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Formulation, Characterization and Effectiveness of Microencapsulated Phytochemicals-derived Essential Oil against Animal-Pathogenic Bacteria: *In Vitro* and *In Silico* Studies

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ABSTRACT

This study aimed to investigate the effectiveness of microencapsulating plant-derived essential oils (EOs) that are rich in total polyphenolic content (TPC), flavonoid content and antioxidant capacity. Specifically, the microencapsulated garlic essential oil (mGEO) and microencapsulated lemongrass essential oil (mLEO) formulated with black soldier fly-based protein (BSF) extract in oil phase solution using spray-drying technique. The results demonstrated the encapsulation process positively influenced the phytochemical characteristics, particle size, zeta potential, and polydispersity index (PDI) of the microcapsules, resulting in an encapsulation efficiency of 84.7 and 20.2%, respectively. The mGEO microcapsules had the highest TPC released *in vitro* after 24h in simulated rumen pH 5.6 (45.0%) and small intestine pH 7.4 (93.3%) buffers when compared with the mLEO microcapsules, which were 50.0 and 77.8%, respectively. Additionally, the released TPC from EO capsules demonstrated *in vitro* antimicrobial activity, particularly the mGEO microcapsules, which exhibited the antimicrobial efficacy against animal pathogens, specifically mastitis-causing bacteria such as *Escherichia coli*, *Enterobacter aerogenes*, *Shigella typhimurium*, *Staphylococcus aureus*, and *Bacillus cereus*. The result of this investigation indicated that the MIC and MBC values were below 100µg/mL, comparable to the concentration of the positive control (e.g., monensin). Moreover, active substance as allicin was predicted and confirmed in terms of bioactive molecule, eco-friendly, *in silico* pharmacokinetic and toxicological properties. This study suggests that microencapsulation technology of phytochemical-derived EOs has a promising future as feed additive for controlling release and combat animal pathogens.

Key words: Microencapsulated additive; Essential oils-feeding; *In vitro* release; *In vitro* antimicrobials; *In silico* ADME, Molecular docking

INTRODUCTION

Nowadays, the emergence of drug-resistant bacteria remains a significant concern for both medical and veterinary communities, posing a major challenge in the effective treatment for pathogenic microbes, which resist to some antibiotics such as streptomycin, chloramphenicol, erythromycin, and chlortetracycline (MacNair et al. 2024). There is a significant increase in searching for novel, safe natural antimicrobial compounds, particularly phyto-derived antimicrobials. Medicinal plants are main and rich source of bioactive compounds, including coumarins,

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flavonoids, phenolics, alkaloids, terpenoids, tannins, lectins, polypeptides, polyacetylenes and essential oils (EOs). Phytochemicals can be extracted from various plant parts, including leaves, twigs, fruits, fruit peels, barks, roots, buds, seeds and flowers (Wanapat et al. 2024; Agrawal et al. 2024). EOs have historically been employed for their antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Corynebacterium pyogenes* and *Mycoplasma* sp. (Rattanachaikunsopon and Phumkhachorn 2010; Issa 2024). In addition to mitigate methane emissions effect which reduce the methanogens population in ruminants (Oke et al. 2009). The mechanism of action of EOs is believed to involve membrane damage throughout the solubilizing capability of the cell's phospholipid bilayer membrane, such mechanism is playing a crucial role in the antibacterial effects (Polo et al. 2020).

The biological activity of black garlic-EO (*Allium sativum*) (GEO) and lemongrass-EO (*Cymbopogon citratus*) (LEO) comprises approximately 60-70% of different hydrocarbon components including polyphenols (e.g., allicin and citral-α, -β), flavonoids, saponins, steroids, tannins, alkaloids, terpenoids, polyphenols, esters, aldehyde and fatty acids (Rajapaksha et al. 2024; Omoboye et al. 2024). Among other constituents, there are: aldehydes, alcohols, and ketones yield several aromatic compounds, including fruity (E-nerolidol), herbal (-selinene), floral (linakool) and citrus (limonene) types (Agrawal et al. 2024). Those components indicated the biological activity of both GEO and LEO such as anti-inflammatory, antiviral, antioxidant, anticarcinogenic, antimutagenic, cholesterol-lowering, and antimicrobial properties, in particular allicin and/or citral components, which are usually interested in manufacturers of various antimicrobial agents, medicines, and food and/or feed additives (Thuong Nhan et al. 2020; Liu et al. 2023; Phupaboon et al. 2024a).

Due to this, the chemical constituents of plant-derived EOs possessing potent antibacterial characteristics with lacking resistance to the elevated temperatures, enzymatic activities, and pH changes in acidic-alkaline environments found in the digestive tracts of humans and animals. In this context, a viable method applied for safety and maintain the bioactivity of these compounds to create microcapsules by spray-drying technology, while the target substances are encased with a uniform or varied coating, yielding diminutive microcapsules. Consequently, researchers have developed encapsulation technology to improve the efficiency of EOs encapsulation, controlled release properties, and deliver potency of medicines or antibiotics to specific targets of animal's diseases. Effective properties of microcapsules usually arising from their engagement with wall materials such as chitin, chitosan, plant-derived protein, and insect-derived protein (Phupaboon et al. 2022). Based on reviewing different references and papers there are no previous articles regarding to evaluation or assessment of the encapsulation of black garlic and lemongrass EOs with chitin derived from BSF-derived protein extract in a lipid-phase solution using the spray-drying technique. Additionally, the hypothesis of this study applied the bioinformatics tools to support the speeding up of drug discovery processes and functional prediction such as molecular docking technique (to evaluate two molecules binding like ligand-protein targets) and *in silico* property (to evaluate the pharmacokinetic and toxicological prediction of compounds or substituents) (Snoussi et al. 2022; Bugnon et al. 2024). Hence, the purposes of this study were aimed to investigate the effect of mGEO and mLEO microcapsules containing allicin compound, mGEO and mLEO were investigated based on physical properties, physiochemical characteristics, and *in vitro* study of the functional properties such as controlling release along with antimicrobial activities, molecular docking prediction using SwissDocking online web, and *in silico* ADME property using SwissADME server.

