

## Acacia Honey Modulates Cell Cycle Progression, Pro-inflammatory Cytokines and Calcium Ions Secretion in PC-3 Cell Line

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### Abstract

**Background:** Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related deaths. Honey has been proven to have a potentiality of being among the functional foods by both *in vivo* and *in vitro* studies.

**Aim:** This study was primarily designed to provide evidence on the apoptotic role of acacia honey on PC-3 cell line.

**Methods:** MTT and mitotic index assay were used to determine the anti-proliferative effects. Cell cycle analysis was conducted on flow cytometer using propidium iodide. Expression of pro-inflammatory cytokines [TNF  $\alpha$  and IL 1 $\beta$ ] and prostate specific antigen were determined by ELISA kits. Calcium ion level was analyzed by colorimetric method.

**Results:** Our results show the anti-proliferative effects of acacia honey on NIH/3T3 and PC-3 cell lines with the IC<sub>50</sub> of 3.7 and 1.9% [v/v] respectively with simultaneous increase in the secretion of calcium ion and down regulation of prostate specific antigen. Significant [p < 0.05] dose-dependent modulation of G0/G1 phase was observed as compared with the control. However, there was an inverse relationship between TNF- $\alpha$  and IL-1 $\beta$  with latter being up regulated. Mitotic index of PC-3 cells decreased as the concentration of honey increases.

**Conclusion:** Apoptotic role of acacia honey on PC-3 cell line may be due to modulation of G0/G1 phase, pro-inflammatory cytokines, calcium ions secretion and down regulation of prostate specific antigen *in vitro*.

**Keywords:** Acacia honey; PC-3 cells; Apoptosis; Cytokines; Calcium ion

### Introduction

Cancer is a disease of multidimensional processes that affects most of the populace in both developed and under developed countries. It is an abnormal cell growth that is characterized by the fact that the cells keep replicating when they are actually suppose to be differentiated. It has been established to be a complex disease caused by abnormal cellular proliferation associated with resistance to normal growth inhibitory signals, uncontrolled activation of growth signals, impairment of apoptosis, promotion of angiogenesis (the development of new blood vessels to support tumor growth), invasion of surrounding tissues and metastasis i.e. spread of tumor cells to distant tissues [1,2].

Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related deaths [3]. The majority of the prostate cancer cells are classified as adenocarcinomas characterized by an absence of basal cells, and uncontrolled proliferation of malignant tumor cells with features of luminal differentiation including glandular formation and the expression of Androgen Receptor (AR) and prostate-specific antigen [4,5].

Cytokines are hydrosoluble extracellular polypeptides or glycoproteins ranging from 8 to 30 kDa [6]. They are produced by several types of cells, at the site of injury, and immune cells, through mitogen-activated protein kinases. Some cytokines can have a pro- (Th1) or anti-inflammatory (Th2) actions, according to the microenvironment in which they are located [6]. Example of pro-inflammatory cytokine are interleukins [IL] 1, 2, 6, 7, and TNF [tumor

necrosis factor]. While anti-inflammatory cytokines include IL-4, IL-10, IL-13, and TGF $\beta$  [transforming growth factor]. Interestingly, most of the pro-inflammatory cytokines tends to play a dual role of apoptosis and carcinogenesis especially at the level of cancer progression. The divalent calcium cation Ca<sup>2+</sup> is used as a major signaling molecule during cell signal transduction to regulate energy output, cellular metabolism, and phenotype as well as apoptosis via direct and indirect mechanisms [7]. Apoptosis, also called programmed cell death or physiological cell death, is an essential and evolutionarily conserved property involved in the maintenance of multicellular organisms and found in all species from worm to man. It is required in many fundamental biological processes including embryonic development, metamorphosis, hormone-dependent atrophy and chemically-induced cell death [8,9].

