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Exploration of the Angiogenic Potential of Honey

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Authors' contributions

Authors RMM and SSB were responsible for the concept, overall coordination of the study, data interpretation and review of the manuscript for intellectual inputs and Author SAK supervised the experimental work, reviewed and analyzed the data, managed the literature searches and reframed the manuscript. Author TAP performed the study, managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The present study was conducted to confirm the angiogenic potential of honey using Chick Chorioallantoic Membrane (CAM), an *in ovo* model and to study its effect on Vascular Endothelial Growth Factor (VEGF) expression in the CAM tissue. Attempts were also made to identify the probable active constituents present in honey that contributed to its angiogenic potential.
Methodology: Honey was evaluated over concentrations ranging from 0.015 to 25% v/v and the extent of angiogenesis was quantified using stereomicroscopy. VEGF expression at transcript level was determined by RT-PCR. Erythropoietin and Heparin were used as positive and negative controls respectively. Four known constituents of honey *viz.*, Glucose, Proline, Vitamin C and Hydrogen peroxide were tested by biochemical methods.
Results: New blood formation was seen at all the concentrations tested, however the pro-angiogenic effect was greater at lower concentrations. These results were significantly greater than that seen with erythropoietin, the positive control. VEGF mRNA expression in CAM tissue also demonstrated similar findings. Among the constituents tested, Vitamin C and Hydrogen peroxide were observed to be associated with the angiogenic effect of honey.
Conclusion: The study thus confirms the pro-angiogenic potential of honey at low

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concentrations. This effect is probably due to the presence of Hydrogen peroxide and Vitamin C and is mediated via alteration in VEGF expression.
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Keywords: Honey; angiogenesis; VEGF; Chorioallantoic membrane; glucose; proline; hydrogen peroxide; stereomicroscopy.

1. INTRODUCTION

Honey has been used in the treatment of acute and chronic wounds since ancient times. Today, with ever increasing bacterial resistance to antibiotics, old remedies are being scientifically re-evaluated. The antibacterial activity of honey has been established in previous studies and it has also been claimed to aid wound healing. Application of dressing containing honey is known to reduce inflammation; debris necrotic tissue reduces oedema and promotes angiogenesis, granulation and epithelialization [1]. Many reports describe the efficacy of honey in aiding the healing of chronic wounds including leg ulcers. Several *in vivo* and *in vitro* studies have demonstrated the direct anti-inflammatory activity of honey and its ability to decrease oxidative stress, by mopping up free radicals arising from burns [2-4] All these studies indicate that honey possesses a broad-spectrum of activity that can positively impact wound healing at a cellular level.

Honey has also been shown to possess pro-angiogenic activity when applied to animal wounds. This may account for the clinically observed rapid development of granulation tissue [5,6], as granules are fibroblasts growing at sites where there are capillary bundles supplying oxygen. Very little research has been published describing the pro-angiogenic effect of honey and the mechanism(s) by which honey stimulates angiogenesis are unknown [7,8]. Many growth factors have been postulated to stimulate one or more steps of the angiogenic process; however, a growing belief is that vascular endothelial growth factor (VEGF) plays a key regulatory role in angiogenesis in both physiological and pathological conditions [9,10].

Honey is a complex natural product, containing more than 400 different substances, e.g. various carbohydrates, organic acids, proteins, amino acids, vitamins, enzymes, aroma substances, mineral substances, pigments, waxes, etc [11]. Of these components, the components responsible for the angiogenesis are not identified yet.

With this background the present study was conducted with an objective to confirm the angiogenic potential of honey using a chick embryo chorioallantoic membrane (CAM) model. CAM is an extra embryonic membrane that is commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors [12]. Further, the VEGF expression in CAM treated with honey was also studied. An attempt was also made to identify the probable active constituents present in honey which could contribute to its angiogenic potential.

