INTRODUCTION

Apoptosis is a pathway of cell death in which cells disintegrate into membrane-bound particles by the activation of enzymes that degrade the cells own DNA, as well as nuclear and cytoplasmic proteins, that are then eliminated by a phagocytosis or by shedding. It is induced by a tightly regulated intracellular program in which cells are destined to die.\(^1\) Apoptosis was initially recognized in 1972 by Kerr and Wyllie. In recent years it has been examined in detail and extracellular signals and intracellular events have been elaborated.\(^2\)

Apoptotic cell death is extremely important for normal cell development and tissue homeostasis. Any dysregulation in this process implicates carcinogenesis and tumour progression.\(^3\) These molecular pathways are controlled by proteins which promote or inhibit activation of a cascade of intracellular cysteine proteases, known as caspases, which cause apoptotic cell death by cleaving a number of structural and regulatory proteins.\(^4,5\)

ABSTRACT

Objective: To compare the combined and isolated growth inhibitory effects of Morarah and Kaltita (herbs) on hepatoma cell lines (Huh-7), through induction of apoptosis or necrosis.

Study Design: Comparative controlled in-vitro study.

Place and Duration of Study: The Molecular Biology Laboratory, The Aga Khan University, Karachi, from June to December 2006.

Methodology: The growth of hepatoma cell lines (Huh-7) was checked by adding Kaltita and Morarah to the cells before culture in a 24 well plate. Six wells were selected and labeled for each of the four variables (controls, Kaltita, Morarah and mixture). After 2 days, cells were studied under an inverted phase contrast microscope and fields were recorded. Approximately four fields per slide of higher intensity were selected randomly to determine the dead cell density, and the procedure was repeated 10 or more times. Frequency and percentages were calculated for dead or alive cells in controls, Morarah, Kaltita and their mixture. Chi-square was used to compare the qualitative variables. P-values < 0.05 were considered significant.

Results: Morarah and Kaltita were found to induce statistically significant (p < 0.001) cell death in hepatoma cell lines (Huh-7). At a magnification of 40x, the controls showed 1% dead cells compared to 91% in Morarah, 83% in Kaltita and 73% in combined mixture of Kaltita and Morarah. At magnification of 20x, the controls showed 4% dead cells compared to 44% in Morarah, 47% in Kaltita and 49% in the combined mixture of Kaltita and Morarah.

Conclusion: Morarah and Kaltita induced cell death in cultured hepatoma cells (Huh-7).

Key words: Hepatoma cell lines. Apoptosis. Necrosis. Morarah. Kaltita. Ferula asafoetida.
coloured resin-like gum, comes from the dried sap extracted from the stem and roots and is used as a spice. The Indo-Pakistani version of this plant is known as 'bing' and 'bingra', and is used for flavour and colour in various lentils. For the present study, Khaltita and Morarah were obtained from Saudi Arabia where it is known as 'Halitza' and 'Yemeni Morarah' respectively. These would add to the wide spread researches going on world wide on natural products to combat cancer. Further, this would enhance the spectrum of drug therapies currently used against cancer and these being food additives, the serious side effects encountered by cytotoxic chemotherapeutics will be avoided.

The combined and isolated effect of natural products has been seen in combating the proliferation of cancer cells. The present study was undertaken to evaluate the synergistic and isolated growth inhibitory effects of Morarah and Khaltita on hepatoma cell lines (Huh-7).

**METHODOLOGY**

The study was conducted at the Aga Khan University Molecular Biology Laboratory from July to December 2006. Hepatoma cell lines (Huh-7) were kindly provided by the Aga Khan University. The cell lines were maintained in the cell culture laboratory of the Aga Khan University, Molecular Biology Laboratory. It was a descriptive, comparative, controlled in-vitro study.

The growth of hepatoma cell lines (Huh-7) was checked by adding the additives, Khaltita and Morarah, individually and combined in a mixture to these cells and the cells were cultured according to given protocol. The flasks of actively growing cells that were 80-90% confluent were selected for experiment. The cell culture medium contained all the additives required by the above cell line. Calcium and magnesium-free phosphate-buffered saline CMF-PBS (10 mL) and trypsin solution 0.1% were used. The procedure was done in a laminar flow hood. The cultures were examined prior to subcultivation, using an inverted phase contrast microscope (100-200x). Signs of microbial contamination were checked. After cell harvesting using the standard protocol, cell culturing was done using the additives.

For cell culturing a 24 well plate was used. All wells were labeled. For each of the four variables (i.e. controls, Khaltita, Morarah and mixture), 6 wells were selected and labeled. Slide covers, used as base for cell cultures, were inserted in all the wells making them the base for the growth of the cells.

