International Journal of Veterinary Sciences and Animal Husbandry

**ISSN:** 2456-2912
VET 2024; 9(1): 1451-1456

www.veterinarypaper.com
Received: 17-10-2023
Accepted: 23-12-2023

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# Comprehensive investigation of beta-caryophyllene for wound healing potential: *In silico* docking on key targets and *in vitro* evaluation on mouse fibroblast cell line

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**DOI:** https://doi.org/10.22271/veterinary.2024.v9.i1t.1155

**Abstract**
Beta-caryophyllene is a natural odorous bicyclic sesquiterpene found in various essential oils known for its anti-inflammatory, antioxidant, analgesic and wound healing properties. In this study, *in silico docking* and *in vitro* analyses have been used to appraise the wound healing potential of beta-caryophyllene (BCP). Molecular docking analysis was performed with Auto Dock V 4.0 using BCP against five receptors such as TGF- β1 (PDB ID- 1PY5), TGF- β2 (PDB ID- 1M9Z), VEGF (PDB ID-3QTK), GSK-3β (PDB ID-1Q5K) and MMP-9 (PDB ID-5UE4) and the binding energy was determined. Cytotoxicity of BCP was evaluated using MTT assay and cell migration ability of BCP was assessed using scratch assay in L929 mouse fibroblast cell lines. *In silico* screening identified strong binding affinities between BCP and crucial wound healing targets with binding energy ranging from -6.84 Kcal/mol to -3.77 Kcal/mol with highest negative binding energy with GSK-3β endorsing its anti-inflammatory property. Subsequent *in vitro* investigations demonstrated the non-cytotoxic nature of BCP up to 320 µM concentration, validating its safety profile. Furthermore, in scratch assay, BCP exhibited a distinguished induction of cell migration in L929 mouse skin fibroblasts with 19.78%, 34.32%, 91.34% wound closure at 12hrs, 24hrs and 48hrs of incubation respectively, emphasizing its positive impact on wound healing processes. This study provides valuable insights into the molecular interactions of BCP and highlights its pledging role as a potential wound healing agent, offering a foundation for further research and therapeutic development.

**Keywords:** Beta-caryophyllene, wound healing, GSK-3β

## 1. Introduction
Wound healing is a dynamic process comprising four overlapping phases: hemostasis, inflammation, proliferation, and remodeling, each orchestrated to restore tissue integrity and functionality (Hunt *et al.*, 2000) [1]. The normal wound healing begins with hemostasis accomplished with the help of growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) from platelets followed by the inflammatory phase with infiltration of neutrophils and phagocytosis. Fibroblasts migrate to commence the proliferative phase and accrete new extracellular matrix (ECM). Finally, crosslinking and organisation of new collagen matrix occurs during the remodelling phase. In non-healing wounds, this effectual and schematised process is lost and a state of chronic inflammation depicted by profuse neutrophil infiltration with associated reactive oxygen species and destructive enzymes were discerned (Diegelmann and Evans, 2004) [2]. Numerous cytokines and growth factors, including glycogen synthase kinase-3β (GSK-3β), matrix metalloproteinases (MMP), interleukin (IL) (inflammatory phase), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tumour necrotic factor- alpha (TNF-α) (proliferative phase) and transforming growth factor beta (TGF-β) (remodelling phase) and others can be referred as biomarkers in the healing of wounds (Lindley *et al.*, 2016) [3]. Various local and systemic factors interrupt the wound healing process, causing delay in the reconciliation of the wound. Prolonged inflammation, excessive reactive oxygen species generation and bacterial infection are some formidable influencing factors in the wound as it can profoundly impair the healing process (Rodrigues *et al.*, 2019) [4].

Consequently, curtailing the imbalance in the cellular responses during wound healing process at the wound site is a crucial facet of effective wound care. Hence wound management possess a serious challenge to the wound care professionals and consume a prodigious deal of healthcare resources around the globe. Henceforth, there is an exigency to explore the novel avenues for combating the infection as well as other aspects to expedite the wound healing.