2. MATERIALS AND METHODS

2.1 Study Drugs

Honey (obtained from Phondaghat Pharmacy, Lot no. 10, date of Pkg. May 2010) was evaluated at 8 different concentrations *viz.,* 0.015, 0.06,0.25,1.5,3,6.25,12.5 & 25% v/v. All

the concentrations were prepared in normal saline solution which acted as vehicle control. Erythropoietin (100µg/ml) procured from local pharmacy was used as the Positive control while Heparin (10U/ml) was used as the Negative control.

2.2 Materials

Eggs: Fertilized eggs of White Leghorn breed chickens (50-60 g) were purchased from local poultry farm, India.

Chemicals: All the chemicals were purchased from Sigma Aldrich, USA and SD Fine chemicals.

2.3 Chick Chorioallantoic Membrane (CAM) Assay

For CAM assay, fresh fertile 6 day old eggs from White Leghorn chickens were used. The eggs were cleaned with 70 % ethanol and study drugs were inoculated through the window made in the blunt side of the eggshell, which was then sealed with parafilm. The vehicle control group was inoculated with sterile normal saline solution, standard group was inoculated with Erythropoietin (100µg/ml) and the test group was inoculated with 0.015, 0.06, 0.25, 1.5, 3, 6.25, 12.5 & 25% of honey. A constant volume of 10 µl was used for inoculation. The eggs were then incubated under condition of constant humidity at 37ºC till day 12.

2.4 Stereomicroscopic Quantification of Blood Vessel Growth

After 12th day of incubation the eggs were removed from the incubator and the CAM tissues were isolated. Images of four non-overlapping regions of the isolated CAM were taken using the stereomicroscope. The average of the count obtained from the analyzed images was compared with that of vehicle control and standard drugs using '3.1 Analysis Software'. The number of blood vessels is directly proportional to the extent of angiogenesis. For counting, a single intact blood vessel was given a score of 1 whereas when bifurcation of single blood vessel was observed a score of 2 was given.

2.5 VEGF Expression Study

From the CAM isolated on 12^{th} day, RNA was extracted using Trizol reagent. cDNA was prepared using First Strand cDNA Synthesis Kit (Fermentas). The cDNA was subjected to PCR with the primers for VEGF corresponded to sequences of exon 3(sense) and exon 7 (antisense) [13] and amplified two splicing variants of avian VEGF, VEGF190 (456 bp) and VEGF165 (381 bp).The primers for VEGF were designed according to the chicken sequence available (GenBank Accession Number AB011078). The primers used were Sense 5′GACCCTGGTGGACATTTTCC3′ & Antisense 5′GTGCGCTCGTTTAACTCAAGC3′. [14] For PCR reaction, 1 µL of cDNA mixture was added to a PCR reaction mix containing 10X PCR buffer, 2 mM dNTP, 10 pM of paired primers, 2 units of Taq polymerase. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and documented. The expression levels were visualized by scanning on a gel documentation system (Syngene Corporation) and the intensity of the expression was determined in terms of the band width.

2.6 Determination of Active Constituents Present in Honey

Honey contains a large number of constituents; however there is limited literature available on their potential role on angiogenesis. In the present study we evaluated only those constituents that are known to be present in higher concentrations in honey.

Carbohydrates: Glucose was estimated by GOD-POD method by using ready to assay kits procured from M/s Transasia Biomedicals Pvt Ltd using a fully automated biochemistry analyser.

Amino Acids: The amount of Proline content in honey was estimated spectro-photometrically [15].

Hydrogen Peroxide (H_2O_2): Concentration of H_2O_2 in honey was estimated in terms of Catalase activity using Beutlers et al Method [16] 1:2000 hemolysate prepared in ethanol was used as source of Catalase.

Vitamin C: Vitamin C in varying concentrations of honey was estimated spectro-photometrically using DNPH method [17].

2.7 Statistical Analysis

The data was analyzed statistically using ANOVA followed by post-hoc test using the Graphpad Instat software Version 3.06. The results obtained with honey were compared with the study controls and $p<0.05$ was considered as level of significance. Pearson's correlation between various active constituents and angiogenesis was analyzed at $p<0.05$. All values in the figures or text are represented as Mean ± SD.

