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Comparative Evaluation of the Antioxidant and Antibacterial Efficacy of Un-encapsulated and Encapsulated Essential Oil of Lemongrass in Alginate – Chitosan Microspheres

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The Alginate-chitosan microspheres encapsulated with LGO prepared by employing ionotrophic gelation technique were investigated by Scanning Electron microscopy (SEM), Fourier Transform Infra Red spectroscopy (FT-IR) and Gas Chromatography Mass Spectroscopy (GC-MS). SEM micrographs observed a spherical morphology with size ranged 1.82-2.08 mm. FT-IR and GC-MS spectral data confirmed the presence of LGO in the prepared microspheres. The microspheres allowed releasing a higher amount of LGO into the simulated intestinal fluid. A significant antibacterial and antioxidant activity of LGO encapsulated microspheres were observed at a lesser concentration compared to the unencapsulated lemongrass oil. This fact was attributed to both the protection of the chitosan barrier and the strong interaction between LGO and chitosan. In conclusion, LGO loaded alginate/ chitosan microspheres have potential applications as a greener agent for medical purposes.

Key words: Lemongrass oil; Chitosan-Alginate Microspheres; Extrusion; Antioxidant ; Antibacterial Activity.

Reactive oxygen species (ROS) and bacterial infections are two threats which the human body, animals and even food are continuously exposed to. ROS may lead to oxidative stress, which has been considered to be related to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS[1]. On the other hand, infections results from bacterial attachment and biofilm formation on surfaces in daily situations. Thus, materials possessing antioxidant and antibacterial activities can be expected to have good potential for applications. Of particular interest would be such materials developed from natural sources.

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Essential oils (EOs) (also called volatile or ethereal oils) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots[2]. Some EOs also are known to have strong antimicrobial activity against a wide variety of pathogens[3]. Besides antibacterial properties, EOs or their isolated components exhibit antioxidant[4], antifungal[4], antiviral[2] and anti insecticidal properties[5].

Lemongrass (*Cymbopogon citratus*), a tall perennial grass comprising of about 55 species, is native to warm region and grows in almost all tropical and subtropical countries . The biologically active constituent of lemon grass is citral constituting more than 75% (w/w) of its essential oil[6]. Several reports describe antimicrobial effects of lemongrass, including activity against both gram-positive and gram-negative bacterial pathogens, and fungi[7]. The effects are attributed in part to the geraniol (alpha-citral) and neral (beta-citral) constituents[8]. In animal study, LGO displayed

anticonvulsant effects, elevated the seizure threshold, or blocked seizure spread[9]. LGO has demonstrated antioxidant and radical-scavenging activity in several experiments[10].

However, LGO exhibits sensitivity to light, heat and oxygen, and has a short storage life if not stored properly. One approach to overcome these problems is to encapsulate LGO in carriers formed from naturally occurring polysaccharides. Among such polysaccharides, chitosan and alginate have been widely used as particulate carriers for encapsulation and controlled release of bioactive compounds[11].

Alginate has attracted increasing attentions as a carrier material due to its excellent biocompatibility, mucoadhesive biodegradability and mild gelation conditions[12]. In presence of multivalent cations such as calcium ions in aqueous media it possesses a unique property of mild gel-formation[13]. However, the slack network of bead results in a major restraint of drug-leakage through the pores during alginate-Ca bead preparation[14]. Conveniently, the mechanical properties and permeability of alginate-Ca bead can be successfully enhanced by a polycation, such as chitosan. Chitosan is a naturally occurring polysaccharide comprising D-glucosamine and N-acetyl-glucosamine with unique polycation characteristics. Upon mixing with the alginate, the strong electrostatic interaction of amino groups of chitosan with the carboxyl groups of alginate leads to the formation of chitosan–alginate complex[15]. The complexation reduces the porosity of alginate beads and decreases the leakage of the encapsulated drugs[16]. On the other hand, this complex exhibits pH-sensitivity that release of macromolecules from alginate beads in low pH solutions is significantly reduced, thereby being used as an oral delivery vehicle[17].

The main objectives of the present work were to study the microencapsulation of LGO, interactions with polymers and their release kinetics with the purpose of evaluating their potential use as natural antibacterial and antioxidant agent for oral administration.

