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EVALUATION OF THE ANTI-ANGIOGENIC AND ANTIOXIDANT ACTIVITY OF INDIAN HONEYS IN COMPARISON WITH MANUKA HONEY FROM NEW ZEALAND

A. A. Naik*, O. D. Juwekar, P. V. Ansulkar, S. D. Deshpande and A. S. Chowdhary

Haffkine Institute for Training, Research and Testing, Acharya donde marg, Parel, Mumbai- 400012, Maharashtra, India

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Correspondence to Author:
A. A. Naik

Scientific Officer,
Haffkine Institute for Training, Research and Testing, Acharya donde marg, Parel, Mumbai- 400012, Maharashtra, India.

E-mail: 7.amruta@gmail.com

ABSTRACT: Honey possesses a wide range of medicinal properties due to presence of phytochemicals including flavonoids and phenolic acids. Free radicals and process of angiogenesis are one of the major contributors to cancer development and the use of phytoconstituents as reactive oxygen species scavengers and as an antiangiogenic moiety has clinical potential and may be having less negative side effects than the existing drugs. This study has explored the phyto-constituents as well as anti-angiogenic and anti-oxidant activity of Indian honey (Kashmir honey and *dhanwantari honey*) in comparison with *manuka* honey. The total phenolic content was measured by folin ciocalteau method and antioxidant activity was estimated by DPPH scavenging assay. Anti-angiogenic activity of honey samples were evaluated by chorioallantoic membrane (CAM) assay. Drabkin's assay was performed in order to check the hemoglobin content of the chorioallantoic membrane sections. Presence of flavonoids and tannins were observed in all honey samples. Higher total phenolic content was found in *kashmir* honey (6662.67 mg GAE /100 gm honey) than *manuka* honey (6078.67 mg GAE /100 gm honey) followed by *dhanwantari* honey (2286 mg GAE /100 gm honey). *Kashmir* honey (IC_{50}= 5.8±13.03 mg/ml) showed most potent anti-oxidant activity followed by *manuka* honey (IC_{50}= 9.1±3.00 mg/ml) and *dhanwantari* honey (IC_{50}=12.3±2.75 mg/ml). All three honey samples showed a dose dependent anti- angiogenic potential in chick embryo model which was then confirmed by dose dependent decrease of hemoglobin observed in drabkin's assay. Thus, we conclude that, among these three honey samples, *kashmir* honey is more potent anti-angiogenic and antioxidan.

INTRODUCTION: Cancer has become an important public health problem with over 8,00,000 new cases occurring every year and is one of the leading causes of death. At any point of time, it is estimated that there are nearly 2.5 million cases in the India with nearly 4, 00,000 deaths occurring due to cancer [1]. One of the important factors responsible for the tumour growth and progression is the process of angiogenesis.

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Excessive angiogenesis (neovascularization) is characteristic of a number of serious diseases including cancer [2]. It is a complex, highly regulated process, involving the sprouting, splitting and remodeling of the existing blood vessels. For tumour to develop in size and achieve metastatic potential, they make an "angiogenic switch" through perturbing the local balance of pro-angiogenic and anti-angiogenic factors [3].

Tumour growth and metastasis are angiogenesis dependent and hence, blocking angiogenesis is one of the strategies to arrest tumor growth. Free radicals produced by cells via various enzymatic and non-enzymatic reactions like respiratory chain reaction, oxidative phosphorylation etc. initiates autocatalytic reactions and damage different bio-

molecules.[4] Oxidative stress is a harmful condition that occurs in excess of ROS and/or a decrease in antioxidant levels which leads to cell / tissue damage and ultimately causes different diseases like coronary diseases, inflammatory disorders, neurological degeneration and cancer. Antioxidants are the one who inhibit oxidative damage to a bio molecule by neutralizing the free radicals. It is being increasingly proposed that reactive oxygen species (ROS) play a key role in human cancer development, especially as evidence is growing that antioxidants may prevent or delay the onset of some types of cancer [5]. Endogenous ROS also plays an important role for cancer cells to induce angiogenesis and tumor growth [6].