3. RESULTS AND DISCUSSION

3.1 Effect on Blood Vessel Formation

Erythropoietin (100µg/ml), the positive control (PC) used in the study demonstrated an increase in the blood vessel formation indicating its pro-angiogenic effect whereas heparin, the negative control (NC) demonstrated an anti-angiogenic effect. Honey showed a dose dependent decrease in the formation of blood vessel with maximum blood vessel formation seen at lower concentrations. At higher concentrations, the number of blood vessels formed was almost similar to the vehicle control indicating minimal effect of angiogenesis at this concentration.

All studied concentrations however demonstrated an increase in the number of blood vessels as compared to normal control and the vehicle control (VC) indicating a pro-angiogenic effect This effect was significantly higher than that seen with erythropoietin (Fig. 1). The stereomicroscopic images are represented in Fig. 2.

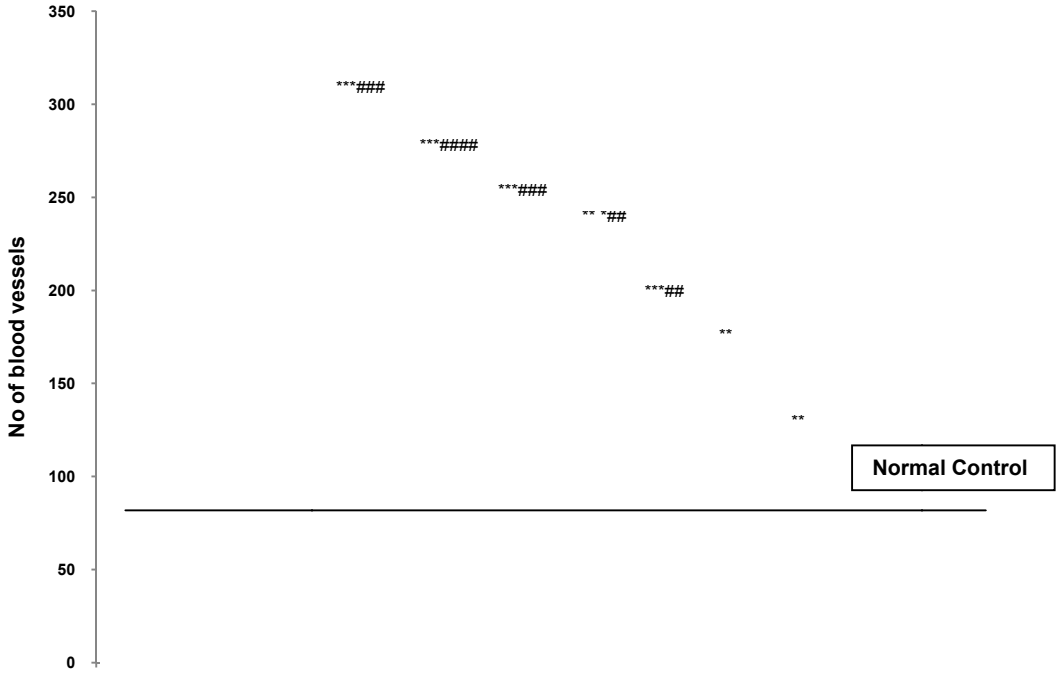
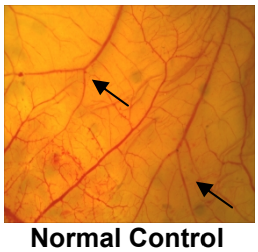
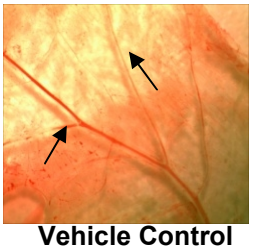


Fig. 1. Effect of honey on blood vessel formation (n=6)

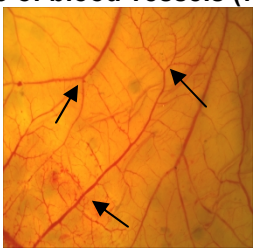
Data represented as Mean ± SD. $^{**}p < 0.01$, $^{**}p<0.01$; $^{***}p<0.001$ as compared to vehicle control; $^{###}p<0.001$, $^{##}p<0.01$ as compared to positive control (ANOVA followed by post-hoc tests); n= number of eggs



