Chapter 3

Materials and Methods

The materials used in the present investigation and their details are presented below:

3.1. Collection of mustard (*Brassica nigra*) seed and garlic (*Allium sativum*)

The *Brassica nigra* seeds were collected from Amolapaam village, Napaam, near Tezpur University (Table 1).

Table 1: Details of the collection location of *Brassica nigra* seeds	
Date	23/01/2020
Location	Amolapaam, Napaam, near Tezpur University
GPS	Latitude: 26; 41; 39.497900000002204 Longitude: 92; 50; 0.988699999987119 Altitude: 15.534 m
Species	*Brassica nigra* (L.) Black mustard

The garlic (*Allium sativum*) cloves were obtained from the local market of Tezpur, Assam, India.

3.2. Biochemical composition of mustard (*Brassica nigra)* Seeds and Garlic (*Allium sativum*)

3.2.1. Moisture content

In a weight balance, 2.0 grams of mustard (*Brassica nigra)* seeds were measured and kept in a glass petri plate at 95- 100^{o} C under pressure ≤ 100mm Hg for 5Hrs (AOAC Official Method 934.01) [1]. The loss of weight to reach a constant weight on dryness was reported as moisture content.

$$\text{Moisture content} = \frac{\text{Weight loss on dryness (g)}}{\text{Weight test portion (g)}} \times 100$$

3.2.2. Percentage of oil in mustard seeds

The percentage of oil yield was calculated by using the following equation,

$$\text{Percentage yield of oil} = \frac{\text{Weight of oil X 100}}{\text{Weight of the sample (g/seeds)}}$$

3.2.3. Ash content

Brassica nigra seed and *Allium sativum* clove samples were burnt to a constant weight in an oven set at 600°C weighing between 3–4 g. The total amount of ash from the weighing differences will be calculated as a weight percentage [1].

3.2.4. Total Carbohydrate

The total carbohydrate content in both mustard seeds and fresh garlic cloves was estimated by the Anthrone method with some slight modifications [2, 3]. One gram of the sample was ground in a mortal pestle and 25 mg of the sample was taken into a boiling tube. Acid hydrolysis of the sample was carried out by boiling in a water bath for 3 hours with 2.5 ml of 2.5N HCl (Merck) and allowed to cool at room temperature. The samples were neutralized with solid Na_2CO_3 (Merck) until effervescence (ceased). The volume of the sample was made up to 50 ml with distilled water and then centrifuged at 3000g for 10 minutes. The supernatant was collected. From the collected supernatant, 0.5 ml and 1 ml samples were taken in a test tube for carbohydrate analysis. The standard preparation was done by taking 0, 0.2, 0.4, 0.8 and 1.0 ml from the dextrose (Merck) stock solution (1mg/ml) with distilled water taken as control. The volume was made up to 1 ml in all the test tubes with distilled water and 4 ml of Anthrone (Merck) reagent (Appendix 1) was then added into the test tubes. The samples were boiled in test tubes in a water bath for 8 minutes. The tubes were then cooled down rapidly and reading was taken at 630 nm wavelength in a spectrophotometer (Thermo Scientific, USA). The experiment was conducted in triplicates.

3.2.5. Crude Fibre content

The crude fibre content of mustard seeds and garlic cloves was estimated following the ISO 6541 [4]. The test samples were first dried to remove the moisture content and then ground to fine powder (as mustard seed consists of more than 10% fatty matter therefore extraction of fatty matter was carried out before the experiment, Appendix 1). Three grams of the test sample was taken and acid and alkaline treatments were carried out. For acid treatment, 200 ml of sulfuric acid (Merck) was mixed with the test sample. A condenser was attached and the mixture was boiled for about 30 minutes. After boiling, 50 ml of cold water was mixed into the mixture and the insoluble residue was separated. The process was repeated with the insoluble residue until the filtrate was neutral to litmus paper. Then the insoluble residue was separated and washed. Next, 200 ml of sodium hydroxide (Merck) was added to the washed residue collected

earlier. Then the mixture was boiled similarly as carried out during acid treatment. After boiling, 50 ml of cold water was added and the insoluble residue was separated. The residue was again washed with 25 ml of sulfuric acid solution (12.5 g of H_2SO_4/L). The residue was again washed with water and collected in the crucible. The residue was then dried in an oven at 130 ± 2 ^oC. The residue was cooled down. After drying, the residue was incinerated in a muffle furnace at 550 ^oC to constant mass. The samples were then weighed.

3.2.6. Crude Fat

The crude fat of both garlic and mustard seeds was determined by the AOAC standard method [1]. Two grams of the ground test sample was taken in a 50 ml beaker and 2 ml alcohol was added and stirred. Ten millilitres of HCl (Merck) were added to the mixture, mixed well and set in a water bath at 70-80 ^oC with frequent stirring during 30-40 minutes. After heating, 10 ml of alcohol was added to the mixture and allowed to cool down. In a Mojonnier fat-extraction apparatus (ABGIL brand) the mixture was transferred and shacked vigorously for 1 minute. Then, 25 ml of petroleum ether (bp<60^oC, HPLC grade, Merck) was added, mixed and centrifuged for 20 minutes at 600 rpm (Thermo Scientific Heraeus Multifuge X1R, USA). The ether-fat part was isolated and repetation of the procedure was carried out until the ether was clear.

3.2.7. Crude protein

The Crude protein in garlic cloves and mustard seed was estimated by the Kjeldahl method AOAC 2.057 [1].

3.3. Physicochemical examination and phytochemical analysis of oil from GMM

3.3.1. Preparation of Garlic mustard oil macerate (GMM)

GMM was prepared according to the process mentioned by Yoo et al. (2012) with some minor modifications [5]. In short, garlic cloves were crushed and paste was made using a mixer grinder (Usha make, Smash, India). The garlic paste was allowed to stay for 10 minutes before mixing with mustard oil (10.0 g/ 40.0 ml) followed by heating (80°C) using a water bath (Hoefer RCB20 PLUS, USA) for 4 hours. After completion, the tube was centrifuged (Thermo Scientific Heraeus Multifuge X1R, USA) at 4000g for 10 minutes. The GMM preparation ends. The GMM/ MO was cooled down for 30 mins and then used

for phytochemical analysis and bioactivity against bacteria, fungus, cytotoxicity, anti-inflammatory activity.

3.3.2. Preparation of garlic toluene extract (GTE)

A normal kitchen blender (Usha Make, Smash) was used to shred 150.0 g of garlic cloves and 300.0 ml of toluene (Merck) was added. After being extracted for the entire night, the filtrate was run through filter paper. Before the two phases formed, 150.0 ml of HPLC grade water (Merck) was mixed and mixed for 24 hours at room temperature. The organic phase was gathered and dried for the current investigation. Following sterile filtration, the dried extract (830.0 mg) was reconstituted in one ml of acetonitrile and used for HPLC analysis [6, 7]. The OSCs in the garlic toluene extract were identified using HPLC and LCMS and the identified compounds were quantified using the peak under the area.