Natural compounds like essential oil have recently gained attention in pharmaceutical industries for their several holistic therapeutic potentials which is mainly attributed to its bioactive constituents. Beta-caryophyllene is a naturally occurring bicyclic sesquiterpene that is widely present in essential oils derived from a variety of fruits, spices, and decorative as well as medicinal plants. Beta-caryophyllene was discovered to produce a full agonist effect on G-protein coupled cannabinoid type 2 (CB2) receptors, a significant therapeutic target in a number of illnesses. Numerous pharmacological properties, including those that are immunomodulatory, antibacterial, hepatoprotective, neuroprotective, nephroprotective, antioxidant, anti-inflammatory and wound healing properties have been documented in experimental investigations (Sharma *et al.*, 2015) [5].

Understanding the interplay between betacaryophyllene and these markers provides insights into its probable mechanisms of action in wound healing. This study aims to bridge the gap between *in silico* predictions and *in vitro* experimental outcomes, shedding light on the multifaceted effects of betacaryophyllene in the context of wound healing.

## 2. Materials and Methods

### 2.1 Test Substances

Beta-caryophyllene (> 95 percent) was procured from M/s Sigma-Aldrich India Ltd., Bangalore, India. They were kept tightly closed and were stored in a dry ventilated area, protected from light.

### 2.2 Cell Line

The cell line used for the study was L929 mouse skin fibroblasts procured from National Centre for Cell Science (NCCS), Pune, India. The L929 mouse skin fibroblasts were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10 percent foetal bovine serum, one percent antibiotic- antimycotic solution containing penicillin-streptomycin and amphotericin B. This cell line was maintained in a humidified incubator at 37 °C with five percent carbon dioxide (CO2). After attaining 70 percent confluency, the cell line was subcultured by enzymatic digestion with 0.25 percent trypsin-1mM EDTA solution and these trypsinised cells were used for the study.

### 2.3 *In silico* Screening of Beta-Caryophyllene with Various Targets of Wound Healing

The *in silico* screening to analyse the interaction of BCP with target proteins in wound healing were done by using AutoDock 1.5.6 (www.mgltools.scripps.edu) according to the method followed by Archana *et al.* (2022) [6]. Chemical structure of BCP as given in figure 1 (SDF files) was obtained from the PubChem Compound Database and Marvin View 17.25.0 (www.chemaxon.com) were used for modifying the ligands (SDF files to. mol2 format) and the SDF file was converted to PDB format using Open Babel.

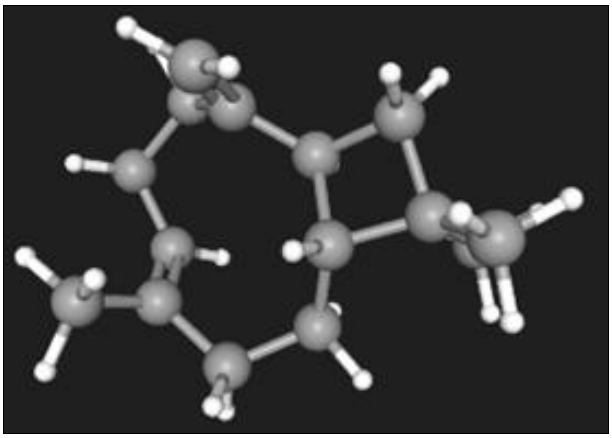

**Fig 1:** Structure of Beta-caryophyllene

Transforming growth factor-β1, TGF- β2, VEGF, GSK-3 and MMP-9 are some of the imperative proteins involved in different phases of wound healing. The crystal structure of receptors such as TGF- β1 (PDB ID- 1PY5), TGF- β2 (PDB ID- 1M9Z), VEGF (PDB ID-3QTK), GSK-3β (PDB ID-1Q5K) and MMP-9 (PDB ID-5UE4) as given in figure 2 was downloaded from the RCSB protein data bank (http://www.rcsb.org).

