

Immunostimulatory Potential of Papain Encapsulated Solid Lipid Nanoparticles

Suriyakala Perumal Chandran*, Kannikaparameswari Nachimuthu

Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, India.

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ABSTRACT

Stimulation of immune function is the primary focus in the field of cancer metastasis treatment. Cancer cells are circulated through the lymphatic system to support metastasis to lymph nodes and adjacent organs. In order to treat metastatic cancer, the immune function should be reactivated to recognize the cancer cells against the natural killer activity and expose the cancer cells to the chemotherapy. Proteolytic enzymes have been explored in cancer therapy including the inhibition of metastasis and improve the body immune mechanism. To achieve this hypothesis, we proposed papain loaded solid nanoparticles to stimulate the immune mechanism by investigating the following; (i) measurement of human lymphoblast cells using TK6 Spleen lymphoblast cell line (ii) proliferation of T cells and secretion of cytokines (IL-2 and IL-4) using T-lymphocytes 8E5 cells (iii) determination of in-vitro cytokines production by activated macrophages. This research evidenced that papain loaded solid lipid nanoparticles possess significant immunostimulatory efficacy, thus it can be proposed as an adjuvant therapy for cancer metastasis.

INTRODUCTION

Most of the cancerous cells are migrated through the lymphatic system to develop metastasis at lymph nodes (LNs) and adjacent organs (Karaman and Detmar, 2014). Cancer-related lymphatics and leaking LNs are responsible for cancer cell migration and invasion, Secondly; the lymphatic system absorbs dietary fats in the intestine and transports them back into systemic circulation (Dixon, 2010). The alarming recognition of lymph node metastasis in cancer has provoked latest investigations to loosen the cellular signals and changes occurred in this multifaceted progression (Cho *et al.*, 2015). Few changes including lymphangiogenesis, development of immunosuppressive cells, up-regulation of cytokines and chemokines, and blood vessel restructure in the lymph node promotes the entry of cancer cells, migration, and survival in the lymph node (Ji, 2017). Though, the fate of cancer cells in the lymph node is contentious (Cady, 2007). Cancer progression and survival are uncontrollable processes responsible for the interplay between cancer cells, normal cells,

and immune mechanisms. Additional factors including cellular modification due to infection, disease-induced stress also responsible for cancer growth or suppression (Senger *et al.*, 1983). Usually, CD4⁺ helper T (Th)1 cells and CD8⁺ cytotoxic T cells (CTL) control cancer development involving their secretion of cytotoxins and interferon (IFN)- γ (Alberts *et al.*, 2002). However other issues such as chronic inflammation may dominate these effects to support cancer growth (Blackwill and Mantovan, 2002; Rakoff-Nahoum, 1996). In order to treat metastatic cancer, the immune function should be reactivated to recognize the cancer cells against the natural killer activity and expose the cancer cells to the chemotherapy. Secondly, the anticancer compounds need to be targeted to the lymphatic system thereby; the escaped metastatic cancer cells will be shattered by anticancer agents without/minimal damage to healthy cells and avoid the first-pass effect.

The therapeutic application of enzymes is considered to be a very promising field due to the numerous health benefits including anticancer, anti-inflammatory, antibacterial, antifungal, and wound healing (Aoyagi, 1996; Enzyme Technology, 2018). Several publications revealed that oral proteolytic enzymes have the capability to enhance the immune mechanism by arbitrating via disturbing the appearance of adhesion molecules, involvement

*Corresponding Author

Suriyakala Perumal Chandran, Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, India.

E-mail: suriyakalabiochemsitry@gmail.com

in the cytokine linkage and impact on protease-activated receptors (Biziulevicius, 2006). The effect of various cellular components of the immune system (granulocytes, NK cells, macrophages and T lymphocytes) and the impact on fabrication and eradication of immune-complex have also been investigated (Wald *et al.*, 2008).