Ayurveda is a natural health care system that originated in India more than 5000 years ago. Its main objective is to achieve optimal health and well-being through a comprehensive approach that addresses mental, physical condition and external environment [7]. With its evolution through centuries it has always fascinated practitioners and researchers for its applications in cancer treatment on a scientifically proven research background. Hence, cancer patients who are already burdened by drug-induced toxic side effects have now turned to seek help from this complementary and alternative medicinal branch hoping for a better cure [8]. Honey has been used as a natural sweetener and traditional medicine since ancient times. In Ayurvedic literature, honey has said to be having property of "*lekhana*" i.e. activity of scrapping [9]. Honey has wide range of medicinal properties and thus an alternative medicine branch, called apitherapy, has been developed in recent years, offering treatments based on honey and other bee products for many diseases [10].

These medicinal properties of honeys are proposed to be derived from phytochemicals especially flavonoids present in them which are found to be varying in proportions with respect to the geographical area, climate and flora of origin [11]. Some of the polyphenols of honey have been evolved as promising pharmacological agents in treatment of cancer [12]. Hamzaoglu et al. reported that tumour implantation in rats was markedly reduced by the application of honey pre- and post-operatively, suggesting that the physico-chemical action, decrease of oxygen availability in the tumour environment, i.e., anti-angiogenic effect prevented the spread of metastatic cells [13].

So far, Indian honeys have not yet been explored with respect to their phytochemical content and also their medicinal properties and hence, objectives of this study was to a) screen the phytochemical contents of honey samples; b) evaluation of antioxidant activity of honey; c) evaluation of their anti angiogenic activity; d) confirmation of its results by performing drabkin's assay.

MATERIALS AND METHODS:

1. Honey samples:

Indian honey samples i.e. Kashmir honey and dhanwantari honey were collected from apiaries by phonda ghat pharmacy, India. Manuka honey was brought from New Zealand.

2. Phytochemical screening[14, 15]:

Phytochemical screening of all three honey samples was carried out by qualitative tests to detect the presence of different phytochemicals like flavonoids, phenols, alkaloids, tannins,terpenoids, steroids, saponins, anthraquinones, phlobatannins and organic acids.

3. Total phenolic content: [16, 17]

The total phenolic content of honey sample was determined by Folin-Ciocalteau method. The standard used for this assay was gallic acid and hence the results were expressed as gallic acid equivalents (GAE). 500µg/ml stock solution of gallic acid was used to prepare concentration range of 20-100µg/ml. 30µl of honey sample (0.1%) were added to 150µl of Folin Ciocalteau Reagent (10%) and 120µl of Na-carbonate (7.5%). Reaction mixture was then incubated in dark for 30 minutes. Absorbance was measured at 765nm.

4. Estimation of anti-oxidant activity[18,19]:

DPPH assay is widely used gold standard assay to test the ability of compounds as a free radical scavenger / hydrogen donors and to evaluate its anti-oxidant potential. Standard used for the assay was butylated hydroxyl toluene (BHT) (0.5%). Concentration range of honey samples was 2-14 mg/ml. 200µl of sample was added to 1800µl of 0.0002% DPPH. The reaction mixture was then incubated in dark for 30 minutes and absorbance

was measured at 517nm on jasco V-630 UV spectrophotometer.

The percentage inhibition was calculated using following formula:

IC (%) = [Abc– Abs/ Abc] x 100

Where Abc= Absorbance of Control

Abs= Absorbance of sample

5.Chorioallantoic membrane (CAM) assay: [20, 21]:
Day 4 embryonated eggs were purchased from central poultry dairy organization (CPDO), Mumbai and incubated at 37^{o}C and 85% humidity for 24 Hrs. After candling, 200µl of different concentrations of honey samples (1.5 -100%) and positive control [1:1=heparin (10U) and hydrocortisone (0.6%)] were inoculated under sterile condition. The eggs were then incubated at 37^{o}C for 48 hrs. After incubation, CAM sections were dissected & were observed for the pattern of vasculature, growth of secondary blood vessels and no. of sprouts were counted.

6. Drabkin's assay [22]:
The dissected CAM sections were then homogenized in 5 ml of chilled normal saline with mortar & pestle. It was centrifuged at 7000 rpm for 30 minutes. 10 ml of Drabkin's reagent was added to 3ml of supernatant. Reaction was incubated at room temperature for 20 minutes and absorbance was measured at 540 nm on jasco V-630 UV spectrophotometer. All above assays were performed in triplicates in order to ensure reproducibility of the result.

