Evaluation of the combined effect of L-Arginine and Honey extracted Polyphenols for wound healing activity in Wistar rats, *in vitro* anti-inflammatory and antioxidant activity

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ABSTRACT

Background and aim: *It has been demonstrated from other studies that arginine and polyphenols both possess and accelerated the rate of wound healing. The purpose of this study was to assess wound healing activities in Wistar rats, antioxidant effects, considering the anti-inflammatory efficacy and capability of the Arginine+Polyphenols (Arg+PP) combination in vitro.*

Method: *Polyphenols were extracted from honey and their constituents, that is, quercetin, kaemferol, caffiec acid, apigenin, naringenin, eppigallocatechin, sinapic acid, syringic acid, vanillin, chicoric acid, were detected by HPLC analysis. On an excision wound in Wistar rats, pharmacological testing of a 10% w/w ointment of L-Arginine+Polyphenols (combined) was performed in comparison to control, standard, L-Arginine, and Polyphenols. Based on factors including wound contraction size and epithelialization time, the effects were assessed. The L-Arginine+Polyphenols (combination) were also assessed for their antioxidant activity using the DPPH radical scavenging activity, nitric oxide scavenging activity, and ferric reducing power, as well as anti-inflammatory activity as measured by anti-proteinase activity and protein degradation.*

Result: *When the animals were treated topically with the combination of Arg+PP (10% w/w), a significant ($p<0.05$) wound healing property was identified with enhanced wound closure. Similarly, the L-Arg+PP combination significantly showed antioxidant activity and In vitro anti-inflammatory action.*

Conclusion: *The current study provided scientific evidence in which the combination of L-Arg+PP possesses remarkable wound healing activity, in vitro antioxidant activity also and anti-inflammatory activities too. It was considered that both, arginine and polyphenols were compatible.*

Keywords: *wound healing, antioxidant effects, honey, polyphenols, L-arginine.*

1. Introduction

Increasing rates of obesity and type II diabetes, an ageing population (particularly in affluent nations with low birth rates), and other health issues are all barriers to wound healing, which is a global medical concern. The problem can occasionally become worse when an excessive exudate production results in the maceration of good skin tissue surrounding the area and impairs wound healing[1].A wound may develop due to pathological processes, occur inadvertently, originate from a disease process, or potentially cause serious impairment or even death[2]. These three layers of skin include various cell types that work in stages to repair wounds or restore the integrity of tissue that has been damaged. These processes of hemostasis, inflammation, proliferation, and remodelling proceed in the proper sequence throughout time without any alterations that can hasten or even impede the healing of cutaneous wounds[3,4]. Reactive oxygen species (ROS) are suddenly generated at the wound site by immune cells. These immune cells play important roles in inflammatory responses and provide defence against invasive microorganisms. Free radicals and ROS harm DNA and proteins and are the main contributors to ageing. The rate of wound healing may be affected by overproduction of ROS which may also causing damage to surrounding cells during inflammation phase[5,6,7]. Antioxidants counteract ROS's negative effects and guard the organism against oxidative stress-induced disease processes as well as oxidative degradation. Oxidative stress is characterised due to the inability of endogenous antioxidants to mitigate oxidative damage to biological targets. Oxidative stress can be brought on by an enhance in ROS/RNS production or reduce in the antioxidant network[8-11]. The amino acid arginine, which Rose categorised as semi-essential, was demonstrated by Seifter *.et.al*. to be an important essential amino acid and the precursor of polyamines associated with tissue regeneration and healing of the wounds. Arginine also stimulates T-cell responsiveness, preventing infection and promoting health[12,13].The hypothesis is that arginine has a regulative role in wound healing. The wound environment controls two routes for arginine metabolism: one through the isoforms of Nitric Oxide Synthase and the other through isoforms of 2-arginase [14]. Secondary metabolites, or physiologically active natural molecules, are abundant in plants and particularly abundant in phenolic and polyphenolic compounds.Vitamins, carotenoids, and phenolic chemicals, which are linked to antioxidant action, are the three main classes of polyphenols. The cosmetics industry has begun to create eco-friendly goods that are rich in green raw materials because of the favourable effects of polyphenols, especially products for therapy and to prevent skin from ageing prematurely. Inhibiting the expression of phospholipases and cyclooxygenases, which are extracted from a variety of plants, can reduce the intensity of the inflammatory process[15] and has been linked to a number of consequences on human health, including lowering the risk of developing cancer and cardiovascular disorders and reducing oxidative stress[16].The use of pharmaceuticals alone or in conjunction with materials produced from nature, such as polyphenols, may lead to the development of new, safer and more effective therapeutic options, or it may be a potential way to boost efficacy while minimizing negative effects. By lessening the longevity of the inflammatory state, topical application of polyphenols (10%) to a wound accelerated healing. This was accompanied by a decrease in TNF-β and IL-1 levels and an increase in IL-10 and arginine levels.Nitric oxide also sped up healing. Therefore, the

aim of our current work was to understand that how polyphenols and l-arginine are used to promote wound healing and have antioxidant and anti-inflammatory effects.

2. Resources and techniques

2.1 Reagents and chemicals

DPPH, Na_2CO_3(sodium carbonate),ascorbic acid, trichloroacetic acid, , trypsin, casein, ferric chloride,ethanol, sodium nitroprusside, potassium ferricyanide, methylene blue solution Griess reagent, sodium hydroxide, Fehling's solution A, phenolphthalein,Fehling's solution B, citric acid, and other chemicals are just a few examples of the chemicals that were used.

2.2 Collection of honey sample

The honey used in this study was purchased from the local vendors of Chorgaliya (Nainital), Uttarakhand and then the honey sample was analyzed by performing some of the physicochemical tests which are as follows:

2.3. Physicochemical evaluation of honey

2.3.1. Estimation of Moisture content

A preweighed petri dish was filled with a five gram sample of honey. The samples were dried to a consistent weight in an oven set at 105° C. On a dry basis, the percentage of moisture was measured, and the weight loss was estimated as follows:

$$\text{Moisture}(\%) = \frac{M_1(\text{weight of the sample+petri dish}) - M_2(\text{wt of the dried sample+petri dish})}{M_1 - M_0(\text{weight of petri dish without sample})} \times 100$$

[17].

2.3.2. Estimation of pH

In order to calibrate a pH metre for use, buffer solutions with a standard pH of 4.0 and 7.0. were used. Then, to analyse the sample's pH, 75mL of distilled water were used to dissolve 10 gms of honey in a beaker. The pH of the fluid was measured immediately by dipping the electrode of the pH metre into it.

2.3.3.Estimation of Ash content

To avoid the risk of loss due to foaming and overflowing, (5g) was precisely weighed into a pre-weighed silica dish and gently pre-heated on a hot plate until the sample turned black and dry. To maintain weight in a furnace, the sample was then ignited at 550°C. After being fired and cooled in a desiccator, the sample was weighed. The outcomes, which are presented as a percentage of Ash, were determined using the formula written below:

$$\text{Ash } \% = \frac{W_3(\text{weight of dish + ash}) - W_1(\text{weight of dish only})}{W_2(\text{weight of honey sample})} \times 100$$

2.3.4. Estimation of Acidity

The honey solution was neutralised with NaOH till pH 8.3 by taking 10g of honey, weighing it, and dissolving it in 75 ml of distilled water. [18].

% Acidity = Volume of sodium hydroxide (NaOH solution) consumed × Exact Weight of Honey.