Papain is a powerful proteolytic enzyme obtained from the unripe fruit of *Carica papaya*. It is widely available and commonly used as a digestive enzyme. However, this enzyme potential and oral bioavailability of papain were hindered by instability at stomach pH. In order to attain maximum therapeutic potential, suitable carrier delivery system has to adopt to encapsulate the papain enzyme (Manu *et al.*, 2011). Numerous carrier delivery system including liposomes, polymeric nanoparticles, micelles, nanoemulsion and lipid nanoparticles have been developed to improve the stability and oral bioavailability of many bio-actives and enzymes. Solid lipid nanoparticles (SLNs) is one of the versatile lipid-based drug delivery systems possesses numerous advantages including, enhanced enzyme stability and by-pass acidic degradation, prolonged drug release and expected to overcome the conventional delivery and to fulfill the requirements for a most favorable drug delivery system (Ce *et al.*, 2011). From our previous study, we optimized the papain loaded solid lipid nanoparticles and investigated the formulation characteristics including crystalline property, thermal behavior, particles size, morphology, *in vitro* drug release profile and *in vitro* cytotoxicity study on human adenocarcinoma cells (HT-29 cell line). In this research, we aimed to analyze the immunostimulatory potential of papain and papain loaded solid nanoparticles to treat metastatic cancer. To achieve this hypothesis, we proposed papain and papain loaded solid lipid nanoparticles to stimulate immune mechanism by investigating the following parameters; (i) measurement of human lymphoblast cells using TK6 Spleen lymphoblast cell line (ii) proliferation of T cells and secretion of cytokines (IL-2 and IL-4) using T-lymphocytes 8E5 cells (iii) determination of in-vitro cytokines production by activated macrophages.

MATERIALS AND METHODS

Papain enzyme was obtained from Kotra Pharma as a compliment sample. Cetyl alcohol, Polyethylene glycol 4000, and other chemicals and reagents were used as an analytical grade.

Preparation of papain loaded solid lipid nanoparticles (P-SLNs)

The solid lipid nanoparticles were formulated by Melt dispersion-ultrasonication technique. In this study papain and carrier material (1:5 ratio) was used and the ratio was optimized from our previous study. Papain enzyme was thoroughly mixed with molten lipidic phase (Cetyl alcohol), and this hot blend was then poured to continuous water phase containing emulsifier (PEG-4000; 2% w/v) at 55°C. The obtained hot dispersion was sonicated with a probe sonicator (5 min at 55°C amplitude, 0.5 frequency) for 1-2 min. The temperature of warm nanoemulsion (o/w) was then reduced to 10-15°C to solidify the emulsion globules into rigid solid particles. The papain encapsulated solid lipid nanoparticles (P-SLNs) were isolated by centrifugation at 10,000 rpm for 10 min. The obtained P-SLNs were dried at room temperature about 24 h and store the nanoparticles container in a cool and dry place.

Preparation of placebo solid lipid nanoparticles

The placebo SLN was prepared by the above-mentioned procedure but without the addition of papain.

Measurement of the proliferation of human lymphoblast cell line

The culture of human spleen lymphoblast TK6 cells (Klungsupya et al., 2015)

Human spleen lymphoblast cells (TK6 cells line) were seeded in vented culture flasks at a density of approximately 5×10^4 flask in 10 ml of TK6 medium (45 ml of RPMI, 5 ml fetal calf serum (10%), 0.5 ml penicillin/strepto mycin and 0.5 ml L-glutamine). Primary cultures were incubated at 37°C in 5% CO₂. The cells were then passaged every two days and reseeded at a density of 5×10^4 .

Papain treatment

At the 7th passage, TK6 cells were seeded in a CellStar 12 well suspension culture plate at a concentration of 5×10^5 cells/well. The cytotoxicity assay of papain and P-SLN was performed using different concentrations of papain (at 20, 40, 60, 80 and 100 µg/ml). Total 5×10^4 cells were exposed to 1 mL of each of the concentrations of papain and P-SLN to a corresponding well with an untreated control. The plate was then incubated (37°C, 5% CO₂) for 24 h and then processed for assessment of cell viability by MTT assay (Klungsupya *et al.*, 2015).