RESULTS AND DISCUSSION: Cancer is a leading cause of death worldwide [23]. With respect to mortality, advances in cancer treatment have not been as effective as those for other chronic diseases[24]. Phytoconstituents as a therapy to scavenge free radicals and to treat disorders like cancer has been proven to be effective. Therefore it is demand of time to use drugs from natural sources like honey to treat cancer.

1. Phytochemical screening:

TABLE 1: PHYTOCHEMICAL SCREENING OF HONEY SAMPLES

Sr.No	Phytochemicals	*Kashmir* Honey	*Dhanwantari* Honey	*Manuka* Honey
1	Phenolics	-	-	-
2	Flavonoids	+	+	+
3	Terpenoids	-	-	-
4	Alkaloids	-	-	-
5	Tannins	+	+	+
6	Organic acids	-	-	-
7	Steroids	-	-	-
8	Saponins	-	-	-
9	Anthraquinones	-	-	-
10	Phlobatannins	-	-	-

[+: present; - : not detected]

Honey has variety of phytochemicals and its composition varies according to the flora from which honey has been collected. In this study as shown in **Table 1,** all three honey samples found to be rich in flavonoids and tannins. Alkaloids, terpenoids, steroids, saponins, etc. were not detected may be due to their less concentrations.

2. Total phenolic content:
Gallic acid is used as a standard for the Folin-Ciocalteau assay **Fig.1**. The total phenolic content was calculated and was found to be of the *kashmir* honey is 6662.67 mg GAE /100 gm honey, that of *manuka* honey is 6078.67 mg GAE /100 gm honey and of *dhanwantari* honey is 2286 mg GAE /100 gm honey. Phenolic compounds and its derivatives are responsible for the functional and medicinal properties of honey. Hence, the total phenolic content in general reflects the therapeutic importance of honey.

3. Estimation of anti-oxidant activity:
Butylated hydroxyl toluene (BHT) is used as standard for DPPH assay **Fig.2** and **3** shows the dose dependent increase in antioxidant activity of all three honey samples. The calculated IC_{50} value of the *kashmir* honey is 5.8$\pm$13.03 mg/ml $\pm$S.D, that of *manuka* honey is 9.1$\pm$3.00 mg/ml

±S.D and of *dhanwantari* honey is 12.3±2.75 mg/ml ±S.D. Potent anti-oxidant activity was shown by both the Indian honey samples. The anti-oxidant potential of a moiety is a reflection of the amount of phytochemicals and phenolic compounds present in it. Hence from the results of the above assays it can be concluded that *kashmir honey* is the most effective out of these three honey samples with respect to antioxidant activity and also because it has higher phenolic content as compared to other honey samples.

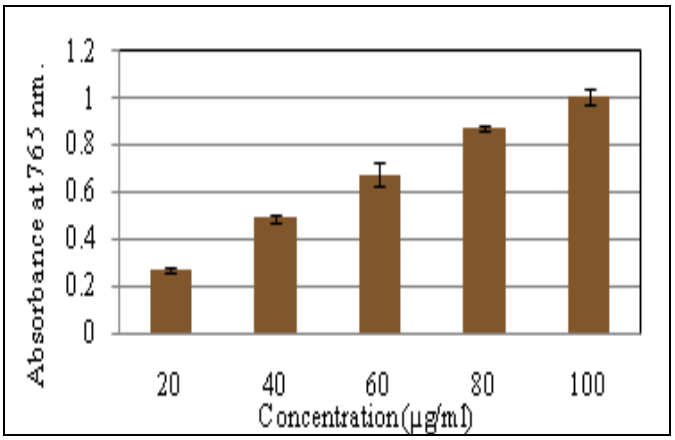

FIG.1: STANDARD PLOT OF GALLIC ACID.