Honey, a natural sweet substance produced by bees through

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regurgitation mechanisms has been proven to have a potentiality of being among the functional foods by both *in vivo* and *in vitro* studies. It is a promising antitumor agent with pronounced anti-metastatic and anti-angiogenic effects [10], antibacterial, anti-inflammatory, immune-stimulant, antiulcer and wound/burn healing effects [11]. Various signaling pathways, including stimulation of tumor necrosis factor-alpha (TNF  $\alpha$ ) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation, mediate the beneficial effects exerted by honey and its major components such as chrysin and other flavonoids [12-16]. However, chronic consumption of honey that was mixed with rat pellets has been shown to have a positive impact on the architecture and integrity of hepatocytes *in vivo* [17]. Its protective role against the kidney dysfunctions induced by sodium nitrite, a known food additives, hepatoprotective, hypoglycemic, reproductive, antihypertensive and of course antioxidant effects has also been reported [18,19]. Honey is produced from many different floral sources and its biochemical and pharmacological activities vary depending on its origin and processing. Honey contains a variety of biologically active compounds such as flavonoids, vitamins, antioxidants as well as hydrogen peroxides [20]. Daily consumption of honey has been shown to have both positive and negative effect on male Wister albino rat [21]. Acacia honey and chrysin the active principle has reduced proliferation of melanoma cells through alterations in cell cycle progression *in vitro* [22]. To the best of our knowledge, so far no study has so far being carryout to establish the links between anticancer activity of acacia honey with modulation of cytokines and possible secretion of calcium ion as a mediator of apoptosis. Therefore, this study was undertaken to provide an evidence on the apoptotic role of acacia honey on PC-3 cell line through the modulation of pro-inflammatory cytokines (TNF  $\alpha$  and IL 1 $\beta$ ) and secretion of calcium ion in an attempt to justify its effects on the expression of prostate specific antigen, mitotic index and anticancer properties *in vitro* (Chart 1).

## Materials and Methods

### Sample collection

Honey, produced by *Apis mellifera* was collected from the northwest frontier of Pakistan during spring season of 2012 from *Acacia modesta* flower and maintained at 4°C until analysis at the industrial analytical centre, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan.

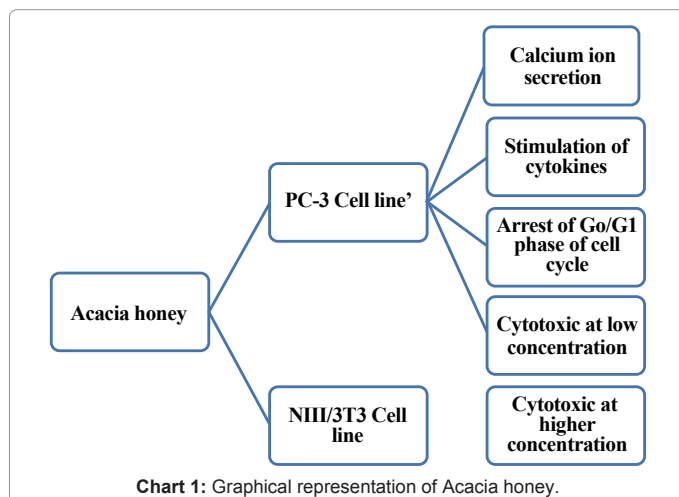


Chart 1: Graphical representation of Acacia honey.

### Cell cultures and treatments

PC-3 Human prostate cancer cell line and NIH/3T3 normal cell lines were generously provided by Dr. Ahmed M. MESAİK of Molecular Immunology Laboratory, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan. The cells were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS). 1% (v/v) L-glutamine, 100 U penicillin and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The cells were passage twice weekly. Acacia honey was added to cells in complete medium after 24 h of growth. Honey was diluted at a final concentration of 0.5, 1.0, 2.0, 4.0 and 8.0 % (v/v) for cancer cell line and 2.0, 4.0, 6.0, 8.0 and 10 % (v/v) for normal cell line. The experiments were in triplicates/duplicates and repeated at least three times along with untreated cells serving as control.

### MTT assay

Anticancer/cytotoxicity of acacia honey on PC-3 and NIH/3T3 cells was evaluated in 96-well flat-bottomed micro plates by using standard MTT (3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyl-tetrazolium bromide) colorimetric assay [23]. Exponentially growing cells were harvested, counted with hemocytometer and diluted with a medium. Cell culture with the concentration of  $1 \times 10^4$  cells/mL were prepared and introduced (100  $\mu$ L/well) into 96-well plates. After overnight incubation, medium was removed and 200 $\mu$ L of fresh medium was added with different concentrations of Honey. After 48 hrs, 200  $\mu$ L MTT (2 mg/mL) was added to each well after aspiration of media and incubated further for 4 hrs. Subsequently, 100  $\mu$ L of DMSO was added to each well after aspiration of media. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm, using a micro plate reader (spectra 340, softmax PRO 4.3.1.LS, Molecular device, USA). The same procedure was followed checking the anticancer activity of honey on PC-3 cells after 24 hrs incubation with honey and another plates 24 hrs after the replacement of honey with just the medium. The cytotoxic effect was recorded as concentration causing 50% inhibition (IC<sub>50</sub>) for PC-3 and NIH/3T3 cells. The percent inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = 100 - (\text{mean of Absorbance of sample} / \text{mean of Absorbance of control}) \times 100$$

