Chapter 5

Discussion

5.1. Biochemical composition of mustard (*Brassica nigra)* seeds and garlic (*Allium sativum*)

During the biochemical analysis of the mustard seeds the moisture content, crude fat, crude protein, crude fibre, total ash, and total carbohydrate were found to be 6.38 ± 0.84 g/100g, 42.39 ± 3.55 g/100g, 21.18 ± 1.35 g/100g, 23.20 ± 1.75 g/100g, 4.24 ± 0.232 g/100g and 25.81 ± 1.25 g/100g, respectively. Again, for the garlic cloves, it was 59.75 ± 1.35 g/100g, 0.27 ± 0.05 g/100g, 6.46 ± 0.31 g/100g, 4.09 ± 0.21 g/100g, 1.22 ± 0.05 g/100g and 32.30 ± 1.25 g/100g, respectively. The moisture content of garlic was earlier reported to be a crucial parameter in determining the period of storage of garlic [1]. For fresh garlic, the percentage of moisture was reported to be between 50-70% which was found to be in the range of our findings [1, 2]. The moisture content for *Brassica juncea* and *Brassica nigra* was reported to be 12.17% and 4.16% which was found to coincide with the moisture content of mustard seeds we have collected [3, 4]. Ash content in *Brassica nigra*, *Brassica juncea* seeds were earlier reported to be in the range of 4-5% [5, 6]. Also, in garlic (*A. sativum*) cloves the ash content was reported to be in the range of 1-2 % [2, 7]. In our finding, the ash content in both the garlic cloves and mustard seeds was found to be in the range of that of the earlier reported findings. Similarly, the total carbohydrate, crude fibre, crude fat and crude protein in the garlic and mustard seeds were found to be in range with that of the earlier reported results [2, 6].

5.2. Physicochemical examination and phytochemical analysis of oil from GMM

5.2.1. Chemical properties

The FFA, PV, AV, IV and SV of GMM were found to be within the limits that were set for edible vegetable oil. The increase in free fatty acids signifies the degradation of triglycerides to free fatty acids [8]. It is reported that factors such as temperature, humidity and light exposure can increase the rate of the degradation process [9]. From our observed results, the FFA levels of both MO and GMM were not found to be significantly different therefore the preparation conditions such as temperature and duration of heating did not affect the integrity of triglycerides at a significant level. PV is a major quality parameter for edible oils, where PV > 10 meq O_2/kg is considered unstable and can easily become rancid, whereas PV < 10 meq O_2/kg indicates the oil is stable against oxidative stress [10]. During our experiment heated mustard oil showed a PV

of more than 10 meq O_2/kg, which suggests a decrease in stability against oxidative stress of mustard oil after heating, whereas non-heated mustard exhibited a PV below 10 meq O_2/kg. The GMM oil showed the lowest PV of 5.29 ± 0.40 meq O_2/kg, which suggests the quality of the oil against oxidative stress has increased which may be due to the presence of garlic-derived compounds, therefore even after heating at the same temperature as that of mustard oil, the PV is significantly lower than that of the mustard oil. Similarly, the AV, IV and SV of GMM and MO which were not found to be significantly different from each other were found to be in the range with that of earlier reports for mustard oil [11, 12]. Even the range of chemical properties of the present finding is found to be in the range of standard values set by FSSAI and USDA [13, 14].

5.2.2. Physical properties

The density of cooking oil ranges from 0.918 to 0.926 g/cm^3 at temperatures between 15 ºC to 25 ºC [15]. With the increase in temperature the density of the oil gradually decreases and maybe due to this, the density of heated mustard oil has dropped to 0.855 g/cm^3. For GMM oil, the density was found to be 0.88 g/cm^3 which was higher than the heated mustard oil and may be due to the presence of garlic compounds in MO. The refractive index of the HMO and GMM were found to be 1.474 and 1.478, respectively, which is slightly higher than the earlier reported value of 1.45-1.46 at 40ºC [16]. The viscosity of heated MO and GMM were found to be 116.23 ± 0.54 and 116.58 ± 0.22 milipoise. It was earlier reported that the viscosity of edible oil after heating decreases, but in the case of mustard oil significant reduction in its viscosity was not observed by Zahir et al. (2017) [17]. The value of viscosity of the HMO and GMM oil was found to be close to the range of that reported by Zahir et al. (2017).

5.2.3. Phytochemical analysis

FTIR analysis is an effective method by which the types and abundance of the functional groups in the given molecule may be determined [18]. As GMM consists of a complex mixture of compounds from both MO and garlic therefore functional group analysis was necessary. Chemical functional groups present in the compounds also give the molecules their characteristic chemical property

along with a potent bioactive nature [19]. During our analysis, freshly prepared GMM displayed minimum transmittance at 722, 1100, 1120, 1164, 1239, 1378, 1418, 1465, 1747, 2854, 2925 and 3008 cm^{-1}. The lowest transmittance at 1378 cm^{-1} for freshly prepared GMM, which is a characteristic peak for C=S stretching (sulfur compounds) [20]. The low transmittance value of fresh GMM may support the fact that a significantly high number of garlic-derived compounds may be present in the macerated oil compared to that of the control oils [21]. When the macerate was stored for more than 10 days, the transmittance percentage at 1378 cm^{-1} (C=S stretching) was found to be significantly reduced by 22.34% compared to the freshly prepared macerate. This phenomenon may have been due to the degradation of the compounds in the GMM due to the long-term storage [22]. As it was earlier reported that garlic derived compounds are highly unstable and degrade rapidly over long-term storage [23]. From the results, it was observed that the wavenumbers and intensity of the peaks in MO, HMO and 10 days stored GMM were very similar and no significant difference was observed. As storage at lower temperatures increases the half-life of the compounds, therefore if the GMM is stored at room temperature then the thiosulfate compounds might start to degrade at a higher rate than compared to -20^{o}C storage which has to be confirmed in future work. During total polyphenol estimation, we observed a significantly higher amount of polyphenol in GMM than compared to MO. This may be due to the presence of the non-polar polyphenols from garlic which became soluble in the MO during preparation of GMM. During the DPPH scavenging activity of GMM, it was observed that the lipophilic part of the oil has higher DPPH scavenging activity than that of the hydrophilic part and a similar observation was reported in earlier works [24-26]. It was earlier reported that compounds such as allicin when present in non-polar solvents and vegetable oil convert to non-polar thiosulfinate compounds such as ajoene and dithiins [27]. These degraded compounds are highly unstable and further degrade to several small organosulfur compounds [28]. The higher antioxidant nature of the sulfur compounds from garlic against hydroxide radical, hydroperoxyl radical, and superoxide radical was earlier reported which may support the fact of a higher DPPH scavenging activity of the lipophilic part of GMM [29]. From HPLC

analysis of GMM, the two major peaks at 3.48 min and 4.08 min coincide with the peaks of that of GTE. However, the area under the peak was found to be higher in the case of GTE than GMM, which may be due to the difference in the solvents used during the preparation of the macerate.

