer, bacterial infections, dyspepsia, intestinal worms, and tuberculosis are among the conditions for which it is used therapeutically [9–11]. Certain plant parts may possess antioxidant qualities due to their inclusion of a variety of antioxidant compounds and phytochemicals. Antioxidants included in this plant include quercetin, B1-rhamnose, sativaside, voghieroside D1 compounds, phytocidin, acrolein, alliin, E-ajoene, Z-ajoene, β -resorcylic acid, pyrogallol, scordinin, gallic acid, rutin, protocatechuic acid, quercetin, and protocatechuic acid [12,13]. These compounds further justify its wide medicinal use and pharmacological importance as an anti-hypercholesterolemic, antihypertensive, antiviral, carminative, stimulant, cholagogue,

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tonic, blood purifier, febrifuge, rubefacient, antibiotic, anti-allergic, aphrodisiac, antifungal, diuretic, antiparasitic, anticoagulant, antirheumatic, and immunostimulatory effects by boosting the mitogenic activity toward thymocytes, mouse splenocytes, and human peripheral blood lymphocytes [14-16]. In present study, Fermented Garlic Honey (FGH) was produced by extracting garlic using Raw Honey (RH) as the solvent, followed by fermentation driven by the natural lactic acid bacteria present in both honey and garlic.

One prominent technique for identifying and measuring complex natural chemicals in plant samples is called untargeted metabolomics [17,18]. Due to its excellent sensitivity, selectivity, and accuracy in identifying natural metabolites, LC-HRMS is frequently employed for untargeted metabolomics [19]. However, this approach analyzes samples and compounds according to the duration of fermentation time used, offering a comprehensive picture of the chemicals that contribute to important biological activities [20].

2. Materials and Methods

2.1 Research Design

The purpose of this investigation was to establish which immunomodulatory chemicals are present in FGH. Immunomodulatory compounds were identified using Fourier transform infrared (FTIR) spectroscopy and liquid chromatography–high-resolution mass spectrometry (LC-HRMS). The LC-HRMS system consisted of a Thermo Scientific™ Vanquish™ UHPLC Binary Pump coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer. Ultimately, Molecular docking studies were performed using Molegro Virtual Docker 7.

2.2 Materials

Materials for this study included 1.5 kg of rubber nectar RH from the Honey Bee Farm (Kediri, East Java) and 0.75 kg of local garlic from Temanggung farmers (Central Java).

2.3 Preparation of Fermented Garlic Honey

Local garlic from Temanggung, Central Java, was first cleaned by removing the roots and then dried thoroughly. Rubber nectar honey was obtained from the Super Honey Bee Farm in Kediri, East Java. Dried garlic and honey are blended in a 1:2 ratio, placed in a closed jar, and fermented for 2, 4, and 6 weeks. The final product, known as FGH, is obtained by filtering the mixture.

2.4 Sample analysis using FTIR

Fourier transform infrared spectrophotometry (FTIR) was employed to identify the functional groups of the active components in RH and FGH. Each sample was analyzed using an Agilent Cary 630 Benchtop FTIR Spectrometer (Santa Clara, CA 95051 United States) over a wavenumber range of 550 to 4,000 cm^{-1} with a resolution of 4 cm^{-1} . Two replicates were performed for each sample. The functional groups detected in the FTIR spectrum of FGH will be compared to those found in the FTIR spectrum of RH.

2.5 Sample analysis using LC-HRMS

FGH samples (FGH at 2, 4, and 6 Weeks) and RH were analyzed using UHPLC coupled with a high-resolution mass spectrometer operating in positive electrospray ionization (ESI) mode. For each sample, 1 mg was dissolved in 1 mL of 70% methanol. The mobile phases used were MS-grade water containing 0.1% formic acid (A) and MS-grade methanol containing 0.1% formic acid (B), employing a gradient method at a flow rate of 0.3 mL/min. The column temperature was set to 40 °C, with a solvent flow rate of 0.3 mL/min. The sample injection volume was 3 μL , and the analysis was performed in positive ESI mode. Mass spectrometry using a spray voltage of 3.30 kV and an ion transfer capillary at 320 °C. The analytical parameters were adjusted using positive ion mode, with spectra acquired in the mass range of 66.7-1000 m/z. For translation, the read compound data was adjusted using reference compounds from MzCloud Mass (<https://www.mzcloud.org/>), ChemSpider (<https://www.chemspider.com/>), and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The LC-HRMS fingerprints, based on feature intensity as a function of m/z ratio and chromatographic time, were used as sample chemical descriptors to characterize and classify FGH using PCA.

2.6 Molecular Docking Study

Molecular docking was performed on compounds identified through LC-HRMS analysis, targeting several receptors, including IL-6 (PDB ID: 1ALU), Caspase-1 (PDB ID: 1RWK), iNOS (PDB ID: 3EBD), and IL-1 β (PDB ID: 6Y8M). The 3D ligand structures were obtained from PubChem and prepared by adding partial charges, while receptor structures were retrieved from the PDB repository and further refined by incorporating missing residues and partial charges. Both the docking simulations and preparation steps were conducted using Molegro Virtual Docker 7. The validation process involved assessing the RMSD value to ensure it remained below 2 Å. Docking evaluation was carried out using the MolDock Score, and interaction analysis was further examined using the Ligand Map feature in Molegro Virtual Docker 7.

3. Result and Discussion

Targeted and non-targeted approaches are the two primary analytical techniques used for authenticating medications and functional foods as well as for fraud prevention. Targeted techniques, on the one hand, concentrate on identifying recognized and particular chemicals (or groups of compounds) that are utilized as primary or secondary markers to address authentication issues. Conversely, untargeted strategies (based on fingerprinting approaches, or metabolomics) have become viable and

effective approaches to authenticity issues. These strategies aim to identify as many instrumental responses as possible without requiring knowledge of the sample components generating these signals [21].