### Determination of cytokines (TNF $\alpha$ and IL 1 $\beta$ ) from the cultured supernatant of PC-3 cells

The experiment was conducted for 24 hrs and 48 hrs incubation period. ELISA kits for specific cytokines were used (R&D systems, USA and Canada) according to the manufacturer's protocol. Each sample was tested in duplicate in each of three or more replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by using a micro plate reader (spectra 340, softmax PRO 4.3.1.LS, Molecular device, USA), and the absorbance readings were converted to pg/ml based upon standard curves obtained with recombinant cytokine in each assay.

### Determination of calcium ion from the cultured supernatant of PC-3 cells

The experiment was conducted for 24 hrs and 48 hrs incubation period as well as 24 hrs after the replacement of honey with just the medium. The calcium ion level in cultured supernatant was quantified using the Cobas kits and Roche/Hitachi 912 automatic analyzer

according to manufacturer's protocols. The principle was based on the complex formed between calcium ion and o-cresolphthalein complexone in alkaline solution. The absorbance was read at 450nm. Each sample was tested in duplicate in each of three or more replicate experiments.

### Determination of prostate specific antigen (PSA) from the cultured supernatant of PC-3 cells

The experiment was conducted for 48 hrs incubation period. ELISA kits specific for PSA (Bio Check, Inc, Vintage Foster City) was used to quantify PSA level in the cultured supernatant according to the manufacturer's protocol. Each sample was tested in duplicate in each of three or more replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by using a micro plate reader (spectra 340, softmax PRO 4.3.1.LS, Molecular device, USA), and the absorbance readings were converted to pg/ml based upon standard curves.

### Mitotic index assay on PC-3 cells

The experiment was conducted for 48 hrs incubation period. Exponentially growing cells were harvested, counted with hemocytometer and diluted with a medium. Cell culture with the concentration of  $1 \times 10^5$  cells/mL was prepared and introduced 1mL/well. 24 hrs after, the various concentrations of the honey were added to the wells except the control that was given only the medium in order to normalized the condition. After 70 h of incubation, a 0.1 mL colcemid solution (10  $\mu$ g/mL) was added to each culture tube and mixed by shaking gently. After 72 h of incubation, cells were harvested by centrifugation, by hypotonic treatment (0.075 M KCl) and fixing in a fresh fixative solution (methanol: acetic acid, 3:1). The fixation step was repeated three times. Slides were air-dried and stained with 5% Giemsa [24]. Mitotic Index was calculated as the proportion of metaphase for 1000 cells as shown below:

Percent mitotic index =  $\frac{\text{Number of metaphase} \times 100}{\text{Total number of cells}}$

### Cell cycle analysis by FACS (fluorescence-activated cell sorting) analysis

PC-3 cells were seeded at density of  $1 \times 10^6$  cells/well in 6-well plates. After 24 h cells were treated with honey as previously described. After 48 hrs treatment and incubation at 37°C and 5% CO<sub>2</sub>, cells were washed with PBS harvested with trypsin and centrifuged, fixed with ice-cold 100 % ethanol overnight. They fixed cells were centrifuged for 1 hr at 300 xg and washed with PBS twice. The cells were then treated with 125  $\mu$ L of 500 U/mL RNase A and incubated at 37°C for 15 minutes and thereafter, 125  $\mu$ L of 5 mg /100 ml Propidium iodide in 1.12% sodium citrate was added and allowed to stand at room temperature for 30 minutes. Cell cycle distribution was analyzed by flow cytometry on a FACSC alibur (Becton Dickinson, Canada, Inc). PI was excited at 488 nm, and fluorescence analyzed at 620 nm. A total of 10,000 events in each sample were acquired. Using CellquestPro software, the percentages of cells at different phases of the cell cycle were determined.

### Statistical analysis

To address the biological variability and stability of the samples, each and every experiment was repeated at least three times and the results were expressed as mean  $\pm$  Standard deviation. Differences

between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc, Chicago, Standard version 10.0.1. P-values < 0.05 were considered statistically significant for differences in mean using the least of significance difference (Lsd).