The proliferation of T-cells and secretion of cytokines

The mitogenic potential of the papain and P-SLN was investigated and compared with standard T-cell mitogen, Con A. Then, estimation of IL-2 and IL-4 secretion in the growth medium by the cells (an index of T-cell activation) is done to assess T-cell activation. Human T-lymphocyte 8E5 cell line was procured from National Centre of Cell Science, India and cultured in RPMI-1640 medium addition with 10% of fetal bovine serum. At 5% µg/ml of confluence, they were harvested by spinning them down at 3000 rpm for 4 min (as they are suspension cells), cell pellet suspended in growth medium and seeded into a 24 well plate in a seeding density of 5×10^4 cells/well. The cells were then incubated for 72 h in the presence (or) absence of a T-cell-specific mitogen (Concanavalin A or Con A from *Canavalia ensiformis*; Cat. No: C5275-5MG; Sigma) and papain. After incubation, the cell culture medium was removed carefully and stored at -20°C for the estimation of IL-2 and IL-4 by MTT assay (Greenfeld *et al.*, 2007).

Measurement of IL-2 and IL-4 produced by activated T-lymphocytes by sandwich (indirect) ELISA

Cytokine (IL-2 and IL-4) release of papain and P-SLNs treated cells were quantified using sandwich (indirect) ELISA method. Wells of ELISA plate (NUNC Maxisorp® cat. No. 442404) was coated with 100 µL of IL-2 polyclonal IgG capture antibody (sc-37496; 1 µg/mL final concentration, Santa Cruz Biotech) in bicarbonate buffer (pH 9.6). Plates were incubated at 4°C overnight to facilitate attachment of antibodies to the plate. After incubation, the coating solution was removed and washed with washing buffer containing NaCl and Tween 20 in PBS, (pH

7.4). The wells were then blocked with 200 μ L of blocking buffer containing 1% BSA Fraction V in PBS (pH 7.4) and incubated at room temperature for 2 h. The plates were washed with washing buffer. The 100 μ L of IL-2 standard and culture medium were added to the assigned wells bicarbonate buffer (0.39-50 ng/100 μ L). Then the plates were incubated for 1 h at 37°C and then, the plates were washed and 100 μ L of IL-2 monoclonal IgG detection antibody (sc-398253; 1 μ g/ml final concentration, Santa Cruz Biotech) in dilution buffer containing BSA and NaCl in PBS (pH 7.4) was added to all the wells except for the well containing the blank and incubated for 1 h at 37°C. After incubation, the wells were washed with washing buffer. And 100 μ L of HRP-conjugated secondary antibody (1:5000 dilution, rabbit anti-mouse IgG-HRP) was added to all the wells. The plate was incubated at 37°C for 30 min. After incubation, the plate was washed thrice with washing buffer and 100 μ L of freshly prepared substrate (TMB in DMSO and citrate phosphate buffer (pH 5) containing H₂O₂) was added to all wells, and incubated in dark 37°C for 15 min for color development. After incubation, the reaction was terminated by treating 50 μ L of H₂SO₄ (2.5 N) per well and the absorbance was measured at 450 nm using ELISA reader. Each sample was analyzed in triplicates (Greenfeld *et al.*, 2007).

Antibodies for IL2 measurement

Capture Ab-IL-2 polyclonal IgG (sc-34796, Santa Cruz Biotechnology); Detection Ab-IL-2 monoclonal IgG (sc-398253, Santa Cruz Biotechnology).

