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IN- VITRO EVALUATION OF NANOCURCUMIN AGAINST MULTI-DRUG RESISTANT BACTERIA

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A natural substance called curcumin, derived from the Curcuma longa plant, is frequently used to fight both gram-positive and gram-negative bacteria.It is poorly soluble in water so it has low bioavailability. The aim of this work was to develop effective nanocurcumin formulation to enhance its water solubility and antibacterial activity against four types of multidrug resistant strains. Nanocurcumin was formulated with different combined stabilizers employing antisolvent nanoprecipitation approach and then evaluated in terms of particle size, zetapotential and transmission electron microscopy. The optimized nanocurcumin formulation containing and Polyvinylpyrrolidone K30 (PVP) and Polyethylene glycol 6000(PEG6000) was selected for the In-vitro antibacterial studies against Staphylococcus aureus ATCC® 43300™, Escherichia coli ATCC® 8739™*, Pseudomonas aeruginosa ATCC® 27853™* and Klebsiella pneumoniae ATCC® 33495™* compared with free curcumin. The prepared nanocurcumin formulation showed mean particle size of 272.15 ± 34.5 nm and polydispersity index (PDI) of 0.423 and high drug content percent ((95.5 ± 1.5 %). The in-vitro antibacterial studies exhibited that the minimum inhibitory concentration (MICs) of free curcumin are 5011, 2511, 10000 and 10000 µg/ml against Staphylococcus aureus ATCC® 43300™*, Escherichia coli ATCC® 8739™*, Pseudomonas aeruginosa ATCC® 27853™* and Klebsiella pneumoniae ATCC® 33495™*, respectively while the MICs of nanocurcumin are 128.8, 549.5, 67.60 and 312.2 µg/ml against Staphylococcus aureus ATCC® 43300™*, Escherichia coli ATCC® 8739™*, Pseudomonas aeruginosa ATCC® 27853™* and Klebsiella pneumoniae ATCC® 33495™*, respectively. It showed highly reduced growth rate of tested strains. Curcumin in nanosized form has remarkable antibacterial activity against Staphylococcus aureus ATCC® 43300™*, Escherichia coli ATCC® 8739™*, Pseudomonas aeruginosa ATCC® 27853™* and Klebsiella pneumoniae ATCC® (MDRO) compared with free curcumin.*

***Keywords**: Curcumin, nanocurcumin, multidrug resistant organism*

INTRODUCTION

Current antibiotics struggle to cure many healthcare-associated illnesses due to multidrug-resistant organisms (MDROs), which is an enormous public health issue[1]. The development of microorganisms that are resistant to antibiotics is mostly caused by repeated drug exposure. As a result, environmental channels including food, animal waste, and water supplies allowed drug-resistant germs to move from animal to human. Accordingly, Antibiotic resistance is developed and is spread by the environment, which implies that multidrug-resistant (MDR) bacteria to move from the environment to clinics[2]. Antibiotics used for therapy have a variety of resistance patterns which piques interest in newly created antimicrobials. Natural compounds such as Atropa belladonna,

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Salixalba and Digitalis purpurea which have been utilized in traditional medicine for centuries in several nations have promise in this area. One of the principal constituents of Curcuma longa a member of the Zingiberaceae family is curcumin. which is derived from the rhizomes[3]. Curcumin (CUR), the primary bioactive component of turmeric, is highly effective against bacteria, fungi, viruses, and inflammation. But further investigation is required to comprehend its antibacterial properties in regard to clinical and multidrug-resistant (MDR) isolates[4]. Nevertheless, biological application of CUR is challenged due to its low aqueous solubility and poor oral absorption and bioavalability. Further, extensive metabolism and rapid systemic elimination are also shortcomings for CUR . In recent years, the development of nanoformulations has made breakthroughs toward enhancing the solubility and the bioavailability of lipophilic drug products[5]. Although CUR is marginally more soluble in alkaline circumstances than it is in neutral or acidic water, its solubility in organic solvents significantly lowers its potential for use in biomedical applications[6]. The significance of nanocurcumin in enhancing the solubility, antioxidant, and antibacterial properties of curcumin in processed foods, as well as its prospective applications in the treatment of cancer, cardiovascular and neurological illnesses, viral and bacterial infections, and others. This due to the high surface area and small particle size of the developed curcumin nanoparticles.[7]. Numerous investigations have demonstrated that curcumin has potent biological activity against both Gram-positive and Gram-negative bacteria as well as broad-spectrum antibacterial action.[8-10]. Several nanocurcumin formulations were designed to enhance water solubility and biological activity.

Bhawana et al. 2011[10] prepared curcumin nanoparticles using wet milling technique have particle size ranged from 2- 40 nm. They found that nanocurcumin exhibited enhanced water solubility and higher antimicrobial effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Penicillium notatum, and Aspergillus niger* when compared with pure curcumin. Bao Hoang Duong et al.[11], developed curcumin nanosuspension using gum arabic as a stabilizer via using high-speed homogenization to enhance curcumin solubility. The results of TEM showed that curcumin nanoparticles small homogeneous particle size of range between 40 to 80 nm and showed enhance aqueous solubility. Further, nanocurcumin formulations were developed by Bahare Salehi et al.[12] for topical application. They demonstrated that nanocurcumin is considered as a promising approach for different skin disorders without toxic effects. They concluded that curcumin nanoformulations can be used for wound management and improved antimicrobial activities at wounded sites.

Hettiarachchi, S.S., et al[13] prepared nanocurcumin without using stabilizer and used as antimicrobial agent. They studied antibacterial activity of nanocurcumin in comparing with free bulk curcumin. They found that nanocurcumin exhibited better antibacterial activity against both Gram-positive and Gram-negative bacteria as compared with free curcumin.

Diffusion and dilution methods are the main two types of laboratory procedures that are available to examine the *in-vitro* susceptibility of bacteria for antimicrobial medicines. Selection of the antimicrobial susceptibility test methodologies is crucial when studying nano/microparticles due to the possibility of sedimentation or the production of turbidity, which might lead to erroneous findings.

