Chapter 2

Review of literature

Garlic is popular as a foodstuff and has high medicinal value in the field of cancer prevention, anti-inflammatory, antimicrobial and antiviral activity. These health benefits of garlic are achieved due to the presence of organosulfur compounds like SAC, thiosulfinate (allicin), AJ, DAS, DADS, DATS, etc. The chemical breakdown of the garlic compounds is well described by Block (1985), according to which intact garlic consists of alliin which gets enzymatically converted to allicin by alliinase once the garlic is crushed [1]. However, these thiosulfinates are found to be rapidly converted to other sulfur-containing compounds depending on the method of processing. Out of these methods, oil maceration of garlic is a processing method that is reported to have high medicinal values. During these processes, the garlic is crushed and mixed in vegetable oil and then further processed. As a traditional practice in Northeast India, similar maceration is prepared with crushed garlic and mustard oil followed by heating and the oil is used during the common cold for massage. However, during our literature search, limited research articles on garlic mustard oil macerate are found. However, heating of garlic macerate in other vegetable oils is reported which might help in understanding the mechanism of breakdown of allicin to other OSCs along with their chemical and bioactivity.

Traditionally, mustard oil is extracted from the dried *B. nigra* seeds by cold press method [2]. Ruth et al. 2021 reported the percentage of oil in *B. nigra* seed upon cold press to be 28%-30%. The results for the physicochemical were FFA (0.37±0.02%) and AV (0.74±0.04 mg/g) [2]. The fatty acid composition which was found in cold press mustard oil were palmitic acid C-16 (2.89%), stearic acid C-18 (1.18%), behenic acid C-22 (0.79%), lignoceric acid C-24 (1.13%) [2]. The MUFAs found in cold press mustard oil were Eicosenoic acid C-20:1 (6.79%), oleic acid C-18:1, Euric acid C22:1 (45.09%), Nervoic acid C-24:1 (1.92%) [2]. It was reported that *B. nigra* consists of chemical constituents which are very similar to *B. juncea* [3]. In research conducted by Rangkadilok et al. (2002), the concentration of sinigrin in brown seeds of *B. nigra* was found to be 31.8 µmol/g dry weight and was found to be significantly high than that of the green seeds (14.3 µmol/g dry weight) [4]. The seeds consist of Glucosinolates, which give the mustard paste its pungent smell [3]. Sinigrin is the most abundant glucosinolate found in *B. nigra* seeds [3]. The *B. nigra* seeds consist of the enzyme myrosinase, which is released after crushing the seeds and it is responsible for the hydrolysis of sinigrin to allyl isothiocyanate (AITC) [3]. AITC was reported to be the key VOC of *B. nigra* essential oil with a concentration of 378.35 mg/ml [5]. Also, the antifungal nature of the EO of

B. nigra was reported against *Aspergillus niger* (MIC=4µl/ml), *Aspergillus ochraceus* (MIC=2µl/ml) and *Penicillium citrinum* (MIC=2µl/ml) [5].

2.1. Physicochemical and phytochemical analysis of garlic macerate in various oil

Lawson et al. (1991) used the HPLC method to identify the compounds in garlic macerate in soyabean oil and hexane [6]. During the study, they reported the presence of vinyl dithiins and (E)- and (Z)- ajoene only when the garlic macerate is prepared in soyabean oil. During the quantification of the compounds, they found about 70% of vinyl dithiins, 18% of dialk(en)yl sulfides and 12% of ajoenes (E- and Z- ajoene). (*E*)-ajoene was found to be the dominant isomer over (*Z*)-ajoene by almost 2 folds. Also, in freshly prepared oil macerate, only (*Z*)-ajoene was found to be present which then with time isomerise to (*E*)-ajoene. They have reported an unknown peak in the garlic macerate in soyabean oil which was later identified as *Z*-10-devinylajoene (*Z*-10-DA) by Yoshida et al. (1998) during garlic macerate preparation in rape seed oil [7]. *Z*-10-devinylajoene is different from Z- ajoene with respect to the substitution of the allyl group by the methyl group flanking a sulfinyl group [7] (Figure 1).

