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FORMULATION, CHARACTERIZATION AND EVALUATION OF METHANOLIC EXTRACT OF *ABUTILON INDICUM* LOADED SOLID LIPID NANOPARTICLES AGAINST MICROORGANISMS CAUSING DIABETIC FOOT AND URINARY TRACT INFECTION

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ABSTRACT

During ancient days it was believed that the herbal remedies are more acceptable and preferable ones in the treatment of diverse sorts of ailments, bacterial and fungal infections, because it was believed that natural remedies are most safer with lesser side effects when compared to synthetic medicines. So the demand for the herbal based formulations has been growing day by day in the world trade market. The main drawbacks of present antibiotic therapies to treat bacterial infections include development of antibiotic resistance due to the indiscriminate use of antimicrobial drugs. Thus herbal extracts might be used as an alternative medicines to reduce the prevalence of pathogens causing diabetic foot and urinary tract infection in human beings. The present study deals with the formulation and evaluation of antimicrobial activity of methanolic extract of *Abutilon indicum* (MEAI) and MEAI loaded solid lipid nanoparticles(SLN's) against wide range of Gram-positive and Gram-negative pathogens that can cause diabetic foot and urinary tract infection. Since this plant is very well known in the traditional system of medicine and reported to possess antidiabetic, antimicrobial, antioxidant, anti inflammatory, wound healing and antifungal activity. Since the plant is dynamic against extensive variety of microorganisms an endeavor has been made to explore the plant against microorganisms bringing on diabetic foot and urinary tract infection. The prepared MEAI and MEAI loaded SLN's were effectively tested for the antimicrobial activity against microorganisms which are responsible for diabetic foot and urinary tract infection such as, *Staphylococcus aureus, Staphylococcus epidermidis, Bacillius subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Proteus mirabilis, Streptococcus pyogenus, Enterobacter, Candida albicans, Aspergillus niger, Streptococcus faecalis* by using modified agar well diffusion method and compared with standard antibiotic. The prepared methanolic extract of *Abutilon indicum* and solid lipid nanoparticles showed the zone of inhibition indicating that the plant used in the formulation was effective in fighting against the wide range of microorganisms due to the vicinity of tannins and also it could be a better alternative to the modern medicine. The present study is particularly aimed to develop to increase the bioavailability and site specificity of the formulation in the treatment of diabetic foot and urinary tract infection.

Keywords: Diabetic foot and urinary tract infection, methanolic extract of *Abutilon indicum*, solid lipid nanoparticles, antimicrobial activity, modified agar well diffusion method.

1. INTRODUCTION

Solid Lipid Nanoparticle (SLN) [**1**] dispersions have been proposed as a innovative type of colloidal drug carrier system which comprising of spherical solid lipid particles in the nanometer ranges, which are dispersed in water or in aqueous surfactant solution. Lipid nanoparticles conquer the membrane stability and drug-leaching problems associated with liposomes and emulsions [**2,3]** and the biodegradation and toxicity problems of polymeric nanoparticles**[7]**, and facilitate prolonged drug release[**4**]. Lipid nanoparticles are prepared from biocompatible lipids and possess excellent biodegradability and lower toxicity [**5**].

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Probably the herbal based drug industry in India is the oldest medical health care system in the world. Herbal medicines although would appear new source for western healers and as well as medical practitioners, the fact is that most of prescribed medicines even today contain herbal extracts.

Medicinal herbs are cheap, safe and also renewable sources which consist of pharmacologically active constituents and are known to produce certain chemical compounds that are naturally toxic to wide range of bacteria and fungi **[6].** Alternative Systems of Medicine namely Ayurveda, Siddha, Unani and Traditional Chinese Medicine have become more popularize in recent years.

