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# *In-Silico* Prediction and *In Vivo* Wound Healing Potential of flower Heads Of *Tagetes patula*

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**ABSTRACT**
*Wound healing refers to a living organism's replacement of destroyed or damaged tissue by newly produced tissue. The present study is focused on the wound healing property of methanolic extract of flower heads of Tagetes patula in animal models. In vivo evaluation of wound healing activity of the extract of Tagetes patula was carried out by excision and incision model using metronidazole as a standard. Reduction in the wound area, % increase in the wound contraction and increase in the tensile strength are indicative of wound healing. The animals treated with the two doses of the extract at2.5% gel and 5% gel have shown significant ($p<0.001$) wound healing property and this might be due to the presence of active constituents like gallic acid, luteolin, quercetin, kaempferol, stigma sterol, tannic acid in the extract. An in vitro anti-oxidant assay was carried out using hydroxyl radical scavenging activity using Ascorbic acid as standard. The extract significantly scavenged the hydroxyl radicals. Molecular docking was done to understand the ligand-binding affinity of the active constituents of the extract, such as gallic acid, luteolin, quercetin, kaempferol, stigma sterol, tannic acid and standard drug metronidazole against PDB ID:5IKQ, IDJS, 4LUV, using ligand fit of maestro 9.1 (Schrodinger Software Inc.). The results revealed that gallic acid, luteolin, kaempferol quercetin, stigma sterol and standard drug metronidazole had shown highest glide scores which indicates a stronger receptor-ligand binding affinity among the various phytochemical constituents present in the extract. An in-silico study of these selected phytochemical constituents was also subjected to ADME by molinspiration. The ADME results revealed that the active constituents of the extract have shown zero violation of the Lipinski's rule indicating the probability of its higher oral bioavailability and TPSA score less than 140 clearly indicating better permeability into the tissues. From the above, methanolic extract of flower heads of Tagetes patula possessed significant wound healing activity and antioxidant activity.*
***KEYWORDS:*** *Metronidazole, Tagetes patula, Docking studies, Schrödinger software, Molinspiration ADME analysis.*

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## INTRODUCTION

Wounds are the physical injuries that result in an opening or breaking of the skin and appropriate method for healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. Wound healing is a physiological process to maintain the integrity of the skin after trauma, either by accident or intent procedure [1]. Wounds are classified as open wounds and closed wounds on underlying cause of wound creation and acute and chronic wound on the basis of physiology of wound healing. Acute wound healing is a well organised process leading to predictable tissue repair where platelets, keratinocytes, immune surveillance cells, micro vascular cells and fibroblasts play a key role in restoration of tissue integrity [2]. *Tagetes patula L.*, Asteraceae, popularly known as French marigold, originated in Mexico. The flowers of *T. patula* are a rich source of lutein and its esters, including flavonoids, a class of secondary metabolites with high therapeutic potential including cardioprotective, anti-inflammatory, antimicrobial and antitumor activities among others [3]. Metrogyl gel is used as standard for wound healing activity. *Invivo* wound healing activity is performed by excision and incision model and *in vitro* antioxidant activity by hydroxyl radical scavenging activity. Molecular docking is a kind of bioinformatics modelling which is used to understand drug-biomolecular interactions for the rational drug design and discovery of the compounds in the *Tagetus patula* flower heads, Ramachandran plot is analysed with procheck along with ADME properties.

## MATERIAL AND METHODS

### Plant collection and drying:

The flower heads of *Tagetes patula* was collected from Hyderabad in the month of November and was identified and authenticated. The flower heads are dried under shade for about six days and coarsely powdered in a mixer grinder. The powdered material was used for extraction process.

### Preparation of methanolic extract of *Tagetes patula*:

Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves extraction followed by evaporation of the solvent. The vapours of the solvent are taken to a condenser and the condensed liquid is returned to the drug for continuous extraction. Continuous extraction process, is nothing but a series of short macerations. The methanolic extracts obtained were evaporated to dryness by keeping at room temperature [4].

### Preliminary phytochemical screening

The extract was subjected to preliminary phytochemical screening to identify various phytoconstituents present in *Tagetes patula.*

### *In-vivo* wound healing activity:

#### *Excision model:*

24 Sprague Dawley rats weighing 180-240 g are divided into four groups and are usedin the study. All the rats will be injected with streptozotocin (55 mg/kg, *i.p*) to induce diabetes. For all theanimals wounds will be created 4 cm long and three surgical sutures were placed 1 cm apart. Group I serves as Diabetic control. The methanolic extract will be prepared in the form of 2.5% and 5% gel and will be applied *topically* on the wounded area for group II and group III animals. Metrogyl gel 2% w/w will be applied as a standard for group IV animals. On $8^{th}$ day and $15^{th}$ day, the animals will be observed for diameter of the wound area for determining the wound healing effect of $7^{th}$ and $14^{th}$ day treatment. Wound area and % of wound contraction were measured in all the groups of animals [5].

