IJPSR (2014), Vol. 5, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 April, 2014; received in revised form, 30 September, 2014; accepted, 10 October, 2014; published 01 November, 2014

ANTI-BIOFILM ACTIVITY OF *EUCALYPTUS GLOBULUS* OIL ENCAPSULATED SILICA NANOPARTICLES AGAINST *E. COLI* BIOFILM

Suhaga Dohare [1*], Shyam Dhar Dubey [1], Manmohit Kalia [1], Preetam Verma [1], Himanshu Pandey [2], Nand Kumar Singh [1] and Vishnu Agarwal [1]

Department of Biotechnology [1], Motilal Nehru National Institute of Technology Allahabad-211004 (U.P.), India
Department of Pharmaceutical Sciences [2], Faculty of Health Sciences, SHIATS Allahabad-211007 (U.P.), India

Keywords:

Eucalyptus globulus oil, *E. coli*, Silica nanoparticles, Biofilm, SEM

Correspondence to Author:

Suhaga Dohare
Ph.D Scholar
Department of Biotechnology,
Motilal Nehru National Institute of Technology (MNNIT),
Teliarganj, Allahabad, Uttar Pradesh - 211004, India

E-mail: suhaga.dohare@gmail.com

ABSTRACT: The inhibitory effect of *Eucalyptus globulus* oil was evaluated against the *E. coli* and the minimum inhibitory concentration (MIC) value of *Eucalyptus globulus* oil was calculated by agar dilution assay, which was found to be 1µl/ml. The *E. coli* biofilm was formed on catheter surface and quantified by crystal violet staining system. The crystal violet quantification method showed maximum biofilm after 48h. The effect of *Eucalyptus globulus* oil was evaluated against *E. coli* biofilm and was found to inhibit the biofilm by 62%. To evaluate the effect of oil containing silica nanoparticle against biofilm system, the *E. globulus* oil was loaded in the silica nanoparticle. The synthesized nanoparticle (SNP) was characterized using scanning electron microscopy (SEM), and data showed the average size of silica nanoparticle 1000 nm. The application of oil loaded silica nanoparticle was done against *E coli* biofilm system and biofilm quantification assay showed 81% reduction in biofilm. The inhibition was further confirmed using light microscopic analysis of biofilm after staining with 0.5% crystal violet. The study is important which can be useful to use *Eucalyptus globulus* oil encapsulated inside the nanoparticle for its potential use in prevention and control of biofilm associated microbial infections and diseases.

INTRODUCTION: Bacterial biofilm are complex sessile microbial communities formed on living and nonliving surface [1-2]. *E. coli* is an opportunistic pathogen responsible for the majority of nosocomial infections and they cause serious infection in its biofilm mode of growth in immunocompromised patients [3]. *E. coli* biofilms also responsible for medical indwelling device related infection, including infection related Foley urinary catheter, intravenous catheter and joint prostheses [4-6].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(11).5013-18

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(11).5013-18

Once the biofilms form on surface, they are up to 1000 times more resistant to antibiotic therapy, than their planktonic counterpart [7-8]. Since year, the conventional antimicrobial therapy was ineffective against *E. coli* related infection due to poor penetration into biofilms matrix, the extracellular polymeric material and increasing resistant to antibacterial agents. Thus emerging multidrug resistant strains created a need for search of new therapeutic alternatives among plants and essential oil which have potent antibacterial properties [9-10].

The exploration of new and natural plant product showing effective antimicrobial activity against *E. coil* and low cytotoxicity and have significantly effect in prevention and controlling of biofilms associated bacterial infection [11-13]. However, the therapeutic uses of essential oil in *E. coli* biofilms

related infection are limited by its low penetration ability as well as poor absorption inside the biofilm matrix. This limitation has been overcome by emerging nanocarriers and nanoparticles since last two decades. The formulation of polymeric nanoparticles based encapsulation that exhibit greater potential efficacy, penetration ability and drug/oil releasing profile.

In present work, the effect of eucalyptus oil was evaluated against *E. coli* and its biofilm. The *Eucalyptus globulus* oil was further encapsulation inside the synthesized silica nanoparticles and assessed the anti- *E. coli* biofilm activity, followed by light microcopy along with control.

