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Research Article

Screening of Critical Variables of *Andrographis paniculata* Extract Loaded onto Chitosan Microparticles

Melissa Yushan Ng[a], Rosnani Hasham[a*], Rahimah Sabtu[a], Mohamad Khairul Hafiz Idris[a], and Illani Abdul Rahman[a]

[a] *School of Chemical Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Johor, Malaysia*

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ABSTRACT

Andrographis paniculata (AP) is a medicinal plant commonly found in Malaysia. However, the main challenges hindering its clinical values is its low bioavailability and solubility in aqueous environment which can be improved by incorporating it into a nanocarrier system. Therefore, this study was conducted to screen the critical variables involved in the development of AP extract-loaded chitosan microparticles. Nine formulations (coded as F1 to F9) at varying chitosan-to-tripolyphosphate (TPP) mass ratio (2:1, 4:1, and 6:1) and reaction time (30, 60, and 90 minutes) were tested utilizing the one-factor-at-a-time (OFAT) technique and characterized by means of their particle size, polydispersity index (PDI), and encapsulation efficiency (EE). The best formulation was further characterized for its zeta potential (ζ-potential), morphology, and stability. F2 was discovered as the best formulation in the first screening with a particle size of 0.595 ± 0.014 µm, PDI of 0.284 ± 0.011, and EE of 81.18 ± 5.53%. Further testing on the formulation revealed a ζ-potential of 6.4 ±1.51 mV with a spherical and smooth-surface microparticles in dispersion. The microparticles were also stable at 4 °C with minimal change in size after 14 days. In conclusion, these results show that entrapment of AP extract into chitosan-TPP microparticles were achievable at good characteristics and stability and could be further studied as a form for delivering therapeutic activities of AP at targeted site.

INTRODUCTION

Nature is abundant with plants that serve particular functions, some for dietary consumption while others serve as medicinal therapy. Medicinal plants refer to plants that contain certain bioactive compounds that are used to cure disease or act as relieving pain as analgesic agents (Okigbo et al., 2008). *Andrographis paniculata* (AP) is a medicinal plant that has been widely used to treat illnesses in China, India, Thailand, and Malaysia due to its promising prospects. This medicinal plant is commonly known as King of Bitters (English), *Fah Talai Jone* (Thai), *Chuanxinlian* (Chinese), and *hempedu bumi* (Malay) in our Malaysian communities.

According to Chinese medicine, AP has cooling properties that is able to relieve internal heat, inflammation, and pain, it is also used as a detoxifier to rid the body of excess toxins (Chao & Lin, 2010). In Ayurvedic medicinal systems, this herb has been used for a variety of ailments such as pre-natal and post-natal care, dysmenorrhea, leucorrhea, and complicated diseases such as malaria, gonorrhea, and jaundice (Okhuarobo et al., 2014). The aerial parts of the plant were commonly used to treat the common cold, hypertension, diabetes, cancer, malaria, and snakebite, especially in Malaysia.

AP has been used to treat various illnesses or to treat wounds. When extracted from the plant, it has been reported to be capable of combatting cancer due to its anti-

*Corresponding Author
E-mail address: yushan@graduate.utm.my (First Author),
r-rosnani@utm.my (Second Author).
School of Chemical Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Johor, Malaysia

cancer activities through the stimulation of lymphocyte proliferation and activation, through its main phytoconstituent, Andrographolide (NCBI, 2021). Andrographolide is a labdane diterpenoid among other derivatives produced by AP, which was able to suppress the proliferation of human colon cancer cells through the Toll-like receptor 4 / nuclear factor - κB/matrix metalloproteinase-9 signaling pathway (Zhang et al., 2014). Andrographolide has extensive medicinal possibilities, however, it has the drawbacks of having poor water solubility and low bioavailability (Yan, Fang, & Du, 2018) which results in poor therapeutic activity (Oseni et al., 2021). Therefore, the drawbacks must be overcome with a suitable delivery system for it to be a novel and potential chemotherapeutic agent. Colon cancer is a cancer that begins in the large intestine, also known as the colon. In Malaysia, it is the second most common cancer in males and the third most common cancer in females (Veettil et al., 2017). Alarmingly, it was reported that colon cancer primarily affects males in the age range of 35-75 (Hassan et al., 2016).