#### *Incision model:*

24 Sprague Dawley rats weighing 180-240 g are divided into four groups and are used in the study.All the rats will be injected with streptozotocin (55 mg/kg, *i.p*) to induce diabetes.

For all the animals the wounds will be created 4 cm long and three surgical sutures were placed 1 cm apart.Group I serves as diabetic control that receives normal saline. The methanolic extract will be prepared in the form of 2.5% and 5% gel and will be applied *topically* on the wounded area for group II and group III animals. Metrogyl gel 2% w/w will be applied as a standard for group IV animals.The sutures will be removed on the eight post-wound day and the treatment was continued. The skin-breaking strength was measured on the $10^{th}$ day [5].

### *In-vitro* anti-oxidant assay:

### Hydroxyl radical scavenging activity

Hydroxyl radical is one of the potent reactive oxygen species in the biological system that reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell.The reaction mixture (1.0 mL) consist of 100 µL of 2-deoxy- D ribose (28 mM in 20 mM $KH_2PO_4$-KOH buffer, pH 7.4), 500 µL of the extract, 200 µL EDTA (1.04 mM) and 200 µM.$FeCl_3$ (1:1 v/v), 100 µL of $H_2O_2$ (1.0 mM) and 100 µL ascorbic acid (1.0 mM) which is incubated at 37°C for 1 h. One millilitre of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) are added and incubated at 100°C for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample[6].

### *In silico* analysis (Molecular Docking studies, Ramachandran plot and ADME by molinspiration)

Molecular docking is anthe mechanistic study by placing a molecule (ligand) into the preferred biding site of the target specific region of the DNA/protein (receptor) mainly in a non-covalent fashion to form a stable complex of potential efficacy and more specificity. Active site generated in schrodinger by PDB protein and ligands is prepared and docking is performed in maestro schrodinger software and visualization is carried out. Ramachandran plot by procheck is aligned and ADME molecular properties are analysed by molinspiration.

## Results

The methanolic extract of *Tagetes patula* was explored for its wound healing activity using animal models and its anti-oxidant activity was screened by *in vitro* anti-oxidant assay. All the results obtained in this study were included below.

### Preparation of methanolic extract of *Tagetes patula*

The methanolic extract of *Tagetes patula* flower heads was prepared by soxhlation technique. The percentage yield of the extract was calculated by using the following formula.

$$\% \text{ yield of extract} = \frac{\text{Amount of extract obtained (grams)}}{\text{Amount of the powder used (grams)}} \times 100$$

= 16/130×100
= 12.3 % w/w.

**Preliminary phytochemical analysis**

The preliminary phytochemical investigation for methanolic extract of *Tagetes patula* flower heads showed the presence of saponins, steroids, alkaloids, flavonoids (quercetin, kaempferol and luteolin), phenols (gallic acid), steroids (stigma sterol), triterpenoids, tannins and carbohydrates.

**Acute toxicity studies**

Methanolic extract of *Tagetes patula* was tested on Swiss albino mice at the dose of 2000 mg/kg bd. wt. *p.o.* The extract did not exhibit any signs of toxicity and mortality even upto 2000 mg/kg. bd.wt. All animals were safe even after 14 days of observation.

***In vivo* wound healing activity:**

Methanolic extract of *Tagetes patula* was investigated for its *in-vivo* wound healing activity in streptozotocin induced diabetic rats using excision and incision wound models. All the results obtained in this study were included below.