This study aimed to study the enhanced water solubility, dissolution rate and antibacterial effect against multidrug resistant microorganisms of nanocurcumin when compared with pure curcumin. In this work nanocurcumin formulations were prepared via antisolvent nanoprecipitation method utilizing different combinations of surfactant stabilizers at weight ratio of 1:1 in an attempt to improve curcumin aqueous solubility and antibacterial efficacy. The stabilizers are polyvinyl pyrrolidoneK30 (PVPK30), lecithin, polyethylene glycol6000 (PEG6000) and sodium lauryl sulphate (SLS). The developed formulations were evaluated for particle size, polydispersity index, drug content and morphology using transmission electron microscopy (TEM). Also, the prepared

nanocurcumin formulations were freeze dried and then evaluated for drug content, saturation solubility and dissolution rates studies. The optimized formulation of nanocurcumin was selected to examine the antibacterial effect of nanocurcumin against multidrug resistant microorganisms.

EXPERIMINTAL MATERIALS

Curcumin (purity > 95%) was purchased from SD Fine-Chem limited Mumbai India. Polyvinylpyrrolidone K30 (PVP, MW 30000) was obtained from Fluka Chemie GmbH, Germany. Poly(ethylene glycol) 6000 (PEG 6000), sodium lauryl sulphate (SLS) were purchased from Adwic, EL-Naser Pharmaceutical Co., Cairo, Egypt. Lecithin soybean was obtained from AppliChem, Germany. Acetone was obtained from PIOCHEM for laboratory Chemicals Co., EGYPT.

Bacterial strain: *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™*, *Klebsiella pneumoniae* ATCC® 33495™* obtained from American type culture collection (ATCC) from microbiologic company (USA).

Preparation of Nanocurcumin

Nanocurcumin different formulations were fabricated using anti-solvent nanoprecipitation method as mentioned previously with slight modifications[9]. Briefly, weighed amount of curcumin was dissolved in acetone (5 mL) to prepare the organic phase. Aqueous phase (antisolvent) was developed by dissolving the combined surfactant stabilizers (1 % wt/v) (stabilizer combination at weight ratio of 1:1) in 20mL of distilled water. The drug solution was added dropwise using a syringe at rate of 0.3 mL/min into the antisolvent aqueous phase on magnetic stirrer (Gallenkamp, Loughborough, UK) at room temperature and at 2000 rpm. The mixture was allowed to stir on magnetic stirrer for 2hrs till complete evaporation of organic solvent and formation of homogenous uniform colloidal dispersion. The composition of different nanocurcumin formulations is presented in **Table 1.**

Characterization of Prepared Nanocurcumin Colloidal Dispersion

Determination of Drug Content in Nanocurcumin dispersion

The prepared nanocurcumin formulations (1 mL) were dissolved in ethanol and sonicated for 2 min to estimate drug content. Then the samples were filtered using Millipore filter of pore size 0.45µm. The content of curcumin was then determined in the filtrate using UV-Vis spectrophotometric analysis **(**Jenway Model 6305, U.K) at 430 nm[14]. The measurements were performed in triplicates.

Particle Size and PDI Measurements

Mean particle size (nm) and polydispersity index (PDI) of prepared formulations were estimated utilizing dynamic light scattering (DLS) by a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) that contains a backscattered light detector measuring at 173°[15]. All the measurements were performed in triplicates.

Transmission Electron Microscopy (TEM)

The developed nanocurcumin formulation was imaged using transmission electron microscopy (JEOL TEM, Model 100 CX II; Tokyo, Japan). A drop of the diluted vesicles from the formulation sample was put to a copper grid with a mesh size of 300 and allowed to sit for one minute to allow some of the vesicles to stick to the carbon substrate after being diluted ten times with distilled water. A piece of filter paper was used to remove extra dispersion, and the grid was then rinsed twice in deionized water for three to five seconds. The sample was then examined under a microscope utilising 10- to 100-fold magnification and an accelerating voltage of 100 kV[16].

Freeze drying Nanocurcumin Formulations

The developed formulations were frozen over night at -80 ±1 ºC and then lyophilized over a period of 48 h using a Free Zone freeze drier (Labconco Inc., Kansas City, MO, USA). Then the samples were stored in tightly closed vials in desiccator for further investigations[17].

Characterization of lyophilized Nanocurcumin Formulations

Determination of Curcumin Saturation Solubility

The saturation solubility of free curcumin and freeze dried nanocurcumin was determined in distilled water. An excess amount of sample (equivalent to 5 mg CUR) was placed in medium in screw-capped glass vial and shaken continuously in thermostatically controlled water bath (Gesellschaft Labor Technik M.B.H.&Co., GFL. Germany) at 50 rpm and 37 °C for 48 hour until equilibrium was attained[18]. Supersaturated suspension was filtered using membrane disc filter (0.45µm) and anlyzed by UV-Vis spectrophotometer at λmax 430 nm to determin drug concentration. All the experiments were performed in triplicates.

***In-vitro* Dissolution Studies**

In-vitro dissolution analysis of the developed freeze dried nanocurcumin formulations and their physical mixtures compared with free curcumin was carried out using a USP type II dissolution apparatus (COPLEY Scientific, NottinghamNG42JY, United Kingdom). Weighed amount of nanocurcumin and their physical mixtures were placed in dissolution medium that consists of 500 ml of phosphate buffer pH 7.4 contains Tween 80 (0.1 % w/v) to maintain sink condition. Dissolution system was kept at 37 °C and agitated at constant rate of 50 rpm[19]. At specified time intervals of 5, 15, 30, 60, 90, and 120 minutes, aliquots of 5 mL were withdrawn and filtered by membrane disc filter (0.45µm). To keep the sink condition, 5 mL aliquots were removed and replaced with an equivalent volume of fresh dissolution media. The drug content in the samples was examined by UV-Vis spectrophotometer at λmax 430 nm. All the measurements were carried out in triplicates.

***In- vitro* antimicrobial activity testing**

Well diffusion method

The well diffusion method is suitable for evaluating the antibacterial activity of plant or microbial extracts utilising Agar cup diffusion.[20]. we assessed the antimicrobial activity of free curcumin and nanocurcumin against Gram-positive and Gram-negative bacteria. The bacteria were produced in sterile saline according to the following procedure: each bacterial strain was cultured for 24 hours in a universal tube containing 20 ml of medium broth. Microbial suspension (0.1 ml/plate) and 15 ml of the appropriate agar medium (15 ml/plate) were placed into 10 cm sterile plastic Petri plates for the bioassay. Using a sterile cork pore, solidified agar were cut (5 cavities/plate) after medium solidification. In-vitro antibacterial assessment, tested samples were pipetted into the cavities (100 µl /cavity) as an optimal formulation of free curcumin and nanocurcumin suspension at concentrations of 50000, 30000, 20000, 10000, 5000, 2500, 1250, 625, and 312.5 µg/ml. After 48 hours, plates were incubated at 28 °C and checked for the inhibitory zone. The inhibitory zone's diameter (mm) surrounding the cavities was measured. 2µg/ml Vancomycin and 2.5µg/ml Gentamycin were used as positive controls and they were pipetted in the cavities (100µl/cavity).

