International Journal of Innovative Technology and Exploring Engineering (IJITEE)
ISSN: 2278-3075 (Online), Volume-9 Issue-1, November 2019

Plant Essential Oils Based Nanoemulsion Formulations and Its Antibacterial Effect on Some Pathogens

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Abstract: ***Antibiotic resistance is the most challenging problem of concern globally and this is invigorating the need of newer antimicrobial products with potential antimicrobial properties. Plant products, especially plant essential oils produce a large array of secondary metabolites as natural antimicrobials. Use of nanotechnology can add advantages to enhance the antibacterial properties of these essential oils. Present study is focused on development of nanoemulsions from plant essential oils and to study their antibacterial activities. Tea Tree Oil, Thyme Oil, Clove leaf and Cinnamon Essential Oils nanoemulsion was formulated using Tween 20 and Tween 80 respectively using probe ultrasonicator. All the formulated Nanoemulsions were then subjected to physicochemical characterization, stability studies and tested for antibacterial activities using Agar-well diffusion method. Stable nanoemulsion formulation with maximum antibacterial activity then subjected to droplet size measurements and polydispersibility index study. Increase in surfactant concentration resulted in reduction in droplet size when ultrasonication time was constant. Cinnamon oil nanoemulsion 20C4 & 80C4 with pdi index 0.573 and 0.382 and droplet size 272.3nm and 133.6 nm respectively demonstrated maximum antibacterial activity in Agar-well diffusion method against S.aureus, E.coli, and S.typhi. When both nanoemulsions were exposed to bacterial growth curve inhibition study. No potential rise in optical density of test pathogens were observed. The inhibition of bacterial growth may be due to killing action of cinnamon oil nanoemulsion formulations to initial bacterial inoculum added to nutrient broth. The study suggests that nanoemulsion formulations from plant essential oils can be used as natural antimicrobials in variety of products.***

Keywords: Agar-well, Bacterial growth inhibition, Droplet size, Essential Oils, Nanoemulsion, polydispersibility index, Tween 20, Tween80, Ultrasonication

I. INTRODUCTION

Microorganisms contribute one of the essential components of the earth and existed on it for more than 3.8 billion years exhibiting great genetic and metabolic diversity, contributing in maintenance and sustainability of ecosystem and also are causative agents of dreadful infections in humans, animals and plants.

These microorganisms have evolved several mechanisms to tolerate selective pressures exerted by various environments and competitive challenges. One of these mechanisms is resistance to antibiotics. In recent times microorganisms evolved the genetic ability to develop and transmit resistance to antibiotics leading to inefficacy of these agents in treatment making use of these antimicrobial agents uncertain from future prospective. [1]Researchers are now warning of a return of pre-antibiotic era; with recent database listing existence of more than 2000 potential resistance |(r-genes) of nearly 400 different types from available bacterial genome sequences. [2] Therefore it is the need of an hour to limit the use of chemical antimicrobial agents and to focus on newer drugs, or formulations of either synthetic or natural origin which can be efficiently used to control microbial population. Despite of all the advancements in area of pharmaceutical chemistry and biotechnology, still plants are used sources of numerous phytomedicines with potential applications in treatment of infectious diseases and ailments. Thousands of different plant species have proven medical importance and these characteristics can be attributed synthesis of limitless phytochemicals with potential antimicrobial activity through specialized metabolic pathways that occur in them. [3] Different parts of the medicinal plants contribute to its medicinal properties, including leaves, stems, barks , fruit ,seed flowers, seeds, rhizomes, tubers, gums, resins and most importantly Essential Oils. Essential oils are normally volatile, rarely colored lipids constituting terepenoids, phenol-derived compound synthesized by many parts of the plants.[4] Present study focuses on use four different Essential Oil which are Tea tree oil, Thyme Oil, Clove leaf Oil & Cinnamon Bark oil. Tea tree Oil is derived from plant -*Melaleuca alternifolia* of native Australian origin. Terpene hydrocarbons mainly monpoterpenes, sesquiterpenes and their associated alcohols contributes to its biological activity.[5] Thyme oil is extracted and distilled form *Thymus vulgaris(L. Lamiaceae*) native to Mediterranean region of Europe, constituting infusions of monpoterpenes, with natural terpenoid thymol and its phenol isomercarvacrol(CVL)showingantitussive,expectorant,antis-pasmodic & antibacterial activities.[6]-14].Clove *(Syzygium aromaticum)* belongs to family Myrtceae. Clove Essential Oil finds application in treatment of acne, asthma rheumatoid arthritis and warts. [15]

