ABSTRACT

Secondary metabolites present in the garlic mustard oil macerate (GMM) may be responsible for the bioactivities claimed in traditional practices. Therefore, the determination of unique compounds in GMM is very important. Traditionally people crush garlic and prepare the macerate (GMM) in boiled ($160^{O}C$) mustard oil but major compounds reported earlier are synthesized by crushing garlic in other vegetable oil and are found highest at $80^{O}C$. Therefore, GMM at $80^{O}C$ and $160^{O}C$ are prepared (GMM80 & GMM160). Three compounds namely, allyl isothiocyanate (AITC), 1-Butene, 4-isothiocyanato- (BITC), 2-vinyl-4H-1,3-dithiin (VD) (excluding the fatty acids) are found to be highly abundant after GCMS analysis of in GMM80. AITC and BITC are the compounds which are reported by earlier research groups to be present in MO. One of these compounds i.e., 2-Vinyl-4H-1,3-dithiin is unique to GMM80 and not found in MO and GMM160. A similar trend was found for antibacterial and antifungal activity where GMM80 is better in many cases. Therefore, it is concluded that GMM80 is better and all further experiments will be carried out with GMM80. Compounds present in GMM80 are also identified by liquid chromatography-mass spectrometry (LC/MS). Two major compounds that were identified are ajoene (AJ) and 2-Vinyl-4H-1,3-dithiin (VD) (which was also detected during GCMS analysis). Since literature suggests AJ and DE production depends on oil quality, temperature, time of heating and garlic to MO ratio, therefore, optimization was carried out with respect to maximum AJ and DE synthesis by feeding different experimental data to response surface methodology (RSM) software. The preparation conditions for optimum production of AJ (garlic and mustard oil ratio 1:2, $55^{o}C$, 4.5 h) and VD (garlic and mustard oil ratio 1:2.22, $77.51^{o}C$, 2.5 h) were standardized. The optimum condition suggested by RSM was validated and used in all future experiments. The concentration of ajoene and vinyl dithiin in optimized GMM was found to increase by 1.75 and 1.83 folds then compared to the initially prepared GMM. This experiment optimized the traditional preparations with respect to two secondary compounds (AJ & DE) production.

Traditionally the GMM oil is applied on the nasal opening with the hope that the VOC will reach the nasal cavity and sinus and prevent cold-related secondary infections caused by bacteria and fungus. To establish the hypothesis, VOCs in optimized GMM were tested against bacteria and fungus. Against *Staphylococcus aureus*, GMM VOC was found to be decreasing staphyloxanthin (SX) which is a potent antibiotic resistance imparting molecule by 44.23 ± 0.14 % compared to MO. The decrease in SX was quantified using UV-visible spectrophotometry, FTIR (SX methanolic extract & bacterial pellet) and scanning electron

microscopy (SEM) analysis. During FTIR analysis, a significant increase in the transmittance was observed at 1645 cm^{-1} (C=C stretching in alkenes present in carotenoids), 1077 cm^{-1} (C-O stretching in ether group or secondary alcohol in staphyloxanthin) and 773 cm^{-1} (C-H bending in hydrocarbon backbone of staphyloxanthin) in the methanolic extracts. For the FTIR analysis of the bacterial pellet, a significant increase in the transmittance was observed for GMM-treated SA at 2947 and 2835 cm^{-1} (C-H stretching for fatty acids in lipids found in cell membrane of *S. aureus*), 1656 cm^{-1} (amide I (C=O) for peptide bond in bacterial cell protein), 1450 cm^{-1} (CH_2 and CH_3 bending found in lipid and proteins) and 1027 cm^{-1} (C-O stretching found in polysaccharide in peptidoglycan layer). During the SEM analysis, the GMM-treated *S. aureus* cells showed to have elongated in size, fused and lost the ability to form a spherical shape which might be due to the increase in membrane fluidity.