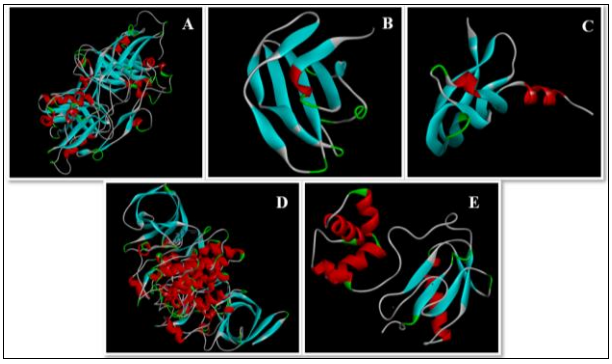


**Fig 2:** Structure of receptor proteins: A.TGF- β1 (PDB ID- 1PY5); B. TGF- β2 (PDB ID- 1M9Z); C. VEGF (PDB ID-3QTK); D. GSK-3β (PDB ID-1Q5K); E. MMP-9 (PDB ID-5UE4)

Using auto-dock tools (version 4.2.6), the receptor and selected ligand PDB files were converted to PDBQT files. Grid box was set on the macromolecules and using the grid parameters, a conf.txt file was prepared. Binding affinity was calculated using VINA (1_1_2_win32.msi) and the results were interpreted using Discovery Studio Visualizer version 16.1.0.15350 (Trott *et al.*, 2010)[7].

### 2.4 *In vitro* evaluation of βeta-caryophyllene for wound healing activity

The cell viability of BCP was studied using MTT assay (Dahham *et al.*, 2015)[8] and the cell migration ability of BCP was assessed using scratch assay (Bolla *et al.*, 2019) [9].

#### 2.4.1 3 - (4, 5 -dimethylthiazol – 2 – yl) - 2, 5 -diphenyl Tetrazolium Bromide Assay (MTT assay)

Trypsinised L929 mouse skin fibroblast cells were seeded at a density of $5 \times 10^3$ cells per well in 200 µL medium and were allowed to attach overnight in a CO2 incubator at 37 °C. Cells were treated with 0.1 percent ethanol and BCP at concentrations of 5, 10, 20, 40, 80, 100, 160, 320 and 640 µM for a period of 24 h (Dahham *et al.*, 2015) [8]. After the

treatment, 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37 °C for 4 h after removing the medium containing BCP and 0.1 percent ethanol. Then, the media with MTT was removed and 200 µL of DMSO was added and read at 570 nm in an microtitre plate reader (Varioskan flash, Thermo Fischer Scientific, Finland) (Gopalakrishnan *et al*., 2019) [10]. The percent inhibition was calculated using the formula below:

Percent viability = (Average absorbance of control cells − average absorbance of treated cells/average absorbance of untreated cells) × 100

The experiment was repeated six times and the effective concentration 50 (EC50) values of BCP was calculated using Graph Pad Prism 5.

### 2.4.2 Scratch assay

The L929 mouse skin fibroblast cells were seeded in 12-well cell culture plate and cells were allowed to attach overnight in a CO2 incubator at 37 °C. Linear scratch was made in confluent cell monolayer using 200 µL pipette tip. Cell debris were washed out with RPMI. Beta-caryophyllene at EC50 of 145.3 µM and cipladine 5 µg/mL (positive control) (Bolla *et al.* 2019) [9] were used for the study. After addition of test substances and cipladine 5 µg/mL, images of cellular gap were captured at $0^{th}$, $12^{th}$, $24^{th}$ and $48^{th}$ h on inverted microscope (Carl Zeiss Primovert, Germany). The cellular gap in the cell monolayer was measured daily using "ImageJ" (Talekar *et al.*, 2017) [11].

