ASSESSMENT OF PHYTO-PHARMACEUTICAL COMPOUNDS PRESENT IN THE FOOD-BASED MEDICINAL FORMULATION OF MACERATED GARLIC (*Allium sativum* L) IN BOILED MUSTARD (*Brassica nigra* L.) OIL

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Chapter 6

Conclusion and Future Perspectives

6.1. Conclusion

The biochemical composition of mustard seeds and garlic cloves was found to be within the range of earlier reported findings for fresh garlic and dried mustard seeds. During the physicochemical analysis, except for peroxide value, all the other parameters such as acid value, saponification value and iodine value were found to be in the range of earlier reports for mustard oil. For the peroxide value, GMM had shown a significantly lower amount of peroxide value than HMO which may be due to the presence of garlic compounds in GMM which scavenges the peroxides.

During the phytochemical analysis of GMM, GCMS analysis showed the presence of allyl isothiocyanate (AITC), 1-butene-4-isothiocyanato and 2-vinyl-4H-1,3-vinyl dithiin to be the major phytochemical other than the fatty acids. Again, during LCMS analysis ajoene, allicin, gallic acid, ajoene dimer and 2-vinyl-4H-1,3-vinyl dithiin were detected. More garlic-derived compounds were detected during LCMS analysis compared to GCMS analysis due to the low thermostability of the compounds. Therefore, LCMS analysis is recommended for the identification of garlic-derived compounds.

During total polyphenolic assay and DPPH scavenging activity, GMM has shown significantly higher activity than compared to MO which may be due to the presence of garlic-derived compounds in GMM oil.

Earlier, the preparation method optimization of GMM was not been reported nor any traditional preparation process is mentioned in any literature. During the present research, based on the overall optimization of GMM to antibacterial activity, antifungal activity, DPPH scavenging activity and amount of ajoene and 2-vinyl-4H-1,3-vinyl dithiin, the final selected optimized condition for preparation of GMM was heating the macerate at 55^{o}C for 2 hours at the ratio of 1:2 of garlic and mustard oil.

The optimized GMM vapour was found to inhibit the growth of *S. aureus, B. cereus, K. pneumoniae* and *E. coli* then compared to MO vapour during vapour diffusion assay. Again, during the agar diffusion method, the GMM has shown a bacteriostatic effect against the four bacterial strains rather than a bactericidal effect. It was observed that GMM vapour was able to inhibit the production of staphyloxanthin in *S. aureus* which was confirmed using UV-vis spectroscopy and FTIR analysis. The reduction in

staphyloxanthin production was found to make the bacteria more susceptible to antibiotics. SEM analysis showed increased membrane fluidity of *S. aureus* cells after exposure to GMM vapour.

During the antibiofilm assay, the GMM oil was found to inhibit the biofilm production in *S. aureus* and *P. aeruginosa*. Also, it was seen that the vapour of GMM inhibits the production of pyocyanin and violacein which are known as marker pigments produced during quorum sensing by *P. aeruginosa* and *C. violaceum*.

For the first time, the study demonstrates a significant antifungal activity of optimized GMM compared to MO against *C. albicans*. Potent antifungal activity of GMM during vapour diffusion assay of optimized GMM may help in developing aroma therapy against upper and lower respiratory tract fungal infections in humans. Additionally, SEM analysis during the direct contact testing verified that *C. albicans*' cell walls were destroyed; as a result, GMM oil may be employed as gel, semi-solids and ointments to test its effectiveness against fungal skin infections. Also, a detailed mechanism study of the compounds present in GMM against *C. albicans* needs to be carried out for the preparation of potential antifungal drugs. The *in-silico* molecular dynamics and membrane dynamics study may also be carried out with the compounds present in the GMM for a better understanding of the molecular interactions.

The results of the cell viability assay indicate that the HEK and THP1 cell lines demonstrated minimal cytotoxicity upon exposure to the highest dose of both MO and GMM oil. The ADME and drug-likeness analysis conducted revealed that none of the eight selected biomolecules violated Lipinski's rule of five. In the context of molecular docking studies, it was observed that sinigrin, vinyl dithiin, ajoene and allicin exhibited the lowest binding energy (expressed in kilocalories per mole) while interacting with a significant proportion of the target protein receptors, including COX2, IL-6, IL-8, TNF α and IL-1β. Surprisingly, it was observed that the downregulation of genes exhibited a similar pattern to the binding energy of biomolecules with proteins as determined through molecular docking investigations. The garlic mustard oil macerate at a concentration of 200 µg/ml as a pre-treatment resulted in a significant reduction in the expression levels of COX-2, IL-1β, TNF-α, IL-8 and IL-6 in the THP-1 monocyte cell line, as compared to the inflammation induced by LPS.

Based on the findings obtained by *in-vitro* and *in-silico* analyses, it was discovered that GMM exhibits superior anti-inflammatory capabilities in comparison to mustard oil. This behaviour may have been detected because of the presence of garlic-derived compounds in the macerate, in conjunction with mustard oil components. However, more validation is required through the testing of individual compounds in isolation or different combinations. In subsequent investigations, it is imperative to elucidate the molecular processes underlying the anti-inflammatory effects of GMM and MO in cellular models. Additionally, the macerate of garlic-mustard oil exhibits significant potential as a novel bio-product for the treatment of nasal congestion. It is imperative to conduct a comprehensive investigation of the stability of the anti-inflammatory property of the macerate under various conditions, including storage time and temperature.

During the transdermal assay, the garlic-derived compounds ajoene, 2-vinyl 4H-1,3-dithiin and allicin present in GMM were found to get transferred through the eggshell membrane which was confirmed by LCMS analysis. Therefore, the traditional practice of massaging the macerate oil in various parts of the body during the common cold may also diffuse the garlic-derived compounds present in the macerate due to the presence of mustard oil which is transdermal.

During the sensory test, the flavour, aroma and overall acceptability of the GMM were rated higher than to mustard oil.

6.2. Future Prospects

Regarding the present research finding, it has been observed that the volatiles of optimized garlic mustard oil macerate preparation have potent effects on antifungal activity. Therefore, in the future, more detailed research may be carried out on different kinds of fungal species (including pathogenic) along with pure compounds from GMM. As the optimization of the GMM preparation was established in the present findings therefore by using techniques such as supercritical CO_2 extraction can be carried out for the higher purity, eco-friendly extraction of the heat-sensitive thiosulfinate compounds. For scale-up purposes strategies such as pilot-scale trials using continuous extractors or supercritical extractors can be utilized. If the stability of the macerate can be achieved in the future for a longer duration, then a viable commercial antifungal and

anti-inflammatory product may be developed. The above future study will be more fruitful if carried out using animal models. The GMM vapour is also found to be potent in staphyloxanthin production inhibitor (44.23 ± 0.14% inhibition), therefore further analysis may be carried out using the same approach as presented above for the antifungal study. GMM was also observed to have a downregulatory effect on some genes coding for pro-inflammatory cytokines, but their application feasibility in animal models and human experiments for reducing nasal congestion during cold may be evaluated in the future. In the present research, we fall short of obtaining the ethical permission for carrying out research by applying GMM on the nasal orifice as well as inhaling GMM vapour by human volunteers to establish traditionally claimed nasal congestion relief during cold. The same experiment may be carried out in the future if ethical permission is obtained.