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ORIGINAL ARTICLE

Preparation, characterization and in vivo wound healing study of topical formulations of curcumin and quercetin loaded PLGA nanoparticles

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Abstract

Different drugs and drug delivery systems have been widely investigated aiming at wound healing. However, the risk of infections is increased until complete healing due to antibacterial resistance against present treatment options and it requires regular wound dressings. The present treatment options are also limited, costly, inefficient, and have side effects Nutraceutical compounds are considered as an alternative to solve these issues. Nutraceuticals like quercetin and curcumin have antimicrobial, anti-inflammatory, and antioxidant activity which helps in wound healing. However, the use of these nutraceuticals resulted in low bioavailability due to poor solubility and abridged target specificity. These issues were resolved by loading curcumin and quercetin into PLGA nanoparticles. Curcumin and quercetin-loaded PLGA nanoparticle formulations have been investigated separately which resulted in increased solubility and improved bioavailability. In the present study, quercetin and curcumin-loaded PLGA nanoparticles were prepared in different ratios 50:50, 75:25, and 25:75 of curcumin and quercetin, respectively, and were characterized for particle size, zeta potential, and polydispersity index. The nanoparticle size was less than 250nm which is considered appropriate for drug delivery. Zeta potential was in the range of -1.83 mV to -3.2 mV that indicates the high stability of nanoformulations. Higher encapsulation efficiency from 64.84% to 88.48% in different formulations was achieved. These three different preparations along with standard drug povidone-iodine 10% were applied for 12 days on cutaneous wounds excised on rabbit's thoracolumbar area. All formulations along with povidone-iodine were compared for wound contraction rate and wound healing time. Two-way ANOVA followed by DMR was applied to data to see the statistical difference between treatments. Wound contraction rate was in the following order T2>T1>T3>T4>C. Wound healing time was C=14 days T1=8 days T2=7 days T3=9 days T4=10 days. Therefore, it can be concluded PLGA loaded nanoparticles of curcumin and quercetin enhance the bioavailability of curcumin and quercetin and their wound healing potential.

Keywords

Nanomedicine
PLGA
Curcumin
Quercetin
Wound healing

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Introduction

Injury of living tissue which results in loss of integrity of the body structures is known as a wound. (Wu et al., 2007) Wounds can be acute and chronic. Acute wounds result from a sudden injury while wounds not healing over a period of time are known as chronic wounds. Delaying in wound healing results in

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chronic wounds and the delayed healing process results in economic losses (Menke et al., 2007). There is a substantial social and financial load linked with wounds that leads to developing newer drugs or formulations for the hastening of wound healing.

Several drugs and therapeutic delivery systems have been practiced accelerating wound healing in different phases of wound healing. However, the present wound healing techniques have augmented the risk of several infections invading during the complete cure of the wound and require regular gauze replacement. Besides this, the existing treatment techniques are quite expensive, inadequate, and incompetent, which leads to the expansion of some new therapeutics' methods to completely, alternate the unachieved clinical needs.

Nutraceuticals like flavonoids are known to be the key constituents present in many plant extracts which have wound healing potential. Significant improvement in cutaneous wound healing has been observed after the topical application of these flavonoids due to their antioxidant, antibacterial, and anti-inflammatory properties (Aslam MS, et al. (2018).

Quercetin, a nutraceutical, belongs to the flavonoid class of natural compounds that possess numerous pharmacological properties including antibacterial, antioxidant, antiplatelet, anticarcinogenic, anti-inflammatory, antiangiogenic, antiviral, antimutagenic, antiradical, antianxiety, antitumor and cognition-enhancing (Bagad and Khan, 2015). In various pharmacological studies, quercetin has the potential activity to inhibit both gram species of bacteria, possibly by disrupting bacterial membrane and by rendering them inactive. Owning to those pharmaceutical properties quercetin is an effective dermal wound healing agent (Vedakumari et al., 2017).

Curcumin is a phenolic agent that has been used as a natural remedy against several diseases due to its antimicrobials, anti-oxidants, anti-tumor, anti-inflammatory properties. It has a potent antimicrobial effect and antioxidant effect. Topical application of curcumin accelerates wound healing (Panchatcharam et al., 2006).

Quercetin and curcumin are two nutraceuticals having an excellent antimicrobial and anti-inflammatory activity that facilitates wound healing. (Güran et al., 2019).