## 3. Results and Discussion

### 3.1 *In silico* screening of beta-caryophyllene with various targets of wound healing

Beta caryophyllene was docked against different proteins of wound healing such as TGF- β1 (PDB ID- 1PY5), TGF- β2 (PDB ID- 1M9Z), VEGF (PDB ID-3QTK), GSK-3β (PDB ID-1Q5K) and MMP-9 (PDB ID-5UE4). Binding energies (Kcal/mol) of BCP obtained from RMSD table are illustrated in table 1 and graphically represented in figure 3. The docked images are given in figure 4. The binding energies of BCP towards TGF- β1, TGF- β2, VEGF, GSK-3β and MMP-9 were -5.2, -4.48 -3.77, -6.84 and -6.41Kcal/mol respectively.

Molecular docking is a reliable and effective computational model for predicting viable binding strategies and understanding the process of ligand binding between proteins and small molecules (Meng *et al.*, 2011) [12]. It was shown that the ligand molecules with the lowest binding energy were the most effective in blocking their respective receptors since a lower docking score (binding energy) is correlated with increased binding affinity (Kariyil *et al.*, 2021) [13]. In our study, BCP exhibited highest negative binding energy with GSK-3β protein indicating a strong interaction with good binding affinity. GSK-3β protein is an imperative monitoring enzyme whose inhibition facilitaes wound healing through b-catanin dependent Wnt signalling pathway and is linked to cell differentiation, cell proliferation, inflammation and glucose metabolism (Harish *et. al.*, 2008) [14]. Protracted inflammation is harmful and can cause the stages of wound healing to become out of control, which can result in severe scarring and one of the arduous issues in the development of a drug for wound healing is achieving balance in the inflammatory cascade (Shukla *et al.*, 2019) [15]. There are several studies on the potential anti-inflammatory activity of BCP (Gertsch *et al.*, (2008); Dahham *et al.* (2015); Gushiken *et al.*, (2022)) [16, 8, 17]. Thus, the anti-inflammatory property of BCP might be due to the inhibition of GSK-3β indicating its possible role in inflammatory phase of wound healing.

**Table 1:** Binding energy (Kcal/mol) of ligands such as BCP against different receptors of wound healing

| Receptors | PDB ID | Binding Energy (Kcal/mol) |
|---|---|---|
| TGFβ1 | 1PY5 | -5.2 |
| TGFβ2 | 1M9Z | -4.48 |
| VEGF | 3QTK | -3.77 |
| GSK-3β | 1Q5K | -6.84 |
| MMP9 | 5UE4 | -6.41 |

