In Vitro Inhibition of MCF-7 Human Breast Cancer Cells by Essential Oils of Rosmarinus officinalis, Thymus vulgaris L., and Lavender x intermedia

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Key words: Breast cancer, Rosmarinus officinalis, Thymus vulgaris L., Lavender x intermedia

ABSTRACT

Background: The essential oils of traditional medicinal plants, including Rosmarinus officinalis, Thymus vulgaris L., and Lavender x intermedia contain anticancer compounds such as lavandulyl acetate, rosmarinic acid and thymol. The aim of this study was to investigate the anticancer effects of the essential oils of R. officinalis, T. vulgaris L., and L. x intermedia on MCF-7 cells.

Methods: Essential oils were prepared from R. officinalis, T. vulgaris L., and L. x intermedia plants. Then, MCF-7 and Hu02 cells were treated with different concentrations of these essential oils for a given time. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine the cellular viability and cytotoxicity in response to treatment with different extract concentrations. The morphological changes were studied by Hoechst and propidium iodide staining. The results were analyzed using the one-way ANOVA and Tukey test.

Results: All three essential oils inhibited the viability of the MCF-7 cell line in a dose-dependent manner. T. vulgaris L. was more potent against MCF-7 cells at 400 µg/ml concentration (IC50 = 48.01 ± 0.94), while R. officinalis was moderate at 50800 µg/ml concentration (IC50 = 47.39±0.91) and the concentration for L. x intermedia was 400 µg/ml (IC50 = 47.39 ± 0.91).


Introduction

Breast cancer is among the most common cancers of women all over the world and represents one of the major health threat that takes the lives of thousands of people every year. Factors affecting fertility, environment, lifestyle, and physical inactivity contribute to this process. Breast cancer ranks first among Iranian women with diagnosed cancers. However, few studies have been conducted regarding clinical and pathologic features and age distribution of this disease in Iran. For over 3500 years, plants have been used to treat cancer, but their antiproliferative potential has only been evaluated since the late 1950s. Hartwell and colleagues used podophyllotoxin and its derivatives as the first anticancer agents for the first time in the late 1960s. The recognition of medicinal plants and extensive research on their essential oils have led to their widespread use in medicine, such that the antimicrobial and antioxidant features of essential oils, especially their anticancer properties, have...
attracted a lot of attention. More than 60% of anticancer compounds are derived from plants, microorganisms, and marine sources. Today, medicinal plants are being considered safe due to their low side effects relative to chemical drugs. The anticancer plants contain a wide range of compounds, such as colchicine, vincristine, and podophyllotoxin, which are mitotic inhibitors binding to mitotic spindle tubular proteins and possessing alkaloids such as vinca alkaloids (Vinca rosea) that are used in chemotherapy. Plants of the mint family (Lamiaceae) can be a rich source of phytochemicals such as phytosterols, flavonoids, carotenoids, and terpenoids that act as antioxidants, annihilating free radicals and stimulating the immune system. These compounds form DNA adducts inhibiting the activity of carcinogens and also blocking metabolic pathways of cancer.

**Thymus vulgaris** is a medicinal plant with therapeutic properties such as antiinflammatory, antiseptic, antitussive, antisPasmodic, and antimucous effects. The essential oil of this plant contains compounds such as eucalyptol (6.23%), camphor (15.14%), camphe (10.54%), α-pinene (8%), carvacrol (18.51%), and thymol (20.35%). Moreover, the plant contains inhibitor compounds for cyclooxygenase and oxidative stress, and the effect of these enzymes on the growth and segregation of cells (including cancer cells) has been confirmed.

**Lavender x intermedia** belongs to Lamiaceae family and is a source of uric acid, oleanolic acid, and betulinic acid. The main components of the essential oils of these plants include linalool (32.8%), linalyl acetate (17.0%), lavandulol acetate (15.9%), alpha-terpineol (6.7%), and geranyl acetate (5%).

**Rosmarinus officinalis** is also a good source of uric acid, oleanolic acid, and betulinic acid. Essential oil of rosemary contains carnosic acid (1%), carnosol (4.6%), rosmarinic acid (4%), uric acid (19.2%), and rosmanol (5%), among which rosmarinic acid is water soluble and other substances are soluble in organic solvents. Rosmarinic acid is a natural phenolic compound that has several biological attributes such as antidepressive, liver tissue protection, antiangiogenesis, antitumor, and anti HIV-1 infection. Several studies have shown anticancer effects of these three plants belonging to Lamiaceae family.