MATERIALS AND METHODS

Microorganisms and culture condition

The Mastitis-causing bacteria used to investigate the antimicrobial activity consisting of Gram (-)ve bacteria including: *Escherichia coli* TISTR 073, *Enterobacter aerogenes* TISTR 1540, *Shigella typhimurium* TISTR 292, while *Staphylococcus aureus* TISTR 029 and *Bacillus cereus* TISTR 678 were used to investigate Gram (+)ve bacteria. Tested microbes were purchased from the Thailand Institute of Scientific and Technological Research (TISTR). All microorganisms were activated into tryptic soy broth and agar (TSB and TSA; Himedia, Thailand) and incubated at 37℃, overnight for cultivating condition. The bacterial-working stock was supplemented with 30% (v/v) glycerol and stored at 4℃ until further analysis.

Essential oil (EO) materials

Both GEO and LEO were collected from independent farms at local market in Khon Kaen province, Thailand. Cold-essential oil extracts extracted under cold-process using Frutelia apparatus (Tefal, Thailand) model no. ZE370138 at room temperature according to the procedure used by Punyauppa-Path et al. (2024). The purity of each EO was measured to be ≥90%.

Formulation and microencapsulation process

The microencapsulation process involved modifications to the procedure established by Phupaboon et al. ((2022, commercial black soldier fly (BSF)-derived protein or chitin extract was selected as the encapsulant material, then combined with both EOs of lemongrass and/or garlic oil extracts in a 1:1 ratio, individually. This formulation comprised a) %20w/v) of BSF-derived protein extract mixed with a) %10v/v) EO solution containing %1)v/v) Tween .80Subsequently, the homogeneous medium underwent processing by the spray-drying technique under the following conditions: operating speed (10mL/min), drying airflow (600L/h), pressure drop (0.75bar), intake temperature (160°C) and output temperature (90°C). Subsequent to the cessation of the spray, the microencapsulated EOs of garlic and lemongrass are called microencapsulated garlic essential oil (mGEO) and microencapsulated lemongrass essential oil (mLEO) in powder forms. Powders was kept in a vacuum-sealed bag and maintained at -20°C until further investigations.

Physical characterization of microcapsules

The physical properties of the microencapsulated EO

formulations were analyzed for particle size, polydispersity index (PDI) and zeta potential. The Zeta sizer Nano ZS (Malvern Instruments Ltd., USA) was used to assess the average diameter, zeta potential, and polydispersity index of all formulations. Each formulation was diluted (1:100) in distilled water prior to assays (Kanpipit et al. 2024). Additionally, the surface morphology of microcapsules was observed in terms of shape and surface structure by using a field-emission scanning electron microscope (FE-SEM; model: TESCAN MIRA, USA) under a surface layer sprayed with gold-palladium and observed microscopically with a 15kV, as described in Phupaboon et al. (2022).

Phytochemical assessment of microcapsules

Phupaboon et al. (2022) described the methodology to analyze the biological properties of microcapsules in terms of phytochemical and antioxidant components, including the total polyphenolics content (TPC) measurement with the Folin-Ciocalteu reagent by reading at 765nm reported in g GAE/g of dry extract (DE) and the total flavonoids content (TFC) measurement with the aluminum chloride reagent by reading at 415nm and reported in g QUE/g DE.

The antioxidant activity was determined using different kinds of free radicals, DPPH- and/or ABTS-radical scavenging activity, treatments were read at 490 and 734nm, respectively, and results reported in inhibition% compared with ascorbic acid data. While the treatments by ferric-reducing antioxidant power (FRAP) capacity were read at 595nm and data reported in g TROE/g DE.

Encapsulation efficiency determination

To quantify encapsulation efficiency (%EE), the total polyphenolic content served as an indirect computational representation of the active ingredient from microcapsules, calculated using the equation (%EE) = (Amount of TPC in extract / Amount of TPC in entrapped) x 100 (Phupaboon et al. 2022).

***In vitro* release study of TPC from microcapsules**

The release of TPC from mGEO and mLEO microcapsules was investigated using a membrane diffusion approach by Phupaboon et al. (2024a) with some modification. The process involved applying a 25µm porous filter bag (F57, Ankom) to weigh 1% (w/v) of each microcapsule into separate screw bottles, which were then suspended in various solutions containing 40mL of a rumen-simulated environment in acetate buffer (pH 5.6) and a small intestine environment in phosphate buffer (pH 7.4). The bottles were incubated in a water bath at 37°C, and 1mL samples were taken in triplicate after 12 and 24 hours of incubation, with 1mL of fresh buffer immediately replacing the sampled solution. The collected samples from various points were subjected to measure the total phenolic content (TPC) by Folin-Ciocalteu reagent at 765nm, and data reported as percentage release of TPC.

