

## Antiproliferative Activity and Apoptosis Induction by Gelam Honey on Liver Cancer Cell Line

**Zakiah Jubri**

Department of Biochemistry  
Faculty of Medicine  
National University of Malaysia  
Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur  
Malaysia.

**Nur Nabilah Najib Narayanan**

Biomedical Science Department  
Faculty of Health Science  
National University of Malaysia  
Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur  
Malaysia.

**Norwahidah Abdul Karim**

Department of Biochemistry  
Faculty of Medicine  
National University of Malaysia  
Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur  
Malaysia.

**Wan Zurinah Wan Ngah**

Department of Biochemistry  
Faculty of Medicine  
National University of Malaysia  
Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur  
Malaysia.

### Abstract

*Gelam honey is a Malaysian monofloral honey produced by Apis mellifera from Melaluca spp. It has high polyphenols content that possesses antioxidant and free radical scavenging activity towards preventing cancer and diseases. This study was to determine the antiproliferative effects of gelam honey on liver cancer, HepG2. MTS assay was carried to obtain IC<sub>50</sub> value of gelam honey towards HepG2 and normal liver, WRL-68 cell lines. The cells proliferation rates were determined by BrdU assay and morphological cell changes were detected by using propidium iodide staining. The IC<sub>50</sub> value of gelam honey towards HepG2 and WRL-68 cells was 25% and 70% respectively. Gelam honey reduced the proliferation of HepG2 at concentrations of 3% to 70%. Morphological analysis for apoptosis detection using fluorescent microscope under 400X magnification producing typical apoptotic characteristic. It showed that gelam honey has antiproliferative activity towards cancer cell by its ability to induce apoptosis.*

**Key words:** Gelam honey; antiproliferation; cancer cells; apoptosis

### 1. Introduction

Apoptosis is a process of programmed cell death. The cells will undergo morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. In contrast to necrosis, the production of apoptotic bodies is engulfed and removed by phagocytic cells before the contents can spill out onto surrounding cells and cause cell damage.

The balance between apoptosis and cell renewal is required to maintain normal state. But cancer cell is lacking of this phenomenon. The cell is continuously dividing and causing cellular and organ dysfunction. Liver cancer has a high frequency rate among the Asians population. It is closely linked to chronic hepatitis B infection that common in Asia. Furthermore, treatment for cancer is not always promising, it has a lot of side effects and recently, a resistance to anticancer drugs has been observed. Therefore, research and development to explore the beneficial effect of natural product is necessary to reduce the use of toxic drugs. Previous study has shown that consumption of natural products associated with low cancer risk (1).

Honey is a natural product produced by honey bees, *Apis mellifera*. It is rich in phenolic compounds such as caffeic acid, caffeic acid phenyl esters, chrysin, galangin, quercetin, kaempferol, acacetin, pinocembrin, pinobanksin, and apigenin and also other antioxidants such as ascorbic acid, amino acids and proteins. Gelam honey is a local Malaysian honey. It is derived from nectar of gelam tree, a plant belonging to the *Myrtaceae* family. Previous studies have claimed that gelam honey contains high level of antibacterial activity largely due to its high content of phenolic compounds (2) and exhibits free radical scavenging activities due to its high antioxidant level (3). These compounds in gelam honey might demonstrate an important finding towards the liver cancer cells. Studies have shown several possible mechanisms mediated the antiproliferative effect of honey towards cancer cells such as involvement in inducing antioxidant effects (4), stimulation of TNF- $\alpha$ , involvement in the inhibition of lipoprotein oxidation (5) and induction of apoptosis and cell cycle inhibition (6). The aim of this study was to determine the antiproliferative effect of gelam honey and its association with apoptosis induction on liver cancer cell, HepG2.

## **2. Materials and Methods**

### **2.1 Honey**

Fresh Malaysian gelam honey was obtained from the Department of Agriculture, Parit Botak, Johor, Malaysia. Gelam honey was irradiated by using the Cobalt-60 source model ELDORADO 8 (Atomic Energy of Canada Limited, Canada) at Malaysian Nuclear Agency in Selangor, Malaysia. Honey was packed in plastic bottles and placed in a box. The box encircles the irradiator at a speed of 1 round/min/5 kGy amounting to 5 circles to produce 25 kGy. The dosage standard calculation is automatically calculated by the irradiator. The irradiated honey was then kept in the dark at room temperature.