MATERIAL AND METHODS:

Microorganism and Culture Conditions

Standard reference strains *Escherichia coli* 25922 procured from ATCC was used in this study. The strain was culture in Luria Bertani broth (Himedia, India) medium containing 10g/l Tryptone, 5g/l Yeast Extract and 10g/l NaCl, and incubated for 24h at 37˚C in orbital shaker (120 rpm) and their growth was assessed at an optical density 600nm (OD_{600}) and this initial culture was further diluted in order to achieve a final concentration of approx. $6.6x10^7$ total cells/ml.

Eucalyptus globulus oil and Triton X-100 were purchased from Sigma Aldrich Company (India), Tetraethylortho-silicate (TEOS 98%) was purchased from Alfa Aesar (India), aqueous ammonia (NH_3, 25%) and absolute ethanol (99 %) were purchased from Merck (India), cetyltrimethylammonium bromide (CTAB) and Dimethyl sulfoxide (DMSO) were purchased from SRL, Luria Bertani agar (LB agar) and Luria Bertani broth (LB broth) were purchased from Himedia Laboratories Pvt. Ltd (India).

Antibacterial activity and Minimum Inhibitory Concentration (MIC) of eucalyptus oil

Agar well diffusion method was used to evaluate the antibacterial activity of eucalyptus oil against gram negative *E. coli* (ATCC 25922). Antibacterial test was performed by preparing LB agar plate and 100 µl inoculum of *E. coli* grown culture was spread on agar plate, well was cut with the help of well borer. Oil was properly dissolved in tween-20 and filled inside the well and incubated for 24h at 37˚C. LB agar culture plate swabbed with same *E. coli* culture without supplement of oil and oil encapsulated silica nanoparticles incubated for 24h at 37˚C was used as control. The experiment was performed in duplicate. Zone of inhibition was measured and assessed the antibacterial activity of oil and oil encapsulated silica as a function of zone of inhibition.

Agar dilution assay was used for determination of MIC of eucalyptus oil [12, 14]. The agar plates were prepared by adding different concentration of eucalyptus oil (0.3 – 50 µl/ml) in sterilized LB agar with the Tween-20 (5µl/ml) to enhance oil solubility. 100 µl of 10^3 cfu of *E. coli* was spread on prepared agar plates, and incubated for 48 h at 37˚C. Plates with Tween-20, but without any plant oil were used as control. Numbers of colonies were counted after 48 h of incubation. MIC of oil was read and was considered to be the lowest concentration of oil required to yield no visible growth of *E. coli*. This experiment carried out in triplicate.

Biofilm formation and quantification

E. coli biofilm formed as a described previously with a certain modification [15-17]. *E. coli* cells were grown overnight in LB broth, at 37˚C, 120 rpm in orbital shaker. Then cell suspension ($6.6x10^7$cfu/ml) was added into each capped tubes with containing sterilized coupon ($0.5cm^2$) of Foley Catheter. The LB broth with sterilized coupon was taken as control. All capped tubes were incubated for at 37˚C without agitation for different time interval (24 h, 48h, and 72h), coupons were transferred into another fresh sterilized capped tubes, washed with sterilized PBS (phosphate buffered saline pH 7.2) and stain with 0.5% crystal violet (w/v) for 15 min., washed with PBS to remove extra stain and stained coupons were dissolved in DMSO. Then biofilm growth was measured by taking absorbance at 570 nm.

Synthesis of silica nanoparticles (SNP) and oil encapsulation

Silica nanoparticles were synthesized as earlier described by Guo *et al* [18], Nooney *et al* [19] and Singh *et al* [20] with a few modifications. Briefly, 35 ml of ethanol and 45 ml of deionized water were mixed in a reaction container, then pH of mixture was adjusted to 11.7 with the addition of 3.5 mL of

ammonium hydroxide, it was then kept on a magnetic stirrer. After 5 min, 1.388 mL of TEOS was added, after 10 minutes 0.274 g of CTAB was added with rapid stirring at room temperature. The solution became slightly turbid after 15 min, indicating hydrolysis and formation of silica. Vigorous stirring was continued for 3 hours. After 3 h reaction was completed, stirring was stopped and the white turbid solution was centrifuged at 15000 rpm for 20 min. The pellet obtained was washed with 70% ethanol and distilled water to remove undesirable particles. The pellet so obtained was collected and dried overnight at 60^0 C. Resulting dried white powder was obtained, this was crushed gently to obtain uniform size silica nanoparticle.