### Results

As seen in figure 1, honey was able to significantly ( $p < 0.05$ ) inhibit the growth of 3T3 cell line in a concentration dependent manner with an IC<sub>50</sub> of 3.7 % (v/v). We used 2, 4, 6, 8 and 10 % (v/v) in order to evaluate the cytotoxicity of honey on normal cell line and from our results; it indicates that the honey is cytotoxic at about 4 % (v/v). Probably, this might be as a result of the generation of hydrogen

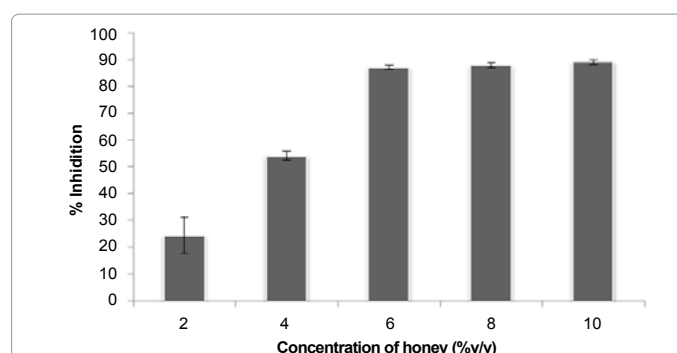


Figure 1: % inhibition on the cytotoxicity of acacia honey on NIH/3T3 normal cell lines with IC<sub>50</sub> of 3.7 % (v/v).

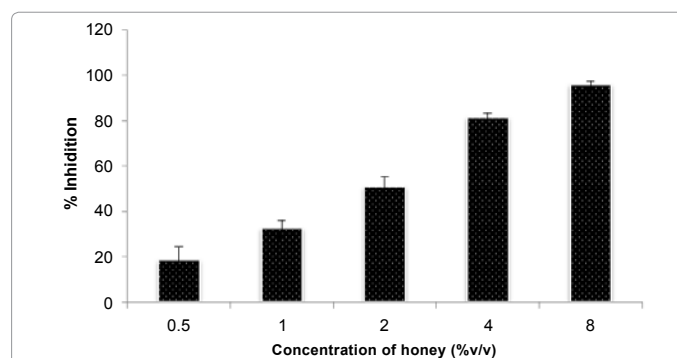


Figure 2: % inhibition on the cytotoxicity of acacia honey on PC-3 cell lines with IC<sub>50</sub> of 1.9 % (v/v) after 48 hrs incubation period.

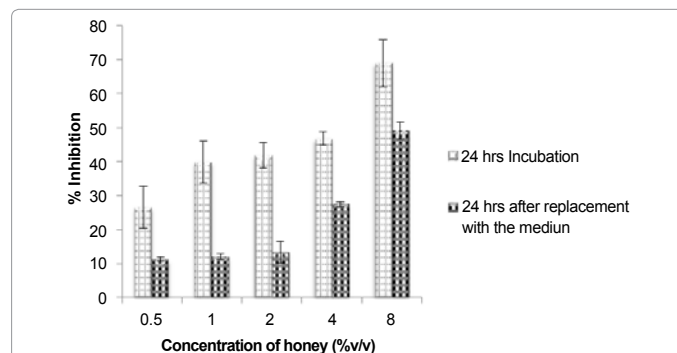


Figure 3: % inhibition on the cytotoxicity of acacia honey on PC-3 cell lines with IC<sub>50</sub> of 4.43 % (v/v) for 24 hrs incubation and >8 % (v/v) after replacement with medium.

peroxide due to oxidation of monosaccharides present in honey which has been implicated in initiating apoptosis.

Figure 2 shows the anticancer activity of honey on PC-3 cell line with an  $IC_{50}$  of 1.9 % (v/v). This indicates that it is more cytotoxic to cancer cells than the normal ones. This might be attributed to the fact that in a disease condition/non-diseases conditions there seem to be different mode of response towards treatments as their metabolisms differ. However, in figure 3, acacia honey was only able to confer its anticancer activity after 24 hrs incubation with an  $IC_{50}$  of 4.43% (v/v). On replacement of honey with a just a medium, there was a reverse of anticancer activity with an  $IC_{50}$  >8.0% (v/v) which was the highest concentration used. By implication, acacia honey seems to be more active against PC-3 cells after long time incubation.

As depicted in figure 4, there was a significant ( $p < 0.05$ ) increase in calcium ion level from 24 to 48 hrs incubation periods with honey. However, the level declined after replacement of the honey with the medium. This indicates that there is a calcium ion involvement on the anticancer activity of acacia honey providing an evidence of apoptotic role since calcium ion is a mediator of programmed cell death.