### **2.2 Cell culture**

HepG2 (ATCC, USA) and WRL-68 cells (ATCC, USA) were maintained in Eagle's Minimum Essential Media (EMEM, Flowlab, Australia) containing 10% fetal calf serum (PAA, Austria) and penicillin/streptomycin (100  $\mu$ g/ml) (Flowlab, Australia) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Stock solution (100%) of honey was stored at room temperature in a dark condition. Prior to use, a serial dilution of gelam honey (Batu Pahat Agricultural Department, Johor, Malaysia) (70%, 50%, 25%, 12%, 6%, 3% and 1%) was made in culture media. When the cells reached confluency, cells were trypsinized, centrifuged and counted by using a haemocytometer. The cell suspension was adjusted to a concentration of  $2 \times 10^4$  cells/ml for MTS and BrdU assay. The cell cultures were divided into two groups; control and treated cells. As a negative control, cells were cultured in EMEM without honey.

### **2.3 MTS assay**

Cell viability studies were performed by using MTS assay. Serial dilutions of honey were made in culture media. Briefly, cells were cultured in 96-well plates and incubated for 24 hours at 37°C in 5% CO<sub>2</sub> incubator. Then, the cultures were treated with honey. After incubation for another 24 hours, MTS was added. The cells were further incubated for another 1-2 hours. Absorbance was measured at 490nm. The percentage viability was calculated by comparing the absorbance of cells treated with honey to the untreated control cells (corresponding to 100% viable cells). The whole procedure was then repeated with different incubation periods of 12 and 48 hours.

### **2.4 BrdU assay**

A 96-well plate was seeded with HepG2 and WRL-68 cells at a uniform density of  $2 \times 10^4$  cells/well. Cells were then incubated for 24 hours in 5% CO<sub>2</sub> at 37°C. Then, the cultures were treated with honey. At the end of the treatment period, cell proliferation rate was measured based on the measurement of BrdU incorporation during DNA synthesis.

Briefly, BrdU labelling solution (10  $\mu$ l/well) was added and incubated for 2 hours at 37°C. Thereafter, FixDenat (200 $\mu$ l/well) was added and incubated for 30 minutes at room temperature. Subsequently, anti-BrdU-POD (peroxidase-conjugated anti-BrdU antibody) solution was added for 90 minutes at room temperature. At the end of the assay, 100 $\mu$ l peroxidase substrate (5-bromo-2'-deoxyuridine labeling) was filled into each well. After 5 minutes of incubation at room temperature, the reaction was stopped with 25 $\mu$ l 1M H<sub>2</sub>SO<sub>4</sub> and absorbance of the samples was assessed. Results were expressed as mean absorbance of the samples in an ELISA plate reader at 450 nm with a reference wavelength of 690 nm.

### 2.5 Propidium iodide (PI) staining

PI was used to stain the nuclear changes of living and apoptotic cells. Briefly, cells (2x10<sup>6</sup> cells/well) were incubated for 24 hours in 5% CO<sub>2</sub> at 37°C with the concentration of 25% gelam honey for HepG2, 70% gelam honey for WRL-68 and 5.5mM H<sub>2</sub>O<sub>2</sub> was used as positive control. Then the cells were further incubated for another 48 hours. After incubation, the cells were harvested and cell pellet was homogenised in 200 $\mu$ l of 1% formaldehyde and incubated for 15 minutes. The cells were washed twice with cold PBS, and then 1ml of 10  $\mu$ g/ml PI was added into each well and incubated at 37°C for 5 minutes in dark to allow nuclear penetration. After being washed with cold PBS, the cells were detected by blue filter (515nm) fluorescent microscope (Olympus Corp., Shibuya-ku, Tokyo, Japan) at 400x magnification.

### 2.6 Statistical analysis

All data are expressed as mean  $\pm$  S.D. (n=6) where 'n' represents the number of samples and differences between control and treated cells statistically analyzed by student t-test. Differences were considered to be statistically significant if  $p < 0.05$ . All statistical analyses were carried out using SPSS for Windows, version 17.0.

## 3. Results and Discussion

MTS assay is used to determine the cell viability in assays of cell proliferation and cytotoxicity. The viability of both HepG2 and WRL-68 were decreased in dose-dependent manner (Figure 1). Cell viability for both HepG2 and WRL-68 cell lines increased significantly after being treated with a low dose of honey which was at 1% and remain increased until the concentration of honey was 12% for HepG2 and 25% for WRL-68 cell as compared to untreated cells. This suggests that the administration of gelam honey at a low dose promotes the growth of both HepG2 and WRL-68 cell. This can be attributed to the fact that honey is rich in antioxidants that will increase the viability of both types of cells (3).