***In vitro* antimicrobial activity of microcapsules**

Both minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were established to study the antimicrobial activity of mGEO and mLEO. Microdilution technique (96-well microplate) by Silva et al. (2023) with some modification was followed. The pathogenic bacteria of *E. coli* TISTR 073, *Ent. aerogenes* TISTR 1540, *S. typhimurium* TISTR 292, *S. aureus* TISTR 029 and *B. cereus* TISTR 678 were used. All tested microbes grown in TSB and TSA medium, incubated at 37°C for 8h, with a final concentration of 10^5 CFU/mL. Microplate containing 100µL of filtered from mGEO and mLEO separately with different concentration ranged from 6.25-100µg/mL with two-fold dilution technique for each was used. Bacterial suspension (20µL) was added followed by 80µL of media broth. Then, microplate was incubated at 37°C for overnight and growth the bacteria on TSA media using drop-plate technique as well as incubation process at the same conditions. Monensin pre mix (Bio Agri Mix, Canada) diluted in media broth, was used as a positive control in terms of antibiotic-feed supplementation. After incubation, the growth of microorganisms in culture plates was determined by the MIC value, the lowest concentration of the extract that inhibits invisible growth of microorganisms after overnight culture. While MBC value is defined as the lowest concentration of the extract that can kill microorganisms without any growth on the culture agar media, as described by Balouiri et al. (2016).

Molecular docking study

Based on the high potent antimicrobial activity of mGEO containing allicin which playing the major role as antimicrobial agent. Allicin compounds (PubChem ID: 65036) was selected as ligand molecules for preliminary screening and to predict the binding site of the energy-minimized target protein structure using SwissDock server under SwissDrugDesign platform, following the methods and parameter setup described by Bugnon et al. (2024). Ligand molecules were prepared by the SwissParam website (http://swissparam.ch/) using the simplified molecular input line entry system (SMILES) platform obtained from the PubChem database. Subsequently, among the chosen five active site protein targets, namely, -3oxoacyl-[acyl-carrier-protein] synthase 1from *E. coli* (PDB ID: 1FJ4), nucleoside diphosphate kinase from *S. aureus* (PDB ID: 3Q8U), glycerophosphodiesteras of *Ent. aerogenes* (PDB ID: 2DXL), crystal structure of 3-hexulose-6-phosphate synthase from *S. typhimurium* (PDB ID: 3F4W), and hemolysin-BL lytic component L1 from *B. cereus* (PDB ID: 7NMQ) were found to have three-dimensional (3D) structures using SWISS-MODEL, and they were retrieved from the RCBS-PDB database. The docking process of allicin with protein targets was conducted utilizing the SwissDock version 2024 free server (http://www.swissdock.ch/), employing the EADock DSS algorithm under Attractive Cavities 2 (AC 2). The AC 2 in SwissDock version 2024 utilizes the Vina Python library (version 1.2.5) to establish a Vina sampling engine and access the scoring functions of AutoDock4, which includes the AC docking score (comprising the CHARMM force field energy and FACTS solvation energy terms) and the SwissParam score (an approximation of the binding free energy).

***In silico* ADME prediction**

The SwissADME program, accessible online (http://www.swissadme.ch/), was utilized to evaluate and predict the drug-likeness and pharmacokinetic characteristics of the allicin compounds (SMILES

notations from PubChem ID: 65036) discovered from garlic-based EO extract. Lipinski's rule of 5, a criterion for assessing drug-likeness, was utilized to evaluate the compounds. Multiple pharmacokinetic parameters were analyzed, encompassing molar refractivity (MR), skin permeability (log Kp), permeability of P-glycoprotein substrate (Pgp), gastrointestinal absorption, blood-brain barrier (BBB) penetration, and cytochrome enzyme inhibition, as described by Snoussi et al. (2022) and Ghannay et al. (2020). The assessments provide significant insights regarding the pharmacological characteristics and appropriateness of the identified compounds for medication development.

Statistical analysis

All investigations were achieved in this study in triplicate (n =3). Data were reported as mean±SD values. Analysis of variant (ANOVA) was used to evaluate the variances among the treatments by Duncan's new multiple range test ($P<0.05$) with IBM SPSS-KKU Statistics Version 27.0 software.

RESULTS

Physical characteristics and phytochemical assessment of microcapsules

As shown in Fig. 1, the FE-SEM microphotography of mGEO (Fig. 1a) and mLEO (Fig. 1b) microcapsules captured at 2000x, there are encapsulated with chitin-derived protein extract obtained from BSF-defatted protein formulated in an oil-liquid solution. The surface morphology of both tested microcapsules had a smooth, glossy appearance, characterized by grooves and various impregnations, with particles displaying variable shapes, including both circular and square forms. Results (Table 1) show the physical characteristics of the mGEO and mLEO microcapsules with particle sizes ranging from 324 to 430nm, with zeta potentials of -12 to -35mV, as well as polydispersity index (PDI) values of 0.32 to 0.42, respectively. Exclusively, the mGEO formulation presented tiny, nano to microparticle size, had a highest zeta potential, and PDI value. Additionally, the results of phytochemical assessment and antioxidant activity of both unencapsulated (in extract solutions, e.g., GEO and LEO) and encapsulated (microcapsule forms, e.g., mGEO and mLEO) were found to be the TPC, TFC, DPPH, and ABTS scavenging inhibition as well as FRAP capacity. These results ranged from 0.39-1.93g GAE/g DE for TPC test, 0.06-0.79g QUE/g DE for TFC test, 49.3-78.1% for DPPH scavenging test, 10.9-15.4%, and 20.2-84.7g TROE/g DE, for ABTS and FRAP scavenging activity respectively. The %EE results of mGEO (84.7%) demonstrated the highest encapsulation efficiency compared with mLEO (20.2%) microcapsule (Table 2).