In figure 5, TNF- $\alpha$  level increased significantly ( $p < 0.05$ ) after 24 hrs incubation at the lowest concentration. However, the level declined with increase in concentration of honey. After 48 hrs incubation, the level was drastically reduced as compared to 24 hrs. This suggests that acacia honey was able to modulate TNF- $\alpha$  expression by acting as an anti-inflammatory agent. This means that more TNF  $\alpha$  is being utilized for apoptotic pathway rather than inflammation which is a key factor for the survival of cancer cells.

Furthermore, from figure 6, IL 1 $\beta$  level increases significantly ( $p < 0.05$ ) at the lowest concentration and start declining with increase in concentration after 24 hrs incubation indicating anti-inflammatory role. Surprisingly, the level elevated after 48 hrs incubation with increase in concentration of honey. This might be as a result of the fact that much of TNF  $\alpha$  has been utilized in the production of IL 1 $\beta$  which also believed to play a key role in the release of calcium ion from endoplasmic reticulum. The sudden increased observed in control might be as a result of the cytokine's role in keeping the cancer cells alive. Therefore, we propose that acacia honey modulates this cytokine towards apoptotic rather than inflammatory role by virtue of the increased in calcium ion level.

From figure 7, the level of prostate specific antigen decreased from

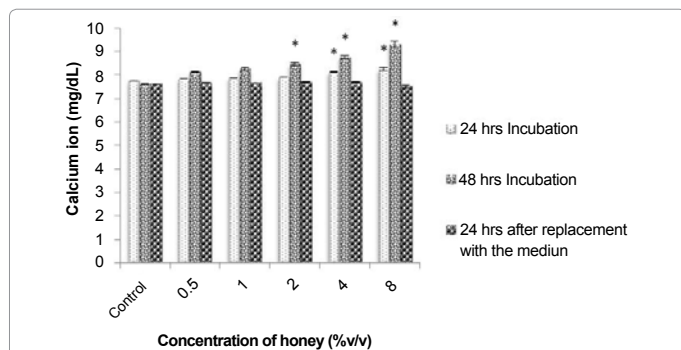


Figure 4: Calcium ion level from cultured supernatant of PC-3 cell lines at different incubation time and replacement of honey with fresh medium.

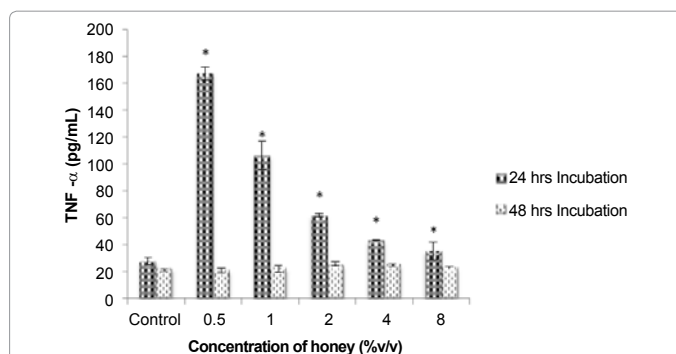


Figure 5: TNF- $\alpha$  level from cultured supernatant of PC-3 cell lines after 24 and 48 hrs incubation periods.

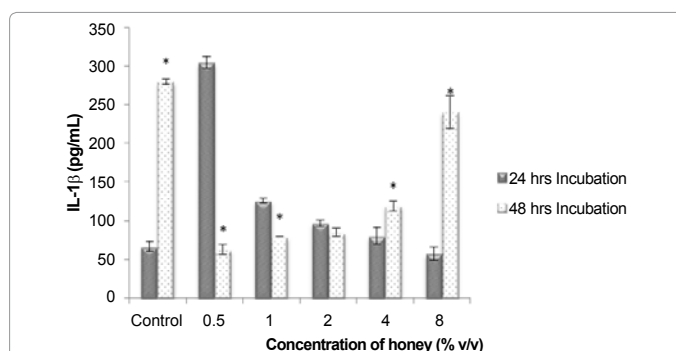


Figure 6: IL-1 $\beta$  level from cultured supernatant of PC-3 cell lines after 24 and 48 hrs incubation periods.