Disc diffusion method

In accordance with Clinical Laboratory Standard Institute, the Kirby-Bauer method and disc diffusion method were used to assess the antimicrobial susceptibility phenotype of the chosen strain (CLSI, 2020). A sterile loop was used. We picked several colonies of the organism to be tested. To make a smooth suspension, the organism was placed in 2 ml of sterile saline and vortexed in the tube. Suspension's turbidity was adjusted to a bacterial density of 0.5 MacFarland (1.5 X 10^8 CFU/ml). After dipping a sterile swab into the solution, extra fluid was squeezed out. To achieve a uniform spread of the inoculum, a Mueller Hinton agar plate's dry surface was infected. This was done by streaking the swab in three separate directions. The agar plate's surface needed to dry for at least 3 to 5 minutes, but no more than 15 minutes, so it was left to sit at room temperature. Using a forceps to distribute each antimicrobial disc at a time, the appropriate antimicrobial-impregnated discs (filter paper soaked with antimicrobial agent) were put on the surface of the agar. After all the discs had been secured, they were left in an incubator at 37 °C for 18 hours. After incubation, the diameters of the inhibitory zones on several discs were measured using a ruler or caliber to the closest millimeter[21]. *In-*

vitro antibacterial evaluation of tested samples (optimum formulation of nanocurcumin and free curcumin suspension at concentration of 50000, 30000, 20000 ,10000 ,5000 ,2500 ,1250, 625, 312.5 µg/ml) were done. The activity of curcumin against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* ® was evaluated.

Determination of minimum inhibitory conc. (MIC) and minimum bactericidal conc. (MBC)

The MIC is the lowest concentration of antimicrobial agent that prevents the organisms' ability to grow visibly, as measured visually by turbidity. Agar cup diffusion methods can also be used. In this approach, the bacteria are inoculated onto the surface of the agar, containing different concentrations (two-fold dilutions) of the antimicrobial agent. After measured the zone of inhibation around each well (X= Radius of each zone), plot X^2 against log conc. of the nanocurcumin and extrapolate the line to obtain MIC. Following the determination of the nanocurcumin's MIC, the MBC was calculated. Four more concentrated dilutions were used, and 5µl aliquots from each well that showed no sign of bacterial growth were plated and incubated for 24 hrs at 37 °C. When 99.9% of the bacterial population is eradicated at the lowest antimicrobial agent concentration, this is known as the MBC endpoint. This was done by inspecting pre- and post-incubated agar plates for microorganisms.[22].

Statistical analysis

All experiments were performed in triplicates and results were expressed as mean values ± standard deviation (SD). T test and Anova were used to compare the effects of free curcumin and nanocurcumin on bacterial growth. The $p < 0.05$ is considered moderate significant, p-value < 0.01 is considered strong significant and p-value < 0.001 is considered very strong significant.

RESULTS AND DISCUSSION

Results

Preparation and characterization of nanocurcumin dispersions

Nanocurcumin formulations containing different combined stabilizers were successfully developed utilizing antisolvent nanoprecipitation method. Visual evaluation of all prepared formulations was carried out on the basis of perception and agglomeration. The results revealed that formulation that prepared using PVPK30 combined with PEG 6000 and SLS showed uniform and homogenous dispersion without any precipitation (Table 1). Also, the other used stabilizer combination exhibited homogenous distribution with slight precipitation.

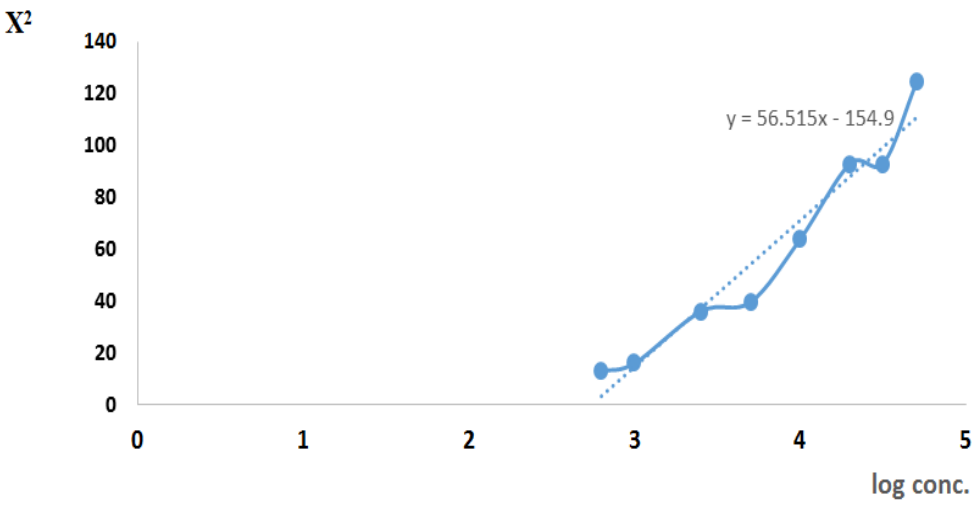

Fig. 1: Standard curve for detection of MIC of nanocurcumin.

Table 1: Composition and characterization of different nanocurcumin formulations containing combined stabilizers (Total stabilizer concentration 1% w/v, combination at wt ratio of 1:1).

Formulation code	Stabilizer combination (1:1 weight ratio)	Physical appearance	Particle size (nm)	PDI	Drug content (%)	Saturation solubility (µg/ml)
F1	PVPK30:PEG6000	Uniform dispersion	272.1 ±34.5	0.423±0.093	95.1 ±0.013	440±0.02
F2	Lecithin :PEG6000	Slight precipitation	399.5±10.75	0.450±0.050	93.12±0.021	150±0.021
F3	PVPK30: SLS	Uniform dispersion	299.3±15.35	0.448±0.037	93.91±0.015	224±0.0023
F4	Lecithin: SLS	Slight precipitation	524.2±10.56	0.598±0.060	92.14±0.043	100±0.022

Determination of Drug Content in Nanocurcumin dispersion

The determined percentage of drug content of the developed nanocurcumin formulations was found in the range of 92.14 ± 0.043 to 95.1 ± 0.013. nanocurcumin that prepared using the combination of PVPK30 and PEG 6000 showed the highest drug content (95.1 ± 0.013 %)(**Table 1**). This result indicates that the utilized method for nanocurcumin preparation is considered appropriated to develop nano-formulation with high drug content.