Even though curcumin and quercetin are safe and efficacious; their clinical uses are restricted because of their poor solubility and bioavailability. Quercetin also behaves the same limitations as curcumin for the satisfactory utilization of clinical employment (Bagad and Khan 2015). Various published data has proved the better antibacterial efficiency of quercetin-loaded nanoparticles as compared to simple quercetin (Kumar et al., 2016). In different pharmacokinetic studies, the greater oral bioavailability of quercetin-loaded PLGA nanoparticles have been observed as compared to quercetin suspension and these particles have demonstrated a sustained release profile of quercetin *in vitro* studies which reduce the dosing frequency (Chitkara et al., 2012). These curcumin-loaded PLGA nanoparticles are also important in the treatment of ovarian cancer by inhibiting the early stage of metastasis (Pandit et al., 2015). To increase permeability, for longer circulation and to enhance the metabolic resistance of curcumin nanoparticles, micelles, phospholipid complexes, and liposomes are prepared. Curcumin-loaded polymeric nanoparticles are used for tumors and they act by preventing the growth of brain tumor cells. The incorporation of quercetin in poly lactide-co-glycolide (PLGA) nanoparticles enhances its antibacterial efficiency and eliminates the solubility, stability, and bioavailability issues.

Curcumin and quercetin have a synergistic antimicrobial and anti-inflammatory effect when given combined (Güran et al., 2019). Therefore, different combinations of curcumin and quercetin loaded PLGA nanoparticles were prepared and their comparative wound healing efficacy was studied among these formulations as well as with standard drug povidone-iodine 10%. The incorporation of curcumin and quercetin in PLGA nanoparticles makes them a very effective therapeutic agent for treating numerous bacterial infections. As quercetin and curcumin are nutraceuticals, so it is devoid of several adverse effects, as compared to other synthetic antibacterials. The preparation method of quercetin and curcumin loaded PLGA nanoparticles is very easy and convenient and can be scaled up to an industrial level with slight modifications.

Materials and Methods

Housing, Feeding, Clinical Examination, and Prophylactic Treatments: All the research work was performed in the laboratories of the Institute of Physiology and Pharmacology, University of Agriculture Faisalabad. All animals were managed uniformly for 3 weeks. The aspect of animal welfare was fulfilled. They were provided clean bedding and were given proper ventilation. A light duration of 8-10 hours was provided to animals. The room temperature was maintained at 25-30 degrees Celsius. Deworming of animals was done a week before the experiment by giving two doses of ivermectin @ 400 micrograms per kg subcutaneously. Freshwater was provided to animals ad the labium. Fresh fodder was provided twice a day to animals. Bedding of wheat straw was provided to animals, which were also replaced on daily basis. For in vivo experimentation formal approval was taken from the institutional animal ethical committee.

Procurement and Grouping of Experimental Rabbits: Four rabbits weighing 1770 grams,1647 grams, 1387, and 1232 grams of either sex were selected for the study considering each animal as a single experimental unit. Animals were perfectly healthy with normal vitals.

Synthesis of Nanoparticles

Chemicals: Poly lactide-co-glycolide (PLGA), Polyvinyl alcohol (PVA**,** Quercetin (97%), Acetone**,** Distilled water.

Preparation of curcumin and quercetin PLGA nanoparticles

(i) Preparation of 5% aqueous PVA solution: First, 5g PVA was weighed properly. Then 100ml distilled water was taken in a neat and clean beaker and poured all the weighted amount of PVA in it. PVA was completely dissolved in distilled water by continuous stirring on a magnetic stirrer at room temperature.

(ii) Preparation of PLGA curcumin and quercetin solution in acetone: 100mg PLGA, 5 mg curcumin, and 5 mg quercetin were weighed accurately. Then 10mL acetone was taken in a screw cap test tube. The first weighted amount of PLGA was added in acetone and was shaken well using a vortex mixer. When PLGA had been completely dissolved, a weighted amount of quercetin and curcumin was added to the solution and was mixed completely on a vortex mixer to form the first formulation in which the curcumin and quercetin ratio is 50:50.

100mg PLGA, 2.5 mg quercetin, and 7.5 mg curcumin were weighed accurately. Then 10mL acetone was taken in a screw cap test tube. The first weighted amount of PLGA was added in acetone and was shaken well using a vortex mixer. When PLGA had been completely dissolved, a weighted amount of quercetin and curcumin was added to the solution and was mixed completely on a vortex mixer to form the first formulation in which the curcumin and quercetin ratio is 75:25.