For oil encapsulation, 10mg of powdered silica nanoparticles were taken in a capped tube and 100µl triton X-100 was added (to increase the porosity). To the above solution 1000µl of *Eucalyptus globulus* oil was added. This solution was stirred continuously for four hours on a magnetic stirrer. Then the suspension was centrifuged at 10,000 rpm for 10 min, pellet so obtained was kept for overnight drying at room temperature. The synthesized silica nanoparticle was characterized by SEM.

Biofilm inhibition assay

Biofilm formed by above mentioned method was inhibited by eucalyptus oil and eucalyptus oil encapsulated SNP by adding different concentration ranges from (0.3 – 50 µl/ml) or its equivalent amount present inside silica nanoparticle. Biofilm on glass cover slips surface without any treatment was used as control. Further samples prepared for light microscopic analysis.

Microscopic analysis

The morphology of synthesized silica nanoparticles was examined by scanning electron microscopy. The sample was mounted to aluminum stubs with double side adhesive carbon tape then gold coated and examined morphology using a scanning electron microscope (SEM) at an acceleration voltage of 15 kV and 10 KX magnifications**.**

Biofilm with and without any treatment were established on glass cover slips surface as described above. For light microscopy, biofilm associated surface were washed with sterilized PBS (phosphate buffered saline pH 7.2) to removed planktonic cells and stain with 0.5% crystal violet (CV w/v) for 10- 15 min., washed with PBS to remove extra stain and observed under light microscopy.

RESULTS:

Biofilm formation and quantification

The biofilm was optimized at different time interval (24h, 48, and 72h) followed by CV quantification assay. The result showed that maximum microbial adhesion and biofilm formation was formed after 48h **(Figure 1).**

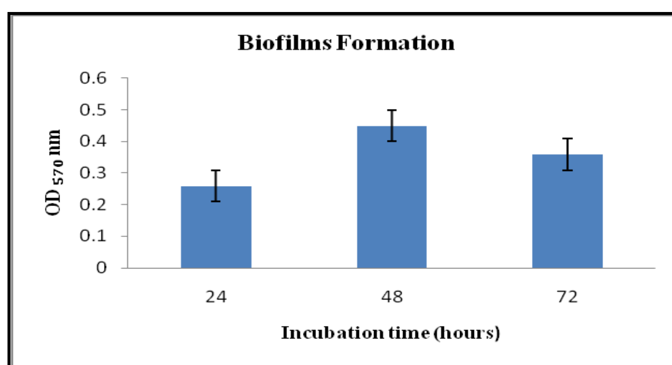

FIG. 1: SHOW THE BIOFILM FORMATION AT DIFFERENT INCUBATION TIMES

Oil encapsulation and SEM analysis

Silica nanoparticle was synthesized by sol gel method under optimized reaction conditions. SEM image **(Figure 2)** revealed that size of silica nanoparticles were 1000 nm, spherical in nature and well dispersed in water, thus providing maximal interface to load optimal amount of drug/oil for delivery. The oil encapsulated silica nanoparticles were achieved by formulation of mixture of silica nanoparticles, Triton X-100, eucalyptus oil. The oil encapsulated silica nanoparticles were formed and further confirmed by antibacterial susceptibility test against biofilms as described in consequent sections.