Particle Size and Size Distribution Measurements

The mean particle size and polydispersity index (PDI) measurements for the developed nanocurcumin formulations are listed in table1. It is observed that the mean particle size of formulations (F1-F4) was found to be ranged from 272.1 ± 34.5 to 524.2 ± 10.56 nm. Formulation that containing PVPK30 combined with PEG6000 (F1) showed the smallest particle size 272.1 ± 34.5 nm (**Figure 2**). Further, **Table 1** shows that the values of PDI are in the range of 0.423±0.093 to 0.598 ± 0.060, that evaluate the uniformity of particle distribution. The results proved that F 1 exhibited the lowest value of PDI (0.423±0.093) which confirms the good uniformity in particle size distribution. So, it was selected for TEM analysis.

Transmission Electron Microscopy (TEM)

TEM images of the selected nanocurcumin formulation (F1) are illustrated in **Figure 3**. The nanoparticles are spherical in shape, non-aggregated and found in nano rage size.

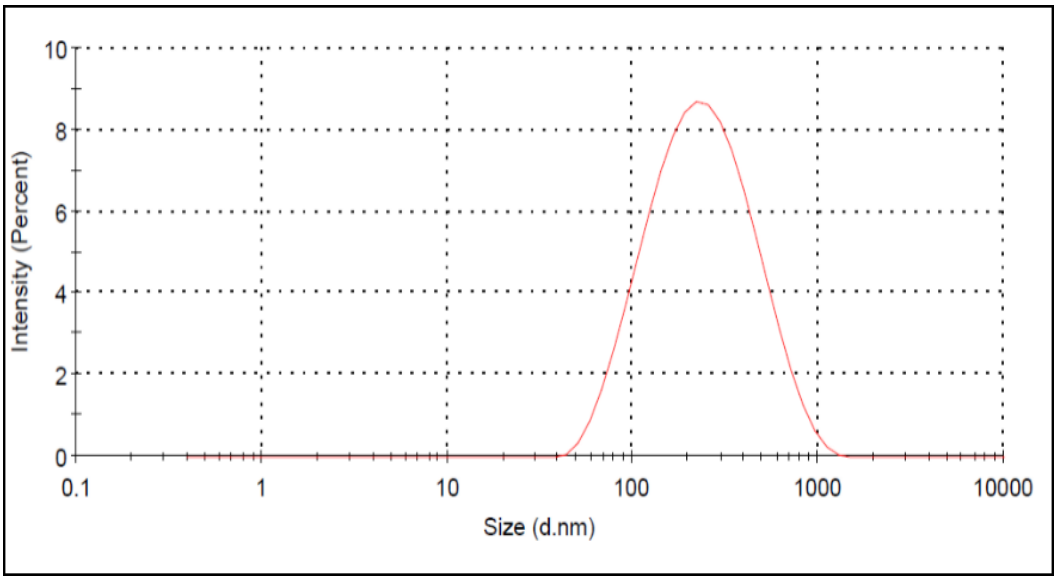

Fig. 2: Size distribution of the prepared nanocurcumin dispersion using PVP and PEG6000 combined stabilizers.

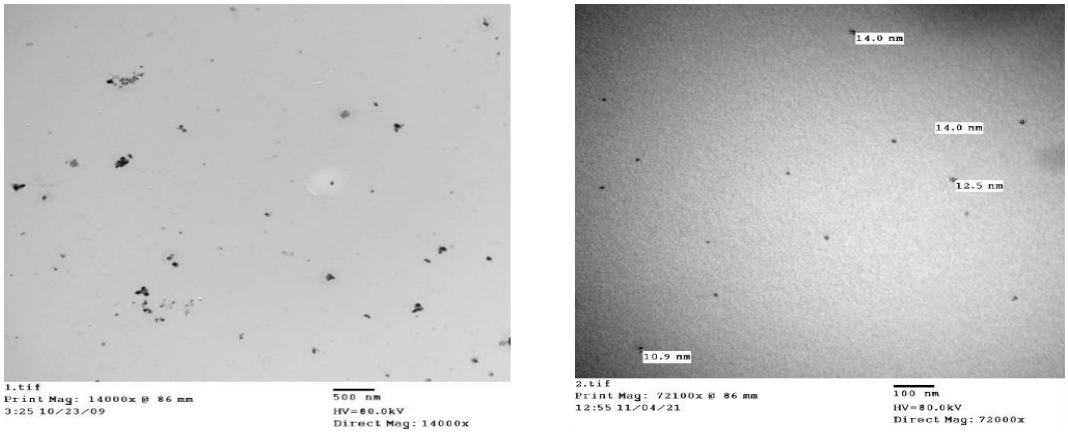

Fig. 3: TEM images of the prepared nanocurcumin dispersion (F1) using PVP and PEG6000 combined stabilizer.

Characterization of lyophilized Nanocurcumin Formulations

Determination of Curcumin Saturation Solubility

The solubility of curcumin in nan-formulation was increased using different mixtures of polymer stabilizers **(Table 1**). It was observed that formulation 1 containing PVPK30:PEG6000 combination showed enhanced curcumin solubility up to100-fold (440 µg/ml) as compared with other utilized combination and free curcumin which exhibited very poor water solubility (4 µg/ml). This finding can be largely attributed to the reduction of particle size and increased surface area which resulting in improved aqueous solubility nanosized curcumin formulation.

***In-vitro* Dissolution Studies**

Figure 4 and 5 illustrate the percentage of curcumin that dissolved as a function of time for nanocurcumin formulations and their physical mixtures in phosphate buffer pH 4.7 and at 37 °C. As seen, all the formulations of nanocurcumin and their physical mixtures showed enhanced dissolution rate as compared with free curcumin. However, nanocurcumin formulations represents improved dissolution as compared with their physical mixtures. This indicate the successful preparation of nanocurcumin using anti-solvent nanoprecipitation method. Also, the highest dissolution was obtained with the formulation that containing PVPK30 and PEG 6000 combination. Where, the dissolution of free curcumin was slow, only 35% of the drug was dissolved within 30 min., while nanocurcumin (F1) exhibited dissolution rate of about 95 % at the same time, (**Figure 4**).

