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Formulation and Evaluation of Ethanolic Extract of Black Fruit (*Haplolobus* sp.) Leaves and Chitosan-Tripolyphosphate Mixture as Antioxidant and Antibacterial Agents

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This study investigated the mixture system comprising an ethanolic extract of black fruit (*Haplolobus* sp.) leaves and chitosan-tripolyphosphate (TPP), hereinafter called extract-chitosan-TPP mixture (ECTM), with a focus on its an antioxidant and antibacterial properties. The solution was prepared by adding the extract to the chitosan solution followed by the addition of TPP and then subsequent sonication. The resulting ECTM was centrifuged and then its encapsulation efficiency was determined based on the phenolic content using gallic acid method. The results showed that the ECTM particle size ranged from 190.7 to 496.8 nm with a polydispersity index of less than 0.5, which indicated that the suspension was homogeneous. The statistical results based on Taguchi method showed that the smallest possible size was 187.08 nm at a chitosan:TPP ratio of 0.2, extract concentration of 15% and ultrasonic time of 10 min. Encapsulation efficiency ranged from 75.45-80.43%. The antioxidant activity, expressed as IC_{50} values, ranged from 0.54 to 25.77, while the antibacterial activity was highest for sample E, showing inhibition of 15.63% against *Staphylococcus aureus* and 14.70% against *Escherichia coli*, relative to the antibacterial activity of chloramphenicol.

Keywords: Black fruit leaf, Chitosan, *Haplolobus* sp., Encapsulation, Tripolyphosphate.

INTRODUCTION

Environment, health and use of natural resources still become major issues worldwide. In waste management efforts, the principles of reduce, reuse and recycle are implemented. Recycling defines as waste reprocessing to make products which are useful in life [1]. Chitosan production from shrimp shell waste represents an important application of recycling principles [2]. Chitosan is a versatile biopolymer obtained through the deacetylation of chitin [3], which is abundantly present in the shells of invertebrates and can be efficiently extracted from shell waste [4]. Structurally, chitosan contains protonated amino groups ($-NH_3^+$) that enable strong electrostatic interactions with negatively charged microbial cell walls and membranes. These interactions disrupt membrane integrity, leading to increased permeability, leakage of intracellular components and ultimately microbial cell death or growth inhibition. Consequently, chitosan exhibits significant antibacterial activity [5,6]. As a polymer, chitosan has the potential to be an encapsulation matrix that easily degraded naturally. This property enables chitosan to function as a slow-release agent, delivering active substances such as natural extracts into the body through nano- or microencapsulation, where chitosan serves as the encapsulating matrix [7,8].

The ethanolic extract of black fruit leaves has been studied to have antioxidant properties. Black fruit is a typical Papuan fruit that has been used since ancestral times to preserve sago [9]. Polar solvent extract (methanol) of black fruit leaves contains phytochemicals, namely alkaloids, flavonoids, saponins and tannins [10]. In this research, the ethanolic extract of black fruit leaves was incorporated into a chitosan solution to enable practical field application. The resulting system produces a mixture comprising extract-loaded nanoparticles along with a fraction of unencapsulated extract. This approach is simple, cost-effective and more practical for field use, as it can be prepared and applied using basic equipment. In practical applications, chitosan and the extract can be directly mixed and applied as an antioxidant and antibacterial agent, for instance, as a natural preservative through immersion in the prepared solution.

Furthermore, this study investigates the encapsulation of the ethanolic extract of black fruit leaves within a chitosan-TPP matrix to obtain particles in the nano size range, followed by evaluation of their antioxidant and antibacterial activities.

EXPERIMENTAL

The black fruit leaves (*Haplolobus* sp.) were collected from Manokwari, West Papua, Indonesia. Commercial chitosan purchased from CV. ChiMultiguna Cirebon, Indonesia (made from shrimp shell, Pharmaceutial Grade, 200 mesh, molecular weight 80 kDa, degree of deacetylation 95.6%). Sodium triphosphate pentabasic (TPP) were purchased from Sigma-Aldrich. Ethanol, Folin-Ciocalteau reagent, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Merck, USA. Polyphenol standards (gallic acid, (+)catechin). Bacterial strains *viz. Escherichia coli* and *Staphylococcus aureus* were acquired from Microbiology Laboratory of Universitas Papua, Manokwari (Indonesia). The other chemicals utilised in this project were of analytical grade.