The aim of the present study was to investigate the cytotoxic effects of essential oils of *T. vulgaris*, *L. x intermedia*, and *R. officinalis* on breast cancer cell lines.

**Methods**

*The MCF-7 Cell line and Hu02 Cells*

The MCF-7 human breast cancer cell line and Hu02 normal human fibroblast cells (control) were purchased from Iranian Biological Resource Center (IBRC), Tehran, Iran. Cell culture materials were obtained from Gibco Company (USA), but the flasks and microplates were acquired from Griner Company (Germany).

**Collection of Plant Samples**

The samples of *T. vulgaris*, *L. x intermedia*, and *R. officinalis*, which were harvested from Boroujerd city of Lorestan Province, were confirmed by (IBRC), Tehran, Iran. Cell culture materials were purchased from Iranian Biological Resource Center (IBRC), Tehran, Iran. Cell culture materials were obtained from Gibco Company (USA), but the flasks and microplates were acquired from Griner Company (Germany).

**Essential Oil Extraction**

The essential oils were extracted using the Soxhlet method. First, 400 g of crushed dried samples from the leaves of each plant was placed in a tray and ethanol was added to it as solvent to make dough. Next, the dough was placed in the thimble inside the Soxhlet apparatus. Then, the ethanol was added slowly to the bottom flask, and the condenser was placed on the apparatus. An electric stove was used to boil solvent inside the flask. Consequently, the essential oil was collected and kept in sealed bottles at +4°C. The process lasted 5-8 hours.

**Cell Culture**

The cells were grown in RPMI-1640 (Gibco, UK) supplemented with 10% PBS (Gibco, UK), 2 mM/L glutamine, 100 unit/mL penicillin, and 100 mg/mL streptomycin (Sigma, US) in 96-well culture plates (Griner, Germany) at 37°C in a 5% CO2 incubator (Heraeus, Hanau, Germany). All experiments were performed using cells from passage 12 or lower.

**MTT Assay**

The cell viability was determined using 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. Briefly, MCF-7 cells were seeded at 10000 cells per well in 96-well culture plates and treated with different concentrations of the extracted essential oils for 24, 48, and 72 hours. Then, the cells were incubated with MTT solution (5 mg/mL in PBS) for 4 h, and the resulting purple formazan was solubilized in 100 μL of DMSO (Sigma). The absorption was measured at 570 nm and 620 nm as a reference using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for four replicates each and were used to calculate the respective means. The percentage inhibition was calculated using the following formula. The IC50 value was determined as the concentration of...
the complex that is required to reduce the absorbance to half of that of the control.

**Hoechst and Propidium Iodide Staining**

The effects of various concentrations of essential oils of *R. officinalis*, *T. vulgaris* L., and *L. x intermedia* on morphological changes were assessed by Hoechst staining. For this aim, we used a fluorescence microscope to examine cell morphology. Briefly, the cells were treated with different concentrations of essential oils of *R. officinalis*, *T. vulgaris* L., and *L. x intermedia* for 24, 48 and 72 hours. The medium was removed and the wells were washed with PBS (Gibson Company). Then, the samples were incubated with 100 µL of PBS + 100 µL of Hoechst solution for 6-8 minutes. Next, the cells were washed by PBS, and their nuclei were examined with a phase contrast microscope (Carl Zeiss, Jena, Germany) and finally photographed. In this stage, the cells appear blue in color. In the next stage, each well was washed with PBS and propidium stain was added. In this stage, this stain is attached to DNA grooves and makes dead cells' nuclei look red.

**Statistical Analysis**

The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey multiple comparison tests, on SPSS 20. The results are presented as mean ± SD. A P value of less than 0.05 was considered statistically significant.

**Results**

The results of the MTT assay are given in Tables 1-4 and Figures 1-4. Comparing the viability of MCF-7 breast cancer cells after 24, 48, and 72 hours showed significant differences among groups treated with various concentrations of *R. officinalis*, *T. vulgaris* L., *L. x intermedia*, and Taxol, which indicates that as the concentration is increased, the viability of MCF-7 cells is significantly decreased. As shown in Table 4, Taxol attenuated the cytotoxic effects of concentrations of essential oils in MCF-7 cells. In Hoechst and propidium iodide staining, live and dead cells respectively appear blue and red in color (figure 5). As the dose is increased, the number of red cells is also increased, which shows apoptosis resulting from the impact of essential oils.