Revised Manuscript Received on November 30, 2019.
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Cinnamon bark Oil is derived from *Cinnamomum zeylanicum*,(family-Lauraceae) and constitutes Cinnamaldehyde (3-phenyl-2-propanol) contributing it its antioxidant, antimicrobial and antiseptic uses.[16] Nano science and nanotechnology are nothing but science and engineering applied on nanometer scale of 10^{-9} meters. From last two decades researchers are focusing on manipulation of matter at the level of single atom or groups to characterize material at nanometer range and its potential applications in field of engineering, science and medicine, revolutionizing field with newer systems and practice. [17] The joint work of biology and nanotechnology has revolutionized biomedical research by exploiting significant properties of materials in nanometer range. Nanoparticles and nanomaterial with functional organic groups are employed as tracers, biomarkers and also as novel drug delivery system.[18] Several years of research in field of drug delivery in thrust of developing newer and promising approaches to enhance the availability and biological activity of natural antibacterial agents and thus considering developments in nanotechnology and emulsion technology, which is influencing the availability of active components in system to demonstrate its effect to combat with problem of antimicrobial resistance.[19] Nanoemulsions can be defined as the homogeneous, thermodynamically stable oil in water(O/W) or water in oil (W/O) dispersions of two immiscible liquids stabilized by interfacial film of surfactants with droplet size in range of 10-500 nm.[20,21] The credit of antimicrobial properties of essential oil nanoemulsion can be attributed to small size of oil droplets with high surface tension which can subsequently fuse with bacterial cell membrane and disrupt it.[22] Nanoemulsions can be formulated by using various high energy and low energy input method, some of which are- Ultrasonication, Phase-inversion temperature(PIT) microfluidisation,solvent evaporation techniques and Hydrogel methods.[23]. Present study focuses on formulations of different oil in water nanoemulsions using Tea tree, Thyme Oil, Clove leaf and Cinnamon Essential Oils using surfactants Tween 20, and Tween 80 respectively, its characterization and study of its antibacterial activity.

II. EXPERIMENTAL DETAILS.

A. Materials and Methods:

For present study - Cinnamon Bark Oil (CBO), Tea Tree (TTO), Clove Leaf Essential (CLO), Pure Thyme Oil (ThO),Tween 20 and Tween 80(s-d fine chemicals) deionized water, were purchased commercially. Hi-sensitivity Test Agar, Nutrient Agar, and Nutrient Broth, from Hi-media Laboratories were used, Standard pure bacterial cultures-*E.coli* (ATCC10799), *S.aureus* (ATCC 6538) and *Salmonella typhi* (NCIM5278) were procured from NCIM_NCL, Pune.

B.Nanoemulsion Formulation

Oil in water Nanoemulsions (O/W) of different plant essential oils were formulated using different oils and Tween 20 and Tween 80 as nonionic surfactants due to its high hydrophilic and Lipophilic Balance(HLB). Initially Coarse Emulsion was prepared with drop wise addition of deionized water to mixture comprising Essential Oil(EO) and Surfactant in ratio of 1:1,1:2,1:3, and 1:4 respectively with simultaneous stirring on magnetic stirrer at 600rpm for approx.60 min. The crude emulsion was formed, and then each crude emulsion was subjected to ultrasonic emulsification using probe ultrasonicator of 120 watts power at 30 KHz frequency and probe diameter of 15mm. the sonicator was set at cycle of 15 seconds. Each crude emulsion was subjected to ultrasonication for 70 min.Sonicator probe generates disruptive forces that reduces the droplet diameter and converts crude emulsion into nanoemulsion. These formulated emulsions were then used for further study.

E.O.	N. E. code	Surfactant Used	Oil: surfactant ratio	% Composition of Different components in Formulations		
				EO(ml)	Surfactant(ml)	Deionized Water(ml)
Tea Tree Oil (TTO)	20 T1	T-20	1:1	3	3	46
	20 T2		1:2	3	6	41
	20 T3		1:3	3	9	38
	20 T4		1:4	3	12	35
	80T1	T-80	1:1	3	3	46
	80T2		1:2	3	6	41
	80T3		1:3	3	9	38
	80 T4		1:4	3	12	35
Thyme Oil (ThO)	20 Th1	T-20	1:1	3	3	46
	20 Th2		1:2	3	6	41
	20 Th3		1:3	3	9	38
	20 Th4		1:4	3	12	35
	80Th1	T-80	1:1	3	3	46
	80Th2		1:2	3	6	41
	80Th3		1:3	3	9	38
	80 Th4		1:4	3	12	35
Clove Leaf Oil(CLO)	20 Cl1	T-20	1:1	3	3	46
	20 Cl2		1:2	3	6	41
	20 Cl3		1:3	3	9	38
	20 Cl4		1:4	3	12	35
	80Cl1	T-80	1:1	3	3	46
	80Cl2		1:2	3	6	41
	80Cl3		1:3	3	9	38
	80Cl4		1:4	3	12	35
Cinnamon Bark Oil(CBO)	20 C1	T-20	1:1	3	3	46
	20 C2		1:2	3	6	41
	20 C3		1:3	3	9	38
	20 C4		1:4	3	12	35
	80C1	T-80	1:1	3	3	46
	80C2		1:2	3	6	41
	80C3		1:3	3	9	38
	80C4		1:4	3	12	35

