



## CELL CYCLE ARREST AND APOPTOSIS INDUCING POTENTIAL OF INDIAN HONEY IN CERVICAL CANCER CELLS

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### ABSTRACT

Honey is a natural product known for its various biological activities like anti-inflammatory, antioxidant, antibacterial, anticancer effects. Cancer is one of the leading causes of death worldwide. Honey due to the presence of phytochemicals in it is found to inhibit the cancer growth. This effect is mediated through cell cycle arrest and induction of an apoptosis. This study focused on the effect of four Indian unifloral honey i.e. Jambhul, Rubber, Litchi and drumstick honey on cervical cancer cell line. The cell cycle arrest were analysed by Propidium Iodide (PI) staining using Flow Cytometer. Further confirmation of apoptosis was done by microscopic examination of stained cells. In order to understand the mechanism of this effect, expression of important marker protein of apoptosis i.e. Active Caspase-3 was analyzed by flow cytometry. All honey samples caused cell cycle arrest in G2M phase in cells. Sub G0G1 peak indicating apoptosis was confirmed by microscopic examination in which apoptic bodies were observed. Prominent increase in expression of Active Caspase-3 protein was seen. Hence, this study concludes that all four Indian honey samples showed potent anticancer activity against cervical cancer.

**KEYWORDS:** Honey, Cell cycle, Apoptosis, Caspase-3.

### INTRODUCTION

As the only available natural sweetener honey was an important food for Homo sapiens. Indeed, the relation between bees and man started as early as stone age. In most ancient cultures, honey has been used for both nutritional and medical purposes. An alternative medicinal branch called Apitherapy which has developed in recent years, is offering treatment based on honey and the other bee products against many diseases. Polyphenols are important group of compounds with respect to the functional properties of honey. Honey compositions and also its different biological effects depend on the botanical origin of honey.<sup>[1]</sup> The quality of honey depends on its chemical composition and floral origin. The composition of active components in plants depends on various factors, particularly on plant biological and climatic conditions. Consequently, it can be reasonably expected that honey properties from different locations should be different.<sup>[2]</sup> Cancer is the most common cause of mortality worldwide. However, most of drugs that are currently used as chemotherapeutic agents are not highly effective.<sup>[3]</sup>

In developing countries, cervical cancer is the most common cancer in women and may constitute up to 25% of all female cancers. Cervical cancer is preceded only by breast cancer as the most common cause of death from cancer in women worldwide.<sup>[4]</sup> The cell cycle is the series of events in which cellular components are doubled, and then accurately segregated into daughter cells. In eukaryotes, DNA replication is confined to a discrete Synthesis or S-phase, and chromosome segregation occurs at Mitosis or M-phase. Two Gap phases separate S phase and mitosis, known as G1 and G2. The central machines that drive cell cycle progression are the cyclin-dependent kinases (CDKs). Cyclin binding allows inactive CDKs to adopt an active configuration. Depending on the severity of the cell cycle defect, checkpoint dysfunction can result in outcomes ranging from cell death to cell cycle reprogramming, which can lead to cancer.<sup>[5]</sup> Apoptosis is highly conserved mechanism by which cells commit suicide. It enables an organism to eliminate unwanted and defective cells through an orderly process of cellular disintegration that has the advantage of not inducing an inflammatory

response.<sup>[6]</sup> The expression of various pro-apoptotic and anti-apoptotic proteins were found to be altered during apoptosis. Activation of caspase 3 has been a hallmark during apoptosis.<sup>[7]</sup> The anticancer effect of honey against cancer has been examined *in vitro* and *in vivo*. Honey is an effective agent for inhibiting the growth of different cancer cell lines *in vitro*.<sup>[8,9]</sup> It is also effective when administered intralesionally or orally in animal models.<sup>[8]</sup> Thus, This study focused on the effect of four unifloral Indian honey samples on cervical cancer cell line i.e. HeLa cell line with respect to cell cycle arrest and apoptosis. Further the apoptosis was confirmed by estimating expression of apoptosis marker i.e. Active Caspase-3 protein.