However, the HepG2 cell viability started to decrease with the increase in honey concentration starting from 25% and 50% for WRL-68cell. The IC<sub>50</sub> of gelam honey towards HepG2 cell was 25% and for WRL-68 cell lines were 70%. Gelam honey exhibited growth inhibition at a lower concentration and more sensitive in HepG2 cells as compared to WRL-68. This finding supported by Jaganathan et al. (2010) on *Ehrlich ascites* carcinoma cells to determine the anticancerous property of honey (7). He suggested that honey has the ability to activate p53 tumour-suppressor gene and that compounds might also have a role as antioxidant property (8,9). Honey treatment on HepG2 and WRL-68 were dependent on the incubation time (Figure 2). HepG2 viability was reduced starting at 12 hours incubation time. The cells viability was reduced 50% at 24 hours. In WRL-68, the cell viability was increased at 12 hours and slightly reduced at 24 hours and drastically at 48 hours. Therefore, 24 hours incubation time was chosen in the study.

Determination of cell proliferation was done by BrdU assay. It was carried out to support the findings on MTS assay. The proliferation of HepG2 cells were decreased with the increased in honey concentration (Figure 3) after 24 hours of incubation starting from 3% of honey concentration and 6% for WRL-68 cells. It was confirmed that gelam honey exhibited antiproliferative activity towards HepG2 cells and higher dose of gelam honey was shown for normal cells. This finding was consistent with an earlier study done by Swellam et al. (2003) which indicated that the administration of honey at any concentrations between 1-25% could inhibit the proliferation of T24, RT4, 253J and MBT-2 cells *in-vitro* and *in-vivo* (10). Previous studies demonstrated that *apigenin* and CAPE (*caffeic acid phenyl ester*), type of phenolic compound in honey, has the ability to inhibit the proliferation of HepG2 cell line (11, 12). The ability of gelam honey to induce apoptosis was shown in figure 4 through the observation of cellular morphological changes with the aid of propidium iodide staining. The morphological changes occurred during the late stage of apoptosis. After 48-hour incubation with gelam honey at its IC<sub>50</sub>, HepG2 cells were observed to display typical apoptosis characteristic by undergoing cell shrinkage.

Figure 4A showed the morphology of untreated HepG2 cells as observed under the fluorescent microscope, to be compared with HepG2 cells treated with H<sub>2</sub>O<sub>2</sub> (4B) as positive control and gelam honey (4C). The administration of both H<sub>2</sub>O<sub>2</sub> and gelam honey can be said to induce apoptosis in HepG2 cells as proven by the reduction in the cell size of treated cells of both groups. Figure 4D showed the morphology of untreated WRL-68 cells to be compared with WRL-68 cells treated with H<sub>2</sub>O<sub>2</sub> (4E) as positive control and gelam honey (4F). WRL-68 cells treated with gelam honey at an IC<sub>50</sub> dose of 70% also displayed cellular shrinkage. The administration of both H<sub>2</sub>O<sub>2</sub> and gelam honey can be said to induce apoptosis in WRL-68 cells as proven by the reduction in the cell size of treated cells. For both HepG2 and WRL-68 cell lines treated with gelam honey, a reduction of more than 50% nuclear size can clearly be observed, whereas this observation is absent in untreated cells of both cell lines.

Previously, we have found that H<sub>2</sub>O<sub>2</sub> at a dose of 5.5 mM was proven to be effective in inducing apoptosis towards both HepG2 and WRL-68 cell lines. Thus, H<sub>2</sub>O<sub>2</sub> at this dose has been used as a positive control in this study. The morphological characteristics exhibited by cells undergoing apoptosis caused by H<sub>2</sub>O<sub>2</sub> were then compared with the morphological characteristics of HepG2 and WRL-68 cells being treated with gelam honey at their respective IC<sub>50</sub> doses.

The apoptotic cell death is one of the mechanisms by which the rate of cell growth is reduced. This is indicated by cell shrinkage (13). Study by Maeno et al. (2000) on HeLa epithelium; U937, human lymphoid murine; NG108-15 and PC12 cells showed that cells which demonstrated apoptotic cell shrinkage, were found to precede cytochrome *c* release, caspase-3 activation, DNA laddering and ultrastructural alterations after insulted with apoptosis inducers (14,15). This reduction in cell volume occurs as a result of K<sup>+</sup>, Cl<sup>-</sup> and organic osmolytes loss (14,16,17). Thus, cell shrinkage is considered a useful indication in the determination of cell death mechanism.