3.3.3. Physicochemical analysis of GMM oil

3.3.3.1. Chemical properties

3.3.3.1.1. Free fatty acid

The oil (2.0±0.2 g) from MO and GMM was taken in a conical flask and 100.0 ml ethanol (Merck) was added and mixed thoroughly which was followed by adding a few drops of phenolphthalein (Merck) [10]. Titration of the solution was then done using the 0.1 N NaOH (Merck) solution with continuous shaking until a slight pink colour persisted.

$$\textbf{Free fatty acid} = \frac{(\boldsymbol{V-B})\times\boldsymbol{M}\times\mathbf{28.2}}{\boldsymbol{W}}$$

V-Volume of NaOH (ml), B- Volume of blank, M- Molarity of NaOH, 28.2- Molecular weight of oleic acid divided by 10, W- Weight of the oil sample.

3.3.3.1.2. Peroxide value

About 5.00 ± 0.05g of mustard oil is weighed and taken in a 250.0 ml conical flask. Then 30.0 ml CH_3COOH-$CHCl_3$ (3:2) was added to the flask and mixed properly to dissolve the oil. Saturated potassium iodide (Merck) solution (Appendix 1) (0.5 ml) was then added and let stand with occasional shaking for 1

minute and 30.0 ml water was mixed into the mixture. Slow titration was done with 0.1 M $Na_2S_2O_3$ (RFCL) with vigorous shaking until the yellow colour was almost gone. After the colour was gone, 0.5 ml 1% starch (Himedia) solution was added and titration was continued with vigorous shaking to release all I_2 (Merck) from $CHCl_3$ (Merck) layer, until the blue colour disappeared (If <0.5 ml 0.1M $Na_2S_2O_3$ is used. Repeat determination with 0.01M $Na_2S_2O_3$). Blank determination was conducted (must be ≤0.1 ml of 0.1M $Na_2S_2O_3$) and subtracted from test portion titration [1].

$$\textbf{Peroxide value}\left(\textbf{milliequivalent}\frac{\textbf{peroxide}}{\textbf{kg}}\textbf{oil or fat}\right) = \frac{\textbf{S} \times \textbf{M} \times \textbf{1000}}{\textbf{g test portion}}$$

Where S- ml $Na_2S_2O_3$ (blank corrected), M- molarity $Na_2S_2O_3$ solution.

3.3.3.1.3. Acid value

Mustard oil and garlic mustard oil macerate were weighed (7.05 g) and taken in a 250.0 ml flask. Then, 50.0 ml alcohol was added which was neutralized by adding 2.0 ml phenolphthalein solution. Titration of the solution was done using 0.25 M NaOH until a faint pink colour persisted for more than 1 minute [1].

$$\textbf{Acid Value} = \frac{\textbf{V} \times \textbf{N}}{\textbf{W}}$$

V- Volume in mL of standard potassium hydroxide or sodium hydroxide used, N- Normality of potassium hydroxide or sodium hydroxide and W- Weight of the sample in grams.

3.3.3.1.4. Iodine value

The mustard oil was passed through the filter paper for the removal of any impurities. The filtered test sample was cooled to 68-71^{o}C and then immediately weighed grams of the sample was poured into a conical flask. About fifteen ml of cyclohexane-acetic acid solvent was added to the oil and swirled

to ensure that it was completely dissolved. About 25.0 ml of Wijs solution (Himedia) was added to the stopper flask containing the oil sample. The flask was then kept for 1-2 hours in the dark at 25± 5ºC. After removal of the flask, 20.0 ml of KI solution was added and mixed properly and 150.0 ml of distilled water was added, mixed and titrated with 0.1 M standard $Na_2S_2O_3$ solution. After the yellow colour disappeared, 1-2 ml of starch solution was added and titration was done until the blue colour disappeared [1].

$$\textbf{Iodine Value (IV)} = \frac{\textbf{(B - S) x M x 12.69}}{\textbf{weight of fat or oil}}$$

Where B- titration of blank (ml), S- titration of test solution (ml) and M- molarity of $Na_2S_2O_3$

3.3.3.1.5. Saponification value

Accurately 5 grams oil was filtered into 250-300 ml Erlenmeyer flask and 50.0 ml alcoholic potassium hydroxide (Merck) solution was pipetted into the flask. The flask was connected to the condenser and boiled until the oil was completely saponified. The sample was cooled and titrated with 0.5 M HCl using phenolphthalein. The same procedure was conducted for blanks [1].

$$\text{Saponification Value} = \frac{28.05\ (\text{B} - \text{S})}{\text{Amount of oil (grams)}}$$

Where B- Amount of 0.5M HCl required by blank and S- Amount of 0.5 M HCl required for test portion.

3.3.3.2. Physical properties

3.3.3.2.1. Determination of the specific gravity of mustard oil and GMM

The specific gravity of MO and GMM was determined by the AOAC method (1990) [1].

$$\text{Specific gravity} = \frac{\text{Density of oil sample (W3} - \text{W1)}}{\text{Density of water (W2} - \text{W1)}}$$

3.3.3.2.2. Determination of viscosity of mustard oil and GMM

The viscosity of the GMM oil was determined using an Ostwald-type gravity flow viscometer using the method described by Fountain et al. (1997) [8].

3.3.3.2.3. Determine the refractive index of mustard oil and GMM

The refractive index of mustard oil and GMM oil will be determined by an automatic digital refractometer (Holmarc Opto-Mechatronics Pvt Ltd.) [9].

3.3.4. Phytochemical analysis

3.3.4.1. FTIR analysis

The mustard oil and garlic mustard oil macerate were combined with potassium bromide (Merck). The produced KBr staphyloxanthin pellets' FTIR spectra were recorded using an FTIR instrument (impact 410, Nicolet, USA). The spectra were scanned with a step size of 1.0 cm-1 in the 4000-400 cm-1 band. The transmittance versus wave number FTIR spectra were plotted [10].

3.3.4.2. Total polyphenol content

Oil from MO/GMM (1.0 ml) was mixed with 5.0 ml of Methanol and vortexed and mixed for 30 min at 141 rpm [11]. The supernatant was collected after centrifugation of the mixture at 3000g for 15 min. In the supernatant (10 µl), 0.6 ml of distilled water was added and 50 µl of 2N Folin Ceocalteu (Himedia) was mixed and vortexed. After 4 minutes, 0.15 ml of 0.75% sodium carbonate (Merck) was added and the volume was adjusted to 1 ml by distilled water and kept in the dark for 2 hours. After the incubation, the absorbance was taken at 750 nm. The experiment was conducted in triplicate.