As during the traditional practice, the GMM is heated at a high temperature (approximately $160^{O}C$) before applying it to the patients. It was seen that the heating of vegetable oils at high temperatures may change their physicochemical and nutritional properties [8]. Therefore, the quality testing of the GMM oil after preparation must be evaluated. For the analysis of the quality of oil, the FFA, PV, AV, IV and SV are estimated. The increase in FFA signifies the degradation of triglycerides to fatty acids [9]. It is reported that factors such as temperature, humidity and light exposure can increase the rate of the degradation process [10]. Omara et al. (2019) reported the change in the physicochemical properties of various commercially available cooking oils upon deep frying of potatoes [11]. During the physicochemical analysis, they checked parameters such as the FFA composition, IV and PV. In the results, they have reported that the hard oils (Fortune Butto, Roki, Tamu, Best fry) are much preferable to the soft oils (Sunseed, Sunny, Sunvita, Sunlite) for deep frying purposes [11]. Again, according to Siddique et al. (2013), palm oil was reported to be more tolerant against oxidation than compared to soybean, sunflower and canola oil which was determined by FTIR analysis [12]. The change in these physicochemical properties of oil after heating makes the oil unsuitable for consumption [13]. Eissa et al. (2023) carried out the biochemical analysis of fresh garlic cloves and dried garlic sheets [14]. In fresh garlic, the

moisture content, crude protein, crude fat, Ash, crude fibre and carbohydrate were found to be 65.2%, 3.01%, 0.54%, 2.28%, 1.16% and 27.81%, respectively [14]. Against for dried garlic sheet the moisture content, crude protein, crude fat, Ash, crude fibre, carbohydrate was found to be 16.14%, 4.85%, 2.66%, 5.62%, 2.48% and 68.25%, respectively [14]. The moisture content of garlic was earlier reported to be a crucial parameter in determining the period of storage of garlic [15]. For fresh garlic, the percentage of moisture was reported to be between 50-70% [15]. For *B. juncea* and *B. nigra* the moisture content was reported to be 12.17% and 4.16% [16, 17]. Ash content in *B. juncea* and *B. nigra* seeds were earlier reported to be in the range of 4-5 % [18, 19]. Biochemical evaluation of *Sinapis alba* and *B. juncea* was evaluated [19]. For *S. alba,* the moisture content, protein, oil, ash, dietary fibre and total carbohydrate content were found to be 5.05%, 36.73%, 31.78%, 4.08%, 5.87%, 16.49%, respectively. And for *B. juncea,* the moisture content, protein, oil, ash, dietary fibre and total carbohydrate content were found to be 4.98%, 32.48%, 36.32%, 3.88%, 6.34%, 16.60%, respectively [19]. Evaluation of antioxidant properties using DPPH scavenging activity is well studied for oil seeds like flaxseed, grapeseed and peanut [20]. During a study led by Tuberoso et al. (2007), it was observed that the lipophilic fraction (LF) of the selected vegetable oils had more DPPH scavenging activity than compared to the hydrophilic fraction (HF). The order of DPPH scavenging activity of various selected oils was flaxseed> grape> maize> peanut> pumpkin> rapeseed> soyabean> sunflower> olive [20].

For the phytochemical analysis, various research groups have used techniques such as LCMS, GCMS, HPLC and FTIR for compound identification in various plant extracts and oils [13, 21-23]. As GMM consists of a complex mixture of compounds from both MO and garlic, therefore, the identification of the compounds is very important for future research and product development purposes. FTIR analysis is an effective method by which the types and abundance of the functional groups in the given molecule may be determined [24]. Functional groups present in the compounds also give the molecules their characteristic chemical property along with a potent bioactive nature [25]. Jamwal et al. (2020) carried out the FTIR analysis of fresh mustard oil (MO) and fried mustard oil [26]. For fresh MO the maximum absorbance upon FTIR was seen at 3008.1, 2922.2, 2852.8, 1743.5, 1463.9, 1417.8, 1377.2, 1236.8, 1160.1, 1119.3, 1098.7, 721.71 cm^{-1}. Again, for fresh MO the maximum absorbance of FTIR analysis was seen at 3006.3, 2921.6, 2852.4, 1745.1, 1710.1, 1654.0, 1463.3, 1416.3, 1377.3, 1236.4, 1160.0, 1119.2, 1099.2, 966.11, 721.69 cm^{-1} [26]. Yeasmin et al. (2021) performed the