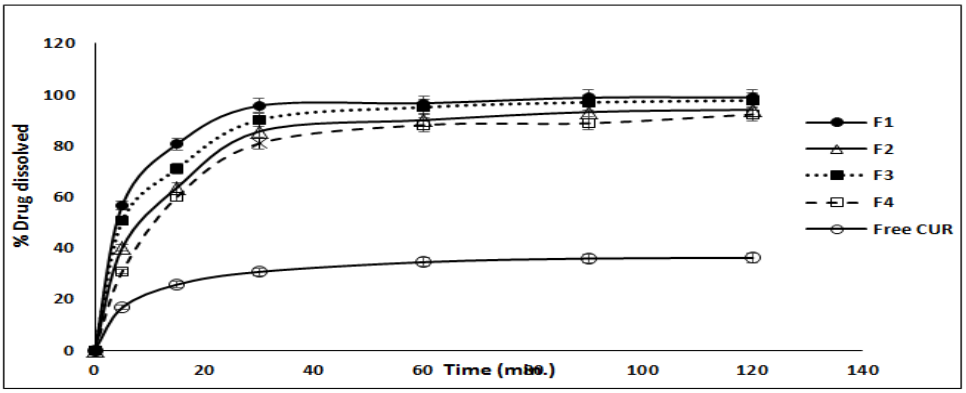

Fig. 4: Dissolution profiles of nanocurcumin stabilized with different combined stabilizers in phosphate buffer pH 4.7 and at37 °C. The experiments were performed in triplicates and the mean ±SD (n=3).

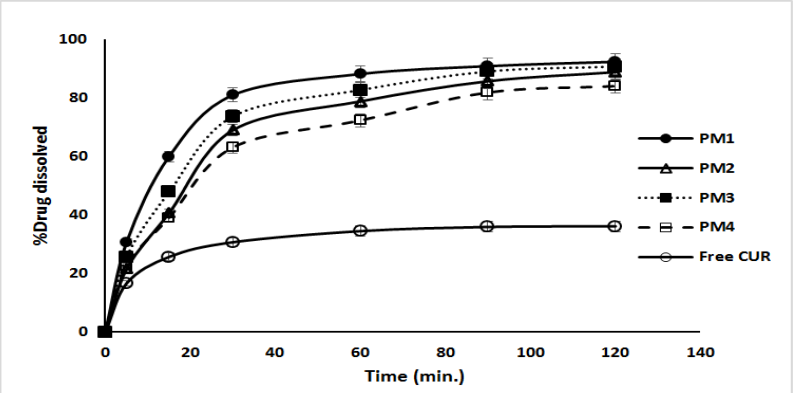

Fig. 5: Dissolution profile of the physical mixtures of curcumin with different combined stabilizers in phosphate buffer pH 4.7 and at 37 °C. The experiments were performed in triplicates and the mean ±SD (n=3). (PM: physical mixture, CUR: curcumin.

In- vitro antimicrobial activity testing

MDROs (*Escherichia coli, Staph aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae*) are the most common bacteria which cause life-threatening human diseases. The nanocurcumin exhibited antibacterial activity against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* as they resulted in clear zones around the free curcumin, nanocurcumin by well (Fig. 6-8) and disc diffusion methods (Fig. 9).

Depending on the types and strains of microorganisms, our investigation revealed variations in the biological activity of curcumin by MIC (Table3). The MICs of free curcumin were 5011, 2511, 10000 and 10000 µg/ml against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™*, respectively. The MICs of nanocurcumin are 128.8, 549.5, 67.60 and 312.2 µg/ml against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™*, respectively. There was a very strong significant in results between free curcumin and nano curcumin (p-value < 0.001).

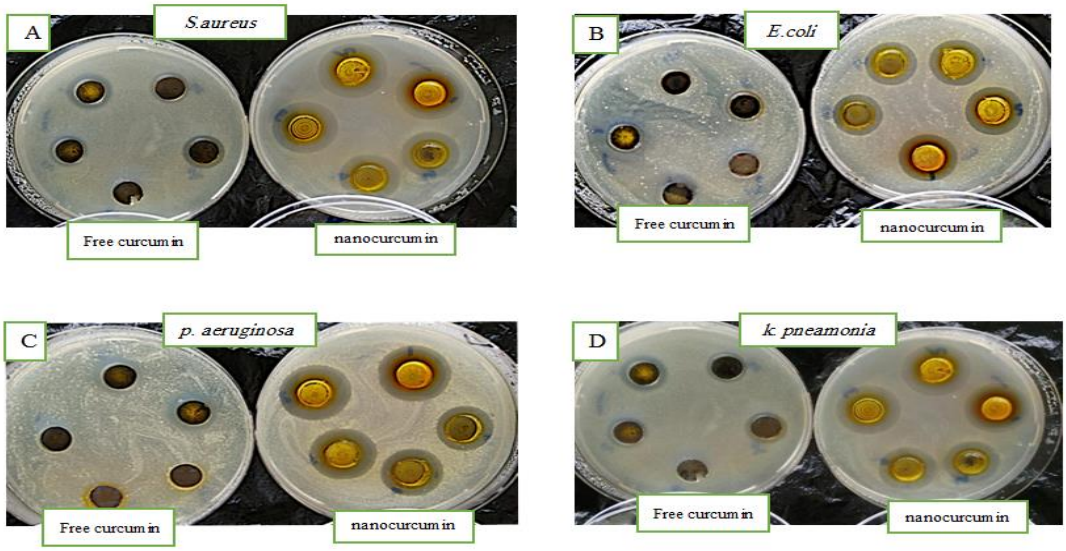

Fig. 6: A, B, C, and D , antibacterial activity of different concentratons of free curcumin and nanocurcumin against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* *and Klebsiella pneumoniae* ATCC® 33495™*evaluated by agar well diffusion.