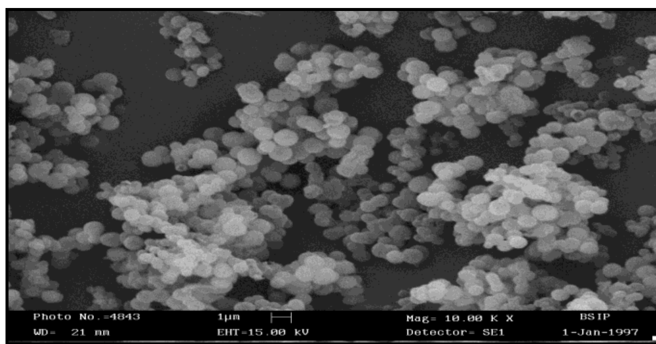

FIG.2: SEM OF SILICA NANOPARTICLES ~ 1000 ± 20 nm

Calculation of MIC value of eucalyptus oil for *E. coli*

The MIC value of eucalyptus oil was assessed against *E. coli.* MIC value of eucalyptus oil was found to be 1 µl/ml. Antibacterial susceptibility test was performed based on agar well diffusion method. The solution 10µg/ml of eucalyptus oil and oil encapsulated SNPs were used in the test. Oil encapsulated SNPs showed Zone of inhibition (ZOI) of 15mm whereas oil showed 9mm. The ZOI of oil encapsulated SNPs was more because of enhanced diffusion and accumulation into microbial cell. SNPs alone were also used to deduce anti- *E. coli* activity. Negligible ZOI was shown by SNPs.

Assessment of eucalyptus oil and oil encapsulated SNP efficacy against *E. coli* biofilm

The decreasing in absorbance at 570 nm in presence of inhibitor **(Figure. 3)** was confirmed the inhibitory effect of eucalyptus oil and oil encapsulated SNP by CV assay. Data demonstrated that 81% and 62% reduction in biofilm in response to oil encapsulated SNP and eucalyptus oil respectively. The effect of eucalyptus oil and oil encapsulated SNPs was further confirmed by light microscopic analysis and data showed reduced biofilm on glass cover slips surface. Figure 4 (a, b, c) shows light microscopy of *E. coli* biofilm and anti-biofilm activity of oil and oil encapsulated SNPs against *E. coli* biofilm.