MATERIAL AND METHODS

Sodium alginate (medium viscosity, 3500 cps for a 2% w/v solution), Chitosan (minimum 85% deacetylated), were obtained from Biocorporals India. All other reagents were of analytical grade obtained from standard companies. Lemongrass oil was purchased from Cyrus Enterprises, Chennai.

Microencapsulation of LGO

ALG/CS microspheres were prepared in a two step procedure based on the ionotropic pre-gelation of polyanion (ALG) with $CaCl_2$ followed by polycationic (CS) crosslinking through an adapted protocol desribed by Gupta *et. al.*,2011 [18]**.** An emulsion (2.4%) of essential oil in 2.5% sodium alginate solution was prepared under stirring using magnetic stirrer homogenizer for 5 minutes at 10 000 rpm. The emulsion was then added drop wise into a solution of 10% $CaCl_2$ in water using a 25-ml 21-gauge hypodermic syringe under constant stirring (300 rpm) to provide an ALG pre-gel. Then 0.75% of chitosan was added drop wise into the pre-gel over 30 minutes whilst stirring. The microsphere suspension was left standing overnight. Blank microspheres were also prepared using the same procedure, excluding the addition of LGO.

Microsphere characterization

Synthesized ALG/CH/LGO microspheres were characterized by SEM, FT-IR, and GC-MS analysis. Further the encapsulation efficiency, *in-vitro* release profile were determined.

Scanning electron microscopy

ALG/CH/LGO microspheres were coated with gold for 5 minutes under vacuum .Morphological examination of beads was performed using scanning electron microscope (JSM-840A,joel Ltd; JAPAN) at 11 kv.

Fourier Transform Infra Red spectroscopy (FT-IR analysis)

The FTIR spectra over the wavelength range 4000 to 400 cm^{-1} were recorded by FTIR spectrophotometer (Nicolet Avatar 660,Nicolet, USA).

Gas Chromatography Mass Spectroscopy (GC/MS)

ALG/CH/LGO microspheres were analysed using GC/MS (Software : GC-MS solution ver.2.53) with two fused silica capillary column VF-5ms of Length:30.0m,Diameter:0.25mm,Film Thickness:0.25um and a flame ionization detector (FID),which was operated in EI mode at -70eV. Ion source temperature and Interface temperature were

set at 200°C and 240°C respectively. 1µl sample bead solution in Helium(99.9995% purity)was injected and analyzed with the column held initially at 50°C for 2 mins and then increased by 10°C/min up to 300°C for 37 mins. Helium was employed as carrier gas with linear velocity of 1.55 ml/min and scan within a range of 40 – 1000 m/z and speed 2000.The relative amount of individual components of the total oil is expressed as percentage peak area relative to total peak area. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds, or by retention indices (RI) and mass spectra.

Evaluation of encapsulation of essential oil

A pre weighed amount for each batch of ALG/CH/LGO microspheres were suspended in methanol (10ml).The Beads were next subjected to vigorous mechanical shaking with a vortex mixer for one minute and subsequently sonicated for one hour with a Branson 1200 ultrasound bath. The filter solutions were analyzed at a wavelength of 273 nm. All loaded determinations were run in triplicate and the mean values were reported.

In vitro release studies

In vitro release of ALG/CH/LGO microspheres profiles was determined as follows. ALG/CH/LGO microspheres obtained from one set of preparations were re-dispersed in 33 ml of 0.01 M phosphate-buffered saline solution (pH 7.4) at a final concentration of 150 mg/ml. Total volume was divided into 33 Eppendorf tubes giving 11 different sets (each set with 3 Eppendorf tubes) for time-dependent release study at time intervals of 0,1, 2,3, 4, 5, 6, 7, 8, 9, 10, 12, 24, 30, 36, 42, 48, 60, 72, 80, 85, 90, 96, 100 and 120 hours. All sets were incubated at 37°C under gentle agitation. At proper time intervals, intake amounts of essential oils in loaded beads were first extracted in methanol and quantified spectrophotometrically.

The release was quantified by equation 1.

Release %= [Released essential oil/Total essential oil encapsulated] ×100 . ...(1)

Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated according to the modified method of Bandoniene,*et al*,[19] using the stable DPPH radical. The methanolic extracts of ALG/CH/LGO microspheres (0.1 ml) were added to 0.5 ml of DPPH of 2 $\times 10^{-4}$ M solution and made up to 1 ml using methanol; the absorbance was read at 515 nm against methanol using a double-beam ultraviolet–visible spectrophotometer (Hitachi U-1100). Simultaneously, the absorbance at 515 nm of the blank sample was measured against methanol. The radical scavenging activities of the tested samples, expressed as percentage inhibition of DPPH, were calculated according to equation 2.

$$\% \text{ Scavenging of DPPH} = [(A_0 - A_s)/A_0] \times 100 \quad ...(2)$$

Where A_0 = the absorbance of the blank sample at 515 nm at time $t = 0$ min

A_s = the final absorbance of the sample.