E.O=Essential Oil,N.E= Nanoemulsion,T-20=Tween 20,

T-80= Tween 80

C: Characterization of Essential Oil Nanoemulsion Formulations: Different Parameters were studied for Characterization of Nanoemulsions.

i)Physicochemical Characterization of Nanoemulsions: The pH of formulated Essential oil Nanoemulsions was tested using pH meter (Systronics-361),Visual appearance and %Transmittance was checked with UV-Visible spectrophotometer (Equiptronics) at 600nm.All the parameters were analyzed in triplicates

ii)Heating –Cooling Cycle: Effect of Heating Cooling on stability of nanoemulsion was studied by keeping each of the respective Nanoemulsion formulation at 40ºC and 4ºC alternatively for around 48hrs time.

This parameter is used to study stability of different Essential Nanoemulsion formulations under study at different temperatures.

iii) Thermodynamic Stability: To prove thermodynamic stability all the formulated nanoemulsions were centrifuged at 10000 rpm for 30 minutes and observed for phase separation if any.

D. Antibacterial Activity:

Antibacterial Activity of Essential Oil Nanoemulsions by Agar Well method: Agar Well Diffusion Method suggested in manual On Antimicrobial susceptibility Testing (Ind.Ass.of Microbiologist) was used to study Antibacterial activity of Essential Oil (EO).For this 100µl of 24 hrs. Old nutrient broth culture of *S.aureus*, *E.coli* and *S.typhi* is inoculated on respective sterilized Hi-sensitivity Agar Plates. Broth culture was spreaded uniformly with sterile cotton swab. Wells were made using sterile borer. To each of the well 100 µl of respective Essential oil Nanoemulsion is added using micropipette aseptically. All the plates were then kept in refrigerator for 30 minute so as to facilitate diffusion of nanoemulsion in media along with bacteriostatic action of low temperature in refrigerator. Culture plates with100µl of Tween 20, Tween 80were and Ofloxacin antibiotic disc (Hi-media) were used as negative and positive controls. All the plates were incubated at 37 ºC for 24 hrs.in bacteriological incubator. Next day zone of inhibition is observed around each Nanoemulsion well and measured using Zone size measurement scale. (Hi-media).

E. Measurement of Droplet Size of Nanoemulsions: Essential oil Nanoemulsion formulation from test oil showing maximum zone of inhibition was selected for measurement of droplet size and polydispersibility index. It was determined using Dynamic Light Scattering (DLS) Technique using 90 plus particle size analyzer (ZS, 90 Malvern Instruments, UK). Before analysis each test Essential Oil Nanoemulsion was diluted with Deionized water to lower viscosity and multiple light scattering effects.

F) Bacterial Growth Curve Inhibition study of Nanoemulsion: Bactericidal activity of selected Nanoemulsion formulation giving maximum zone of inhibition in Agar well assay is investigated using *S.aureus, E.coli and S.typhi* inhibition studies. For this assay each bacterial culture is inoculated in 50 ml of sterilized nutrient broth in three separate flasks. Growth is monitored at every 4 hr. using UV –visible spectrophotometer until optical density is read 0.13 at 600nm (OD of 0.13 corresponds to $1*10^8$ CFU/ml bacteria). 1ml of selected Nanoemulsion 20T2, 80Th3, 20C4, 80C4, and 20Cl2 is added to respective 49 ml of Nutrient broth containing 0.1 ml of bacterial culture *S.aureus, E.coli and S.typhi* respectively. Flask containing Nutrient broth and Nanoemulsion, which is lacking bacterial culture is taken as positive control while flask containing nutrient broth and Nanoemulsion and lacking inoculum taken as negative control. All these flasks are kept in rotary shaker incubator at 200 rpm to ensure uniform mixing.

III.RESULTS AND DISCUSSIONS:

i) Physicochemical Characterization: Surfactant concentration found to affect the visual appearance of all test oil nanoemulsions except very low effect on cinnamon oil Nanoemulsions. Nanoemulsions with higher surfactant concentration showed higher % Transmittance. This rise in % Transmission may be due to reduction in droplet diameter with increase in surfactant concentration. (Table II) No noticeable change in pH is observed in all 32 nanoemulsions which mostly varied in range of 6.0 to 6.5

ii) Stability of Nanoemulsion: Increase in surfactant concentration considerably affected the stability of Essential oil nanoemulsions. Stability of Test oil nanoemulsions under study was increased by energy input during ultrasonication. Amongst all eight nanoemulsions of Tea Tree oil 20T2, 20T3, 20T4 was found to be thermodynamically stable when centrifuged at 10000 rpm for 30min. while in case of Thyme oil nanoemulsions, 20Th3,20Th4, &80Th3 exhibited same stability. In Clove oil nanoemulsions 20Cl2 & 80Cl4 Nanoemulsion was found to be thermodynamically stable.(Table II)