2.3.5. Estimation of Sugars

A sample of 5g honey was dissolved in 100 ml of D. H2O. 3-4 drops of phenophthelien were added, proceeded by NaOH solution, which was increased until the solution turned pink. After the solution had been treated with HCl until its colour had returned, then added 200 ml of distilled water. Each of 5 ml that is, Honey solution, Fehling sol A, and Fehling sol B were taken in conical flask. After that, the mixture was cooked for two minutes and then, methylene blue indicator (two to three drops) was added to it during boiling. After that, titration was done with honey solution and the end point was indicated by the formation of brick red colour. It was observed how much honey solution was utilized in the titration.

$$\text{Reducing Sugar \%} = \frac{\text{Fehling solution constant } 0.051 \times \text{total amount of solution}}{\text{Weight of sample solution x capacity of titrate}} \times 100$$

Once more, 100ml of d.H2O was added to the 5g of honey solution in a beaker. After adding a couple of phenolphthalein drops, add Sodium hydroxide solution until the solution color turns into pink. Following the addition of HCl until the solution returned to its original colour, 200ml (volume 1) of pure distilled water was to be added. 50ml of the aforementioned solution was obtained, to which 5g of citric acid was added, heated for 10 minutes, and then cooled. After that, distilled water and neutralised sugars were added to get the final volume up to 200 ml (volume2). The Fehling solution A and B were added to 5ml of the honey solution, which was then heated with methylene blue (2-3 drops) and titrated with honey solution for 2 minutes until it turned brick redcoloured. The amount of honey solution ustilised was noted[17,18].

$$\text{Total sugars} = \frac{\text{Fehling solution constant } 0.051\text{x}200\text{x}200\text{x}100}{5\text{x}50\text{x}\text{Vol.of Honey Solution (which is to be used for titration)}}$$

Non-reducing sugars (Sucrose) = total amount of sugars – reducing sugars.

2.3.6 Estimation of total phenolic content

Honey solutions of concentration of 5g in 50ml were centrifuged and then filtered with the help of filter papers. After that, 2.5ml was taken from the 0.2 N reaction solution of Folin-Ciocalteau reagent and 2ml was taken from theNa_2CO_3 solution and then both were added to 0.5 ml of the prepared solution. After the incubation of two hours of the reaction mixture's absorbance was evaluated at 760nm in UV/Vis spectrophotometer and compared to a methanol blank. The standard curve was developed for the concentration range between 0mg to 200 mg/100 ml for gallic acid.

2.3.7 Estimation of total flavonoid content

0.2 g in 1 ml honey solution was centrifuged and then filtered using filter papers. Then, 4 ml of distilled water + 0.3 ml of $NaNO_3$ solution with a concentration of 15 g/100 ml were added to 1 ml of the prepared solution. Then, 4 ml of NaOH solution (4 g/100 ml) and 0.3ml of aluminium chloride solution (10gm/100ml) were added. With distilled water, the mixture was diluted to 10 ml, mixed, and then let to stand for 15 min. A UV/Vis spectrophotometer was used to compare the reaction mixture's absorbance at 415 nm to a methanol blank. Based on

the quercetin solutions standard curve and presented as quercetin equivalents in mg/100gm of the honey sample, the total flavonoid amount was also deterzmined [19].

2.4. Isolation of polyphenols from honey

Ethyl acetate was used as an extracting agent for the extraction of polyphenols. After using HCl to bring the honey solutions with a concentration of 10g/50ml to pH 2, 30% sodium chloride was added to completely saturate the solution. Three volumes of ethyl acetate, one of 50 ml and two of 25 ml each, were used to extract the resultant solutions after they had been filtered to remove any solid particles. The extracts were mixed properly and then vacuum vaporised at 40°C. 5cc of methanol was added to the leftovers, which were then stored at 18 °C. Before being analysed using high-performance liquid chromatography (HPLC), all samples were filtered using Millex-LCR (PTFE) filters with a pore size of 0.45 m[20].

2.5. Characterization of polyphenols

For the chromatographic studies of phenolic substances, high-performance liquid chromatography (HPLC-SHIMADZU, Lab Solutions) was employed. Flavonoids like kaempferol, quercetin, chrysin, hesperetin, galangin, and naringenin were identified at 330 nm, while phenolic acids like caffeic, chlorogenic, p-coumaric, ferulic, gallic, sinapic, and syringic acid were discovered at 280nm. Acetonitrile was used as the mobile phase, while a solution of 2.5 g/100 ml acetic acid served as the gradient, during gradient elution, which was done at a rate of flow of 1ml/min. The solvent solution was linearly increased from 3% acetonitrile to 8% for ten minutes, 15% for twenty minutes, 20% for thirty minutes, 30% for forty minutes, and 40% for fifty minutes. The column was ultimately isocratically cleaned before the subsequent injection.At a temperature of 25°C, the chemicals under investigation were separated on a 250 x 4.5 mm RP18 Lichrosorbcolumn[19].

2.6. Preparation of ointment

L-arginine and the polyphenols found in honey were used to create an oil in water emulsion. To make 100 g of ointment, the ingredients were stearic acid (14%, weight for weight), triethanolamine (1.2%, weight for weight), glycerol (13.5%, weight for weight), propylene glycol (5%, weight for weight), and oleic acid (10%, weight for weight). The following four formulations were created: ointment base, polyphenol 10% (w/w), Arg+PP 10%, and arginine 10% (w/w) (combination). The ointment created in this way had a neutral pH. During experiments, this ointment was kept in collapsible tubes and kept in a cool, dry location. Likewise, no polyphenols or L-arginine were used in the preparation of the blank ointment[21,22].

2.7. Evaluation of ointment

2.7.1 Appearance- Appearance was evaluated by color, odor, and smoothness.

2.7.2 pH- The pH analysis for ointment was determined by using pH meter. With the use of a standard buffer solution, the pH metre was calibrated. About 1gm of prepared ointment was diluted in distilled water after this the pH meter was immersed in container, reading was taken.

2.7.3 Spreadability- Spreadability is a term to express the donator extend of the area to which the ointment radially spread on application to the skin or affected part. It is calculated in terms of the time it takes for two slides to separate from the ointment, or in seconds. Because it will take less time to separate two slides, spreadability is claimed to be preferable. It was calculated using the equation [23].

$$S\,(spreadability) = M(Weight\ attached\ to\ the\ higher\ slide)\ \times L\ /\ T(time\ required\ to\ fully\ separate\ the\ slide\ from\ one\ another)$$

2.7.4 Extrudability- The extrudability of several formulations was calculated in terms of weight in grams to meet the requirement of extruding an ointment from a collapsible tube in 10 seconds.

2.7.5 Viscosity -A 250 ml beaker that was clean and dry was used to collect the test sample to be observed, and the viscosity of the sample was assessed using the viscometer's spindles 1 to 4 in accordance with standard operating procedure. Using a Brookfield viscometer, their rheological parameters were also evaluated at 250 C[24].

2.8.IR spectra

FTIR spectra were used to identify the compounds' functional groups. A Perkin Elmer FTIR spectrometer with a resolution of $4cm^{-1}$ was used to capture the spectra. The spectrum of arginine, polyphenols and Arg+PP was recorded immediately. The spectra were captured and processed using OMNIC 32 software[25].

2.9.Development of excision wounds

The Institutional Animal Ethics Committee approved the study, which employed white adult Wistar rats weighing 250–300 g. Diethyl ether was used to make the rats unconscious using an open mask technique, and then the dorsal hairs were removed with hair removal cream. After that, 70% ethanol was used to sanitise the area. The excision dorsal wounds were created using a 10mm diameter skin biopsy punch.

2.10Experimental design

Thirty injured rats were kept in individual cages, separated into five different groups of 6 rats in every group. For 15 days, First groups animals received topical treatment with ointment base, group 2 animals received topical treatment with betadine (10%), group 3 animals received topical treatment with arginine ointment (10%), group 4 animals received topical treatment with honey extracted polyphenols ointment (10%), and group 5 animals received topical treatment with Arg+PP (combined) ointment (10%).

