INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-1, Jan-Mar-2013 Coden : IJABPT Copyrights@2013

ISSN : 0976-4550

Received: 22nd Nov-2012 *Revised: 27th Nov-2012* *Accepted: 27th Nov-2012*

Research article

EFFECTS OF THE COMPONENT OF SUNFLOWER OIL BASED NANOEMULSIONS AGAINST MEAT-BORNE PATHOGENS

K.Bradeeba*[1] and P.K.Sivakumaar[1]

[1]Annamalai University University, Science Faculty, Departmentof Microbiology,Annamalai nagar, Chidambaram-608002,Tamil Nadu,
phone number:7598122938
*Corresponding author: apeedarpwp@gmail.com

ABSTRACT: Today's consumer looking for wholesome food without chemical preservatives. These can be classified as novel compounds obtained from sunflower oil based nanoemulsions that delay microbial growth of pathogens and spoilage organisms in food. Sunflower oil was screened for the development of a surfactin based nanoemulsion formulation. On further screening for antibacterial activity sunflower oil-based nanoemulsion designated as AUSN-1 showed highest activity against *Pseudomonas aeruginosa* followed by *Bacillus cereus*, *Proteus mirabilis*, *Acinetobacter baumannii* , *Bacillus sp and Aeromonas sp*. AUSN-1 also demonstrated a high bacteriostatic and bactericidal con (%) against *Pseudomonas aeruginosa, Bacillus cereus* respectively.Their role in controlling food pathogens and mechanisms of action. This work suggests the possible use of nanoemulsion as preservatives in meat products.

Keywords: Preservatives, Sunflower oil, AUSN-1, *Pseudomonas aeruginosa*

INTRODUCTION

In foods, spoilage due to the presence of bacterial and fungal infection has been a major concern for decades in the food industry as it cause a considerable loss, worldwide (Negi *et al.,* 2005). Moreover, the increased awareness on the ill-effects of synthetic chemicals present in foods has also led to the search for other alternative non toxic antimicrobial compounds (Shan *et al.,* 2007). Meat is the most perishable of all important food since it contain sufficient nutrient needed to support the growth of microorganisms (Okonko *et al., 2010*).

As an effective alternative, in the recent years "nanotechnology" based techniques or tools are used during cultivation, production, processing, or packaging of the food products and the food is termed as "nanofood" (Cho *et al.,* 2008; weiss *et al.*, 2006). In nanofood; nanoparticles are often used for imparting color and flavour improvement, better storage and preservation, pathogen detection, antimicrobial properties, intelligent packaging, etc.

Among these [Nanoparticles] a few research groups have presented data regarding the antimicrobial properties of certain micro and nanoemulsions (Paula *et al.,* 2007) Nanoemulsions are water-in- oil formulations that have a broad spectrum of antimicrobial activity. These compounds are produced by mixing a water immiscible liquid phase into an aqueous phase by high stress mechanical extrusion that yields a uniform population of droplets ranging in diameter from 200 to 800 nm. They have broad biocidal efficacy and represent a new generation of disinfectants that disrupt selectively the membranes of isolated prokaryotic cells and viruses, but does not affect eukaryotic cells present in the tissue. Numerous reports have documented the bactericidal activity against numerous clinical pathogens, which include *H.influenzae*, *Bacillus cereus*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Vibrio cholerae.* Moreover, its inhibitory activity against filamentous fungi including *Microsporum* spp., *Trichophyton mentagro-phytes*, *Trichophyton rubrum*, *Aspergillus fumigatus*, *Fusarium oxysporum* and *Epidermophyton floccosum* has also been documented (Hamouda *et al.,* 1999).

However, in food microbiology there are an only a few reports on the bactericidal affect of these nanoemulsion against food borne pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Al-Adham, *et al.,* 2000) *Listeria monocytogenes* (Ferreira *et al.,* 2010).

A detailed study on the antimicrobial activity of these nanoemulsions against meat borne pathogens and its potential use as a preservative in the food industry has not been done earlier.