***Excision wound model:***

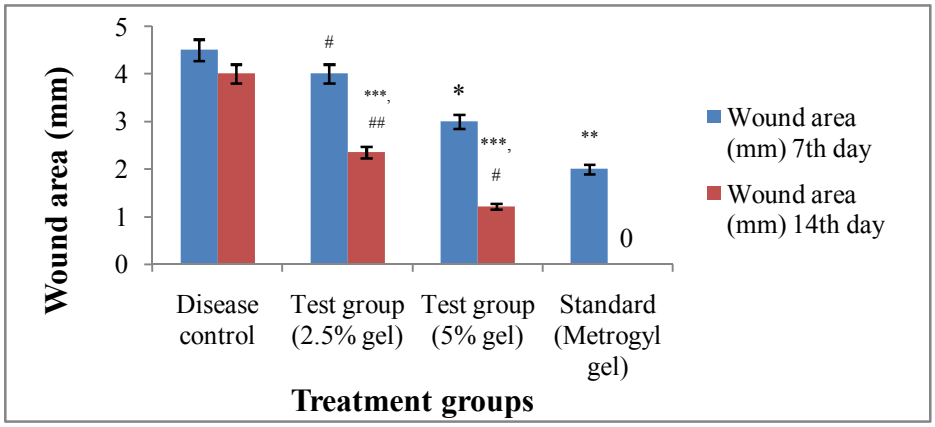



**Figure 1:** Effect of METP on excision wound parameters (wound area)

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were expressed as ( *=p<0.01, **= p<0.001, ***=p<0.0001) vs disease control group and (#=p<0.001, ## =p<0.0001) vs standard group, ns = non significant.

The METP treated groups at doses (2.5% gel and 5% gel) showed significant decrease in the wound area when compared to wounded diabetic control group. The Metrogyl treated group showed significant decrease in the wound area when compared to wounded diabetic group (Figure1).

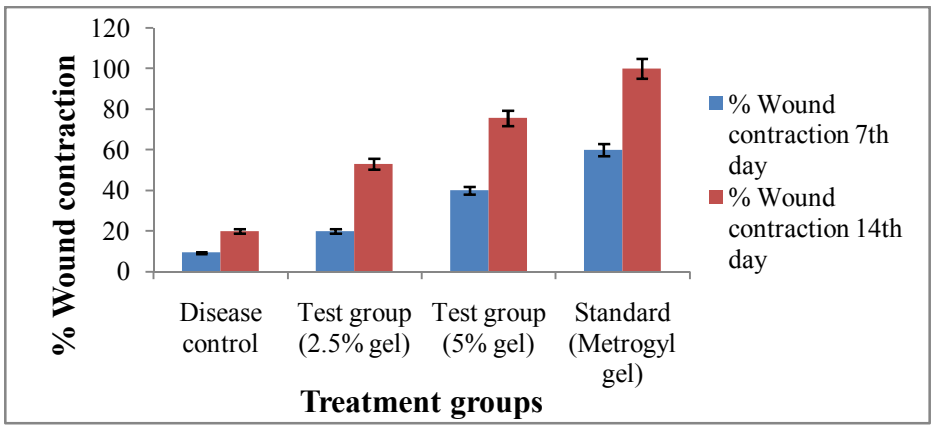



**Figure 2:** Effect of METP on excision wound parameter (% wound contraction)

The METP treated groups at doses (2.5% gel and 5% gel) showed significant increase in the wound contraction when compared to wounded diabetic control group. The Metrogyl treated group showed higher wound contraction when compared to wounded diabetic group (Figure2 &3).