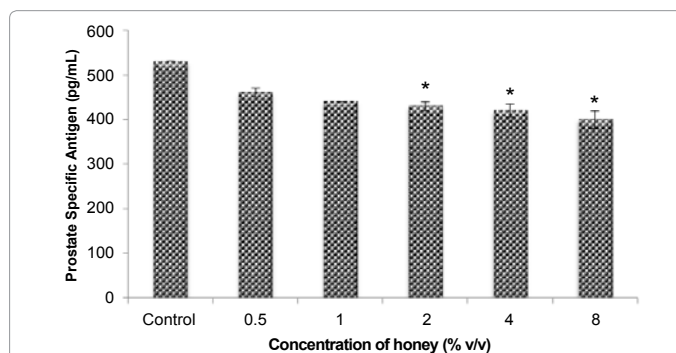


Figure 7: Prostate specific antigen level from cultured supernatant of PC-3 cell lines after 48 hrs incubation period.

2-8% (v/v) of honey indicating an effect on its expression. Similarly in figure 8, mitotic index of PC-3 cell line decreases significantly ( $p < 0.05$ ) in a concentration dependent manner. In fact we could not detect appreciable number of cells at 8 % (v/v). This is not surprising because the  $IC_{50}$  was 1.9% (v/v).

Arrest of cell cycle phases is one of the important steps towards anticancer activity. Our results (Figure 9 a,b,c) indicate that acacia honey was able to modulate G0/G1 phase significantly ( $p < 0.05$ ) by altering its progression to other phases in a concentration dependent with concomitant decreased in % S phase and G2/M phase as compared with control. This means that honey was able to arrest the cancer cell proliferation at G0/G1 phase due to the observed % DNA contents.

## Discussion

Antioxidants activity of honey has been found to be as a result of the synergistic effects of wide range of compounds present in it [12]. The diversity and concentration of these components is dependent on the botanical and geographical origin of honey. Moreover, it is also influenced by the variation in climate and availability of plant sources for the honeybees to harvest nectar within a specific region [25]. Therefore, honey originating from different floral sources differs in their chemical compositions. Our findings show that acacia honey was able to act as an anti-proliferative agent by its ability to inhibit the growth of PC-3 cell line *in vitro*. This is in support with reports that acacia honey reduces the proliferation of other cell lines like melanoma cells [22]. Basically, various polyphenols are reported in honey. Some of which are Caffeic acid, Caffeic acid phenyl ester, Chrysin, Galangin, Quercetin, Acacetin, Kaempferol, Pinocembrin, Pinobanksin and Apigenin. These have evolved as promising pharmacological agents in treatment of cancer [26]. Characterization of acacia honey revealed three (3) phenolic acids (p-hydroxybenzoic, ferulic and t-cinnamic acid), abscisic acid and five (5) free flavonoids (pinobanksin, apigenin, pinocembrin, chrysin and acacetin) [27]. Polyphenols and phenolics have been reported to inhibit cancer-related pathways and processes including prostate cancer [28,29]. High phenolic and hydroxymethylfurfural (HMF) contents of honey resulted in the growth inhibitory effects of cancer cells [30], and flavonoids have been reported to induce cell death of various cancer cell lines [31]. It has previously been reported that a Japanese pure unfractionated honey significantly reduced the proliferation of the human bladder cancer cells, T24 [32]. It has been reported that other type honey shows no significant cytotoxic effect on normal human cell lines [33]. The cytotoxic effect of honey may be attributed to the fact that it may contain pyrrolizidine alkaloids which are considered as toxic compounds [34].

Inhibition of apoptosis can be seen as the basis of abnormal cell growth and calcium ion has been implicated to play a vital role via direct and indirect apoptotic pathways. The involvement of  $Ca^{2+}$  in cell death has been recognized very early in the history of programmed cell death, with the demonstration *in vitro* that  $Ca^{2+}$  ionophores, which are the molecules capable of transporting  $Ca^{2+}$  across membranes down its electrochemical gradient, are highly toxic to cells [35]. In the present study we demonstrate that anti-proliferative effects of acacia honey is being accompanied by stimulation of calcium ion and thus, implicating its anticancer activity in relation to the induction of apoptosis via the calcium ion dependent pathway.

Pro-inflammatory cytokines are one of the major key players

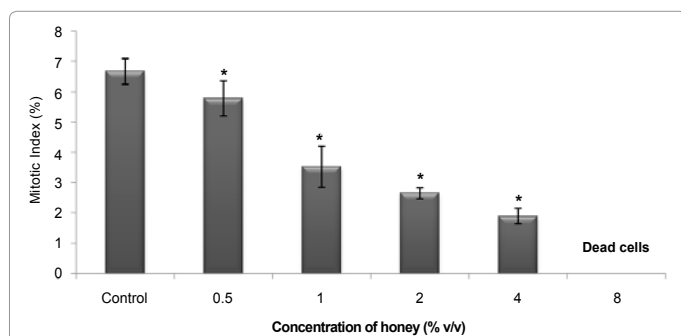


Figure 8: % mitotic index of PC-3 cell lines after 48 hrs treatment with different concentration of honey.