Taking all these into account in the present work, development of sunflower oil based nanoemulsion system was studied. This was followed by the critical evaluation of its antimicrobial activity against selected food borne pathogens. The following were the major objectives to be considered under the present study. 1. Development of nanoemulsion using sunflower oil. 2. Testing the antimicrobial activity of nanoemulsion against selected meat borne pathogens.

MATERIAL AND METHODS

Development of emulsions

The oil-in-water (O/W) nanoemulsion has an oil phase of sunflower oil (16% v/v of the total emulsion), ethanol (2%), and triton X-100 (2%). Therefore, this phase represents 20% (v/v) of the emulsion. The components of the oil phase were mixed and kept for 1 h at 86 °C. After this, the water phase was added, and the mixture was emulsified.

Agar well diffusion method

The selected bacterial isolates were inoculated into 10 mL of serial nutrient broth and incubated at 37◦ C for 16-18 hours. Using a sterile cotton swab, the nutrient broth cultures were swabbed on the surface of sterile nutrient agar plates. Agar wells prepared with the help of sterilized cork borer. Different concentration of extracts (100% and 50%) was added to different wells in the plate. The plates were incubated in an upright position at 37°C for 24 hours.

Effect of nanoemulsion on the growth of pathogens (agar well diffusion method)

Agar well diffusion method was followed for the evaluation of antibacterial activity of known nanoemulsion *viz.*, 100%, and 50% Nutrient agar medium was prepared and autoclaved at 15 Ib pressure for 20 minutes and cooled to 45^0C, poured in sterile petriplates and allowed for solidification. Cell suspension (10^6 cell ml^{-1}) of the selected test organisms *viz., Bacillus cereus, Proteus mirabilis, Acinetobacter baumannii, Aeromonas sp*, *Pseudomonas aeruginosa was* prepared separately. "L" rod was used to spread the cell suspension. The stainless steel cork borer used to make the well, each well was filled with the 100%and 50% of nanoemulsion concentration was prepared. The plates were incubated at 37^0 C for 24 hours; observed for the zone of inhibition. Diameter of inhibition zones was measured and expressed in mm.

Time-kill assay

Five replicate microtiter plates, each containing two fold serial dilutions of nanoemulsions were prepared and inoculated with the test pathogens. Negative control wells without bacteria were included on each plate. Plates were incubated at 37°C, and 10-fold serial dilutions were plated in triplicate onto nutrient agar plates, after 1-7 h. After a further 7 h of incubation at 37°C, colonies were enumerated and recorded as the average cfu/ml.

Minimum bacteriostatic concentration

Minimum bacteriostatic were determined using a modified method developed by Smith-Palmer *et al*. (1998). Tubes with different volumes of tryptic soy broth were prepared. The tryptic soy concentration of the broth in the tubes was prepared to give a final concentration of 3% after addition of Nanoemulsion. These tubes were inoculated with 0.5ml of the prepared bacterial cultures. An appropriate volume of the nanoemulsion to be tested was added so that the final volume in the tube was 10ml. Inoculated tubes were incubated at 28±2˚C. After 24 h, tubes were observed for growth both visually and through the measurement of absorbance at 600 nm. Samples (100-ml) from those tubes showing no growth were placed on tryptic soy agar (Difco Laboratories) and incubated at 35^0C for 24h. Duplicate sets of tubes were prepared each time and each experiment was repeated three times.

Minimum bactericidal concentrations

Minimum bactericidal concentrations were determined using a modified method developed by Smith-Palmer *et al.* (1998). The minimum bactericidal concentration was the lowest concentration at which bacteria failed to grow in tryptic soy broth and showed no growth when 100-ml samples were plated on to tryptic soy agar (Smith- Palmer *et al.,* 1998). The minimum bactericidal concentrations for these nanoemulsions were determined by pour plate method using tryptic soy agar medium with 0.5ml of *E. coli* strain inoculum and added sanitizer. The final volume in the plate was maintained at 15ml with a 3% concentration of tryptic soy medium and 1.5% agar.