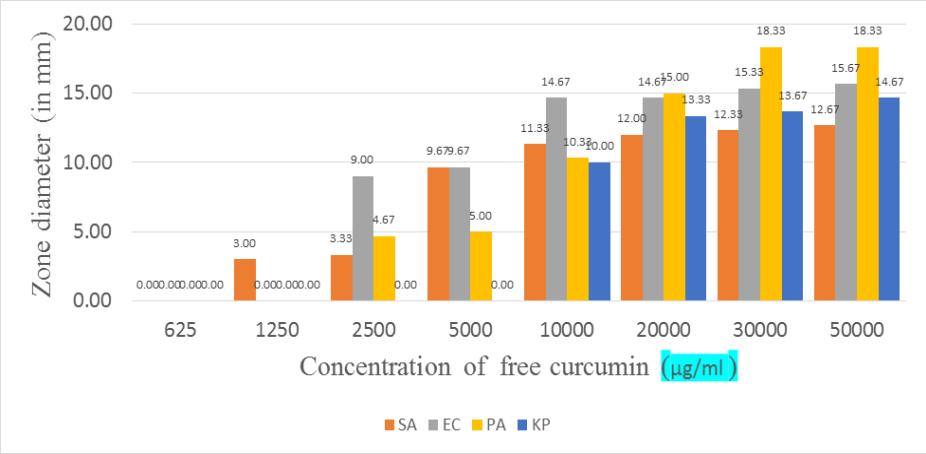

Fig. 7: Antibacterial activity of different concentrations of free curcumin against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™*evaluated by agar well diffusion.

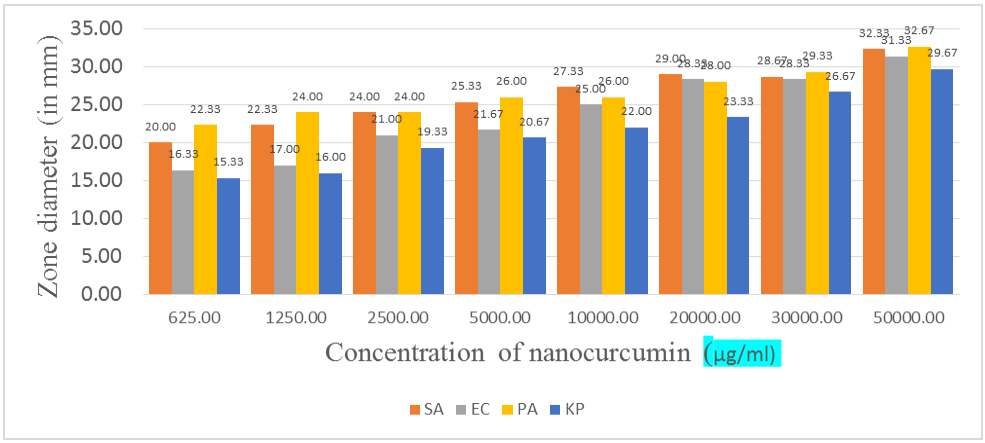

Fig. 8: Antibacterial activity of different concentrations of nanocurcumin against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* evaluated by agar well diffusion.

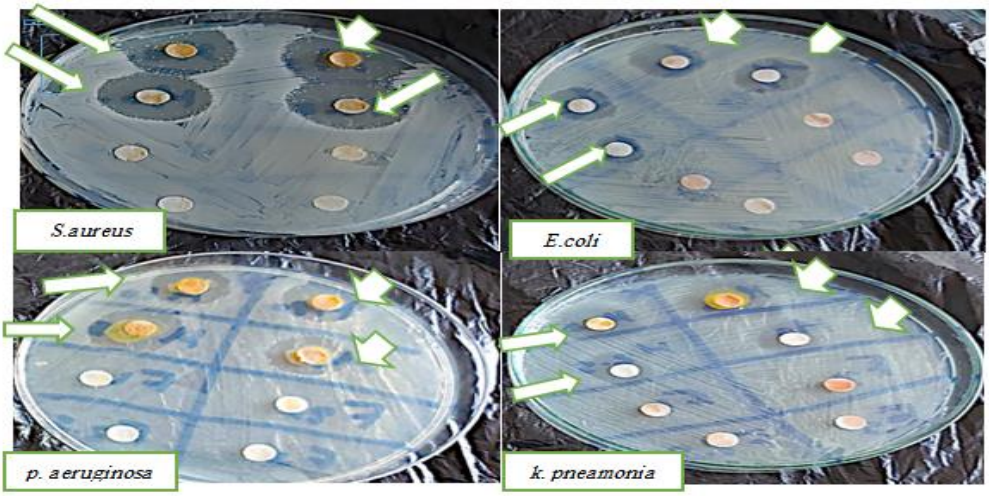

Figure 9: Antibacterial activity of different conc. Of nanocurcumin (White arrow) with inhibition zone and different conc. of free curcumin with no inhibition zone against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™*evaluated by agar disc diffusion.

Table 2: Antibacterial effect of blank (dis. Water) , free-CUR, positive control antibiotic AB (vancomycin for s.aureus and gentamycin for G-ve strain) and CUR-NS against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* evaluated by agar well diffusion.

Microorganism	**Zone of inhibition ± SD (mm) (n=3)**				**p.value 1 (CUR-NS and antibiotic)**
	blank	**Free-CUR**	**Antibiotic (positive control)** (2µg/ml vancomycin and 2.5µg/ml gentamycin)	**nanocurcumin**	
Staphylococcus aureus* ATCC® 43300™	0	10	19 ±1	17.8 ± 0.8	(p> 0.05)
Escherichia coli* ATCC® 8739™	0	12	17 ± 0.7	18 ± 0.4	(p> 0.05)
Pseudomonas aeruginosa* ATCC® 27853™	0	11.5	18 ±0.5	18 ± 0.4	(p> 0.05)
Klebsiella pneumoniae ATCC® 33495™	0	11	17 ± 0.5	16 ± 1	(p> 0.05)

Note: * p > 0.05 was considered to be not significant
Vancomycin for *Staphylococcus aureus* ATCC® 43300™*. Gentamycin for *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™*
Abbreviations: SD standerd deviation.

Table3: Determination of MIC and MBC of free curcumin and nanocurcumin against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* using agar well diffusion method.

Antimicrobial agent	**Mean of MIC µg/ml** ±SD				**Meam of MBC µg/ml** ±SD			
	Staph aureus	*E.coli*	*pseudomonas*	*klebsellia*	*Staph aureus*	*E.coli*	*pseudomonas*	*klebsellia*
Free curcumin	5011 ± 9	2511 ± 8.5	10000 ± 9.5	10000 ±8	10000 ±9.5	5000 ±8.5	10000 ±9.4	20000 ±7
nanocurcumin	128.8 ± 1.5	549.5 ± 1	67.60 ± 2	316.2 ±1.8	128.8 ±1.5	549.5 ±2	67.60 ± 1.9	316.2 ± 1.9
p.value	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Note: p-value < 0.001 was considered to be very strong significant.
Abbreviations: SD standerd deviation.