The diabetic foot syndrome is one of the most important diabetic complications and it is said to be most important cause among diabetic patients during hospitalization [**7].** It was found that the most frequent etiological microorganism responsible for diabetic foot infection is Staphylococci **[8, 9, 10].** Infections of the foot

are very common in people who are enduring with diabetes mellitus. Most of the diabetic foot infections occur in a foot ulcer, which plays a significant role in the worsening of the ulcers and can ultimately leads to the formation of gangrenous wounds **[11].** If this infection is untreated, it can spread to underlying tissues, including bone also. The diabetic foot infection is the major clinical consequence leading to lower extremity amputation, and also about 60 percent of all amputations in developed countries **[12].**

From the bacteriological studies important investigations says that the diabetic foot infections were complex and also polymicrobial in nature. Diabetic foot ulcer is a result of repeated aerobic, anaerobic or fungi infections either alone or in combination **[13].** Aerobic microorganisms (Gram+ve) such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are seen as pathogens in diabetic wounds **[14].** Aerobic microorganisms (Gram–ve) such as the *Citrobacter sp., Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), Acinetobacter* and *Serratia sp.* are often cultured from a diabetic wound**[15].** Most predominant Gram - positive anaerobes that are isolated from diabetic wounds include the *Peptostreptococcus* species, *Clostridium* species along with other *Propionibacterium* species**[16].** The most common Gram – negative anaerobes such as *Bacteroides fragilis*, *Fusobacterium* species, *Prevotella* species and *Veillonella* species are isolated from diabetic wounds **[17].** Clinically most common significant fungi cultured from Diabetic Foot ulcers is *Candida* spp**[18].**The most common *Candida* species isolated from Diabetic Foot ulcers include *Candida albicans, Candida tropicalis, Candida guilliermondii and Candida pseudotropicalis***[19].** Microbial Communities of cells growing on a surface and embedded in a self-synthesized matrix composed of extracellular polymeric substances leads to biofilm formation**[20].** Most predominant clinically significant fungi cultured from DF ulcers is *Candida* spp.

Urinary tract infection (UTI) is a infection of bacteria that can affects any part of the urinary tract. When it affects the lower urinary tract it is called as a simple cystitis (a bladder infection) and when it affects the upper urinary tract it is called as pyelonephritis (a kidney infection). Urinary Tract Infections are among the most common infections of the bacteria which are most prevalent extra intestinal and affecting the people of all group ages from neonates to old age **[21].** Approximately 150 million individuals are diagnosed with UTI in every year globally. It is estimated that about 35% of healthy females suffer with UTI infection at some stage during their life. About 5% of women in each and every year suffer with the problem of painful urination (dysuria) and urinary frequency. The incidence of UTI is greater in female than in male, which may be either due to the anatomical predisposition or urothelial mucosal adherence to the mucopolysaccharide lining or other host factors **[22].**

The most common cause of UTI is Gram negative bacteria that belong to the family Enterobacteriaceae. Members of this family include *E.coli*, *Klebsiella, Enterobacter* and *Proteus*. Also Gram positive *Staphylococcus* sp. plays a role in the infection **[23].** *E.coli* is one of the most common bacteria capable of causing infection in humans, particularly urinary tract infections **[24].** Nowadays, drug resistance is a big growing problem in treatment of different types of infectious diseases like tuberculosis, malaria, diarrhea, urinary tract infections etc. According to the Goldman and Huskins **[25]** the improper and uncontrolled usage of various antibiotics resulted in the resistance to microorganisms, which became a major health problem throughout the world.

2. MATERIALS AND METHODS

2.1 Plant material:

The leaves of *Abutilon indicum* (PARC/2014/2287) were collected from in and around Tirupathi, India in the month of March 2014 and it was identified and authenticated. The taxonomical identification and authentication of the plant was done by Dr. P. Jayaraman, Director, National Institute of Herbal Medicine, Plant Anatomy Research Centre, Chennai. The voucher specimen is preserved in laboratory, Department of Pharmacognosy, Sree Vidyanikethan College of Pharmacy for further reference.

2.2 Extraction of *Abutilon indicum* leaves:

The leaves of *Abutilon indicum* (Malvaceae) were collected, washed and shade dried. After complete drying, they were powdered and passed through a 60 mesh sieve and stored in air tight container. Dried powdered drugs were used to prepare extract. About 50 g of shade dried powder of plant material was extracted with methanol by using soxhlet extraction technique. The solvent was evaporated and immediately the accurate weight of the extract was noted. Extractive value was calculated with reference to the air dried based drug.