As of date, none of the commercialized drugs with AP were intended for specific target sites nor intended for colon cancer treatment. Despite the many research-backed evidence showing that andrographolide is capable of inducing cancer cell apoptosis, it is not recognized as a prescribed drug in combatting cancer, particularly, colon cancer. This is due to the lack of clinical trials to test its drug safety and efficacy, many of the testing done in previous research involves mostly *in vitro* testing, which is inconclusive to determine its actual effect towards humans. The technique of nanoencapsulation is a promising approach for the development of medicine targeting cancer cells, which could serve as an alternative method for cancer therapy rather than chemotherapy which causes discomfort to the patient (Canadian Cancer Society, 2021).

Before developing a delivery system that delivers the prepared drug-loaded microparticles to the desired target site, it is desired to formulate a suitable microparticle that has the optimal size and properties for better absorption into the cells, taking advantage of the microflora of the colon, where biodegradable enzymes are present, it is favorable to use biodegradable polymers for colon-specific drug delivery, has been a more site-specific approach compared to other approaches (Philip & Philip, 2010).

Chitosan (CS) is a linear polycationic copolymer of β (1–4) linked 2-acetamide-2-deoxy-β-D-glucopyranose and 2-amino-2- deoxy-β-D-glucopyranose derived from deacetylation of chitin, the constituent of the exoskeletons of animals, especially crustaceans (crabs, shrimps, and lobster), mollusks and insects. CS is widely used in the pharmaceutical field and has been gaining increasing importance as a potential formulation excipient, to binding, disintegrating and tablet coating properties due to its biocompatible, biodegradable, and non-toxic (Ruiz-Caro & Veiga-Ochoa, 2009; Sari et al., 2019; Pantic et al., 2020).

Hence, this paper aims to encapsulate AP extract with chitosan (AP-CS) using a microencapsulation method to achieve particles with a small size.

MATERIALS AND METHOD

Materials

The raw of AP was purcahed from Ethno Resources Sdn Bhd (Sungai Buloh, Selangor, Malaysia. The raw material was undergone pre-treatment processing stages which are cleaning, drying, and grinding (40 mesh) for the purpose of size reduction. Ethanol, acetic acid, sodium hydroxide solution, hydrochloric acid, chitosan, sodium tripolyphosphate, sodium carbonate, Folin-Ciocalteu reagent, and gallic acid were purchased from Merck, Germany. Distilled water has been used throughout the experiment.

Extraction

The ultrasound-Assisted Extraction (UAE) method has been employed for the extraction of AP. In detail, 5 g of the AP powder was soaked in 50% ethanol with a sample-to-solvent ratio of 1:10. The beaker was put in a bath sonicator for 15 minutes. Then, the sample was centrifuged at 5000 rpm to retrieve the supernatant. Next, by using a rotary evaporator the extract was concentrated. The remaining excess moisture has been removed by oven drying at 45 °C before being stored until further use.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu method described by Zhang et al. (2006) (with some modifications.) Briefly, 2 mg of AP dry extracts were dissolved in 2 mL of 50% ethanol to give a final concentration of 2 mg/mL. Extract solution (30 µL) was then mixed with 15 µL Folin-Ciocalteu reagent, 60 µL of 10% (w/v) aqueous solution of sodium carbonate, and 195 µL of distilled water in a 96-well plate. The sample was then incubated for 60 minutes in a dark place, and the absorbance was measured at a wavelength of 765 nm by using a Bio-Tek ELX808 microplate reader. A series of gallic acid standard solutions were prepared with concentrations ranging from 0 to 1.00 mg/mL. The results were expressed as mg gallic acid equivalents over dry weight (mg GAE/ g DW).

