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Short communication

A novel green gelatin–agar microencapsulation system with *P. urinaria* as an improved anti-*A. niger* model

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ABSTRACT

In this study, a novel green microencapsulation system was used to develop *Phyllanthus urinaria* (PU) extract containing microcapsules. Agar was used with gelatin as the wall matrix materials of microcapsules to prevent the use of toxic crosslinker formaldehyde. Microencapsulated PU extract was developed to improve the potential antifungal activities of PU water extracts. The active components and surface morphology of PU extract containing microcapsules were analyzed by liquid chromatography/mass spectrometry and scanning electron microscopy, respectively. The in vitro release study demonstrated that approximately 80% of drug was released after 120 h. PU loaded microcapsules were shown to have a stronger anti-*Aspergillus niger* activity than the free drug.

1. Introduction

Administration of herbal medicine through microparticulate drug delivery system possesses high potential for various applications in therapeutic and pharmaceutical fields (Ajazuddin, 2010). Microencapsulation is a micro-packaging technique used for microcapsule production by surrounding small particles of solids, droplets of liquids or dispersions of solids in liquids within polymer coatings to give small capsules useful for various applications (Lam, Gambari, et al., 2012; Lam, Lee, Kok, et al., 2012; Lam, Lee, Wong, et al., 2012; Lam, Yuen, et al., 2012). Gelatin, as a biodegradable polymer, is required a crosslinker formaldehyde to improve the general properties of microcapsules (Cheng et al., 2009; Huang, Cheng, Yu, Tsai, & Cham, 2007). However, formaldehyde can cause respiratory stimulations (Kulle, 1993) and allergic contact dermatitis (O'Quinn & Barrett Kennedy, 1965). Therefore, agar was integrated with gelatin as wall matrix materials in microcapsule production to avoid these problems. Agar is a natural polysaccharide obtained from seaweed. Agar molecules form double helices, which subsequently crosslink with gelatin molecules to give a gel network at <30 °C.

Medicinal herbs are useful to treat human ailments for several centuries. *Phyllanthus urinaria* (PU) is the herbal species of the genus *Phyllanthus* (Euphorbiaceae). PU can neutralize the acetaminophen induced hepatotoxicity (Hau et al., 2009), promote the anti-oxidant and anti-inflammatory activities (Fang, Rao, & Tzeng, 2008). Phyllanthus plants have growth inhibition toward various microorganisms (Dabur et al., 2007). Few literatures studied the potential antifungal activities of PU water extracts. In this study, we attempt to integrate PU extract into agar–gelatin based microparticulate system (Lam, Lee, Kok, et al., 2012). The anti-*Aspergillus niger* (*A. niger*) activities of microencapsulated PU extract was investigated.

2. Experimental

2.1. Materials

Agar and gelatin were obtained from Sigma–Aldrich, Germany. PU extract was provided by Bioactive Technologies Ltd., HK.

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Preparation method and characterizations of PU extract have been reported previously (Hau et al., 2009). Pure olive oil was purchased from Easy Creation Asia Limited, HK. All the other chemical reagents were supplied from Sigma, Germany.

2.2. Preparation of microcapsules

Agar and gelatin were dissolved in deionized water and well mixed together at of 50–70 °C. PU extract was poured into the agar–gelatin mixture and continuously mixed for 5 min. Pure olive oil with 1% (v/v) Span 80 as a surfactant was added to the agar–gelatin–drug mixture followed by continuous stirring to form the water in oil emulsion. The emulsion was then subject to a homogenizer (BioSpec Products, USA) to form the homogeneous emulsion. The emulsion was allowed to cool down until <25 °C and continuously stirred for 3 h. The precipitates were subsequently washed by acetone, followed by deionized water. The resulting microcapsules were suction-filtered and stored in the desiccator (Lam, Lee, Kok, et al., 2012).

2.3. Drug loading efficiency

Fifty milligrams of PU extract loaded microcapsules were dissolved in 50 mL deionized water by heat. The completely dissolved microcapsules in water were then centrifuged. The supernatant was heated at 70 °C to remove the water so as to determine PU extract content in microcapsules. The drug loading was determined by weight after cooling.

2.4. Particle size

Ten milligrams of PU extract loaded microcapsules were dispersed in 1 mL of 1 mM KCl solution. The particle size of microcapsules was determined using a particle size analyzer (MALVERN, ZETASIZER 3000 HAS, United Kingdom).