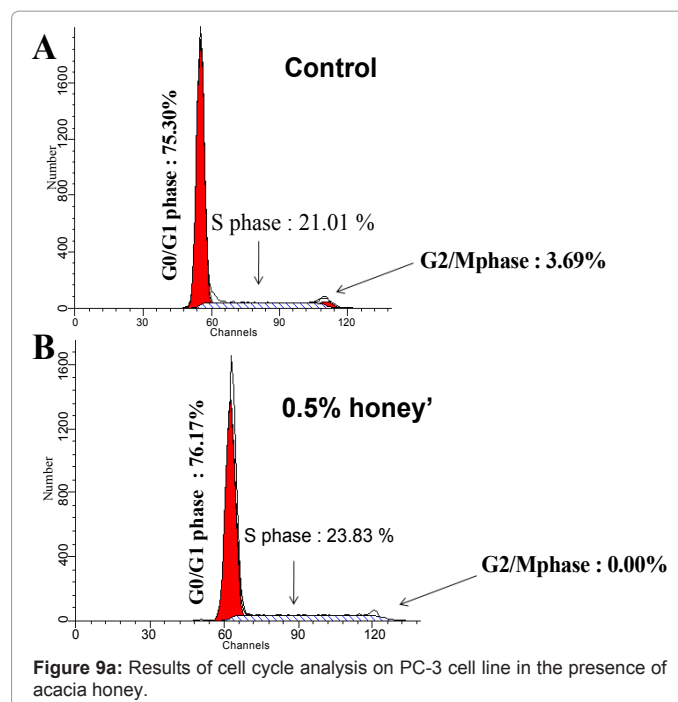


Figure 9a: Results of cell cycle analysis on PC-3 cell line in the presence of acacia honey.

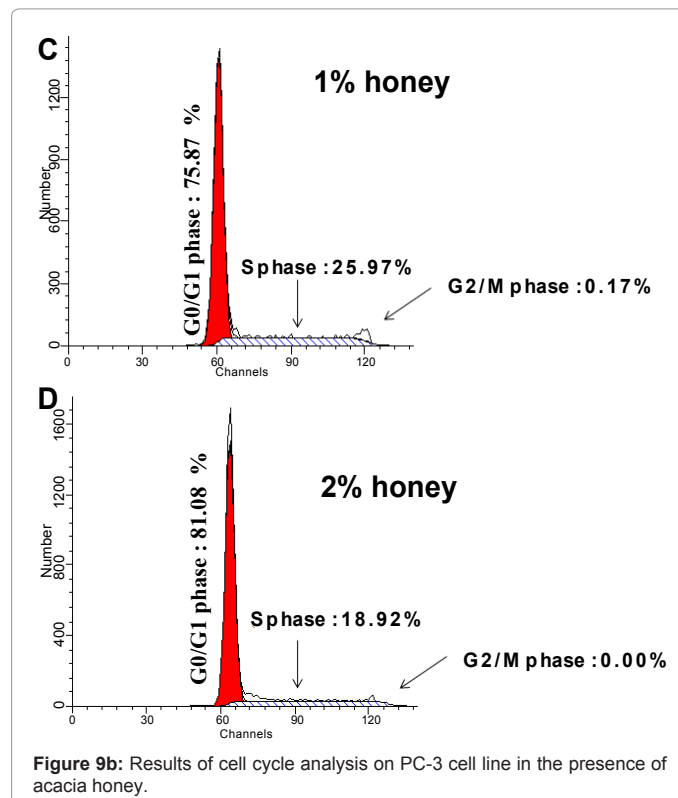
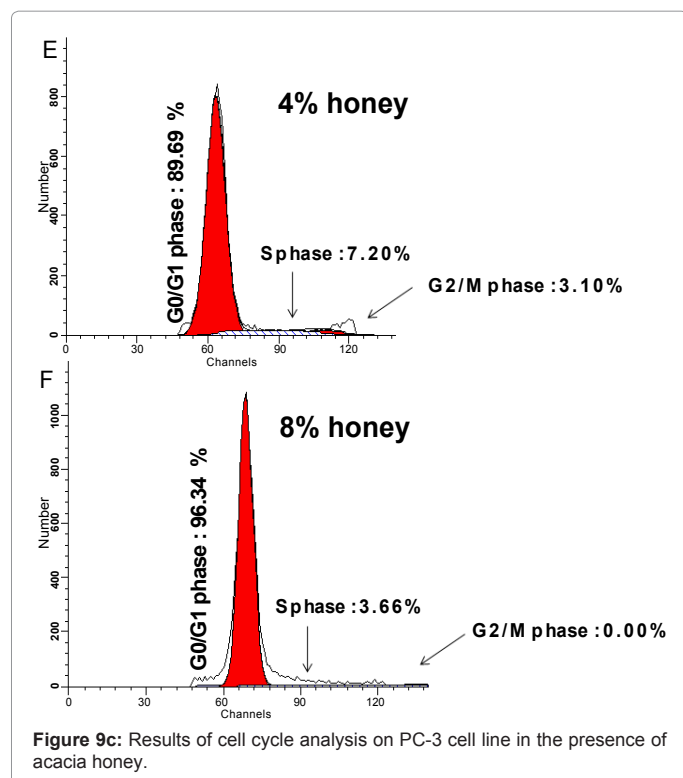