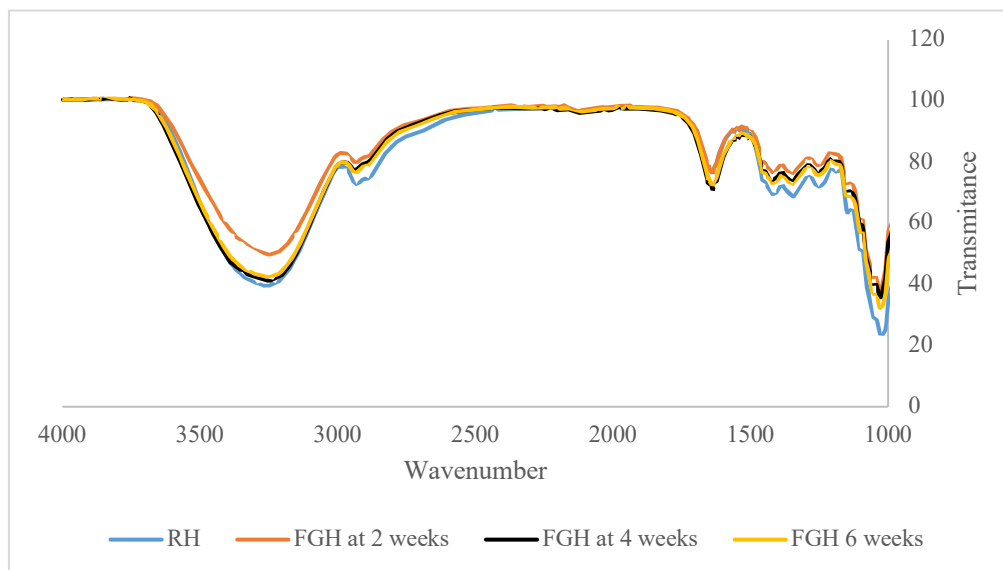


Fig. 1. FTIR Spectra of Raw Honey and Fermented Garlic Honey at 2, 4, and 6 Weeks

The FTIR spectra of RH and FGH at 2, 4, and 6 Weeks profiles show similar wavelengths and peak shapes, namely at wave numbers 771.55893-864.74238 can be interpreted as the anomeric region of carbohydrates or δ (C-H) (especially in the sugar structure); 896.42475-918.78878 is interpreted as ν (C-C) in the carbohydrate structure, δ (C-H); 1025.01791-1028.74524 is interpreted as ν st (C-O) in the C-OH group or ν st (C-C). 1339.97795-1341.84162 is defined as δ (-OH) in C-OH group; 1420.11572 is defined as δ st (O-H) in C-OH + δ (C-H) in alkene group; 1638.16498 is defined as δ st (O-H) in H₂O; 2931.55121-2935.27855 is defined as ν s and as (C-H) in CH₂ and CH₃ groups; 3244.64759-3268.87528 is defined as ν st (O-H) in H₂O. Where ν —stretching vibrations, δ —deformation vibrations, s—symmetric, as—asymmetric, st—strong.

Table 1. FTIR peak values and functional groups of Raw Honey and Fermented Garlic Honey at 2, 4, and 6 Weeks

Wavenumber (cm ⁻¹)	Types and Shapes
800	Anomeric region of carbohydrate or vibrational deformation(δ); (C-H) (particularly in the carbohydrate structure)
900	Vibrational stretching (ν); (C-C) in the carbohydrate structure, vibrational deformation (δ); (C-H)
1000	Strong vibrational stretching (ν st); (C-O) from C-OH bond or strong vibrational stretching (ν st); (C-C) in the carbohydrate structure, vibrational deformation (δ) (C-H)
1150	Vibrational stretching (ν); (C-H) in the carbohydrate structure
1250	Vibrational stretching (ν) (C-H) in the carbohydrate structure or vibrational stretching (ν) (C-O) in the carbohydrate structure
1300	Vibrational deformation (δ); (-OH) from C-OH bond
1400	Strong vibrational deformation (δ st) (O-H) from C-OH+ vibrational deformation (δ); (C-H) from alkene
1600	Strong vibrational deformation (δ st); (O-H) from H ₂ O
2900	Symmetric vibrational stretching (ν s) and asymmetric (ν as); (C-H) from CH ₂ and CH ₃
3260	Strong vibrational stretching (ν st); (O-H) from H ₂ O

Additional testing was conducted using LC-HRMS. Samples of RH and FGH at 2, 4, and 6 Weeks were examined using the LC-HRMS method to discover non-targeted chemicals and forecast their biological activity as immunomodulators. Based on the substances discovered in the RH, these compounds were identified, and the results were obtained through a literature data search for immunomodulator content.

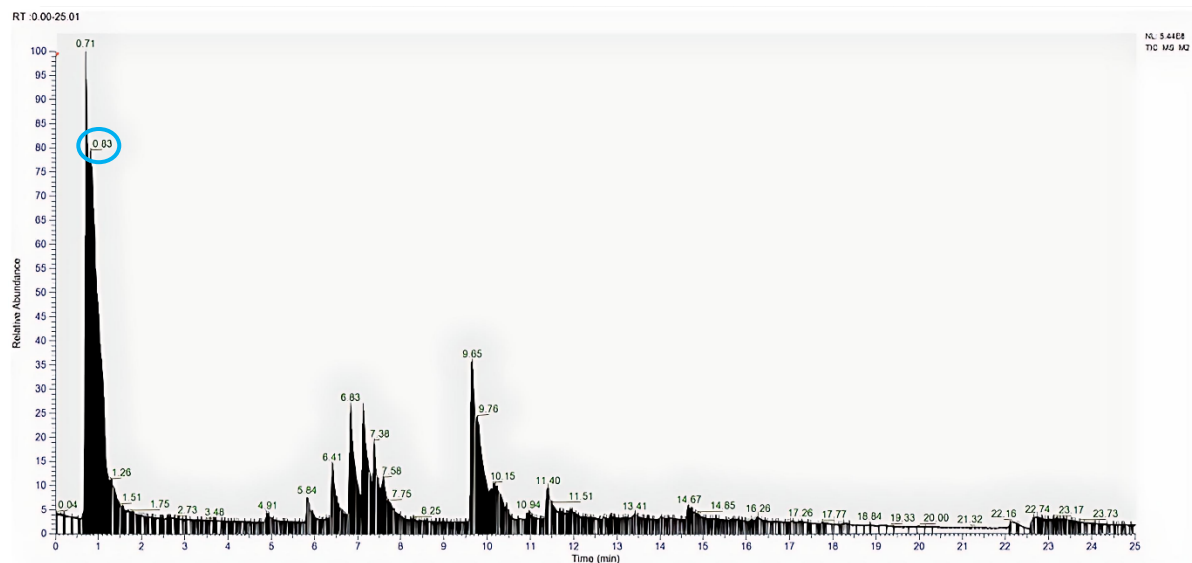


Fig. 2. LC-HRMS chromatogram of Raw Honey (RH) (Red circle: GSMC; Blue Circle: Meglutol; Yellow Circle: GSAC; Purple Circle: SAC)