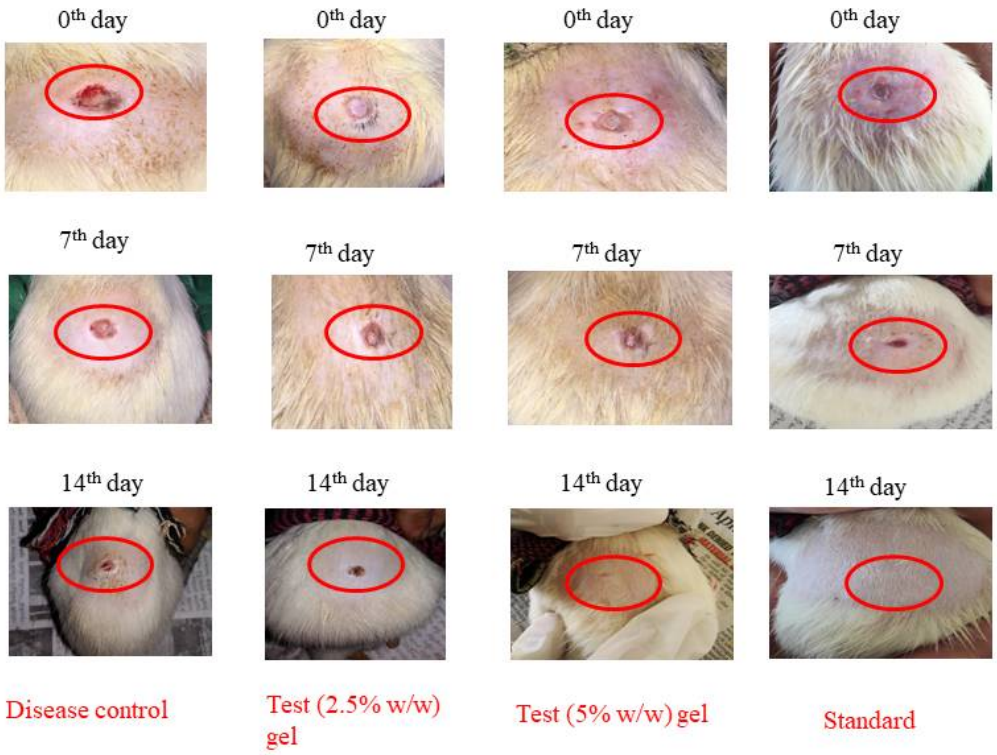



**Figure 3:** Effect of METP on wound area in excision wound model

***Incision wound model:***

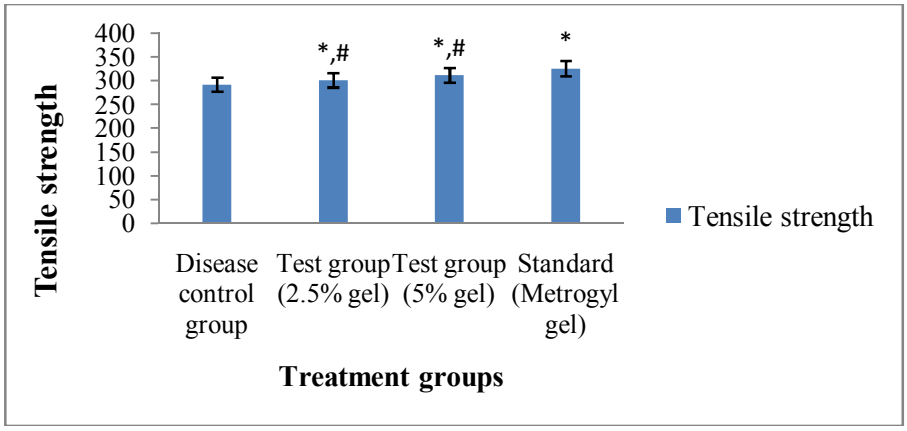



**Figure 4:** Effect of METP on tensile strength in incision wound model

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were expressed as (*=p<0.0001) vs disease control group and (#=p < 0.0001) vs standard group.

The METP treated groups at doses (2.5% gel and 5% gel) showed significant increase in the tensile strength (p<0.001) when compared to wounded diabetic control group. The metrogyl treated group showed significant increase in the tensile strength (p<0.001) when compared to wounded diabetic group.

***In vitro* antioxidant assay**

The methanolic extract of *Tagetes patula* flower heads was subjected to *in vitro* antioxidant activity using Hydroxyl radical scavenging assay.

**Hydroxyl radical scavenging assay:**

The *in vitro* antioxidant activity was performed using hydroxyl radical scavenging assay.

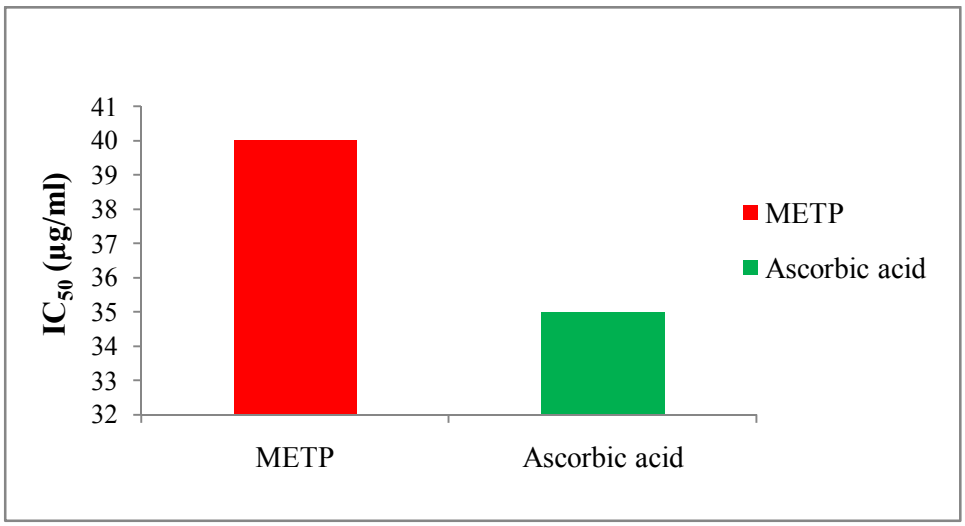



**Figure 5:**Effect of METP on hydroxyl radical scavenging assay

The $IC_{50}$ value of the METP was 40 µg/ml and the standard drug ascorbic acid was 35 µg/ml. From the results it is clear that the METP showed antioxidant activity.