2.5. Identification of PU extract in microcapsules

To identify major components from PU extract in microcapsules, liquid chromatography/mass spectrometry (LC–MS) analysis was performed with Agilent 1200 G1956B LC/MSD with ES-API source. PU extract (5 mg/mL) and the extract from microcapsules containing PU extract (5 mg/mL) were diluted to 100× and then filtered with a 0.22 μm nylon microfilter (Millipore). The solvent system consisted of 10 mM ammonium acetate in deionized water, pH 3.6 (solvent A) and acetonitrile (solvent B). The gradient profile was as follows: sample analysis (0–10 min, 20–50% B; 10–25 min, 50–95% B; 25–45 min, 95–95% B; 45–60 min, 95–20% B; and column wash and equilibration (70–80 min, 65–65% A). The chromatograms were extracted as total ion current (TIC) chromatograms. The flow rate was adjusted to 0.7 mL/min, and 20 μL aliquot of reconstituted sample was injected onto a 150 mm × 2.1 mm i.d., 5 μm, Agilent Zorbax Eclipse XBD C18 column at 40 ± 0.8 °C.

2.6. SEM

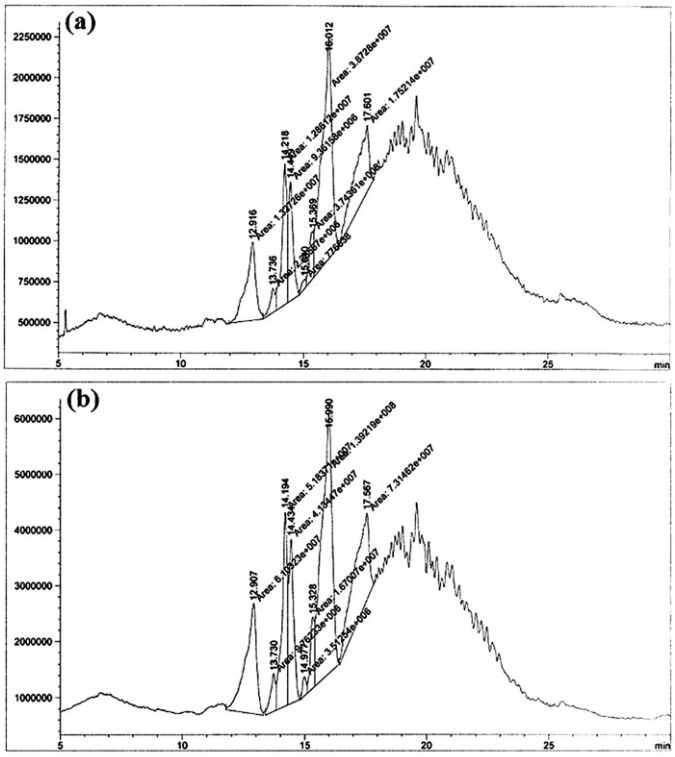
Microcapsules were attached to SEM stubs using a twisted adhesive tape and gold coated prior to SEM observation. The morphology of microcapsules was evaluated using a scanning electron microscope (JEOL JSM-6490, USA).

2.7. In vitro release study

Microcapsules were suspended in 100 mL phosphate medium (pH 7.4) and incubated at 100 rpm for 120 h at room temperature. Aliquots were taken at desired time intervals and centrifuged. Fresh medium was added to maintain the total volume of the system. The supernatant was heated at 70 °C to evaporate the medium in order to determine the released PU extract content. The drug was then weighted after cooling.

2.8. Minimum inhibitory concentrations (MICs)

A. niger was adjusted to a concentration of 1×10^3 cells/mL. The MICs of PU extract and PU extract loaded microcapsules were determined by Potato Dextrose Broth (PDB) dilution method. The diluted *A. niger* at a concentration of 1×10^3 cells/mL was added to test tubes. Various concentrations of PU extract and PU extract loaded microcapsules were added from a starting concentration of 1000 μg/mL and then diluted serially. Terbinafine was used as the



(c)

Peak #	RetTime [min]	Sig	Type	Area	Height	Area %
1	12.916	1	MM	1.32726e7	4.81060e5	13.4678
2	13.736	1	MF	2.28567e6	1.52329e5	2.3193
3	14.218	1	FM	1.28612e7	8.70955e5	13.0503
4	14.449	1	FM	9.36158e6	7.35886e5	9.4992
5	15.040	1	MF	7.76638e5	6.07780e4	0.7881
6	15.369	1	FM	3.74361e6	2.94252e5	3.7987
7	16.012	1	FM	3.87280e7	1.36667e6	39.2975
8	17.601	1	MM	1.75214e7	4.14562e5	17.7791
Totals :				9.85508e7	4.37650e6	