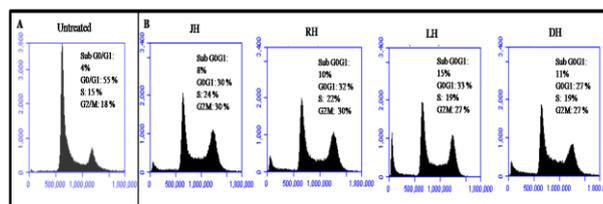
## MATERIALS AND METHODS

- 1. Collection of honey samples:** Four different types of unifloral Indian honeys i.e. Jambhul, Rubber, Litchi and Drumstick honey were procured from standard apiaries of India. They were stored at room temperature in sterile conditions.
- 2. Cell line:** Human cervical cancer cells i.e. HeLa cell line was maintained as monolayer cultures in DMEM cell culture media supplemented with 10% fetal bovine serum, 1X Antibiotic-Antimycotic solution. The cell cultures and experimental plates were maintained at 37°C in 5% CO<sub>2</sub>.
- 3. Cell cycle analysis<sup>[10]</sup>:** Cell cycle analysis of cervical cancer cells treated with honey samples was performed using a Flow Cytometer (Backmen FC-CXP 500). Cells were seeded in 6 well plates and were allowed to adhere overnight at 37°C in 5% CO<sub>2</sub>. Cells were treated with IC<sub>50</sub> concentrations of honey (w/v) for 48 Hrs. Then cells were trypsinized and were fixed with chilled 70% ethanol. Control cells i.e. untreated cells were processed exactly as honey treated cells. After fixation, cells were washed twice with 1X sterile PBS (pH 7.4) and were centrifuged at 1000 rpm for 10 minutes. Cells were then resuspended in 1X sterile PBS. Solution of RNase A (Sigma-Aldrich, USA) and Propidium Iodide (PI) (Sigma-Aldrich, USA) reconstituted in 1X sterile PBS were added to the cells and incubated for 30 min at 37°C. Post incubation, cells were acquired on Flow Cytometer. Cells were analysed using CXP 500 software provided with the Backmen Flow Cytometer. Assay was performed in triplicates.
- 4. Microscopic examination<sup>[11]</sup>:** After being washed with cold 1X PBS (pH 7.4), cells stained with Propidium Iodide (PI) were observed under fluorescent microscope. Assay was performed in triplicates.
- 5. Active Caspase-3 assay by Flow Cytometry<sup>[12]</sup>:** This assay was performed using PE- Conjugated Rabbit Anti- Active Caspase-3 Antibody (BD pharmingen). Cells treated with IC<sub>50</sub> concentrations (w/v) of all four honey samples for 48 Hrs. were trypsinized and were fixed with chilled 1% paraformaldehyde for 15 minutes at 4°C. Cells were washed twice with FACS I buffer containing 1X

PBS with sodium azide and fetal bovine serum. Control cells were processed exactly as honey treated cells.  $1 \times 10^6$  cells were suspended in 100  $\mu$ l FACS buffer II containing saponin, 1X PBS with sodium azide and fetal bovine serum for 15 minutes. 20  $\mu$ l of PE- Conjugated Rabbit Anti- Active Caspase-3 Antibody was added to cells as per the instructions given in the product sheet. After incubation of 45 minutes, cells were washed twice with FACS buffer I and were centrifuged at 1000 rpm for 10 minutes. Cells were then resuspended in 0.5 mL of chilled FACS buffer I and were acquired on Flow Cytometer. Analysis was done by using CXP 500 software provided with the Backmen Flow Cytometer. Assay was performed in triplicates.

## RESULTS

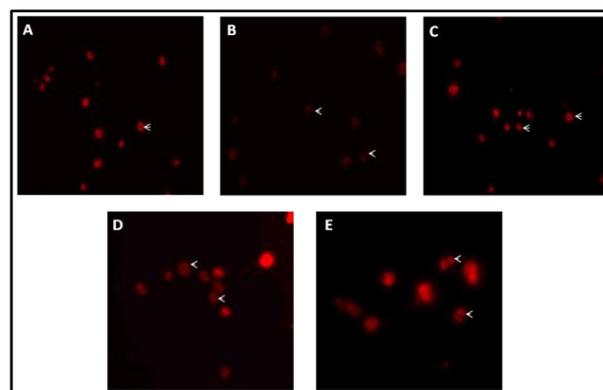
- 1. Cell cycle analysis:** Effect of four Indian unifloral honey samples were studied by staining cervical cancer cells i.e. HeLa cells with propidium iodide and were analysed on Flow Cytometer.



**Fig. 1: Effect of IC<sub>50</sub> concentration of all four Indian honey samples on cell cycle in HeLa cells.**

A: Untreated HeLa cells; B: HeLa cells treated with Jambhul Honey (JH), Rubber Honey (RH), Litchi Honey (LH) and Drumstick Honey (DH).