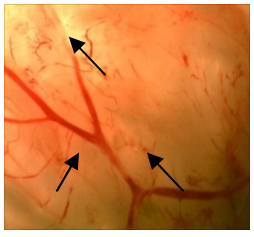
Normal Control
No of blood vessels (72)



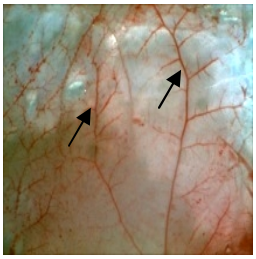
Vehicle Control
No of blood vessels (75)



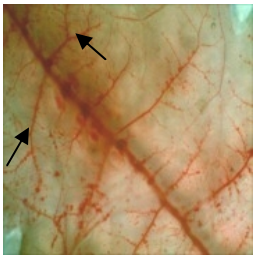
Positive Control
No of blood vessels (87)



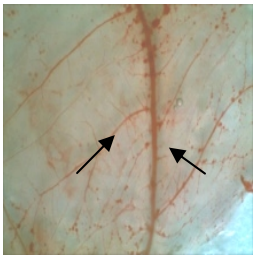
Negative Control
No of blood vessels (48)



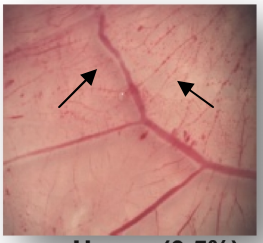
Honey (0.015%)
No of blood vessels (250)



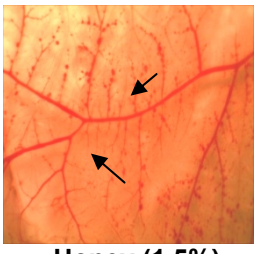
Honey (0.0625%)
No of blood vessels (234)



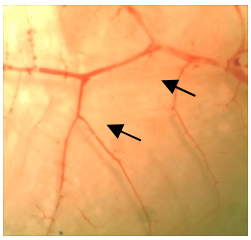
Honey (0.25%)
No of blood vessels (198)



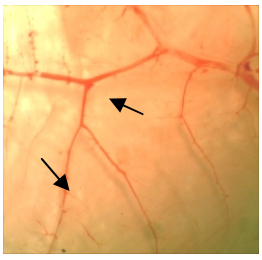
Honey (0.5%)
No of blood vessels (172)



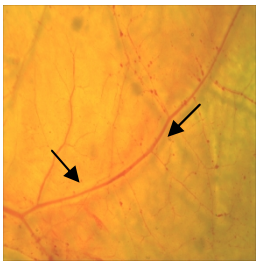
Honey (1.5%)
No of blood vessels (153)



Honey (6.25%)
No of blood vessels (102)



Honey (12.5%)
No of blood vessels (88)



Honey (25%)
No of blood vessels (78.9)

Fig.2. Stereomicroscope Images of CAM

3.2 Expression

To determine the mechanism of action of honey, VEGF expression at transcript level was determined by RT-PCR. VEGF expression was studied only at selected concentrations *.i.e.,* 1.5, 6.25 and 12.5% due to financial constraints. VEGF levels were expressed after normalization with GAPDH expression, a house keeping gene to normalize the cDNA concentration for all the primers. The PCR products were run on agarose along with 100bp ladder (well 1). In well 3 cDNA of Erythropoietin (positive control) treated CAM showed two distinct bands, one of 456 bp corresponding to VEGF 190 and the second of 381 bp corresponding to VEGF 165, of which VEGF 165 is the dominant band. Although these bands were also seen in the Heparin (Negative control- well 2) as well as saline treated CAM (well 4), they were not as distinct. In case of honey, 2 bands were expressed however they were most distinct at the 1.5% concentration as compared to the higher concentrations. The results are summarized in Fig. 3.