2.3 Microorganisms:

The microorganisms used in this study were *Staphylococcus aureus, Staphylococcus epidermidis, Bacillius subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Proteus mirabilis, Streptococcus pyogenus, Enterobacter, Candida albicans, Aspergillus niger and Streptococcus faecalis.*

2.4 Preliminary Phytochemical Screening of methanolic extract of *Abutilon indicum* (MEAI)

The Preliminary phytochemical studies were conducted for the methanolic extract of *Abutilon indicum* leaves to find out the presence of various phytoconstituents like carbohydrates, alkaloids, glycosides, flavonoids, phenolic compounds, steroids, triterpenoids and tannins as per the standard procedures **[26].**

3. Preparation of *Abutilon indicum* loaded Solid lipid nanoparticles

The methanolic extract of *Abutilon indicum* solid lipid nanoparticles (SLN's) were prepared by using

Ultrasonic homogenization technique **[27]**. Stearic acid, Soya lecithin and MEAI were mixed in ethanol as organic solvent and then this mixture was allowed to heat for 70^0C. 2% Tween 80 was dissolved in Phosphate buffer (P^H 7.4) and then this mixture was heated upto 70^0c. Finally the aqueous phase was added to the organic phase drop wise during the process of homogenization (20000 rpm for 20 min).This whole mixture was then sonicated for 20 min to obtain Solid lipid nanoparticles. The SLN formulations and compositions are shown in the table 1.

Table 1: Formulation design of SLN'S

A-SLN1		**A-SLN2**	
MEAI : SA : SL **(1:4:1)**		MEAI : SA : SL **(5:4:2)**	
Composition	**Quantity**	**Composition**	**Quantity**
MEAI leaves	100mg	MEAI leaves	500mg
Stearic acid	400mg	Stearic acid	400mg
Soyalecithin	100mg	Soyalecithin	200mg
Ethanol	25ml	Ethanol	25ml
Tween 80 (2%)	30ml	Tween 80 (2%)	30ml
Phosphate Buffer (P^H 7.4)	45ml	Phosphate Buffer (P^H 7.4)	45ml

MEAI - Methanolic extract of *Abutilon indicum* leaves
SA - Stearic acid
SL - Soya lecithin

4. Characterization of solid lipid nanoparticles

4.1 Determination of P^H

The P^H was determined by using digital P^H meter. 50ml of herbal formulation was taken in beaker. Then the bulb of the P^H meter was dipped into the formulation and the P^H was measured precisely [**28]**

4.2 Determination of Viscosity

The viscosity of herbal formulation was determined by using digital Brookfield viscometer. 150ml of poly herbal formulation is taken into 250ml of beaker and then the tip of viscometer was dipped into the beaker containing formulation and its viscosity was measured. Viscosity was measured to ensure the better delivery of the formulation **[29]**.

4.3 Determination of Refractive Index

Refractive index of selected formulations was determined using an Abbe type refractometer.

4.5 Measurement of particle size, polydispersity index and zeta potential

Particle size distribution of *Abutilon indicum* loaded Solid lipid nanoparticles A-SLN1 and A-SLN2 was determined by laser scanning technique by using Nanopartica SZ-100 (Horiba company, Japan) instrument after appropriate dilution with double distilled water. The mean particle size, polydispersity index and zeta potential were calculated for A-SLN1 and A-SLN2 formulations maintained at 25^0C and polydispersity index used to measure the size distribution population of nanoparticles **[30]** and the results were shown in the figures 2-5.

4.6 Scanning Electron Microscopy

The SEM analysis was carried out using a scanning electron microscope (LEO, 435 VP, U.K). Prior to examination, samples were mounted on an aluminium stub using a double sided adhesive tape and making it electrically conductive by coating with a thin layer of gold (approximately 20 nm) in vacuum. The scanning electron microscope was operated at an acceleration voltage of 5 KV and resolution of 4000 **[31]**.