(d)

Peak #	RetTime [min]	Sig	Type	Area	Height	Area %
1	12.907	1	MM	6.10323e7	1.97260e6	15.3906
2	13.730	1	MF	9.76233e6	6.78597e5	2.4618
3	14.194	1	MF	5.18371e7	3.49729e6	13.0719
4	14.434	1	FM	4.13447e7	2.95428e6	10.4260
5	14.977	1	MF	3.51254e6	3.49056e5	0.8858
6	15.328	1	MF	1.67007e7	1.27654e6	4.2115
7	15.990	1	FM	1.39219e8	4.66705e6	35.1070
8	17.567	1	MM	7.31462e7	1.57538e6	18.4454
Totals :				3.96555e8	1.69708e7	

Fig. 1. LC–MS analysis of microencapsulated PU: (a) MS spectrum of microencapsulated PU extract; (b) MS spectrum of control PU extract detected by ES-API; (c) relative peak area % of microencapsulated PU extract; and (d) relative peak area % of control PU extract.

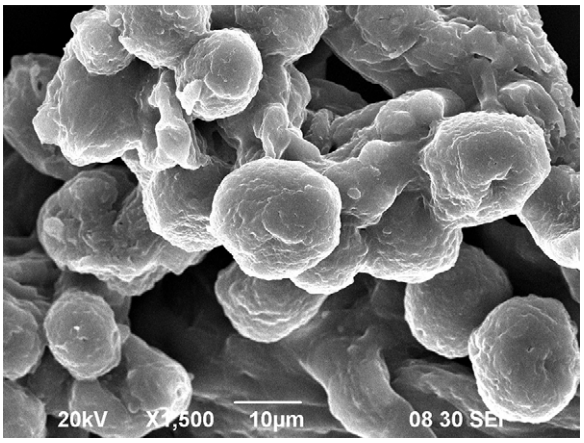


Fig. 2. SEM image of PU extract containing microcapsules.

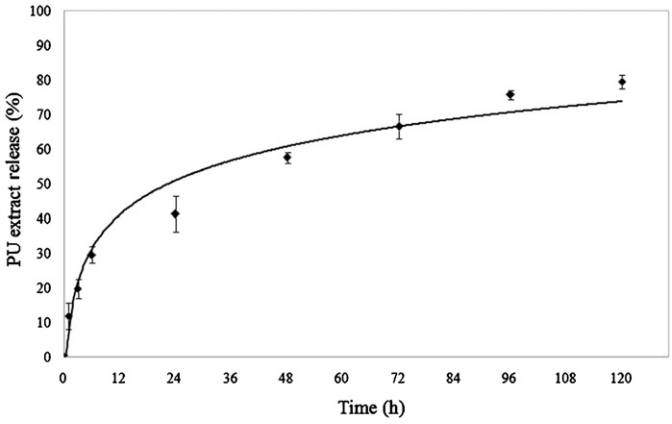


Fig. 3. Controlled release of PU extract loaded microcapsules.

positive control while blank microcapsules (without drugs) were used as a negative control. They were then incubated at 25 °C for 48 h.

2.9. Antifungal study

A. niger at a concentration of 1×10^3 cells/mL was placed on agar plates. Cotton fabric was cut in circular shape with 1 cm in diameter. Cotton stripes, loaded with PU extract or PU extract microcapsules, were placed on the agar surface. Terbinafine was used as a positive control. The plates were incubated in an incubator at 25 °C for 7 days.

3. Results and discussion

3.1. Drug loading efficiency and particle size

The mean drug loading efficiency and drug loading of PU extract loaded microcapsules were 69.08% and 15.86 ± 1.25 mg of PU extract per gram of microcapsules, respectively. The mean particle size was 19.11 ± 2.58 μm.

3.2. Identification of PU extract in microcapsules

The LC–MS analysis showed a tight alignment of common peaks between microencapsulated PU extract and control PU extract, a retention time window of 2–30 min in two TIC (total ion current) chromatograms (Fig. 1a and b) was almost identical. The microencapsulated PU extract was well characterized to be the same as the control PU extract according to their typical relative area percentages (Fig. 1c and d). LC–MS study revealed the existence of PU extract in microcapsules.