Extraction of black fruit leaves: The collected black fruit leaves and fruits were washed thoroughly with distilled water and air-dried at room temperature. A total of 1000 g of leaves and 115.80 g of fruits were processed. The fruit flesh was separated from the peel, dried, and finely powdered. Extraction of the leaf and fruit powders was performed by the maceration method using 96% ethanol as solvent. The resulting extracts were concentrated to obtain 70.48 g of leaf extract and 41.40 g of fruit extract.

Formulation of extract-chitosan-TPP mixture: The formulation of ethanolic extract of black fruit leaves-chitosan mixture was carried out by mixing a 1% (w/v) chitosan solution prepared in 1% (v/v) acetic acid with the extract solution prepared in 70% ethanol, followed by magnetic stirring for 1 min. The resulting chitosan-extract mixture was added dropwise to the tripolyphosphate (TPP) solution at a rate of 1 drop/min and subsequently subjected to ultrasonication for a predetermined duration. The detailed formulation parameters are given in Table-1. This process resulted in the formation of chitosan-based nanocapsules along with residual unencapsulated ethanolic extract. The final system, consisting of nanocapsules and free extract, was referred to as the extract-chitosan-TPP mixture (ECTM).

Detection method

Phenolic test: The phenolic test was estimated qualitatively and quantitatively according to the literature [11] with some modifications. The phenolic content was assessed in two conditions: extract solutions without encapsulation and those that remained unadsorbed in the ECTM mixture. To analyze the non-adsorbed phenolics, the ECTM mixture was centrifuged, and the supernatant was collected for further analysis. For qualitative analysis, the presence of phenolic compounds was qualitatively identified using a 1% $FeCl_3$ solution. In brief, 1 mL of the test sample was mixed with two drops of $FeCl_3$. A strong blackish-green colour indicated the presence of phenolic compounds [12].

For the quantitative assessment, 1 mL of test solution was pipetted and 0.4 mL of Folin-Ciocalteau reagent was added. The mixture was agitated and allowed to stand for 4 to 8 min. Following this, 4.0 mL of Na_2CO_3 solution was introduced, and the mixture was shaken until homogeneous followed by the addition of distilled water to make up the total volume to 10 mL and the solution was left for 2 h at room temperature. The phenolic content was determined by comparing the absorbance of the test sample with that of a gallic acid standard, which was prepared using the same procedure as the sample.

Antioxidant assay: The antioxidant test on ECTM was carried out using the DPPH method according to the literature [13].

Antibacterial assay: The disc diffusion method was used to evaluate the antibacterial activity of ECTM on agar medium. *S. aureus* and *E. coli* were selected as the test organisms, and antibacterial activities were recorded at 24 h intervals over a period of 72 h.

Statistical analysis: Particle size data were analysed in Minitab® 20.4 (64-bit) using the Taguchi method to determine the smallest particle size obtained.

RESULTS AND DISCUSSION

In the encapsulation system formulation, particle size is an important factor which influences the active compound delivery system. Smaller particle sizes are preferred in this case since they have enthalpy and entropy characteristics that favour the interactions between nanoparticles and living cells [14]. The particle size and polydispersity index (PI) values of the ECTM formulations are summarized in Table-2. All formulations exhibited average particle sizes below 1000 nm, falling

TABLE-1
DETAIL OF ETHANOLIC EXTRACT-CHITOSAN-TPP (ECTM) MIXTURE FORMULATIONS

ECTM sample code	Chitosan:TPP ratio	Cons. extract (%)	Ultrasonic time (min)	Cons. chitosan (%)	Cons. TPP (%)	Vol. chitosan (mL)	Vol. TPP (mL)	Vol. extract (mL)
A	0.2	5	1	1	5	4.5	4.5	0.5
B	0.2	10	5	1	5	4.5	4.5	0.5
C	0.2	15	10	1	5	4.5	4.5	0.5
D	1	5	5	1	1	4.5	4.5	0.5
E	1	10	10	1	1	4.5	4.5	0.5
F	1	15	1	1	1	4.5	4.5	0.5
G	5	5	10	1	0.2	4.5	4.5	0.5
H	5	10	1	1	0.2	4.5	4.5	0.5
I	5	15	5	1	0.2	4.5	4.5	0.5

TABLE-2
PARTICLE SIZE AND POLYDISPERSITY INDEX (PI) OF ETHANOLIC EXTRACT-CHITOSAN-TPP (ECTM) MIXTURE

Sample code ECTM	Chitosan:TPP ratio	Cons. extract (%)	Ultrasonic time (min)	Particle size (nm)	PI
A	0.2	5	1	283.7	0.290
B	0.2	10	5	199.1	0.374
C	0.2	15	10	190.7	0.311
D	1	5	5	496.8	0.162
E	1	10	10	426.5	0.405
F	1	15	1	435.5	0.415
G	5	5	10	407.7	0.390
H	5	10	1	379.6	0.415
I	5	15	5	349.1	0.484