physicochemical and GCMS analysis for the identification of fatty acid composition in mustard oil and blended mustard oil with rice bran oil [27]. Specifically for mustard oil the moisture content, density, specific gravity, RI, viscosity, FFA, PV, IV, AV and unsaponifiable matter were 0.05±0.002%, 0.8867±0.3 at 25^{O}C, 0.9021±0.01 at 25^{O}C, 1.4652±0.001 at 25^{O}C, 61.30±0.02 cst, 0.15±0.006 (as oleic acid %), 2.84±0.13 (meq/kg), 121.3±0.32 g/100g, 0.4±0.02 % and 1.5±0.10%. The fatty acid composition of mustard oil upon GCMS was Hexadecanoic acid methyl ester (13.99%), 9,12-Omadecadienoic acid (Z, Z)-, methyl est (18.903%), 9-Octadecenoic acid (Z)-, methyl ester (9.506%), Octadecanoic acid. Methyl ester (11.696%), 9.12-Octadecadknoic acid, methyl ester, (E) (13748%), 8.11.14-Ecosatrienoic acid, methyl ester (10.464%), Heneicosanoic acid. Methyl ester (10.338%), 13-Docosanoic acid. Methyl ester (11.347%) [27]. From the earlier mentioned literature by Lawson et al. (1991), by using HPLC compounds such as ajoenes and vinyldithiins were identified in the garlic oil macerates [6]. They have mentioned that the high-temperature environment of GCMS leads to the degradation of compounds such as ajoene and allicin for which methods such as HPLC and LCMS may be used for compound identification in garlic macerated oils. Block et al. (1988) have mentioned that the use of GCMS causes thermal degradation of ajoene and allicin which leads to the formation of vinyldithiins [28].

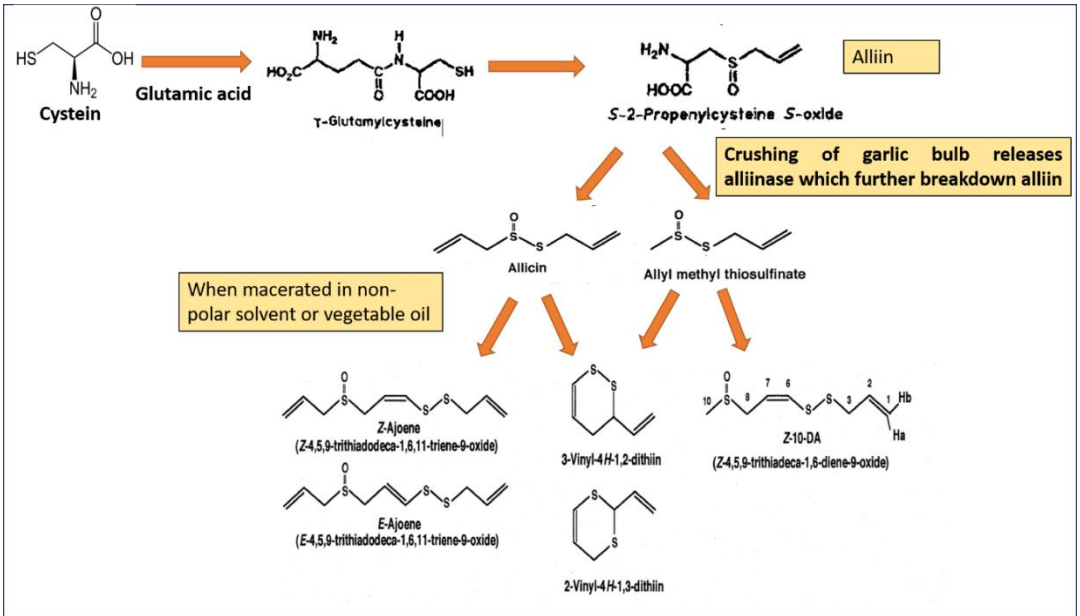

Figure 1: Flowchart of the chemical breakdown of allicin in non-polar solvents and vegetable oils (Image courtesy: Singha, J., Dutta, N., and Saikia, J.P. A novel volatile staphyloxanthin biosynthesis inhibitor against *Staphylococcus aureus*. *Microbial Pathogenesis*: 107489, 2025).