3.3.4.3. Antioxidant activity

Mustard oil/ garlic mustard oil macerate is mixed with methanol (1:1) (500 µl: 500 µl) [12]. The mixture was vortexed for 10 sec, shaken at 141 rpm for 30 minutes and shaken for 10 sec. Centrifuged at 3000 g for 15 minutes. The hydrophilic fraction (HF) was collected and the lipophilic fraction (LF) was dissolved in ethyl acetate (Merck). Both the

HF and LF parts were tested for DPPH scavenging activity. Extract (HF, LF and GMM) (10 µl) was mixed with 1 ml of DPPH (Merck) (0.04mM) and incubated at RT for 1 hour. The absorbance was taken at 517 nm. The experiment was conducted in triplicate.

3.3.4.4. Thin Layer Chromatography

The TLC plate (Silica 60 F_{254} TLC, Merck) was activated by heating at 80^{o} C in a hot air oven (Genaxy, India). The GMM, MO and raw garlic in ethyl acetate were loaded in the plate along with the standard and then dried. The plates were then developed in HPLC-grade ethyl acetate. After the plate was developed, it was dried and then visualized under UV light and stained with I_2.

3.3.4.5. High-Performance Liquid Chromatography

The GMM oil was homogenized in acetonitrile in a proportion of 1+5 (w/v) for 30 minutes. The mixture was centrifuged at 3000 g for 20 minutes at 4^{o}C and, the supernatant was collected. The extraction was repeated 3 times. The supernatants were pooled together. The extract was then filtered through a 0.2 µm PTFE membrane filter (Himedia). The garlic mustard oil sample was analyzed by analytical high-performance liquid chromatography (Waters Corporation, USA) using a C18 column (150 x 4.6mm) [13]. The eluent was acetonitrile: water (70:30) at a flow rate of 0.9 ml/min. The eluate was monitored for its purity as well as its identity at 254 nm in the UV detector.

3.3.4.6. Liquid chromatography-mass spectrometry (LCMS) analysis

The LCMS analysis was performed on an Agilent 6100, Germany with a C18 reverse phase column (2.1X 50 mm, 1.8 µm). The sample elution was performed in an isocratic manner with LCMS-grade water and acetonitrile in a 30:70 proportion for 15 minutes. The water consists of 0.1% formic acid and ammonium format respectively. A sample volume of 5 µL was injected, maintaining a flow rate of 0.2 ml/minute. The ionization was done with positive ESI mode for the mass range of 100-1000 m/z [14]. The LCMS results were analyzed with the help of MestReNova software v14.1.1-24571 [15].

3.3.4.7. Gas chromatography-mass spectrometry (GCMS) analysis

The chemical analysis of the oil of MO and GMM constituents was performed according to the method suggested by Sobrinho and team using an Agilent 8890 GC for gas chromatography and 59777B GC/MSD for mass spectrometry with slight modifications [16]. Column: Rtx-5MS (Cross bond, 5% diphenyl/95% dimethyl polysiloxane) measuring 30m x0.25 mm x 0.25 m df; carrier gas: He (24.2 mL/min, in constant linear velocity mode); injector temperature of 250 ^{0}C, split mode (1:100); and detector temperature of 250 ^{0}C. The column temperature was set at 35-180 ^{0}C at 4 ^{0}C each minute, then 180-280 ^{0}C at 17 degrees Celsius per minute, then 280 ^{0}C for 10 minutes. At an electron impact of 70 eV, mass spectra were obtained. One µl of oil was injected. The chemicals were identified by comparing their mass spectra to those in the National Institute of Standards and Technology (NIST) and published literature.

3.4. Optimization of garlic mustard oil macerate

3.4.1. Optimization of garlic mustard oil macerate using response surface methodology

3.4.1.1. Experimental design and process optimization

RSM was applied to optimize the conditions for optimum bioactive compound production [17]. The experimental design was developed using CCD [18]. The CCD in the experimental design consisted of 23 factorial points, 6 axial points (α=2) and 3 replicates of the central points (Table 2). All the 17 samples were used for HPLC analysis for quantification. The independent variables were temperature (X1, ℃), reaction time (X2, hours) and oil volume (X3, multiplied by raw garlic weight) and the dependent variables are ajoene (Y1, µg/g) and 2-vinyl-4H-1,3, dithiin (Y2, µg/g) (Table 3). The coding of the variables was conducted using the formula mentioned below:

$$xi = \frac{(Xi - Xo)}{\Delta Xi}$$

Where xi is the coded value of an independent variable, Xi is the real value of an independent variable, Xo is the real value of an independent variable at the centre point and ΔXi is the step change value.

For RSM procedure experimental data were analysed using Design Expert 13 [19]. The quadratic polynomial equation is:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

Where Y is the dependent variable of ajoene content; βo is a constant, βi, βii and βij are regression coefficients and Xi, Xj are levels of the independent variable. The statistical analysis of the model was performed by analysis of variance (ANOVA). The significance of each term in the polynomial was assessed statistically by the F-value at a probability (P) of 0.01 or 0.05. The three-dimensional response plots were generated using Design Expert 13 software.

Table 2: The independent variables used during RSM represented Coded and uncoded format

Symbol	Independent variables	Code variable levels				
		-2	-1	0	1	2
X1	Temperature (°C)	20	40	60	80	100
X2	Time (Hours)	2	4	6	8	10
X3	Oil volume (multiplied by the weight of Garlic in grams) (ml)	1	2	3	4	5

Table 3: Representing 17 different conditions for the garlic mustard oil macerate preparation designed by Central Composite Design.

Sl. No.	Factor 1 Temperature (°C)	Factor 2 Time (hours)	Factor 3 Oil volume (ml)
1	55.00	4.50	4.00
2	70.00	3.00	3.00
3	85.00	4.50	2.00
4	100.00	3.00	3.00
5	70.00	3.00	3.00

6	55.00	4.50	2.00
7	55.00	1.50	4.00
8	85.00	1.50	2.00
9	70.00	6.00	3.00
10	40.00	3.00	3.00
11	85.00	1.50	4.00
12	70.00	3.00	3.00
13	70.00	3.00	5.00
14	70.00	3.00	1.00
15	70.00	0.00	3.00
16	85.00	4.50	4.00
17	55.00	1.50	2.00

3.4.1.2. High-Performance Liquid Chromatography activity analysis of experimental designs

The methodology mentioned in section 3.3.15 was used for HPLC analysis. The extract was then passed through a 0.22 µm nylon-66 syringe filter and used for HPLC analysis. Upon the LCMS analysis of the macerate, we found the signature of a protonated cyclic dimer and a cyclic trimer of polyamide 66 with an m/z value of 453.3 and 679.4, respectively which was the contamination from the nylon-66 syringe filter (Figure 2) [20]. Therefore, afterwards, we used a 0.22 µm PTFE (Himedia) syringe filter for all the experiments.