Total bacterial population in beef, mutton and pork

Total bacterial population was determined at different time intervals during storage. Each samples (boiled, 10 g) coated with nanoemulsion was homogenized by using a lab-blender stomacher-400 (laboratory equipment, London, UK) in 90 ml of sterile peptone water. Serial dilutions were prepared and spread-plated on sterile petriplates and plates were incubated at 35 ± 1°C at different time intervals (24, 36 and 48 h). Counts were reported as $\log_{10}$ cfu/g of meat.

Microscopic observations

Morphology and structure of the emulsions were studied using the transmission electron microscopy (TEM) TOPCON 002B operating at 200 kV and of a 0.18 nm capable point-to-point resolution. Combination of bright field (BF) imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the emulsions and to determine the amorphous or crystalline character of their components. In order to perform the TEM observations, the concentrated emulsion was first diluted in water (1/10), a drop of the diluted emulsion was then directly deposited on the holey film grid and observed after drying. The emulsion appears dark and the surroundings are bright, a "positive" image is seen. The direct observation also enabled us to perform selected area electron diffraction (SAED) to check the crystallinity of the emulsion core components (Guinebretière et al.,2002; Louchet et al., 1988).

Histopathological staining in meat muscle:

Xylen I	-	10 minutes
Xylen II	-	10 minutes
Alcohol-50%	-	rinse
Alcohol-100%	-	rinse
Washed with water		
Haematoxyles	-	15 minutes
Washed with water rinse with 1% alcohol (blueing colour)		
Fosine	-	5 minutes

RESULTS AND DISCUSSION

Antimicrobial activity of AUSN-1against certain meat borne pathogens

In the present study antimicrobial activity as determined by well diffusion method showed that gram positive organisms were found to be more susceptible to nanoemulsions, when compared to the gram negative organisms. This high degree of resistance offered by these selected gram-ve bacteria as observed in our present study is in agreement with the earlier reports of (Hamouda *et al.,* 2000); reported entric Gram-ve bacilli such as *C. freundii, E. cloacae, E. coli, P. mirabilis, P. aeruginosa, S. typhimurium, S. dysenteriae*, and *S. aureus* were resistant to 10% 8n8 (a formulation of nanoemulsion). Vaara, (1993) reported that this resistance offered by enteric Gram-negative bacilli is attributed to its cell wall lipopolysaccharide (LPS).

Table 1. Antimicrobial activity of Nanoemulsion against certain meat spoilage pathogens by well diffusion method

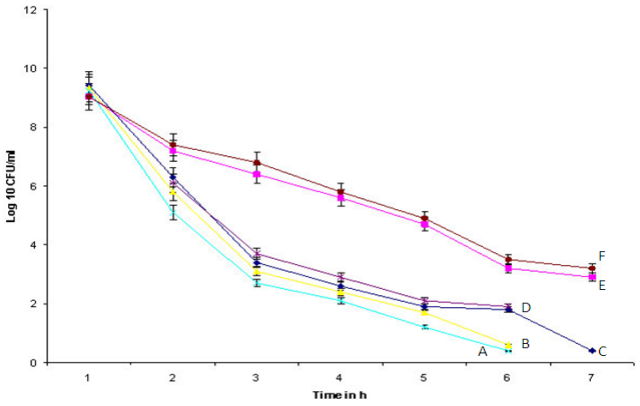
Organism	Zone of inhibition(mm)		
	Nanoemulsion concentration		
	50	100	SN
Acinetobacter baumannii	15.47 ± 0.12	22.42 ± 0.22	19.17 ± 0.03
Bacillus sp	11.34 ± 0.13	18.64 ± 0.22	14.41 ± 0.11
Bacillus cereus	27.12 ± 0.02	32.33 ± 0.12	18.62 ± 0.02
Pseudomonas aeruginosa	30.14 ± 0.14	34.26 ± 0.12	30.12 ± 0.11
Proteus mirabilis	25.67 ± 0.12	31.35 ± 0.11	23.45 ± 0.05
Aeromonas sp	-	9.74 ± 0.22	18.64 ± 0.02

*The value is the diameter (mm) across the zone of inhibition and the agar well (5mm Values are mean ±SD of three replicates from one representative of each experiment was carried out in 3 times, and similar results were obtained each time, within a column different letters.SN-sodium nitrate