2.11. Measurements of wound contraction and photography

All of the wounds were digitally photographed at predetermined intervals, namely on days 0, 3, 6, 9, and 12 following wounding. Following a planimetric determination of the wound area on each of these different days, Wilson's formula was used to determine the percent wound contraction as given:

$$\%\ \text{wound contraction} = \frac{\text{wound area on day zero} - \text{wound area on particular day}}{\text{wound area on day zero}} \times 100$$

2.12. Assessment of invitro anti-oxidant activity

2.12.1 DPPH anti-oxidant radical scavenging activity

Standard, arginine, polyphenol, and Arg+PP samples were dissolved in pure ethanol for the DPPH of anti-oxidant radical scavenging experiment and then introduced right away to 3.9ml of ethanol based DPPH solution (0.1mmol/L), where they were vigorously agitated. After half an hour at room temperature, the anti-oxidant capacity was estimated using the formula below and represented as a percentage of DPPH lowered. The absorbance at the 517nm was then measured: DPPH% is calculated as, $\left(\frac{\text{AB (abs.of DPPH sol.with ethanol blank)} - \text{AS(abs.of DPPH sol.after interation with sample at different con.)}}{\text{AB (abs.of DPPH sol.with ethanol blank)}}\right) \text{x } 100$

The proportion of DPPH reduced versus the concentration of each sample was shown on a graph with a SC50 value[26,27] .

2.9.2 Nitric oxide scavenging activity

2ml of sodium nitroprusside (10mM) was made in phosphate buffer saline and applied to various concentrations of sample, namely standard, arginine, polyphenols, Arg+PP (0.5ml) with 150 minutes of incubation at room temperature. The same reaction combination with and without the extract served as the standard and the control, respectively. About 0.5ml of Griess reagent was added after the incubation, and then absorbance was calculated at 490 nm [28].

$$\% \text{ Inhibition} = \frac{[\text{NO}\bar{2}]\text{Control} - [\text{NO}2]\text{sample}}{[\text{NO}2]\text{Control}} \times 100$$

2.9.3 Ferric reducing power

Using potassium ferricyanide and ferric chloride, this method assessed the ability of various substances to reduce ferric compounds. Separate mixtures of the ensuing serial concentrations were made: 2.5ml of 1% potassium ferricyanide, 0.2M phosphate buffer (pH level of 6.6), and 2.5ml of each of the following: standard, arginine, polyphenols, and Arg+PP. After 20 minutes of incubation at temp. of 50°C, 2.5 Ml of 10% trichloroacetic acid (TCA) to the solutions it was also added. Then 2.5 mL of the mixture was blended with 0.5 milliliter of $FeCl_3$ (1%), followed by an equal volume of distilled water. Later then 30min. of incubation at temp. of 25°C, the solution was tested for absorbance at 700 nm. The graph was used to calculate the EC50 value, or effectual concentration, of the test sample which is to be reduced iron by 50%[28].

2.10 Estimation of invitro anti-inflammatory activity

To the test solution, 1ml egg albumin solution (1mM), 2.8ml phosphate saline buffer (pH 6.4), and 1ml of varied L-arginine, polyphenol, and L-Arg+PP concentrations ranging from 100-500 g/ml or standard acetylsalicylic acid concentrations of 100 and 200 g/ml were added. After that, the mixture produced was incubated at 27°C.By putting the reaction mixture in a water bath set at 70 °C for 10 minutes, denaturation was induced and turbidity was produced. The turbidity was measured spectrophotometrically at 660 nm after cooling. Since no

medication was added to the control, the percentage inhibition of denaturation could be estimated. Each experiment was run three times, and the average was calculated[29].

2.10.2 Anti-proteinase action

Using dilution series of L-arginine, polyphenols, L-Arg+PP, or standard to generate four concentrations (100-500/mL), the proteinase inhibitory effect was assessed. The reaction mixture was composed of 0.06 mg of trypsin, 1 ml of 20 mMTrisHCl buffer (pH 7.4), and 350 l of diluted L-arginine, polyphenols, L-Arg+PP, or standard at varied concentrations. One millilitre of 0.8% (w/v) casein was then added after the mixture had been incubated at 37°C for five min. The process was then stopped by adding 2ml of 70% $HClO_4$ after the incubation of mixture for about another 20 minutes. The resulting turbid suspension was then centrifuged, and by using buffer as a reference,the protein-hydrolyzed supernatant's absorbance was assessedat 210 nm.The procedure was carried out three times. The formula, that is,

$$\% \text{ proteinase inhibitory action} = \frac{(\text{absorbance blank} - \text{absorbance sample})}{\text{blank}} \times 100$$

was used to compute the % inhibition of proteinase activity. To get the IC50 values, a plot ofthe dosage inhibition curve in linear range betweenconcentration vs. proteinase inhibitory actionwas used. The concentration that was employed varied from 100 to 500 g/mL. The standard was acetylsalicylic acid[30].

3. Statistical evaluation

The data is shown as mean Standard Deviation. For the statistical evaluation, One-way ANOVA and the Tukey's test was employed post hoc for paired mean comparisons (GraphPad Prism 5). Statistically significant differences were shown by values of $p < 0.01$*, $p < 0.001$**and $p < 0.0001$***.

4. Results

4.1 Physicochemical analysis of honey

The following are the outcomes of the physicochemical examination of honey:

Table 1. Values of Physicohemical test of honey

S. No.	Name of test	Values
1.	Moisture content	7.5%
2.	pH	4.39
3.	Ash content	0.29%
4.	Acidity	11.41meq/kg
5.	Total Sugars	73.41%
6.	Sucrose	7.26%
7.	Total phenolic content	171.59mg/100ml
8.	Total flavonoid content	89.27mg/100ml

4.2Characterization of Honey extracted polyphenols

The HPLC retention times of phenolic compounds were used to define and identify them, and they were compared to the standard reference retention times. (Fig.1,2). The compounds found to be present were, gallic acid, epigallocatechin, apigenin, naringenin, quercetin, kaemferol, syringic acid, vanillic acid, pinocembrim, galangin, ferulic acid, coumaric acid, synaptic acid, procatechin, vanillin, caffiec acid, hydroxycinnamic acid, quinic acid, chicoric acid and epicatechin.