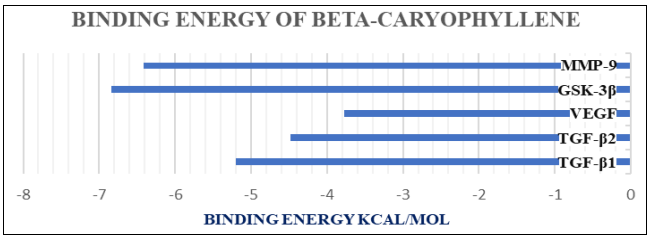


**Fig 3:** Graphical representation of binding energy of beta-caryophyllene with different receptors of wound healing

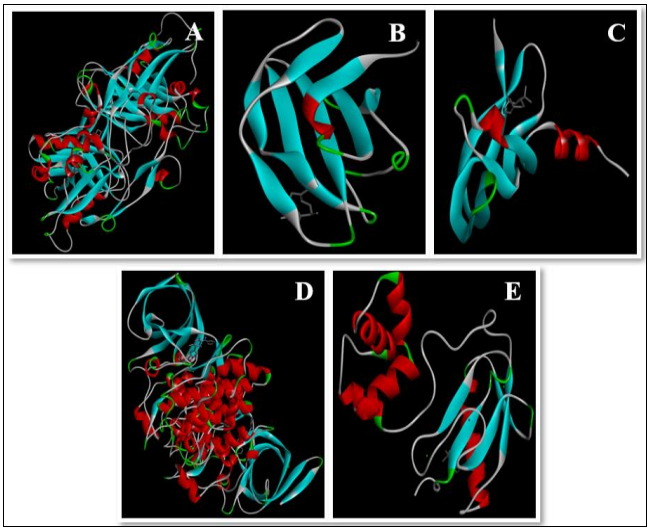


**Fig 4:** Docked images of Beta-caryophyllene with A. TGF- β1, B. TGF- β2, C. VEGF, D. GSK-3β and E. MMP-9

### 3.2 *In Vitro* evaluation of βeta-caryophyllene for wound healing activity

#### 3.2.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT assay)

The results of MTT assay are presented in figure 5 and graphical representation of percent cell viability of BCP is illustrated in figure 6. The BCP displayed maximum percent mean viability at a concentration of 10 µM. Among the various concentrations, the mean cell viability was lower at 640 µM. The results are depicted in table 2. The effective concentration 50 ($EC_{50}$) for BCP in L929 cells was calculated to be 145.3 µM. The percentage viability of L929 cells at the highest treated concentration (640 µM) of BCP was observed to be 59.14 ± 1.03. However, BCP decreased the cell viability less than 70 percent only at 640 µM. The results of the MTT assay revealed that the treatment with BCP showed a decrease in the percent mean cell viability in a concentration dependent manner from 10 to 640 µM with a minimum 70 percent cell viability at 320 µM.

The cytotoxicity assay is predicated on the notion that assessing a biological material's biological and therapeutic significance can be aided by early toxicity screening. Evaluating the plant material's cytotoxic effects on cells or an

*in vivo* model is essential because certain plant metabolites may be harmful to cells due to intermolecular interactions within the cell (Bolla *et al.*, 2019) [9]. According to ISO (1997) [18], 70 percent cell viability is the minimum value required to consider an agent to be non-cytotoxic. Consequently, it could be concluded that BCP was noncytotoxic at concentration up to 320 µM. In an *in vitro* study conducted by Dahham *et al.* (2015) [8], BCP affected the 50 percent cell viability of 3T3-L1 cells only at 530 µM concentration indicating its low toxicity towards normal cell lines. In another study conducted by Ahmed *et al.* (2022) [19], BCP was found to be non-toxic up to 100 µM concentration in A549 and BEAS lung cancer cell lines. These studies were in coherent with the results of the present study.

**Table 2:** Percent mean cell viability of various concentrations of BCP in L929 mouse skin fibroblast cells estimated using MTT, %

| Concentration | Beta caryophyllene |
|---|---|
| 10 | 98.07 ± 0.40 |
| 20 | 93.57 ± 1.03 |
| 40 | 91.65 ± 1.02 |
| 80 | 80.29 ± 1.40 |
| 160 | 75.28 ± 1.58 |
| 320 | 70.91 ± 1.53 |
| 640 | 59.14 ± 1.03 |

** Significant at 0.01 level; ns non-significant

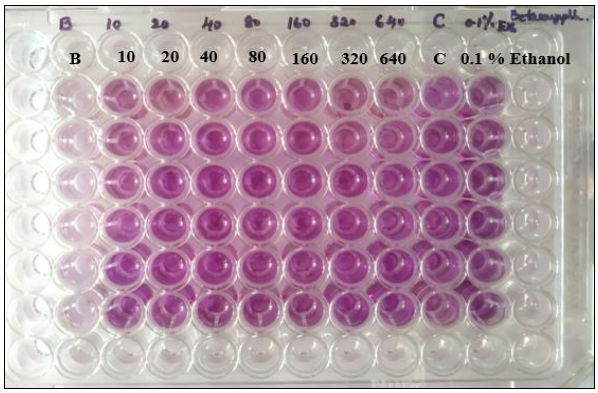


**Fig 5:** Cell viability of BCP in L929 mouse skin fibroblast cells determined using MTT: From left to right: B - Blank, C- Control; cells treated with various concentrations at 10, 20, 40, 80,160, 320 and 640 µM.