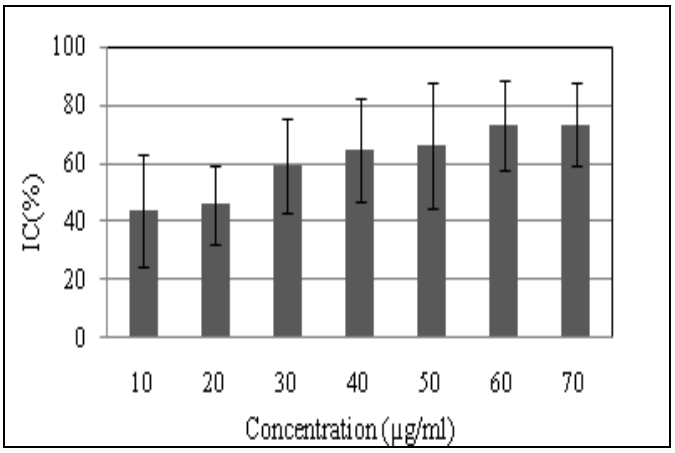

FIG.2: DPPH ASSAY OF BUTYLATED HYDROXYL TOLUENE.

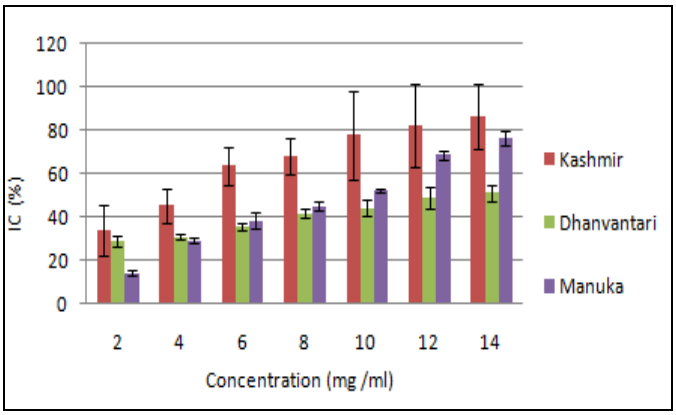

FIG. 3: DPPH ASSAY OF HONEY SAMPLES.

4. Chorioallantoic membrane (CAM) assay for evaluation of anti- angiogenic potential of honey samples:

The original Chorioallantoic membrane (CAM) assay was described by experimental embryologists more than 50 years ago and has long been a mainstay for the study of embryonic organ development and angiogenesis[20].

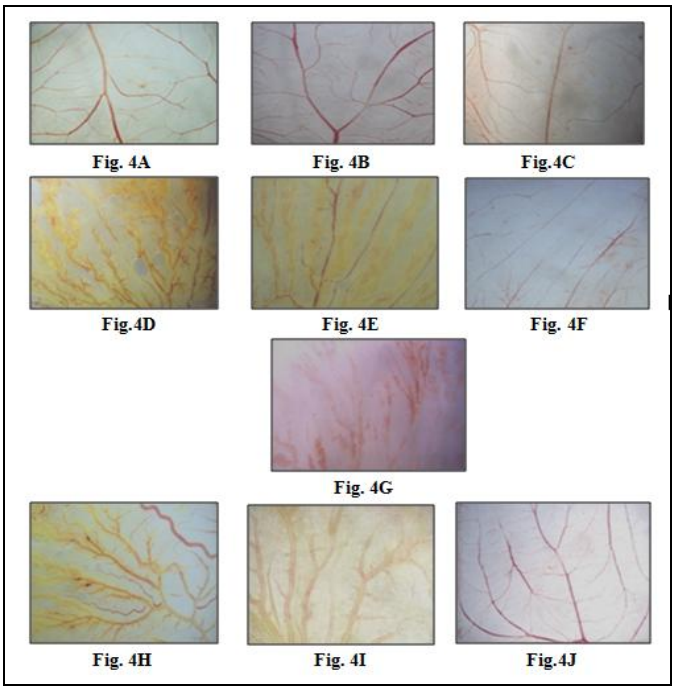

FIG. 4: CAM SECTIONS SHOWING ANTI-ANGIOGENIC EFFECT OF HONEY SAMPLES.

4A: 1.5% of honey sample; 4B: 3.5% of honey sample; 4C: 6.25% of honey sample; 4D: 12.5% of honey sample; 4E: 25% of honey sample; 4F: 50% of honey sample; 4G: 100% of honey sample; 4H: Negative control; 4I: Positive control; 4J: Untreated.