Table 1 shows the antimicrobial activity of AUSN-1 at different dilution (100%, 50%) evaluated against *Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis*, *Acinetobacter baumannii* and *Aeromonas sp* . Among the different dilution tested the undiluted (100%) based AUSN-1 show the highest inhibition activity the selected test pathogens.Undiluted (100%) AUSN-1 exhibited the highest inhibitory effects against *Pseudomonas aeruginosa* (34.26mm), *Bacillus cereus* (32.33mm), *Proteus mirabilis* (31.35mm), *Acinetobacter baumannii* (22.42mm),*Bacillus sps* (18.64), *Aeromonas sp* (9.74mm) respectively.AUSN-1 at 50% also showed moderate inhibitiory activity against *Pseudomonas aeruginosa* (30.14mm), *Bacillus cereus* (27.12mm), *Proteus mirabilis (*25.67mm*), Acinetobacter baumannii (*15.47*mm)* and *Bacillus sp (*11.34*)*. While the nanoemulsion at 50% concentration did not show any show any zone of inhibition against Aeromonas sp.

Time kill study of nanoemulsion against certain food borne pathogens

The nanoemulsion was evaluated for antimicrobial activity based on time kill study and the resulted are presented in Fig. 1.



*Values are a mean of three replications. Error bars indicate the minimum significant difference (5%) for comparing treatments on each sampling time. A-*Pseudomonas eruginosa*, B- *Bacillus cereus, C-Acinetobacter baumannii* D-*Proteus mirabilis*, E-*Bacillus sp*, F-*Aeromonas sp*

Fig. 1 Time-kill study on antimicrobial activity of Nanoemulsion against certain meat spoilage bacterial pathogens

However the decrease in activity of nanoemulsion, when diluted or at reduced concentration as observed in the present study is in agreement with the previous reports of Zhang *et al.,* (2008); reported that reduce in activity of nanoemulsion is due to the fact that addition of water led to significant changes in nanoemulsion structure that affected its antimicrobial property (Hou *et al*., 2007).

Nanoemulsion exhibited the highest inhibitory effect against *Pseudomonas aeruginosa,* followed by *Bacillus cereus* respectively, in which a 100% reduction in bacterial population was observed. While *Proteus mirabilis*, *Acinetobacter baumannii* offered a high degree of resistance for nanoemulsion, which recorded only a 50% reduction on the bacterial population.Time kill study on the antimicrobial activity of nanoemulsion against selected meat spoilage pathogens has showed that viability was lost almost completly after an expose of 6-7h. These results are in agreement with the previous reports of Al-Adham *et al.,* (2000); reported that an o/w microemulsion ethyoleate emulsified by Tween 80 and pentanal gave a 5 log reduction in the population of *S.aureus* and *P.aeruginosa*.

Minimum bacteriostatic and bactericidal concentration of nanoemulsion against selected food borne bacterial pathogens

The bacteriostatic and bactericidal concentrations of nanoemulsion were evaluated against selected meat borne pathogens and the results are presented in Fig. 2.

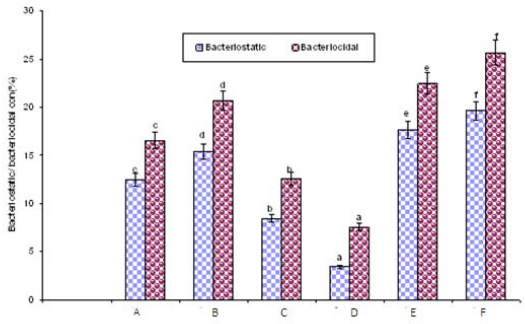


Fig. 2 Minimum bacteriostatic and bactericidal concentration of nanoemulsion on certain selected meat

Spoilage bacterial pathogens

A–*Acinetobacter baumannii*, B-*Baciiius sp*, C-*Bacillus cereus*, D- *Pseudomonas eruginosa*,,E-*Proteus mirabilis*, F-*Aeromonas sp*. The minimum bacteriostatic concentration was the lowest concentration at which bacteria failed to grow in tryptic soy broth but showed growth when 100-ml samples were plated onto tryptic soy agar. The minimum bactericidal concentration was the lowest concentration at which bacteria failed to grow in tryptic soy broth and showed no growth when 100-ml samples were plated onto tryptic soy agar.Mean values in the same column that are followed by the same, regular case letters are not significantly deferent ($P< 0.05$).