4.7 Fourier Transform Infrared (FTIR) spectroscopic analysis

The FTIR analysis of MEAI and MEAI loaded SLN's were carried out by using Agilent Resolution Pro instrument(US). The FTIR spectra of MEAI and MEAI loaded SLN's were recorded using FTIR spectrophotometer in the range of 4000-650 cm^{-1} **[32]**.

5. Determination of antimicrobial activity

The screening of Anti-microbial efficacy of the MEAI was performed on various micro organisms by using Agar well diffusion method **(33)**. The agar plates were prepared by pouring 20 mL of sterile molten Mueller-Hinton (MH) agar (Himedia Lab Pvt. Ltd, Mumbai, India). The bacterial cultures were prepared by adding the seed culture in the autoclaved agar medium followed by pouring into petri plates. The solid agar medium was gently punctured with the aid of 8mm sterile cork borer to make a proper well. 50µl of MEAI (50mg/ml) and MEAI loaded SLN's (500mg/ml) were added in the pre labelled wells together with reference antibiotic i.e. streptomycin. Here the MEAI served as test and streptomycin served as standard. The reference antibiotic was used in the concentration range of 100µg/ml. It was taken care that the sample should be placed at the level of cavity. The diffusion of extract was allowed for 1hr at room temperature on a sterile bench. Then the Petri plates were incubated for 48 hrs at 37^0 C. After 48 hrs the plates were observed for the presence of inhibition of bacterial growth and that was indicated by clear zone of inhibition of bacterial growth around the wells. The size of inhibitory zone was measured in mille meters (mm). Minimum Inhibitory Concentration (MIC) was determined **(34).**

6. RESULTS

6.1 Preliminary Phytochemical screening

Table 2: Preliminary Phytochemical Analysis of Methanolic extract of *Abutilon- indicum* leaves

S. No.	**Phytoconstituents**	**MEAI**
1	Alkaloids	+
2	Aminoacids	_
3	Glycosides	+
4	Carbohydrates	+
5	Flavonoids	+
6	Phenolic groups	+
7	Resins/gums	+
8	Saponins	+
9	Steroids	+
10	Tannins	+
11	Terpenoids	+

MEAI indicates Methanolic extract of *Abutilon indicum*

"+" indicates Present
"-" indicates Absent

6.2 Determination of P^H, Viscosity, and Refractive index (R.I) of A-SLN1 and A-SLN2

PH and viscosities of the formulation are in same range without any much deviation, PH of the herbal formulations are found to be nearly neutral i.e. 7. The viscosities of the prepared formulations are found in Brookfield viscometer and are in optimum range ≥ 210 mpa. The refractive indeces of the formulations are nearly same without much deviation. The results were shown in the table 3.

Table 3: P^H, Viscosity, and Refractive index (R.I) of A-SLN1 and A-SLN2

S. No	Formulation	PH	Viscosity (mpa)	R.I
1.	A-SLN1	6.96	210.68	1.406
2.	A-SLN2	7.12	239.01	1.598

6.3 Scanning electron microscopy(SEM)

The SEM photograph (Figure 1) of optimised formulation reveals that A-SLN2 was spherical and moderately uniform in size.

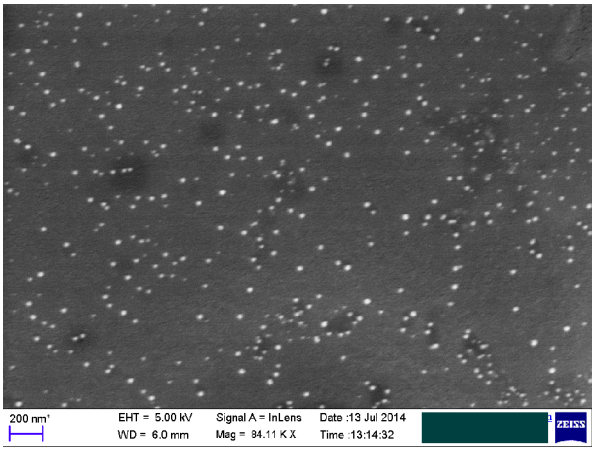

Fig 1: SEM analysis of Solid lipid nanoparticles

6.4 Particle size determination

The measurement of zeta potential allows for prediction about the storage stability of colloidal particles, as the particle aggregation will be less to the charged particles. For the prepared SLNs the Zeta Potential (mV): particle size and polydispersity index are tabulated in Table 3.