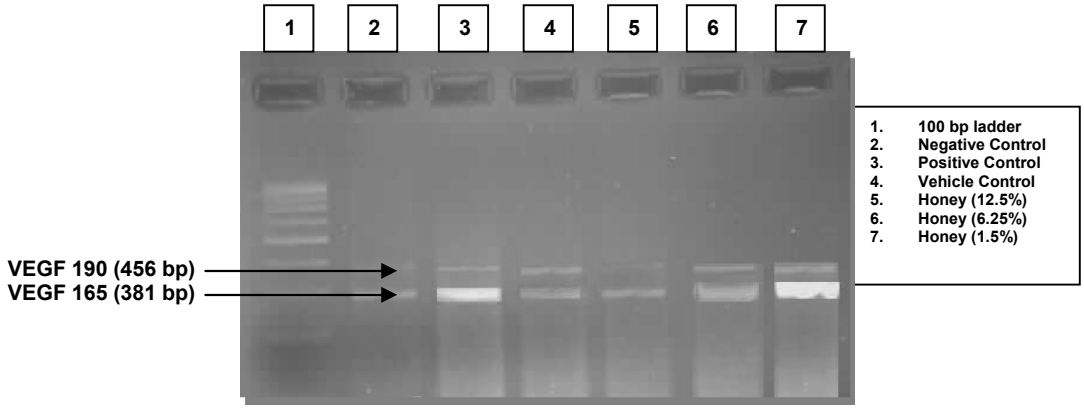


Fig. 3. Gel Electrophoresis of mRNA showing VEGF Expression

3.3 Determination of active Constituents Present in Honey

A concentration dependent increase in glucose content was seen with increasing concentrations of honey (Fig. 4.1). Hydrogen peroxide was estimated in the different concentrations of honey in terms of Catalase activity. A decrease in H_2O_2 concentration was observed with increasing concentrations of honey (Fig. 4.2). However proline and Vitamin C content increased with increasing concentrations of honey (Fig. 4.3 & Fig. 4.4).

As seen in Fig. 5 a positive correlation between angiogenesis and catalase activity was observed ($r = 0.8389$; $p=0.0047$) and a negative correlation between Glucose ($r= -0.6943$; $p=0.0380$), Proline ($r = -0.9722$; $p = 0.0001$) and Vitamin C ($r = -0.8838$; $p= 0.0016$) was seen.