The result reveals that the undiluted nanoemulsion exhibited the highest reduction against *Pseudomonas aeruginosa (86.42),* followed by *Bacillus cereus* (80.63), *Proteus mirabilis* (78.63), Acinetobacter baumannii (68.22), *Bacillus sp* (57.34), *Aeromonas sp* (50.42).Nanoemulsion exhibited moderate bacteriostatic and bacteriocidal concentration (%) against, Proteus mirabilis, *Acinetobacter baumannii* ,*Bacillus sps* and *Aeromonas sp* did not show any bacterial population. Among the different dilution studied, the undiluted showed highest inhibition potency against all the tested pathogens followed by 50% when compared to control. Results on the bacteriostatic and bactericidal activity of nanoemulsion have showed that it has more bacteriostatic activity, when compared to the bactericidal activity. However, there has been no earlier report on the bactericidal or bacteriostatic activity of the nanoemulsion.

Influence of Sunflower oil based nanoemulsion treatment on the total heterotrophic bacterial population of beef, mutton and pork

The total heterotrophic bacterial population as influenced by nanoemulsion treatment was compared with a standard antibiotic (Amphicillin at 1000ppm) and the results are tabulated in Table 2 higher reduction in the heterotrophic population of beef was observed at 12 h and between the two samples tried the highest inhibitory effect was showed on beef sample (2.56 log10 cfu/g) at 12 h.A higher reduction in the heterotrophic population of mutton and pork were observed at 12 h (1.27 log10 cfu/g and 2.10 log10 cfu/g).it is interesting to note that nanoemulsion treatment is found to have 100% more activity when compared to the standard antibiotic; Amphicilin.

Table 2. Influence of nanoemulsion treatment on the total heterotrophic bacterial population on Mutton,Beef and Pork

Food product	Treatment	Total heterotrophic bacterial population (Log 10 Cfu/ml in different time periods in (h)					
		6	12	24	36	48	60
Mutton	Nanoemulsion	4.56 ± 0.22^{aB}	2.56 ± 0.04^{aA}	4.98 ± 0.22^{aB}	5.98 ± 0.02^{aC}	7.98 ± 0.16^{aD}	8.14 ± 0.02^{aE}
	Amphicillin	7.89 ± 0.12^{bB}	5.64 ± 0.22^{bA}	7.98 ± 0.22^{bC}	9.64 ± 0.22^{bD}	10.54 ± 0.22^{bE}	13.81 ± 0.11^{bF}
	Control	9.54 ± 0.22^{cA}	12.22 ± 0.02^{cB}	14.84 ± 0.22^{cC}	16.84 ± 0.12^{cD}	18.98 ± 0.22^{cE}	20.42 ± 0.08^{cF}
Beef	Nanoemulsion	2.52 ± 0.02^{aB}	1.27 ± 0.22^{aA}	3.44 ± 0.22^{aC}	3.98 ± 0.02^{aD}	5.98 ± 0.12^{aE}	6.44 ± 0.22^{aF}
	Amphicillin	5.84 ± 0.22^{bB}	3.66 ± 0.03^{bA}	5.42 ± 0.04^{bB}	$7.24\pm0.0.04^{b}_{C}$	9.04 ± 0.08^{bD}	10.64 ± 0.44^{bE}
	Control	7.22 ± 0.04^{cA}	10.24 ± 0.08^{cB}	12.44 ± 0.16^{cC}	13.02 ± 0.04^{cD}	14.98 ± 0.12^{cE}	15.88 ± 0.22^{cF}
Pork	Nanoemulsion	1.56 ± 0.22^{aB}	2.10 ± 0.04^{aA}	4.00 ± 0.22^{aB}	4.55 ± 0.02^{aC}	6.38 ± 0.16^{aD}	$8..86\pm0.02^{aE}$
	Amphicillin	3.99 ± 0.10^{bB}	5.64 ± 0.22^{bA}	$6..91\pm0.22^{bC}$	7.78 ± 0.22^{bD}	9.54 ± 0.22^{bE}	12.11 ± 0.11^{bF}
	Control	6.77 ± 0.12^{cA}	7.34 ± 0.02^{cB}	10.44 ± 0.22^{cC}	12.84 ± 0.12^{cD}	15.18 ± 0.22^{cE}	21.49 ± 0.08^{cF}