Preparation of AP-CS Microparticles

AP loaded onto chitosan microparticles were prepared according to the ionic gelation method described by Chen et al. (2016) with minor adjustments. An 8 mg of CS powder was added to 4 mL of 0.2% acetic acid solution with constant magnetic stirring for 6 hours. A 2 mg of AP extract was then dissolved in 1 mL of 50% ethanol to make a final concentration of 2 mg/mL. A portion of 2 mg of sodium tripolyphosphate (TPP) was dissolved in 2 mL of distilled water to obtain a negatively charge electrolyte. Thereafter, 2 mL of the AP solution was added to 4 mL of CS solution. set of parameters of TPP solution was then added dropwise into the AP-CS solution slowly over a magnetic stirrer for a certain time at 500 rpm. The CS to TPP mass ratio of 2:1, 4:1, 6:1 (Abosabaa, ElMeshad, & Arafa, 2021), and reaction time; 30 minutes, 60 minutes, 90 minutes (Vaezifar et al., 2013) were studied to determine the optimal parameters to prepare desired microparticles of specific size (Table 1).

Table 1 Formulation matrix and the varied parameters

Formulation	Mass Ratio of CS:TPP	Stirring Time (minutes)
F1	2:1	30
F2	4:1	30
F3	6:1	30
F4	2:1	60
F5	4:1	60
F6	6:1	60
F7	2:1	90
F8	4:1	90
F9	6:1	90

Characterization of AP-CS Microparticles

Particle Size, Polydispersity Index, and Zeta Potential

The particle size, polydispersity index, and zeta potential of the AP-CS microparticles has been measured based on the dynamic light scattering (DLS) technique by using Litesizer 500 Analyzer (Anton Paar). The parameters for measurement and calculation were set as follows: 1.33 material refraction index, 90 °C measurement angle, 25 °C, and water as the dispersant. The sample has been diluted 10-fold with distilled water. Each sample was analysed in triplicate, and results have been expressed in nm ± SD.

Encapsulation Efficiency (%)

For the EE%, the collected supernatant was analyzed for its drug content by following Folin-Ciocalteu method using a constructed calibration curve (y = 4.8966x+0.0456, R^2 = 0.999) of gallic acid (0-0.5 mg/mL). Briefly, 30 µL of the sample (supernatant and pellet) solution was mixed with 15 µL Folin reagent, 60 µL of 10% (w/v) aqueous solution of sodium carbonate, and 195 µL of distilled water in a 96-well plate. The 96-well plate was then put in a dark condition to incubate for 60 minutes before having its absorbance measured at a wavelength of 765 nm using an Elisa microplate reader (Epoch, BioTek). The readings were done in triplicate for the entrapment efficiencies, and results were expressed in % ± SD. The drug EE% was calculated by using the following formula:

$$\text{Encapsulation Efficiency (\%)} = \frac{W_{Total(AD)} - W_{Free(AD)}}{W_{Total(AD)}} \times 100$$

Morphological Structure

The morphology of the AP-CS microparticles was studied by using Transmission Electron Microscopy (TEM) (HRTEM 120kV). This evaluation was performed on the formulation yielding the smallest particle size. The microparticle dispersion was spread on a 200-mesh copper grid coated with a carbon membrane for about 3 minutes. Then, by using filter paper the excess droplets were removed. The grid was then placed above a drop of phosphotungstic acid solution (2%, w/w) for about 2 minutes. The grid was then observed under a Hitachi H-7110 electron microscope.

Stability Study

For the stability test, the selected formulation was stored at chiller temperature (4 ± 2 °C), and room temperature (25 ± 2 °C) for a duration of two weeks as adapted from the studies of Katas, Raja, and Lam (2013). The microparticles were measured for particle size and polydispersity index were determined to evaluate the physicochemical stability after two weeks. All results were reported as the mean value ± SD.

RESULTS AND DISCUSSION

Total Yield of Extraction

The total yield of extraction highly affected by choice of parameter since its measured to evaluate the efficiency of an extraction technique in extracting specific components from the plant material. The total yield is expressed as the weight percentage of the dried crude extract over the weight of the raw material (Zhang et al., 2007). The value obtained for AP yield from the plants was 7%.