20 Th1	Milky White	12	6.0	+	-
20 Th2	Milky White	12	6.1	+	-
20 Th3	Translucent	17	6.0	+	+
20 Th4	Transparent	79	6.3	+	+
80Th1	Milky White	15	6.0	-	-
80Th2	Milky White	12	6.0	-	-
80Th3	Translucent	14	6.0	+	+
80 Th4	Translucent	14	6.0	+	-
20 Cl1	Milky Yellow	14	6.1	-	-
20 Cl2	Milky Yellow	15	6.0	+	+
20 Cl3	Translucent	18	6.0	-	-
20 Cl4	Transparent	85	6.0	-	+
80Cl1	Milky Yellow	12	6.2	-	-
80Cl2	Milky Yellow	14	6.1	-	-
80Cl3	Milky Yellow	13	6.0	-	-
80Cl4	Transparent	85	6.0	+	+
20 C1	Milky	15	6.0	-	-
20 C2	Yellowish White	14	6.0	-	-
20 C3	Yellowish Milky	15	6.0	+	-
20 C4	Off White	14	6.0	+	+
80C1	Milky White	15	6.0	-	-
80C2	Milky White	17	6.0	-	-
80C3	Milky White	14	6.0	-	-

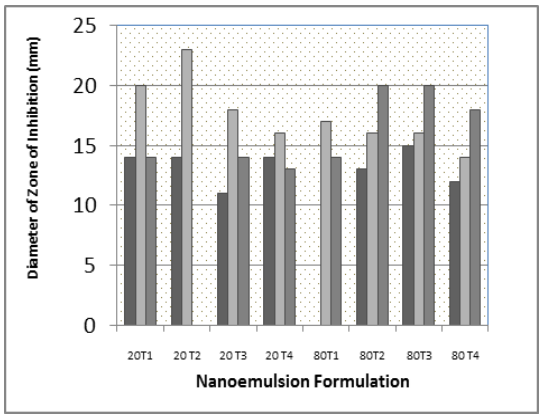
%T= %Transmittance, C- Centrifugation,+=stable, -=unstable

iv) Antibacterial Activity of Nanoemulsion by Agar-Well Diffusion Method: All formulated Essential Oil Nanoemulsion were screened for its Antibacterial Activity against *S.aureus, E.coli & S.typhi*. Agar-well diffusion experiment demonstrated antibacterial activity of all Essential Oil (EO) Nanoemulsions. Table III depicts diameter of zone of inhibition (mm) for each Nanoemulsion against bacteria under study.

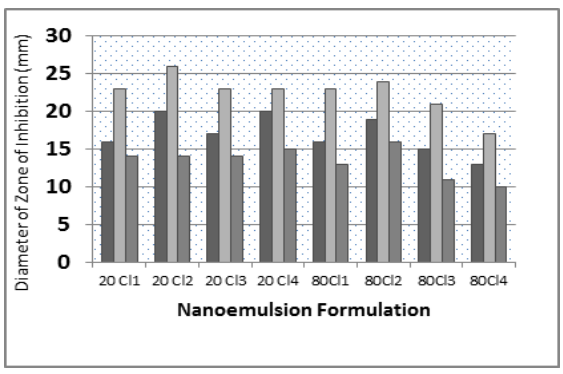
Table III: Antibacterial Activity of Essential Oil Nanoemulsion Formulations.

N.E.	Diameter of Zone of Inhibition in(mm)		
	S.aureus	*E.coli*	*S.typhi*
20T1	14mm	20mm	14mm
20 T2	14mm	23mm	NZ
20 T3	11mm	18mm	14mm
20 T4	14mm	16mm	13mm
80T1	NZ	17mm	14mm
80T2	13mm	16mm	20mm
80T3	15mm	16mm	20mm
80 T4	12mm	14mm	18mm
20 Th1	26mm	14mm	18mm
20 Th2	15mm	13mm	NZ
20 Th3	16mm	NZ	24mm
20 Th4	16mm	NZ	23mm
80Th1	17mm	15mm	36mm
80Th2	NZ	NZ	22mm
80Th3	16mm	22mm	23mm
80 Th4	14mm	15mm	17mm
20 Cl1	16mm	23mm	14mm
20 Cl2	20mm	26mm	14mm
20 Cl3	17mm	23mm	14mm
20 Cl4	20mm	23mm	15mm
80Cl1	16mm	23mm	13mm
80Cl2	19mm	24mm	16mm
80Cl3	15mm	21mm	11mm