*Different letters after values indicate that there is a significant difference at a P value of 0.05 as determined by DMRT. The small letters compare among bacterial combinations and capital letters compare among different time periods.

Though, results pertaining to the influence of nanoemulsion treatment on total heterophic bacterial and of beef mutton and pork are less. Teixeira *et al., (*2007) reported that o/w nanoemulsions were active against five food borne pathogens including *S.aureus, Ecoli, P.aeruginosa, S.typhi, Listeriamonocytogenes.*

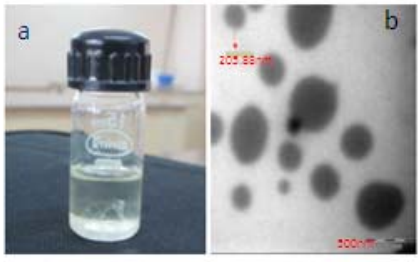
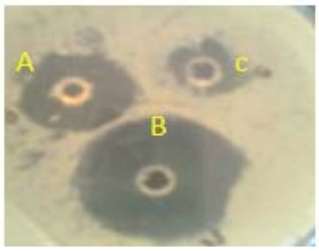


Fig.3 a)Nanoemulsion b) Transmission electron micrograph of Nanoemulsion



(A) : 50% nanoemulsion
(B) : 100% nanoemulsion
(C) : 100% sodium nitrate

Fig.4 ANTIIMICROBIAL ACTIVITY (SHOWING ZONE OF INHIBITION) OF NANOEMULSION AGAINST *Pseudomonas aeruginosa*

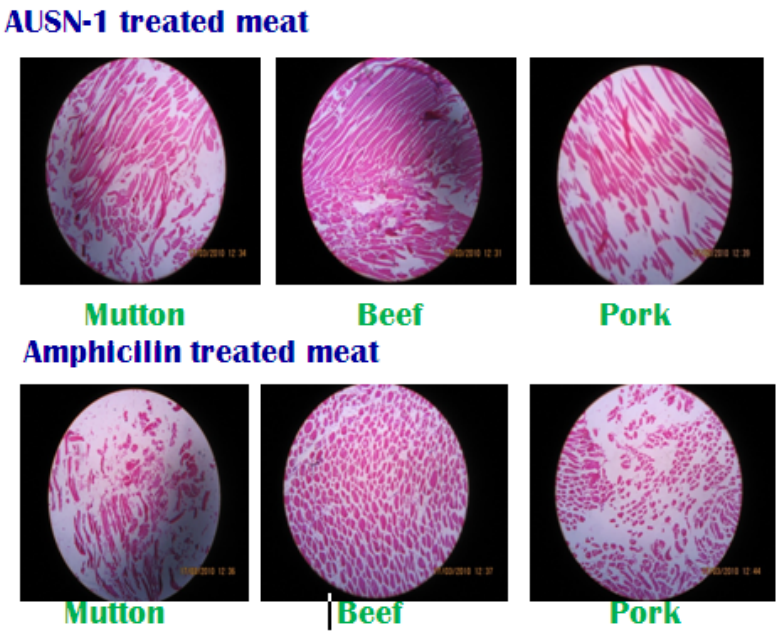


Fig.5 HISTOLOGICAL PHOTOGRAPHS OF MEAT SHOWING GROSS TISSUE DAMAGE. ALL HISTOPATHOLOGY IS SHOWN AT 40 X MAGNIFICATION

CONCLUSION

In conclusion this work suggests the possible use of nanoemulsion as preservatives in food products.