Antibodies for IL4 measurement

Capture Ab-IL-4 polyclonal IgG (sc-1260, Santa Cruz Biotechnology); Detection Ab-IL-4 monoclonal IgG (sc-12723, Santa Cruz Biotechnology); HRP-conjugate (secondary antibody) - rabbit anti-mouse IgG-HRP (sc-358914 Santa Cruz Biotechnology) – common to both IL-2 & IL-4.

Standards

IL-2 standard protein (sc-4879, Santa Cruz Biotech); IL-4 standard protein (sc-4595, Santa Cruz Biotech).

Determination of *in-vitro* cytokines production by activated macrophages

Macrophages, significant components in the human immune surveillance mechanism, respond actively to inflammation by producing pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6; high levels of these cytokines can cause systemic complications. TNF- α is a pleiotropic inflammatory cytokine and can stimulate the production or expression of IL-1 β and IL-6.

Cell culture and seeding of RAW264.7

RAW264.7 cell line was procured from National Centre of Cell Science, Pune and cultured in DMEM medium addition with 10% FBS. When confluence, the culture was trypsinized and seeded into a 96 well plate in a seeding density of 5×10^4 cells per well and then incubated at 37°C and CO₂ overnight to allow attachment.

Papain treatment

Serial dilutions of the papain, P-SLN and salicylic acid

(500, 250, 125, 62.5, 31.25, 15.625, 7.812 and 3.906 μ g/mL respectively) were prepared and added to each well. Untreated cells were served as a negative control. Then the plates were incubated for 24 h and investigate the potential of cell viability.

Determination of cell viability

At the end of drug exposure, the growth medium was aspirated from each well and 50 μ L of MTT solution (5 mg/ml) was added to each well. The plate was incubated for 4 h at 37°C in dark condition to facilitate the formation of formazan crystals. After incubation, 200 μ L of acidified DMSO was added to each well to dissolve the formazan crystals to give a purple color. Total 25 μ L of glycine buffer was added to each well and the absorbance was recorded in a plate reader at 570 nm. The IC₅₀ value of the test samples was calculated from the graph of absorbance along Y-axis against the concentration along X-axis.

In vitro anti-inflammatory activation of RAW264.7 cells

RAW264.7 cells were cultured in a 12-well plate in triplicate at a seeding density of 5×10^5 cells/well. The volume of medium per well was 1 mL. The cells were then stimulated with bacterial (*E. coli*) lipopolysaccharide (L5418-2ML Sigma-Aldrich) at 2 μ g/mL per well and the cells were incubated for 18 h at 37°C in the presence of salicylic acid and papain. After the incubation period, the growth medium from each well was collected for TNF-estimation. Grouping of cells was done as follows; Group 1 = cell control; Group 2 = LPS-control (2 μ g/ml); Group 3 = LPS control + papain drug (50 μ g/ml); Group 4 = LPS control + SLN Trial-3 (50 μ g/ml); Group 5 = LPS control + salicylic acid (2.5 mM).

TNF- α Assay

TNF- α level was analyzed by ELISA sandwich method. A captured antibody solution added to each well in 96-well plate and incubated at 4°C overnight. The plate was washed repeatedly using wash buffer solution (300 μ l), and then incubated for 1 h in a shaker. Approximately 1 μ l of assay buffer solution was added to each standard and sample well. Plates were shaken by orbital shaker (at 25°C for 1 h). Then the plate was washed few times and subsequently, 100 μ l of the dilute Avidin-HRP solution was added to each well and incubated for 30 min at 25°C in an orbital shaker. The plate was washed again, and then added 100 μ l of substrate solution, incubated for 10 min under the dark room. The reaction was stopped by adding 100 μ l of stop solution and the absorbance was measured (Soromou *et al.*, 2012). TNF- α estimation was done by sandwich ELISA as mentioned before using the following; capture antibody-TNF- α polyclonal IgG (sc-1348; 1 μ g/ml final concentration, Santa Cruz Biotechnology); standard protein-TNF- α (hBA-158; sc-4564, Santa Cruz Biotechnology); detecting antibody-TNF- α monoclonal IgG (sc-133192; 1 μ g/ml final concentration, Santa Cruz Biotechnology); secondary antibody-HRP-conjugate (1:5000 dilution, rabbit anti-mouse IgG-HRP; sc 35891 Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

The P-SLNs have formulated by melt dispersion-ultrasonication technique with same formulation and processing variables which were optimized from our previous study (Chandran

et al., 2018) and investigated the efficacy solid lipid nanoparticle as a colloidal carrier system to enhance the immunomodulatory action as compared with pure papain.