Table 3: Zeta potential, particle size and polydispersity index of SLN Formulations.

S. No	Formulation	Size average (nm)	Polydispersity index (PI)	Zeta potential
1.	A-SLN1	169	2.021	-9.1Mv
2.	A-SLN2	293	1.861	-10Mv

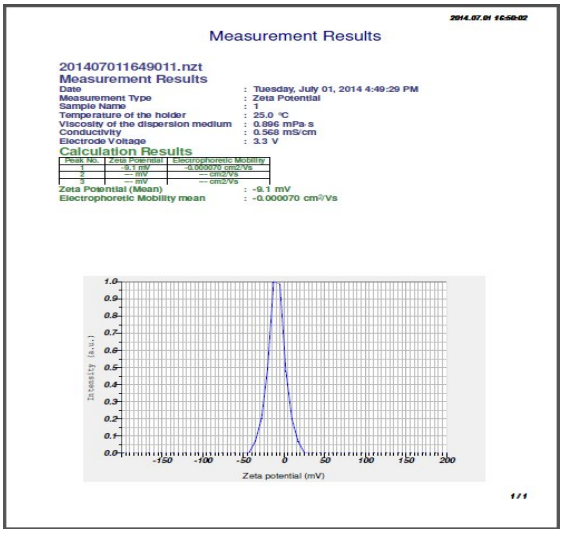

Fig 2: Zetapotential for A-SLN2

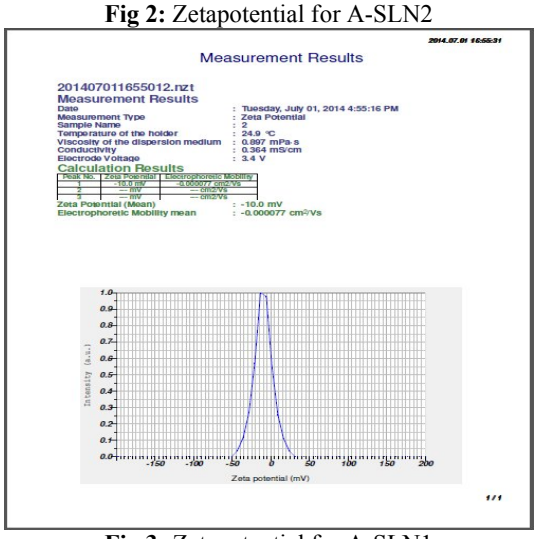

Fig 3: Zetapotential for A-SLN1

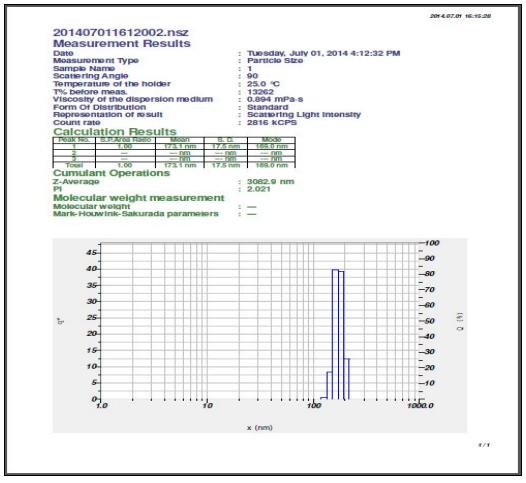

Fig 4: Particle size and PI of A-SLN2

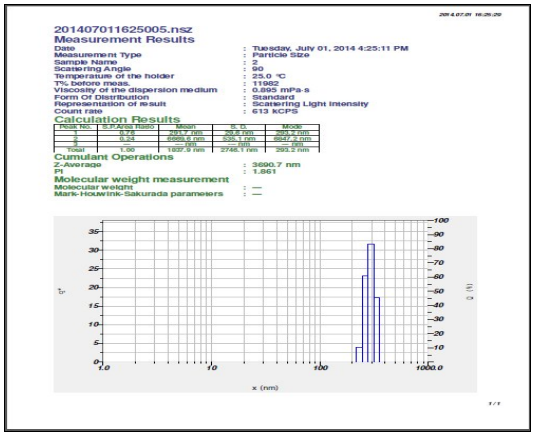

Fig 5: Particle size and PI of A-SLN1

6.5 FT-IR Analysis

The FT-IR spectroscopy used to investigate the interactions between lipid, extract and other excipients. From the FT-IR spectra of MEAI, optimised formulation of SLN it is confirmed that there is decrease in intensity of peak at 3333 cm^{-1} corresponding to OH group and 2972 corresponding to CH stretching compared to FT-IR spectra of MEAI was observed. Absence of strong peak at 1616 cm^{-1} and dual peaks at 1394, 1334 cm^{-1} indicates COOH group present in MEAI was involved in hydrogen bonding. These predictions ensures that the interactions between lipid molecules of SLN with the MEAI. The FTIR spectra of MEAI (Fig 6) and MEAI loaded SLN (Fig 7) were recorded using FTIR spectrophotometer in the range of 4000-650 cm^{-1} and the interpretation results were shown in the table 4.

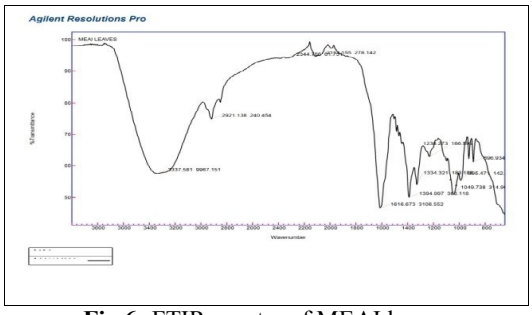