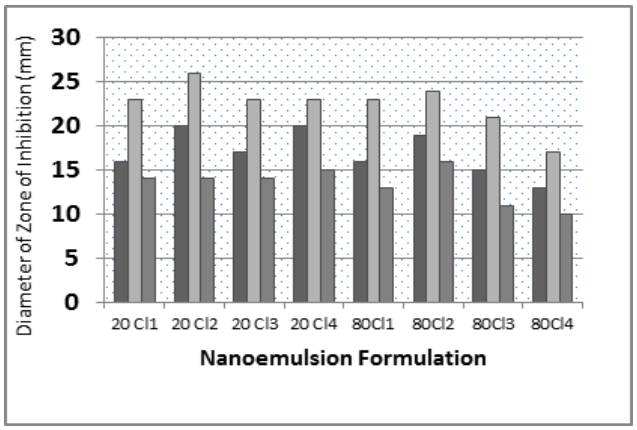
NZ= No Zone



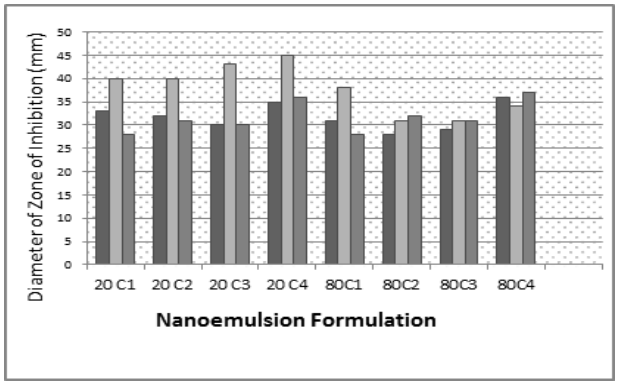
Graph I



Graph II



Graph III



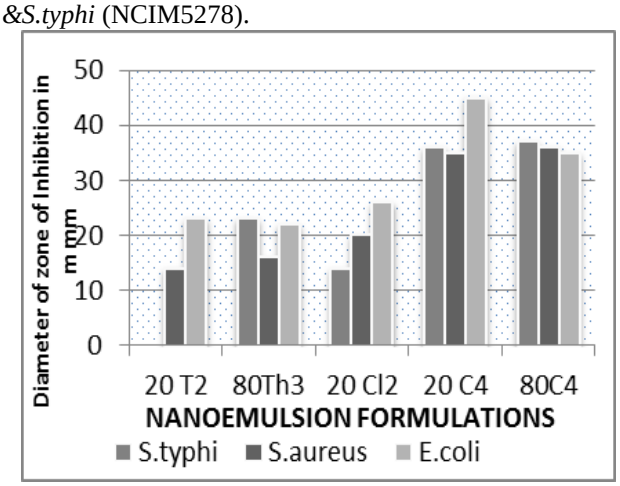
Graph IV

Graph I- Antibacterial activity of Tea Tree Oil Nanoemulsions
Graph II- Antibacterial activity of Thyme Oil Nanoemulsions
Graph III- Antibacterial activity of Clove Oil Nanoemulsions
Graph IV- Antibacterial activity of Cinnamon Oil Nanoemulsions

Graph I, II, III, & IV depicts the antibacterial activity of Tea tree oil, Thyme oil, Clove oil & Cinnamon oil nanoemulsions respectively. In all thermodynamically stable Tea tree oil nanoemulsion (20T2, 20T3, 20T4) formulations, 20T2 was found to demonstrate maximum antibacterial activity.

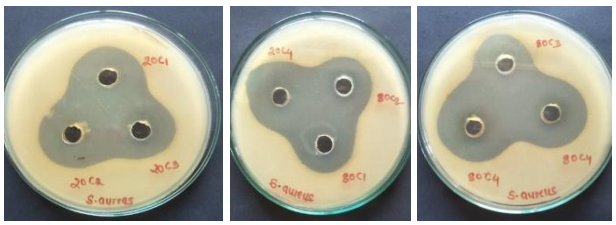
While in case of all stable Thyme oil nanoemulsions (20Th3, 20Th4, &80Th3) 80Th3 demonstrated maximum antibacterial activity against test organisms.While thermodynamically stable Clove oil nanoemulsions (20Cl2 & 80Cl4), 20Cl2 demonstrated maximum antibacterial activity. Thermodynamically stable Cinnamon oil Nanoemulsions 20C4 & 80C4 demonstrated potential antibacterial activity against test organisms, *S.aureus* (ATCC 6538), *E.coli* (ATCC10799) and *Salmonella typhi* (NCIM5278) with zone diameter of 35mm, 45mm &36mm respectively in case of 20C4 and 44mm, 34mm, 37mm in case of 80C4. Graph V depicts Comparative analysis of antibacterial activity of Nanoemulsion formulation showing maximum antibacterial activity in each of oil type.