LCMS analysis of GTE consists of two major peaks at retention times of 0.826 and 0.98 minutes. The mass spectrum of the peak at retention time 0.826 min exhibits major ion peaks at 325 (100%) dimeric species of allicin [$(All_2S_2O_3)_2 + H^+$] and further degradation of the dimeric species forms ajoene [m/z = 235 (30.77%)] [30]. The mass spectrum of the peak at 0.98 min has characteristic peaks at m/z 145 (100%) and 237 (32.54%) which confirms the presence of acrolein dimers (2-vinyl-4H-1,3, dithiin or 3-vinyl-4H-1,3-dithiin, m/z=145) and 2-Propenyl 1-(2-propenylsulfinyl) propyl disulfide (molecular weight = 236.4 g/mol, m/z [M+ H^+]= 237), respectively [31, 32]. At a retention time of 1.26 min, MS data showed a signature peak of vinyl dithiin (m/z 145.00) and an unknown fraction with m/z value of 217.00. The MS data of the peaks at retention times 1.93 min and 2.35 min, revealed a major peak at m/z value of 217.00 and 274.00, respectively which did not match with any known compounds in garlic according to our literature survey. For GMM, major peaks were found at retention times (RT) of 0.80, 0.85 and 0.98 min. The mass spectrum at RT 0.80 showed peaks at m/z value of 169.10 (100.00%) (gallic acid) and 235 (62.40%) (protonated ajoene). The peak at RT 0.85 had m/z values of 235.00 (100.00%) (protonated ajoene), 163.00 (90.50%) (allicin), 169.10 (90.09%) (gallic acid) and 145 (22.89%) (2-vinyl-4H-1,3, dithiin or 3-vinyl-4H-1,3-dithiin) [31, 32]. At RT 0.98 min, the m/z value was 145.00 (100.00%) (2-vinyl-4H-1,3, dithiin or 3-vinyl-4H-1,3-dithiin), 163.00 (23.64%) (allicin). At a retention time of 0.98 min presence of vinyl dithiin was found with the signature peak of m/z=145. This may suggest that during the heating process of GMM, thermal degradation of allicin (m/z=163) had occurred leading to the synthesis of acrolein dimers (2-vinyl-4H-1,3-dithiin and 3-vinyl-4H-1,3-dithiin, m/z=145) and ajoenes (m/z=235) [31, 33]. The mass spectrum of the third peak at a retention time of 1.15 min has shown the major peaks at m/z 195, 241 and 259 which can be considered as the diagnostic ion for glucosinolates present in

mustard oil [34]. The m/z value obatined during LCMS analysis for mustard oil (m/z value 169, 337, 338, 59, 178) were also detected during the LCMS analysis of GMM, which suggest the presence of MO compounds in GMM. Although limited information was found regarding the compounds detected in MO during our literature suvey.

Upon GCMS analysis, several peaks were observed for GMM. One prominent peak was found at retention time 6.125 which was found to be allyl isothiocyanate (AITC) with ionic table 99.0 (999), 41.0 (620), 39.0 (618), 72.0 (391), 45.0 (110), 71.0 (110). AITC is formed due to the enzymatic conversion of sinigrin which is a glucosinolate in mustard oil [35]. At 17.56 min a peak was found and the MS data confirmed the compound to be 2-vinyl-4H-1,3, dithiin (molecular weight=144 g/mol) with ionic table 72.0 (999), 71.0 (987), 111.0 (484), 144.0 (376), 45.0 (357) and 39.0 (176). Compounds like allicin and ajoene that were found during LCMS analysis were not found during GCMS analysis, which may be due to the compound's degradation of vinyl dithiin at high temperatures [36]. The details of the compounds detected in the GCMS analysis of GMM and MO other than AITC and 2-vinyl-4H-1,3, dithiin are listed in Table 5. In an earlier study by Abu-Lafi et al. (2004) and Sowbhagya et al. (2009), 2-vinyl-4H-1,3, dithiin and 3-vinyl-4H-1,2, dithiin was reported upon GCMS study of garlic and its essential oil [37, 38]. Similarly, AITC and 1-Butene-4-isothiocyanato were also reported earlier for seed oil of *Brassica juncea*, *Brassica napus and Brassica nigra* species [39, 40].

The presence of organo-sulfur compounds (OCSs) which were detected by LCMS and GCMS analysis in GMM may enhance its bioactive properties such as DPPH scavenging, antibacterial, antifungal and anti-inflammatory. Due to this, significantly higher total polyphenol and DPPH scavenging activity was observed during our experiment.

5.3. Optimization of garlic mustard oil macerate

This finding forces us to optimize the preparation parameter which suggests a correlation of biochemical and antimicrobial activity with ajoene and vinyl dithiin concentrations in GMM. The formation of organo-sulfur compounds (OCSs) is mostly

dependent on the polarity of the solvent, the reaction parameters (heating temperature, reaction time and amount of edible oil) and the kind of fatty acids in the oil and shaking of the macerate [41, 42]. Only a few related articles were available on the optimization of OSCs in garlic macerated in soybean and rice bran oil [41, 43]. Moreover, ajoene and 2-vinyl dithiin have been identified as the major OSCs in GMM, which suggests that the mixture will likely have significant health advantages. Using response surface methodology, the optimized condition for ajoene was at a temperature of 55 ^{o}C, a reaction time of 4.5 hours and an oil volume of 2.00 factor of weight of garlic; and for 2-vinyl-4H-1,3, dithiin, the conditions were a temperature of 77.51 ^{o}C, a reaction time of 2.22 hours and oil volume of 2.25 factor of weight of garlic. In earlier research conducted by Yoo and team, during optimization of garlic macerated in soyabean oil the range of Z- and E- ajoene was found to be 35.48- 720.79 µg/g of garlic juice and 15.13- 256.42 µg/g of garlic juice which was found to be significantly lower than the prepared formulations in the present research [41]. The results imply that the preparation process during our research provided a much higher ajoene yield than the work published by Yoo and colleagues, which may be attributable to the reduction in allicin formation due to vortex and sonication [43]. In earlier research by Yoo and team, at optimum conditions for E- ajoene (98.80 ^{o}C, 6.87 hours and oil volume of 2.57 factor of weight of garlic) the yield was 225.75 ± 9.7 µg/g of garlic juice and for Z-ajoene (42.24 ^{o}C, 9.71 hours and oil volume of 3.08 factor of weight of garlic) the yield was 833.59 ± 59.1 µg/g of garlic juice [41]. Nazin and team have reported that the yield of E-ajoene (172.0 µg/g of garlic) and Z-ajoene (476.0 µg/g of garlic) is best in rice oil when heated at temperature of 80 ^{o}C at 15% garlic in oil [44]. Also, it was observed that the concentration of E- and Z- ajoene may vary depending on the nature of the vegetable oil used while preparing the macerate [45]. Also, it has been reported that at the extraction condition of ½ w/w garlic oil, 37 ^{o}C, 6 hours in olive or sunflower oil the yield of vinyl dithiin was 133 mg/100 g of fresh garlic vinyl dithiin and upon microwave irradiation the yield increased to 486 mg/100 g of raw garlic [43]. The yield of vinyl dithiin after microwave irradiation reported by Dethier and the team matched close to our yield of 2-vinyl-4H-1,3, dithiin at the optimized conditions [43]. We were able to enhance the ajoene and vinyl dithiin synthesis using our findings at substantially lower heating temperatures and for a shorter amount of time. It was found that the concentration of ajoene and vinyl dithiin in optimized GMM was found to increase by

1.75 and 1.83 folds, respectively compared to the initially prepared GMM. Furthermore, previous studies have optimized garlic-containing composites based on their antioxidant properties, total polyphenolic content and solvent selection for extraction, but research on the optimization of ajoene and vinyl dithiin, which are reported to have significant bioactive roles in reducing inflammation, tumour suppression, lowering hypertension, antibacterial, antifungal and antiviral properties, is very limited [46-55]. From the results of optimization of GMM with respect to antibacterial, antifungal and DPPH scavenging activity, various optimal parameters were found. For antibacterial activity by vapour diffusion assay against *S. aureus* it was 2- and 4-hour heating at 80^{o}C in water bath at the ratio of 1:4 and 1:8 of garlic and mustard oil. Upon agar diffusion assay, the optimal condition for GMM preparation based on its antifungal activity against *C. albicans* was 55^{o}C water bath heating for 1 hr with ratio of garlic: MO (1:2). This optimal condition was like that of the optimal condition found upon response surface methodology (55^{o}C water bath heating for 4.5 hr with ratio of garlic: MO (1:2)). But again, during vapour diffusion assay against *C. albicans* the optimal condition found was 2-hour heating at 80^{o}C and 30 second heating at 160^{o}C in the ratio of 1:2 of garlic and mustard oil. Also, during optimization through DPPH scavenging activity the optimal condition was heating GMM at 100^{o}C for 4 hours at the ratio of 1:4 of garlic and mustard oil. From all the various approaches of optimization of GMM, the condition which was considered best was heating the macerate at 55^{o}C for 2 hours at the ratio of 1:2 of garlic and mustard oil. This optimal condition was selected since results during RSM and antifungal activity by agar diffusion matched and was reproducible.