2.2. Optimization of garlic macerate in various vegetable oils

As the bioactive potential of garlic-derived OSCs in vegetable oil was discovered, researchers have worked on the enhancement of these OSCs through various optimization processes. It was stated that the garlic OSCs formation in vegetable oil significantly depends on the type of fatty acids present in the oil along with dependent variables such as the temperature of heating the macerate, duration of heating and garlic to mustard oil ratio [29, 30]. During our literature survey, we did not find any research on the process optimization of garlic macerate in mustard oil. Only a few related articles were available on the optimization of OSCs in garlic macerated in oils like soybean, olive and rice bran oil [29, 31]. In the research conducted by Yoo et al. (2014), optimization of the formation of *E*- and *Z*- ajoene in garlic soyabean oil macerate was done [29]. During the research, they mixed the garlic paste in soyabean oil and then vortexed the mixture for a few minutes [29]. For the optimization, RSM was followed using CCD [29]. The dependent variables that were considered while optimization was the ratio of garlic and oil (1:2-1:5), the temperature of heating (20ºC-100 ºC) and the duration of heating (2hr-10hr). HPLC was used for the quantification of *E*- and *Z*- ajoene at 254 nm. The optimized conditions for *E*- and *Z*- ajoene were found to be 98.80 ºC, 6.87hr and garlic to oil ratio of 1:2.57 and 42.24 ºC, 9.71hr and garlic to oil ratio of 3.08, respectively [29]. Yoo et al. (2012) determined ajoene isomers in garlic macerated in soyabean oil, corn oil, olive oil, grape seed oil, rice bran oil, perilla oil, flaxseed oil and sesame oil. In the results it was found that maximum amount of ajoene got produced in rice bran oil (792.9 µg/g of garlic juice) followed by olive oil (785.1 µg/g of garlic)> sesame oil (752.7 µg/g of garlic)> flax seed (734.8 µg/g of garlic)> corn seed oil (673.4 µg/g of garlic)> perilla oil (641.4 µg/g of garlic)> grape seed oil (544.4 µg/g of garlic)> soyabean oil (543.7 µg/g of garlic) [32]. 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 a temperature of 80ºC at 15% garlic in oil [33]. Optimization of the formation of vinyldithiins in garlic macerate using edible vegetable oil (linseed, colza, soybean, olive, sunflower, grape seed, corn, peanut seed) was done by Dethier et al. (2013) [31]. During their research Spanish garlic sunflower oil and olive oil were found to be the best for the formation of vinyldithiins then compared to other selected garlic and vegetable oils [31]. The optimized condition for the vinyldithiins formation, garlic to oil ratio of 1:2 was heated at 37ºC for 6 hours which yielded 133 mg of vinyldithiins from

100g of fresh garlic [31]. It was also found that with a change in the type of vegetable oil, the concentration of vinyldithiins varies significantly [31]. Therefore, during GMM preparation the OSCs which are present may vary from the earlier reported results and thus process optimization of this traditional medicine becomes important. From the earlier finding it was observed that the formation of ajoene and vinyl dithiin in different vegetable oils used are significantly different. Therefore, it was hypothesised that during GMM oil preparation the OSCs which maybe present may very as due to the difference in fatty acid composition of mustard oil.