Findings indicated that gelam honey and H<sub>2</sub>O<sub>2</sub> have the ability to induce apoptosis towards HepG2 and WRL-68 cells. Cell death by apoptosis could be distinctly identified and differentiated from healthy or necrotic cells through the unique morphological characteristics that were observed. Propidium iodide is a nuclear stain that is supposedly unable to enter apoptotic cell membrane. However, in this study PI has the ability to penetrate into HepG2 and WRL-68 cells membrane after being exposed to 1% formaldehyde before observation was done under fluorescent microscope. Then, apoptosis characteristics were easily identified by comparing the cell size between treated and untreated cells for HepG2 and WRL-68.

#### **4. Conclusion**

Malaysian gelam honey has the ability to reduce the proliferation rate of liver cancer cells at low dose but not affecting the normal liver cells. This antiproliferation effect is associated with the ability of gelam honey to induce apoptosis in liver cancer cells.

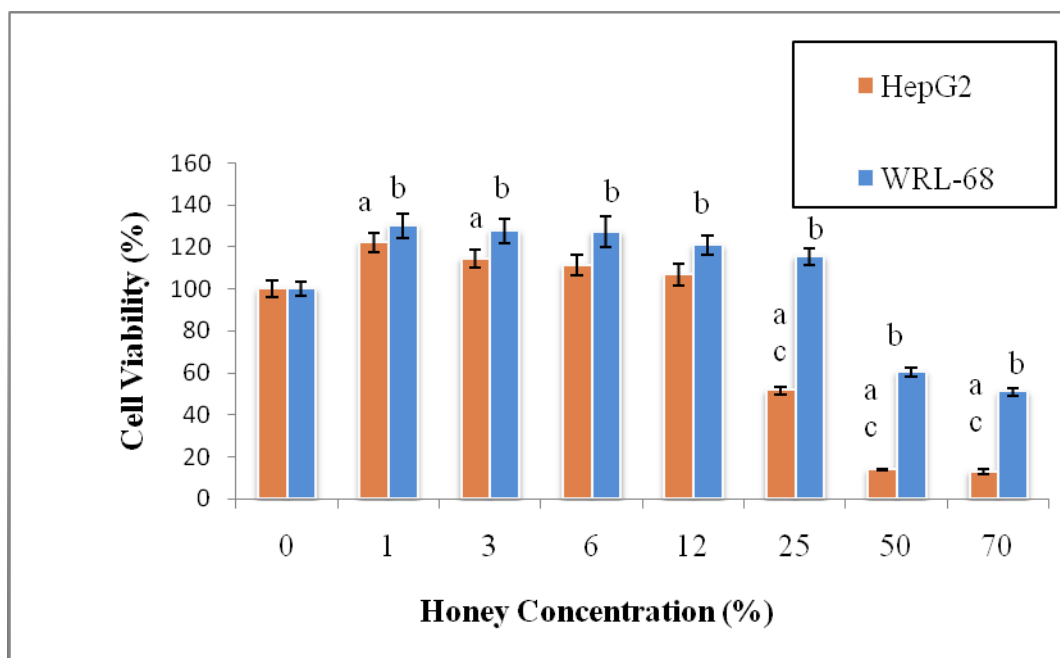
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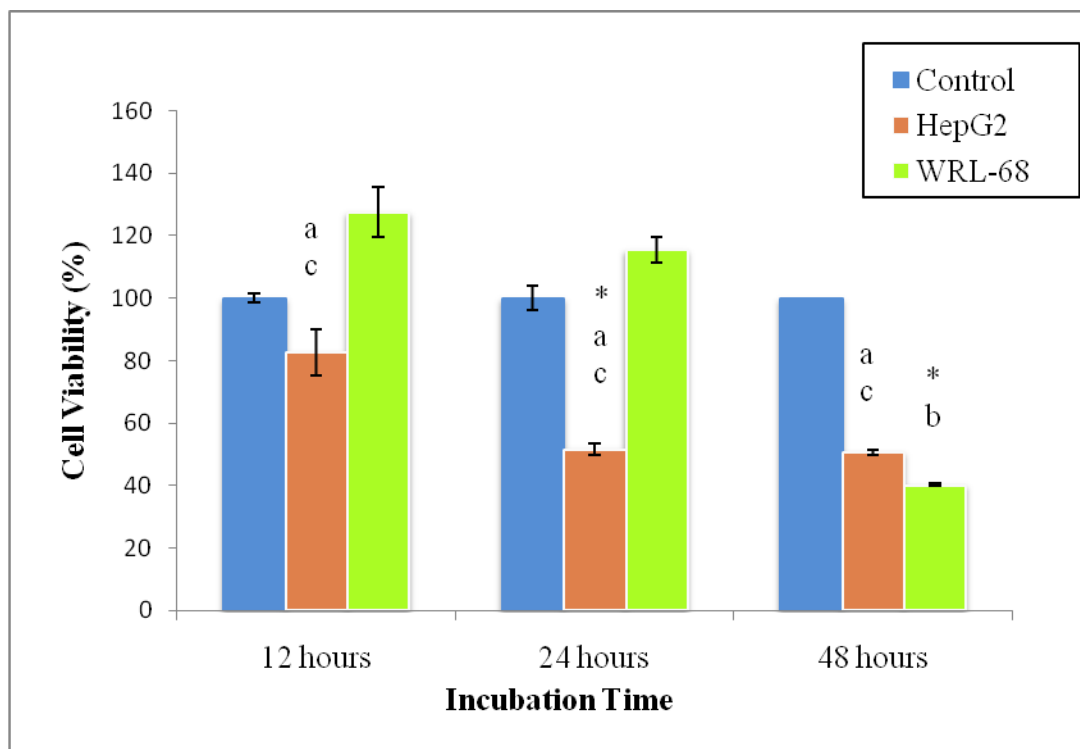
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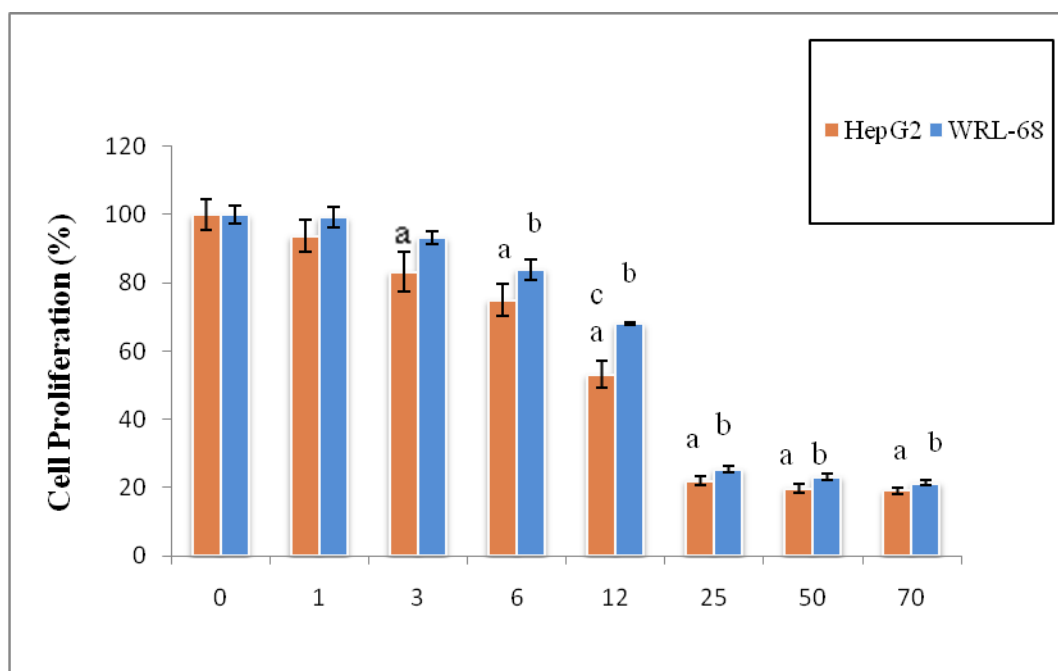
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**Figure 1:** The viability of HepG2 and WRL-68 cell treated with different concentration of gelam honey. Data were expressed as mean  $\pm$  SD of 3 replicates. a indicates significant difference compared to HepG2 at 0 concentration (control). b indicates significant difference compared to WRL-68 at 0 concentration (control). c indicates significant difference compared to WRL-68 of the same honey concentration. Significance is taken at  $p < 0.05$ .