From Graph Cinnamon oil nanoemulsion with surfactant Tween20, ie20C4 and with Tween 80, i.e. 80C4 demonstrated potential antibacterial activity as compared to 20T2, 80Th3, & 20Cl2 .20T2 formulation showed least antibacterial activity in all five nanoemulsion formulations against. *S.aureus* (ATCC 6538), *E.coli* (ATCC10799) ,&*S.typhi* (NCIM5278).



Graph V- Comparison of Antibacterial activity of Selected Oil Nanoemulsions

The usage of nanoemulsion as an antimicrobial agent is another and promising development. The nanoemulsion combines with lipid-containing microorganisms due to the electrostatic attraction among the droplets of emulsion which possesses a cationic charge and anionic charge on the pathogen surface. [27] When nanoemulsions combine through the microbes, they destabilize the pathogen lipid film, bringing about cell lysis.[28] The nanoemulsion found to inhibit movement of microbes e.g. *Staphylococcus aureus*, *Escherichia coli*.[29] The plant-based oils of clove, and thyme loaded nanoemulsion was showed antimicrobial activity against an extensive variety of gram negative and gram positive microbes.[30]



(a)Antibacterial activity of Cinnamon oil Nanoemulsion on *S.aureus* (ATCC 6538)

(b)Antibacterial activity of Cinnamon oil Nanoemulsion on *E.coli* (ATCC10799)

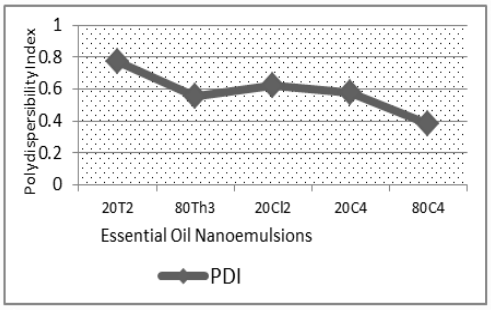
(c)Antibacterial activity of Cinnamon oil Nanoemulsion on *S.typhi* (NCIM5278)

v) Droplet Size of Nanoemulsions:

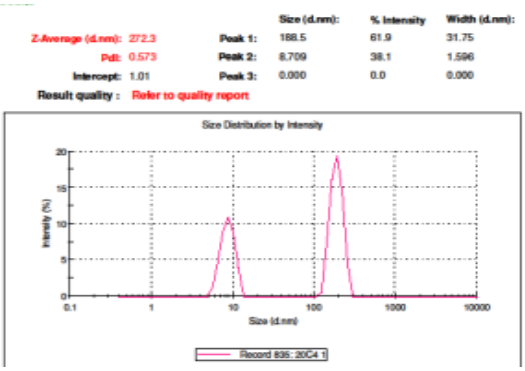
The droplet size and pdi of selected nanoemulsions were analyzed using photon correlation microscopy using Malvern Zetasizer**.** on comparing polydispersibility index(pdi) and droplet size of selected nanoemulsion formulations showing maximum antibacterial activity from each of test oil type, lowest pdi 0f 0.382 was reported in Cinnamon oil Nanoemulsion 80C4 with droplet diameter of 133nm. Followed by 20C4 with pdi of 0.573 and droplet diameter of 272.3nm indicating 80C4 and 20C4 as monodispersed as compared to 20T2, Th3 and 20Cl2.20T2 nanoemulsion showed highest pdi and droplet diameter in all five nanoemulsions selected for analysis which can be directly correlated with least antibacterial activity of this formulation. Table IV shows Droplet size measurement of Test Oil Nanoemulsion. The higher polydispersity index indicates lower uniformity of droplet size of nanoemulsion [31] Polydispersity values near 1.0 are indication of a polydispersed system [32]. All the nanoemulsions had low polydispersity index values which indicate the overall stability and uniformity of the formulation Also potential antibacterial activity of Cinnamon oil nanoemulsion formulations can be correlated with its monodispersed nature and lower droplet diameter along with antibacterial content present in cinnamon oil.

Table IV: Droplet size measurement of Test Oil Nanoemulsion

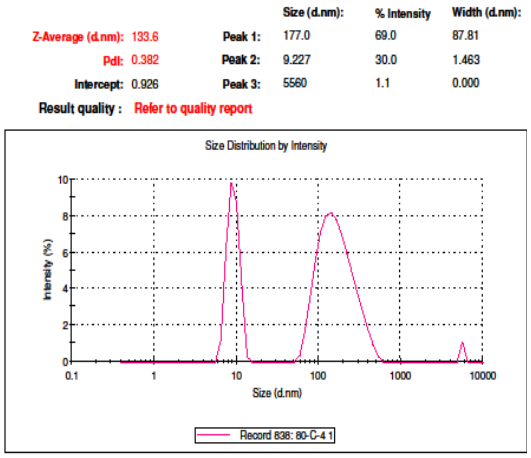
Nanoemulsion code	Pdi Index	Droplet diameter (nm) z-average
20T2	0.774	460.8
80Th3	0.553	285.9
20Cl2	0.622	303.3
20C4	0.573	272.3
80C4	0.382	133