Discussion

Curcumin has a variety of biological and pharmaceutical application such as antibacterial, antiviral, antioxidant and ant inflammatory. Concerning to the antibacterial effect, Curcumin triggers numerous pathways of cell death in microbial pathogens[23]. Although having multiple actions, the shortcomings such as poor bioavailability, low water solubility, photodecomposition, short shelf-life, and rapid elimination challenge its pharmaceutical impacts. Curcumin is classified as BCS (Biopharmaceutical Classification System) IV drug due to poor solubility in an aqueous phase. The antibacterial effect of curcumin is limited because of high lipophilicity and low permeability. Nanocurcumin formulations enhance water solubility, bioavailability, permeability, prolonged, physical stability, and antimicrobial effects[24&25]. In the present investigation, nanocurcumin formulations are fabricated utilizing anti-solvent nanoprecipitation method with different combinations of stabilizers. Acetone is used as a safe solvent which has high solubility for curcumin. it has low boiling point 56 ^{o}C and it does not form azeotrope with water so can be easily evaporated from the nanocurcumin dispersion. Different stabilizers were used in combinations (Table 1) and drug content in the developed formulations was found to be more than 90%. This may result in improved biocompatibility and safety since the presence of lower amounts of inactive additives during the preparation. The results of DLS showed that the prepared formulations have nanosized range of (Table 1) with narrow ,uniform size distribution according to the values of P.D.I. this may be due to that presence of polymers like PVPK30, lecithin and PEG6000 as stabilizers which are considered crucial as they provide an advantage of preventing drug solubilization and Ostwald ripening that may be observed with surfactant with small molecules[26]. Particle size of the developed formulations ranged from 272.1 ± 34.5 for nanocurcumin stabilized by PVPK: PEG6000 combination to 524.2 ± 10.56 nm for that contains Lecithin: SLS. Nanocurcumin with PVPK30 :PEG 6000 as combined stabilizer exhibited the smallest particle size. This result may be attributed to the presence of PVPK30. PVPK 30 is vinyl pyrrolidone polymer and is known be adsorbed on the surface of the drug particle and prevent the crystal growth and particle aggregation, this result is matching with previous report[27]. The fabricated nanoformulations were freeze-dried and then evaluated for saturation solubility and dissolution rate profiles. The results of saturation solubility (Table 1) proved that nanocurcumin formulation 1 exhibited the highest curcumin solubility (440 µg/ml) compared to other nanoformulations. This result may be due to the smallest particle size of nanocurcumin containing PVPK30:PEG6000, it was reported in previous literature that the saturation solubility increases with decreasing particle size[28]. These results are in agreement with that obtained by Samar A. Afifi et al.[14]. Further, the results showed that nanocurcumin containing PVP:PEG6000 with particle size of 272.1 ± 34.5 exhibited the highest dissolution rate as about 95% of drug dissolved in 30 minutes whereas, formulation containing lecithin:PEG6000 of 399.5 ± 10.75 presented the least drug dissolution rate as about 80% of drug dissolved at the same time. This finding may be related to small particle size and high surface area of the drug particles in the nanocurcumin formulation. Therefore, the reduction in particle size resulted in an increase in the dissolution rate. The effect of used stabilizer combinations on drug dissolution can be arranged according to this order

PVPK30:PEG6000>PVPK30:SLS>Lecithin:PEG6000>Lecithin :SLS

Based on the aforementioned experimental findings, formulation of nanocurcumin that containing PVPK30 :PEG6000 combined stabilizer was selected for further investigation as antibacterial agent.

The zone of inhibition was determined in millimeters using the well and disc diffusion technique to test the bactericidal activity of free curcumin and nanocurcumin at various doses. In Well diffusion method at conc. 50000 µg/ml, *Staphylococcus aureus* ATCC® 43300™* was found to exhibit maximum zones of inhibition of 32.3 and 16 mm for nanocurcumin and free curcumin, respectively. There was a very strong significant in results between free curcumin and nano curcumin (p-value < 0.001). At same conc. *Escherichia coli* ATCC® 8739™* was found to exhibit maximum zones

of inhibition of 31.3 and 15.6 mm for nanocurcumin and free curcumin, respectively. There was a very strong significant in results between free curcumin and nano curcumin (p-value < 0.001). While *Pseudomonas aeruginosa* ATCC® 27853™* found to exhibit maximum zones of inhibition of 32.6 and 18.3 mm for nanocurcumin and free curcumin, respectively. There was a very strong significant in results between free curcumin and nano curcumin (p-value < 0.001). At 50000 µg/ml, *Klebsiella pneumoniae* ATCC® 33495™*was found to exhibit maximum zones of inhibition of 29.6 and 14.6 mm for nanocurcumin and free curcumin, respectively. There was a very strong significant in results between free curcumin and nano curcumin (p-value < 0.001) (Figure 6-8). Nanocurcumin has a larger surface area than free curcumin, so there is greater surface contact with microbes which increases its antibacterial effect. When compared to its free form, curcumin in nano form has benefits, especially in terms of water solubility and bioavailability. In disc diffusion method the results are nearly same to well diffusion method. nanocurcumin was more effective against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli ATCC*® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* *and Klebsiella pneumoniae* ATCC® 33495™* with a zone of inhibition diameter of 29.3, 19, 25, 15.3 mm respectively at conc 50000 µg/ml than free curcumin with zone diameter 12.3, 11, 0, 3 mm respectively (Figure 9).

As concluded by Basniwal, Buttar et al. 2011, the order of the various bacteria that were inhibited by nanocurcumin at a 200 g/mL concentration was *S. aureus* > *P. aeruginosa* > *coli.* For *S. aureus*, the diameter of the inhibitory zones at 400 g/mL of curcumin demonstrated highest efficacy. According to the findings, the chosen Gram-positive bacteria were more sensitive than the chosen Gram-negative bacteria. It's possible that this is due to differences in the composition and structure of their cell membranes. Gram-negative bacteria have an outer phospholipidic membrane, whereas Gram-positive bacteria have an outer peptidoglycan layer; when curcumin interacts with either of these, different sorts of interactions take place. The order of the organisms on which nanocurcumin had the greatest effect in this study was *P. aeruginosa* > *S. aureus* > *E. coli*> *and then K. pneumoniae*. Therefore, the source of bacterial strains as well as the bacterial membrane structure have an impact on the antibacterial efficacy of nanoparticles.[29].