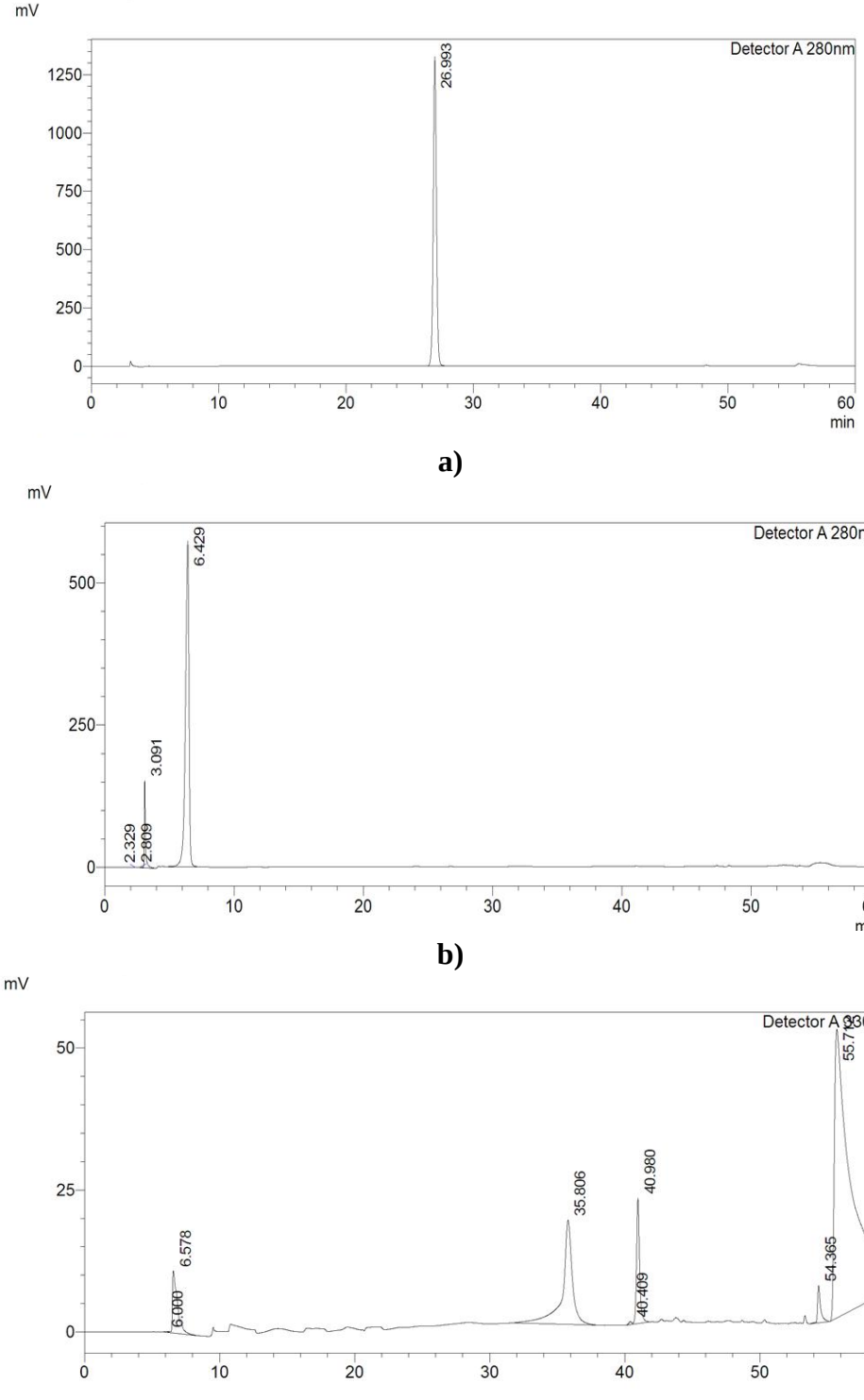


Fig.1 HPLC chromatogram of standard a) Vanillin, b) Gallic acid, c) Quercetin

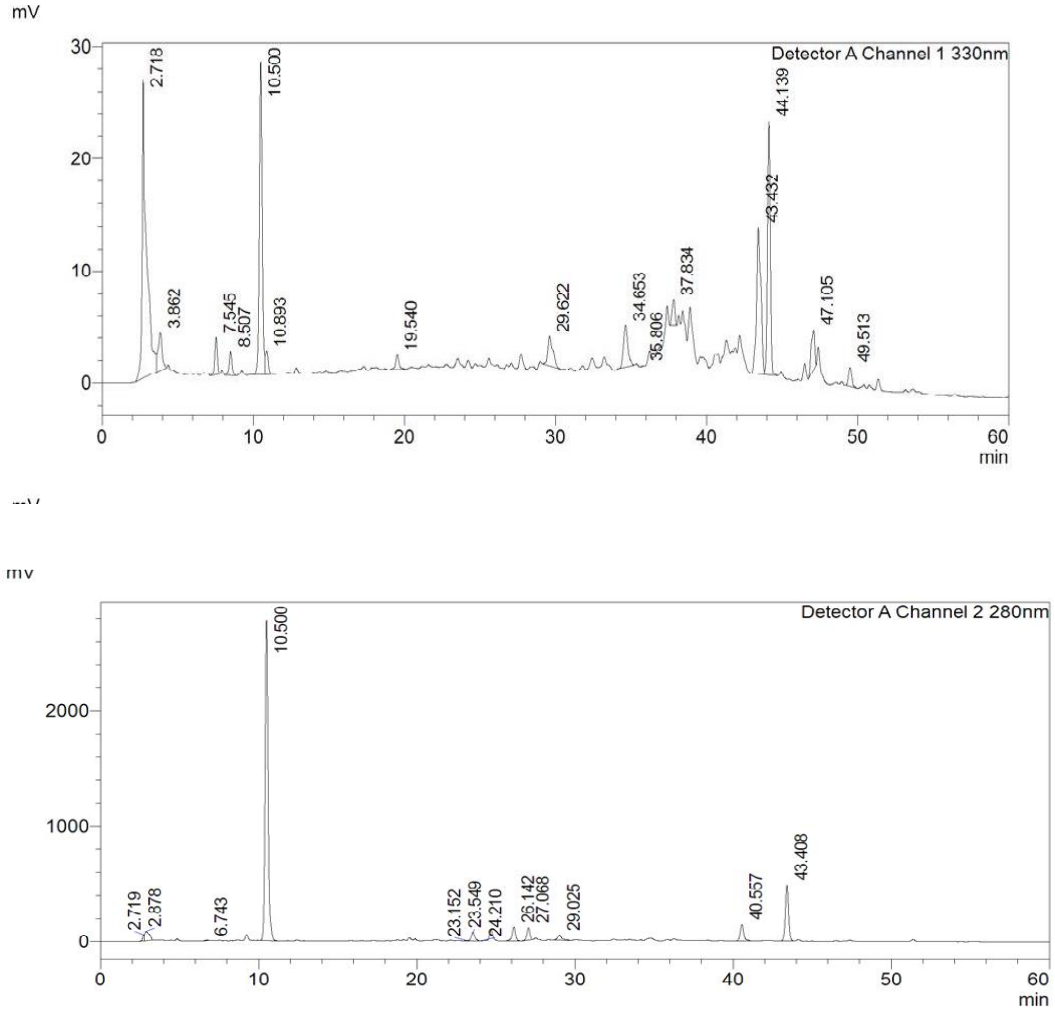


Fig 2. HPLC chromatogram of polyphenols at λ=330nm and λ=280nm.

4.3Evaluation of ointment

4.3.1 Appearance- Appearance of Ointment base and L-Arginine ointment was white whereas that of polyphenol ointment and L-Arg+PP (combined) ointment was pale yellow with characteristic odor.

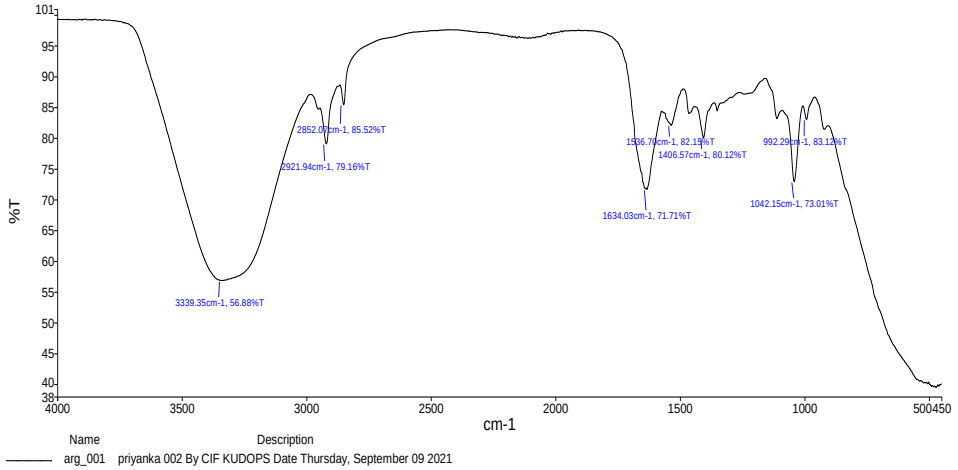
4.3.2 pH- The pH of L-Arginine ointment was 8.89, polyphenol ointment was 7.39, and L-Arg+PP (combined) ointment was 8.60, according to a pH metre.