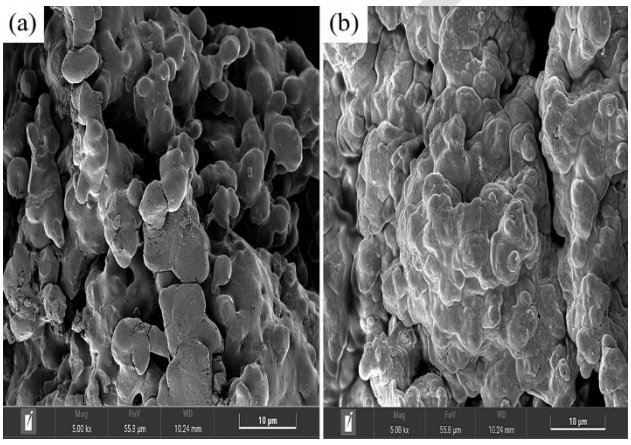

Fig. 1: FE-SEM micrographs of (a): the mGEO and (b): mLEO microcapsules captured at 5000x.

Table 1: Physical characteristics of the mGEO and mLEO microcapsules

Formula	Particle size (nm)	Zeta potential (mV)	PDI
mGEO	324.1±4.1[a]	-12.8±0.4[a]	0.32±0.0[a]
mLEO	430.2±3.3[b]	-35.2±0.6[b]	0.42±0.1[b]

[a,b] Significant differences between groups were observed at a P-value <0.05, with data reported as mean ± SD of triplicates.

***In vitro* TPC release profiles of microcapsules**

Fig. 2 presents the summary of an *in vitro* TPC release% obtained from both mGEO and mLEO microcapsules in a simulated rumen juice (acetate buffer) at pH 5.6 and a simulated intestinal juice (phosphate buffer) at a pH 7.4 for 12 and 24h of post-incubation. Particularly, %TPC release of mGEO in simulated rumen juice with acetate buffer (pH 5.6), was 42.3% for 12h and 75.0% for 24h, while with a simulated intestinal juice (phosphate buffer) at pH 7.4 were 82.1 and 93.3% at 12 and 24h, respectively. In addition, another result was found: the %TPC release from mLEO microcapsules in acetate buffer (pH 5.6) was 27.8% for 12h and 50.0% for 24h, along with 52.9% at 12h and 77.8% at 24h-post incubation in a simulated intestinal juice (pH 7.4) condition. Interestingly, no effects on the amount of TPC released in both buffers used with mGEO and mLEO after 12 and 24h of incubation period, however, mGEO revealed higher %TPC release rate in pH 7.4 than pH 5.6 after 12h and 24h of incubation periods.

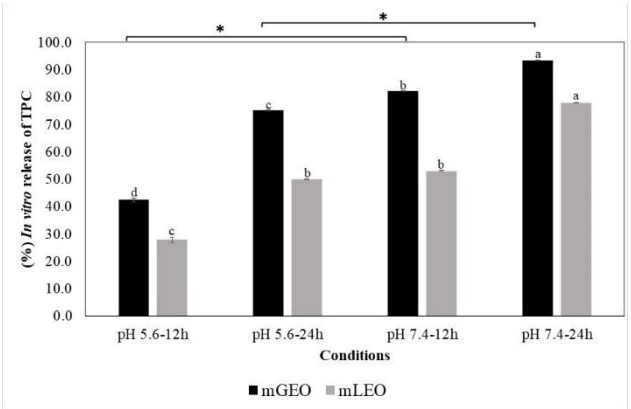

Fig. 2: *In vitro* release profiles of TPC from the mGEO and mLEO microcapsules in a rumen simulated buffer at pH 5.6 and a simulated intestinal buffer at pH 7.4 under different incubation times. Different letters [a,b,c,d] in the same group indicate a significance at $P<0.05$ and * at $P<0.01$, with data reported as mean±SD of triplicates.

***In vitro* antimicrobial activity**

The antimicrobial activity of mGEO and mLEO was calculated and achieved by MIC and MBC values. Both types of microcapsules were tested against five animal-pathogenic bacteria, as shown in Table 3. From the obtained data, the mGEO showed high activity with lowest

Table 2: Physiochemical and phytochemical characteristics in unencapsulated and encapsulated forms obtained of the GEO, LEO, mGEO and mLEO

Formula	TPC (g GAE/g)	TFC (g QUE/g)	DPPH (%)	ABST (%)	FRAP (g TROE/g)	(%EE)
GEO	0.81±1.7^{c}	0.09±6.8^{b}	51.5±8.4	10.9±0.9	12.3±2.5^{c}	-
LEO	0.39±2.3^{d}	0.06±4.2^{b}	49.3±2.9	12.7±0.5	17.5±9.5^{b}	-
mGEO	1.93±5.2^{a}	0.79±2.0^{a}	87.8±4.2	15.4±1.0	17.3±7.2^{b}	84.7±0.2^{a}
mLEO	0.96±1.0^{b}	0.10±1.5^{b}	78.1±7.2	12.8±0.5	20.6±1.6^{a}	20.2±0.1^{b}

^{a,b,c,d} Significant differences between groups were observed at a P-value <0.05, with data reported as mean± SD of triplicates.