Graph VI: PDI of Nanoemulsions



(a)



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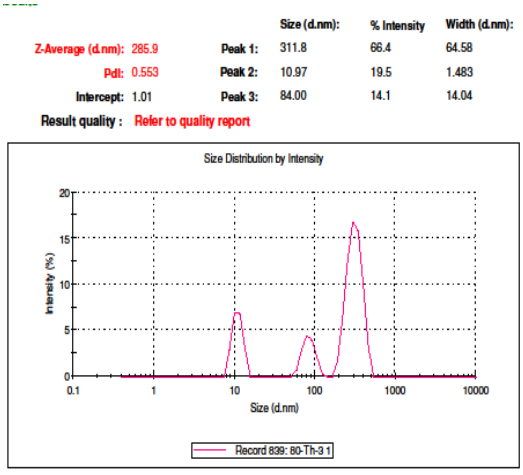
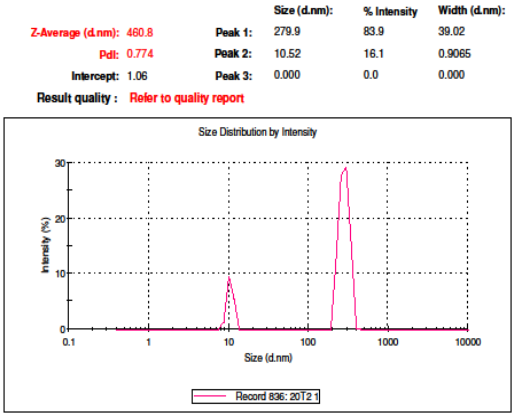


(c)

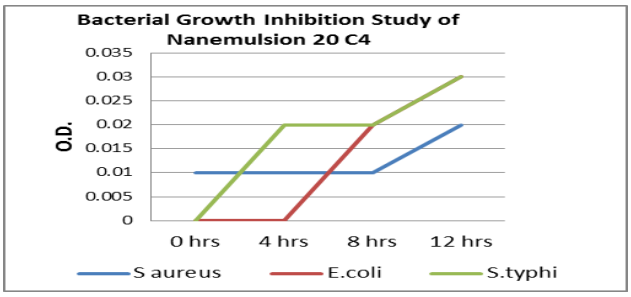


(d)

Graph VII:Droplet Size report of Essential Oil Nanoemulsions-(a) 20C4 (b) 80C4 (c) 80 Th3 (d) 20T2 USING Malvern Zetasizer.UK

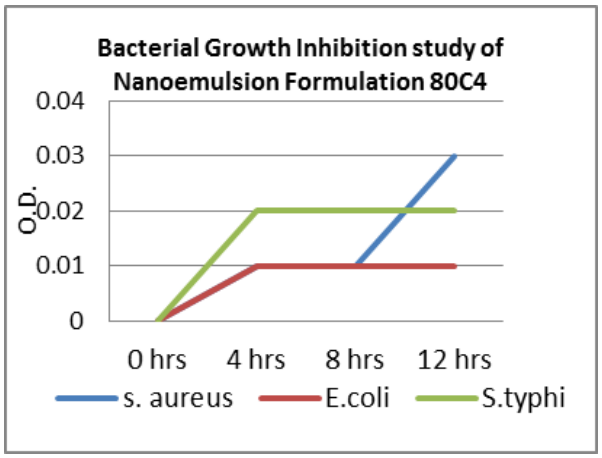
vi) Bacterial Growth Curve Inhibition study of Nanoemulsions: From the bacterial growth inhibition study of 20C4, it is observed that, *S.aureus* is inhibited strongly and optical density of broth does not rise in logarithmic manner and is restricted to 0.01 till 12 hrs. After 12 hr. Only slight rise of 0.02 OD is seen . *E.coli* is also found to be inhibited on exposure to Nanoemulsion 20C4 as O.D. remains stationary to 0.02 at the end of 8 hrs. Only rise of 0.01 is seen at the end of 12hrs .*Salmonella typhi* is also found to get inhibited by 20C4 nanoemulsion with only rise of 0.01 OD after 4 hrs. And then after growth is found to be restricted to 0.02.From all the above readings and observations, it can be concluded that there is rapid rise in optical density of nanoemulsion exposed nutrient broth containing bacterial inoculum. The inhibition of bacterial inoculum. The inhibition of bacterial growth may be due to killing action of 20C4 to initial bacterial inoculum added to nutrient broth.