**Figure 2:** The viability of HepG2 and WRL-68 treated with 25% (HepG2 IC<sub>50</sub>) of honey concentration at 12, 24 and 48 hours incubation period. Data were expressed as mean ± SD of 3 replicates. a indicates significant difference compared to control. b indicates significant difference compared to control. c indicates significant difference compared to WRL-68 of the same incubation time. \* indicates significant difference compared to previous incubation period in the same cell type. Significance is taken at p<0.05.



**Figure 3:** The effect of different honey concentration on the proliferation of HepG2 and WRL-68 cell. Data were expressed as mean ± SD of 3 replicates. a indicates significant difference compared to control. b indicates significant difference compared to control. c indicates significant difference compared to WRL-68 of the same honey concentration. Significance is taken at p<0.05.

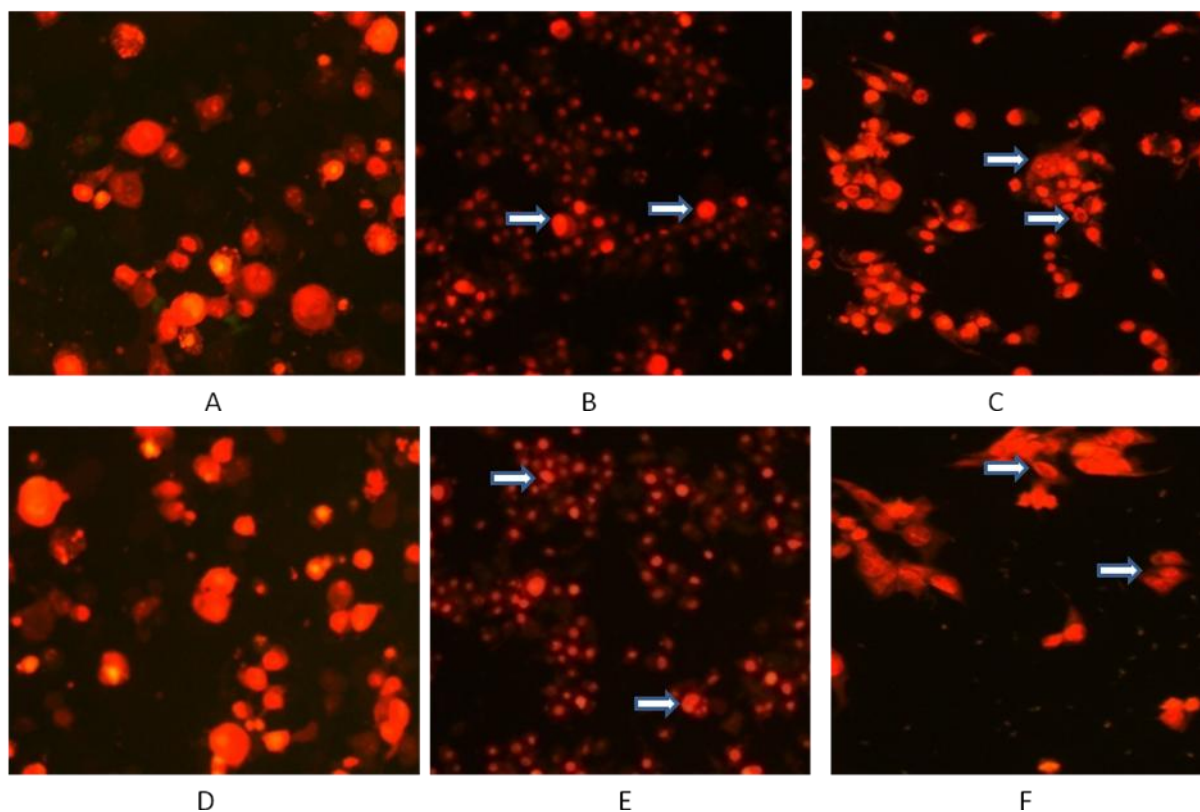


Figure 4: Morphological analysis for apoptosis detection was done using fluorescent microscope under 400X magnification in HepG2 cells (A=untreated, B=H<sub>2</sub>O<sub>2</sub>, C=Gelam honey) and in WRL-68 cells (D=untreated, E=H<sub>2</sub>O<sub>2</sub>, F=Gelam honey) after 48 hours of incubation. White arrows indicate cell shrinkage.