2.3. Bioactive properties of thiosulfinate compounds present in garlic oil macerate

Extensive work on the different bioactivity of ajoene and vinyl dithiin was done by various research groups. Yoshida and colleagues have also reported the antimicrobial activity of the *Z*-10-DA against various gram-positive bacteria (*B. cereus, B. subtilis, S. aureus, Mycobacterium phlei, micrococcus luteus*), gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa, Xanthomonas maltophilia*) and yeast (*Candida valida, Hansenniaspora valbyensis, Kluyveromyces lactis, Saccharomyces cerevisiae*) [7]. The MIC values of Z-10-DA against gram-positive bacteria, gram-negative bacteria and fungi were in the range of 2-150 µg/ml, 75-17 µg/ml and 10-75 µg/ml, respectively [7]. For Z- ajoene, the MIC values against gram-positive bacteria, gram-negative bacteria and fungi were in the range of 5-120 µg/ml, 70-113 µg/ml and 10-20 µg/ml, respectively. For E- ajoene, the MIC values against gram-positive bacteria, gram-negative bacteria and fungi were in the range of 10-250 µg/ml, 150-200 µg/ml and 15-50 µg/ml [7]. *Z*-10-DA was found to have higher antimicrobial activity than compared to *E*- and *Z*- ajoene, which suggests that the substitution by the methyl group can be effective against microbial growth [7]. Ohta et al. (1999) reported ajoene to have in-vitro antibacterial activity against three selected strains of *Helicobacter pylori* (MIC=10 to 25 µg/ml) [34]. Jakobsen et al. 2012 reported ajoene (100 µg/ml) treatment causes the inhibition of rhamnolipid produced by *P. aeruginosa* [35]. Rhamnolipid is found to cause necrosis of polymorphonuclear leukocytes (PMNs) and under the treatment of ajoene, no PMN necrosis was observed after staining with propidium iodide stain [35]. Ajoene was also found to inhibit the quorum sensing (QS) ability of *P. aeruginosa* during in vitro and in vivo studies. At the concentration of 80 µg/ml of ajoene, the expression of *rhlA* was reduced by 12 folds and that of *lasB* by 5 folds. This suggests that ajoene may target the Rhl QS system [35]. According to the study conducted by Naganawa et al. (1996), ajoene was reported to have a broad-spectrum antibacterial activity

[36]. At the concentration of 20 µg/ml, ajoene inhibited the growth of gram-positive bacteria, such as *B. cereus, Bacillus subtilis, Mycobacterium smegmatis and Streptomyces griseus*, *S. aureus* and *Lactobacillus plantarum* and gram-negative bacteria, such as *E. coli*, *K. pneumoniae* and *Xanthomonas maltophilia* with MICs range between 100-160 µg/ml. Ajoene (20 µg/ml) also inhibited the fungal growth of *Saccharomyces cerevisiae*. They have put the argument that the disulfide bond present in ajoene was responsible for its antimicrobial activity which was confirmed by reacting cysteine with the disulfide of the ajoene molecule thus reducing the antimicrobial activity of ajoene [36]. Xue et al. (2018) reported a synergistic antimicrobial effect of ajoene and two metal oxides (i.e., Al_2O_3 and TiO_2) against *Campylobacter jejuni* (reduced by 8 log10 CFU/mL). The mechanism of action of the synergistic effect of ajoene and metal oxide was determined by RNA sequencing and confocal micro-Raman spectroscopy. The mechanism involves a two-step process: at first, ajoene was found to cause physical injury in the bacterial cell membrane and also decrease its the resistance towards stress; in the second step the metal oxide nanoparticle targets this weekend cells and finally disintegrate the bacterial cell membrane [37]. In terms of antifungal activity, ajoene (20 µg/ml) inhibited the growth of *A. niger* and *C. albicans* [38].