**Effect of Essential Oils on the Viability of Cells**

In vitro cytotoxic effects of the essential oils on MCF-7 breast cancer cell line viability were evaluated by MTT assay and compared with the result of Taxol, a commercially available anticancer drug, the cytotoxicity of which is shown in Table 1. The comparison of cell viability at different essential oil concentrations (1000, 800, 400, 200, 100 µL/mL) and Taxol (10, 1, 0.1, 0.01, 0.001µM) disclosed similar viability rates. As shown in Figure 1, the cytomorphological effects of essential oils on MCF-7 cells at different concentrations include activation of an intracellular suicide program characterized by morphological changes like cytoplasmic and cellular shrinkage, oxidative stress, coiling, and biochemical responses leading to apoptosis. It is quite obvious from the results that the apoptosis rate of MCF-7 cells is increased with increase in the concentration of essential oil. A dose-dependent increase in cell inhibition was seen after 24, 48, and 72 h incubation. The IC₅₀ for *T. vulgaris* was 400 µg/mL (48.01 ± 0.94). The complete inhibition (90%) of breast cancer cells was obtained at the maximum concentration of 1000 µg/mL. These results highlight the dose- and time-dependent increase in cytotoxicity. The IC₅₀ value suggests that the essential oil proves to be a promising drug for chemotherapeutic treatment.

Figure 2 shows that the cytotoxic effect of *L. intermedia* essential oil is dose- and time-dependent at 1000 µg/mL (P < 0.05), but at lower concentrations (800, 400, and 200 µg/mL) the decrease in cell viability is only dose-dependent.

Also, figure 3 demonstrates a dose- and time-dependent cytotoxic effect of *T. vulgaris* essential oil at 1000 and 800 µg/mL (P < 0.05), but only a dose-dependent effect at lower concentrations (400, 200, and 100 µg/mL).

The cytotoxic effect of *R. officinalis* essential oil is dose- and time-dependent at 1000 µg/mL (P < 0.05), but only dose-dependent at lower concentrations (800, 400, 200, and 100 µg/mL) (figure 4).

**Table 1. 50% Inhibitory Concentrations (% v/v) for Taxol Against MCF-7 Cells (P < 0.05)**

<table>
<thead>
<tr>
<th>Time</th>
<th>0.001 µg/mL</th>
<th>0.01 µg/mL</th>
<th>0.1 µg/mL</th>
<th>1 µg/mL</th>
<th>10 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>75.39 ± 0.16</td>
<td>67.45 ± 0.52</td>
<td>55.99 ± 0.86</td>
<td>51.73 ± 1.21</td>
<td>48.31 ± 1.02</td>
</tr>
<tr>
<td>48 h</td>
<td>63.98 ± 0.61</td>
<td>56.33 ± 0.69</td>
<td>47.55 ± 0.73</td>
<td>33.86 ± 0.97</td>
<td>26.91 ± 1.01</td>
</tr>
<tr>
<td>72 h</td>
<td>68.90 ± 0.22</td>
<td>49.94 ± 0.70</td>
<td>38.19 ± 0.82</td>
<td>27.62 ± 0.95</td>
<td>21.88 ± 1.31</td>
</tr>
</tbody>
</table>
### Table 2. 50% inhibitory concentrations (% v/v) for *L. intermedia, T. vulgaris,* and *R. officinalis* Against MCF-7 Cells (P < 0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>400 µg/mL</th>
<th>800 µg/mL</th>
<th>1000 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. intermedia 24 h</td>
<td>83.61 ± 0.59</td>
<td>75.11 ± 0.72</td>
<td>66.09 ± 0.91</td>
<td>51.23 ± 1.03</td>
<td>44.52 ± 1.11</td>
</tr>
<tr>
<td>48 h</td>
<td>69.25 ± 0.51</td>
<td>57.89 ± 0.66</td>
<td>42.90 ± 0.89</td>
<td>29.74 ± 1.05</td>
<td>23.80 ± 1.14</td>
</tr>
<tr>
<td>72 h</td>
<td>61.28 ± 0.86</td>
<td>59.37 ± 0.75</td>
<td>44.93 ± 0.87</td>
<td>29.44 ± 0.91</td>
<td>20.89 ± 0.97</td>
</tr>
<tr>
<td>T. vulgaris 24 h</td>
<td>69.31 ± 0.70</td>
<td>59.81 ± 0.85</td>
<td>48.01 ± 0.94</td>
<td>40.22 ± 1.03</td>
<td>34.29 ± 1.06</td>
</tr>
<tr>
<td>48 h</td>
<td>47.25 ± 0.71</td>
<td>38.07 ± 0.79</td>
<td>30.86 ± 0.69</td>
<td>23.79 ± 0.82</td>
<td>18.95 ± 1.99</td>
</tr>
<tr>
<td>72 h</td>
<td>49.27 ± 0.65</td>
<td>38.94 ± 0.78</td>
<td>31.19 ± 0.60</td>
<td>19.94 ± 0.74</td>
<td>15.72 ± 0.88</td>
</tr>
<tr>
<td>R. officinalis 24 h</td>
<td>75.41 ± 0.77</td>
<td>68.15 ± 0.89</td>
<td>55.62 ± 0.79</td>
<td>47.39 ± 0.91</td>
<td>40.21 ± 1.04</td>
</tr>
<tr>
<td>48 h</td>
<td>59.91 ± 0.66</td>
<td>46.73 ± 0.66</td>
<td>38.55 ± 0.80</td>
<td>25.33 ± 0.84</td>
<td>21.07 ± 1.02</td>
</tr>
<tr>
<td>72 h</td>
<td>57.34 ± 0.72</td>
<td>49.18 ± 0.83</td>
<td>38.99 ± 0.75</td>
<td>23.65 ± 0.80</td>
<td>18.48 ± 0.96</td>
</tr>
</tbody>
</table>