TABLE-3
RESULTS OF ANOVA ANALYSIS (ANALYSIS OF VARIANCE)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chitosan:TPP Ratio	2	81486.0	40743.0	760.07	0.001
Extract concentration	2	8856.6	4428.3	82.61	0.012
Ultrasonic Time	2	973.3	486.6	9.08	0.099
Error	2	107.2	53.6		
Total	8	91423.1			

within the range of 190.7-496.8 nm. The formulations obtained in this study can be categorized as nanoparticulate systems [15]. In addition to particle size, the polydispersity index (PI) values range from 0, indicating a completely homogeneous system, to 1, representing a highly polydisperse distribution [16]. In present study, the PI values of ECTM were below 0.5, ranging from 0.162 to 0.487, suggesting a relatively uniform particle size distribution across all formulations.

In this study, three critical parameters affecting the encapsulation of black fruit leaf ethanol extract were systematically evaluated, namely the chitosan:TPP ratio, extract concentration and ultrasonication time. Each parameter was investigated at three different levels, enabling the application of an L9 orthogonal array within the Taguchi experimental design framework [17]. This approach allowed efficient optimization of particle size while minimizing the number of experimental runs.

The influence of these parameters on particle size was statistically examined using analysis of variance (ANOVA), and the results obtained from Minitab® are summarized in Table-3. The analysis revealed that both the chitosan:TPP ratio and extract concentration exerted a statistically significant effect on particle size ($p < 0.05$), whereas ultrasonication time did not show a significant contribution within the selected range. These findings were further supported by the Taguchi response analysis presented in Table-4, which ranked the chitosan:TPP ratio as the most dominant factor influencing particle size, followed by extract concentration, with ultrasonication time having the least impact.

The pronounced effect of the chitosan:TPP ratio on nanoparticle size is consistent with earlier reports [18,19]. Nanoparticle formation through ionic gelation depends on the electrostatic interactions between the positively charged amino groups of chitosan and the negatively charged phosphate groups of TPP. Achieving a stable nanoparticle system with minimal size requires an optimal balance between these interacting species. Previous studies have shown that particle size decreases as the chitosan:TPP ratio increases from 2:1 to 8:1 [19], while other investigations reported an optimum ratio of 5:1, with larger particles forming at both lower (3-4) and higher (6-7) ratios [18].

TABLE-4
ANALYSIS RESULTS BASED ON TAGUCHI MODEL
(RESPONSE TABLE FOR MEANS)

Level	Chitosan:TPP ratio	Extract concentration	Ultrasonic time
1	224.5	396.1	366.3
2	452.9	335.1	348.3
3	378.8	325.1	341.6
Delta	228.4	71.0	24.6
Rank	1	2	3

Phenolic content: Phenolic content analysis was performed on both the initial ethanolic extract of black fruit leaves and the unencapsulated extract remaining after the encapsulation process to confirm the presence of phenolic compounds and to determine encapsulation efficiency. Qualitative analysis of the free extract showed a positive response for phenolics, indicated by the formation of a blackish-green colouration. Quantitative estimation of phenolic compounds encapsulated within the chitosan nanoparticles is presented in Fig. 1, with values ranging from 7.65 to 17.00 mg GAE/g, confirming effective retention of phenolics within the nanoparticle matrix.

The encapsulation efficiency values, ranged from 75.45% to 80.43%, demonstrating efficient entrapment of phenolic compounds in the chitosan-TPP system (Fig. 1b). The high encapsulation efficiency can be attributed to favourable electrostatic interactions and hydrogen bonding between phenolic compounds and the chitosan polymer network. These results are consistent with previous studies. Sulaeman *et al.* [20] reported an encapsulated phenolic content of 3.81 mg GAE/g with an efficiency of 81.69% for propolis encapsulated by spray drying, while Pudziuvelyte *et al.* [21] obtained 17.927 mg GAE/g for phenolics encapsulated in chitosan from acerola byproducts.