5.4. Bioactivity of optimized garlic mustard oil macerate

5.4.1. Antibacterial activity

During the MIC and MBC determination assay against four selected bacterial species, optimized GMM showed antibacterial activity till the maximum dilution of GMM/8 (0.125% v/v) against *S. aureus.* After the assay, bacterial samples were collected from below the oil samples and plated in a fresh media plate for the determination of MBC. The MBC results suggested that till the maximum concentration of GMM (100% v/v), the bacteria below the oil had shown growth in fresh MHA. Therefore, we may suggest that the optimized

GMM oil is not bactericidal rather it is bacteriostatic. As from the earlier report, it was seen that bacterial growth in more than 4 times the MIC is considered to be bacteriostatic [56]. When gram-positive bacteria *S. aureus* and *B. cereus* were treated with GMM oil, the pink colouration of the cell membrane by safranin was observed which might be due to the disruption of the peptidoglycan layer of the bacterial cell. Upon vapour diffusion assay against *S. aureus* by GMM vapour, reduction in staphyloxanthin pigment was confirmed using UV-Vis and FTIR analysis. The FTIR analysis of the bacterial cells sheds light on the fact that after the treatment of GMM volatiles not only there is a decrease in the staphyloxanthin production but also a decrease in the membrane integrity of *S. aureus*. This disruption in membrane integrity is proposed with respect to significant changes as found in non-staphyloxanthin peaks like 3437, 2085, 1645 (membrane phospholipids), 1077 (correspond to polysaccharides) and 773 cm^{-1} (membrane phospholipids) in the FTIR data of *S. aureus* cells treated with GMM volatile (Figure 48 (B)) [57]. Along with this FTIR data, SEM image analysis also supported the theory that *S. aureus's* membrane fluidity changes when exposed to volatiles of GMM due to a change in membrane structure. Therefore, our finding coincide with the previous report of Valliammai et al that the increase in membrane fluidity in *S. aureus* enhances sensitivity to the membrane targeting antibiotic polymyxin B [58]. Also, much previously done work has reported that the membrane damage led to higher susceptibility of *S. aureus* towards antibiotics [59, 60]. Therefore, during the current investigation, a greater zone of antibiotic inhibition may have been also influenced by the increase in membrane fluidity. As non-polar garlic VOCs are reported to bind with the cell membrane and penetrate the plasma membrane which causes membrane rupture and the leakage of vital cell components like proteins [61-64]. The disruption of the bacterial cell wall of *Corynebacterium glutamicum*, *S. aureus, E. coli, Erwinia caratovora, Pseudomonas syringae, Xanthomonas campestris, Magnoporthe grisea, Fusarium proliferatum* by garlic extract was earlier reported in various research articles [61, 65, 66].

5.4.2. Antibiofilm and anti-quorum sensing analysis

After observing the reduction in staphyloxanthin production of the GMM oil against *S. aureus*, the antibiofilm activity of the GMM was also analyzed. In the preliminary screening of the bacteria for biofilm production in Congo red agar plates, *S. aureus* was found to produce a significant amount of biofilm due to the production of exopolysaccharide and form slime (black). Due to this agar diffusion and vapour assay in CRA was carried out to check the inhibition in biofilm production. During both assays, we found that GMM oil showed a significant reduction in biofilm production which was marked by reduced dark pigmentation on the CRA plates compared to MO. The vapour of GMM was found to significantly reduce the dark precipitate formation by *S. aureus* which may be a strong correlation with the enhanced antibiotic susceptibility by gentamycin, kanamycin and tetracycline. Also, the impairment of biofilm production by *S. aureus* may be responsible for the reduced production of staphyloxanthin which was earlier suggested by a few research groups [67, 68]. The ring biofilm inhibition assay showed a reduction in biofilm production in a dose-dependent manner for both GMM and MO, were for GMM no ring was observed from the concentration of 25 µl/ml. This finding helps in the establishment of biofilm inhibition by GMM oil against *S. aureus*. Against, *P. aeruginosa* the GMM vapour was found to reduce the pyocyanin production which is known to be a crucial virulence factor [69]. Also, during the broth dilution method, a reduction in the pyocyanin was observed for GMM and MO in a dose-dependent manner. During the microscopic assay, inhibition in the biofilm was observed for GMM at 100 µl/ml then followed by MO (100 µl/ml). Also, a reduction in the carbohydrate concentration in the EPS produced by *P. aeruginosa* was observed at the concentration of 100 µl/ml of GMM> 200 µl/ml> 200 µl/ml GMM> 100 µl/ml MO. Violacein is a pigment produced by *Chromobacterium violaceum* which is a marker pigment for quorum sensing [70]. The GMM and MO vapour showed a positive inhibition in the violacein production during vapour diffusion and broth dilution method. It is reported that ajoene, allicin and isothiocyanate present in mustard oil are responsible for anti-QS activity. The disulfide bond containing compounds from garlic and mustard oil is found to downregulate the expression of key genes involved in QS

pathways [71]. The compounds down-regulate *lasR* which further down-regulates the expression of *phzM* (which encodes pyocyanin), *pslB* (a key role for the synthesis of biofilm matrix polysaccharide) and *chiC* (responsible for chitinase production) [72].

5.4.3. Antifungal activity

The optimized GMM had shown a significantly higher antifungal activity against *C. albicans* during both agar and vapour diffusion assay. During the experiment, the VOCs from both MO and GMM were found to be highly antifungal. GMM showed higher antifungal activity than MO, which may be due to the compounds from MO such as AITC and 1-Butene-4-isothiocyanato, along with oil-soluble garlic compounds such as vinyl dithiin and ajoene. Previous studies on the antifungal activity of GMM VOCs against *C. albicans* have never been reported. Essential oil from clove and cinnamon during vapour diffusion assay is reported to be effective against *C. albicans* by 60% and 70%, respectively; and against *Aspergillus flavus* by 90% and 75%, as reported by Lopez et al. 2005 [73]. Another research which is carried out using the method of vapour diffusion by Delespaul et al. 2000 for 37 essential oils out of which *Cymbopogon martini* (L.)*, Cymbopogon nardus* (L.)*, Thymus vulgaris, Satureja montana and Chenopodium ambrosioides* showed maximum efficacy against 8 selected mould strains [74]. Prior reports by Isshiki et al. 1992, Dhingra et al. 2009, indicate that AITC found in essential oil of mustard species effectively inhibits the growth of fungi associated with both human pathogenesis and food spoilage, such as *Aspergillus glaucus*, *Aspergillus parasiticus, Aspergillus niger, Aspergillus flavus, Penicillium citrinum and C. albicans* [75, 76]. Allicin vapour (50 mM) is earlier reported by Schier et al. 2023, to inhibit the growth of *Mucor racemosus* (area of ZOI=1965 mm^2) and *Rhizopus stolonifer* (area of ZOI=2405 mm^2) during vapour diffusion method [77]. Fungal infection in the nasopharyngeal region has been observed to be linked with bronchiolitis in infants, while *C. albicans* infections in the mouth and nasopharyngeal region are known to affect individuals with weakened immune systems [78, 79]. The use of essential oils for aromatherapy is known to be the one of oldest medicines in the world and is known to be effective against bacterial, fungal and viral

infections [79]. Thus, the GMM volatiles may be employed as aromatherapy to treat fungus-related respiratory tract illnesses in future research works. Agar diffusion is a straightforward and inexpensive method for detecting the MIC and MBC against bacteria, according to Balouiri et al. 2016 [80]. Therefore, to determine the MIC and MFC against *C. albicans*, we employed a similar technique in our study. GMM/8 dilution (12.5 %, v/v) was found to be the lowest concentration at which no fungal growth was observed under the oil and thus it was considered the MIC and GMM/4 (25 %, v/v) was found to be the lowest concentration at which fungicidal activity was observed and thus considered to be the MFC. Nystatin (10µg) has shown a zone of inhibition of 22±0.57 mm. Mirabadi et al. 2019 have reported that 30 µl of garlic essential oil at the concentration of 250, 500 and 1000 µg/ml concentration gave a ZOI against *C. albicans* with diameter of 9.83±1.09, 19.23±1.66 and 35.66±3.48 mm, respectively by agar diffusion method [81]. Prajapati et al. 2021 reported the ZOI diameter of various concentration of garlic oil (10%, 20%, 30%, 40%, 50%) to be 12, 14, 18, 21 and 24 mm, respectively [82]. Garlic essential oils were earlier reported to have a significant antifungal activity during radial diffusion assay against species such as *A. tenuissima* by 100% at 1000 ppm concentration [83]. Essential oils are developed into formulations such as gels, semi-solids and ointments which are then used to counter fungal skin infections [84]. Similarly, GMM oil may be developed into gels and ointments and then tested against fungal skin infections. During the poison food assay, the radius of the fungal colony was found to increase in dose dose-dependent manner. Li et al. 2014 reported the MIC and MFC of garlic oil against *P. funiculosum* by using poisoned food assay to be 0.0625 and 0.125 % (v/v), respectively [85]. Again Li et al. 2016 reported the MIC value of garlic oil against *C. albicans* to be 0.35 µg/ml which was lower in concentration compared to our results which may be due to a higher concentration of garlic-derived VOCs in garlic oil than compared to GMM oil [86]. Under both conditions, the cells were found to be ovular in structure with smooth cell walls. Likewise, *C. albicans* cell wall damage and cytoplasmic content leakage were observed upon the treatment of garlic aqueous extract were reported [87, 88]. SEM analysis on the effect of