The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of percentage scavenging effect against extract concentration.

Determination of antibacterial activity

Clinical isolates of human pathogenic gram negative bacterial strains *E.coli* and *Pseudomonas aeruginosa* were obtained from Ramachandra Medical College, Porur. The clinical isolates were identified based on the culture and biochemical characteristics described by standard method. Pure culture was maintained on nutrient agar slant.

100 ml of trypticase soy broth medium was prepared, sterilized by autoclaving. After sterilization, a loopful of respective bacterial strain was inoculated into the broth, incubated at 37 ^{0} C for 24hours.

Screening of synthesized microspheres for antibacterial activity was done by Nutrient Broth dilution method. Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in $\log_2$ serial dilutions (two fold).

The antibacterial diffusion assay was carried out using Broth Dilution method as described by the National Committee for Clinical Laboratory Standards [20]. *E.coli and Pseudomonas aeruginosa* (1×10^8 CFU/ml) was suspended in each nutrient broth (100 ml), to which the pure essential oils and alginate-chitosan-LGO microspheres at different contents were added. The

cell concentration was controlled as 1×10^6 CFU/ml when samples were injected in the nutrient broth. The culture medium was maintained at 37°C with shaking. Growth of the cells was estimated by measuring the absorbance of the culture medium at 600 nm using a UV/VIS spectrophotometer (Specgene, Techne Inc., U.K.). Growth of the cells was evaluated by dry cell weight (DCW) determined from a calibration curve of DCW versus absorbance at 600 nm. The percent of growth inhibition was defined as the following by eq. 3.

$$\% \text{ Inibition} = \frac{Abs_{control} - Abs_{essential}}{Abs_{control}} \times 100 . \quad ...(3)$$

Where $Abs_{control}$ and $Abs_{essential}$ are the absorbance of control and essential oils at 600 nm respectively. In this study, "control" means the experimental result of the cell growth with injection of the cell only.

Evaluation of Biofilm inhibition assay

Synthesized microspheres and LGO at different concentrations was evaluated against clinical isolate of *E.coli* and *Pseudomonas aeruginosa* adopting bio film inhibition spectrophotometric assay. An overnight culture of *E.coli* and *Pseudomonas aeruginosa* in Trypticase Soy broth was diluted in the ratio 1:100 in respective fresh medium and grown for another hour. 5ml of diluted strains was added into 6 well titre plate and different concentrations of synthesized alginate-chitosan-essential oil beads and essential oil was added and incubated at 37° C for 3 days.

Qualitative biofilm assay by SEM analysis

Smear technique is applied in which sterile glass slide of size 2x2 cm wiped with ethanol is taken with cover slip. 1-2 drop of culture is pippeted on glass slide and a thin smear was made. Later it was air dried at room temperature for 5-6 hours and then the morphology and surface structure of the culture treated with microspheres were observed using SEM photographs. Samples were placed on an aluminum stub with a conductive copper tape and then covered with a gold layer under an argon atmosphere. SEM machine was operated at a vacuum of the order of 10-5 torr. The accelerating voltage of the microscope was kept in the range 10-20 kV.

RESULTS AND DISCUSSION

Characterization of ALG-CS microspheres loaded with LGO

The ALG-CS microspheres loaded with LGO was carried out successfully using a multistep process of o/w emulsion, gelification and solvent removal[18]. The morphology of the LGO loaded alginate/chitosan microspheres were characterised by scanning electron microscopy. As shown in figure 1, the microspheres had a spherical shape and showed surface cracks probably caused by partial collapsing of the polymer network during drying[15]. The particle size of the microspheres ranged from 1.22 to 1.88 mm. This is in concurrence with C. Dima *et al*., (2013) report on the size of the encapsulated coriander essential oil-loaded microspheres prepared in alginate/chitosan systems at a ratio of 1:2 was found to be 1056 µm[21].