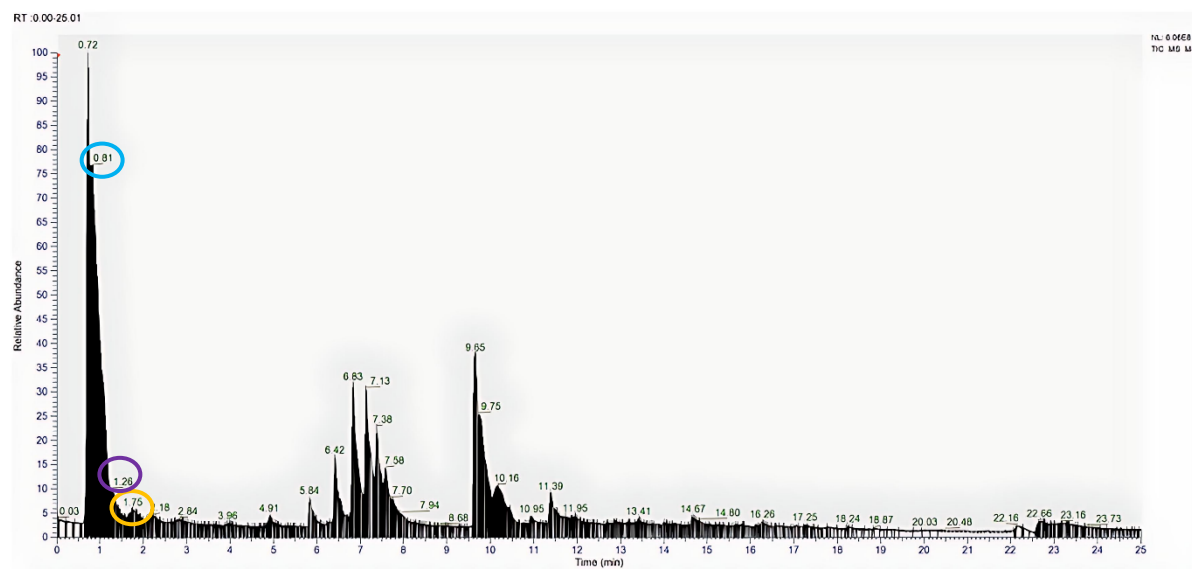


Fig. 3. LC-HRMS chromatogram of Fermented Garlic Honey (FGH) at 2 Weeks (Red circle: GSMC; Blue Circle: Meglutol; Yellow Circle: GSAC; Purple Circle: SAC)

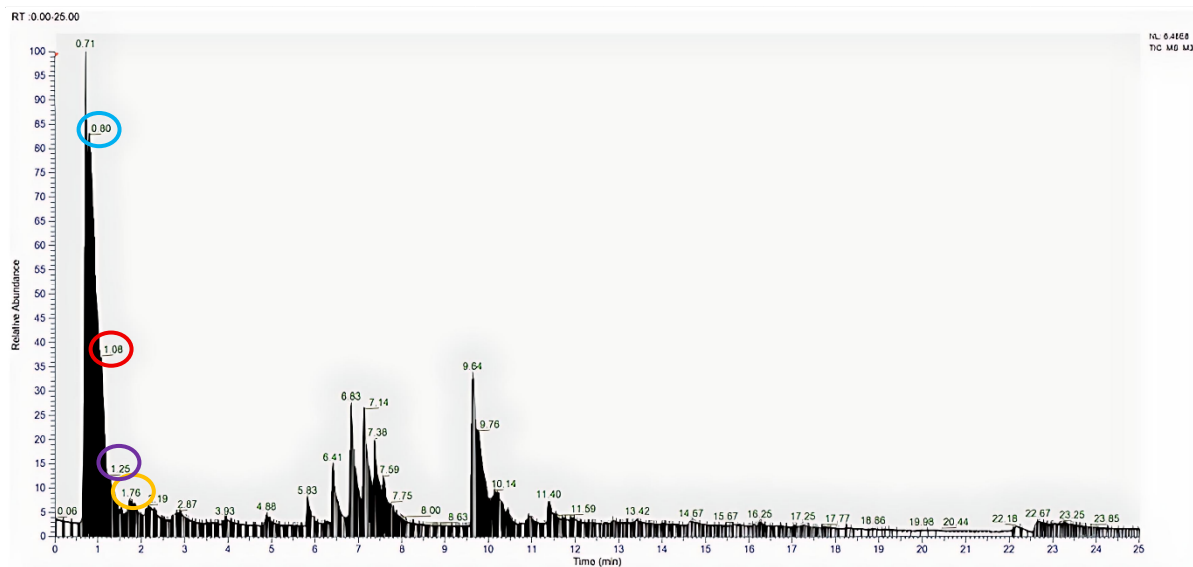


Fig. 4. LC-HRMS chromatogram of Fermented Garlic Honey (FGH) at 4 Weeks (Red circle: GSMC; Blue Circle: Meglutol; Yellow Circle: GSAC; Purple Circle: SAC)