Weber et al. (1992) did a detailed antiviral study of the garlic compounds and stated that the ajoene had shown the maximum anti-viral activity against viruses like herpes simplex virus type 1 (HSV1), herpes simplex virus type 2 (HSV2), parainfluenza virus type 3 (PV3), vaccinia virus (VV), human rhinovirus type 2 (HRV 2) which was followed by allicin, allyl methyl thiosulfinate and methyl allyl thiosulfinate [39]. E-ajoene at the concentration of 500, 250 and 125 µg/ml reduced the HSV-1 growth by 2.6, 1.6 and 1.2 $\log_{10}$, respectively. At the concentration of 500 and 250 µg/ml of ajoene, the inhibition of PV3 growth was observed by 2.9 and 1.1 $\log_{10}$, for HRV-2 the growth inhibition was 1.1 and 0.7 $\log_{10}$ and for VV the growth reduction was 1.7 and 1.0 $\log_{10}$. At 126 µg/ml concentration of E-ajoene, the growth inhibition of VSV and HSV-2 was found to be 2.6 and 1.0 $\log_{10}$ [39]. In a study conducted by Walder et al. (1997), synthetic ajoene was found to exhibit antiviral activity against human immunodeficiency virus (HIV)-1 (IIIB) [40]. During the study, ajoene was found to protect Molt-4 cells from HIV-1 infection and reduce the cell death of CD4 T-cells [40]. The 50% cytotoxicity concentration (CTC50%) and 50% effective inhibitory concentration (EIC50%) for ajoene was found to be 1.88 µM and about 0.35 µM, respectively, before and after HIV-1 infection. During the mechanism study, ajoene was found to inhibit virus adsorption which is

crucial for viral replication [40]. The proteases of SARS CoV-2 namely, main protease (Mpro) and papain-like protease (PLpro) are found to be responsible for its pathogenesis. Shekh et al. 2023 reported that three garlic-derived compounds namely, E-ajoene, S-(3-pentanyl)-L-cysteine-sulfoxide and 1-propenyl allyl thiosulfinate were found to have high affinity with both proteases of SARS CoV-2. The garlic compounds were found to modify the active site cysteine thiols of the proteases through S-thioallylation, S-thioallyl sulfinyl propenylation and S-thiopropenylation and E-ajoene to be a potential dual protease targeting covalent inhibitor of SARS CoV-2 [41].

Traditionally GMM oil is applied in the nasal orifice which reduces nasal congestion. As nasal congestion is mainly caused due to inflammation therefore the GMM may consist of compounds that may be anti-inflammatory. In an *in-vitro* assay with LPS-activated RAW 264.7 macrophage cells conducted by Lee et al. (2012), four garlic-derived sulfur compounds (*E*- and *Z*- ajoene and oxidized sulfonyl derivates of ajoene) have shown significant anti-inflammatory activity [42]. The compounds were found to downregulate the expression of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin-1β (IL-1β) and interleukin-6 (IL6). The research group also reported a reduction in the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in the cells [42]. Again, in 2020 the anti-inflammatory effect of allicin and Z-ajoene in LPS-activated RAW 264.7 macrophage was done [43]. It was reported that allicin and Z-ajoene downregulated the expression of pro-inflammatory cytokines IL1β, IL6 and IL1β and upregulated the expression of inflammatory cytokine IL10. A reduction in phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) protein along with modification in S-thiolation in Cys108, Cys367 and Cys687 [43]. Similarly, ajoene was found to downregulate the expression of COX2 expression (ic50 value: 3.4 μM) with the mechanism of action the same as that of the NSAID indomethacin [44]. The research group also pointed out that the anti-inflammatory effect of garlic compound in down-regulating the COX2 expression may reduce the risk of gastrointestinal carcinomas [44]. The compounds that are reported to have anti-inflammatory activity were also found to have potent anticancer activity [45-47].

As traditionally GMM oil is used for massaging purposes during the common cold, mustard oil may help in the transdermal delivery of the compounds through the stratum corneum. Therefore, to check the transdermal activity of the macerate, a skin permeation study may be used to validate the transfer of the compounds in GMM through the membrane. As the

use of stratum corneum in humans, mice and other animals is subjected to ethical clearance therefore various research groups have used the eggshell membrane for transdermal activity [48]. The eggshell membrane consists of high keratin, readily available and cheap therefore it has been a good alternative as a transdermal membrane [49]. During the research conducted by Ansari et al. (2006), it was found that the diffusion of drugs such as metronidazole and diclofenac through eggshell membranes followed Fick's first law and non-Fickian mechanism [48].

2.4. References

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