***In silico* analysis**

**a) Molecular docking studies**

**A. Structure based drug design**

Initially, the protein downloaded from PDB wasprepared by removing chains B, C and D. Water molecules present in all the chains were removed. Energy minimization was done. Later molecules drawn using chemdraw were converted to mol format and ligprep was created. Grid generation was done by removing crystal ligand metformin and the structures were docked against 1NOI protein.

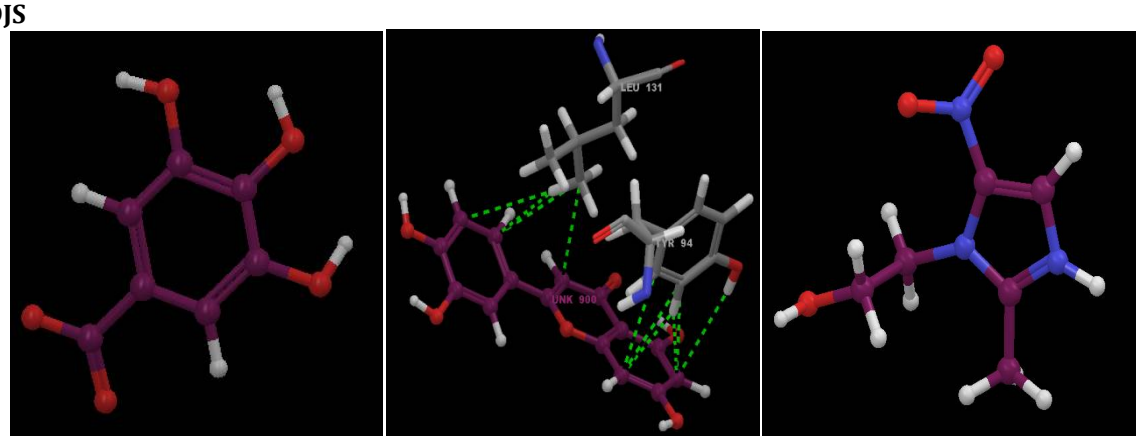
**B. Schrodinger XP-docking results**

XP docking indicates that some of our compounds have good binding ability (PDB ID: 5IKQ, 1DJS, 4LUV).