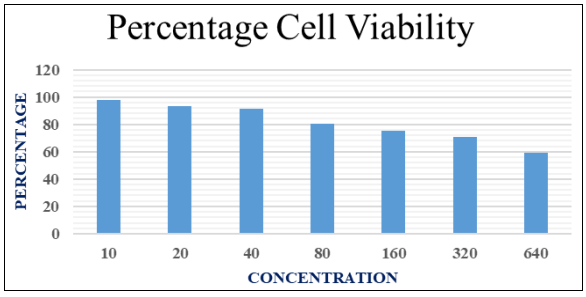


**Fig 6:** Graphical representation of percent cell viability of BCP in L929 mouse skin fibroblast cells assessed using MTT

### 3.2.2 Scratch assay

The results of the scratch assay are displayed in table 3 and figure 7. Reduction in mean cellular gap between 0th, 12th, 24th and 48th hr was significantly ($p$<0.01) different for media control, positive control and BCP. The mean cellular gap between media control, positive control and BCP was non-significant ($p$<0.01) on 0th hr. On 12th, 24th and 48th hr, there was significant ($p$<0.01) difference of cellular gap between media control, positive control and BCP. The percentage wound closure (cell migration) at different time intervals in media control, positive control, BCP treated cells have been graphically represented in figure 8 and tabulated in table 4. The percentage wound closure increased in media control, positive control and BCP on 0th, 12th, 24th and 48th hr. There was significant ($p$<0.01) difference in wound closure percentage on 12th, 24th and 48th hr between media control, positive control and BCP. The results of the scratch assay indicated that BCP treated L929 cells at its $EC_{50}$ value, closed the gap created by the scratch by 91.34 percent in 48 hrs. In the positive control, 99.85 percent of the gap was closed at 48th hr. The results revealed that L929 cells migrated better towards the artificially created scratch when treated with BCP as compared with untreated cells. This suggested that BCP augmented wound healing by inducing the migration of fibroblasts.

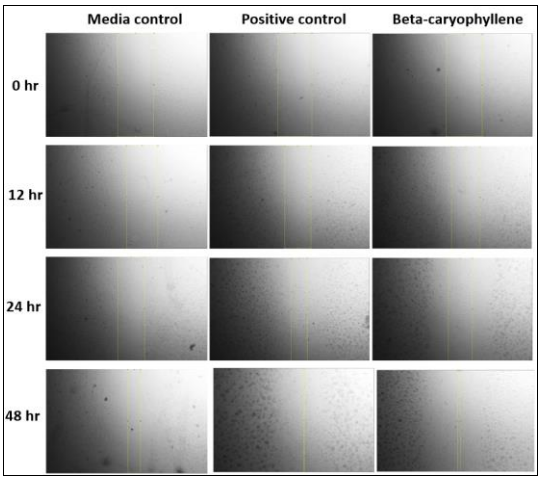


**Fig 7:** Show the media, positive control and beta-caryophyllene

Fibroblasts are essential for the early phases of wound healing because they actively proliferate, migrate to the wound site, and stimulate the production of new extracellular matrix (ECM) and thick actin myofibroblasts. Hence, fibroblasts' migratory and proliferative capacities are essential for wound healing (Li *et al.*, 2004) [20]. In an *in vitro* wound healing study conducted by Koyama *et al.* (2019) [21], BCP exhibited an increased cell migration in primary cultured fibroblasts and they suggested that activation of cannabinoid receptor 2 might have led to an upsurge in chemotactic responses which resulted in increased migration of cells. This was in coherent with the findings of the present study.