Table 3: *In vitro* antimicrobial activity of the released TPC from mGEO and mLEO microcapsules to investigate in animal-pathogenic bacteria testing

Evaluation	MIC (µg/mL)					MBC (µg/mL)				
	Ec	*Ea*	*St*	*Sa*	*Bc*	*Ec*	*Ea*	*St*	*Sa*	*Bc*
mGEO	25	25	25	50	50	50	50	50	100	100
mLEO	50	50	50	100	100	100	100	100	100	100
Monensin	25	25	25	25	25	50	50	50	50	50

Note: *Ec*, *E. coli* TISTR 073; *Ea*, *Ent. aerogenes* TISTR 1540; *St*, *S. typhimurium* TISTR 292; *Sa*, *S. aureus* TISTR 029; *Bc*, *B. cereus* TISTR 678

Table 4: Molecular docking analysis results of allicin ligand in various active sites (protein PDB) of *E. coli* (a); *S. aureus* (b); *Ent. aerogenes* (c); *S. typhimurium* (d); and *B. cereus* (e)

Protein PDB ID	Microbes	Protein targets	Unique protein chains	AC score (kcal/mol)	Swiss-Param score (kcal/mol)
1FJ4	*E. coli*	-3oxoacyl-[acyl-carrier-protein] synthase 1	1 (12,182 atoms)	-14.99	-6.02
3Q8U	*S. aureus*	Nucleoside diphosphate kinase	1 (7,566 atoms)	-14.71	-6.01
2DXL	*Ent. aerogenes*	Glycerophosphodiesteras	1 (4,343 atoms)	-16.12	-6.27
3F4W	*S. typhimurium*	Crystal structure of 3-hexulose-6-phosphate synthase	1 (3,694 atoms)	-11.14	-5.92
7NMQ	*B. cereus*	Hemolysin BL lytic component L1	1 (3,498 atoms)	-18.89	-6.56

concentration (25 to 50µg/mL) to inhibit 50% of tested microbes (*E. coli*, *Ent. aerogenes*, and *S. typhimurium*) compared to monensin (positive control). mGEO represented the lowest MBC values (50 to 100µg/mL) against *S. aureus* and *B. cereus*. Whereas the mLEO exhibited less effectiveness in MIC values (50 to 100µg/mL), while MBC values were 100µg/mL against the tested microbes.

Molecular docking profiles

The binding interactions of allicin as a ligand molecule [SMILES ID: C=CCSS(=O)CC=C] docked in various protein active sites by SwissDock server as shown in Fig. 3(a-e), which interact by using hydrogen bonds, ionic bonds, cation, hydrophobic, and stacking interactions. The binding affinity (AC docking score) values of allicin ligand are ranged from -11.14 to -18.89 kcal/moL against to five PDB-protein targets (1FJ4, 3Q8U, 2DXL, 3F4W, and 7NMQ) of pathogenic bacteria. In particular in 7NMQ, it was found that the highest an AC docking score of -18.89 kcal/moL against hemolysin-BL lytic component L1 from *B. cereus*, followed by -16.12 kcal/moL docked with glycerophosphodiesteras protein of *Ent. aerogenes*. In addition, the SwissParam score, which is the binding free energy calculated from the weight sum of the polar and nonpolar terms showed a high docking score at -6.56 and -6.27kcal/moL in terms of binding energy with various targets: hemolysin-BL lytic component L1 of *B. cereus* and glycerophosphodiesterase of *Ent. aerogenes*, while other protein targets were -6.02, -6.01, and -5.92kcal/moL obtained from -3oxoacyl-[acyl-carrier-protein] synthase 1, nucleoside diphosphate kinase, and crystal structure of 3-hexulose-6-phosphate synthase, respectively (Table 4).

***In silico* ADME profiles**

Moreover, the selection of the allicin ligand was predicated on its significant antimicrobial efficacy and its binding affinity, as indicated by the AC docking score. The characteristics of molecules, including their phytochemical properties, lipophilicity, drug-likeness, pharmacokinetics, toxicity prediction, and compatibility with medicinal chemistry, were elucidated using the SwissADME server tool, as detailed in Table 5. The outcome of this component exhibits a significant number of rotatable bonds, a molar

Table 5: SwissADME profiles of allicin ligand based on antimicrobial reagent related to molecular properties, drug-likeness, pharmacokinetics and toxicity prediction

Entry	ADME properties
Physicochemical Properties/Lipophilicity/Drug-likeness	
Ligand	Allicin
SMILES	C=CCSS(=O)CC=C
Formula	$C_6H_{10}OS_2$
Molecular weight (g/mol)	162.27
Num. heavy atoms	9
Num. arom. heavy atoms	0
Fraction Csp3	0.33
Num. rotatable bonds	5
Num. H-bond acceptors	1
Num. H-bond donors	0
Molar refractivity	45.88
TPSA (Å2)	61.58
Lipinski's rule	Yes
Bioavailability score	0.55
Consensus Log P	1.61
Pharmacokinetics/Toxicity prediction	
GI absorption	High
Log K_p (skin permeation; cm/s)	-6.36
Blood–brain barrier permeant	Yes
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
AMES toxicity	No
Hepatotoxicity	No
hERG I/II inhibitors	No