In order to examine the relationship between the components of microspheres, the polyelectrolytes interactions and LGO entrapment were investigated, since it has been well established that the carboxyl group (COO^-) of the anionic polymer may interact with the amino group ($-NH_3^+$) of the chitosan and form an ionic complex between the two compounds[15]. Changes in the FTIR spectra with respective to the absorption bands of the amino groups, carboxyl groups and amide bonds were monitored.

FT-IR spectrum of LGO Figure 2 (A), alginate Figure 2 (B) and beads of LGO /alginate are presented in Figure 2 (C) respectively. The spectrum revealed that the prepared LGO /alginate beads contained alginate due to the characteristic peak of alginate at 3400 cm^{-1} and 1600 cm^{-1} corresponding to O-H stretching and carbonyl stretching respectively. Moreover, the characteristic peak of EO at 2900 cm^{-1} corresponding to C-H stretching was also found. Thus, the prepared beads were confirmed the presence of LGO.

Whereas the FT-IR spectra for chitosan Figure 2 (D) showed the broadband at around 3446 cm^{-1} attributes to –sNH and –OH stretching vibration. The weak band at 2873 cm^{-1} may be due to –CH– stretching in chitosan. The bands at 1659 and 1590 $cm^{"1}$ represent amide and amine groups of chitosan, respectively, and the bands at 1070 cm^{-1} are assigned to the skeletal vibration of C–O

Table 1. GC-MS identified compounds of alginate-chitosan-lemongrass oil microspheres

Peak	R.Time	Area	Area %	Name
1.	4.881	495874	0.22	1,7,7-Trimethyltricyclo[2.2.1.0,2,6]heptane
2.	5.106	930938	0.41	Alpha-pinene
3.	5.447	2297268	1.01	Camphene
4.	5.596	18223	0.01	1-[(1,2-epoxy-3-hydroxy)propyl]cyclohexane
5.	5.866	178495	0.08	Exo-Isocamphane
6.	5.910	74720	0.03	(+)-Beta-Phellandrene
7.	6.015	257553	0.11	Isocamphane
8.	6.124	25436	0.01	(-)-P-Menth-3-ene
9.	6.216	3845333	1.69	6-Methyl-5-hepten-2-one
10.	6.571	373909	0.16	n-Caprylaldehyde
11.	6.753	26035	0.01	Alpha-terpinene
12.	6.918	224600	0.10	o-Cymene
13.	6.985	10483423	4.60	D-Limonene
14.	7.110	2382167	1.04	trans- Ocimene
15.	7.229	1537314	0.67	Cis-Ocimene
16.	7.467	115003	0.05	2,6-Dimethyl-1-nonen-3-yn-5-ol
17.	7.518	69985	0.03	Gamma –terpinene
18.	7.784	245276	0.11	4-Nonanone
19.	7.994	249787	0.11	Alpha-Terpinolen
20.	8.120	380360	0.17	Alpha-Naginatene
21.	8.233	137206	0.06	Perllene
22.	8.275	1741185	0.76	Beta-linalool
23.	8.405	981762	0.43	Delta1.,alpha-cyclohexaneacetaldehyde3,3-dimethyl-(Z)
24.	8.936	432352	0.19	Trans-caran,4,5,epoxy
25.	8.997	1492838	0.65	7-methyl-3-methylene-6-octen-1-ol
26.	9.135	4478951	1.96	(R)-(+)-Citronellal
27.	9.286	3328057	1.46	cis-Verbenol
28.	9.426	337079	0.15	2,6,-Dimethyl-1-nonen-3-yn-5-ol
29.	9.519	1885037	0.83	Borneol
30.	9.585	6031917	2.64	Carane, 4,5-epoxy-,trans
31.	9.644	686421	0.30	L-Carveol
32.	9.764	342263	0.15	Octahydro-2H-chromen-2-one
33.	9.866	889477	0.39	alpha-Terpenol
34.	9.982	1062744	0.47	Capraldehyde
35.	10.325	1206693	0.53	beta-Citronellol
36.	10.530	82249450	36.06	beta-Citral
37.	10.675	8581775	3.76	trans-Geraniol
38.	10.960	56929322	24.96	alpha-Citral
39.	11.596	7082061	3.10	(Z)-3-cyclpropyl-7-(2-methoxyethyl)norcarane
40.	12.015	421255	0.18	Citronellol acetate
41.	12.335	788852	0.35	(+)-cyclosativene
42.	12.417	8528703	3.74	Geraniol acetate
43.	12.591	1004097	0.44	beta-Elemene
44.	13.025	5490730	2.41	Caryophyliene
45.	13.497	1012546	0.44	alpha-Caryophyliene
46.	14.019	388629	0.17	1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa [e]azulene
47.	14.218	733446	0.32	gamma-Cadinene
48.	14.264	1139355	0.50	beta-Cadinene
49.	14.340	350752	0.15	alpha-Farnesene
50.	14.667	918147	0.40	Elemol
51.	15.113	2049427	0.90	Caryophyliene oxide
52.	15.449	170758	0.07	Decalin
53.	15.629	207873	0.09	Cubenol
54.	15.984	114360	0.05	alpha-Eudesmol
55.	24.071	679318	0.30	Monoethylhexyl phthalate