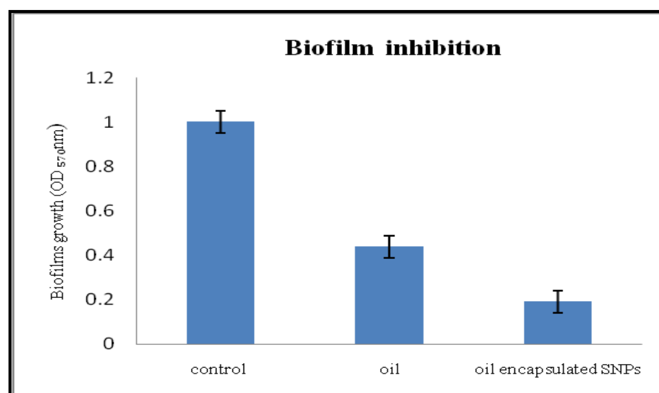

FIG. 3: BIOFILM INHIBITION THROUGH OIL AND OIL ENCAPSULATED SNP

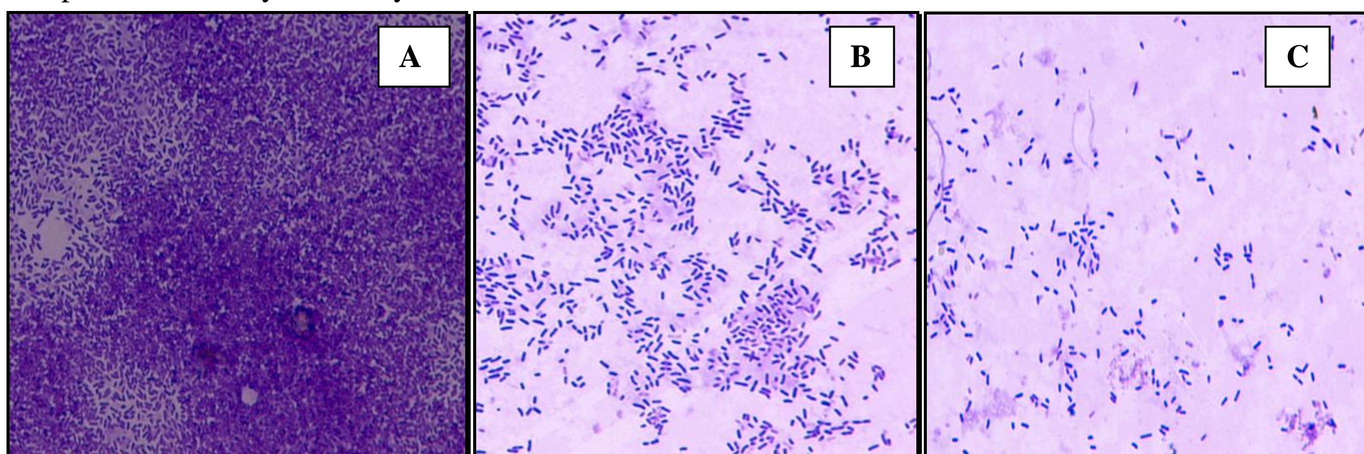

FIG. 4: LIGHT MICROSCOPY IMAGES OF BIOFILM (A) CONTROL WITHOUT ANY TREATMENT (B) BIOFILM TREATED WITH OIL (C) BIOFILM TREATED WITH OIL ENCAPSULATED SNP.

DISCUSSIONS: *Eucalyptus* is one of the most important and widely used genera and its species well known for as medicinal plant because of their antimicrobial and pharmacological properties.

Eucalyptus globulus oil is the main furnisher of essential oil and finds many applications as prevention and treatment of microbial associated infections and diseases, such as ulcer, measles, encephalitis, inflammation, respiratory and sinus infection, viral infection (herpes), bronchitis, rheumatism and arthritis, migraines and diabetes [10, 21]. *Eucalyptus globulus* oil showed antibacterial activity against both Gram positive and Gram negative bacteria (*E. coli)* [10]. *E. coli* is responsible for uropathogenic infections and medical device associated biofilm infections and diseases. Biofilm formed by *E. coli* are more resistant to antibiotic than planktonic cells. The recalcitrant property of *E. coli* biofilm is continuous rise up due to repeat use of antibiotic drugs. In this context, it is urgent need to search new alternative antimicrobial agent which that can inhibits the growth of biofilm associated microbes, and leave potent therapeutic effect. In current study, we evaluated the anti bacterial and antibiofim activity of oil encapsulated silica nanoparticles.

In present study results clearly demonstrate that oil encapsulated SNP act as enhanced antibacterial and antibiofilm agent against *E. coli* biofilm. In our study oil encapsulated SNP is a potentially strong biofilm inhibitor compare to eucalyptus oil can be explained on the basis of releasing proper amount of active components of eucalyptus oil inside the cells and biofilm of *E. coli* by using silica nanoparticles. The study supports the finding of Agarwal *et al* [12], who was demonstrated the *Candida albicans* biofilm inhibition in response to eucalyptus oil. In another study the normal growth of *C. albicans* has also shown to inhibited by eucalyptus oil [13]. The eucalyptus oil possess potent antimicrobial and antibiofilm effect due to presence of active component such as monoterpinol and eucalyptol (1, 8 – cineole). The results not only examined potential utility of eucalyptus oil in antibacterial infections but also encouraging potential application of oil encapsulated SNP in treatment of clinical antibiofilm, include medical prosthetic devices associated infections and diseases. Further oil encapsulated SNP also can use against other pathogenic microbes and the SNP can use carrier of other essential oils which have reported antimicrobial property.