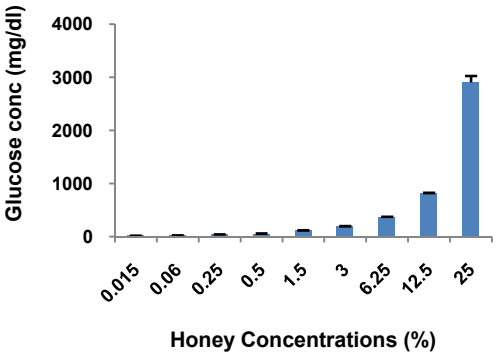


Fig 4.1. Glucose

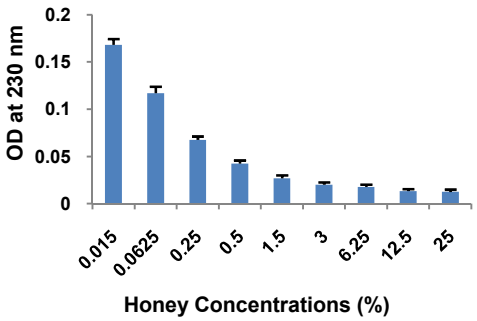


Fig 4.2. Catalase

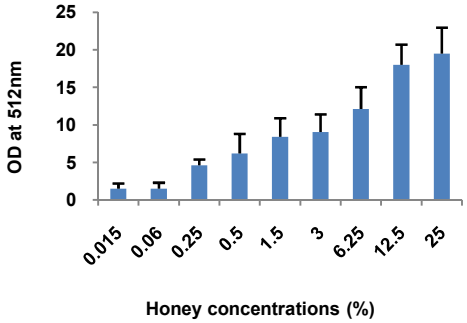


Fig 4.3 : Proline

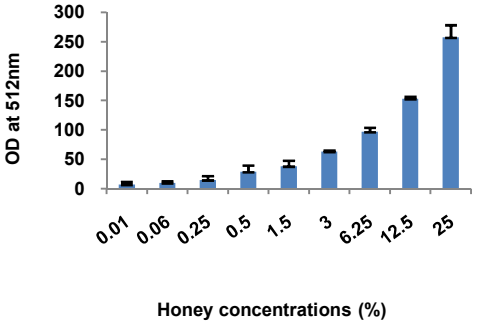
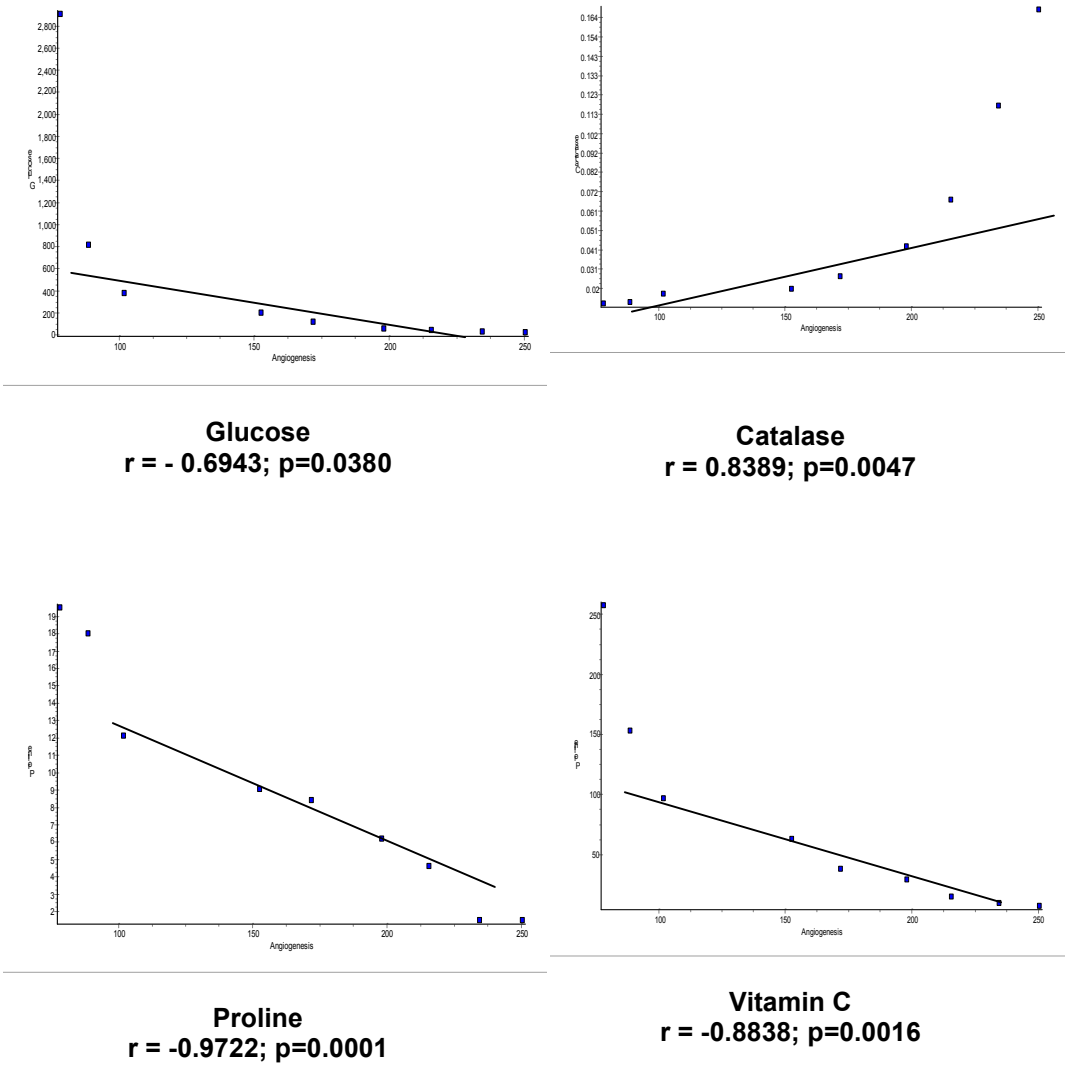