Table 2. Percentage inhibition of *E.coli* and *Pseudomonas aeruginosa* at various concentrations of lemongrass oil and chitosan/alginate lemongrass oil microspheres

Essential oil	Concentration [µl/5ml]	*E.coli* Inhibition percentage[%]	*P. aeruginosa* Inhibition percentage[%]
Lemongrass oil	2	22.11	14.48
Lemongrass oil	3	46.37	55.89
Lemongrass oil	4	67.48	65.89
Lemongrass oil-Chitosan-Alginate Bead	0.75 g beads (2 µl LG oil)	53.82	55.72
Lemongrass oil-Chitosan-Alginate Bead	1.15 g beads (3 µl LG oil)	55.09	58.86
Lemongrass oil-Chitosan-Alginate Bead	1. 5 g beads (4 µl LG oil)	75.08	76.61

stretching. Comparing Figures 2 (D) and 2 (E), the peak at 1590 cm^{-1} for primary amine bending has been shifted to new positions, 1580 cm^{-1} and 1562cm^{-1}, most probably due to the electrostatic interactions occurring for $–NH_2$ group of chitosan with alginate/lemongrass oil.

GC- MS analysis was performed to evaluate the composition of LGO encapsulated in chitosan/alginate microspheres as shown in the Table 1. The GC-MS analysis demonstrated the presence of main chemical components of lemongrass oil viz., 24.96% alpha-citral , 36.06% beta-citral, 3.76% trans-geraniol, 3.74% geraniol-acetate, 4.60% D-limonene, 1.96% (R)-(+) citronellol, 0.18% citronellol acetate, 1.53% beta-citronellol and 0.41% alpha-pinene respectively (table 1) in the LGO encapsulated chitosan-alginate microspheres. This study reflects that encapsulation did not affect the chemical stability of the biologically active constituent of lemongrass viz., citral (24.96% alpha- citral, 36.06% beta-citral) of its essential oil. *C.C.Liolis et al* (2009) in his study established successful encapsulation of carvacol, thymol, β-cymene and γ-terpinene the major constituents of the essential oils from *Organum dietamnus L* in phosphatidyl choline based liposomes by GC-MS analysis[22]

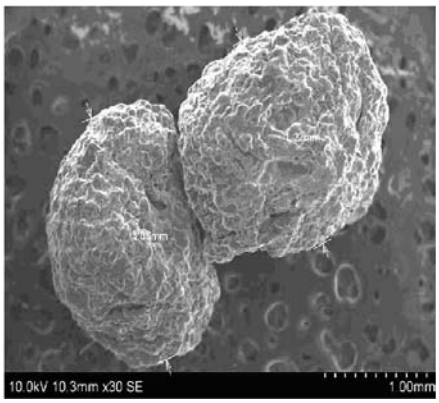

Fig. 1. SEM image of alginate-chitosan-lemongrass oil microspheres (ALG/CH/LGO)

Quantitative analysis of the LGO loaded alginate-chitosan microspheres (ALG/CH/LGO)

The entrapment efficacy of the ALG/CH/LGO micropsheres prepared using 2.5% w/v ALG, 0.75% W/V CH and 1.0% w/v calcium chloride was determined using UV-Vis spectroscopy and 93% of successful entrapment of LGO was demonstrated. This is in accordance with the previous report where the entrapment of amoxicillin was maximum by using 2% w/v alginate concentration, which was attributed to stable gel complex in presence of chitosan and Ca++ [23].Also Abreu *et al*., 2010 presented high encapsulation efficiency of lisinopril using calcium chloride as crosslinking agent[24]. C. Dima *et al*., (2013) reported a maximum entrapment efficiency of 97% of coriander essential oil encapsulated in alginate/chitosan systems at a size of 1056 µm[21]. The high values of the encapsulation efficiency, in what regards the alginate/chitosan microspheres, are probably owed to the microsphere surface, too, that is coarser as shown in figure 1.