100mg PLGA, 2.5 mg curcumin, and 7.5 mg quercetin were weighed accurately. Then 10mL acetone was taken in a screw cap test tube. The first weighted amount of PLGA was added in acetone and was shaken well using a vortex mixer. When PLGA had been completely dissolved, a weighted amount of quercetin and curcumin was added to the solution and was mixed completely on a vortex mixer to form the first formulation in which the curcumin and quercetin ratio is 25:75.

(iii) Formulation of nanoparticles: PLGA nanoparticles with the addition of quercetin and curcumin had been prepared by following the solvent displacement method with minor changes. First, 20mL PVA solution was taken in a beaker and put on a magnetic stirrer under 120rpm at 25°C. Then, by using micropipette PLGA curcumin and quercetin solution was added in PVA solution drop by drop slowly and steadily for homogenous distribution of drug and polymer in the formulation for three different formulations separately. As a result, dispersions of nanoparticles of three different formulations were prepared (Pool et al., 2012).

(iv) Purification of quercetin PLGA nanoparticles: After freshly preparing the dispersion of nanoparticles, it was kept on a magnetic stirrer for continuous stirring for 4 hours at 40°C, so that the organic phase (acetone) was completely removed. The remaining dispersion was then centrifuged at 25000rpm for half an hour at 25°C. The pellet of nanoparticles was settled down. The supernatant obtained after ultracentrifugation was discarded. Then the settled down pellet was dispersed in distilled water on a vortex mixer. The dispersion was sonicated for 10 minutes for complete homogenization (Tefas et al., 2015).

Characterization of quercetin PLGA nanoparticles: The previously prepared nanoparticles were characterized by the following parameters; Particle size, Zeta potential, and Polydispersity Index (PDI). Zetasizer Nano-ZS90 was used to measure particle size polydispersity index (PDI) of curcumin and quercetin loaded PLGA nanoparticles by dynamic light scattering method. Zeta potential was analyzed by electrophoretic light scattering.

Encapsulating efficiency: For the determination of encapsulating efficiency, first, a standard curve was generated with the help of different dilutions of pure curcumin and quercetin separately. The different dilutions of concentrations 5mcg/mL, 10mcg/mL, 15mcg/mL, 20mcg/mL, 25mcg/mL, were prepared in methanol. The maximum wavelength (λmax) of curcumin and quercetin was determined by taking a wavelength scan at a UV-visible spectrophotometer. The maximum wavelength (λmax) was 420 nm for curcumin and 300nm for quercetin. Then the encapsulating efficiency was measured by the ultracentrifugation method followed by ultraviolet-visible spectrophotometric analysis. Curcumin and quercetin loaded nanoparticles were centrifuged first at the speed of 25000rpm for 30minutes at 25°C and then the resulting supernatant was analyzed at 420 nm for curcumin and 300nm for quercetin and by using an ultraviolet-visible spectrophotometer. The concentration of curcumin was determined with the help of a previously constructed standard curve. Then the concentration of curcumin in nanoparticles was determined by subtracting the theoretical concentration of curcumin nanoparticles in each formulation dispersion from supernatant concentration. The encapsulating efficiency was calculated by following the given formula; (Bagad and Khan 2015). Similarly, the concentration of quercetin in nanoparticles was determined by subtracting the theoretical concentration

of quercetin nanoparticles in each formulation dispersion from supernatant concentration.

% Encapsulating Efficiency = (Curcumin weight in nanoparticles)/(Initial weight of curcumin used)×100

% Encapsulating Efficiency = (Quercetin weight in nanoparticles)/ (Initial weight of Quercetin used) ×100

Excision of Wounds and treatment protocol: For wound excision, the thoracolumbar area was shaved properly with an electrical clipper. Then the area was scrubbed with a general cleansing agent. For this purpose methylated spirit and tincture of iodine were used. Local anesthesia through infiltration was given before surgical excision wounds inflicted on rabbits to fulfill the requirement. Antiseptic solution soaked cotton swabs were used to make the area germ-free. The surgical area was marked with a permanent marker and a scale was used to mark 1.5 cm. Four wounds of 1.5 cm were created on lateral sides of the animal at an appropriate distance of 2 cm from the midline and one wound was created over the dorsal midline of thoracolumbar regions by restraining the animal in sternal recumbency. All wounds were marked with numerical letters as 1, 2, 3, 4, and 5 to differentiate the treatment applied to them.

Formulations and the standard drug were applied to wounds from the day on which surgical excision wounds were given to animals as shown in Fig. 1. Healing was observed by monitoring wounds daily and recorded at 3-day intervals. The proper post-operative care was done by keeping them separate to avoid scratching and vitals were monitored for general health status.