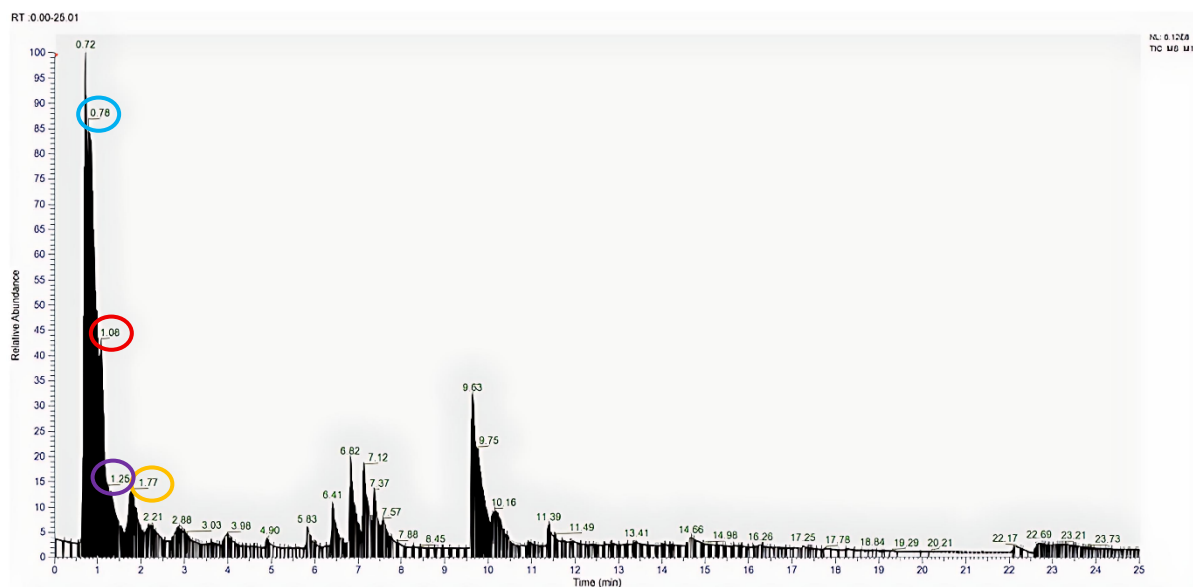


Fig. 5. LC-HRMS chromatogram of Fermented Garlic Honey (FGH) at 6 Weeks (Red circle: GSMC; Blue Circle: Meglutol; Yellow Circle: GSAC; Purple Circle: SAC)

Table 2. Chemical Structure and MS-MS spectra

No	Structure	MS Spectra-1	MS Spectra-2
Meglutol			
1			
Gamma-Glutamyl-S-Methylcysteine (GSMC)			
2			
Gamma-Glutamyl-S-Allylcysteine (GSAC)			
3			
S-Allyl-L-cysteine (SAC)			
4			

The LC-HRMS investigation revealed a number of compounds that were lacking in the RH but identical in FGH samples. Specific to FGH at 2, 4, and 6 Weeks, a number of chemicals were detected, including Gamma-Glutamyl-S-Allylcysteine, Gamma-Glutamyl-3-(Allyldisulfanyl) Alanine, Gamma-Glutamyl-3-[(1E)-1-Propen-1-yl sulfinyl] Alanine, And Gamma-Glutamyl-S-Methylcysteine. RH and FGH contain the following chemicals, which have been found to have immunomodulatory activity:

- (1) Meglutol (3-hydroxy-3-methylglutaric acid), a hypolipidemic compound found naturally in a wide range of foods, is found in all four types of honey. In the cholesterol biosynthesis pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is an enzyme that meglutol competitively inhibits to prevent the synthesis of cholesterol. During the duration of an eight-week therapy period, meglutol administration was found to effectively reduce plasma LDL cholesterol concentrations in individuals with familial hypercholesterolemia (type IIa or hyper-beta-lipoproteinemia) [22]. Many foods with high meglutol concentrations have been identified as dietary supplements and may help regulate LDL-C levels and improve cardiovascular health.
- (2) Gamma-Glutamyl-S-allylcysteine (GSAC) constitute an array of dipeptide intermediates corresponding to the synthesis of S-alk(en)yl-L-cysteine sulfoxides (ACSOs), among which γ -glutamyl-S-allyl-cysteine (GSAC) is prevalent with good water solubility. GSAC is a peptide that is easily soluble in water and lacks the pungent flavour of garlic, giving it an advantage over the volatile organosulphur compounds found in garlic [23]. This is consistent with this study, which detected GSAC in the LC-HRMS analysis of FGH, considering honey contains water about 18–24%, which can dissolve a number of GSAC from garlic [24]. The key sulfur-containing natural constituent in intact garlic is γ -Glutamyl-S-allyl-L-cysteine which on fermentation in 15–20% ethanol mixture at room temperature for up to twenty months, is hydrolyzed and deglutamized to water soluble S-allyl-L-cysteine, S-allylmercap to-L-cysteine, and S-methyl-L-cysteine by the enzyme γ -glutamyl transpeptidase (also known as γ -glutamyl transferase). Although few research have studied the antiglycative effect of garlic extract, to our knowledge, investigations addressing the antiglycative role of GSAC are rare. In the present work, the antiglycative effect of GSAC was examined in the bovine serum albumin (BSA)/glucose system. The antiglycative efficacy of GSAC was established using protein glycation product and glycated-BSA chemical structure assays. GSAC awards may raise focus in the health-care industry, particularly in study and treatment of diabetes and other AGE-related disorders. Our findings indicate that GSAC may inhibit proinflammatory cytokines that contribute to inflammation-related diseases in the body, in addition to its immunoboosting properties [25].

Table 3. Names, Retention time (Rt), and Molecular Weight (MW) values for the compounds identified from RH and FGH