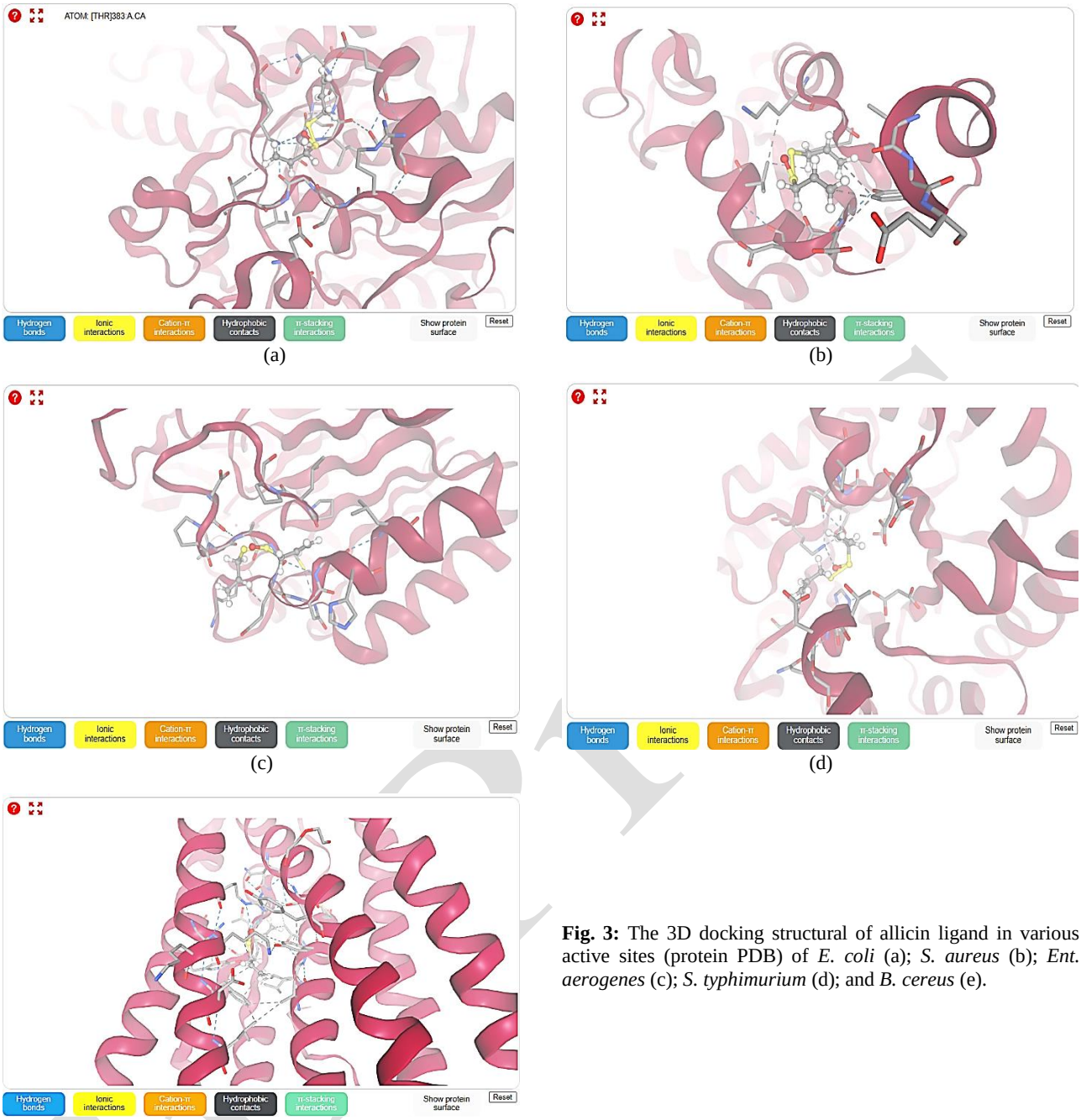

Fig. 3: The 3D docking structural of allicin ligand in various active sites (protein PDB) of *E. coli* (a); *S. aureus* (b); *Ent. aerogenes* (c); *S. typhimurium* (d); and *B. cereus* (e).

refractivity of 45.88 and a TPSA of 61.58 Å^2. It also demonstrates a low count of H-bond acceptors and donors, adhering to Lipinski's rule of 5. Consequently, it appears to possess favorable oral bioavailability, rated at 0.55, alongside a lipophilicity score (consensus Log P) of 1.61. Their gastrointestinal (GI) absorption was notably high, demonstrating the ability to traverse the blood-brain barrier (BBB) and exhibiting skin permeation at a rate of -6.36cm/s. This particularly indicates a minimal to nonexistent impact on the central nervous system (CNS). Furthermore, the allicin compound was not detected in the substrate for permeability glycoprotein (P-gp), indicating that there is a minimal likelihood of efflux from the cell. Furthermore, this compound did not impede all the examined cytochrome P450 isoenzymes, which are essential in the biotransformation of drugs via O-type oxidation reactions.