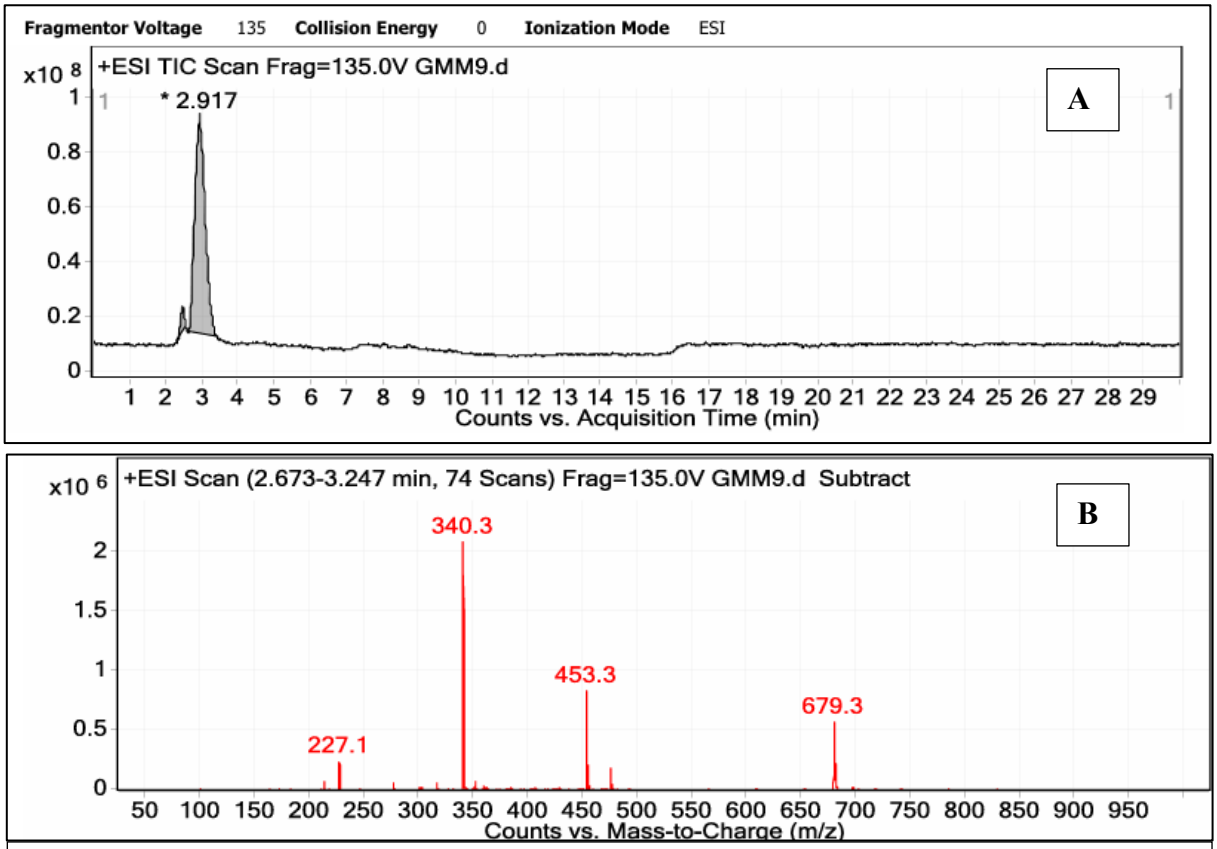

Figure 2: (A) Liquid chromatogram of GMM, (B) mass spectrometric data at retention time between 2.67 and 3.24 minutes.

3.4.2. Optimization of GMM based on antibacterial activity and antifungal activity

3.4.2.1. Quantification of the amount of GMM required for antibacterial and antifungal activity during vapour diffusion assay

Vapour diffusion assay was carried out by the method suggested by Clemente et al. (2016) with slight modification [21]. For the antibacterial assay, on Mueller Hinton Agar (MHA) plates 100 µl of bacterial broth (10^8 CFU/ ml bacterial cells) was spread. Various volumes (500, 250, 100, 50 µl) of GMM/MO oil was loaded on the autoclaved filter paper and the lid was closed and the plate was sealed with two parafilm. For the antifungal activity, the method suggested by Magaldi et al. [22] was used. At first, a Petri plate with Sabouraud dextrose agar (SDA) was inoculated with 100 µL of fungal culture (0.5 MacFarland). Then, in the middle of the Petri plate lid, autoclaved filter paper (Whatman No.1) was placed and different amount of the GMM sample was poured (1000, 500, 250, 100 µl) and the Petri plate was then

sealed with two parafilm tapes and incubated at 35 °C. After the incubation of 24 hours. Mustard oil was taken as control and for blank, no sample was given in the lid. The experiment was repeated three times.

3.4.2.2. Optimization of GMM based on antibacterial activity against *S. aureus*

3.4.2.2.1. Antibacterial activity by vapour diffusion method

Vapour diffusion was carried out by the method suggested by Clemente et al. 2016 with slight modification [21]. On the MHA (Himedia) plates, 100 µl of bacterial broth (10^8 CFU/ ml bacterial cells) was spread. With a micropipette, 750 microliters of oil were loaded in an autoclaved filter paper (Whatman no. 1) on the lid of the Petri plate (Tarsons) and the lid was closed and the plate was sealed with two parafilm (Bemis). The plates were incubated at 37^o C for 24 hours.

3.4.2.3. Optimization of GMM based on antifungal activity against *C. albicans* MTCC 410

3.4.2.3.1. Antifungal activity by vapour diffusion method

Vapour diffusion was carried out by the method suggested by Clemente et al. 2016 with slight modification [21]. On the Sabouraud dextrose agar (Himedia) plates, 100 µl of *C. albicans* MTCC 183 broth (10^6 CFU/ ml fungal cells) was spread. With a micropipette, 300 microliters of oil were loaded in an autoclaved filter paper on the lid of the Petri plate the lid was closed and the plate was sealed with two parafilm. The plates were incubated at 28 ^{o}C for 48 hours.

3.4.2.3.2. Antifungal activity by agar diffusion method

Agar diffusion was carried out by the method suggested by Cotton et al. 2019 with slight modification [23]. On the Sabouraud dextrose agar (Himedia) plates, 100 µl of *C. albicans* MTCC 183 broth (10^6 CFU/ ml bacterial cells) was spread. With a micropipette, 30 microliter of oil was loaded over the agar and

the plate was sealed with two parafilm. The plates were incubated at 28 ° C for 48 hours.

3.4.2.4. Preliminary preparation of GMM

The preliminary GMM was prepared as mentioned in Table 4.