	Formula	MW	RT	Raw Honey	Fermented Honey		Garlic
					2	4	6
Meglutol	C6 H10 O5	162.05241	0.773	✓	✓	✓	✓
Bis(4-ethylbenzylidene)sorbitol	C24 H30 O6	414.20389	11.397	✓	✓	✓	✓
2-Amino-1,3,4-octadecanetriol	C18 H39 N O3	317.29201	9.769	✓	✓	✓	✓
6-(alpha-D-glucosaminy)-1D-myo-inositol	C12 H23 N O10	341.13115	0.75	✓		✓	
Oleamide	C18 H35 N O	281.27144	14.67	✓	✓	✓	✓
(2S)-3-Phenyl-2-([[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl]amino)propanoic acid	C15 H21 N O7	327.13153	1.303	✓	✓	✓	✓
Lactide	C6 H8 O4	144.04205	0.928	✓	✓	✓	✓
Phenyl D-glucopyranosiduronic acid	C12 H14 O7	270.07373	0.775	✓	✓	✓	✓
Melezitose	C18 H32 O16	504.16886	0.834	✓			
1-([[(3S,4S,5R)-2,3,4-Trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl]-2-pyrrolidinecarboxylic acid	C11 H19 N O7	277.11597	0.786	✓	✓	✓	✓
A-Lactose	C12 H22 O11	342.11536	0.785	✓			
1,5-Anhydro-1-(2,4,6-trihydroxyphenyl)hexitol	C12 H16 O8	288.08406	0.774	✓	✓	✓	
N-acetyl-L-2-amino adipic acid	C8 H13 N O5	203.07887	1.03	✓	✓	✓	✓
L-phenylalanine	C9 H11 N O2	165.07897	1.319	✓	✓	✓	✓
Choline alfoscerate	C8 H20 N O6 P	257.10268	0.772	✓	✓		
Glutaryl carnitine	C12 H21 N O6	275.13689	1.062	✓	✓		
Bis(methylbenzylidene)sorbitol	C22 H26 O6	386.17261	10.451	✓	✓	✓	✓
C14-dihydroceramide	C32 H65 N O3	511.49663	16.258	✓	✓	✓	✓
Hexadecanamide	C16 H33 N O	255.2563	14.264	✓			
3-(6-Hydroxy-7-methoxy-1,3-benzodioxol-5-yl)propanoic acid	C11 H12 O6	240.06299	0.776	✓	✓	✓	✓
Bis-beta-D-fructofuranose 1,2':2,3'-dianhydride	C12 H20 O10	324.10478	1.108	✓			
N,N-Bis(2-hydroxyethyl)dodecanamide	C16 H33 N O3	287.24581	9.958	✓	✓	✓	✓
C16-dihydroceramide	C34 H69 N O3	539.52806	17.049	✓	✓	✓	
Linoleamide	C18 H33 N O	279.2563	14.023	✓			✓
Lauramide	C12 H25 N O	199.19362	11.715	✓	✓	✓	✓
1-(beta-D-ribofuranosyl)thymine	C10 H14 N2 O6	258.08519	0.805	✓			
N-(3-Carboxypropanoyl)-5-hydroxynorvaline	C9 H15 N O6	233.08962	0.755	✓		✓	✓
Fructoselysine	C12 H24 N2 O7	308.15764	0.704	✓	✓	✓	✓
Phenylglyoxylic acid	C8 H6 O3	150.03153	0.69	✓			
Fmet	C6 H11 N O3 S	177.04578	0.804		✓	✓	✓
L-(+)-arginine	C6 H14 N4 O2	174.11136	0.847		✓		
Methyl alpha-aspartylphenylalaninate	C14 H18 N2 O5	294.12102	2.859		✓	✓	✓
(2S)-4-Methyl-2-([[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl]amino)pentanoic acid	C12 H23 N O7	293.14712	1.063		✓	✓	✓
Gamma-Glutamyl-3-(allyldisulfanyl)alanine	C11H18 N2 O5 S2	322.06508	3.965		✓	✓	✓
Linamarin	C10 H17 N O6	247.10507	0.757		✓	✓	✓

Gamma-Glutamyl-S-methylcysteine	C9 H16 N2 O5 S	264.07781	1.046	√	√	√
1-[(3-Carboxypropyl)amino]-1-deoxy-beta-D-fructofuranose	C10 H19 N O7	265.11571	0.877	√		
Gamma-Glutamyl-3-[(1E)-1-propen-1-ylsulfinyl]alanine	C11 H18 N2 O6 S	306.08748	0.975	√	√	
(2S)-3-(1H-Imidazol-4-yl)-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl}amino)propanoic acid	C12 H19 N3 O7	317.12126	0.736	√	√	√
N2-Succinyl-L-glutamic acid 5-semialdehyde	C9 H13 N O6	231.07402	0.803	√	√	√
Gamma-Glutamyl-S-allylcysteine	C11 H18 N2 O5 S	290.09325	2.189		√	√
L-Glutamic acid	C5 H9 N O4	147.05284	0.754		√	√
S-Allyl-L-cysteine	C6 H11 N O2 S	161.05097	1.129		√	√
(2S)-3-Methyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl}amino)butanoic acid	C11 H21 N O7	279.13167	0.894		√	
.Alpha.-aminoadipic acid	C6 H11 N O4	161.06851	0.706		√	√
Cetoniacytone A	C9 H11 N O5	213.06337	0.856			√
Lotaustralin	C11 H19 N O6	261.12102	0.798			√
(2S)-3-(4-Hydroxyphenyl)-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl}amino)propanoic acid	C15 H21 N O8	343.12568	1.01			√
Met-glu	C10 H18 N2 O5 S	278.09374	1.251			√

- (3) Gamma-Glutamyl-S-methylcysteine (GSMC) is a Garlic's cholesterol-lowering impact may be attributed in part to a decrease in hepatic cholesterol production [26]. This compound ability to decrease triacylglycerol may be attributed in part to its inhibitory effect on fatty acid production. Among the water-soluble compounds, S-alk(en)ylcysteines (including SAC) inhibited cholesterol production rates in a dose-dependent manner, with a maximum inhibition of 40-60% achieved at 2.0-4.0 mmol/L. Glutamate derivatives of S-alk-(en)ylcysteines (such as GSAC and GSMC) reduced production by 20-35% [27].
- (4) S-Allyl-L-cysteine (SAC) is the most abundant sulfur-containing amino acid found in *Allium* plants, such as garlic. It is produced by the enzyme γ -glutamyl transpeptidase, which hydrolyzes and deglutamylates GSAC, a phytochemical found in intact garlic. SAC has been found to be the primary active ingredient and most common organosulfur compound in fermented garlic, including black garlic [28]. According to reports, SAC can scavenge reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), peroxynitrite anion ($ONOO^-$), and hydroxyl radicals ($\cdot OH$). SAC's anti-oxidative abilities have been related to its ability to scavenge ROS/RNS due to the allyl group in its structure, therefore protecting cells against lipid peroxidation, protein oxidation and nitration, as well as mitochondrial damage. Chelate metal ions, like Fe^{2+} and Cu^{2+} , promote antioxidant defense, inhibit pro-oxidant enzymes like NADPH oxidase and nitric oxide synthase, inhibit the NF- κ B inflammatory pathway, and boost NO levels in endothelial cells, leading to anti-inflammatory responses [29,30]. The thiol group found in SAC is the nucleophile that gives it its antioxidant qualities. It quickly targets and neutralizes electrophilic sites to make them less reactive. It is a stable, water-soluble antioxidant that is less toxic than other garlic constituents and readily absorbed by the brain and other tissues [31,32]. SAC possesses biological and pharmacological activities that include cancer prevention, neuroprotection, hepatoprotection, antidiabetic, cardioprotection, anti-asthmatic, and nephroprotection [33]. The neuroprotective effect of SAC administration was due to its ability to reduce the increase of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA, TNF- α , and COX-2 protein expression as an indicator of inflammation [34-36]. Additionally, SAC suppresses the activation of NF- κ B in human T cells caused by TNF- α and hydrogen peroxide, indicating a strong anti-oxidant and anti-inflammation function of SAC. SAC's inhibition of NF- κ B, partly by preventing oxidative modification of LDL, further supports the role of advanced glycation end product in preventing atherogenesis and reducing the risk of heart disease and stroke [37-39].