4.3.3 Spreadability-Spreadability of L-Arginine ointment was found to be 6sec, polyphenol ointment 5sec, and of L-Arg+PP (combined) ointment was 3sec.

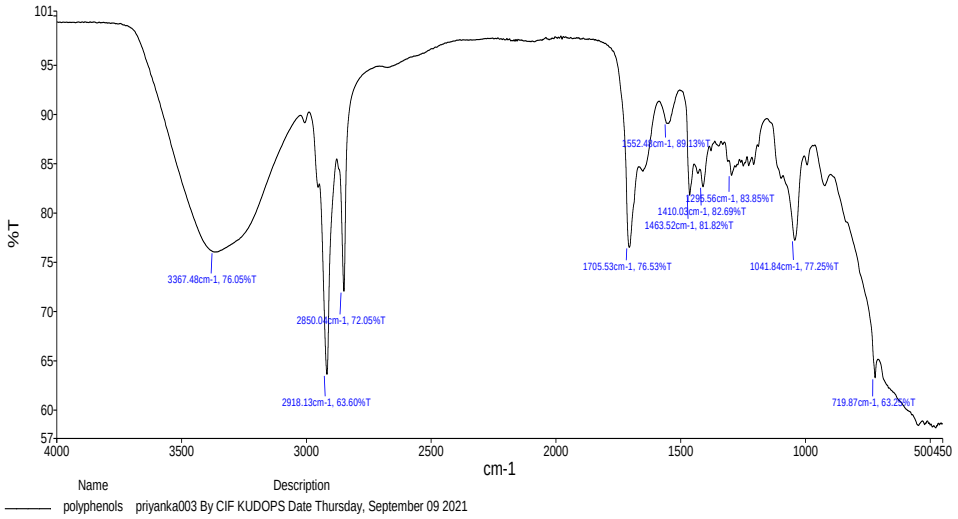
4.3.4 Extrudability- The extrudability of L-Arginine ointment was found to be 26gm, polyphenol ointment 27gm, and of L-Arg+PP (combined) ointment was 24gm.

4.4IR spectra of formulation

The FTIR spectra of L-Arginine, Polyphenols and combination of L-Arg+PP are shownin Fig 3a, 3b and 3c. The characteristic peak of L-Arginine wasobserved at 3333.7 (N-H stretch), 1643.46 (C-N stretch), 1351.43 (C=N stretch). The spectrum of Polyphenols showed the peak at 3367.48 (O-H stretch), 1705.53 (C=O stretch) and in combination of L-Arg+PP at 3337.72 (NH stretch), 3337.72 (OH stretch), 1643.46 (C-N stretch).



(a)



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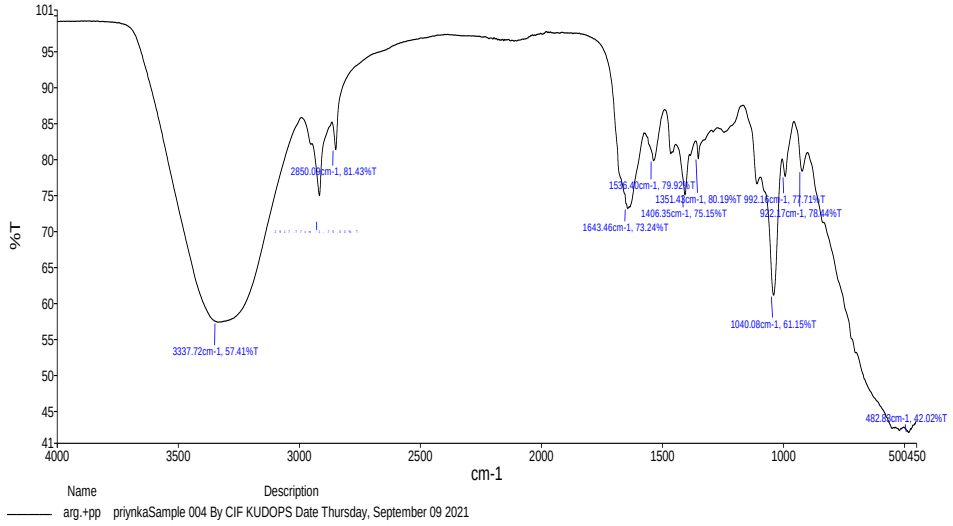


(c)

Fig 3. FTIR spectra of L-arginine, Polyphenols, combination of L-Arg+PP

4.5 Epithelialization period evaluation

The epithelialization period was reported to be lowest in L-Arg+PPcombination group ascompared to control and other ointment treated groups. Comparatively observing, the combination group experienced scab formation and shedding earlier.

Treatment Groups	Epithelialization period (Mean+SEM)
Control (Ointment base)	11.33±0.210
Standard (Betadiene 10%w/w)	5.667±0.333***
Test 1 (L-Arginine 10%)	6.000±0.258***
Test 2 (Polyphenols 10%)	7.167±0.307***
Test 3 (L-Arg+PP 10%)	4.333±0.210***

Table 2. Data showing epithelialization period of control, standard, L-arginine, polyphenol, L-Arg+PP groups.Values are depicted as Mean ± SE.