**5IKQ**

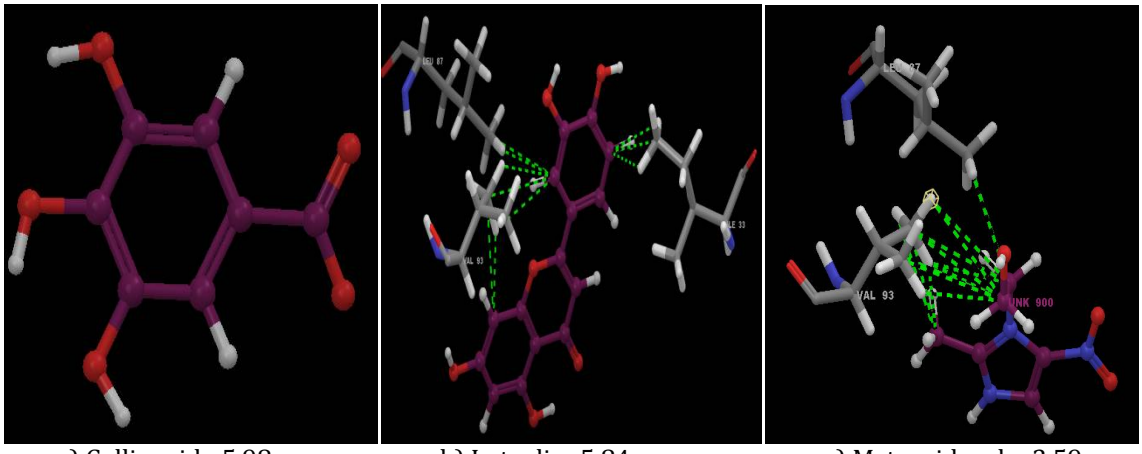

a) Luteolin -12.76 b) Gallic acid - 8.60 c) Metronidazole -6.51

**1DJS**




a) Gallic acid - 5.36 b) Kaempferol -4.35 c) Metronidazole -3.60

**4LUV**




a) Gallic acid - 5.98 b) Luteolin -5.84 c) Metronidazole -3.59

**Figure 6:** Docking visualization of protein a) 5IKQ b) 1DJS c) 4LUV

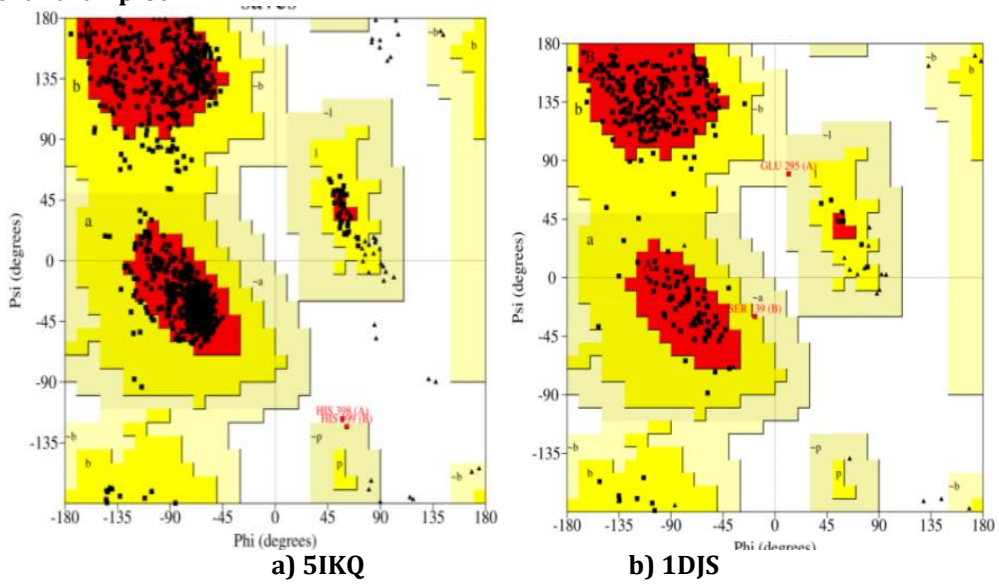
**Table 1:**Docking results of METP of protein (PDB ID: 5IKQ, 1DJS, 4LUV)

| S. No | Name of the compound | Glide score | | |
|---|---|---|---|---|
| | | 5IKQ | 1DJS | 4LUV |
| 1 | Gallic acid | -8.60 | -5.36 | -5.98 |
| 2 | Luteolin | -12.76 | -4.19 | -5.84 |
| 3 | Quercetin | -7.62 | 4.08 | -4.45 |
| 4 | Kaempferol | -8.21 | -4.35 | -5.17 |
| 5 | Stigmasterol | --- | --- | -3.08 |
| 6 | Metronidazole | -6.51 | -3.60 | -3.59 |

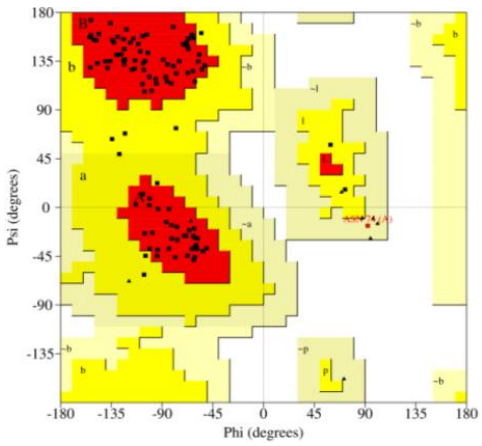
Docking studies of METP and standard metronidazole were observed against COX inhibitor, FGF promoter and inhibitor of protein-protein interactions (PDB ID: 5IKQ, 1DJS, 4LUV). The parameters analysed in the study includes glide score. The results show that gallic acid and luteolin are having highest glide score.

The more negative glide score indicates the better binding affinity

**b) Ramachandran plot**




**a) 5IKQ** **b) 1DJS**




**c) 4LUV**

**Figure 7:** Ramachandran plot of protein a) 5IKQ b) 1DJS c) 4LUV by Procheck

**Table 2:** Ramachandran plot statistics by procheck

| Protein | Favoured regions (%) | Additional allowed regions (%) | Generously allowed regions (%) | Disallowed regions (%) |
|---|---|---|---|---|
| **5IKQ** | 90.2 | 9.6 | 0.1 | 0.1 |
| **1DJS** | 86.9 | 12.4 | 0.7 | 0.0 |
| **4LUV** | 89.6 | 9.4 | 0.6 | 0.0 |

**c) *In-silico* ADMET analysis by molinspiration**

**Table 3:** ADME properties of compounds from *Tagetus patula* by molinspiration

| Compound | MW | nON | nOHNH | nV | nrotb | TPSA | miLogP |
|---|---|---|---|---|---|---|---|
| Gallic acid | 170.12 | 5 | 4 | 0 | 1 | 97.98 | 0.59 |
| Luteolin | 286.24 | 6 | 4 | 0 | 1 | 111.12 | 1.97 |
| Quercetin | 302.24 | 7 | 5 | 0 | 1 | 131.35 | 1.68 |
| Kaempferol | 286.24 | 6 | 4 | 0 | 1 | 111.12 | 2.17 |
| Stigmasterol | 412.70 | 1 | 1 | 1 | 5 | 20.23 | 7.87 |
| Metronidazole | 171.16 | 6 | 1 | 0 | 3 | 83.88 | -0.47 |

MW = Molecular weight, nON = number of hydrogen bond acceptors, nOH = number of hydrogen bond donors, nV = number of violation of Lipinski's rule of five, nrotb = number of rotatable bonds, TPSA = Total Polar Surface Area and miLogP = Octanol-water partition coefficient logP.