DISCUSSION

This study aimed to find a natural EO sources, with antimicrobial characteristics to substitute antibiotics usually used as treatment for animal pathogenic bacteria, in particular *E. coli*, *Ent. Aerogenes*, *S. typhimurium*, *S. aureus*, and *B. cereus*. Such microbes are main cause of diseases in livestock and aquaculture infection. The tropical plant-derived essential oils (e.g., black garlic and lemongrass) were chosen due to their widespread availability and ease of cultivation throughout Thailand. Furthermore, these plants, in particular garlic extract have been documented to exhibit antimicrobial properties due to rich-phytochemical constituents, including the majority component of allicin, γ-glutamyl-S-allylcysteine and allicin transformed products such as diallyldisulfide and vinyldithiins in terms of polyphenolic compounds, flavonoids, and other antioxidant compounds (Roy et al.

2006; Wanapat et al. 2024).

This research investigates encapsulating bioactive-edible ingredients in particle form resulted from spraying technique to get efficient delivering of antimicrobial agents. This is innovative treatment strategy, currently gaining widespread interest as an alternative to antibiotics. With respect for the physical and phytochemical characteristics of the mGEO and mLEO in microparticle form, results represent that mGEO microcapsules had the highest %EE (84.7%). Such data compared and agreed with findings of Tavares et al. (2021) and Thuong Nhan et al. (2020), who found that the %EE of mGEO in β-cyclo-dextrin was at 82.0% and mLEO formulated by maltodextrin mixed with Arabic gum was at 84.8% by using spray-drying technique. Due to the high molecular weight, high branched nature, and the composition includes various acidic polysaccharides of chitin from BSF-based protein extract Thuong Nhan et al. (2020).

Previous studies have demonstrated that microencapsulation can facilitate controlled release and bioaccessibility of bioactive compounds (such as polyphenols and flavonoids) in food, feed and supplementation, resulting in gastro-resistant microparticles, modify medication solubility and minimize phytochemical incompatibilities (Phupaboon et al. 2024a). Particularly, food applications and feed supplementations, both require using of microencapsulation to enhance the stability of EOs. Mechanism of enhancing stability is usually depending on the quality of wall materials associated with their capacity to preserve the phytochemical compounds and guarantee efficient delivery to the target site (Ibrahim and Hassen 2021). The current finding of this study showed non-significant differences in the amount of *in vitro* TPC released in acetate (pH 5.6) and phosphate (pH 7.4) buffers between the mGEO and mLEO at 12 and 24h of incubation, however, the mGEO revealed high TPC release rate with pH 7.4 at 12 and 24h periods. Ibrahim and Hassen (2021) conducted a previous study and found that encapsulated mimosa (*Acacia mearnsii*) tannin, formulated with palm oil and sunflower oil, released tannins after 24h in rumen (pH 5.6) at 18.3 and 15.6%, while in small intestine condition (pH 7.4) at 23.7 and 18.6% in simulated buffers. Furthermore, microencapsulated cinnamon essential oils (CEOs), coated with ammonium salt and gum arabic, exhibited a gradual release, with release percentage ranging from 19.7 to 49.8% after 30 days (Liu et al. 2023).

For *in vitro* antimicrobial activity, the most striking result to emerge from obtained data is that mGEO had potent effect with lowest concentration (25 to 50µg/mL) for MIC and (50 to 100µg/mL) for MBC values compared with monensin premix as a positive control. The results of the current study are consistent with previous observations, which suggested that plant secondary metabolites, particularly, polyphenolic and flavonoid compounds, such as resveratrol, baicalein, biochanin A, silybin, kaempferol, quercetin, catechin gallates, chebulinic acid, and curcumin, are widely used in medical and pharmaceutical applications including antibacterial agents (Ashraf et al. 2023). These chemicals augment the efficacy of antibiotics against resistant pathogens *Campylobacter jejuni*, *Mycobacterium smegmatis*, *Chlamydia* spp., *Mycobacterium*, and *S. aureus* through several methods, one prominent one being the attenuation of efflux pump (EP) activity, functioning as efflux pump inhibitors (EPIs), and exhibiting substantial EPI activity against pathogenic bacteria (Angelini 2024). Possible explanation is might be that plant antimicrobial peptides (AMPs) classified into distinct groups according to their amino acid sequences, cysteine motifs, disulfide bond positions, and secondary structural characteristics (Slavokhotova and Rogozhin 2020). The main families of antimicrobial peptides encompass: defensins, thionins, α-hairpinins (peptides resembling hairpins), hevein-like peptides, knottins, snakins, lipid-transfer proteins, and cyclotides. Additionally, there are other types of exist peptides not listed above, including those characterized by atypical cysteine motifs, those devoid of disulfide bonds, cyclic peptides that lack a cysteine knot, and peptides that exhibit a high abundance of glycine, histidine, and alanine (Santos-Silva et al. 2020). According to Zhao et al. (2021), who studied that the assessment of encapsulation efficiency, controlled release, and antimicrobial efficacy of GEO-vegetable oil (VO) microcapsules demonstrated that GEO was effectively encapsulated and gradually released, exhibiting significant antibacterial activity against both *E. coli* and *S. aureus*. Leimann et al. (2009), who reported the microencapsulated LEOs presented the MIC values to *E. coli* (22.32mg/mL) and to *S. aureus* (2.79mg/mL) meaning that the encapsulation process did not cause any deterioration in the essential oil. Another study of Issa (2024) demonstrated that the unencapsulated-EOs forms (e.g., thyme, mint, and lavender), in particular thyme oil, which showed that MIC and MBC values at 9µg/mL to inhibit *E. coli*, *S. Newport*, *S. aureus*, *S. aureus* MRSA, *K. pneumoniae*, *C. pseudotuberculosis* STS and *C. pseudotuberculosis* STG. Moreover, numerous studies have demonstrated the importance of administering an optimal concentration of unencapsulated and/or microencapsulated plant-based phytonutrients obtained from mangosteen peels, lemongrass, banana leaf, red dragon fruit peels, and tung oil (*Vernicia fordii*) during *in vitro* ruminal fermentation. Their action enhances nutrient degradability, improves dietary protein utilization, reduces enteric methane production, increases the ratio of propionate, and acetate (C2) to propionate (C3) concentrations, as well as acts as an antimicrobial inhibitor to combat the methanogen population (Phupaboon et al. 2024b; Prachumchai et al. 2024; Dagaew et al. 2024; Suriyapha et al. 2024; Prommachart et al. 2024).