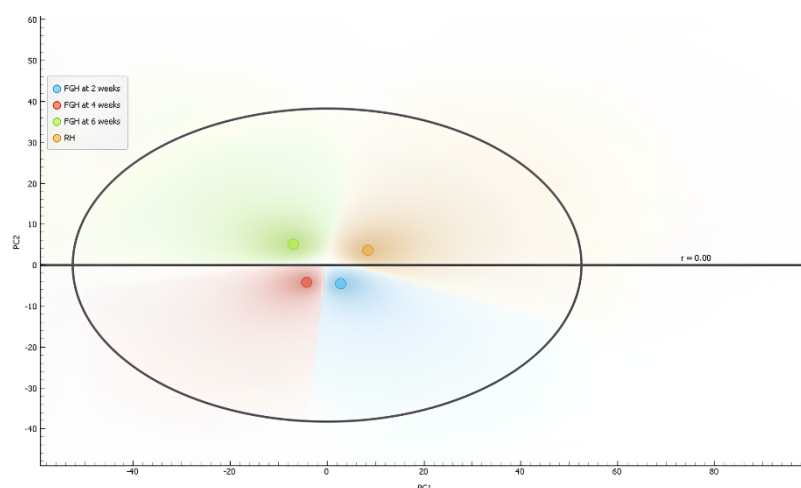


Figure 6. PCA of LC-HRMS data of RH and FGH

Further testing was carried out using LC-HRMS. Table 2 summarizes the report from the LC-HRMS analysis. The sample spectra clearly distinguish between the chemical compositions of RH and FGH. The primary peak appears to increase between RT 1 and 3 (Fig. 1). The trial lasted 20 minutes, however there was no noticeable peak at 15 minutes. PCA was used to evaluate the potential of LC-HRMS fingerprints as discriminative chemical markers for the classification of honey samples.

All honey samples, both with and without garlic fermentation, displayed comparable FTIR spectra and LC-HRMS chromatogram profiles. PCA was used to group the honey samples according to the fermentation period in order to assess the potential of LC-HRMS fingerprints as discriminatory chemical markers for honey sample categorization. PCA is an unsupervised multivariate technique that shows how samples are grouped according to how closely their metabolite compositions match. Furthermore, PCA can extract information and reduce data to identify variable combinations that best characterize the data set. The PCA score plot of PC1 against PC2 using LC-HRMS metabolic fingerprints is displayed in Figure 6. High similarity in metabolite profiles is shown by adjacent clusters. The bioactivity of the chemicals found in each honey sample's LC-HRMS was evaluated in silico.

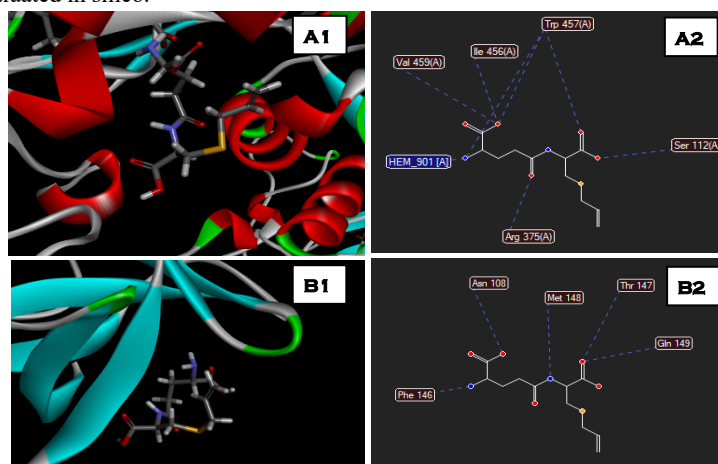
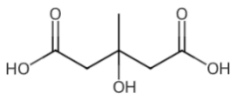
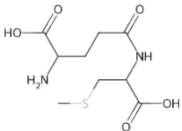
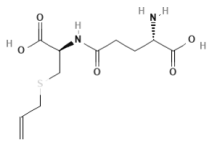
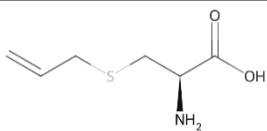


Figure 7. 3D (1) and 2D (2) visualization of binding interactions by using Molegro Visualizer 2021 software; (A) protein-ligand interactions between iNOS and Gamma-Glutamyl-S-Allylcysteine (-98.0139 kcal/mol) 3D; (B) protein-ligand interactions between IL-1 β and Gamma-Glutamyl-S-Allylcysteine (-78.7374 kcal/mol)

Four potential proteins, IL-6, CASPASE-1, iNOS, and IL-1 β , were chosen for molecular docking because they are commonly employed as therapeutic targets to modify immune responses in the treatment of inflammation-related disorders [40,41]. As shown in Table 4, the docking score (kcal/mol) of the optimal binding pose was calculated for each ligand against the chosen proteins. The docking scores for meglutol, GSMC, GSAC, and SAC ranged from -106.685 to -39.4957 kcal/mol. Notably, GSAC achieved the most favorable binding with iNOS (-98.0139 kcal/mol), while other interactions (e.g., GSMC with IL-1 β) also showed strong binding (-79.3964 kcal/mol).