The possible use of Lemongrass oil microspheres in medical purposes imposes serious researches regarding their release capacity in certain physicochemical conditions. In this direction , the in vitro drug release profiles of lemongrass oil -alginate/chitosan microspheres at an acidic pH of 7.4 was studied. As shown in figure 4 the release of the LGO was demosntrated to occur in a controlled manner. This can not only prevent potential drug loss in an acid environment (e.g. the gastric fluid) but also controls drug release in the GI tract. In total 80% of LGO was released in

total over 120 hours. Therefore more amount of the drug will be absorbed into the blood system and in turn transported from the blood circulatory system to the site of the infection or stress efficiently than when LGO is not encapsulated in the microspheres.

Antioxidant activity

Antioxidants inhibit lipid peroxidation by a well known mechanism i.e., free radical scavenging. One of the fast methods to evaluate the antioxidant activity is the scavenging activity on DPPH, a stable free radical and widely used index. In the current study the antioxidant capacity of unencapsulated form of LGO and its encapsulated form were analyzed by DPPH assay. After performing the DPPH test it has been concluded that the EC_{50} value for DPPH radical scavenging ability of unencapsulated form of LGO was 110µg/ml which in concurrence with the Sin Yen Sah *et al.*, recent studies where he demonstrated the lemongrass oil had the DPPH radical scavenging activity at an EC_{50} of 192 µg / ml[25]. While so, encapsulation of LGO into ALG-CS microspheres resulted in DPPH radical scavenging activity at a significantly lesser EC_{50} value of 67 µg /ml. This might be attributed to the better protection of LGO against antioxidation compared to the unencapsulated essential oils. This strong protective effect in alginate-chitosan micropsheres would be attributed to the amphiphilic properties