The proliferation of Human lymphoblast cell line

The treatment of papain and P-SLN on proliferation of Human lymphoblast TK6 cell line showed the percentage proliferation was 97.05 and 98.22% which was treated with 20 $\mu\text{g}/\text{mL}$ concentration of papain and P-SLN respectively, whereas the proliferation rate 91.33% and 92.5% was achieved at 100 $\mu\text{g}/\text{mL}$

mL concentration of papain and P-SLN respectively. These results (Fig. 1) revealed that there was no significant difference on the rate of proliferation which was treated the cells with papain at 20 $\mu\text{g}/\text{mL}$ of papain and P-SLN respectively whereas, the percentage of proliferation was decreased with papain and P-SLN at 100 $\mu\text{g}/\text{mL}$ concentration. The papain and P-SLNs did not seem to stimulate the proliferation of lymphoblast cells considerably at increasing the dose compared to the control. It was revealed that the papain and P-SLNs do act as a mitogen to the lymphoblast cells and do not show any toxicity to the cells, indicating it is safe for use.

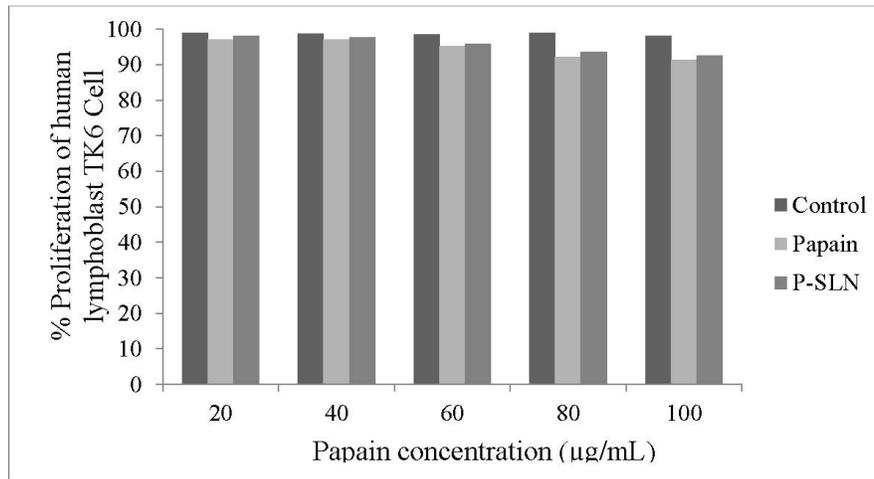


Fig. 1: The percentage proliferation of human lymphoblast (TK6 cells) treated with different concentration of Papain and PSLNs.

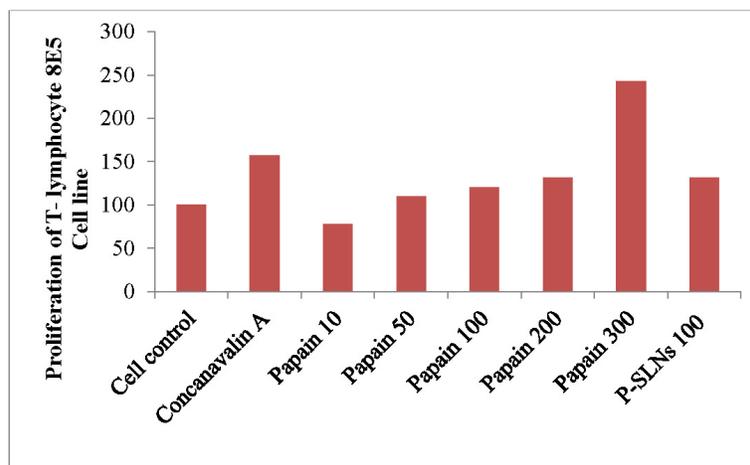


Fig. 2: Proliferation of T-lymphocyte 8E5 Cell line treated with different concentration of papain and P-SLNs.