Fig 4.4 : Vitamin C

Fig. 4. Evaluation of active constituents present in honey



Glucose
r = - 0.6943; p=0.0380

Catalase
r = 0.8389; p=0.0047

Proline
r = -0.9722; p=0.0001

Vitamin C
r = -0.8838; p=0.0016

Fig. 5. Correlation between angiogenesis (No of new blood vessel formation) and active constituents

4. DISCUSSION

Honey has long been documented as having healing properties [18,19] and more recent research has shown that honey can be effective for clearing infections in a wide range of wounds, including abscesses, surgical wounds, traumatic wounds, burns, and ulcers of varied etiology. Hepsen *et al* have described the inhibitory effect of propolis (which is collected by bees and used as a cement in hive construction) on corneal neovascularisation in a rabbit corneal model, while Molan generalised that honey'…promotes tissue regeneration through stimulation of angiogenesis and the growth of fibroblasts and epithelial

cells' [20-21]. The present study was thus conducted to confirm the angiogenic potential of honey and to study its effect on VEGF expression. Attempts were also made to identify the probable constituents among the major constituents of honey i.e., carbohydrates, amino acids, vitamins and enzymes contributing to its angiogenic potential.

The angiogenic potential of honey was explored in the CAM Model by quantitative estimation of the new blood vessels using stereomicroscopy. The effect of honey was evaluated over a concentration range of 0.015 to 25%. Initially we had planned the study using 6 concentrations of honey *viz*. 100%, 50%, 25%, 12.5%, 6.25% and 1.5%. However, with the higher concentrations *viz*. 100% and 50% we repeatedly faced the problem of contamination which was possibly due to its high sugar content. UV sterilization would probably have prevented this issue, but, due to lack of access to the same, we had to limit our study to slightly lower concentrations (0.015 to 25%). Although the results of the higher concentrations (50 & 100%) is not reported in this paper due to incomplete [n=3] data; we observed an anti-angiogenic effect of honey even at these concentrations. A dose dependent significant decrease in blood vessel formation in CAM as compared to the vehicle control was seen with select concentrations tested, with maximal pro-angiogenic effect seen at the lowest concentration tested *i.e.,* 1.5%.

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic growth factors and plays a significant role in both development and homeostasis. It plays an important role in the activation of endothelial cells during the initial steps of angiogenesis and also in the maintenance of the differentiated state of blood vessels [22]. Thus, the extent of angiogenesis depends on the extent of VEGF expression. Erythropoietin, the standard used in the study caused an increase in the number of blood vessels confirming its pro-angiogenic activity whereas heparin demonstrated an anti-angiogenic effect by decreasing the number of new blood vessel formation. The VEGF mRNA expression of Erythropoietin treated CAM showed expression of two prominent VEGF bands of 456 bp and 381 bp respectively, as compared to bands observed with heparin treated CAM and saline treated CAM, proving its angiogenic effect. All the concentrations of honey tested showed expression of VEGF bands of 456 bp and 381 bp with maximum expression observed at 1.5% concentration. These results were in line with that obtained with the stereomicroscopy thus confirming the pro-angiogenic effect of honey at the lowest concentrations. Literature reveals that CAM cells express at least two VEGF isoforms, $VEGF_{165}$ and $VEGF_{190}$. Also the $VEGF_{165}$ is dominant isoform in CAM over the other isoform $VEGF_{190.}$ Also in our study $VEGF_{165}$ was expressed dominantly as compared to $VEGF_{190}$ in CAM treated with honey with maximum expression seen at lower concentrations.