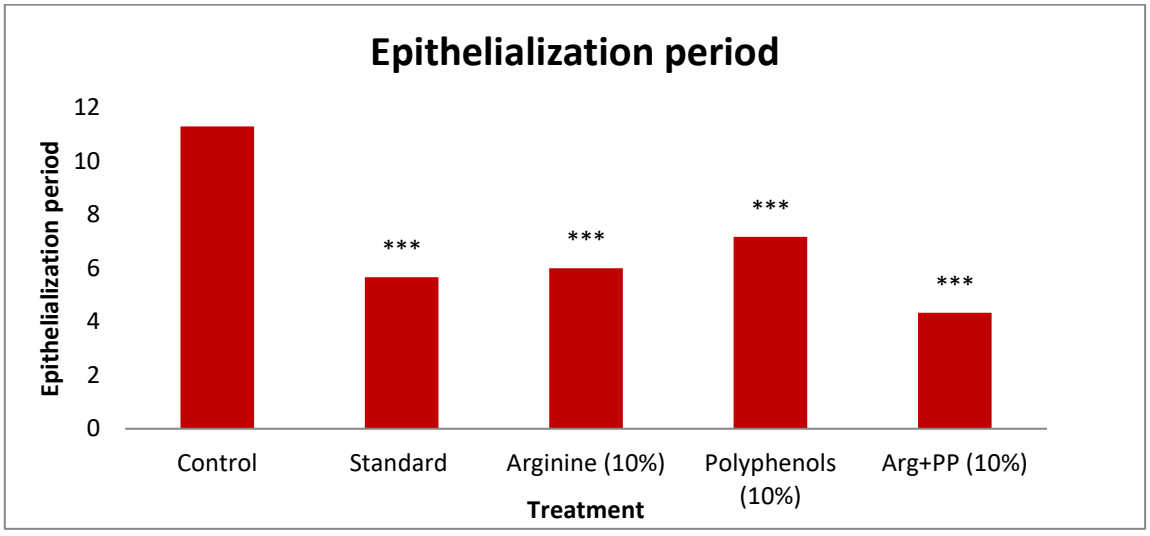


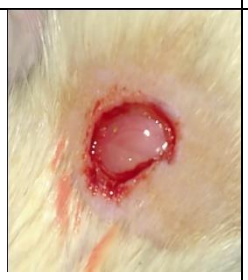
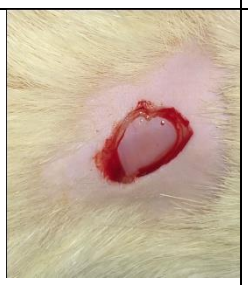
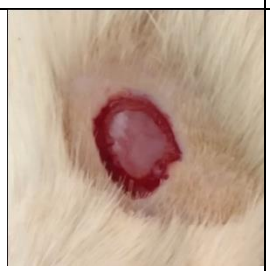
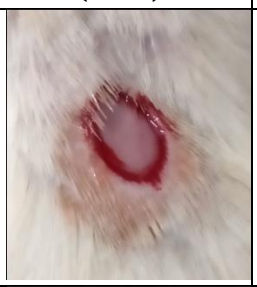
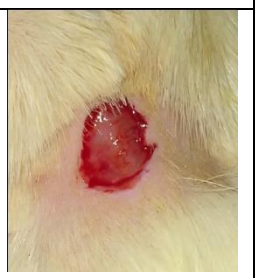
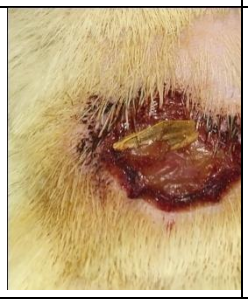
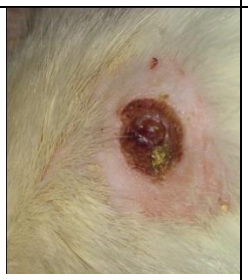
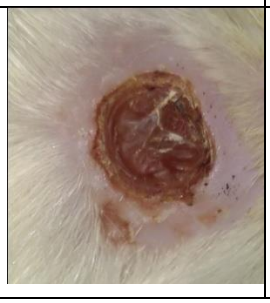
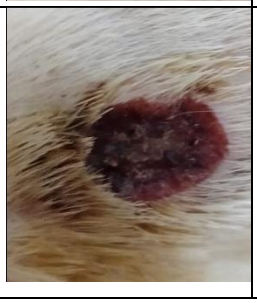
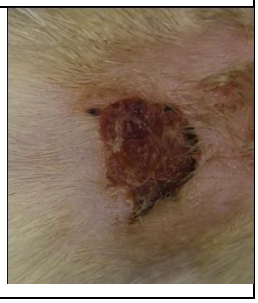
Fig 4. Effects ofcontrol, standard, arginine, polyphenol, Arg+PP groupson epithelialization period.

4.6*Wound closure effect of PP+ARG combination*

As shown by the percent wound contraction, topical use of the PP+ARG combination (ointment) significantly reduced the size of the wound. In comparison to the other groups, except standard, wound closure was more pronounced in combinationofPP+ARGtreated wounds after 5th day.

S.No.	Time (Days)	Control (Ointment base)	Standard (Betadiene 10%w/w)	Test 1 Arginine (10%w/w)	Test 2 Polyphenols (10%w/w)	Test 3 (Arg+PP) (10%w/w)
1.	Day 0	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
2.	Day 3	9.650±0.5627	7.117±0.0945 ***	7.817±0.0600 ***	7.983±0.0477 ***	7.500±0.1549 ***
3.	Day 6	9.067±0.0333	6.917±0.0477 ***	7.550±0.0670 ***	7.800±0.0447 ***	7.317±0.0654 ***
4.	Day 9	7.350±0.1708	4.133±0.1022 ***	5.500±0.3098 ***	5.800±0.3474 **	5.383±0.3260 ***
5.	Day 12	5.933±0.1687	0.750±0.0763 ***	2.450±0.0922 ***	3.750±0.5156 ***	1.817±0.1108 ***
6.	Day 15	4.350±0.2986	0.016±0.0166 ***	0.183±0.0542 ***	0.983±0.3027 ***	0.116±0.0307 ***

Table 2: Effects of control, standard, arginine, polyphenol, Arg+PP groups on % wound healing. Results of the data are expressed as mean ± SEM (n=6) **p<0.01, ***p<0.001 as compared to the respective control group.

Time (Days)	Control Group	Standard	Arginine (10%)	Polyphenols (10%)	ARG+PP (10%)
Day 0					
Day 3					

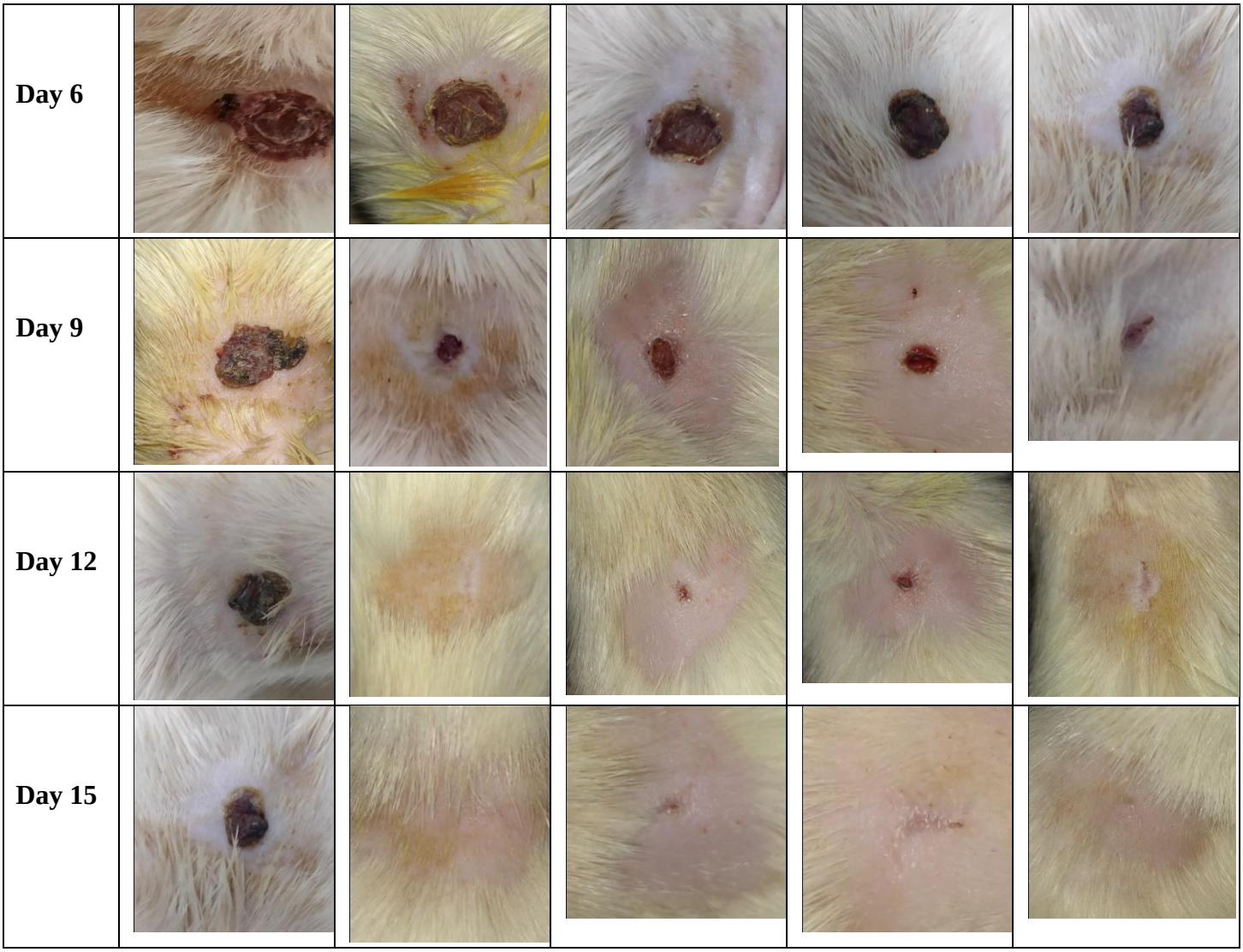


Fig 5. The control, standard, L-Arginine (10%), Polyphenols (10%), and L-Arg+PP (10%) treatment groups' representative wound photos. Ruler measurements are made in millimetres.The percentage of the original area used to measure the wounds' healing over time.