Nowadays, molecular docking techniques serve as essential instruments in the identification of potent molecules during the virtual screening of extensive databases, aimed to forecasting protein-ligand interactions with respect to antimicrobial efficacy (Ghannay et al. 2020; Snoussi et al. 2022). The finding of the current study is consistent with Roy et al. (2006) who found that *in vitro* antimicrobial testing suggested that the mixture of crushed garlic and black cumin seeds (*Nigelia sativum*) has antimicrobial effects on *E. coli* and *S. aureus* by using the main compounds of garlic extract (allicin) and other components such as diallyldisulfide and vinyldithiins, which were identified by GC-MS and LC-MS/MS analysis. Snoussi et al. (2022) conducted an evaluation of the phytochemical composition, as well as the antioxidant, antimicrobial, antibiofilm and anti-quorum sensing properties of hairy garlic (*Allium subhirsutum*) aqueous

extract, utilizing both *in vitro* and *in silico* methodologies. *In silico* pharmacokinetic and toxicological predictions revealed that, among the sixteen identified compounds, fourteen exhibited significant potential as drug candidates and may serve as lead compounds for subsequent development and drug design. Navinraj et al. (2023) demonstrated that nimbolide, a tetranortriterpenoid (limonoid) compound derived from the leaves of *Azadirachta indica*, was evaluated both *in vitro* and *in silico* for its antimicrobial efficacy against *Fusarium oxysporum* f. sp. *cubense*, *Macrophomina phaseolina*, *Pythium aphanidermatum*, and *Xanthomonas oryzae* pv. *oryzae*. The affinity of nimbolide for various protein targets in bacteria, fungi, and insects was validated using *in silico* approaches through modeling on the SWISS-MODEL server, followed by molecular docking conducted with the SwissDock server. Docking of homology-modeled protein structures indicated that the majority of selected target proteins exhibit a higher affinity for the furan ring of nimbolide. Furthermore, the stability of the optimal protein–ligand complex was verified through molecular dynamics simulation. The investigation conducted by Ghannay et al. (2020) elucidated the forecasting of novel trifluoromethylated compounds through an analysis of their physicochemical parameters, including lipophilicity and bioactivity score, alongside their pharmacokinetic characteristics such as absorption, distribution, metabolism, and excretion (ADME). This encompasses aspects like plasma protein binding (PPB), the ability to penetrate the blood–brain barrier (BBB), human intestinal absorption (HIA), cellular permeability (PCaco-2), permeability in Madin–Darby canine kidney cells (PMDCK), P-glycoprotein (P-gp) efflux, as well as the roles of CYP inducers, substrates, and inhibitors, skin permeability (PS) and toxicological profiles, including mutagenicity, carcinogenicity, acute toxicity, environmental impact, and cardiotoxicity (hERG inhibition), all employing *in silico* computational methodologies. The results obtained offer valuable insights into the pharmacotherapeutic potential and toxicity of the molecules under examination, demonstrating a commendable alignment between *in vitro* antimicrobial activity and the predicted properties.

Conclusion

This study successfully encapsulated plant-derived essential oils (EOs) extracted from garlic (GEO) and lemongrass (LEO). The formulation involved microencapsulation of mGEO and mLEO through spray-drying, using mixture of chitin and BSF-derived protein extract in an oil-based solution. The phytochemicals exhibited abundant concentration of TPC and TFC leading to significant antioxidant capacity. Post spray-drying, the encapsulated essential oils revealed generally well-preserved state, accompanied by an elevated of TPC aligned encapsulation efficiency. The mGEO microcapsules demonstrated significant *in vitro* TPC release in simulated rumen (pH 5.6) and small intestine (pH 7.4) conditions. Additionally, they exhibited potent antimicrobial activity against the animal pathogens: *E. coli*, *Ent. aerogenes*, *S. typhimurium*, *S. aureus*, and *B. cereus*, with MIC and MBC values below 100µg/mL. Results indicate the potency of mGEO microcapsules retained allicin component as an alternative, eco-friendly edible antibiotic to control animal infections, which confirmed by molecular ligand-target docking interaction and *in silico* property.

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Author's Contribution: SP conceived and designed the experiment. SP and NK performed the study, and conducted lab analyses. SPP, FJH and CK supervised and coordinated the experiments and CK provided and supported the materials. SP performed statistical analyses of experimental data and prepared the manuscript format. SP, FJH, and SS prepared the manuscript draft. All authors critically revised the manuscript and approved the final version.

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