garlic essential oil against *C. albicans* is found to be scanty during our literature survey. AITC was found to inhibit biofilm production in *C. albicans* at concentrations more than 1mg/ml, but no cell disruption was reported [89]. Therefore, during the SEM analysis, other GMM VOCs may be assigned for cell structure disruption of *C. albicans*. The *in-silico* molecular docking analysis of volatile compounds of GMM and MO with N-myristoyltransferase was conducted. N-myristoyltransferase is earlier reported to be the target protein against *C. albicans* as it play a crucial role in signal transduction and required for growth [90]. The molecular docking analysis of eight selected molecules present in GMM with 1nmt supports the facts that the combination of compound found in GMM may be responsible for the higher antifungal activity against *C. albicans* compared to MO. In research conducted by Shaweta et al. 2021, Z-ajoene showed lowest binding energy of -5.07 Kcal/mol with 1, 3-beta-glucan synthase (PDB ID: 4m80) which is a target molecule for black fungus [91]. Again, S- allyl cysteine and alliin had shown as significant lower binding energy against three selected fungal protein receptors with PDB ID 5TZ, 4UYM and 2Y7L [92]. Diallyl disulfide, diallyl trisulfide and fluconazole were seen to form complex with the sterol 14α-demethylase (CYP51) protein of *C. albicans* [93].

5.4.4. Cell viability assay against HEK293 normal cell line, THP-1 cell line and MCF7 breast cancer cell line

The cell viability assay against a normal cell line was conducted to check its cellular cytotoxicity. From the cell viability assay against HEK293 and THP-1 cell lines at the maximum concentration of 400 µg/ml for GMM and MO, we observed no significant cytotoxicity. Whereas, very high cellular cytotoxicity was observed for both GMM and MO against the MCF7 breast cancer cell line in a dose-dependent manner. From figures 84 and 85, it can be observed that at each concentration of MO and GMM (25, 50, 100, 200, 400 µg/ml) significantly high cellular cytotoxicity was observed against MCF7 cell lines compared to HEK and THP1 cell lines. According to research conducted by Li et al. 2002, Z-ajoene had antiproliferative activity against breast cancer (MCF7), nasopharyngeal carcinoma (KB), hepatocellular carcinoma (Bel 7402), gastric carcinoma (BGC823), colon carcinoma (HCT), Hela cell (Hela), whereas had

lower cytotoxicity against normal marsupial kidney cells (PtK2) [94]. Diallyl trisulfide which is a OSC of garlic is earlier reported to induce apoptosis in the MCF7 breast cancer cell line through upregulation of pro-apoptotic Bax and p53 protein [95].

5.4.5. Anti-inflammatory activity

LPS is recognized for its ability to trigger the production of various pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α, IL-8, IL-10 and IL-12 [96]. Building on our *in-silico* investigation into how our compounds interact with various pro-inflammatory molecules, our subsequent research focus is aimed at determining whether our compound can effectively diminish the LPS-induced secretion of these pro-inflammatory cytokines in THP-1 macrophages. Through an examination of the available literature, it was observed that there is a lack of previous research investigating the anti-inflammatory properties of garlic mustard oil macerate. Nevertheless, many scientific investigations have been undertaken to explore the anti-inflammatory characteristics of substances derived from garlic. Previous studies have indicated that garlic extracts possess the ability to reduce the concentrations of IL12, TNFα, IL1α, IL8, IFNγ and IL2, while concurrently enhancing the synthesis of IL-10 in both whole blood and human placental explants [97, 98]. In previous studies using RAW264.7 macrophages exposed to lipopolysaccharide (LPS) stimulation, it has been observed that Z-ajoene and allicin exhibit the ability to decrease the gene expression of IL-1β, IL6 and IL2β, while concurrently enhancing the expression of IL10 [49]. From the results, it also observed that upon MO treatment, the Cox2 expression was upregulated. This observation could potentially be linked to the well-documented up-regulatory effects of AITC present in mustard oil, on COX2 expression [99, 100]. Based on this observation, it is plausible to assert that the chemicals found in GMM may have the ability to not only mitigate the overexpression of LPS-induced COX-2, but also counteract the upregulation induced by AITC. Given the documented COX-2-mediated inflammatory response to AITC in THP-1 cell lines, future research work could focus on the utilization of AITC as an agent for initiating inflammation. Z-ajoene and E-ajoene along with other sulfur

analogues from garlic are reported to have anti-inflammatory effects against LPS-induced inflammation [50]. Although the analysis and observation are currently based on the semi-quantitative analysis, therefore in the future qPCR and western blotting analysis must be carried out to confirm the findings. From the molecular docking analysis of the compounds present in GMM high binding efficiency with COX-2, IL1β, IL6, IL8 and TNF α was observed. Z- ajoene integrated with Cox2 with the lowest minimum binding energy out of all the selected compounds via pi-sulfur, alkyl, van der Waals and pi-alkyl interactions. Again, allicin was also found to bind strongly with a minimum binding energy of -4.9 Kcal/mol with hydrogen bonds at Trp C:387 and His C:388, alkyl, pi-alkyl and van der Waals interactions. Again, 1-butene-4-isothiocyanato was found to bind more efficiently (-4.2 Kcal/mol) to Cox2 through hydrogen bonding (Arg B:469), followed by van der Waals interaction. AITC binds to Cox2 through weak interaction of van der Waal, alkyl and pi-alkyl giving the binding affinity of -3.7 Kcal/mol. Sinigrin which is a precursor to AITC present in mustard oil was found to bind strongest with Cox2 through several hydrogen bonding (Asn C:34, Asn C:39, Gln C:461, Gly C:45, Try C:130, Cys C:47 and Ser C:49), allyl and van der Waals interaction giving the binding energy of -7.6 Kcal/mol. Similarly, highest binding affinity with Cox2 protein was observed in case of 3-vinly dithiin, E-ajoene and 2-vinly dithiin giving the binding energy of -5.3, -4.9 and -5.0 Kcal/mol.

5.4.6. ADME and drug-likeness analysis

Consequently, the ADME and drug-likeness investigations were conducted for all the major compounds in GMM. The compound's bioavailability may be impeded by one or more infringements of Lipinski's rule [101]. The findings of our study indicated that none of the eight compounds that were chosen for analysis were found to violate Lipinski's criterion. All molecules in the dataset possess a molecular weight below 500 dal, a hydrogen bond acceptor count less than 5, a hydrogen bond donor count less than 10 and a logP value below 5. Except for sinigrin, it was observed that all the other 7 compounds had a significantly high absorption throughout the gastrointestinal tract. Consequently, even though sinigrin demonstrated the best binding affinity with

all the chosen proteins during molecular docking research, effective bioactivity may not be attained because of limited GI absorption. In terms of blood-brain barrier permeability (BBB), AITC, allicin, 2-vinyl-dithiin and 3-vinyl-dithiin exhibited high permeability and other compounds did not pass through the BBB. P-glycoprotein is associated with the efflux of substrate drugs from the cells, thus decreasing the efficient activity of the drug [102, 103]. Except for sinigrin, no other compound is observed to be a Pgp substrate. Again, E-and Z-ajoene was found to be an inhibitor for CYP2C9 protein which breaks down steroid hormones and fatty acids [104].