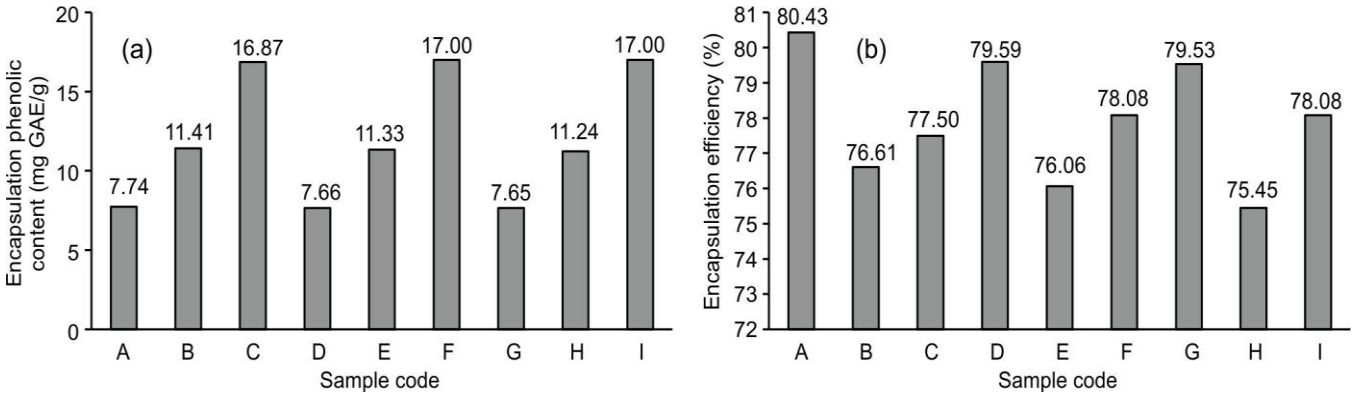

Fig. 1. Encapsulated phenolic content (a) and encapsulation efficiency as phenolic content (b)

Antioxidant activity: The antioxidant activity of the ECTM samples was evaluated using the DPPH radical scavenging assay and the results are shown in Fig. 2. The IC_{50} values ranged from 0.54 to 25.77%, indicating variable yet appreciable antioxidant potential among the samples. Comparable results were reported by Poli *et al.* [22], where ethyl-methyl ketone (90.26%) and ethyl acetate (90.05%) extracts exhibited DPPH scavenging activity exceeding 50%. The antioxidant response observed in the present study is further supported by positive flavonoid results obtained during phytochemical screening, confirming the presence of phenolic constituents responsible for radical scavenging. At a concentration of 1000 μg/mL, the extracts effectively inhibited free radicals, consistent with the behaviour of primary antioxidants. The DPPH method is widely used to evaluate antioxidant activity based on the ability of compounds to donate hydrogen atoms to stabilize free radicals [23].

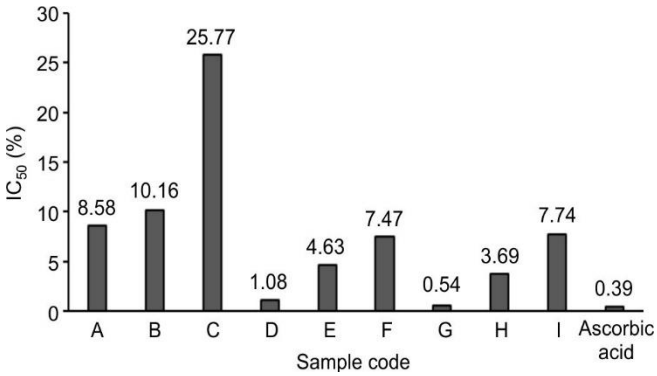

Fig. 2. IC_{50} results of antioxidant activity using the DPPH method

Among the ECTM formulations, samples G and D demonstrated particularly strong antioxidant activity, as reflected by IC_{50} values close to that of the positive control, ascorbic acid, which is a potent antioxidant [24]. Similar improvements in antioxidant activity following encapsulation have been reported in earlier studies. Bagheri *et al.* [25] showed that chitosan-nettle essential oil nanoparticles exhibited enhanced DPPH scavenging compared to the unencapsulated oil, attributed to improved stability of phenolic compounds. Likewise, Lan *et al.* [26] reported that cinnamaldehyde encapsulated in chitosan nanoparticles (encapsulation efficiency ≈ 74.39%) retained antioxidant activity with an IC_{50} value of 223.44.

The antioxidant capacity of the encapsulated system is influenced not only by the stabilization of phenolic compounds within the chitosan matrix but also by the inherent radical scavenging ability of chitosan itself [3], although this intrinsic activity is relatively modest [27]. Chitosan scavenges DPPH radicals primarily through hydrogen donation, leading to the formation of more stable macromolecular radicals via reactions with superoxide radicals and hydroxyl anions [3].