Table 4: Describing the parameters for preliminary GMM preparation

Sl No.	Mustard oil (ml)	Garlic (g)	Temperature (°C)	Time of heating (Hr)
1	40	10	80	4 hours
2	40	10	160	1 minute
3	40	10	160	1 minute

3.4.2.5. Preparation of GMM based on the ratio of garlic and mustard oil

Table 5: Details of the preparation method for GMM based on ratio of garlic and mustard oil

Sample ID	Garlic (gram)	Mustard oil (ml)	Ratio	Temperature (°C)	Time of heating
G160/2/1	3	6	1:2	160	1 minute
G160/4/1	3	12	1:4	160	1 minute
G160/18/1	3	24	1:8	160	1 minute
G160/16/1	3	48	1:16	160	1 minute

3.4.2.6. Preparation of GMM based on time of heating of macerate at 160°C

Table 6: Details of the preparation method for GMM based on the time of heating of GMM at 160°C

Sample ID	Garlic (gram)	Mustard oil (ml)	Ratio	Temperature (°C)	Time of heating (second)
G160/4/0	3	12	1:4	160	0
G160/4/30	3	12	1:4	160	30
G160/4/60	3	12	1:4	160	60
GR160/4/120	3	12	1:4	160	120
GR160/4/180	3	12	1:4	160	180

3.4.2.7. Preparation of GMM based on time of heating of macerate at 80°C

Table 7: Details of the preparation method for GMM based on the time of heating of GMM at 80°C

Sample ID	Garlic (gram)	Mustard oil (ml)	Ratio	Temperature (oC)	Time of heating (Hours)
G80/4/0	10	40	1:4	80	0
G80/4/1	10	40	1:4	80	1
G80/4/2	10	40	1:4	80	2
G80/4/4	10	40	1:4	80	4
G80/4/8	10	40	1:4	80	8
G80/4/16	10	40	1:4	80	16

3.5. Bioactivity of optimized GMM

3.5.1. Antibacterial activity

3.5.1.1. Bacterial culture

For the antibacterial assay, four bacterial samples were taken, i.e., *S. aureus* MTCC 3160, *B. cereus* MTCC 430, *K. pneumoniae* MTCC 618 and *E. coli* MTCC 40. All the bacterial samples were stored in glycerol stock at -80 °C. The bacterial culture was maintained in Mueller Hinton broth at 37 °C.

3.5.1.2. Agar diffusion

Agar diffusion was carried out by the method suggested by Cotton et al. (2019) with slight modification [23]. On the Mueller Hinton Agar (Himedia) plates, 100 µl of bacterial broth (10^8 CFU/ ml bacterial cells) was spread. With a micropipette, 30 microliters of oil were loaded over the agar and the plate was sealed with two parafilms. The plates were incubated at 37 °C for 24 hours.

3.5.1.3. Vapour diffusion

Vapour diffusion was carried out by the method suggested by Clemente et al. 2016 with slight modification [21]. On the Mueller Hinton Agar (Himedia) plates, 100 µl of bacterial broth (10^8 CFU/ ml bacterial cells) was spread. With a micropipette, 750 microliters of oil were loaded in

an autoclaved filter paper on the lid of the Petri plate the lid was closed and the plate was sealed with two parafilms. The plates were incubated at 37^{o} C for 24 hours.

3.5.1.4. Minimum inhibitory concentration and minimum bactericidal concentration

The GMM was diluted using MO (as MO did not show any antifungal activity during agar diffusion) to the factors 2, 4, 8, 16 (GMM/2, GMM/4, GMM/8, GMM/16). Agar diffusion was carried out by a method suggested by Cotton et al. 2019 with slight modification [23]. On the MHA (Himedia) plates, 100 µl of bacterial broth (10^8 CFU/ ml bacterial cells) was spread. With a micropipette, 30 microliters of oil were loaded over the agar and the plate was sealed with two parafilms. The plates were incubated at 37 ^{o} C for 24 hours. The lowest dilution of the GMM that inhibited visible bacterial growth was considered the MIC. With the help of a sterile loop, the zone under the oil was pricked and spread in a sterile MHA plate. The concentration where no bacterial colony was seen was the MBC. Gentamycin (Himedia) (10 µg) was taken as a positive control. All the experiment was conducted in triplicate.

3.5.1.5. Gram staining

For gram staining Gram's staining kit (Himedia) was used. The bacteria from near the zone of inhibition was taken from an inoculating loop and was smeared in a glass slide and heat fixed. Followed by the standard gram staining method. The slides were then observed under the microscope (Labomed Vision 2000) at 4X, 10X 40X and 100X (immersion oil).

3.5.1.6. Staphyloxanthin inhibition by GMM against *S. aureus*

The antibacterial assay was carried out using the method described by Clemente et al. 2016 with some modifications [21]. In short, *S. aureus* MTCC 3160 (100 µl, 10^6 CFU/ ml) was spread on MHA petri plates (90 X 15 mm) before boring three wells (6 mm) followed by pouring gentamycin, tetracycline and kanamycin (10 µl; 1mg/ml) in the wells.

The plates were then allowed to incubate for 30 minutes to ensure complete diffusion of the antibiotic solutions at 4^{o}C. These plates were inverted and the lid was loaded with a double-folded, autoclaved filter paper (Whatman no. 1, diameter 125 mm) with 500 µl GMM absorbed into it. The Petri plate was then sealed with two parafilm tapes and incubated at 37^{o}C for 24 hours. This arrangement will allow volatiles from GMM to interact with *S. aureus* above. The zone of inhibition was recorded before carrying out a one-way ANOVA.

3.5.1.6.1. Inhibition percentage of staphyloxanthin

To investigate the suppression of staphyloxanthin by *S. aureus*, bacteria were collected from Petri plates treated with mustard oil and GMM vapour. The bacteria were rinsed with PBS and centrifuged for 10 minutes at 6000 rpm. The staphyloxanthin was extracted from the pallet using methanol (Merck) and the OD was measured at 450 nm with a spectrophotometer (Thermo Scientific Multiskan Go) [24].

% of staphyloxanthin inhibition = [(Control $OD_{462\ nm}$ – Treated OD_{462nm})/ Control $OD_{462\ nm}$] X 100

3.5.1.6.2. Fourier-transform infrared spectroscopy (FTIR) analysis

Alterations in the membrane of *S. aureus* after exposure to GMM and MO vapour were analyzed by FTIR analysis. Treated *S. aureus* MTCC 3160 was collected from the Petri plates washed with PBS (pH 7.0) and centrifuged at 6000 rpm for 10 minutes. The bacterial cells were then subjected to an FTIR spectrometer (NICOLET, USA) and the spectral scan was carried out from the range of 4000-400 cm^{-1} [10]. The treated and untreated bacterial cells' methanolic extracts of staphyloxanthin were combined with potassium bromide (KBr). The produced KBr staphyloxanthin pellets' FTIR spectra were recorded using an FTIR instrument (impact 410, Nicolet, USA). The spectra were scanned with a step size of 1.0 cm^{-1} in the 4000-400 cm^{-1} band.