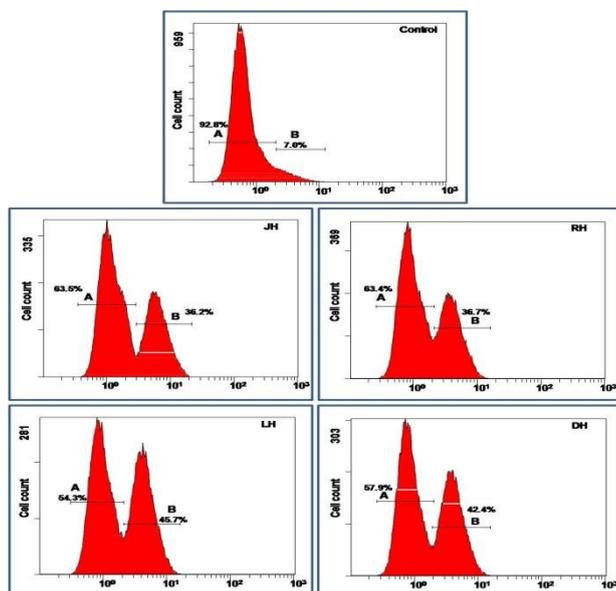
- 2. Microscopic examination:** For further confirmation of apoptosis, microscopic examination of HeLa cells stained with Propidium Iodide (PI) were done.



**Fig. 2: Microscopic examination (20X) of HeLa Cells treated with IC<sub>50</sub> concentration of four Indian honey samples A: Untreated HeLa cells (arrow indicating normal cell morphology); B: HeLa cells treated with Jambhul honey; C: HeLa cells treated with Rubber honey; D: HeLa cells treated with Litchi honey; E: HeLa cells treated with Drumstick honey (arrow indicating apoptotic bodies).**

### 3. Active Caspase-3 assay by Flow Cytometry:

Active Caspase-3 protein expression in HeLa cells treated with all four Indian unifloral honey samples were analysed by Flow Cytometer with CXP 500 software.



**Fig. 3: Flow cytometry analysis of HeLa Cells treated with IC<sub>50</sub> concentration of four Indian honey samples. [Control: Untreated HeLa cells; JH: HeLa cells treated with Jambhul honey; RH: HeLa cells treated with Rubber honey; LH: HeLa cells treated with Litchi honey; DH: HeLa cells treated with Drumstick honey.**

### DISCUSSION

All over the world, scientists have great interests in the anticancer properties of natural compounds. It's because they are believed to be relatively less toxic and have been used as natural remedies since ancient times. More than 25% of drugs used in last 20 years were directly derived from plants, while another 25% were chemically altered natural products.<sup>[12]</sup> Honey finds a role in both domestic and medicinal applications. It has been widely used as sweetener since ancient times. Composition of honey varies depending upon the geographical conditions and the nectar sources.<sup>[13,14]</sup> Honey of various types has been demonstrated to show anticancer effect on different types of cancers. Recent studies on human breast and cervical,<sup>[15]</sup> oral and osteocarcinoma<sup>[9]</sup> cancer cell lines using Malaysian Jungle Tualang honey showed significant anticancer activity. Honey has also been shown to have antineoplastic activity in an experimental bladder model *in vivo* and *in vitro*.<sup>[8]</sup> Honey is thought to mediate these anticancer effects due to its important components such as Chrysin<sup>[16]</sup> and other Flavonoids.<sup>[17]</sup> The positive scientific evidence for anticancer properties of honey is growing. Among the mechanisms suggested for this activity are inhibitions of cell proliferation, induction of apoptosis, and cell-cycle arrest.<sup>[18]</sup> Sadia Afrin *et al.* (2018) studied the effect of treatment of manuka honey on colon cancer cell lines for 48 Hrs. by

measuring apoptosis and cell cycle arrest as well as apoptosis protein expression. They found that treatment caused cell cycle arrest at G<sub>2</sub>M phase in cells and also the apoptosis were induced which was confirmed by increase in protein expression of Caspase-3.<sup>[19]</sup> In our study, we found that treatment of all four unifloral Indian honey samples caused cell cycle arrest in cervical cancer cell line (HeLa). It was observed that treatment of these honey samples for 48 Hrs. caused cell cycle arrest in G<sub>2</sub>M phase. It was observed that this treatment also resulted into induction of apoptosis which was indicated by a sub G<sub>0</sub>G<sub>1</sub> peak (Figure No. 1). Further in order to confirm the apoptosis induced by honey treatment, microscopic examination of HeLa cells stained with Propidium Iodide were done by using fluorescent microscope. Apoptosis i.e. shrinkage of cells and presence of apoptic bodies were seen in HeLa cells treated with all four honey samples (Figure No. 2: marked with arrows). In order to understand the mechanism of action of this apoptosis inducing activity, further we did expression study of protein playing important role in apoptosis i.e. Caspase-3. Caspases, a family of cysteine proteases, are involved in apoptotic pathways. In particular when Caspase-3, is activated it has many cellular targets that produce the morphological features of apoptosis.<sup>[20]</sup> In our study, it was observed that the expression of Active Caspase-3 which is an important apoptic protein and strong indicator of apoptosis, was prominently increased in HeLa cells treated with all four Indian honey samples as compared to untreated HeLa cells (Figure No. 3).