Honey is a concentrated aqueous solution of inverted sugars, but it also contains a very complex mixture of other saccharides, proteins, enzymes, amino acids, organic acids, polyphenols, and carotenoid like substances, maillard reaction products, vitamins and minerals [23]. Carbohydrates constitute about 95 to 97% of the dry weight of honey [24]. Fructose and glucose are the most predominant sugars present and are responsible for most of the physical and nutritional characteristics of honey [25]. Hence to identify the probable constituents present in the honey responsible for its angiogenic potential we detected the presence of carbohydrates, mainly glucose. However we observed that the glucose content in honey increased with increasing concentrations of honey and we had obtained pro-angiogenic effect of honey at lower concentrations. Based on these observations we can conclude that glucose may not be responsible for the angiogenic effect of honey. Hence the other major carbohydrate *i.e.,* fructose may be evaluated further to explore the role of carbohydrates in angiogenesis.

Approximately 18 essential and non-essential amino acids are present in honey. Proline is the primary amino acid, and lysine being the second most prevalent [26]. In our study we evaluated proline content in honey to evaluate the role of amino acids in angiogenesis. It was observed that proline content increased with increasing concentrations of honey. Increased angiogenic effect was observed at lower concentration of honey in our results whereas the proline content at this concentration was found to be the least as compared to higher concentrations. Thus we assume that presence of proline in honey may not contribute to its angiogenic potential.

Honey contains trace amounts of Vitamins and minerals. As vitamin C is known to play a vital role in wound healing, we decided to estimate its levels in honey to explore its role in angiogenesis. Vitamin C levels were found to be increased with increasing concentrations of honey in our study and there are experimental studies wherein anti-angiogenic effect has been reported at high doses of Vitamin C [27]. In our study too, we found that as the concentration of honey decreased, the pro-angiogenic potential increased depicting the role of Vitamin C in angiogenesis. It has been reported that the vitamin C content of honey, which is typically at least three times higher than that in serum, [28] could be of particular importance because of the essential role of this vitamin in collagen synthesis thereby contributing to angiogenesis. Thus, it is possible that at low concentrations of honey, the inhibitory effect of Vitamin C on angiogenesis decreases resulting in the pro-angiogenic effect seen. Hence we postulate that trace amounts of Vitamin C may be responsible for the angiogenic effect exhibited by honey.

Another constituent which we looked upon in evaluating the angiogenic potential of honey was hydrogen peroxide as it is a commonly used antiseptic. Hydrogen peroxide was estimated in terms of Catalase activity in the different concentrations of honey. It was observed that as the concentration of honey increased, the H_2O_2 concentration decreased. Literature has long suggested that the rate of hydrogen peroxide production by glucose oxidase in honey depends largely on the degree of honey dilution [29] and that little or no production of hydrogen peroxide occurs in full strength honey. This is in agreement with our results of angiogenesis which showed greater angiogenesis at lower concentrations of honey. Thus we can infer that the presence of hydrogen peroxide in honey would contribute majorly to its pro-angiogenic effect.

The study thus confirms the pro-angiogenic potential of honey which was highest at the lowest concentration tested, which is probably due to the presence of H_2O_2 and that the angiogenic effect is mediated *via* VEGF expression. This is a first study to report that the angiogenic potential of honey is mediated *via* VEGF expression and that vitamin C and H_2O_2 may be responsible for its angiogenic effect. Further studies, to investigate VEGF upregulation and vascular morphometric changes in the CAM tissue on exposure to honey are necessary to confirm or refute this observation. Effect of other constituents' *viz.,* proteins, enzymes, other amino acids, organic acids, polyphenols, and carotenoid present in honey may also be explored as we have evaluated the role of only select constituents in our study.

5. CONCLUSION

The study thus confirms the pro-angiogenic potential of honey at low concentrations. This effect is probably due to the presence of Hydrogen peroxide and Vitamin C and is mediated via alteration in VEGF expression.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

We thank Amruta Nachankar, Shrutika Pednekar, Puja Indurkar and Preeti Solanki for their technical assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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