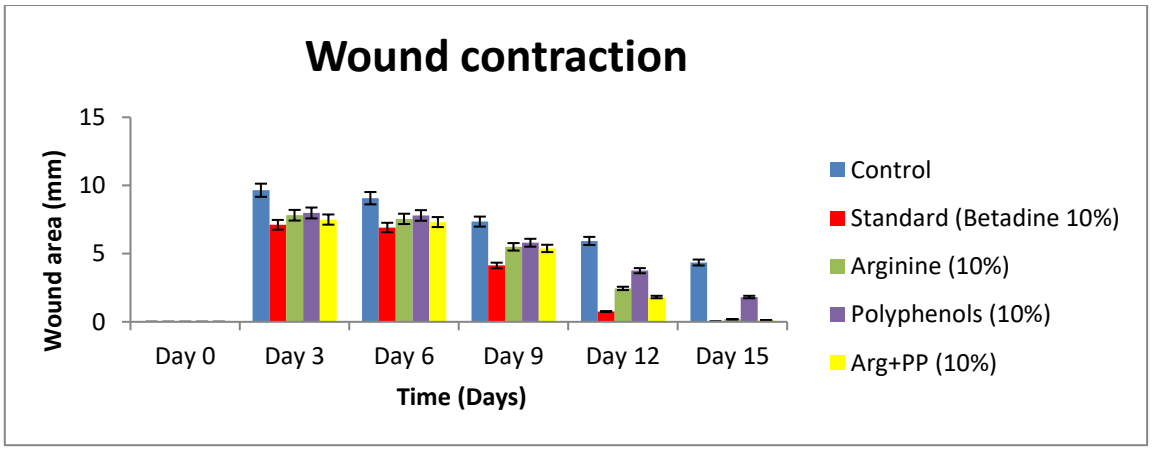


Fig 6. % Wound healing by control, standard, L-arginine ointment, polyphenol ointment, L-Arg+PP ointment on days 3, 6, 9, 12 and 15.

4.7 DPPH radical scavenging activity

The combination of Arg+PP possessed significant DPPH scavenging activity with SC50 of 22.74µg/ml when compared with standard, l-arginine and polyphenols which SC50 was found to be 25.31µg/ml, 27.63µg/ml and 29.04µg/ml.

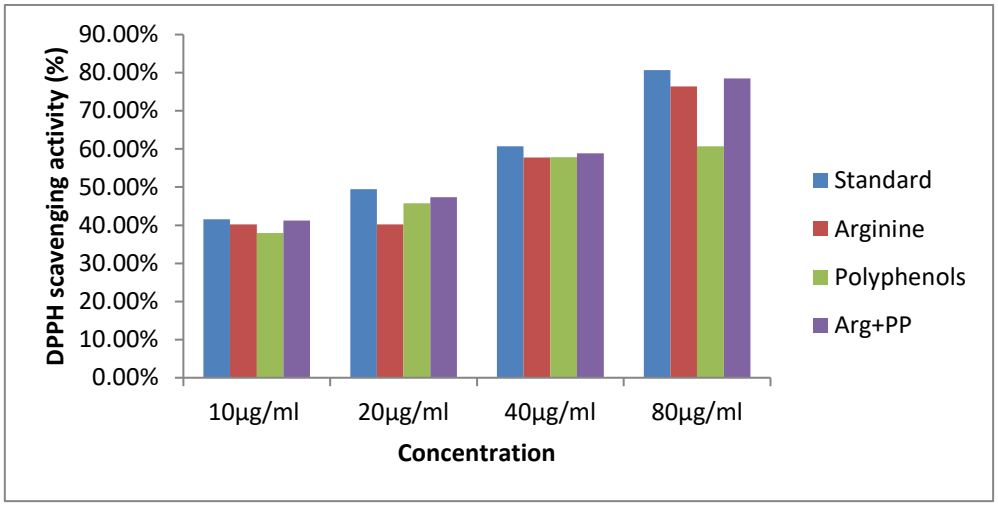


Fig 7. Effect of various standard concentrations, including L-arginine, polyphenols, and ascorbic acid, on DPPH radical scavenging assay

4.8 Nitric oxide scavenging activity

The combination of Arg+PP possessed significant Nitric oxide scavenging activity with IC50 of 22.34µg/ml when compared with standard, l-arginine and polyphenols which IC50 was found to be 30.8µg/ml, 35.3µg/ml and 26.80µg/ml.

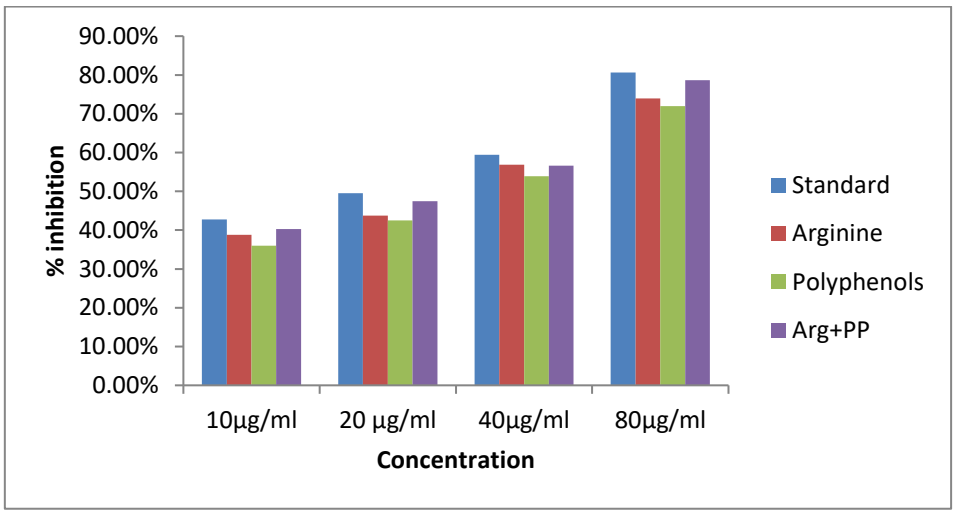


Fig 8. Effect of various standard concentrations, including L-arginine, polyphenols, and ascorbic acid, on the Nitric Oxide Scavenging Assay

4.9 Ferric reducing power

The combination of Arg+PP possessed significant ferric reducing power with IC50 of 22.17µg/ml when compared with standard, l-arginine and polyphenols which IC50 was found to be 5.23µg/ml, 17.5µg/ml and 29.04µg/ml.

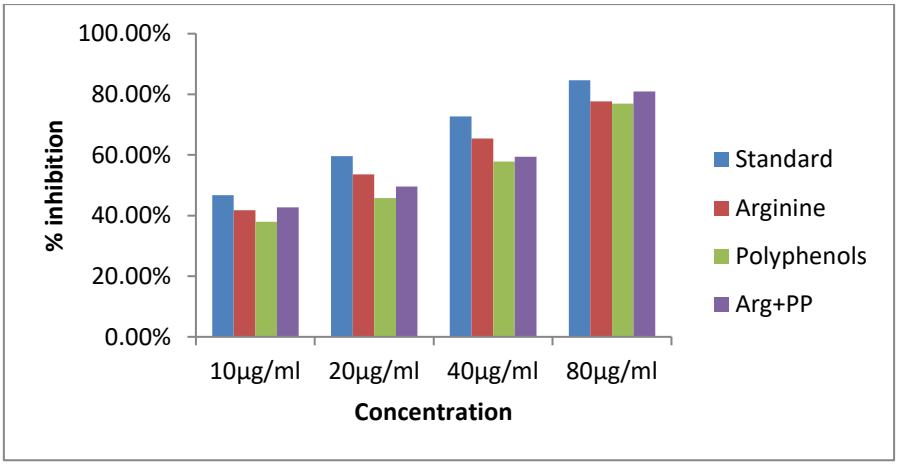


Fig 9. Effect of various standard concentrations, including L-arginine, polyphenols, and ascorbic acid, on Ferric reducing power assay

4.10 Effect on Protein Denaturation

The combination of Arg+PP possessed significant protein denaturation activity with IC50 of 34.90µg/ml when compared with standard, l-arginine and polyphenols which IC50 was found to be 34.4µg/ml, 37.35µg/ml and 47.87µg/ml.