Antibiotic sensitivity of *S. aureus* (SA) under the exposure of GMM VOC to three antibiotics (gentamycin, kanamycin, tetracycline, 10 ng each) were tested and found to be significantly higher than untreated and MO VOC treated cells. Similarly, against *Candida albicans* (CA) high antifungal activity was recorded which is optimized using agar diffusion and vapour diffusion assay along with minimum inhibitory concentration (MIC=GMM/8 dilution) and minimum fungicidal concentration (MFC=GMM/4 dilution) determination. Bioactivity against SA and CA was validated by *in-silico* molecular docking with literature-suggested molecular targets with GMM compounds detected from GCMS and LCMS analysis. This research successfully establishes the possible role of the VOCs in the traditional preparation of GMM in reducing microbial load in nasal and sinus cavities with a hint of possible mechanisms. Similarly, biofilm production inhibition was also found to be positive with GMM against several bacterial species such as *S. aureus*, *Pseudomonas aeruginosa and Chromobacterium violaceum*.

Traditionally people apply GMM oil also in the top of the skin covering the nose and believe it provides relief in nasal congestion which leads to the opening of the nasal passage. During cold this nasal congestion mostly happens due to acute inflammation and steroidal anti-inflammatory spays are found to be able to reduce the congestion quickly via anti-inflammatory activity. Therefore, it is important to examine whether GMM has anti-inflammatory activity or not. The anti-inflammatory testing on the THP-1 cell line also demands a cytotoxicity assay. Therefore, cytotoxicity against HEK 293 and THP-1 cells and then the anti-inflammatory activity of GMM on cell lines were tested with respect to the expression of pro-inflammatory cytokines and Cox2 during lipopolysaccharide (LPS) induced inflammation. The IC_{50} values

of GMM against HEK 293 and THP-1 cells were found to be >400 µg/ml. During the anti-inflammatory activity, GMM (200 µg/ml) significantly down-regulates the expression of all the selected cytokines than that of LPS-treated cells. Also, MO (200 µg/ml) significantly down-regulates the expression of all the expression compared to the LPS-treated cells except Cox2. Again, GMM down-regulates the expression of IL1β, IL8 and Cox2 expression compared to the MO-treated cells, whereas MO down-regulates the expression of TNFα and IL-6 expression compared to the GMM-treated cells. The possible mechanism behind the anti-inflammatory activity of GMM is also evaluated by carrying out an in-silico molecular docking study of GMM compounds with the selected pro-inflammatory cytokines (IL1β, IL8, Cox2, TNFα, IL-6) of inflammatory response pathways. This finding establishes the possible positive role of GMM in reducing cold-based congestion and scientific validation of the traditional practice.

Traditionally GMM is also applied as massage oil in the palm and feet along with the back part of the body as part of cold and fever relief. This suggests that it is necessary to study whether GMM compounds can pass through the skin barrier or not. This study is also necessary to establish anti-inflammatory activity in the nose as GMM is applied on the top of the nose skin and the site of inflammation is inside the mucosal membrane. Permeability through the skin for any compound is studied under transdermal activity. Before carrying out human trials, permeability activity at the preliminary level is studied on the eggshell membrane as this membrane mimics some of the characteristics of human skin. Therefore, the transdermal permeability of the GMM oil through the egg membrane is studied and found that the oil diffuses through the membrane. The GMM compounds (AJ and VD) are also found to be permeable through the egg membrane as identified by LC-MS without hampering the membrane structure.

The physicochemical analysis of the optimized GMM was carried out to see the quality of the oil and the degradation occurred during the preparation process. The physicochemical properties of GMM oil were found to be in the range standardized by the USDA and FSSAI for edible cooking oil. Finally, a sensory accessibility test for the GMM oil was carried out with 30 volunteers using a 9-point hedonic scale for parameters such as colour, aroma, flavour and overall acceptability. During the experiment, it was found that the volunteers found the flavour and aroma of the GMM oil significantly better than MO.