## DISCUSSION

### Wound healing activity

The elevated blood glucose level in diabetes may cause endothelial damage with potential occlusion of capillary vessels as well as hyperglycaemia induced leukocyte dysfunction, decrease chemotaxis and phagocytosis resulting in impaired wound healing. The impaired healing in diabetic rats is predominantly due to hypoxia, fibroblast and epidermal cell dysfunction, impairment in angiogenesis and increased levels of metalloproteases [7]. The increase in the production of reactive oxygen species, lipid peroxidation and ineffective scavenging has a vital role in skin lesions and in modulation of fibroblast proliferation[8]. The various phytochemical active constituents identified in the methanolic extract of *Tagetes patula* flower heads were saponins, steroids, alkaloids, flavonoids, phenols, carbohydrates, triterpenoids and tannins. The quercetin (flavonoid) shows significant increase in wound contraction by enhanced epithelisation and possible mechanism of improved wound healing by quercetin is due to ability of quercetin to improve the tissue anti-oxidant levels [9]. Luteolin (flavonoids) causes increase in the wound contraction by increase in collagen synthesis, promote the cross-linking of collagen, decrease the degradation of soluble collagen, accelerate the conversion of soluble collagen to insoluble collagen, and inhibit the catabolism of soluble collagen [7]. Gallic acid (phenol)causes increased wound contraction by increase in the level of collagen leading to proliferation of fibroblast cells fastening the epithelialization and remodelling phases, may potentiate wound healing by quenching free radicals formed during the inflammatory phase. Tannic acid (tannin) promote wound healing by chelation of free radicals, promoting

contraction of the wound, increasing the formation of capillary vessels and fibroblasts and it also induces keratinocytes proliferation wound, increasing the formation of capillary vessels and fibroblasts, and it also induces keratinocytes proliferation [10]. Alkaloid shows significant increase in percentage closure by enhanced epithelialization and enhanced collagen synthesis [11]. Saponin faster wound contraction may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes, and may increase the gap junctional intracellular communication in fibroblasts, and induces a more rapid maturation of granulation tissue as reported [12].Lupeol (triterpenoid)causes increase in the contraction of the wound by the better formation of granulation tissue with marked proliferation of fibroblasts, increased vascularization, and deposition of collagen fibres[13].

The METP at two dose levels 2.5% gel and 5% gel significantly reduced the wound area and increased the % wound contraction. The possible mechanism by which METP enhanced the wound healing processes might be due to presence of saponins, steroids, alkaloids, flavonoids, phenols, carbohydrates, triterpenoids and tannins present in *Tagetes patula* flower heads. Metrogyl gel is an antibiotic that can be used to eliminate both anaerobic and aerobic bacteria. It helps maintain a moist wound environment. Metrogyl mainly acts in the proliferative phase & inhibits the metalloproteases [14].

Increased LPO and decreased antioxidant protection frequently occurs when ratio of ROS produced exceeds the level of antioxidants available. Such a reaction may lead to cytotoxicity, allergy, mutagenicity, carcinogenicity and delayed diabetic wound healing [15]. Saponin shows an increase in tensile strength which may be due to the increase in collagen concentration and stabilization of the fibres[12].Flavonoid causes increase in the tensile strength which may be due to increase in the collagen synthesis further leads to collagen synthesis [16]. Alkaloids shows higher tensile strength may be due to the increase in collagen concentration and stabilization of the fibres. A healing tissue synthesizes collagen, which is a constituent of growing cell. The higher tensile strength indicates better healing of wounds [11]. Phenolscauses increase in the tensile strength by increased collagen level and stabilization of the collagen fibers [17].

The METP at two dose levels 2.5% gel and 5% gel significantly increased the tensile strength. The possible mechanism by which METP enhanced the wound healing processes might be due to presence of saponins, steroids, alkaloids, flavonoids, phenols, carbohydrates, triterpenoids and tannins present in *Tagetes patula* flower heads.