**Table 3:** *In vitro* evaluation of wound healing activity of BCP using scratch assay in L929 mouse skin fibroblast cells: comparison of cellular gap between groups and between hrs

| Group | 0th hr | 12th hr | 24th hr | 48th hr |
|---|---|---|---|---|
| Media control | $175.23^{A} \pm 1.32$ | $153.32^{aB} \pm 0.89$ | $131.38^{aC} \pm 1.04$ | $66.21^{aD} \pm 1.43$ |
| Positive control | $174.36^{A} \pm 0.78$ | $132.31^{cB} \pm 1.51$ | $85.03^{cC} \pm 1.76$ | $0.79^{cD} \pm 0.01$ |
| BCP | $176.97^{A} \pm 1.39$ | $141.67^{bB} \pm 0.86$ | $115.29^{bC} \pm 1.12$ | $15.38^{bD} \pm 0.29$ |

** Significant at 0.01 level; ns non-significant
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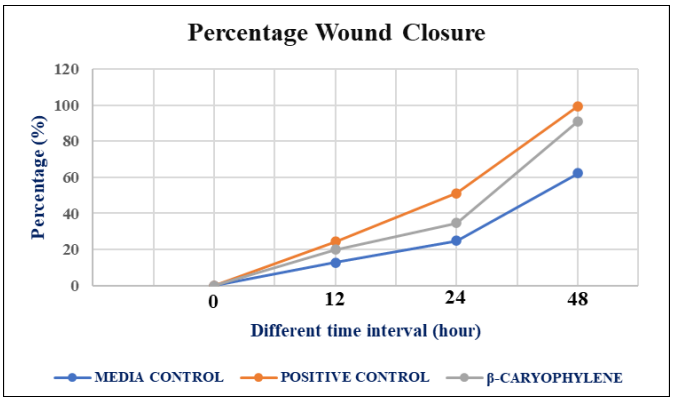


**Fig 8:** Graphical representation of percentage wound closure at different time intervals in media control, positive control and BCP treated L292 cells

**Table 4:** Percentage wound closure at different time intervals in media control, positive control, BCP treated L292 cells- comparison between groups at different hrs

| Group | 12TH Hr | 24th Hr | 48th hr |
|---|---|---|---|
| Media Control | $12.56^{c} \pm 0.41$ | $24.81^{c} \pm 0.48$ | $62.56^{c} \pm 0.48$ |
| Positive Control | $24.45^{a} \pm 0.43$ | $51.19^{a} \pm 0.29$ | $99.85^{a} \pm 0.15$ |
| Beta caryophyllene | $19.78^{b} \pm 0.49$ | $34.32^{b} \pm 0.37$ | $91.34^{b} \pm 0.59$ |

** Significant at 0.01 level

Means having different letter as superscript differ significantly within a column

## Conclusion

In conclusion, this comprehensive investigation underscores the potential of BCP as a promising candidate for wound healing applications. The *in silico* docking study revealed robust binding affinities with key targets, particularly the GSK-3β protein, suggesting a molecular basis for its therapeutic effects. Importantly, the *in vitro* analysis demonstrated the safety of BCP up to 320 µM, a crucial aspect for its clinical translation. Moreover, the observed enhancement of cell migration in L929 mouse skin fibroblasts further supports its positive impact on wound healing processes. The significance of this study lies in bridging the gap between computational predictions and experimental validations, providing a solid foundation for future research and potential clinical applications of BCP in wound healing. The identified molecular interactions and demonstrated cellular responses contribute valuable insights to the field, opening avenues for targeted drug development. Future perspectives involve deeper exploration of the underlying mechanisms driving BCP's efficacy, potentially through additional *in vivo* studies and clinical trials. Further investigations into its broader applicability, optimal dosage, and formulation are essential for translating these findings into effective therapeutic interventions.

## Conflict of interest statement

The authors declare that there is no actual or potential conflict of interest that could inappropriately influence in this work.

## Acknowledgement

The authors express their sincere gratitude to College of Veterinary and Animal Sciences, KVASU, Mannuthy, Thrissur, Kerala for providing all research facilities for the successful completion of the study.

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