The proliferation of T-cells and secretion of cytokines

The standard T-cell mitogen Con A stimulated the proliferation of cells which was indicated by 156.78% viability compared with control group. The papain also demonstrated a dose-dependent stimulation of T-cell proliferation (77.98%–242.29% viability) compared with cell control (Fig. 2). The T-cell proliferation of P-SLNs showed 131% of cell viability at 100 $\mu\text{g}/\text{mL}$ concentration of papain. This gives a strong indication of the activation of T-cells by the papain and P-SLNs which can be proved by the further estimation of IL-2 and IL-4 secreted in

the cell culture medium by the activated T-cells. Hence, from the proliferation assay, it can be concluded that P-SLN has the potential to stimulate proliferation of T-cells by *in-vitro* compared to the papain. Cytokines are significant signaling compounds, produced by immune cells and affect immune function. IL-2 also referred as T cell growth factor (TCGF), is a glycosylated alpha-helical polypeptide, which is needed for T-cells proliferation, natural killer cells (NK) cytolytic action, differentiation of regulatory T cells, modulation of T helper (Th) cell differentiation and activation-induced cell death (Thèze, 1998). IL-4 contributes

in several B-cells activation precursors and other cell types. It is a co-stimulator of DNA production. It persuades the expression of class-II MHC molecules on resting B-cells. It enhances both secretion cell surface of IgE and IgG1. It also regulates the expression of the low-affinity Fc receptors; IL-4 is secreted (Hurdoyal *et al.*, 2017).

Estimation of cytokine offers a useful technique for the precise analysis of cytokines such IL-2, and IL-4 and the immunomodulatory effect was investigated by ELISA method.

Con A stimulated the T-cells evident by the release of IL-2 (3.84 ng/mL) and IL-4 (6.21 ng/mL) when compared to cell control [IL-2 (0.072 ng/mL) and IL-4 (0.24 ng/mL)]. Papain was also found to stimulate the T-cells in a dose-dependent manner (10-300 µg/mL). PSLNs treated cells were also showed significant release IL-2 & IL-4 from T-cells as compared to pure papain at the same concentration (100 µg/ml). It revealed that the carrier delivery system such as solid lipid nanoparticles possesses the capability to stimulate IL-2 & IL-4 from T-cells then pure papain (Fig. 3).

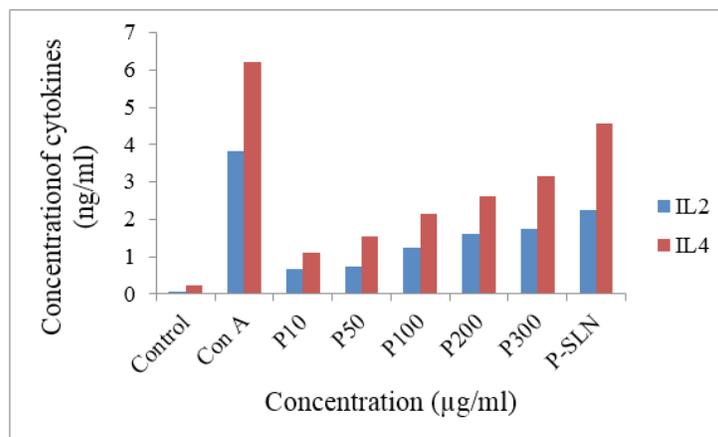


Fig. 3: Secretion of IL-2 and IL-4 treated with different concentration of papain and P-SLNs.