**Antioxidant activity**

Hydroxyl radical is thought to initiate cell damage ***in vivo***. Thus, removing hydroxyl radical is very important for the protection of living systems. The hydroxyl radical is an extremely reactive free radical formed in biological system and has been implicated as a highly damaging species, capable of damaging almost every molecule found in living cell [18].

Phenolsshows reducing property by acting as electron donors &suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition [6]. Beta sitosterol (steroid)shows antioxidant property by its redox properties, which allow them to act as reducing agents, hydrogen donors and single oxygen quenchers[19]. Kaempferol (flavonoid) shows reducing property by acting as the chelators of iron ions [20]. Tannic acid (tannins)shows hydroxyl radical scavenging activity by inhibiting the reduction of iron and thus preventing the deoxyribose damage [21]. Hydroxyl radicals were effectively scavenged and 2-deoxyribose was prevented from degradation by lupeol (triterpenoid). Beta carbolene (alkaloid)scavenged hydroxyl radical by reacting with hydroxyl radical and prevents the DNA damage [22]. Saponins scavenge the hydroxyl radical by inhibiting the hydroxyl radical generation thus preventing the degradation of deoxyribose of DNA [23].

The ability of METP to quench hydroxyl radicals seems to inhibit the reduction of ferric ions, prevent the breakdown of DNA, prevention of process of lipid peroxidation. The scavenging activity of METP might be due to presence of saponins, steroids, alkaloids, flavonoids, phenols, carbohydrates, triterpenoids and tannins present in *Tagetes patula* flower heads.

Molecular docking:

Molecular docking continues to holds great promise in the field of computer based drug design which screens small molecules by orienting and scoring them in the binding site of a protein. The docking analysis of isolated compounds from methanolic extract of*Tagetus patula* and standard drugs like metronidazole were carried out using Schrödinger software. The various constituents identified in the plant extract aregallic acid, luteolin, quercetin kaempferol, stigma sterol, tannic acid and standard drug Metronidazole were subjected to docking against PDB ID: 5IKQ, IDJS, 4LUV

The highest glide scores were observed with gallic acid, luteolin, quercetin kaempferol, stigma sterol, tannic acid and standard drug metronidazole with almost all the selected proteins with PDB ID: 5IKQ, IDJS, 4LUV.

The glide scores of the gallic acid, luteolin, quercetin, kaempferol, were found to be more than the glide score of standard drug metronidazole stating that the compounds might have same affinity or greater affinity to bind to the proteins. These results clearly indicate that the chemical constituents mentioned above might have shown similar mechanism to that of the standard drug metronidazole in reducing wounds. The proteins identified namely 5IKQ, IDJS, 4LUV are modeled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is evident from the Ramachandran plot that predicted models have most favourable regions, additionally allowed regions, generally allowed regions and disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted models are of good quality. According to Ramachandran plot a good quality model would be expected to have over 90 % in the most favoured region.Proteins like 5IKQ, IDJS, 4LUV showed almost 90% favoured regions which clearly indicate that the selected models in the present study are of good quality.

**Molinspiration ADME analysis:**

Molinspiration molecular properties were calculated on the bases of Lipinski's rule and its components. Lipinski's rule of five is to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it an orally active drug in humans. In the present study, all the compounds that are docked has lower molecular weight so that they are easily absorbed, diffused and transported. The selected active constituents like gallic acid, luteolin, quercetin, kaempferol and standard drug metronidazole has zero violations except stigma sterol which has one violation out of five. Any compound with zero violation clearly indicates the probability of its higher oral bioavailability.

Topological polar surface area (TPSA) allows the prediction of transport properties of drug candidates in the intestines and blood-brain barrier. The TPSA score in all the selected active constituents of the extract and standard drugs metronidazole was found to be less than 140 which clearly indicated better permeability into the tissues.

Molinspiration ADME enables the computation of key physicochemical, pharmacokinetic, drug-like and related parameters for one or multiple molecules. Number of H-bond acceptors should be in a range of 0-10 and number of H-bond donors should be 0-5. All the selected active constituents in the present study were found to be within the range. A negative value for ilogP means the compound has a higher affinity for the aqueous phase (it is more hydrophilic); when ilogP equals 0 the compound is equally partitioned between the lipid and aqueous phases; a positive value for ilogP denotes a higher concentration in the lipid phase (i.e., the compound is more lipophilic). In the present study all the active constituents have shown a positive ilog p- value clearly indicating a higher concentration in the lipid phase.

## CONCLUSION

The present study revealed that the methanolic extract of *Tagetes patula* possess wound healing activity and antioxidant activity. However further studies are required to confirm the exact mechanism of action and to isolate the phytochemical constituents responsible for these activities.

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