Table 4. Molecular docking score (kcal/mol) and interaction residues of the most bioactive compounds with 4 probable target proteins based on the best protein-ligand binding position

Native Ligands / Compounds	Chemical Structure	Putative Targets	PDB ID	Docking Score Value	Interacting Residues
L-Tartaric Acid		IL-6	1ALU	-38.9726	Arg179, Arg182
3-(2-Mercapto-acetyl-amino)-4-oxo-pentanoic acid		CASPASE-1	1RWK	-101.908	Arg179A, Gly238A, Gln283A, Ser339B, Arg341B
(2S)-2-methyl-2,3-dihydrothieno[2,3-f][1,4]oxazepin-5-amine		INOS	3EBD	-64.5536	Ser112, Arg375, Trp457
4-[(5-bromopyridin-2-yl)amino]-4-oxobutanoic acid		IL-1 β	6Y8M	-66.4585	Thr147, Met148, Gln149
Meglutol		IL-6	1ALU	-39.4957	Gln175, Arg182
		CASPASE-1	1RWK	-77.9367	Arg179A, Ser236A, Ser339B, Arg341B, Ser347B
		INOS	3EBD	-60.4538	Arg375, Ile456, Trp457
		IL-1 β	6Y8M	-62.4714	Asn108, Phe146, Met148
Gamma Glutamyl-S-Methylcysteine		IL-6	1ALU	-35.6509	Arg179, Arg182
		CASPASE-1	1RWK	-106.685	Arg179A, Ser236A, Gly238A, Cys244A, Gln283A, Arg341B
		INOS	3EBD	-87.1683	Arg375, Ile456, Val459
		IL-1 β	6Y8M	-79.3964	Asn108, Phe146, Thr147, Gln149
Gamma-Glutamyl-S-Allylcysteine		IL-6	1ALU	-27.8682	Gln175, Arg179
		CASPASE-1	1RWK	-100.714	Arg179A, Ser236A, His237A, Gly238A, Cys285A, Ser339B
		INOS	3EBD	-98.0139	Ser112, Arg375, Trp457, Val459
		IL-1 β	6Y8M	-78.7374	Asn108, Phe146, Thr147, Met148, Gln149
S-Allyl-L-Cysteine		IL-6	1ALU	-37.9548	Gln175
		CASPASE-1	1RWK	-82.3709	Arg179A, Ser339B, Arg341B
		INOS	3EBD	-68.9493	Trp457
		IL-1 β	6Y8M	-54.3447	Asn108, Phe146

According to the literature, Caspase-1 is an important regulator of innate immunity, playing a critical role in the activation of the inflammasome (NLRP3) and the production of pro-inflammatory cytokines. Caspase-1 expression is elevated in immunological organs such as the spleen, lymph nodes, and thymus as a result of the inflammatory-mediated immune response that occurs following infection or tissue damage [42]. The discovery of innovative non-peptide small-molecule caspase-1 inhibitors is a key technique for combating excessive caspase-1 activation induced by inflammatory conditions [43]. Increased caspase-1 activation promotes the development of IL-18 and IL-1 β , two cytokines that start the inflammatory process. Numerous inflammatory conditions, including obesity, diabetes, dyslipidemia, hypertension, traumatic brain injury, and cerebrovascular illness, are exacerbated by abnormal expression of NLRP3. Endoplasmic reticulum stress and oxidative stress induced by the overexpression of IL-1 β can lead to diabetes, pancreatic cell death, altered T cell activation, and disruption of islet β -cell function [44-46]. Inflammatory cytokine production is linked to NO synthase (iNOS) [47]. The primary bioactive components of FGH are Meglutol, GSMC, and GSAC. Targeting cytokine expression with natural chemicals to alter the overall immune response may be a promising therapeutic approach. Based on the findings described above, the compounds meglutol, GSAC, GSMC, and SAC identified in FGH are expected to alter

immune response via many signaling pathways. In particular, in inflammatory illnesses, their activities on immune system components are associated with a pro-inflammatory state characterized by the release of cytokines [48]. Cytokines regulate the immune response to infection or inflammation, while also controlling inflammation through a complex network of interactions. This understanding serves as the foundation for the use of herbal medicines such as FGH in the treatment of immune-related diseases [49, 50]. Our results suggest that FGH is expected to alter immune responses in the treatment of inflammation-related disorders; however, further in vitro or in vivo research is needed to support this finding.

4. Conclusion

Using FTIR and LC-HRMS techniques, we obtained detailed qualitative and semi-quantitative profiles of raw honey (RH) and fermented garlic honey (FGH). The results indicate that FGH, a combination of honey and garlic, has potential as an immunomodulatory agent, as evidenced by the presence of compounds such as meglutol, GSMC, GSAC, and SAC; however, the remaining three components were only identified in fermented garlic honey. Docking simulations indicate favorable interactions between these bioactive molecules and the target receptors IL-6, CASPASE-1, iNOS, and IL-1 β . In conclusion, FGH is predicted to have the ability to modulate immunological responses in the treatment of inflammation-related diseases; however, more in vitro or in vivo studies are required to confirm this.