Fig 6: FTIR spectra of MEAI leaves

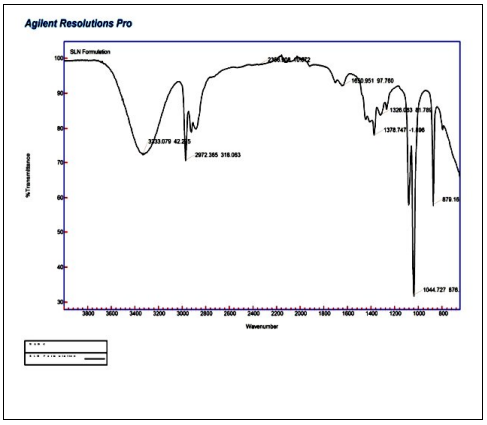

Fig 7: FTIR spectra of SLN formulation

Table 4: FTIR interpretation of MEAI and SLN

S. No	Functional group	MEAI Leaves	SLN formulation
1	O-H stretch	3337 cm^{-1}	3333 cm^{-1}
2	C-H stretch	2921 cm^{-1}	2972 cm^{-1}
3	C≡N	2334 cm^{-1}	2356 cm^{-1}
4	C=O stretch	1616 cm^{-1}	1650 cm^{-1}
5	C-O stretch	1397 cm^{-1}	1326 cm^{-1}
6	C-H def(aromatic)	896 cm^{-1}	879 cm^{-1}

Antimicrobial activity

The results of modified agar well diffusion method (Table 5) showed that the prepared MEAI and MEAI loaded SLN's having inhibitory effect on the microorganisms which are responsible for the diabetic foot and urinary tract infection. The anti-microbial activity of MEAI and MEAI loaded SLN's have been comparable to that of marketed antibiotic (Streptomycin). The diameter of zones of inhibitions were given in the table no.5 and the zones of inhibition by MEAI and MEAI loaded SLN's against the microorganisms responsible for diabetic foot and urinary tract infection such as *Staphylococcus aureus, Escherichia coli, Bacillius subtilis, Enterobacter, Proteus mirabilis, Proteus vulgaris, Staphylococcus epidermidis, Pseudomonas aeruginosa, Enterobacter, Streptococcus pyogenus, Klebsiella pneumonia, Candida albicans, Aspergillus niger, Streptococcus faecalis* were showed in the fig numbers 8,9,10,11,12,13,14,15,17,18,19,20 respectively.