Samples were prepared as 1µg/1µl with sterilized water and 10 µl was used for each well. In brief hepatoma cells were seeded at a density of 0.05 x 106 cells per well of 24-well cell culture plates in the presence of minimum essential medium (Invitrogen, Carlsbad, CA), with 10 µl prepared sample of each Khaltita and Morarah in separate wells, whereas, 5 µl of each in the wells labeled as mixture, was added. In wells labeled as controls, 10 µl of water was added. After 2 days, cells were studied under a immunoflorecent microscope (inverted phase contrast microscope) and fields recorded. Two to four fields per slide of higher intensity were selected randomly to determine the dead cell density, and the procedure was repeated 10 or more times.

Frequency and percentages were calculated for dead or alive cells in controls, Morarah, Khaltita and their mixture. Chi-square or test of proportions was used to compare the qualitative variables. P-values less than 0.05 were considered significant.

**RESULTS**

The comparative analysis revealed the apoptotic action of substances when these were added separately and as a mixture.

The cell culture showed death in large groups of confluent cells with cellular detachment as well as individual cell death or in small groups. The effects of Khaltita, Morarah and their mixture on hepatoma cell lines Huh-7 at magnifications of 20x and 40x are described in Table I and II. The controls showed 4% dead cells compared to 44% (p < 0.001) in Morarah, 47% (p < 0.001) in Khaltita and 49% (p < 0.001) in the combined mixture. Similarly at a magnification of 40x the controls showed 1% dead cells, compared to 91% (p < 0.001) in Morarah, 83% (p < 0.001) in Khaltita and 73% (p < 0.001) in the combined mixture of Khaltita and Morarah. These results were found to be statistically significant (p < 0.001).

Figure 1 shows various patterns of cellular death at 40 x magnification induced by Khaltita, Morarah and a mixture of both. Khaltita and mixture shows cell death in small groups of cells and in some individual cells. Morarah containing plate shows cell death in comparatively larger groups of cells in confluence. There was no cell death seen in controls at this magnification.

**Table I:** The effects of Khaltita and Morarah at 20x and 40x magnification on hepatoma cell lines (Huh-7).

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Number of fields</th>
<th>(20x)</th>
<th></th>
<th>Dead (%)</th>
<th>(40x)</th>
<th></th>
<th>Dead (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H2O)</td>
<td>(n=11)</td>
<td>405</td>
<td>390 (96)</td>
<td>15 (4)</td>
<td>196</td>
<td>194 (99)</td>
<td>02 (1)</td>
</tr>
<tr>
<td>Morarah</td>
<td>(n=18)</td>
<td>455</td>
<td>254 (56)</td>
<td>201 (44)</td>
<td>129</td>
<td>12 (9)</td>
<td>117 (91)</td>
</tr>
<tr>
<td>Khaltita</td>
<td>(n=15)</td>
<td>362</td>
<td>193 (53)</td>
<td>169 (47)</td>
<td>72</td>
<td>12 (17)</td>
<td>60 (83)</td>
</tr>
<tr>
<td>Mixture (Morarah-Khaltita)</td>
<td>(n=10)</td>
<td>868</td>
<td>452 (52)</td>
<td>416 (48)</td>
<td>70</td>
<td>19 (27)</td>
<td>51 (73)</td>
</tr>
</tbody>
</table>
Figure 2 shows various patterns of cellular death at 20x magnification induced by Khaltita, Morarah and a mixture of both. Morarah showed death in almost all cells and dispersion of these cells. The mixture showed both live and dead cells with some loss of cohesiveness. Khaltita showed equivocal cell death with retained cohesiveness.

### DISCUSSION

The effects of Khaltita, Morarah and their mixture on hepatoma cell lines Huh-7 when compared with the controls showed up to 91% dead cells in Morarah, Khaltita and their mixture whereas, only 0.8% in controls in a specified time. The results were significant at a p-value of < 0.001.

The cell death pattern observed in case of Morarah, was in the form of large groups of confluent cells with cellular detachment. This points towards the fact that the action of these substances resulted in damaging the structural components as well as the denaturing of enzymes relating this effect to the pattern of cellular death that occurs in necrosis. The addition of Khaltita and their mixture resulted in cell death, individually as well as in clumps. Individual cell death amongst group of cells is the pattern observed in apoptosis while necrosis is the gross and histologic correlate of cell death occurring in clumps of cells in the setting of irreversible exogenous injury. Necrotic cells are unable to maintain membrane integrity and their contents often leak out. The morphologic appearance of necrosis is the result of the denaturing of intracellular proteins and enzymatic digestion of the cell. It is a well known fact that apoptosis and necrosis sometimes coexist and in this way they share some common features and mechanisms. This shows that there is involvement of both the processes of cell death, i.e. necrosis as well as apoptosis.