As shown in **Fig. 4**, the vasculature of the CAM sections after treatment with honey samples in the concentration range of 1.5% to 100% indicates decrease in the branching & sprouting of blood vessels and also shedding of vasculature.

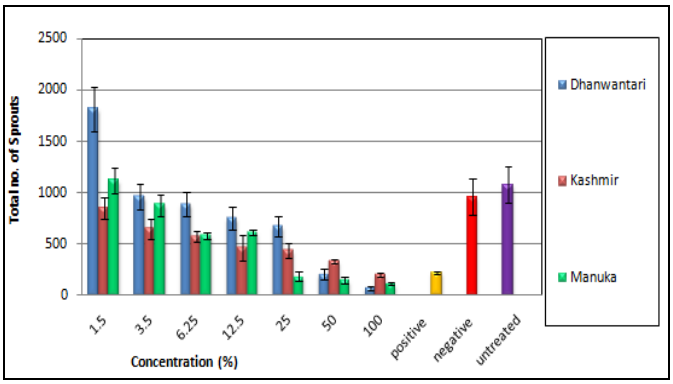

FIG. 5: CAM ASSAY REPRESENTING ANTI – ANGIOGENIC ACTIVITY OF HONEY SAMPLES.

As **Fig.5** indicates sprouting of CAM sections treated with increasing concentrations of honey samples which shows decrease in total number of sprouts on blood vessels in dose dependent manner. Among these three, *kashmir* honey has shown greater activity at lower concentrations (1.5% to 12.5%) than *manuka* honey followed by *dhanwantari* honey.

5. Drabkin's assay:

In order to find out hemoglobin content of the CAM sections treated with honey samples, drabkin's assay was performed. It was carried out to confirm the dose dependent anti-angiogenic behaviour of honey samples in the CAM assay. As shown in **Fig. 6**, dose dependent decrease was observed in haemoglobin content of CAM sections treated with increasing concentrations of honey samples which confirms the findings of CAM assay.

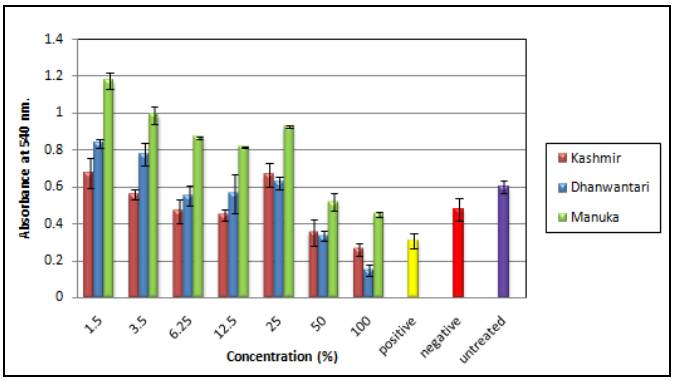

FIG.6: HAEMOGLOBIN CONTENT OF CAM SECTIONS DETECTED BY DRABKIN'S ASSAY

From the above study we observed that both the Indian honeys i.e. *kashmir* honey, *dhanwantari* honey and *manuka* honey contains flavonoids and tannins along with high total phenolic content.All three honey samples possess very good anti-angiogenic and antioxidant activity.

As it has been stated in previous literature that depending on the geographical variations, the phytochemical constituents of honey changes and thus its medicinal properties too. This study was the first attempt so far to discover and explore these properties and phytochemical contents of Indian honeys. Since reactive oxygen species and event of angiogenesis are interlinked and together responsible for the tumour growth, it will be of great importance to include honey as a potential drug which can encounter both these processes and helps for the tumour regression. In the near future, these Indian honey samples can be characterized by exploring and quantifying the phytochemicals present in it. It will be of very much great interest to decipher the mechanisms involved in their mode of action in order to use apitherapy as a leading branch of therapy in field of oncology.

CONCLUSION: All three honey samples possess very good dose dependent anti-angiogenic and antioxidant activities. Among these three samples, *Kashmir* honey is more potent than other two at lower concentrations.

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