On exposure to 80C4 nanoemulsion, the growth of *S.aureus* is found to be restricted as there is no rise in the optical density of nutrient broth containing 100µl of nutrient broth beyond 0.01 at the end of 8hrs. The rise of only 0.01 OD is seen at the end of 12 hrs. This proves that 80C4 has restricted the growth and multiplication of *E.coli*. *S.typhi* is also inhibited by 80C4 and growth is seen restricted to 0.02 till 12 hrs. Thus it can be concluded that the reason for restricted rise in OD is due to killing action of 80C4 nanoemulsion formulation on initial bacterial inoculum exposed to it.



Graph VIII Bacterial growth inhibition Study of Cinnamon oil Nanoemulsion 20C4

On exposure to 80C4 nanoemulsion, the growth of S.aureus is found to be restricted as there is no rise in the optical density of nutrient broth containing 100µl of nutrient broth beyond 0.01 at the end of 8hrs. The rise of only 0.01 OD is seen at the end of 12 hrs. This proves that 80C4 has restricted the growth and multiplication of *E.coli. S.typhi* is also inhibited by 80C4 and growth is seen restricted to 0.02 till 12 hrs. Thus it can be concluded that the reason for restricted rise in OD is due to killing action of 80C4 nanoemulsion formulation on initial bacterial inoculum exposed to it. Comparison of antibacterial activity of all 32 nanoemulsion formed using Tea tree, Thyme, Clove leaf & Cinnamon Essential Oil demonstrated maximum antibacterial activity in stable Nanoemulsions 20T2, 80Th3, 20 Cl2, 20C4, and 80C4 respectively in each of oil type.



Graph IX Bacterial growth inhibition Study of Cinnamon oil Nanoemulsion 80C4

Comparison of diameter of zone of inhibition obtained in these nanoemulsion formulations demonstrated maximum antibacterial activity in Cinnamon oil Nanoemulsion formulation 80C4 with zone diameter of 44mm ,34mm, and 37mm followed by 20C4 of 35 mm, 45mm,and 36mm against standard test cultures *S.aureus, E.coli & S typhi* respectively. Nanoemulsion formulations 20T2,80 Th3, and 20Cl2 was also found to exhibit antibacterial effect in Agar well Diffusion experiment but not to the extent of that of Cinnamon oil Nanoemulsion formulations.(Table III). From the bacterial growth inhibition study of 20C4, it is observed that, *S.aureus* is inhibited strongly and optical density restricted to 0.01 till 12 hrs. After 12 hrs. Only slight rise of 0.02 OD is seen .E *coli* is also found to be inhibited as OD remains stationary to 0.02. Only rise of 0.01 is seen at the end of 12hrs.*Salmonella typhi* is also found to be inhibited by 20C4 nanoemulsion with only rise of 0.01 OD after 4 hrs. Growth is found to be restricted to 0.02., it can be concluded that there is no rapid rise in optical density of nutrient broth containing bacterial inoculum exposed to Cinnamon oil nanoemulsion 20 C4 and 80 C4 respectively.. The inhibition of bacterial growth may be due to killing action of tested Cinnamon oil nanoemulsions to initial bacterial inoculum added to nutrient broth. The results obtained can be directly co-related with data obtained from polydispersibility index (pdi) and droplet size measurement studies. (Graph VII). Lowest pdi 0f 0.382 was reported in Cinnamon oil Nanoemulsion 80C4 with droplet diameter of 133nm, followed by 20C4 with pdi of 0.573 and droplet diameter of 272.3nm indicating 80C4 and 20C4 as monodispersed as compared to 20T2, 80 Th3 and 20Cl2. Also potential antibacterial activity of Cinnamon oil nanoemulsion formulations can be correlated with its monodispersed nature and lower droplet diameter along with antibacterial substance content present in Cinnamon Essential Oil. Cinnamon oil nanoemulsion demonstrated higher antimicrobial activity against four tested bacterial cultures- *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and *Staphylococcus aureus*, even at far lower concentrations.[33]

IV. CONCLUSIONS

All plant essential oil demonstrated antibacterial activity in Agar-well diffusion method but Cinnamon oil based Stable nanoemulsions- 20C4 & 80C4 with droplet diameter in 272.3 nm and 133.6 nm demonstrated maximum diameter of zone of inhibition, indicating potential bactericidal activity against *S.aureus* and *E.coli and S typhi.* This study illustrates that surfactant type, oil: surfactant ratio had significant effect on droplet diameter and polydispersibility index of nanoemulsions along with antibacterial substance present in oil. Moreover Cinnamon oil nanoemulsion can be as used as substitute for chemical antimicrobial compounds in hand washes, face washes, mouthwash as surface sterilizers, for sterilization of catheters etc. Monodispersed nanoemulsion formulations with lower droplet diameter tend to be more antibacterial as compared to polydispersed nanoemulsion with higher droplet diameter Thus Essential oils Nanoemulsions are one of the innovative antibacterial formulations

ACKNOWLEDGMENTS:

We deeply acknowledge R.C.Patel college of Pharmacy, Shirpur, Maharashtra for providing consultancy in droplet size measurement & pdi studies.

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