5.4.7. Transdermal activity

The diffusion cell that was prepared in the laboratory had the limitation of using a new setup for each incubation time. However, it was seen that for 2 ml of oil of MO and GMM, it took approximately 2 hours of incubation time at 37ºC. The eggshell membrane was found to retain approximately 17.55 mg of oil during the diffusion and the rest of the oil either got transferred or retained in the cap of the donor compartment. The FTIR results showed the transmittance for untreated eggshell membranes at 3459, 1635, 1400, 1091 and 648 cm^{-1}. The 1635 cm^{-1} corresponds to collagen (C=O stretching) which is one of the major components of the eggshell membrane [105], 1400 cm^{-1} corresponds to symmetrical vibration of carboxylate which are mainly found in proteins and lipids [106], 1091 cm^{-1} is found to correspond to antisymmetric C-O-C stretching usually present in glycosidic linkage in IR spectra of di- and polysaccharides or glycoproteins in the membrane structures [107] and 648 cm^{-1} which is assigned for in-plane bending for carbonyl group [108]. The FTIR results for the MO/GMM oil-treated eggshell membrane showed minimum transmittance at peaks which we observed during the FTIR analysis of MO and GMM oil. Moreover, peaks corresponding to the eggshell membrane were not seen which may be due to the ~95% abundance of oil by weight in the membrane. During LCMS analysis of the oil diffused through the membrane after 2 hours of incubation has shown the presence of allicin (m/z=163.100) at retention time of 2.77 minutes which was not observed at the same retention time for MO. Again, at retention time of 7.43 minutes the presence of 2-vinyl-

4H-1,3-dithiin (m/z=145.100) and ajoene (m/z=235.100) were not detected for MO at the same retention time. This finding supports the fact that during the application of GMM oil in traditional practice, the mustard oil helps in the transfer of the garlic derived OSCs through the skin. Although for further confirmation of the results the transdermal assay must be carried out using human or animal stratum corneum in the future research.

5.4.8. Sensory acceptability test

During the sensory test, it was seen that the means of the relative score for the colour of both MO and GMM were not significantly different which supports the fact that the preparation process of GMM did not change the colour of the oil. The volunteers scored significantly higher for the aroma and flavour of GMM in the MO. This finding helped in understanding the quality of GMM to be better in terms of flavor and aroma which may be due to the presence of garlic compounds in the oil.

5.5. Reference

[1] Sunanta, P., Pankasemsuk, T., Jantanasakulwong, K., Chaiyaso, T., Leksawasdi, N., Phimolsiripol, Y., Rachtanapun, P., Seesuriyachan, P., and Sommano, S.R. Does curing moisture content affect black garlic physiochemical quality? *Horticulturae*, 7(12): 535, 2021.

[2] Essa, H.A., Shehata, A.N., AboZaid, A.M., Moawad, R.K., Abdelmaguid, N.M., Saleh, O.S., and Ibrahim, W.A. Chemical Characteristics, Mineral Contents and Color evaluation of Fresh Garlic Cloves and Dried Garlic Sheet. *Egyptian Journal of Chemistry*, 66(3): 323-331, 2023.

[3] Tithi, D., Amin, M., Azad, H., and Rahman, M. Effect of different containers on moisture content and germination of mustard seed. *Journal of Science and Technology*, 8, 2010.

[4] Danlami, U., Orishadipe Abayomi, T., and Lawal, D.R. Phytochemical, nutritional and antimicrobial evaluations of the aqueous extract of Brassica Nigra (Brassicaceae) seeds. *Am. J. Appl. Chem*, 4(161.10): 11648, 2016.

[5] Cserhalmi, Z., Márkus, Z., Czukor, B., Baráth, Á., and Tóth, M. Physico-chemical properties and food utilization possibilities of RF-treated mustard seed. *Innovative Food Science & Emerging Technologies*, 1(4): 251-254, 2000.

[6] Abul-Fadl, M., El-Badry, N., and Ammar, M. Nutritional and chemical evaluation for two different varieties of mustard seeds. *World Applied Sciences Journal*, 15(9): 1225-1233, 2011.

[7] Kahyaoğlu, D.T. Comparison of the antioxidant activity of garlic cloves with garlic husk and stem: Determination of utilization potential of garlic agricultural wastes. *Türk Tarım ve Doğa Bilimleri Dergisi*, 8(2): 463-469, 2021.

[8] Santos, K.A., de Aguiar, C.M., da Silva, E.A., and da Silva, C. Evaluation of favela seed oil extraction with alternative solvents and pressurized-liquid ethanol. *The Journal of Supercritical Fluids*, 169: 105125, 2021.

[9] Frega, N., Mozzon, M., and Lercker, G. Effects of free fatty acids on oxidative stability of vegetable oil. *Journal of the American Oil Chemists' Society*, 76: 325-329, 1999.

[10] Borompichaichartkul, C., Luengsode, K., Chinprahast, N., and Devahastin, S. Improving quality of macadamia nut (Macadamia integrifolia) through the use of hybrid drying process. *Journal of Food Engineering*, 93(3): 348-353, 2009.

[11] Khan, A., Sankhyan, P., and Kumar, S. Biochemical characterization of Mustard Oil (Brassica campestris L.) with special reference to its fatty acid composition. *Asian J. of Adv. Basic Sci*, 1(1): 1-9, 2013.

[12] Budhathoki, N., and Punmagar, M. Analysis of Mustard and Sunflower oil available in local market of Baglung Bazaar. *Pragnya Sarathi प्रज्ञा-सारथि*, 22(1): 37-43, 2024.

[13] Sehgal, S., Swer, T.L., Bhalla, A., and Chandrakanth, N. Quality and Safety of the Frying Oils used in Small or Medium-sized Food Enterprises in West Delhi, India. *Oriental Journal of Chemistry*, 37(3): 547, 2021.

[14] Al-Fatlawi, A.M., and Abbas, N.M. Investigating peroxides and acid value in used edible vegetable oils. *The Iraqi Journal of Agricultural Science*, 41(4): 123-132, 2010.

[15] Weast, R.C. CRC handbook of chemistry and physics. 1986.

[16] Chakraborty, S., Gupta, S.S., Sengupta, A., and Ghosh, M. Quality ascertain of different mustard oil samples obtained from the local market of West Bengal, India. *Asian Journal of Dairy and Food Research*, 37(2): 138-143, 2018.

[17] Zahir, E., Saeed, R., Hameed, M.A., and Yousuf, A. Study of physicochemical properties of edible oil and evaluation of frying oil quality by Fourier Transform-Infrared (FT-IR) Spectroscopy. *Arabian Journal of Chemistry*, 10: S3870-S3876, 2017.

[18] Chen, Y., Mastalerz, M., and Schimmelmann, A. Characterization of chemical functional groups in macerals across different coal ranks via micro-FTIR spectroscopy. *International Journal of Coal Geology*, 104: 22-33, 2012.

[19] Kalgutkar, A.S., Gardner, I., Obach, R.S., Shaffer, C.L., Callegari, E., Henne, K.R., Mutlib, A.E., Dalvie, D.K., Lee, J.S., and Nakai, Y. A comprehensive listing of bioactivation pathways of organic functional groups. *Current drug metabolism*, 6(3): 161-225, 2005.

[20] Nagarajan, D., and Kumar, T.R. Fourier transform infrared spectroscopy analysis of garlic (Allium). *International Journal of Zoology Studies*, 2(6): 11-14, 2017.

[21] Nandiyanto, A.B.D., Oktiani, R., and Ragadhita, R. How to read and interpret FTIR spectroscope of organic material. *Indonesian Journal of Science and Technology*, 4(1): 97-118, 2019.

[22] Stoia, M., Istratie, R., and Păcurariu, C. Investigation of magnetite nanoparticles stability in air by thermal analysis and FTIR spectroscopy. *Journal of Thermal Analysis and Calorimetry*, 125: 1185-1198, 2016.

[23] Fujisawa, H., Suma, K., Origuchi, K., Kumagai, H., Seki, T., and Ariga, T. Biological and chemical stability of garlic-derived allicin. *Journal of agricultural and food chemistry*, 56(11): 4229-4235, 2008.