The results obtained from our study shows that the nanocurcumin have got a very good antibacterial activity against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli* ATCC® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* in dose dependent manner.

Numerous microorganisms have already been studied to see how well nanocurcumin works.

As shown in the (Table 2), effect of nanocurcumin was found less than effect of vancomycin with p.value : p > 0.05 (not significant) against Staphylococcus aureus ATCC® 43300™*, and more than effect of gentamycin with p.value : p > 0.05 (not significant) against Escherichia coli ATCC® 8739™*, and nearly equal to gentamycin with p.value : p > 0.05 (not significant) against Pseudomonas aeruginosa ATCC® 27853™*, and less than gentamycin with p.value : p > 0.05 (not significant) against Klebsiella *pneumoniae ATCC® 33495*™*.

Studies in the literature show that for the same microorganism tested using the same method, nanocurcumin had a lower MIC than curcumin. In other publications, the MIC value was only provided for one species and one reference strain[30&31] . Sometimes too low concentrations of curcumin have been used to determine its antimicrobial activity for instance. At the maximum levels of 64 µg/mL, 100 µg/mL, 128 µg/mL, 156 µg/mL, 256 µg/mL, 330 µg/mL and 375 µg/mL[32-38]. Hence, there is still a need for extensive research of the effects of curcumin against a large number of microbial strains and species.

A clearly stronger antibacterial effect of nanocurcumin against Gram-positive and Gram-negative bacteria than free curcumin where observed (Table 3). It shows decrease in MIC of nanocurcumin (which indicated that a reduction in size of nanoparticles caused improvement in their bioavailability and solubility due to decrease in size of particles). This nanocurcumin strongly inhibited the growth of *Pseudomonas* aeruginosa ATCC®

27853™* (MIC = 67.5 µg/mL) more than others reference strains. The median MICs of nanocurcumin against the *Staphylococcus aureus* ATCC® 43300™* and *Klebsiella pneumoniae* ATCC® 33495™*reached 128.8 and 312.2 µg/mL, respectively.

Similar results were reported by Dai, Lin et al. 2022, Basniwal, Buttar et al. 2011 and Shariati, Asadian et al. 2019, results showed a broad-spectrum inhibitory effect of nanocurcumin against MDR[39-41]. Ożarowski et al. 2020 discovered that curcumin can modify gene expression and prevent bacterial DNA replication. Additionally, it damages the bacterial cell membrane and lessens microbial motility[4].

Conclusion

In this study nanocurcumin formulations were prepared using combined stabilizers via antisolvent nanoprecipitation method . Nanocurcumin formulation stabilized by PVPK30 and PEG6000 at weight ratio of 1:1 exhibited the smallest particle, highest saturation solubility and dissolution rate as compared with other combinations. Nano-curcumin exhibited higher aqueous solubility and presented a higher dissolution rate within 30 min when compared with free curcumin. Also, antibacterial activity against *Staphylococcus aureus* ATCC® 43300™*, *Escherichia coli ATCC*® 8739™*, *Pseudomonas aeruginosa* ATCC® 27853™* and *Klebsiella pneumoniae* ATCC® 33495™* was strongly enhanced.

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التقييم المختبري لمادة النانو كوركومين ضد البكتيريا المقاومة للأدوية المتعددة

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الكركمين هو مركب طبيعي من نبات كركم لونجا يستخدم على نطاق واسع كعوامل مضادة للميكروبات ضد البكتيريا موجبة وسالبة الجرام. إنه ضعيف الذوبان في الماء لذا فهو يتمتع بتوافر حيوي أقل. كان الهدف من هذا العمل هو تطوير تركيبة نانوكركمين فعالة لتعزيز قابليتها للذوبان في الماء ونشاطها المضاد للبكتيريا ضد أربعة أنواع من السلالات المقاومة للأدوية المتعددة. تمت صياغة نانوكركمين مع المثبت المركب Polyvinylpyrrolidone K30 (PVP) وفول الصويا الليسيثين باستخدام نهج الترسيب النانوي المضاد للمذيبات ثم تم تقييمه من حيث محتوى الدواء وحجم الجسيمات والمجهر الإلكتروني للإرسال (TEM). تم تقييم التأثير المضاد للبكتيريا في المختبر ضد المكورات العنقودية الذهبية ATCC® 43300™* و ميكروب القولون النوذجي ATCC® 8739™* و الزائفة الزنجارية ATCC® 27853™* و الكلبسيلة الرئوية ATCC® 33495™* ومقارنته بالكركمين الحر. تم تطوير الكركمين النانوي الذي يحتوي على نسبة عالية من الدواء (١,٥٪± ٩٥,٥) بنجاح. أظهر الكركمين النانوي المحضر متوسط حجم جسيم قدره ٢٧٢,٢٥ ± ٣٤,٥ نانومتر ومؤشر التشتت المتعدد (PDI) يبلغ .٤٢٣. أظهرت الدراسات المضادة للبكتيريا في المختبر أن الكركمين الحر هو ٥٠١١ و ٢٥١١ و ١٠٠٠٠ و ١٠٠٠٠ ميكروغرام / مل ضد المكورات العنقودية الذهبية ATCC® 43300™* و ميكروب القولون النوذجي ATCC® 8739™* و الزائفة الزنجارية ATCC® 27853™* و الكلبسيلة الرئويه ATCC® 33495™* ، على التوالي ، في حين أن اقل تركيزات تثبط البكتريا من النانوكركمين هي ١٢٨,٨ و ٥٤٩,٥ و ٦٧,٦٠ و ٣١٢,٢ ميكروغرام / مل ضد المكورات العنقودية الذهبية ATCC® 43300™* و ميكروب القولون النوذجي ATCC® 8739™* و الزائفة الزنجارية ATCC® 27853™* و الكلبسيلة الرئوية ATCC® 33495™* ، على التوالي. يتمتع النانوكركمين بمعدل نمو منخفض للغاية للسلالات المختبرة. يحتوي الكركمين النانوي على نشاط مضاد للجراثيم ملحوظ ضد المكورات العنقودية الذهبية ATCC® 43300™* و ميكروب القولون النوذجي ATCC® 8739™* و الزائفة الزنجارية ATCC® 27853™* و الكلبسيلة الرئوية ATCC® 33495™* عند مقارنته بالكركمين الحر.