Total phenolic content

The gallic acid calibration curve as depicted in **Figure 1** has been used to determine the total phenolic content (TPC) in the plant extract.

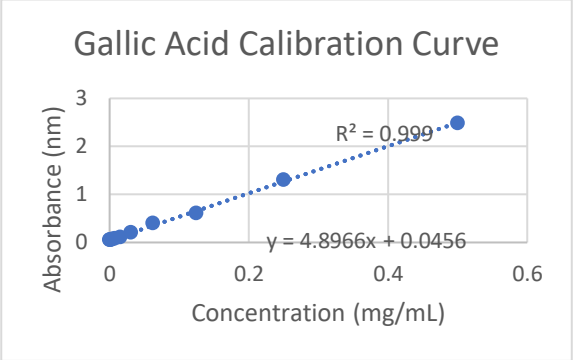

Figure 1 Gallic acid calibration curve (0-0.5 mg/mL)

The TPC was analyzed to determine the amount of phenolic compounds in a plant that has redox properties, which allows their antioxidant properties. TPC of the extract was calculated based on the regression equation of the calibration curve (y=4.8966x+0.0456, R^2 = 0.999) and the results were expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg GAE/g DW). The TPC value was recorded to be 13.832 ± 0.85 mg GAE/g DW. This value was higher compared to the TPC value obtained in a previous study by Salleh et al. (2014) who performed the experiment using the same subject AP, which is 7.78 mg GAE/ g DW, the TPC value obtained when using 50% ethanol was two times higher than the TPC value obtained using 70% methanol as the solvent for extraction of herbal actives.

Particle Size and Polydispersity Index of AP-CS Microparticles

The effect of the mass ratio of CS: TPP and stirring time (min) were varied to find the optimum parameter yielding the stable formulation with the smallest particle size and polydispersity index. The stirring speed was set at a constant 500 rpm for all formulations. All AP-CS prepared in this study exhibited milky characteristics when TPP was added into the solution, which indicates particle formation (Pedroso-Santana & Fleitas-Salazar, 2020). The resulting mixture had turbidity in appearance and could flow freely when its containers were tilted.

According to **Table 2**, F1, F4, and F7 showed the particle size was consistently large even for different reaction times (30, 60, and 90 minutes). This finding is

similarly reported with the study done by Hussain et al. (2016), where it was reported that the size of their formulated microparticles was also the largest at a mass ratio of 2:1 due to aggregation of the microparticles. It can be explained that when the mass ratio of CS: TPP increased, the lower concentration of TPP used could reduce the occurrence of larger particles by minimizing aggregation that leads to the formation of larger particles. The large particle size of Formulation F1 (72.712 ± 33.590 µm ± SD) could be attributed to the mishandling of the preparation of the sample, and the agglomeration of particles into particles with a large radius (Ashraf et al., 2018) which in turns affects the data range of the sample. The high value of standard deviation shows the range of sizes of agglomerated particles that exist in the analyzed samples.

The PDI of a microparticle indicates the heterogeneity of a sample based on particle size, where a low PDI (<0.3) indicates homogeneity and uniformity of the microparticle system and more than that (>0.3) would indicate a heterogenous system consisting of different sizes (Mudalige et al., 2019). The relatively low polydispersity indices of formulation F2, F3, F4, F6, and F9 obtained from the study reveals that the samples exhibit a consistent average size. Formulation F5 with a polydispersity index of 0.335 ± 0.025 implies that the system is heterogenous and consists of particles with varying sizes. The particles in F5 usually exhibits a consistent average size of 13.259 µm, the smallest particle size detected was 8.149 µm and the largest was 18.369 µm existing within the same sample.