Antibacterial activity: The antibacterial activity of the ECTM samples was evaluated using the disc diffusion method and the results are presented in Fig. 3. The diameter of the inhibition zones ranged from 0.00 to 0.41 cm against *S. aureus* and 0.03 to 0.38 μm against *E. coli*, indicating varying antibacterial efficacy among the samples. Overall, ECTM samples A–I exhibited stronger inhibitory effects against *E. coli* than against *S. aureus*. This difference can be attributed to the structural characteristics of the bacteria; *E. coli* is a Gram-negative bacterium with a thinner peptidoglycan layer, making it more susceptible to antibacterial agents, whereas *S. aureus* is Gram-positive and possesses a thicker cell wall that provides greater resistance.

When compared with 5% NaCl solution, which showed no inhibitory effect on either bacterium, all ECTM samples demonstrated superior antibacterial activity, confirming the intrinsic antimicrobial potential of the encapsulated system. Relative to the standard antibiotic chloramphenicol, sample E exhibited the highest antibacterial effectiveness, achieving 15.63% inhibition against *S. aureus* and 14.70% against *E. coli*. These results highlight the notable antibacterial contribution of the ECTM formulations. The observed antibacterial activity is consistent with previous findings indicating that chitosan-extract systems benefit from the strong antimicrobial properties of chitosan [28]. Chitosan inhibits bacterial growth through electrostatic interactions between its positively charged NH_3^+ groups and the negatively charged bacterial cell membrane, leading to increased membrane permeability and leakage of intracellular components [29]. Furthermore, nanoscale particles exhibit enhanced antibacterial activity due to their ability to penetrate bacterial membranes and disrupt cell walls more effectively than larger particles [25]. Notably, sample G demonstrated the best overall balance of antibacterial and antioxidant activity among all ECTM samples, suggesting its potential suitability for multifunctional bioactive applications.

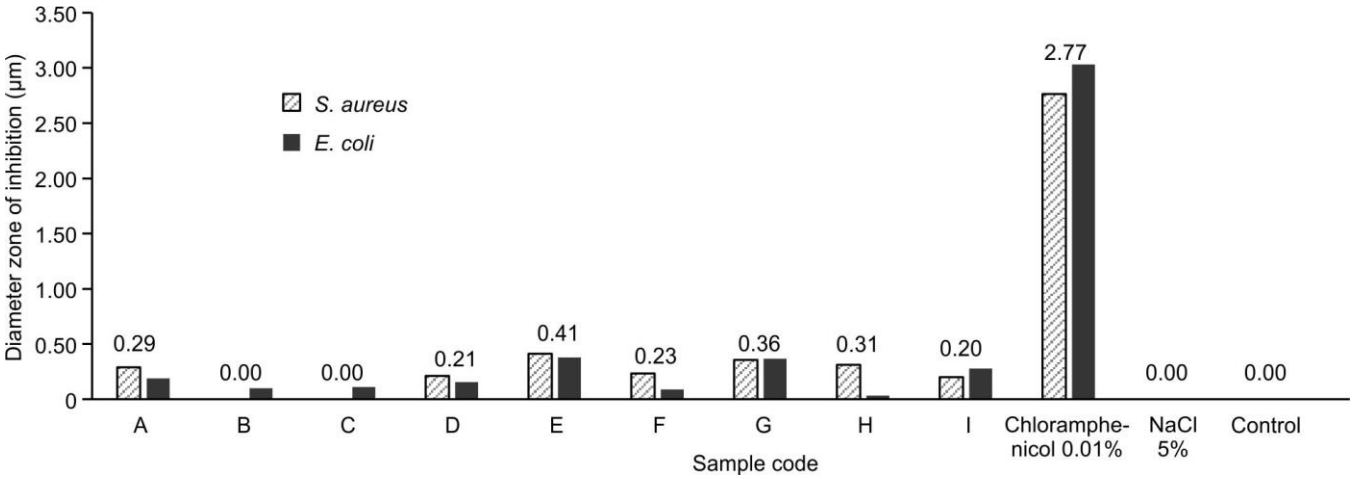

Fig. 3. Antibacterial activity results of different formulations based on disc diffusion method

Conclusion

This study confirms the successful encapsulation of black fruit leaf ethanolic extract within a chitosan-TPP nanoparticle system, producing stable submicron particles with good size uniformity. The chitosan:TPP ratio and extract concentration were identified as the key factors influencing particle size. High encapsulation efficiency (>75%) ensured effective retention of phenolic compounds, resulting in significant antioxidant and antibacterial activities. Enhanced activity against *E. coli* compared to *S. aureus* was observed, with sample G exhibiting the best overall performance. The results highlight the potential of this eco-friendly system for bioactive delivery and antimicrobial applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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