As mentioned earlier Khaltita and Morarah belong to the family of plant called *Ferula asafoetida*. Typical asafoetida contains about 40-64% resin, 25% endogeneous gum, 10-17% volatile oil, and 1.5-10% ash. The resin portion is known to contain asareninotannols 'A' and 'B', ferulic acid, umbelliferone and four unidentified compounds. The constituents present in Khaltita and Morarah have been researched extensively. Ferulic acid has been shown to suppress cancer of the digestive tract (tongue, esophagus, stomach, intestine and colorectal cancers) lung carcinogenesis, liver cancer and breast cancer. Umbelliferone or 7-hydroxy coumarin, resin portion of *Ferula asafoetida*, is a widespread natural product of this family. Coumarin and its derivatives are all considered phenylpropanoids. Coumarin has been used in the treatment of lymphedema. Naturally occurring coumarins (NOCs) are anti-carcinogenic in the mouse skin model. Umbelliferone derivatives 4MU (4-methylumbellierone) act by suppressing the over expression of hyaluronan synthases, prevent and strip the hyaluronan coating on the surface of the cells, exposing them to chemicals that can modify programmed cell death. In other words, 4MU actively targets and sensitizes infected T-cells for anticancer drugs, making the agents work better.

Cell lines exhibit immortality. One of the important mechanisms of tumour cell immortality has been

![Table II: Comparison of dead cell density between Morarah/Khaltita and controls.](image1)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Dead (%) at 20x</th>
<th>p-value</th>
<th>Dead (%) at 40x</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>15 (4)</td>
<td>&lt; 0.001</td>
<td>02 (1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Morarah</td>
<td>201 (44)</td>
<td></td>
<td>117 (91)</td>
<td></td>
</tr>
<tr>
<td>Control (H₂O)</td>
<td>15 (4)</td>
<td>&lt; 0.001</td>
<td>02 (1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Khaltita</td>
<td>169 (47)</td>
<td></td>
<td>60 (83)</td>
<td></td>
</tr>
<tr>
<td>Control (H₂O)</td>
<td>15 (4)</td>
<td>&lt; 0.001</td>
<td>02 (1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mixture (Morarah/Khaltita)</td>
<td>416 (48)</td>
<td>&lt; 0.05</td>
<td>51 (73)</td>
<td></td>
</tr>
</tbody>
</table>

*p-value < 0.05 was considered as significant.*

![Figure 1: Effect of Khaltita and Morarah on Huh-7 cell lines at magnification 40x.](image2)

![Figure 2: Effect of Khaltita and Morarah on Huh-7 cell lines at magnification 20x.](image3)
through the inhibition of apoptosis. In this way the cancer cells acquire the ability of unlimited growth.\(^2\) One of the mechanisms of immortality researched thoroughly is that Huh-7 show increased expression of key Hedgehog(\(Hh\)) pathway genes (\(IHH\), PTCH, and GLI). Among several pathways, it has been found that there are significant alterations in the expression of key genes in the (\(Hh\)) pathway. Signaling by the \(Hh\) pathway is crucial to normal embryonic growth and development of many tissues, including the liver. The reactivation of \(Hh\) in adults have been associated with cancers. These include rare familial syndromes, such as Gorlin’s syndrome (basal cell nevus syndrome), and also common cancers such as small-cell lung cancer and prostate and pancreatic cancer. The tumour cell may depend on \(IHH\) for their growth i.e. they might be ‘addicted’ to \(IHH\) – and could therefore be susceptible to the effects of agents that block signaling by \(IHH\).\(^20\) The ability of plants to influence programmed cell death in cancerous cells in an attempt to arrest their proliferation has been the topic of much research lately. However, this is the first ever study which has been done on Khalitita and Morarah, which are resin like gum extracted from the stem and roots and are used as spices. Since programmed cell death is a highly conserved mechanism of self-defense, also found to occur in plants, therefore, it is natural to assume that chemicals must exist in them to regulate programmed cell death and hence they can likely be proved to be an important source of agents that will modulate programmed cell death.\(^21\)

These plants have shown the capability to induce cell death in these malignant cells. Thus, we may hypothesize, that even in these immortalized cells either the death programme has been activated or the cell has been deprived of the basic raw materials for cell growth through modulation of gene expression.

**CONCLUSION**

Morarah and Khalitita, both combined and isolated, induced cell death on hepatoma cell lines (Huh-7) via apoptosis/necrosis induction. These results may add to the wide spread researches going on world wide on natural products to combat cancer. Being commonly used as food additives, the serious side affects encountered by cytotoxic chemotherapeutics will be negated and these will also enhance the spectrum of drug therapies currently used against cancer. Further research at molecular levels is required to find out which genes have been knocked out and which ones have been enhanced.

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**REFERENCES**