### CONCLUSION

From this study we conclude that, all four Indian honey samples caused cell cycle arrest and induced apoptosis in cervical cancer cells and thus possess potent anticancer activity.

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### REFERENCES

1. Stefan Bogdanov *et al.*, Honey for Nutrition and Health: A Review, *Journal of the American College of Nutrition*, 2008; 27(6): 677–689.
2. Jose Miguel *et al.*, Contribution of honey in nutrition and human health: a review, *Mediterr J Nutr Metab*, 2010; 3: 15–23.
3. Gottesman, 2002: Gottesman, M.M., 2002. Mechanism of cancer drug resistance. *Annu. Rev. Med.*, 2002; 53: 615–627.
4. Eileen M. Burd, Human Papillomavirus and Cervical Cancer, *Clinical microbiology reviews*, 2003; 16(1): 1–17.
5. Kevin J. Barnum *et al.*, Cell Cycle Regulation by Checkpoints, *Methods Mol Biol.*, 2014; 1170: 29–40.

6. Jacobson MD *et al.*, Programed cell death in animal development, *Cell*, 1997; 88: 347-354.
7. MICHAEL, O.H, The biochemistry of apoptosis, *Nature*, 2000; 407: 770-776.
8. Swellam T *et. al.*, Antineoplastic activity of honey in an experimental bladder cancer implantation model: in vivo and in vitro studies, 2003; *Int J Urol* 10: 213–219.
9. Gashm *et. al.*, Antiproliferative effect of Tualang honey on oral squamous cell carcinoma and osteosarcoma cell lines, *BMC Complement. Altern. Med.*, 2010; 10: 1–8.
10. Saravana Kumar Jaganathan *et. al.*, Honey constituents and their apoptotic effect in colon cancer cells, *Journal of ApiProduct and ApiMedical Science*, 2009; 1(2): 29 – 36.
11. Clement Yedjou *et. al.*, Basic Mechanisms of Arsenic Trioxide (ATO)- Induced Apoptosis in Human Leukemia (HL-60) Cells, *Journal of Hematology & Oncology*, 2010; 3: 28.
12. Vuorelaa, P. *et. al.*, Natural products in the process of finding new drug candidates, *Curr. Med. Chem.*, 2004; 11: 1375–1389.
13. Anupama *et. al.*, Sensory and physicochemical properties of commercial samples of honey, *Food Research International*, 2003; 36(2): 183–191.
14. Nor Hayati Othman, Does Honey Have the Characteristics of Natural Cancer Vaccine? *Journal of Traditional and Complementary Medicine*, 2012; 2: 276-283.
15. Agustine *et.al.*, Tualang honey induces apoptosis and disrupts the mitochondrial membrane potential of human breast and cervical cancer cell lines, *Food and Chemical Toxicology*, 2011; 49: 871–878.
16. Woo *et.al.*, Chrysin induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells, *Biochem Biophys Res. Commun*, 2004; 325: 1215-1222.
17. Jaganathan *et.al.*, Studies on the phenolic profiling, anti-oxidant and cytotoxic activity of Indian honey: in vitro evaluation, *Nat Prod Res.*, 2010; 24: 1295-1306.
18. Nor Hayati Othman, Honey and Cancer: Sustainable Inverse Relationship Particularly for Developing Nations—A Review, *Evidence-Based Complementary and Alternative Medicine*, 2012; Article ID 410406, 10 pages.
19. Sadia Afrin *et.al.*, The inhibitory effect of Manuka honey on human colon cancer HCT-116 and LoVo cell growth. Part 1: the suppression of cell proliferation, promotion of apoptosis and arrest of the cell cycle, 2018, *Food Funct.*, 2018; 9: 2145–2157.
20. Siu-wan ip *et.al.*, Bee Venom Induced Cell Cycle Arrest and Apoptosis in Human Cervical Epidermoid Carcinoma Ca Ski Cells, *Anticancer research*, 2008; 28: 833-842.