Silica nanoparticles were formed by sol gel method. The sol gel method involves hydrolysis and condensation of TEOS (metal alkoxide) in the presence of ammonia as catalyst. The larger size of silica nanoparticles formed due to continuous aggregation of nuclei [22-23]. The study is in support was the finding of Strober *et al* [24], who reported that silica nanoparticles produced, size ranging from 50 to 2000 nm from aqueous alcohol solutions of silica alkoxides in presence of ammonia. The sized and morphology of synthesized silica nanoparticle was determined by SEM. The average size of SNP was 1000nm nm and spherical in shape **(Figure 2).**

Antibacterial susceptibility test was performed by agar well diffusion method against *E. coli*. Oil encapsulated SNP formulation showed larger zone of inhibition (15 mm) whereas oil showed lower (9 mm). Higher the antibacterial activity of oil encapsulated silica nanoparticle may be due to effect of nanoparticle and enhanced diffusion. The MIC value of oil was 1µl/ml: at 1µl/ml and higher concentration no visible growth was observed. Inhibition of growth was depended on concentration of oil. Eucalyptus oil and oil encapsulated SNP further checked against *E. coli* biofilm. CV assay analysis showed the 50 µl/ml concentration of oil encapsulated SNP was able to reduce 81.4% whereas, 62.4% concentration of eucalyptus oil. Oil encapsulated SNP shows 19% more inhibition than eucalyptus oil may be due to impact of nanoparticle and improved penetration of oil encapsulated SNP into *E. coli* biofilm. Further antibiofilm activity of oil and oil encapsulated SNP was proved by light microscopy. Microscopy micrograph of control biofilm, and treated biofilm revealed that most reduction to biofilm constituents was caused by eucalyptus oil encapsulated SNP, (Figure 4 A, B, C) suggesting that oil encapsulated SNP penetrated the biofilm matrix and interference in formation of biofilm constituents and metabolic process of *E. coli* biofilm.

CONCLUSIONS: Eucalyptus oil encapsulated SNPs were successfully formed using mixture of eucalyptus oil and triton x-100. *In vitro* antibiofilm activity was determined against *E. coli*. The antibiofilm effect of eucalyptus oil and oil encapsulated SNP was found to be 62 and 81% respectively. The superior antibiofilm activity of eucalyptus oil encapsulated SNPs may be due to combined result of nano size and use of silica nanoparticles as carrier. The current research work suggests the potent efficacy of oil against *E. coli* biofilm can be improved by SNP.

In the present work oil encapsulated SNP exhibits reproducible result against biofilm. In the context, in the future to deduce the efficacy of oil encapsulated SNP against other biofilm forming microbes and explore its therapeutic effect *in vivo* against biofilm.

ACKNOWLEDGEMENTS: Authors are thankful to Department of Science and Technology (DST), Government of India, for financial support.

REFERENCES:

1. Hancock V, Witso IL and Klemm P: Biofilm formation as a function of adhesin, growth medium, substratum and strain types. International Journal of Medical Microbiology 2011; 301(7):570-576.
2. Hou S, Burton EA, Simon KA, Blodgett D, Luk YY and Ren D: Inhibition of *Escherichia coli* biofilm formation by self-assembled monolayers of functional alkanethiols on

gold. Applied and Environmental Microbiology 2007; 73(13):4300-4307.