In-vitro cytokines production by activated macrophages

TNF- α is a vital cytokine responsible for inflammatory reaction by triggering nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), cytokine, and adhesion molecule inducer (Bastian Hoesel). TNF- α inhibitory potential estimation is important in anti-inflammatory agent screening since this cytokine is a significant mediator of the inflammatory process (Sandhiutami *et al.*, 2017). This research revealed that papain can inhibit pro-inflammatory cytokines of TNF- α , and it was similar to standard. The IC₅₀ values of papain, P-SLNs, and salicylic acid were determined by TNF- α ELISA assay using RAW264.7 cells were 250 µg/ml, 250 µg/ml, and 500 µg/ml respectively (Table 1).

Table 1: The IC₅₀ values of papain P-SLNs and salicylic acid on RAW264.7 cells.

S. No.	Sample	IC ₅₀
1	Papain drug	>250 µg/mL
2	P-SLNs	>250 µg/mL
3	Salicylic acid	>500 µg/mL

The lipopolysaccharides (LPS) treated Raw 264.7 cells have been widely utilized to investigate the inflammatory responses (Pradeep Kumar *et al.*, 2017; Lee *et al.*, 2013). The investigation of papain and salicylic acid effect towards the secretion of TNF- α revealed that both treatments were able to dose-dependently reduce TNF- α concentration in LPS-induced RAW264.7 cells. LPS stimulated the production of TNF- α in the cells (2.767 µg/mL) when challenged with it as compared to cell control (0.367 µg/mL). Salicylic acid, a standard anti-inflammatory agent, prominently reduced TNF- α production (1.160 µg/mL) as compared to the LPS control. Pure drug and P-SLN were

also found to reduce TNF- α production (2.433 & 2.055 µg/mL respectively); the anti-inflammatory activity of P-SLN was found to higher than the pure papain (Fig. 4). Hence, the data show that the pure drug and SLN both possess anti-inflammatory activity concerned to TNF- α production, the papain with SLN carrier system have more prominent in anti-inflammatory activity over the pure papain.

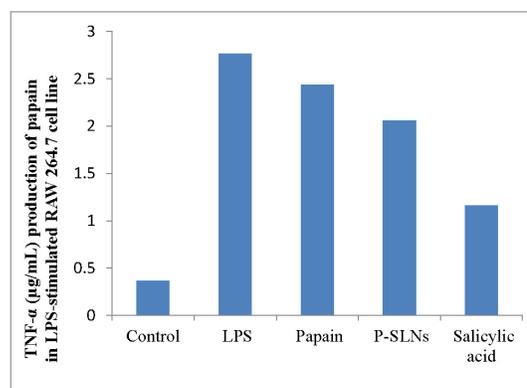


Fig. 4: TNF- α production of papain, P-SLNs and standard (Salicylic acid) in LPS-stimulated RAW 264.7 cell line.

CONCLUSION

Reactivation of immune function is the primary focus in the treatment of cancer metastasis. The immune function is drastically compromised during the metastasis stage and cancer cells take this advantage to appear and disseminate through the lymphatic system to support metastasis to the adjacent organs. In order to treat metastatic cancer, the immune function should

be reactivated to recognize the cancer cells against the natural killer activity and expose the cancer cells to the chemotherapy. It concluded that the advantageous effects related to anti-inflammatory potential attributed to papain might be associated with the inhibition of chemical mediators. Papain loaded solid nanoparticles has proven that it has a potential cytotoxic effect on human colorectal adenocarcinoma cells and immunomodulatory action. This research showed the favorable immunostimulatory mechanism against P-SLNs treatment. These results will enlighten us to proceed to *in vivo* study to confirm the lymphatic targeting efficiency of P-SLNs and immunostimulatory potential against metastatic cancer.

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