The transmittance versus wave number FTIR spectra were plotted.

3.5.1.6.3. Scanning electron micrography (SEM) analysis

The treated (GMM/MO) and untreated (control) bacterial cells were rinsed in PBS (pH 7.4) and fixed for 2 hours at 4 C in 2.5% (v/v) glutaraldehyde. After washing the fixed cells in PBS (pH 7.4), they were dehydrated for 5 minutes in escalating acetone concentrations (30%, 50%, 70% and 90% v/v), followed by 1 minute in 100% acetone. Samples were air-dried and mounted on stubs with double-sided carbon tape before being coated with a thin layer of platinum with an ion sputter JFC 1100 and inspected in a JEOL S-3000 H scanning electron microscope at 20Kv [25].

3.5.1.6.4. Molecular docking analysis of GMM volatile compounds with Dehydroxysqualene synthase (*CrtM*) protein

For the docking study of dehydroxysqualene synthase CrtM work done by Negi and the team was followed [26]. The ligand structures (Allyl isothiocyanate (CID: 5971), ajoene (CID: 5386591), allicin (CID: 65036), 2-vinyl-4H-1,3, dithiin (CID: 133337), sinigrin (CID:23682211)) were downloaded from PubChem database. The 3D crystal structure of dehydroxysqualene synthase CrtM (ID: 2ZCO) was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) protein databank. Molecular docking analysis was done by using Autodock Tools v 1.5.7 molecular interaction study PyMol v 2.5.5 and BIOVIA Discovery Studio visualizer v 4.5 [27].

3.5.2. Antibiofilm analysis

3.5.2.1. Screening of bacteria producing biofilm

For screening of the bacteria for biofilm production slime mold production in Congo red agar plate assay was performed. For our study,

we took the four bacterial strains *S. aureus* MTCC 3160, *B. cereus* MTCC 430, *K. pneumoniae* MTCC 618 and *E. coli* MTCC 40.

3.5.2.2. Antibiofilm activity against *S. aureus*

3.5.2.2.1. Agar diffusion assay

The experiment was carried out in a similar manner as that mentioned in section 3.5.1.2 except Congo red agar was used.

3.5.2.2.2. Vapour diffusion assay

The experiment was carried out in a similar manner as that mentioned in section 3.5.1.3 except Congo red agar was used.

3.5.2.2.3. Ring-biofilm inhibition assay

A loop full of overnight culture of *S. aureus* was added in trypticase soy broth with 1% glucose and different concentration of GMM and MO was added to the media (6.25 to 100 µl/ml) and incubated for 24 hours at 37^{o}C with shaking at 225 rpm. The media was removed and the tube was washed with PBS (pH 7.4). By using crystal violet (0.1%) the tube was stained and the tubes were allowed to dry after the excess stain was decanted. In an inverted position, the tubes were then air-dried and biofilm ring formation was observed [28].

3.5.2.3. Antibiofilm activity against *Pseudomonas aeruginosa* MTCC 2297

3.5.2.3.1. Vapour diffusion assay

The experiment was carried out in a similar manner as that mentioned in section 3.5.1.3.

3.5.2.3.2. Microscopic analysis

The antibiofilm activity of GMM was validated through light microscopy [29]. In a 24-well plate, *P. aeruginosa* was cultured containing a 1 x 1 cm glass slide for biofilm production. During the inoculation, GMM/MO oil (100 µl/ml) was added to the well. After 48 hours of incubation, the glass slides were washed with PBS and stained with 0.4% crystal violet stain and then observed under a light microscope (Labomed, Vision 2000) at 400X magnification. DMSO (1%) was taken as a negative control.

3.5.2.3.3. Estimation of total carbohydrate in EPS

In 15 ml falcon tubes, *P. aeruginosa* was cultured in LB broth with GMM/MO treatment of 100 and 200 µl/ml concentration. For the isolation of EPS from the biofilm, the planktonic cell was washed with normal saline. Then the EPS sample (100 µl) was dissolved in 900 µl of water 5 ml of 98% concentrated sulfuric acid and 1 ml of phenol was added to the test tubes. The yellow colouration was then measured spectrophotometrically at 490 nm with H_2SO_4 and phenol as a blank [30].

3.5.2.3.4. Pyocyanin inhibition

For the quantification of pyocyanin, the *P. aeruginosa* culture was centrifuged at 10,000 rpm for 15 minutes. Then, in 5 ml of the supernatant, 3 ml chloroform was added which was followed by re-extraction with 1 ml of 0.2N HCl. The colour change was detected using a spectrophotometer at 520 nm [30].

3.5.2.4. Violacein inhibition assay in *Chromobacterium violaceum* MTCC 12472

3.5.2.4.1. Vapour diffusion assay

The experiment was carried out in a similar manner as that mentioned in section 3.5.1.3.

3.5.2.4.2. Violacein inhibition

In a 15 ml falcon tube, *C. violaceum* ($O.D._{600}$ = 0.4) was added in 5 ml tryptic soy broth consisting of GMM/MO (8, 16, 32, 64 µl/ml). Tubes were then cultured at 28°C for 30 hours and 1 ml culture from each tube was taken and centrifuged at 8000 rpm for 10 minutes for the collection of bacterial pellets. Then 100% DMSO was added for dissolving the violacein pigment and the absorbance was taken at 585 nm. Then the violacein inhibition was calculated [31].

3.5.3. Antifungal activity

3.5.3.1. Vapour diffusion assay

Vapour diffusion was carried out by the method suggested by Clemente et al. 2016 with slight modification [21]. On the Sabouraud dextrose agar plates, 100 µl of C. albicans MTCC 183 broth (106 CFU/ ml fungal cells) was spread. With a micropipette, 300 microliters of oil were loaded in an autoclaved filter paper on the lid of the Petri plate the lid was closed and the plate was sealed with two parafilm (from 300 ml GMM vapour diffused in 44.53 cm3, Petri plate with 90mm X 13 mm with 5mm of height decrease due to culture media). The plates were incubated at 28 ^{o}C for 48 hours. MO was used as a negative control.

3.5.3.2. Agar diffusion assay

Vapour diffusion was carried out by the method suggested by Cotton et al. (2019) with slight modification [23]. On the Sabouraud dextrose agar (Himedia) plates, 100 µl of C. albicans MTCC 183 broth (106 CFU/ ml fungal cells) was spread. With a micropipette, 30 microliters of oil were loaded over the agar and the plate was sealed with two parafilms. The plates were incubated at 28^{o}C for 48 hours.