Wound Healing Evaluation: Following parameters were used to evaluate wound healing:

Wound contraction rate: "The percentage reduction in original wound size" is known as wound contraction rate Measurement scale was used to measure the wound dimensions. Reading was taken in millimeters.

Wound Healing time: Healing starts right from the time of wound infliction and it continues till the time at which complete regeneration of damaged tissue occurs. Healing time was evaluated on daily basis. The wounds were visually inspected daily. Daily observations of estimated daily healing time were added till the day in which scar tissue dropped off (Madhumathi et al., 2010).

Statistical Analysis: Two-way ANOVA followed by DMR was applied to data to see the statistical difference between treatments.

Results

Characterization of Nanoparticles: The behavior of nanoparticles-based drug delivery systems depends on the physical and chemical properties of the nanoparticles, like entrapment efficiency, zeta potential, Polydispersity index, and particle size. Results of characterization are shown in Table 1.

Wound contraction rate: Wound contraction rate in curcumin and quercetin (75:25) loaded PLGA nanoparticles treated wound was highest T2 followed by T1, T3, and T4. This trend remained consistent throughout the study period shown in Fig. 2. The results were statistically analyzed by applying two-way ANOVA followed by DMR. As ($p<0.05$) hence proved statistically significant.

Wound healing time: Wound healing time was C=14 days T1=8 days T2=7 days T3=9 days T4=10 days as shown in Fig. 3.

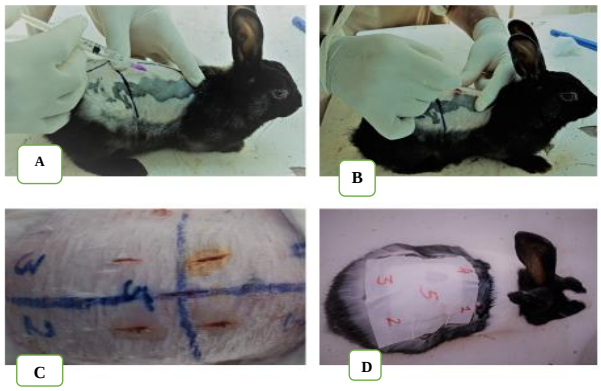

Fig. 1: Application of drugs.

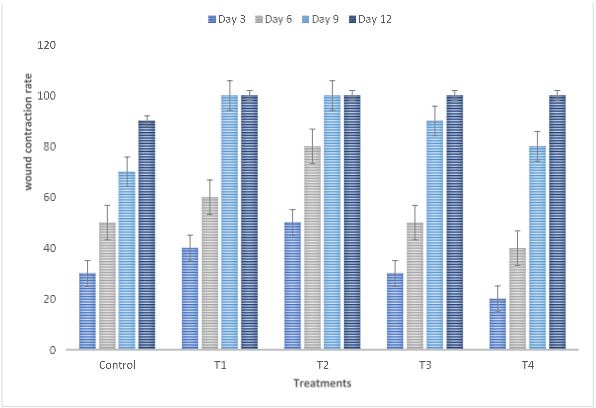

Fig. 2: Wound Contraction rate.

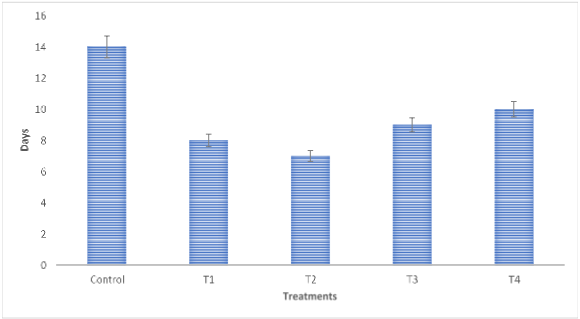

Fig. 3: Effect of different treatments on the healing time of wounds.

Table 1: Characterization of Nanoparticles

Sr. No	Formulation	Size(nm)	Zeta Potential(mV)	PDI	Encapsulating Efficiency
1	Curcumin and Quercetin loaded PLGA nanoparticles (50:50)	243.3	-1.83	0.131	C =86.25% Q =74.4%
2	Curcumin and Quercetin loaded PLGA nanoparticles (75:25)	216.3	-3.2	0.136	C=88.48% Q=77.7%
3	Curcumin and Quercetin loaded PLGA nanoparticles (25:75)	215.5	-3.7	0.138	C= 64.84% Q= 83.85%

Discussion

Zeta Size: Zeta size is an important parameter for the characterization of nanoparticles. The prepared formulation exhibited a diameter of 243.3 nm, 216.3, and 215.5 of 1st 2nd, and 3rd formulations respectively, which lies within the range of 10-1000nm confirming nanoparticles synthesis in the prescribed size range (Verma et al., 2010) If the size of the nanoparticle is small it shows an increase in surface area and ultimately an increase in drug release. Smaller particles are more likely to aggregate during storage and transportation. The extent to which a polymer is degraded is also dependent on size like degradation of PLGA increase with the increase in particle size *in vitro* (Redhead et al., 2001).