Table 5: Anti-microbial sensitivity result of the MEAI leaves and SLN's

Microorganisms	Zone of inhibition (mm)			
	MEAI	Streptomycin	SLN's	Streptomycin
Staphylococcus aureus	24±0.57	22.66±0.33	18.33±0.33	18.66±0.33
Escherichia coli	22±0.57	24.33±0.33	24.33±0.33	21.66±0.33
Bacillius subtilis	15.33±0.88	18.66±0.66	24.66±0.33	24.33±0.66
Proteus mirabilis	19±0.57	20.33±0.33	19±0.57	21.33±0.88
Proteus vulgaris	24.33±0.33	24.33±0.33	15.33±0.66	16.33±0.33
Staphylococcus epidermidis	24.66±0.33	26.33±0.33	21±0.57	23.66±0.33
Pseudomonas aeruginosa	20.66±0.88	23.66±0.33	23.66±1.0	24.33±0.33
Enterobacter	23.66±0.33	25.33±0.33	22±0.57	23.66±0.33
Streptococcus pyogenus	25.66±0.33	25.33±0.33	18.33±0.33	19.66±0.33
Klebsiella pneumonia	23±0.57	24.33±0.57	22±0.57	21±0.57
Candida albicans	21±0.57	23±0.57	23.33±0.33	26.66±0.33
Aspergillus niger	18.33±0.33	20.66±1.20	25.33±0.33	26.66±0.33
Streptococcus faecalis	20.66±0.33	20.66±0.33	21.66±0.33	22.33±0.33

MEAI : Methanolic Extract of *Abutilon indicum,* ± **:** Mean standard deviation of three replicates

Petriplates showing the zone of inhibition against the microorganisms responsible for Diabetic foot and Urinary tract infection