Table 2 Particle size and polydispersity index of AP-CS microparticles

Formulation	Particle Size µm ± SD	Polydispersity Index (PDI) ± SD
F1	72.712± 33.59	0.385 ± 0.079
F2	0.836± 0.040	0.217 ± 0.084
F3	0.595 ± 0.0139	0.284 ± 0.011
F4	4.338 ± 1.850	0.298 ± 0.060
F5	13.259± 5.110	0.335 ± 0.025
F6	1.448 ± 0.096	0.277 ± 0.024
F7	42.203± 2.67	0.387 ± 0.11
F8	1.597 ± 0.270	0.327 ± 0.011
F9	0.711± 0.056	0.280 ± 0.022

In terms of reaction time, the general trend in our studies suggests that higher reaction times tend to lead to larger sizes of particles formed. 30 minutes of reaction time overall produces the smallest microparticles. Previous study has shown that 30 minutes is sufficient for cross-linking of active loaded CS and TPP solution. By extending the reaction duration to 120 minutes, the particle size was reported to have significantly increased in particle size, which has been explained due to the swelling of nanospheres during the mixing process (Tilkan & Özdemir, 2018).

Overall, a higher CS: TPP mass ratio results in a more homogenous particle dispersion in particles formed using the 6:1 ratio, whereas the size is consistently large for particles formed using higher mass concentrations of CS (2:1, 4:1).

Encapsulation Efficiency of AP-CS Microparticles

Encapsulation Efficiency is another important parameter to measure the amount of encapsulated active ingredient within the microparticles. The EE% is attributed to the concentration of the polymer in the system, where an increase in polymer concentration increases the density of the system and thus assist in the formation of crosslinks (Sari et al., 2016). **Table 3** shows the EE% of all the formulations. It shows the EE of all the formulations were in the range of 70-88%, which is an acceptable range (Jarudilokkul, et al., 2011). In their work encapsulating a protein by using the IG method, their reported EE was in similar ranges (75.39-82.64%). In particular, the desired formulation (F3) yielding the smallest size had an EE of 81.18 ± 5.53%, which is an acceptable EE as supported by the findings of another study (Tepsatian & Kittigowittana, 2017), where their formulation of oolong tea extract loaded AP-CS had an EE% of 79%. The factors affecting the encapsulation efficiency of a microparticulate system include concentration of the polymer, solubility of polymer in solvent, rate of solvent removal, and solubility of the organic solvent in water (Jyothi et al., 2010). The high EE% and large sizes of some of the formulation (F1, F5, F7) correlates with the magnitude of chitosan concentration that causes the chitosan molecules to be bound to each other thus increasing intermolecular crosslinking bonds, which will in turn increase the EE% and also the molecular size (Zahrani et al., 2017). Based on screening on particle size, PDI, and EE%, F3 collectively showed the smallest particle size with a stable PDI value and high EE%.

Table 3 EE(%) of AP-CS microparticles

Formulation	Encapsulation Efficiency, % ± SD
F1	81.18 ± 1.73
F2	70.92 ± 0.43
F3	81.18 ± 5.53
F4	87.44 ± 0.43
F5	80.93 ± 0.00
F6	73.17 ± 1.56
F7	81.93 ± 3.78
F8	72.42 ± 3.13
F9	84.18 ± 2.17

Zeta Potential of AP-CS Microparticles

The zeta potential of a microparticle system is important in determining its colloidal stability. It is also a measure of the effective electric charge on the microparticle's surface. Zeta potential that are greater than 30 mV tends to be homogenous and display monodispersity, while values that are near 0 and smaller than 5 mV could lead to agglomeration of the particles (Gumustas et al., 2017). The zeta potential of formulation F3 was observed to be 6.4 ± 1.5, which indicates that the system may be prone to agglomeration. The zeta potential of the system is positive due to the amino groups present in the system from CS.

Morphological Structure of AP-CS Microparticles

The morphology of the AP-CS microparticles was observed by using a TEM. The micrographs of the microparticle system of formulation F3 showed some aggregation of the microparticles as can be inferred from **Figure 2** below. According to Ashraf et al. (2018), when the isolated and dispersed microparticles are accumulated, it can be assumed that a large microparticle with high radius. As a result, the aggregation of the microparticles can be physically assumed by the growth of particle size in the microcomposites. From the micrographs, the AP-CS microparticles had a round spherical shape and a smooth

surface, implying that the actives were successfully encapsulated using the ionic gelation method. These findings align with the morphology that typically results from AP-CS microparticles synthesized using the ionic gelation technique, as can be inferred from the findings of a previous publication (Raj et al., 2018).