[24] Espin, J.C., Soler-Rivas, C., and Wichers, H.J. Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2, 2-diphenyl-1-

picrylhydrazyl radical. *Journal of Agricultural and Food chemistry*, 48(3): 648-656, 2000.

[25] Valavanidis, A., Nisiotou, C., Papageorgiou, Y., Kremli, I., Satravelas, N., Zinieris, N., and Zygalaki, H. Comparison of the radical scavenging potential of polar and lipidic fractions of olive oil and other vegetable oils under normal conditions and after thermal treatment. *Journal of Agricultural and Food Chemistry*, 52(8): 2358-2365, 2004.

[26] Tuberoso, C.I., Kowalczyk, A., Sarritzu, E., and Cabras, P. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. *Food Chemistry*, 103(4): 1494-1501, 2007.

[27] Ramirez, D.A., Locatelli, D.A., González, R.E., Cavagnaro, P.F., and Camargo, A.B. Analytical methods for bioactive sulfur compounds in Allium: An integrated review and future directions. *Journal of Food Composition and Analysis*, 61: 4-19, 2017.

[28] Sheikh Raisuddin, S.A., Fatima, M., and Dabeer, S. Toxicity of anticancer drugs and its prevention with special reference to role of garlic constituents. *Annals of Phytomedicine*, 7(1): 13-26, 2018.

[29] Nishimura, H., Takahashi, T., Hanny Wijaya, C., Satoh, A., and Ariga, T. Thermochemical transformation of sulfur compounds in Japanese domestic Allium, Allium victorialis L. *BioFactors*, 13(1-4): 257-263, 2000.

[30] Fujisawa, H., Suma, K., Origuchi, K., Seki, T., and Ariga, T. Thermostability of allicin determined by chemical and biological assays. *Bioscience, biotechnology, and biochemistry*, 72(11): 2877-2883, 2008.

[31] Calvey, E.M., Roach, J.A., and Block, E. Supercritical fluid chromatography of garlic (*Allium sativum*) extracts with mass spectrometric identification of allicin. *Journal of chromatographic science*, 32(3): 93-96, 1994.

[32] Calvey, E.M., White, K.D., Matusik, J.E., Sha, D., and Block, E. Allium chemistry: identification of organosulfur compounds in ramp (Allium tricoccum) homogenates. *Phytochemistry*, 49(2): 359-364, 1998.

[33] Calvey, E.M., Matusik, J.E., White, K.D., DeOrazio, R., Sha, D., and Block, E. Allium Chemistry: supercritical fluid extraction and LC− APCI− MS of thiosulfinates and related compounds from homogenates of garlic, onion, and ramp. Identification in garlic and ramp and synthesis of 1-propanesulfinothioic acid S-allyl ester. *Journal of Agricultural and Food Chemistry*, 45(11): 4406-4413, 1997.

[34] Paul, S., Geng, C.-A., Yang, T.-H., Yang, Y.-P., and Chen, J.-J. Comparative study of the glucosinolate profiles in turnip from four agroclimatic zones of china and neighboring countries. *Journal of Food Measurement and Characterization*, 13: 2798-2811, 2019.

[35] Okulicz, M. Multidirectional time-dependent effect of sinigrin and allyl isothiocyanate on metabolic parameters in rats. *Plant foods for human nutrition*, 65: 217-224, 2010.

[36] Block, E., Iyer, R., Grisoni, S., Saha, C., Belman, S., and Lossing, F.P. Lipoxygenase inhibitors from the essential oil of garlic. Markovnikov addition of the allyldithio radical to olefins. *Journal of the American Chemical Society*, 110(23): 7813-7827, 1988.

[37] Abu-Lafi, S., Dembicki, J.W., Goldshlag, P., Hanuš, L.r.O., and Dembitsky, V.M. The use of the 'Cryogenic'GC/MS and on-column injection for study of organosulfur compounds of the Allium sativum. *Journal of Food Composition and Analysis*, 17(2): 235-245, 2004.

[38] Sowbhagya, H., Purnima, K.T., Florence, S.P., Rao, A.A., and Srinivas, P. Evaluation of enzyme-assisted extraction on quality of garlic volatile oil. *Food chemistry*, 113(4): 1234-1238, 2009.

[39] Zhou, Q., Tang, H., Jia, X., Zheng, C., Huang, F., and Zhang, M. Distribution of glucosinolate and pungent odors in rapeseed oils from raw and microwaved seeds. *International Journal of Food Properties*, 21(1): 2296-2308, 2018.

[40] Ayadi, J., Debouba, M., Rahmani, R., and Bouajila, J. Brassica Genus Seeds: A Review on Phytochemical Screening and Pharmacological Properties. *Molecules*, 27(18): 6008, 2022.

[41] Yoo, M., Lee, S., Kim, S., and Shin, D. Optimizing conditions for E-and Z-ajoene formation from garlic juice using response surface methodology. *Food science & nutrition*, 2(5): 605-611, 2014.

[42] Hibi, T. *Method of processing garlic and preparing ajoene-containing edible oil products*. 1997, US Patent 5,6112,077, filed May 22, 1995, and issued March 18, 1997.

[43] Dethier, B., Hanon, E., Maayoufi, S., Nott, K., and Fauconnier, M.-L. Optimization of the formation of vinyldithiins, therapeutic compounds from garlic. *European Food Research and Technology*, 237: 83-88, 2013.

[44] Naznin, M.T., Akagawa, M., Okukawa, K., Maeda, T., and Morita, N. Characterization of E-and Z-ajoene obtained from different varieties of garlics. *Food chemistry*, 106(3): 1113-1119, 2008.

[45] Yoo, M., Lee, S., Kim, S., and Shin, D. Validation of HPLC method for determination of E-and Z-Ajoene in oil-macerated garlic juice. *Food Science and Biotechnology*, 21(1): 107-111, 2012.

[46] Sasmaz, H.K., Kadiroglu, P., Adal, E., Sevindik, O., Aksay, O., Erkin, O.C., Selli, S., and Kelebek, H. Optimization of black garlic production parameters using response surface methodology: Assessment and characterization of bioactive properties. *Journal of Applied Research on Medicinal and Aromatic Plants*, 34: 100477, 2023.

[47] Ramirez, D.A., Locatelli, D.A., Torres-Palazzolo, C.A., Altamirano, J.C., and Camargo, A.B. Development of garlic bioactive compounds analytical methodology based on liquid phase microextraction using response surface design. Implications for dual analysis: Cooked and biological fluids samples. *Food chemistry*, 215: 493-500, 2017.

[48] Sasmaz, H.K., Adal, E., Kadiroğlu, P., Selli, S., Uzlasir, T., and Kelebek, H. Optimization of complex coacervation parameters for the production of encapsulated black garlic using response surface methodology. *Journal of Food Science*, 88(11): 4424-4439, 2023.

[49] Hitchcock, J.K., Mkwanazi, N., Barnett, C., Graham, L.M., Katz, A.A., Hunter, R., Schäfer, G., and Kaschula, C.H. The Garlic Compound Z-Ajoene, S-Thiolates COX2 and STAT3 and Dampens the Inflammatory Response in RAW264. 7 Macrophages. *Molecular Nutrition & Food Research*, 65(3): 2000854, 2021.

[50] Lee, D.Y., Li, H., Lim, H.J., Lee, H.J., Jeon, R., and Ryu, J.-H. Anti-inflammatory activity of sulfur-containing compounds from garlic. *Journal of medicinal food*, 15(11): 992-999, 2012.

[51] Kaschula, C.H., Hunter, R., and Parker, M.I. Garlic-derived anticancer agents: Structure and biological activity of ajoene. *Biofactors*, 36(1): 78-85, 2010.

[52] Beppu, H., Matsumoto, Y., Fujino, T., Itani, Y., Sumitho, T., Higashiguchi, T., Chihara, T., Tamai, I., Sonoda, S., and Shimpo, K. Evaluation of the effects of the ingestion of ajoene, a sulfur containing compound derived from oil-macerated garlic, on metabolic

parameters, abdominal circumference, and blood pressure in Japanese metabolic syndrome patients. A pilot study. *Journal of Analytical Bio-Science Vol*, 33(5), 2010.