3. Ghanwate NA: Biofilm eradication studies on uropathogenic *E. coli* using ciprofloxacin and nitrofurantoin. International Journal of Pharmaceutical and Biomedical Research 2012; 3(2):127-131.
4. Bonkat G, Widmer AF, Rieken M, van der Merwe A, Braissant O, Müller G, Wyler S, Frei R, Gasser TC and Bachmann A: Microbial biofilm formation and catheter-associated bacteriuria in patients with suprapubic catheterization. World Journal of Urology 2013; 31(3):565-571.
5. Nowatzki PJ, Koepsel RR, Stoodley P, Min K, Harper A, Murata H, Donfack J, Hortelano ER, Ehrlich GD and Russell AJ: Salicylic acid-releasing polyurethane acrylate polymers as anti-biofilm urological catheter coatings. Acta Biomaterialia 2012; 8(5):1869-1880.
6. Hall-Stoodley L, Costerton JW and Stoodley P: Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology 2004; 2(2):95-108.
7. Dohnt K, Sauer M, Müller M, Atallah K, Weidemann M, Gronemeyer P, Rasch D, Tielen P and Krull R: An *in vitro* urinary tract catheter system to investigate biofilm development in catheter-associated urinary tract infections. Journal of Microbiological Methods 2011; 87(3):302-308.
8. Kasimanickam RK, Ranjan A, Asokan GV, Kasimanickam VR and Kastelic JP: Prevention and treatment of biofilms by hybrid-and nanotechnologies. International Journal of Nanomedicine 2013; 8:2809.
9. Upadhyay RK, Dwivedi P and Ahmad S: Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. Asian Journal of Medical Sciences 2010; 2(3):152-158.
10. Bachir RG and Benali M: Antibacterial activity of the essential oils from the leaves of *Eucalyptus globulus* against *Escherichia coli* and *Staphylococcus aureus*. Asian Pacific Journal of Tropical Biomedicine 2012; 2(9):739-742.
11. Rukayadi Y, Lau KY, Zainin NS, Zakaria M and Abas F: Screening antimicrobial activity of tropical edible medicinal plant extracts against five standard microorganisms for natural food preservative. International Food Research Journal 2013; 20(5):2905-2910
12. Agarwal V, Lal P and Pruthi V: Prevention of *Candida albicans* biofilm by plant oils. Mycopathologia 2008; 165(1):13-19.
13. Agarwal V, Lal P and Pruthi V: Effect of plant oils on *Candida albicans*. Journal of Microbiology, Immunology and Infection 2010; 43(5):447-451.
14. Hammer KA, Carson CF and Riley TV: In-vitro activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida spp*. Journal of Antimicrobial Chemotherapy 1998; 42:591–595.
15. Zalewska-Piatek BM, Wilkanowicz SI, Piatek RJ and Kur JW: Biofilm formation as a virulence determinant of uropathogenic *Escherichia coli* Dr+ strains. Polish Journal of Microbiology 2009; 58(3):223-229.
16. Nakao R, Ramstedt M, Wai SN and Uhlin BE: Enhanced biofilm formation by *Escherichia coli* LPS mutants defective in hep biosynthesis. PloS One 2012; 7(12):e51241.
17. Almeida C, Azevedo NF, Santos S, Keevil CW and Vieira MJ: Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH). PloS One 2011; 6(3):e14786.
18. Guo H, Qian H, Sun S, Sun D, Yin H, Cai X, Xuepeng Cai, Liu Z, Wu J, Jiang T and Liu X: Hollow mesoporous silica nanoparticles for intracellular delivery of fluorescent dye. Chemistry Central Journal 2011; 5(1):1.
19. Nooney RI, Thirunavukkarasu D, Chen Y, Josephs R and Ostafin AE: Synthesis of nanoscale mesoporous silica spheres with controlled particle size. Chemistry of Materials 2002; 14(11):4721-4728.
20. Singh LP, Agarwal SK, Bhattacharyya SK, Sharma U and Ahalawat S: Preparation of silica nanoparticles and its beneficial role in cementitious materials. Nanomaterials and Nanotechnology 2011; 1(1):44-51.
21. Dakov T: Antimicrobial effect of essential oil isolated from *Eucalyptus globulus* Labill. from Montenegro. Czech Journal of Food Sciences 2011; 29(3):277-284.
22. Bogush GH, Tracy MA and Zukoski Iv CF: Preparation of monodisperse silica particles: control of size and mass fraction. Journal of Non-Crystalline Solids 1988; 104(1):95-106.
23. Ibrahim IA, Zikry AAF and Sharaf MA: Preparation of spherical silica nanoparticles: Stober silica. Journal of American Science 2010; 6(11):985-989.
24. Stöber W, Fink A and Bohn E: Controlled growth of monodisperse silica spheres in the micron size range. Journal of Colloid and Interface Science 1968; 26(1):62-69.

How to cite this article:
Dohare S, Dubey SD, Kalia M, Verma P, Pandey H, Singh NK and Agarwal V: Anti-Biofilm Activity of *Eucalyptus Globulus* Oil Encapsulated Silica Nanoparticles against *E. Coli* Biofilm. Int J Pharm Sci Res 2014; 5(11): 5013-18.doi: 10.13040/IJPSR.0975-8232.5 (11).5013-18.

All © 2014 are reserved by International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **ANDROID OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)