3.5.3.3. Minimum inhibitory concentration and minimum fungicidal concentration determination by agar diffusion assay

The GMM was diluted using MO (as MO did not show any antifungal activity during agar diffusion) to the factors 2, 4, 8, 16 (GMM/2, GMM/4, GMM/8, GMM/16). Agar diffusion was carried out by a method suggested by Cotton et al. 2019 with slight modification [23]. On the Sabouraud dextrose agar plates, 100 µl of C. albicans MTCC 183 broth (10^6 CFU/ ml fungal cells) was spread. With a micropipette, 30 µl of oil was loaded over the agar and the plate was sealed with two parafilm and incubated at 28^{o}C for 48 hours. The lowest dilution of the GMM that inhibited visible fungal growth was considered as the MIC. With the help of a sterile loop, the zone under the oil was pricked and spread in a sterile SDA plate. The concentration where no fungal colony

was seen was the MFC. Nystatin (10 µg) was taken as a positive control. All the experiment was conducted in triplicate.

3.5.3.4. Lactophenol blue staining

Lactophenol blue stain was prepared according to standard procedure [32]. The fungal after the MIC of GMM treatment was stained with prepared lactophenol blue stain and then observed under the microscope.

3.5.3.5. Poison food assay

Poison food assay was carried out by adding the oil of the GMM and MO in the Sabouraud dextrose agar media before solidification with slight modification [33, 34]. For the experiment, the concentration of oil in the media was 0 (control),1.25, 2.5, 5, 10 and 20 µl/ml. In the centre of the plate, 10 µl of C. albicans with 0.5 MacFarland concentration was loaded. The plates were then incubated at 28^{o}C. The minimum concentration at which no fungal growth was observed was considered as MIC. During the storage, the diameter of the fungal colony was also measured to check the growth inhibition by different concertation of GMM and MO compared to the control. The experiment was conducted in triplicate.

3.5.3.6. Scanning electron microscopy

Fungal cultures from the zone of inhibition were collected in a glass cover slip and then fixed for 2 hours in 2.5% glutaraldehyde solution at 4^{o}C. The samples were washed with PBS three times to remove any unwanted debris. Later, the samples were serially dehydrated using ethanol percentages of 20, 40, 60, 80, 90 and 100% (v/v). The samples were then air dried; coated with platinum using auto-fine coater and examined under a scanning electron microscope (6390lv, Jeol, Japan) at an accelerating voltage of 20kV. The image mode was a secondary electron image with a working distance of 10 mm [25].

3.5.3.7. Molecular docking analysis of GMM volatile compounds with N-myristoyltransferase (*nmt*) protein

For the docking study of N-myristoyltransferase work done by Meenambiga and the team was followed [35]. The ligand structures Allyl isothiocyanate (CID: 5971), (E)- ajoene (CID: 5386591), (Z)-ajoene (CID: 9881148), allicin (CID: 65036), 2-vinyl-4H-1,3, dithiin (CID: 133337), 3-vinyl-4H-1,2, dithiin (CID: 150636), sinigrin (CID:23682211)) and 1-Butene-4-isothiocyanato (CID: 90479303) were downloaded from PubChem database. The 3D crystal structure of N-myristoyltransferase NMT (ID: 1nmt) was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) protein databank. The grid size was set to 80×80×80 xyz points with a grid spacing of 0.503 Å and the grid centre was designated at dimensions (x, y and z): 53.83, 23.92 and 31.33. Molecular docking analysis was done by using Autodock Tools v 1.5.7 molecular interaction study PyMol v 2.5.5 and BIOVIA Discovery Studio visualizer v 4.5 [27].

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

3.5.4.1. GMM sample preparation for cell culture

The oil samples, weighing 100 mg, were initially dissolved in dimethyl sulfoxide (DMSO, Merck) in the presence of 1% tween 20 (Merck), resulting in a concentration of 100 mg/ml. The solution was then filtered using a 0.22 µm PTFE (Himedia) syringe filter. Subsequently, a volume of 10 microliters of the produced samples was solubilized in 1 ml of the cell culture media, resulting in a final concentration of 1 mg per ml for the sample, 1% dimethyl sulfoxide (DMSO) and 0.01% tween 20.

3.5.4.2. Cell culture and cell viability assay

For cell culture and cell viability assay method described by Majumder et al 2019 was followed with slight modifications [36]. For 12 hours, 5000 MCF7 breast cancer cells and HEK 293 cell lines were cultured in 96-well plates with 10% FBS-containing DMEM. Around 10,000 THP-1 cells were seeded into each well of a 96-well plate and after 48 hours

of treatment with 5 ng/ml of 1-phorbol-12-myristate-13-acetate (PMA), the cells had differentiated into macrophage-like cells. Dulbecco's phosphate buffer saline was used to wash the cells before they were allowed to rest in the RPMI-1640 medium for 24 hours. On the day of the experiment, cells were treated with different concentrations of GMM and MO ranging from 25-400 µg/ml in triplicate and were incubated at 37^{o} C and 5 % CO2 incubator for 24 hours. After incubation, in each well 15µl of MTT (5 mg/ml of PBS) was added and again incubated for three hours. After incubation, the media was decanted and cell lysis was done using MTT lysis solution (MTT lysis solution: 11g SDS, 0.2M HCL, 50% isopropanol). The absorbance of the lysate was measured using 96-well plate readers (Multiskan Go, Thermo Scientific) at 595nm. The percentage of viability of cells was measured by comparing them with control samples.

3.5.5. Anti-inflammatory activity against THP-1 cell line

3.5.5.1. Treatment for studying the effect of GMM/ MO on gene expression

For studying gene expression, cells were treated with concentrations of 200 µg/ml GMM and MO for 4 hours after a resting period in RPMI media. Following the treatment, inflammation was induced in cells by 100 ng/ml lipopolysaccharide (LPS) treatment for 2 hours.

3.5.5.2. Preparation of cDNA synthesis (first-strand)

To each tube, 0.5 µl of oligo dT and dNTP is added and the volume of the tube is made to 6 µl by adding nuclease-free water. The mixture was incubated at 65^{o}C for 5 minutes, followed by incubation in ice for 5 minutes. To the mixture, 2 µl of 5X RT buffer, 0.5 µl of reverse transcriptase enzyme, 0.5 µl of RNase inhibitor and 0.1 M DTT were added. The PCR reaction was carried out.

3.5.5.3. Semi-quantitative PCR

Semi-quantitative polymerase chain reaction analyses were performed with the generated cDNA. A total of 20 µl of PCR mix was prepared by combining 2 µl of 10X buffer with 0.8 µl of dNTP, 0.5 µl of forward primer, 0.5 µl of reverse primer, 4 µl of cDNA and nuclease-free water.