### Table 3. 50% inhibitory concentrations (% v/v) for *L. intermedia, T. vulgaris,* and *R. officinalis* against Hu02 Cells (P < 0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>400 µg/mL</th>
<th>800 µg/mL</th>
<th>1000 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. intermedia 24 h</td>
<td>99.83 ± 0.14</td>
<td>90.63 ± 0.98</td>
<td>76.24 ± 0.69</td>
<td>61.44 ± 0.52</td>
<td>57.85 ± 0.48</td>
</tr>
<tr>
<td>48 h</td>
<td>82.63 ± 0.73</td>
<td>74.46 ± 0.84</td>
<td>67.32 ± 0.71</td>
<td>53.28 ± 0.62</td>
<td>48.41 ± 0.54</td>
</tr>
<tr>
<td>72 h</td>
<td>78.37 ± 0.64</td>
<td>73.92 ± 0.65</td>
<td>61.87 ± 0.76</td>
<td>46.63 ± 0.99</td>
<td>42.97 ± 0.79</td>
</tr>
<tr>
<td>T. vulgaris 24 h</td>
<td>98.54 ± 0.35</td>
<td>87.46 ± 0.68</td>
<td>68.38 ± 0.88</td>
<td>52.84 ± 0.77</td>
<td>49.76 ± 0.49</td>
</tr>
<tr>
<td>48 h</td>
<td>85.33 ± 0.47</td>
<td>77.59 ± 0.81</td>
<td>62.81 ± 0.63</td>
<td>46.93 ± 0.48</td>
<td>43.41 ± 0.78</td>
</tr>
<tr>
<td>72 h</td>
<td>76.79 ± 0.92</td>
<td>70.63 ± 0.75</td>
<td>56.12 ± 0.75</td>
<td>40.82 ± 0.56</td>
<td>35.88 ± 1.19</td>
</tr>
<tr>
<td>R. officinalis 24 h</td>
<td>98.35 ± 0.83</td>
<td>89.32 ± 0.72</td>
<td>75.87 ± 0.63</td>
<td>60.98 ± 0.86</td>
<td>56.97 ± 0.85</td>
</tr>
<tr>
<td>48 h</td>
<td>90.26 ± 0.51</td>
<td>81.47 ± 0.94</td>
<td>63.91 ± 0.84</td>
<td>49.61 ± 0.58</td>
<td>46.35 ± 0.93</td>
</tr>
<tr>
<td>72 h</td>
<td>83.61 ± 0.69</td>
<td>75.84 ± 0.91</td>
<td>58.72 ± 0.93</td>
<td>42.19 ± 0.89</td>
<td>39.94 ± 0.97</td>
</tr>
</tbody>
</table>

### Table 4. 50% inhibitory concentrations (% v/v) for *L. intermedia, T. vulgaris,* and *R. officinalis* essential oils + Taxol against MCF-7 cells (P < 0.05).