Conflicts of interest

There are no conflicts to declare

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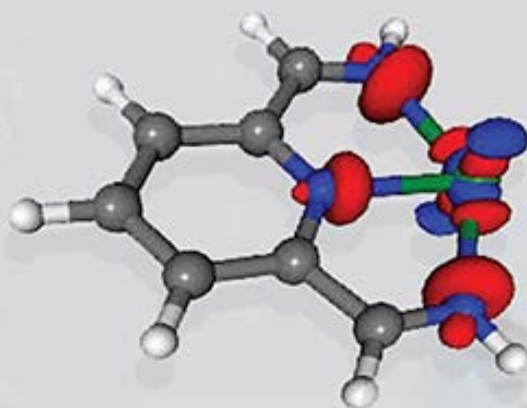
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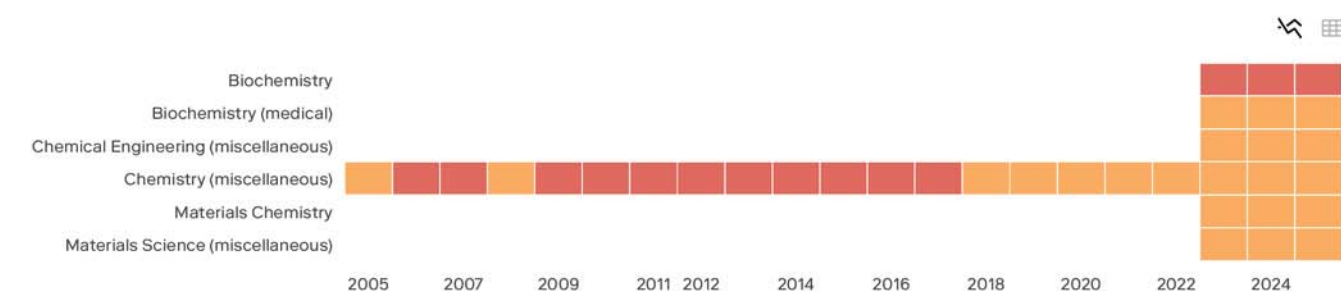
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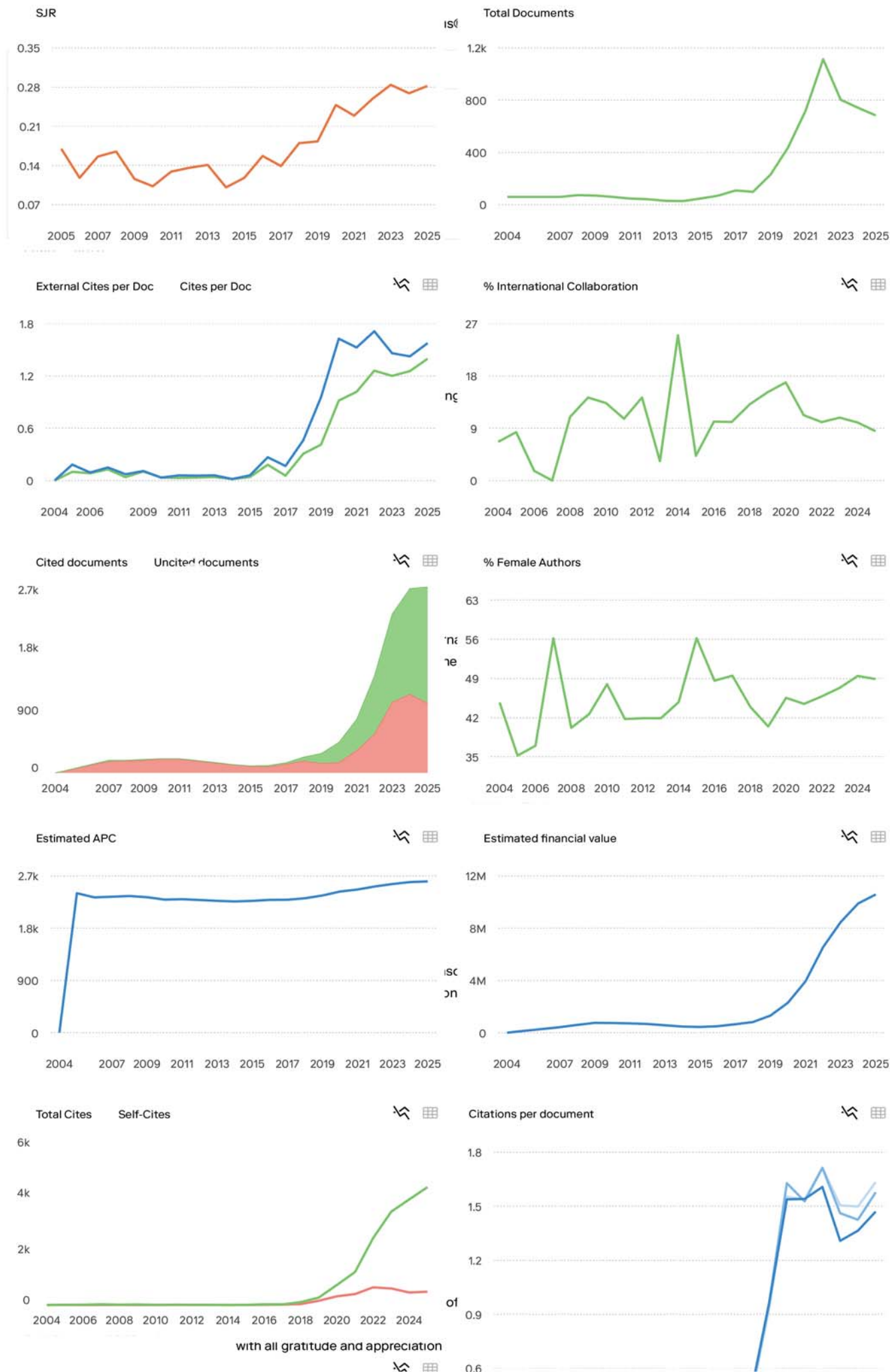
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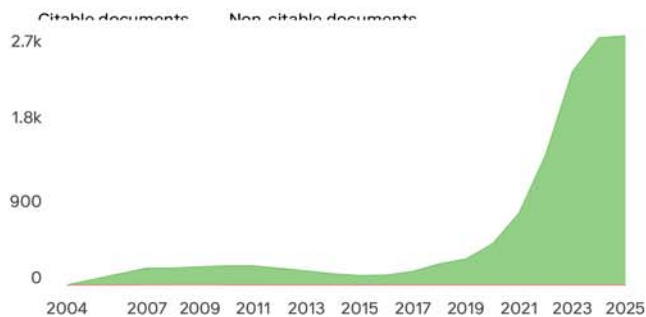
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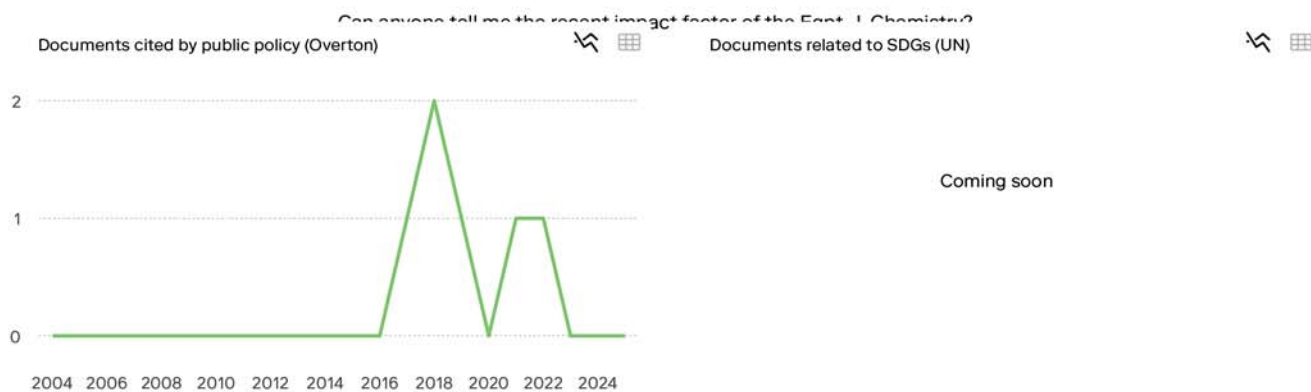
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