Initial denaturation of 95°C for 5 minutes, run for 36 cycles (denaturation at 94°C for 30 sec, annealing temperature specific for primers, extension at 72°C for 30 sec). The sequence of the forward and reverse primers used for the study is mentioned in the supplementary material (Table 8).

Table 8: Sequence of the forward and reverse primers used for gene amplification

Primer	Forward	Reverse	Amplicon size
β-Actin	CCCTTCATTGACCTCAACTACA	ATGACAAGCTTCCCGTTCTC	429 bp
TNF-α	CCAGGGACCTCTCTCTAATCA	TCAGCTTGAGGGTTTGCTAC	106 bp
COX-2	GTTCCAGACAAGCAGGCTAATA	CCACTCAAGTGTTGCACATAATC	78 bp
IL-8	ACATACTCCAAACCTTTCCACCC	CAACCCTCTGCACCCAGTTTTC	151 bp
IL-1β	GGTGTTCTCCATGTCCTTTGTA	GCTGTAGAGTGGGCTTATCATC	125 bp
IL-6	CACTCACCTCTTCAGAACGAAT	GCTGCTTTCACACATGTTACTC	107 bp

3.5.5.4. ADME (Absorption, Distribution, Metabolism and Excretion) and drug-likeness analysis

Ertl et al. (2000) [37] found that the logP (partition coefficient), molecular weight acceptor and donor hydrogen atoms in a molecule are directly related to the ADME and drug-likeness analyses. These rules are also known as Lipinski's rule of five [38]. In the present study, the molecular properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness and medical chemistry analysis for each compound were estimated using SwissADME [39]. In our earlier works, during GCMS and LCMS analysis, we found that Allyl isothiocyanate (AITC), ajoene, ajoene, allicin, dithiin, sinigrin and 1-butene-4-isothiocyanato were the major volatile compounds found in GMM [40] and therefore for the *in-silico* analysis we selected the compounds.

3.5.5.5. Molecular docking analysis of GMM volatile compounds with Cox2, IL1β, IL6, TNFα and IL-8

The ligand structures allyl isothiocyanate (CID: 5971), (E)-ajoene (CID: 5386591), (Z)-ajoene (CID: 9881148), allicin (CID: 65036), 2-vinyl-4H-1,3, dithiin (CID: 133337), 3-vinyl-4H-1,2, dithiin (CID: 150636), sinigrin (CID:23682211) and 1-butene-4-isothiocyanato (CID:

90479303) were downloaded from PubChem database. The 3D crystal structures of Cox2 (ID: 4cox) [41], IL1β (ID: 1itb) [42], IL6 (ID: 1n26) [43], TNFα (ID: 2az5) [44] and IL-8 (ID: 4xdx) [45] were downloaded from RCSB protein databank. The details of the coordinates of the grid box and the size of the grid box used are described in Table 9. Also, Autodock Tools v.1.5.7 was used for the molecular docking analysis, PyMol v.2.5.5 for the molecular interaction investigation and BIOVIA Discovery Studio v.4.5 for the visualization [27].

Table 9: The details of the protein used for molecular docking, PDB code, grid box coordinate and size.

Protein	PDB code	Coordinates of a grid box	Size of a grid box
Cox2	4cox	X: 42.049328 Y: 33.540308 Z: 35.576820	X: 80 Y: 80 Z: 80
IL1β	1itb	X: 36.541524 Y: 4.668280 Z: 14.920035	X: 80 Y: 80 Z: 80
IL6	1n26	X: 22.199151 Y: 48.826406 Z: 76.002159	X: 80 Y: 80 Z: 80
IL8	4xdx	X: 15.730860 Y: -6.276681 Z: -11.143801	X: 80 Y: 80 Z: 80
TNFα	2az5	X: -13.680024 Y: 71.594360 Z: 26.988780	X: 80 Y: 80 Z: 80

3.5.6. Transdermal activity

3.5.6.1. Preparation of the eggshell membrane

For the transdermal study eggshell membrane was used [46]. For the preparation of the eggshell membrane, a small hole was made in the lower bottom of the fresh raw egg to remove the inner contents. The eggshell was then submerged into 0.1 N HCl for 3 hours to dissolve the eggshell and the membrane was then washed with distilled water (Figure 3A).

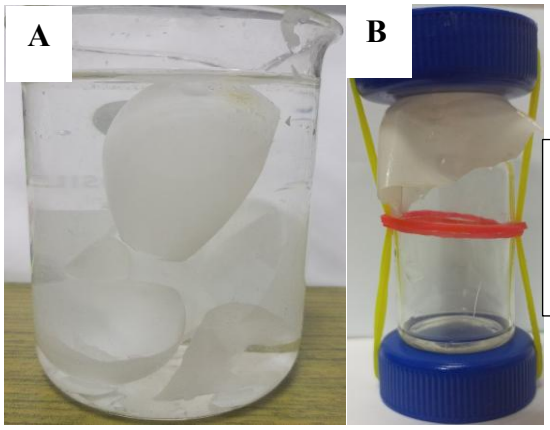

Figure 3: (A) Prepared eggshell membrane; (B) diffusion cell prepared in laboratory.

3.5.6.2. In-vitro drug release

The diffusion cell was prepared in the lab by using a 50 ml Eppendorf tube cap, rubber bands and glass culture tubes. A precise hole (diameter=17 mm, area = 226.98 mm^2) was made in the cap using a metal rod. The prepared egg membrane was then placed in the opening of the glass culture tube and the cap was put over it and fixed using a rubber band (Figure 3B). The eggshell membrane was sandwiched between the cells. The maximum capacity of each of the donor and receiver compartments was 3.0 ml and 35.0 ml, respectively. The surface of the epidermis exposed to the solution was 226.98 mm^2. The donor medium consisted of 1 ml of the GMM oil and the receptor medium consisted of 5.5 ml of ethanol: pH 7.4 phosphate-buffer saline (PBS) (1:1) to maintain the human physiological pH. The diffusion cell was then incubated at 37^o C water bath. The limitation of our prepared diffusion cell was that for each time interval separate diffusion cells were needed. At appropriate intervals (0, 0.5, 1, 2 hours) the diffused oil was collected by dissolving the sample in acetonitrile (HPLC grade, Merck). The permeated amount of the sample was run through LCMS analysis. The membrane was washed three times using cotton-containing ethanol. The membrane was then subjected to FTIR analysis.

3.5.7. Sensory acceptability test

A sensory acceptability test was conducted using the method suggested by Moura et al. (2019) and 30 volunteers, including students and employees from Tezpur University (Assam) and the ages ranged from 20 to 50 [47]. The samples

were assessed on a 9-point hedonic scale, with the descriptive phases ranging from 9 ("like extremely") to 1 ("Dislike extremely"), for qualities like the colour, aroma, flavour and overall acceptability of the product. The data were analyzed using One-way ANOVA.

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