MEAI leaves SLN's

Fig 8: Extract and SLN's against *Staphylococcus aureus*

Fig 9: Extract and SLN's against *Escherichia coli*

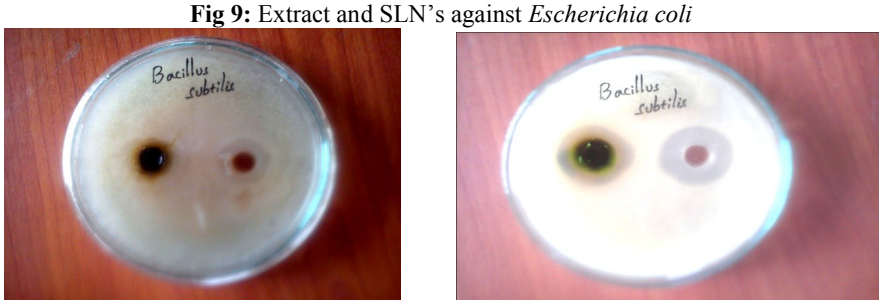

Fig 10: Extract and SLN's against *Bacillus subtilis*

Fig 11: Extract and SLN's against *Proteus mirabilis*

Fig 12: Extract and SLN's against *Proteus vulgaris*

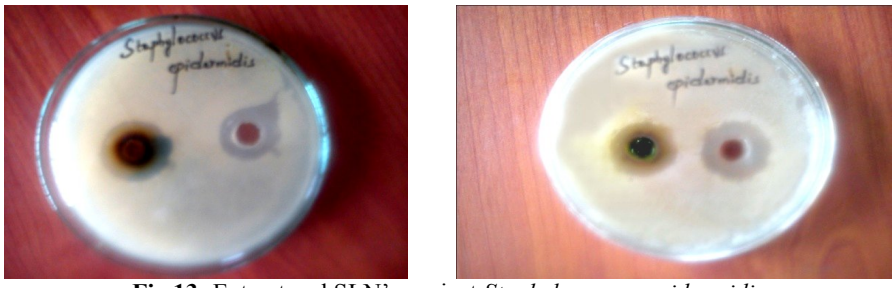

Fig 13: Extract and SLN's against *Staphylococcus epidermidis*

Fig 14: Extract and SLN's against *Pseudomonas aeruginosa*

Fig 15: Extract and SLN's against *Enterobacter*

Fig 16: Extract and SLN's against *Streptococcus pyogenus*

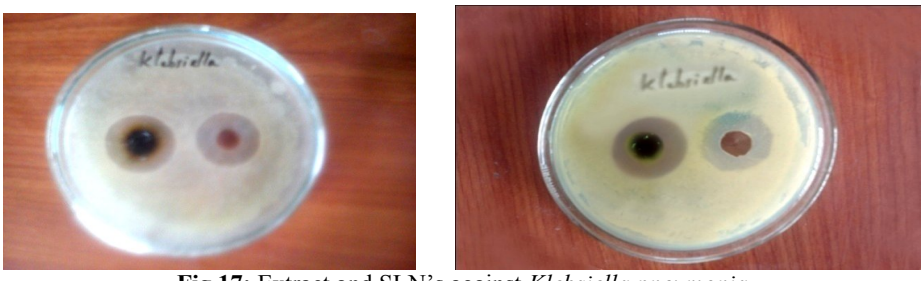

Fig 17: Extract and SLN's against *Klebsiella pneumonia*

Fig 18: Extract and SLN's against *Candida albicans*

Fig 19: Extract and SLN's against *Aspergillus niger*

Fig 20: Extract and SLN's against *Streptococcus faecalis*

5. DISCUSSION

Solid lipids could be effectively utilized for a drug to prolong the release and to eliminate the systemic toxic effects due to their controlled release and targeting properties. Stearic acid, a saturated monoacid triglyceride was used as a solid matrix in which the drug was incorporated. Soya lecithin, a phospholipid was then coated on the surface of the lipid matrix which was used as a surfactant. Tween80 (1% and 2%) was used as a co-surfactant and also stabilizing agent. It has been reported that the size of the SLNs decreases as the ratio of phospholipids to stearic acid increases**[35].** In order to understand this effect, two formulations of SLNs with different ratios of MEAI**:** SA**:** SL (Methanolic extract of *Abutilon indicum* **:** Stearic acid **:** Soya lecithin), i.e., 1**:**4**:**1, and 5**:**4**:**2 were prepared to achieve minute particles size, which are hereafter referred as A-SLN1, A-SLN2 respectively. The details of composition of two SLN formulations have been given in Table 2 and the results of particle sizes obtained have been expressed in Table 3. The decrease in particle size during nanoparticles formation is associated with an increase in surface area. The excessive surfactant molecules present rapidly cover the new surfaces, which reduce the surface tension and thereby facilitate the particle partition during emulsification **[36].** Hence, an increase in the concentration of surfactant results in decrease in particle size of the SLN. The concentration of soya lecithin was slightly increased in A-SLN 2 than in A-SLN 1.

Herbal remedies are more acceptable and preferable ones in the treatment of different types of diseases, bacterial and fungal infections. There are many medicinal plant extracts have been known to possess the antimicrobial activity and are used for the treatment of microbial infections due to the presence of certain active chemical constituents like tannins etc which are effectively fight against wide range of both Gram-positive and Gram-negative microbes.

The main drawback of current antibiotic therapies to treat bacterial infections include development of antibiotic resistance due to the indiscriminate use of antimicrobial drugs. Thus herbal extracts might be used as an alternative medicines to reduce the prevalence of pathogens causing diabetic foot and urinary tract infection in human beings. Natural herbal remedies are considered as most safer than the allopathic drugs due to the lesser side effects like local irritation, contact dermatitis, photosensitivity, itching, pruritus skin peeling, redness of the skin etc. The preliminary phytochemical investigation revealed the presence of various chemical constituents in the MEAI was reported in the Table 1.

6. CONCLUSION

Methanolic extract of *Abutilon indicum* extract was found to be successfully loaded to stearic acid-SLN by a hot homogenization followed by ultrasonication method. A-SLN's exhibited enhanced antibacterial effect compared to MEAI against all tested microorganisms in-vitro. The formulated A-SLN's possess spherical shape and moderately uniform in size. The nanoparticle system would be an effective carrier for oral delivery of *Abutilon indicum.*

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