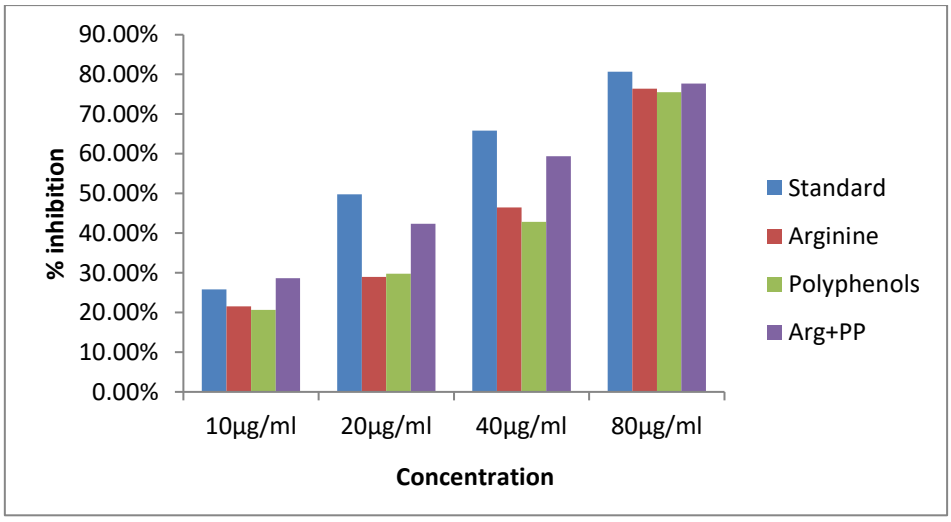


Fig 10. Anti-inflammatory activity of standard, L-arginine, polyphenols and L-Arg+PP for different concentrations using Inhibition of Protein denaturation method.

4.11 Anti-proteinase action

The combination of Arg+PP possessed significant anti-proteinase activity with IC50 of 37.19µg/ml when compared with standard, l-arginine and polyphenols which IC50 was found to be 21.9µg/ml, 47.56µg/ml and 24.30µg/ml.

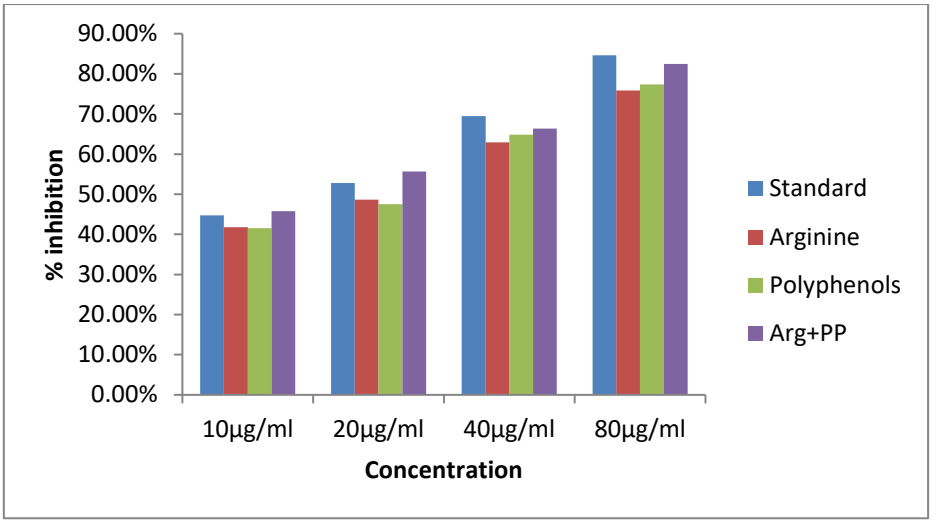


Fig 11. Anti-inflammatory activity of standard, L-arginine, polyphenols and L-Arg+PP for different concentration using Anti-proteinase action method.

5. Discussion

In this study, the effect of L-Arg+PP on wistar rats' ability to repair wounds was investigated.The polyphenols used were extracted from honey which was characterised by HPLC analytical technique and ethyl acetate was used as extracting agent. The compatibility between L-arginine and polyphenols was evaluated by IR spectroscopy and the resultant spectra showed the compatibility between arginine and polyphenols. Then the topical formulation, that is, ointment of arginine, polyphenols and combination of L-Arg+PPwere formulated and then its pharmacological ability to treat wounds was assessed. The epithelialization period and % wound contraction were measured from the wound healing activity.In example, polyphenols have anti-inflammatory actions via scavenging free radicals, controlling cellular processes in inflammatory cells, and regulating the actions of the enzymes responsible for metabolising arachidonic acid (phospholipase A2, COX),arginine, and other proinflammatory compounds. Inhibiting the generation of ROS-producing enzymes, scavenging ROS, or upregulating or protecting antioxidant defences of antioxidant activity, such as scavenging radicals and metal ion chelation capabilities, are all examples of polyphenol antioxidant activity. Polyphenol wound healing activity depends on its natural antioxidant properties which can efficiently eliminate excess ROS during the inflammatory stage and also lessen the inflammatory response by targeting particular enzymes.Additionally, arginine possess its effects by NO synthesis. NO appears to affect the translational or post-translational levels of protein or collagen synthesis. NO both *in vitro* and *in vivo* up-regulates the metalloproteinase matrix metalloproteinase-2 (gelatinase), which is largely produced by fibroblasts and expressed principally during the remodelling phase of wound healing.

% inhibition was seen when the combination of Arg+PP'swas evaluated for*in vitro*anti-inflammatory andantioxidant properties. To measure the antioxidant activity, the DPPH radical scavenging test, ferric reducing power assay, and Nitric oxide scavenging assay were employed to for which various concentration of arginine, polyphenols, and Arg+PP groups against ascorbic acid i.e. standard were prepared. The *in vitro* anti-inflammatory activity was

also carried out for various concentrations of standard, arginine, polyphenols, and Arg+PP groups, and the methods utilised were protein denaturation method and anti-proteinase action.

6. Conclusion

The combined effects of L-Arg+PP (Polyphenols) on topical wound healing were examined in the current study. In conclusion, L-Arg+PP dramatically sped up the healing process for excision wounds in rats. According to the current study, the combination significantly sped up wound healing compared to applying L-arginine and polyphenols separately. L-arginine and polyphenols are compatible with one another, according to the results of the combo treatment. Combination therapy may be more beneficial in complex circumstances where a single pharmacological treatment is ineffective.It was assume that the combination of L-Arg+PP enhances the wound healing by synthesize the NO and by the regulation of cellular activities in inflammatory cells.In *invitro* antioxidant study, the combination of L-Arg+PP exhibited the highest % inhibitory action in comparison to individual action of L-arginine and polyphenols, evidenced by the present study. Similarly, the combination of L-Arg+PP exhibited significantly higherantiinflammatory activity in comparison to individual action of L-arginine and polyphenols.

Conflict of Interest

None

Acknowledgements

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