3.3. SEM

The SEM image (Fig. 2) demonstrates that PU extract loaded microcapsules were approximately spherical in shape with certain level of lumping. The particle sizes of microcapsules ranged from 11.82 to 24.54 μm.

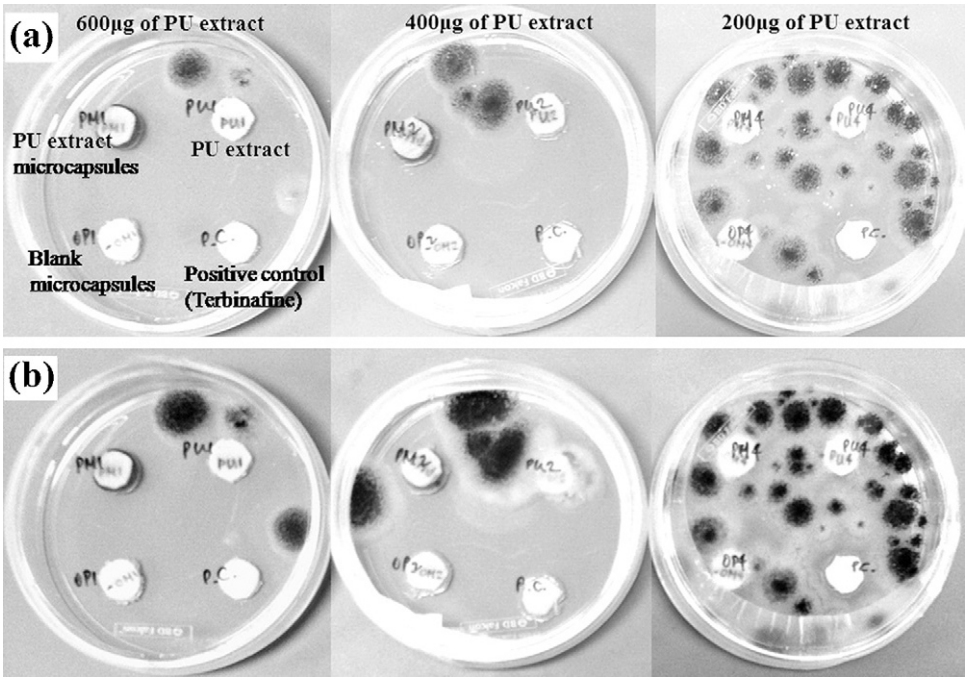


Fig. 4. Growth inhibition of PU extract containing microcapsules toward *A. niger*: (a) after incubation for 4 days and (b) 7 days.

Table 1
MICs of PU extract containing microcapsules and PU extract.

	MIC (μg/mL)		
	Positive control (terbinafine)	PU extract microcapsules	PU extract
A. niger	1.25	600	>1000

3.4. In vitro release study

In the release study, the percentage of drug release was investigated for 120 h (Fig. 3). After the first 6 h, there was a burst release of PU extract from microcapsules, with approximately 30% of drug release. Afterwards, another 10% of drug was released during the fast release period (up to 12–24 h). The slow release of drug could be observed during 24–96 h, with about 75% of drug release after 96 h. After 120 h, PU extract release reached a plateau at around 80%.

3.5. MICs and antifungal study

Table 1 shows that PU extract loaded microcapsules possessed a stronger anti-*A. niger* activity (MIC = 600 μg/mL) compared to PU extract (MIC > 1000 μg/mL). The same amount of blank microcapsules did not show any anti-*A. niger* effect. Fig. 4 demonstrates a dose dependent trend in anti-*A. niger* activity. Microcapsules containing 600 μg and 400 μg PU extract exhibited a stronger anti-*A. niger* activity than the free drug. This might be associated with the gradual release of PU extract which continuously inhibited the growth of *A. niger*. It was supposed that the released drug could diffuse in the soft agar and therefore promote the inhibitory effect to neighbor sites. However, free PU extract might only reduce the fungal growth at the beginning. No any observable growth inhibition of 200 μg PU extract loaded microcapsules could be found.

4. Conclusions

Here we report the development and characterization of agar–gelatin based microcapsules containing PU extract. A safe and economic approach using agar and gelatin as the wall matrix materials of microcapsules was proposed, which could be an alternative method for microcapsule formation eliminating toxic crosslinker formaldehyde. It was concluded that the microencapsulated PU extract possessed a stronger anti-*A. niger* activity than the free drug.

Acknowledgement

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