Figure 9b: Results of cell cycle analysis on PC-3 cell line in the presence of acacia honey.

during chronic inflammation which favored the survival of cancer cells. Of such type are TNF  $\alpha$  and IL 1 $\beta$ . It has been reported that pro-inflammatory cytokines are capable of promoting proliferation, invasion and angiogenesis of prostate cancer, and of several other solid tumors [36-38]. Our results show the anti-inflammatory role of acacia honey by its ability to down regulate the level of TNF  $\alpha$  in PC-3 cell



line. This supports the anti-inflammatory role of honey as previously described [11]. The level of IL-1 $\beta$  was observed to be high in our present study. However, it has been established that TNF  $\alpha$  stimulates other cytokines including IL 1 $\beta$  which up regulates the level of some protein kinases such as extracellular regulated kinase (ERK). ERK has been found to phosphorylate the calcium ion channel of the endoplasmic reticulum thereby causing the depolymerisation of cytoskeletons leading to calcium ion release. The released calcium ion activates calpain which further activates procaspase-12 that will ultimately stimulate the execution caspases of an apoptotic pathway like procaspase 3 and 7 [39-41]. We therefore propose that, acacia honey modulates TNF  $\alpha$  towards the production of IL 1 $\beta$  which corroborates with an increase in calcium ion level and anticancer activity against PC-3 cell line in a concentration dependent manner.

Prostate-specific antigen (PSA), a member of the kallikrein protein family, is widely used as a surrogate marker in the early diagnosis and management of prostate cancer [42]. PSA is a serine protease with chymotrypsin-like activity [43], and is largely found in prostatic tissue and seminal plasma. In prostate cancer, excess PSA spills into circulation where it exists as a mixture of various molecular forms, including free PSA and several PSA protease inhibitor complexes [44]. Our finding indicates that acacia honey was able to decrease the level of PSA in a concentration dependent manner. PSA has been established to play a significant role in prostate tumor growth by regulating various pro-angiogenic and anti-angiogenic growth factors [45]. It has also been reported to stimulate the generation of reactive oxygen species in prostate cancer [46]. This further supports our findings that show the antioxidant potentials of acacia honey due to the down regulation of PSA *in vitro*.

Cell cycle arrest especially at G0/G1 phase induces apoptosis. In this study, we observed that acacia honey induced a dose-dependent

G0/G1 phase arrest. The inhibition of cell growth could be a result of the induction of apoptosis that may be mediated by cell cycle arrest. Therefore we hypothesize that acacia honey mediated inhibitory effects of cell growth of PC-3 cells might be due to perturbation in the cell cycle, which may possibly lead to programmed cell death. The antiproliferative effect of honey on human (A375) and murine (B16-F1) melanoma cell lines was found to be mediated by G0/G1 cell cycle arrest and induction of hyperploid progression [22,14].

## Conclusion

We therefore conclude that anticancer activity and apoptotic role of acacia honey on PC-3 Cell line may be due to modulation of G0/G1 phase of cell cycle, TNF- $\alpha$ , IL-1 $\beta$ , calcium ions secretion and down regulation of prostate specific antigen *in vitro*.

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