[53] Choi, J.-A., Cho, S.-N., Lim, Y.-J., Lee, J., Go, D., Kim, S.-H., and Song, C.-H. Enhancement of the antimycobacterial activity of macrophages by ajoene. *Innate Immunity*, 24(1): 79-88, 2018.

[54] Terrasson, J., Xu, B., Li, M., Allart, S., Davignon, J.L., Zhang, L.h., Wang, K., and Davrinche, C. Activities of Z-ajoene against tumour and viral spreading in vitro. *Fundamental & clinical pharmacology*, 21(3): 281-289, 2007.

[55] Walder, R., Kalvatchev, Z., and Apitz-Castro, R. Selective in vitro protection of SIVagm-induced cytolysis by ajoene,[(E)-(Z)-4, 5, 9-trithiadodeca-1, 6, 11-triene-9 oxide]. *Biomedicine & pharmacotherapy*, 52(5): 229-235, 1998.

[56] Gonzalez, N., Sevillano, D., Alou, L., Cafini, F., Gimenez, M.-J., Gomez-Lus, M.-L., Prieto, J., and Aguilar, L. Influence of the MBC/MIC ratio on the antibacterial activity of vancomycin versus linezolid against methicillin-resistant Staphylococcus aureus isolates in a pharmacodynamic model simulating serum and soft tissue interstitial fluid concentrations reported in diabetic patients. *Journal of Antimicrobial Chemotherapy*, 68(10): 2291-2295, 2013.

[57] Askoura, M., Yousef, N., Mansour, B., and Yehia, F.A.-z.A. Antibiofilm and staphyloxanthin inhibitory potential of terbinafine against *Staphylococcus aureus*: in vitro and in vivo studies. *Annals of Clinical Microbiology and Antimicrobials*, 21(1): 1-17, 2022.

[58] Valliammai, A., Selvaraj, A., Muthuramalingam, P., Priya, A., Ramesh, M., and Pandian, S.K. Staphyloxanthin inhibitory potential of thymol impairs antioxidant fitness, enhances neutrophil mediated killing and alters membrane fluidity of methicillin resistant *Staphylococcus aureus*. *Biomedicine & Pharmacotherapy*, 141: 111933, 2021.

[59] Alipiah, N.M., Shamsudin, M.N., Yusoff, F.M., and Arshad, A. Membrane biosynthesis gene disruption in methicillin-resistant *Staphylococcus aureus* (MRSA) as potential mechanism for reducing antibiotic resistance. *Indian journal of microbiology*, 55: 41-49, 2015.

[60] Kim, W., Zou, G., Hari, T.P., Wilt, I.K., Zhu, W., Galle, N., Faizi, H.A., Hendricks, G.L., Tori, K., and Pan, W. A selective membrane-targeting repurposed antibiotic with activity against persistent methicillin-resistant *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences*, 116(33): 16529-16534, 2019.

[61] Chen, C., Liu, C.-H., Cai, J., Zhang, W., Qi, W.-L., Wang, Z., Liu, Z.-B., and Yang, Y. Broad-spectrum antimicrobial activity, chemical composition and mechanism of action of garlic (Allium sativum) extracts. *Food Control*, 86: 117-125, 2018.

[62] Diao, W.-R., Hu, Q.-P., Zhang, H., and Xu, J.-G. Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel (Foeniculum vulgare Mill.). *Food control*, 35(1): 109-116, 2014.

[63] Chen, Z., He, B., Zhou, J., He, D., Deng, J., and Zeng, R. Chemical compositions and antibacterial activities of essential oils extracted from *Alpinia guilinensis* against selected foodborne pathogens. *Industrial crops and products*, 83: 607-613, 2016.

[64] Cui, H., Zhang, X., Zhou, H., Zhao, C., and Lin, L. Antimicrobial activity and mechanisms of *Salvia sclarea* essential oil. *Botanical studies*, 56(1): 1-8, 2015.

[65] Perry, C.C., Weatherly, M., Beale, T., and Randriamahefa, A. Atomic force microscopy study of the antimicrobial activity of aqueous garlic versus ampicillin against

Escherichia coli and Staphylococcus aureus. *Journal of the Science of Food and Agriculture*, 89(6): 958-964, 2009.

[66] Mihoub, M., Aloui, A., El May, A., Sethom, M.M., Ben Ammar, A., Jaafoura, M.H., Kacem, R., and Landoulsi, A. Effects of garlic extract on cell wall of Corynebacterium glutamicum. *Annals of microbiology*, 60: 637-643, 2010.

[67] Vijayakumar, K., Muhilvannan, S., and Arun Vignesh, M. Hesperidin inhibits biofilm formation, virulence and staphyloxanthin synthesis in methicillin resistant *Staphylococcus aureus* by targeting SarA and CrtM: An in vitro and in silico approach. *World Journal of Microbiology and Biotechnology*, 38(3): 1-12, 2022.

[68] Yehia, F.A.-z.A., Yousef, N., and Askoura, M. Exploring Staphylococcus aureus virulence factors; special emphasis on Staphyloxanthin. *Microbiology and Biotechnology Letters*, 49(4): 467-477, 2021.

[69] Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A.J., Forbes, A., Perkins, A.V., Davey, A.K., Chess-Williams, R., Kiefel, M.J., and Arora, D. Cellular effects of pyocyanin, a secreted virulence factor of Pseudomonas aeruginosa. *Toxins*, 8(8): 236, 2016.

[70] Cheng, K.-C., Hsiao, H.-C., Hou, Y.-C., Hsieh, C.-W., Hsu, H.-Y., Chen, H.-Y., and Lin, S.-P. Improvement in violacein production by utilizing formic acid to induce quorum sensing in Chromobacterium violaceum. *Antioxidants*, 11(5): 849, 2022.

[71] Fong, J., Yuan, M., Jakobsen, T.H., Mortensen, K.T., Delos Santos, M.M.S., Chua, S.L., Yang, L., Tan, C.H., Nielsen, T.E., and Givskov, M. Disulfide bond-containing ajoene analogues as novel quorum sensing inhibitors of Pseudomonas aeruginosa. *Journal of medicinal chemistry*, 60(1): 215-227, 2017.

[72] Li, W.-R., Ma, Y.-K., Shi, Q.-S., Xie, X.-B., Sun, T.-L., Peng, H., and Huang, X.-M. Diallyl disulfide from garlic oil inhibits Pseudomonas aeruginosa virulence factors by inactivating key quorum sensing genes. *Applied microbiology and biotechnology*, 102: 7555-7564, 2018.

[73] Lopez, P., Sanchez, C., Batlle, R., and Nerin, C. Solid-and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. *Journal of agricultural and food chemistry*, 53(17): 6939-6946, 2005.

[74] Delespaul, Q., de Billerbeck, V.G., Roques, C.G., Michel, G., Marquier-Viñuales, C., and Bessière, J.-M. The antifungal activity of essential oils as determined by different screening methods. *Journal of essential oil research*, 12(2): 256-266, 2000.

[75] Dhingra, O., Jham, G., Rodrigues, F., Silva Jr, G., and Costa, M. Fumigation with essential oil of mustard retards fungal growth and accumulation of ergosterol and free fatty acid in stored shelled groundnuts. *Journal of Stored Products Research*, 45(1): 24-31, 2009.

[76] Isshiki, K., Tokuoka, K., Mori, R., and Chiba, S. Preliminary examination of allyl isothiocyanate vapor for food preservation. *Bioscience, biotechnology, and biochemistry*, 56(9): 1476-1477, 1992.

[77] Schier, C., Gruhlke, M.C., Reucher, G., Slusarenko, A.J., and Rink, L. Combating Black Fungus: Using Allicin as a Potent Antifungal Agent against Mucorales. *International Journal of Molecular Sciences*, 24(24): 17519, 2023.

[78] Shibata, R., Zhu, Z., Kyo, M., Ooka, T., Freishtat, R.J., Mansbach, J.M., Pérez-Losada, M., Camargo, C.A., and Hasegawa, K. Nasopharyngeal fungal subtypes of infant bronchiolitis and disease severity risk. *EBioMedicine*, 95, 2023.