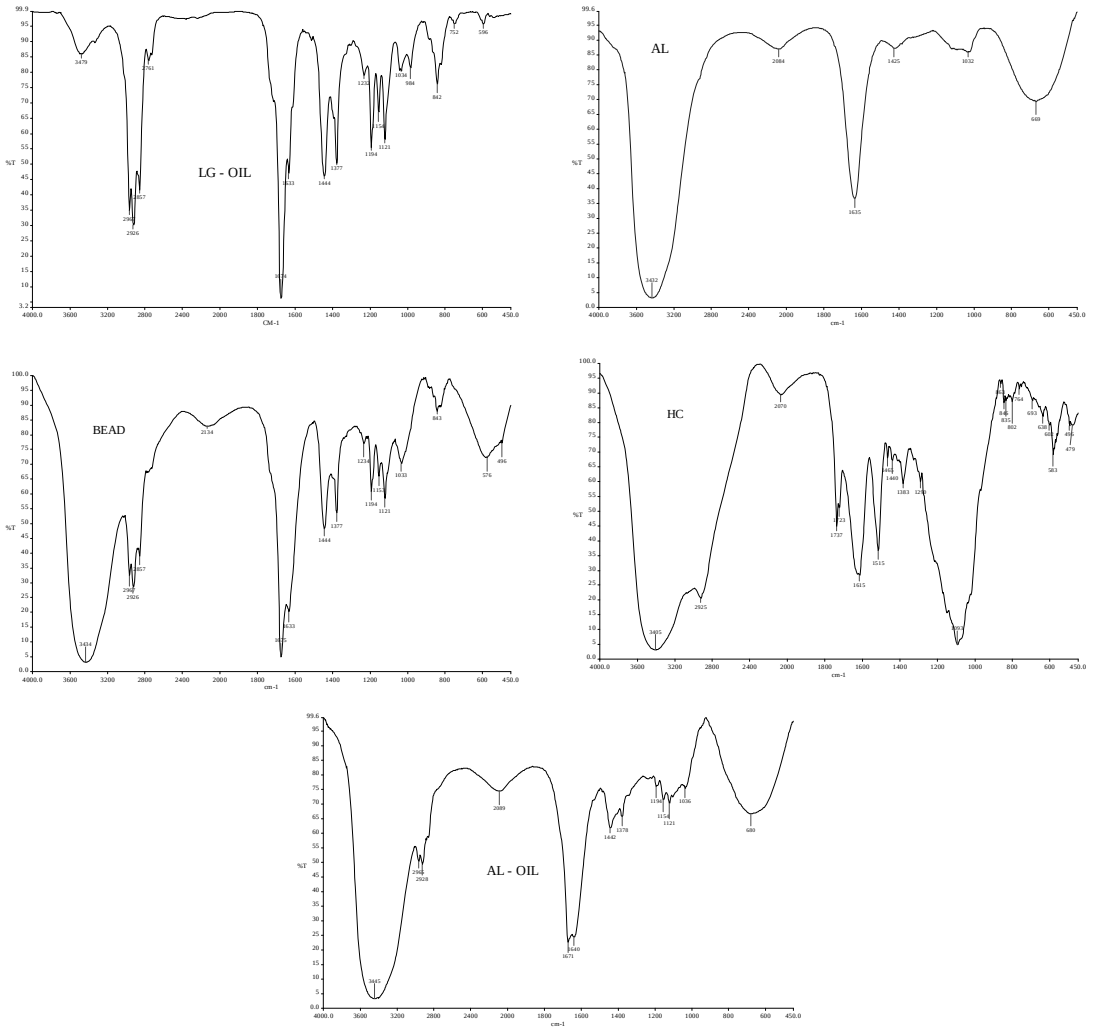

Fig. 2. FTIR spectra of (A) lemongrass oil (B)blank alginate beads (C)lemongrass oil encapsulated alginate microspheres (D)chitosan (E)lemongrass oil encapsulated alginate –chitosan microspheres

of phenolic constituents.It is generally assumed that an increase in the number of hydroxyl group in a phenol enhances the hydrogen donor ability and inhibition of oxidation. Thus the encapsulation technique maintain the antioxidant power of essential oil. So these microspheres can be used in the human body as a well accepted antioxidant drug formulation.

Antimicrobial activity

Antimicrobial activity was performed by nutrient broth culture of two bacterial strains (Gram-negative) *E.coli* and *Pseudomonas aeruginosa*. Variations of the cell concentration with injection of the essential oils and alginate/ chitosan bead in nutrient broth are represented in table 2. Inhibition percentage of cell growth with various injection amounts of the essential oils and alginate/chitosan bead was evaluated from the results.

As indicated in table 2, LGO and its encapsulated form demonstrated inhibition of *E.coli* and *Pseudomonas aeruginosa* in a dose dependent manner. The inhibition percentage of *E.coli* was 22.11%, 46.37% and 67.48% respectively at various concentrations of lemongrass oil. While so in the case of ALG/CH/LGO microspheres 53.82%, 55.09%, 75.08% inhibition was observed with the increase in lemongrass oil encapsulated respectively. Similarly, the inhibition percentage of *Pseudomonas aeruginosa* at various concentrations of lemongrass oil were 14.48%, 55.89% and 65.89% respectively, whereas in the case of ALG/CH/LGO microspheres 55.72%, 58.86%, 76.61% inhibition was observed with the increase in lemongrass oil encapsulated respectively. Thus the encapsulated form of lemongrass oil was the efficient antimicrobials against both *E.coli* and *Pseudomonas aeruginosa*. Studies have already illustrated the antimicrobial activity of LGO is known to the presence of α-citral (geraniol) and β-citral (neral) biologically active constituents[8]. The biologically active constituent of lemongrass viz., citral (24.96% alpha-citral, 36.06% beta-citral) of its essential oil was not subjected to any fluid dynamic stress during encapsulation suggesting the retention of antimicrobial properties of lemongrass against gram-negative bacteria pathogens[7]. F.Donni *et al*., (2011) demonstrated higher antimicrobial activity of the nanoencapsulated terpenes on *E.coli*, *L.delbrueckii* and *S.Cervisiae* inoculated in orange and pear juices at lower to the volumes of unencapsulated mixture[26].C.C.Liolis *et al* (2009) established the successful encapsulation of major constituents of the essential oils from *Organum dietamnus L* in phosphatidyl choline based liposomes as the possible improvement of their antimicrobial activity against selected microbia[22]. Thus our study also clearly demonstrates that encapsulation of LGO improves their antimicrobial activity.