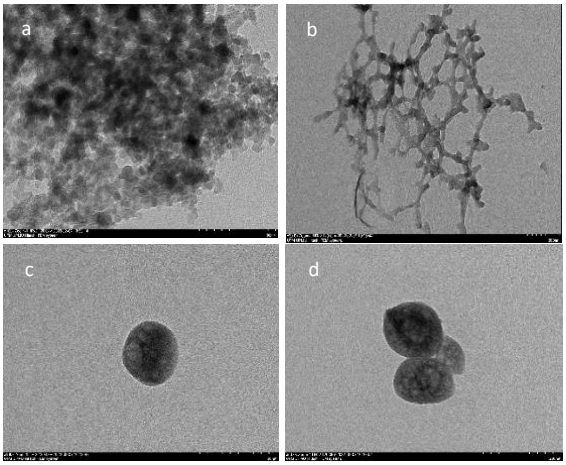

Figure 2 Morphology of AP-CS microparticles ((a) x30k, (b)x30k, (c) x100k, (d) x100k)

Stability of AP-CS Microparticles

Stability tests are important to predict the shelf life of the product. To determine the physical stability of the AP-CS microparticles, the formulations have been further stored at a temperature of 4 °C, and 25 °C for two weeks. From **Table 4**, it can be observed that the size of microparticles increases over time depending on the storage conditions. The two conditions were selected to observe the effect of thermo-responsive and suffering a pH variation at room temperature, which will lead to decreased stability and the loss of other important properties (Pedroso-Santana & Fleitas-Salazar, 2020).

Table 4 Change in particle size of AP-loaded CS microparticles after 2 weeks at different temperature for formulation F3

Temperature (°C)	Particle size, µm ± SD	Polydispersity Index (PDI), ± SD
Initial	0.595 ± 0.014	0.284 ± 0.011
4	0.602± 0.0054	0.2617 ± 0.019
25	0.616± 0.0262	0.2739 ± 0.056

These results are in alignment with the studies reported by (Katas et al., 2013), where their studies show that after conducting the stability test for a maximum of 14 days, there was a significant increase in particle size due to degradation of the particles compared to storing at 4 °C. Thereby suggesting that the stability test conducted for 14 days was sufficient to prove that the microparticle systems are best stable at a temperature of 4 °C rather than ambient temperature. Based on these results, our findings suggest that the optimal storage temperature for microparticles prepared by the ionic gelation method would be 4 °C.

CONCLUSION

In general, this study was carried out to extract and encapsulate AP in a mild condition. From the data obtained, the extract recovery was acceptable and the TPC was moderately high. The mass ratio of CS: TPP and the reaction time had effects on the size of the microparticles formed. The objective of this study was achieved, where the extract recovered was successfully encapsulated by optimizing the parameters to determine the smallest size, and later characterized. Overall, the formulation having a high CS: TPP ratio and 30 minutes of reaction time produced the smallest size in average. The selected formulation, F3 had a positive zeta potential as well as a spherical shape and smooth surface. The EE% of all formulations were sufficiently high and the stability tests conducted show that the microparticles prepared were most stable at 4 °C over a 2-week basis. These findings show that the chitosan microparticles prepared were an acceptable candidate for delivery to the colon as the size of the formulation selected was below the human colon carcinoma pore cut-off size of 0.400-0.600 µm.

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References

Abosabaa, S., ElMeshad, A. N., & G Arafa, M. (2021). Chitosan nanocarrier entrapping hydrophilic drugs as advanced polymeric system for dual pharmaceutical and cosmeceutical application: A comprehensive analysis using Box–Behnken Design. *Polymers*, *13*(5), 677.