Zeta potential: Zeta Potential is the charge present on the surface of a particle. The storage stability of a colloidal dispersion is affected by zeta potential. Zeta potential describes the electric barriers that hinder the nanoparticles from agglomeration and aggregation. If a particle acquires very low zeta potential it may likely aggregate because electric repulsion would not be adequate. For a stable nanosuspension, the required value of zeta potential should be -30mV for electrostatically stable suspension and -20mV for sterically stabilized systems. If a system of nanosuspension has zeta potential has a value of 20mV it shows that nanosuspension is likely to aggregate quickly and has short-term stability. Zeta potential provides information about storage conditions and predicts the discarding time of nanosuspension. If the zeta potential value varies between -5 to 5 it shows that the suspension will have fast aggregation. In the present study, the zeta potential value of the prepared formulation was -1.83mV,-3.2 mV, and -3.8 mV respectively. As the PLGA polymer is uncharged it gives negative zeta potential near to neutrality. Its value is negative because PLGA polymers acquire a slightly negative zeta potential which is likely to be due to the ionization of glycolic acid in PLGA polymer. The negative zeta potential is beneficial in drug delivery and prolonging the circulation time property (Jacobs et al., 2000).

This rule applies to nanosuspensions which have pure electric stabilization and have stabilized with low molecular weight. This will not be valid for preparations that use stabilizers with high molecular weight. In a suspension, every single molecule is covered by opposite charges and forms a stern layer. Exterior to this stern layer there is a cloud-like area made by the hydrated counter ions. This cloud-like area is called a diffuse electric layer. A layer at the exterior of this layer is called the shear plane. Zeta potential is theoretically defined as the potential at this shear plane. An electric double layer plays a vital role in the stability and is formed by the stern layer and a portion of the diffuse layer (Gong et al., 2010).

Polydispersity Index: Polydispersity index is defined as the ratio of the size difference to the mean diameter of the particle. It gives information about the homogeneity of particles in a suspension and its range varies between 0-1. If the value of PDI is close to zero it shows that the suspension is homogenous and if the value is greater than 0.3 it shows the heterogeneity of suspension. High values of PDI show that there is much variation in particle size. In the present study, the PDI of curcumin and quercetin loaded nanosuspension was found to be 0.36,.27, and .138 and which shows that solution is homogenous. The results are comparable with (Müller and Peters, 1998) who describe .25-.5 as broad distribution.

Encapsulating Efficiency: Entrapment efficiency gives an idea about the percentage drug that is successfully entrapped/adsorbed into nanoparticles. The encapsulation efficiency of the prepared formulation was 64.84%-88.48% that is good encapsulating efficiency.

Wound Contraction Rate: The wounds, which were treated with curcumin and quercetin T2 (75:25) loaded PLGA nanoparticles were highest followed by T1, T3, and T4. This trend was consistent throughout the study period. Wound healing was higher in combinations of curcumin and quercetin because they have synergistic effects and results are comparable with (Kundur et al., 2019) who also described the synergistic effect of curcumin and quercetin. Curcumin is more efficacious than quercetin described by (Heeba et al., 2014) therefore T2 has the maximum amount of curcumin which's why it shows the highest wound contraction rate followed by T1, T3, and T4. The results are also comparable with (Miah et al., 2017) who described ethanol extract of curcumin shows better wound healing than standard drug povidone-iodine.

Wound Healing Time: Wound healing time was C=14 days T1=8 days T2=7 days T3=9 days T4=10 days. The

results are comparable with (Panchatcharam et al., 2006) who describes curcumin and with (Vedakumari et al., 2017) who describes quercetin increases wound healing by acting at every phase of wound healing.

Curcumin and quercetin loaded PLGA nanoparticles increase the bioavailability of curcumin and quercetin and which results in an increase in their wound healing potential.

Conclusion: It can be concluded based on all the above studies that curcumin and quercetin loaded PLGA nanoparticles increase the bioavailability of curcumin and quercetin and which results in an increase in their wound healing potential.

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