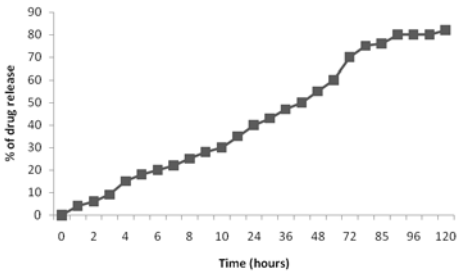

Fig. 3. In vitro drug release profile of LGO

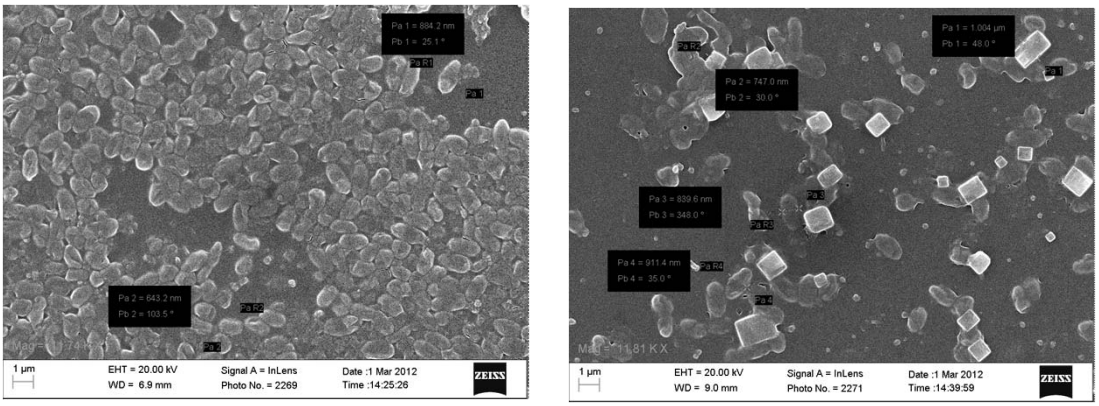

Fig. 4. SEM image of biofilm of *Pseudomaonas aeruginosa* before (A) and after0treatment of Alginate-chitosan-lemongrass oil (B)

Qualitative biofilm assay by SEM analysis

SEM results support the percentage inhibition data (table 2) and demonstrate that *Pseudomonas aeruginosa* grew and developed normal biofilms in control, but biofilm formation was significantly impaired when administered with ALG/CH/LGO microspheres. In the control biofilm, the cells formed a dense aggregate (Figure 5A), whereas, after ALG/CH/LGO microspheres treatment, cells were scattered and shrinked (Figure 5B).

Similar observation by scanning electron microscopy (SEM) of the two Gram negative bacteria, *E. coli* 0157:H7 strain EDL 933 and *Salmonella enterica subsp enterica serovar Typhi* strain ATCC 19430 was demonstrated when exposed to mustard EO (allyl isothiocyanate is the main component) evidence an imperfect and unfinished cell shape[27]. Such differences on cell damage were also verified on *E. coli* O157:H7 cells treated with cinnamaldehyde, Sporan and Sporan +acetic acid which appeared wrinkled and shrunken, including morphological rod alterations. In addition, some cells treated with cinnamaldehyde or Sporan + acetic acid were transparent. Similarly, SEM images of treated *Salmonella* cells showed morphological alterations. When compared to untreated cells, all treated cells collapsed and appeared empty of contents[28]. However, our study clearly reports that encapsulation can also enhance the mechanism of antimicrobial action and decrease the concentration of antimicrobial compound needed for inhibition via increased LGO delivery to the membranes of microorganisms in aqueous environments.

CONCLUSION

This study aimed to elucidate the physico-chemical characteristics of ALG/CH/LGO microspheres and their antioxidant as well as antimicrobial activity. The encapsulation did not affect the chemical stability of the biologically active constituent of lemongrass retaining its antioxidant and antimicrobial activity. The ALG/CH microspheres encapsulated with LGO were the most effective antimicrobials against both *E.coli* and *Pseudomonas aeruginosa*. Encapsulation also enhanced the mechanism of antimicrobial action and decreased the concentration of antimicrobial compound needed for inhibition via increased LGO delivery to the membranes of microorganisms in aqueous environments. The results obtained encourage the use of lemon grass oil encapsulated in alginate/chitosan microspheres for the controlled release of Lemongrass oil (LGO) for oral administration as an antibacterial and antioxidant agent.

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