Ashraf, M. A., Peng, W., Zare, Y., & Rhee, K. Y. (2018). Effects of size and aggregation/agglomeration of nanoparticles on the interfacial/interphase properties and tensile strength of polymer nanocomposites. *Nanoscale Research Letters*, *13*(1), 1-7.

Canadian Cancer Society (2021). Side effects of chemotherapy. In Canadian Cancer Society. Retrieved June 10, 2021, from https://www.cancer.ca/en/cancer-information/diagnosis-and-treatment/chemotherapy-and-other-drug-therapies/chemotherapy/side-effects-of-chemotherapy/?region=on.

Chao, W. W., & Lin, B. F. (2010). Isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). *Chinese Medicine*, *5*(1), 1-15.

Chen, S., Guo, F., Deng, T., Zhu, S., Liu, W., Zhong, H., Yu, H., Luo, R., & Deng, Z., (2016). Eudragit S100-coated chitosan microparticles co-loading tat for enhanced oral colon absorption of insulin. *AAPS PharmSciTech*, *18*(4), 1277-1287.

Gumustas, M., Sengel-Turk, C. T., Gumustas, A., Ozkan, S. A., & Uslu, B. (2017). Effect of polymer-based microparticles on the assay of antimicrobial drug delivery systems. In *Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics* (pp. 67-108). Elsevier.

Hassan, M. R. A., Ismail, I., Suan, M. A. M., Ahmad, F., Khazim, W. K. W., Othman, Z., ... & Mustapha, N. R. N. (2016). Incidence and mortality rates of colorectal cancer in Malaysia. *Epidemiology and Health*, *38*.

Hussain, Z. A. H. I. D., & Sahudin, S. H. A. R. I. Z. A. (2016). Preparation, characterisation and colloidal stability

of chitosan-tripolyphosphate microparticles: Optimisation of formulation and process parameters. *International Journal of Pharmacy and Pharmaceutical Sciences*, *8*(3), 297-308.

Jarudilokkul, S., Tongthammachat, A., & Boonamnuayvittaya, V. (2011). Preparation of chitosan microparticles for encapsulation and release of protein. *Korean Journal of Chemical Engineering*, *28*(5), 1247-1251.

Jyothi, N. V. N., Prasanna, P. M., Sakarkar, S. N., Prabha, K. S., Ramaiah, P. S., & Srawan, G. Y. (2010). Microencapsulation techniques, factors influencing encapsulation efficiency. *Journal of microencapsulation*, *27*(3), 187-197.

Katas, H., Raja, M. A. G., & Lam, K. L. (2013). Development of chitosan nanoparticles as a stable drug delivery system for protein/siRNA. International Journal of Biomaterials, 2013.

Mudalige, T., Qu, H., Van Haute, D., Ansar, S. M., Paredes, A., & Ingle, T. (2019). Characterization of nanomaterials: Tools and challenges. *Nanomaterials for food applications*, 313-353.

Okhuarobo, A., Falodun, J. E., Erharuyi, O., Imieje, V., Falodun, A., & Langer, P. (2014). Harnessing the medicinal properties of Andrographis paniculata for diseases and beyond: a review of its phytochemistry and pharmacology. *Asian Pacific journal of tropical disease*, *4*(3), 213-222.

Okigbo, R. N., Eme, U. E., & Ogbogu, S. (2008). Biodiversity and conservation of medicinal and aromatic plants in Africa. *Biotechnology and Molecular Biology Reviews*, *3*(6), 127-134.

Oseni, B. A., Azubuike, C. P., Okubanjo, O. O., Igwilo, C. I., & Panyam, J. (2021). Encapsulation of AND in poly (lactide-co-glycolide) microparticles: Formulation optimization and in vitro efficacy studies. *Frontiers in Bioengineering and Biotechnology*, *9*, 61.

Pantic, M., Horvat, G., Knez, Z., Novak, Z. (2020). Preparation and characterization of chitosan-coated pectin aerogels: Curcumin case study. Molecules, 25, 1187; doi:10.3390/molecules25051187.