[79] Buckle, J. Clinical aromatherapy and AIDS. *Journal of the Association of Nurses in AIDS care*, 13(3): 81-99, 2002.

[80] Balouiri, M., Sadiki, M., and Ibnsouda, S.K. Methods for in vitro evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis*, 6(2): 71-79, 2016.

[81] Mirabadi, M., Azadeghan Qomi, H., and Didehdar, M. In vitro activities of garlic essential oil against Candida species. *Tabari Biomedical Student Research Journal*, 1(1): 12-17, 2019.

[82] Prajapati, M., Shah, M., Ranginwala, A., Agrawal, P., Acharya, D., and Thakkar, S. Antifungal effects of tulsi, garlic, cinnamon and lemongrass in powder and oil form on Candida albicans: An: in vitro: study. *Journal of Oral and Maxillofacial Pathology*, 25(2): 306-312, 2021.

[83] Muy-Rangel, M.D., Osuna-Valle, J.R., García-Estrada, R.S., Martín-Hernández, S., and Quintana-Obregón, E.A. In vitro antifungal activity of garlic essential oil (Allium sativum L.) against Alternaria tenuissima. *Revista mexicana de fitopatología*, 36(1): 162-171, 2018.

[84] Abd Rashed, A., Rathi, D.-N.G., Ahmad Nasir, N.A.H., and Abd Rahman, A.Z. Antifungal properties of essential oils and their compounds for application in skin fungal infections: Conventional and nonconventional approaches. *Molecules*, 26(4): 1093, 2021.

[85] Li, W.-R., Shi, Q.-S., Liang, Q., Huang, X.-M., and Chen, Y.-B. Antifungal effect and mechanism of garlic oil on Penicillium funiculosum. *Applied microbiology and biotechnology*, 98: 8337-8346, 2014.

[86] Li, W.-R., Shi, Q.-S., Dai, H.-Q., Liang, Q., Xie, X.-B., Huang, X.-M., Zhao, G.-Z., and Zhang, L.-X. Antifungal activity, kinetics and molecular mechanism of action of garlic oil against Candida albicans. *Scientific reports*, 6(1): 22805, 2016.

[87] Ghannoum, M.A. Studies on the anticandidal mode of action of Allium sativum (garlic). *Microbiology*, 134(11): 2917-2924, 1988.

[88] Ashrit, P., Sadanandan, B., Shetty, K., and Vaniyamparambath, V. Polymicrobial Biofilm Dynamics of Multidrug-Resistant Candida albicans and Ampicillin-Resistant Escherichia coli and Antimicrobial Inhibition by Aqueous Garlic Extract. *Antibiotics*, 11(5): 573, 2022.

[89] Raut, J.S., Bansode, B.S., Jadhav, A.K., and Karuppayil, S.M. Activity of allyl isothiocyanate and its synergy with fluconazole against Candida albicans biofilms. *J. Microbiol. Biotechnol*, 27(4): 685-693, 2017.

[90] Chen, S.-H., Sheng, C.-Q., Xu, X.-H., Jiang, Y.-Y., Zhang, W.-N., and He, C. Identification of Y118 amino acid residue in Candida albicans sterol 14α-demethylase associated with the enzyme activity and selective antifungal activity of azole analogues. *Biological and Pharmaceutical Bulletin*, 30(7): 1246-1253, 2007.

[91] Sharma, S., Sharma, A., and Gupta, U. Molecular Docking studies on the Anti-fungal activity of Allium sativum (Garlic) against Mucormycosis (black fungus) by BIOVIA discovery studio visualizer 21.1. 0.0. *Annals of Antivirals and Antiretrovirals*, 5(1): 028-032, 2021.

[92] Bouamrane, S., Khaldan, A., Alaqarbeh, M., Sbai, A., Ajana, M.A., Bouachrine, M., Lakhlifi, T., and Maghat, H. Garlic as an effective antifungal inhibitor: A combination of reverse docking, molecular dynamics simulation, ADMET screening, DFT, and retrosynthesis studies. *Arabian Journal of Chemistry*, 17(3): 105642, 2024.

[93] Pereira, R., Mendes, J.d.F.S., dos Santos Fontenelle, R.O., Rodrigues, T.H.S., dos Santos, H.S., Marinho, E.S., Marinho, M.M., and de Morais, S.M. Antifungal activity, antibiofilm, synergism and molecular docking of Allium sativum essential oil against

clinical isolates of C. albicans. *Research, Society and Development*, 10(12): e313101220457-e313101220457, 2021.

[94] Li, M., Ciu, J.-R., Ye, Y., Min, J.-M., Zhang, L.-H., Wang, K., Gares, M., Cros, J., Wright, M., and Leung-Tack, J. Antitumor activity of Z-ajoene, a natural compound purified from garlic: antimitotic and microtubule-interaction properties. *Carcinogenesis*, 23(4): 573-579, 2002.

[95] Malki, A., El-Saadani, M., and Sultan, A.S. Garlic constituent diallyl trisulfide induced apoptosis in MCF7 human breast cancer cells. *Cancer biology & therapy*, 8(22): 2174-2184, 2009.

[96] Tucureanu, M.M., Rebleanu, D., Constantinescu, C.A., Deleanu, M., Voicu, G., Butoi, E., Calin, M., and Manduteanu, I. Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor. *International journal of nanomedicine*: 63-76, 2018.

[97] Hodge, G., Hodge, S., and Han, P. Allium sativum (garlic) suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. *Cytometry: The Journal of the International Society for Analytical Cytology*, 48(4): 209-215, 2002.

[98] Makris, A., Thornton, C., Xu, B., and Hennessy, A. Garlic increases IL-10 and inhibits TNFα and IL-6 production in endotoxin-stimulated human placental explants. *Placenta*, 26(10): 828-834, 2005.

[99] Moilanen, L.J., Laavola, M., Kukkonen, M., Korhonen, R., Leppänen, T., Högestätt, E.D., Zygmunt, P.M., Nieminen, R.M., and Moilanen, E. TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Scientific Reports*, 2(1): 380, 2012.

[100] Kim, S.-J., Park, H.-J., and Youn, H.-S. Allyl isothiocyanate suppresses lipopolysaccharide-induced expression of inducible nitric oxide synthase, but not induced expression of cyclooxygenase-2. *Molecular & Cellular Toxicology*, 8: 149-154, 2012.

[101] Lipinski, C.A., Lombardo, F., Dominy, B.W., and Feeney, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*, 64: 4-17, 2012.

[102] Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., and Willingham, M.C. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences*, 84(21): 7735-7738, 1987.

[103] Gatlik-Landwojtowicz, E., Äänismaa, P., and Seelig, A. Quantification and characterization of P-glycoprotein− substrate interactions. *Biochemistry*, 45(9): 3020-3032, 2006.

[104] Sangkuhl, K., Claudio-Campos, K., Cavallari, L.H., Agundez, J.A., Whirl-Carrillo, M., Duconge, J., Del Tredici, A.L., Wadelius, M., Rodrigues Botton, M., and Woodahl, E.L. PharmVar GeneFocus: CYP2C9. *Clinical Pharmacology & Therapeutics*, 110(3): 662-676, 2021.

[105] Tiong, W.H., Damodaran, G., Naik, H., Kelly, J.L., and Pandit, A. Enhancing amine terminals in an amine-deprived collagen matrix. *Langmuir*, 24(20): 11752-11761, 2008.

[106] Lu, Y., and Miller, J.D. Carboxyl stretching vibrations of spontaneously adsorbed and LB-transferred calcium carboxylates as determined by FTIR internal reflection spectroscopy. *Journal of colloid and interface science*, 256(1): 41-52, 2002.

[107] Nikonenko, N.A., Buslov, D.K., Sushko, N.I., and Zhbankov, R.G. Investigation of stretching vibrations of glycosidic linkages in disaccharides and polysaccarides with use of IR spectra deconvolution. *Biopolymers: Original Research on Biomolecules*, 57(4): 257-262, 2000.

[108] Catauro, M., Barrino, F., Dal Poggetto, G., Crescente, G., Piccolella, S., and Pacifico, S. New SiO2/caffeic acid hybrid materials: Synthesis, spectroscopic characterization, and bioactivity. *Materials*, 13(2): 394, 2020.