REFERENCES

Al-Adham, I. S. I.,Khalil, E .,Al-Hmoud, N.D., Kierans, M.,Collier,P.J., (2000). Micro emulsions are membrane-active, antimicrobial, self-preserving systems. *Journal of Applied Microbiology*.89**,** p. 67-70.

Cho, Y.,Kim, C.,Kim, N.,Kim, C.,Park, B. (2008). Some cases in applications of nanotechnology to food and agricultural systems. Biochip. J. 2(3), 183–185.

Ferreira, J.P.,Alves, D., Neves, O., Silva, J., Gibbs ,P.A., Teixeira, P.C.(2010). Effects of the components of two antimicrobial emulsions on food-borne pathogens. Food Control.21,27–230.

Guinebretière, S., Briançon, S.,Fessi, H.,Teodorescu, V.S., Blanchin, M.G. (2002). Nanocapsules of biodegradable polymers: preparation and characterization by direct high resolution electron microscopy. Mater. Sci. Eng. C 21,137–142.

Hamouda, T.,Baker, J.R.Jr.2000. Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. Journal of Applied Microbiology. 89, 397–403.

Hamouda, T.,Hayes, Cao,M.M.,Tonda,Z.,Johnson,R,.,Wright, K-D.C et al. (1999). A novel surfactant nanoemulsion with broad-spectrum sporicidal activity against Bacillus species. Journal of Infection Diseases.180,1939–1949.

Hou, L., Y.,Shi, P., Zhai,.,Le, G. (2007).. Inhibition of food borne pathogens by Hf-1, a novel antibacterial peptide from the larvae of the housefly (Musca domestica) in medium and orange juice. Food Control.18,1350–1357.

Louchet, F.,Verger-Gaugry, J.L.,Thibault-Desseaux.,Guyot,P.,(1988). Microscopie électronique en transmission In Les Techniques de l'Ingénieur. 875-10.

Negi, P.S., Chauhan, A.S.,Sadia,G.A.,.Rohinishree,Y.S.,Ramteke,R.S. (2005). Antioxidant and antibacterial activities of various seabuckthorn (Hippophae rhamnoides L.) seed extracts.Food chem.92,119-124.

Okonko I.O. Ukut i.,OE, Ikpoh IS, Nkang AO, Udeze AO, Babalola TA, Mejeha Ok, Fajobi EA.(2010). Assessment of bacteriological quality of fresh meats Sold in calabar metropolis, nigeriaejeafche.9(1),89-100.

Paula C. Teixeira, Gonçalo, Leite, Ricardo, Domingues, Joana Silva, Paul, Gibbs, Joao Paulo Ferreira, (2007). Antimicrobial effects of a micro emulsion and a nanoemulsion on enteric and other pathogens and biofilms. International Journal of Food Microbiology.118,15–19.

Shan, B.,Cai,J.D- Brooks .,Corke, H. (2007). The in vitro antibacterial activity of dietary spice and medicinal herb extracts. J. Food Microbial. Vol,117,p .112-119.

Smith-palmer, A. J.,Stewart .,Fyfe,L. (1998). Antimicrobial propertiesof plant essential oils and essences against five important food borne pathogens. J. Lett. Appl. Microbial,vol,26,p 118-122.

Teixeira, P. C.,Leite, G. M., Domingues, R. J.,Silva, J.,Gibbs, P. A., Ferreira,J.P. (2007). Antimicrobial effects of a micro emulsion and a nanoemulsion on entericband other pathogens and biofilms. International Journal of Food Microbiology.118,15–19.

Vaara, M, (1993). Outer membrane permeability barrier to azithchromycin, clarithr omycin, and roxithromycin in gram-negative enteric bacteria. Antimicrob. Agents Chem other,vol,37,p 354–356.

Weiss, J.,Takhistov, P.,McClements, D.J.(2006).Functional materials in food nanotechnology. J Food Sci,vol, 71,R107–R116.

Zhang, H., Feng,F.,Zhan, J.Li, X.,Wei,H.,Wang, H. Li, H.,Zheng, X.(2008). Formulation of food grade micro emulsions with glycerolmonolaurate: effects of short-chain alcohols, polyols, salts and nonionic surfactants. European Food Research and Technology.226 ,613–619.