Pedroso-Santana, S., & Fleitas-Salazar, N. (2020). Ionotropic gelation method in the synthesis of microparticles/microparticles for biomedical purposes. *Polymer International*, *69*(5), 443-447.

Philip, A. K., & Philip, B. (2010). Colon targeted drug delivery systems: a review on primary and novel approaches. *Oman Medical Journal*, *25*(2), 79.

Raj, P. M., Raj, R., Kaul, A., Mishra, A. K., & Ram, A. (2018). Biodistribution and targeting potential assessment of mucoadhesive chitosan microparticles designed for ulcerative colitis via scintigraphy. *RSC Advances*, *8*(37), 20809-20821.

Ruiz-Caro, R., & Veiga-Ochoa, M. D. (2009). Characterization and dissolution study of chitosan freeze-dried systems for drug controlled release. Molecules, 14, 4370-4386; doi:10.3390/molecules14114370

Salleh, L. M., Hartati, H., Jamaludin, R., Yunus, M. A. C., Yakub, H., & Aziz, A. A. (2014). Antioxidant activity and total phenolic contents in methanol extracts from Swietenia mahagoni and Andrographis paniculata. *Jurnal Teknologi*, *69*(4).

Sari, R., Setyawan, D., Retnowati, D., Pratiwi, R. (2019). Development of andrographolide-chitosan solid dispersion system: Physical characterization, solubility, and dissolution testing. *Asian Journal of Pharmaceutics,* 13(1): 5

Sari, R., Feriza, M., & Putri, A. N. A. (2016). Polymeric particulate system of carboxymethyl chitosan-diterpen lactone fraction of Andrographis paniculatas Nees: Characterization and in vitro release study. *International Journal of PharmTech Research*, *9*(1), 120-127.

Tepsatian, P., & Kittigowittana, K. (2017). Encapsulation Efficiency of Oolong Tea Chitosan Microparticles for Cosmetic Applications. *Walailak Journal of Science and Technology (WJST)*, *14*(9), 677-685.

Tilkan, M. G. Y., & Özdemir, N. (2018). Investigation of the parameters affecting the release of flurbiprofen from chitosan microspheres. *Brazilian Journal of Pharmaceutical Sciences*, *53*.

Vaezifar, S., Razavi, S., Golozar, M. A., Karbasi, S., Morshed, M., & Kamali, M. (2013). Effects of some parameters on particle size distribution of chitosan microparticles prepared by IG method. *Journal of Cluster Science*, *24*(3), 891-903.

Veettil, S. K., Lim, K. G., Chaiyakunapruk, N., Ching, S. M., & Hassan, M. R. A. (2017). Colorectal cancer in Malaysia: Its burden and implications for a multiethnic country. *Asian Journal of Surgery*, *40*(6), 481-489.

Yan, Y., Fang, L. H., & Du, G. H. (2018). AND. In *Natural Small Molecule Drugs from Plants* (pp. 357-362). Springer, Singapore.

Zahrani, K., Imansari, F., Utami, T. S., & Arbianti, R. (2017, July). Release Profile of Andrographis paniculata leaf extract nanocapsule as α-Glucosidase inhibitors. In *IOP Conference Series: Materials Science and Engineering* (Vol. 214, No. 1, p. 012020). IOP Publishing.

Zhang, J., Li, Y., Gao, W., Repka, M. A., Wang, Y., & Chen, M. (2014). AND-loaded PLGA-PEG-PLGA micelles to improve its bioavailability and anticancer efficacy. *Expert Opinion on Drug Delivery*, *11*(9), 1367–1380.

Zhang, Q., Zhang, J., Shen, J., Silva, A., Dennis, D. A., & Barrow, C. J. (2006). A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. *Journal of Applied Phycology*, *18*(3), 445-450.

Zhang, S. Q., Bi, H.M., and Liu, C.J., Extraction of bio-active components from *Rhodiola sachalinensis* under ultrahigh hydrostatic pressure. *Separation and Purification Technology,* 2007. 57(2): p. 277-282.