<table>
<thead>
<tr>
<th>Time</th>
<th>200+ 0.001 µg/mL</th>
<th>400+ 0.01 µg/mL</th>
<th>800+ 0.1 µg/mL</th>
<th>1000+ 1 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. intermedia + Taxol 24 h</td>
<td>79.24 ± 0.67</td>
<td>68.57 ± 0.84</td>
<td>62.40 ± 0.88</td>
<td>46.84 ± 0.96</td>
</tr>
<tr>
<td>48 h</td>
<td>62.53 ± 0.49</td>
<td>51.33 ± 0.59</td>
<td>36.67 ± 0.73</td>
<td>25.93 ± 0.85</td>
</tr>
<tr>
<td>72 h</td>
<td>54.37 ± 0.36</td>
<td>52.48 ± 0.72</td>
<td>41.06 ± 0.69</td>
<td>24.57 ± 0.74</td>
</tr>
<tr>
<td>T. vulgaris + Taxol 24 h</td>
<td>48.05 ± 0.67</td>
<td>52.61 ± 0.77</td>
<td>40.54 ± 0.88</td>
<td>38.17 ± 0.92</td>
</tr>
<tr>
<td>48 h</td>
<td>43.28 ± 0.54</td>
<td>35.81 ± 0.65</td>
<td>27.36 ± 0.58</td>
<td>20.66 ± 0.74</td>
</tr>
<tr>
<td>72 h</td>
<td>37.95 ± 0.48</td>
<td>33.76 ± 0.53</td>
<td>28.29 ± 0.52</td>
<td>16.28 ± 0.63</td>
</tr>
<tr>
<td>R. officinalis + Taxol 24 h</td>
<td>70.95 ± 0.82</td>
<td>64.55 ± 0.80</td>
<td>50.99 ± 0.95</td>
<td>42.38 ± 0.88</td>
</tr>
<tr>
<td>48 h</td>
<td>52.08 ± 0.63</td>
<td>44.38 ± 0.72</td>
<td>34.58 ± 0.76</td>
<td>21.47 ± 0.79</td>
</tr>
<tr>
<td>72 h</td>
<td>55.41 ± 0.14</td>
<td>45.67 ± 0.84</td>
<td>32.08 ± 0.81</td>
<td>20.98 ± 0.83</td>
</tr>
</tbody>
</table>

**Figure 1.** The results of one of the triplicate MTT tests
Figure 2. The results of one of the triplicate MTT tests

Figure 3. The results of one of the triplicate MTT tests

Figure 4. The results of one of the triplicate MTT tests.

Figure 5. Morphologic changes in MCF-7 and Hu02 cells treated with essential oils at 100μg/mL. a: Untreated MCF-7 cells; b: Hu02 control cells; c-e: MCF-7 cells treated with T. vulgaris for 24, 48, and 72 hours; f-h: MCF-7 cells treated with R. officinalis for 24, 48, and 72 hours; i-k: MCF-7 cells treated with L. intermedia for 24, 48, and 72 hours; l-n: MCF-7 cells treated with 100 + 0.001 μg/mL essential oils of T. vulgaris + Taxol, R. officinalis + Taxol, and L. intermedia, respectively.
**Discussion**

Recently, many studies have been conducted on the anticancer and cytotoxic effects of herbal medicines as they are associated with low side effects. In this study, we evaluated the cytotoxic and inhibitory effects of *R. officinalis*, *T. vulgaris* L., and *L. x intermedia* essential oils against MCF-7 human breast cancer cells. Studies on the cytotoxic and inhibitory effects of rosemary essential oil on various cancer cell lines, including HL60, K562, MCF-7, MDA-MB-468, M14, A375, NCI-H82, DU-145, Hep-3B, and MDA-MB-231, has demonstrated that these effects vary depending on the cell lines. 36, 31 Research has also found that the flavonoids in rosemary inhibit the expression of Cox-2 by activating PPAR-γ. Cox-2 is an enzyme that is upregulated in malignant and premalignant cancers such as breast, colon, pancreas, lung, stomach, head and neck, skin, and pharynx. 32 Applying the essential oil of *R. officinalis* to the skin of mice prevents benzopyrene covalent binding to DNA and inhibits epidermal tumor formation. Moreover, the uric acid in *R. officinalis* prevents the binding of benzopyrene to epidermal cell DNA, and TPA to the membrane of these cells. Uric acid inhibits NF-κB pathway in cancer cells possibly by suppressing the p65 component of NF-κB and, thus, downregulating oncogenes such as Cox-2 and MMP-9, Cyclin D1, C-Jun, and C-Fas. Also, the carnosol in *R. officinalis* leaves functions in a similar manner and inhibits the activity of NF-κB. 33 In line with our findings, the anticancer properties of the compounds of the essential oils of *R. officinalis* and *L. x intermedia* have been demonstrated by other studies.

Regarding anticancer properties of *T. vulgaris*, Sertel and colleagues demonstrated in vitro inhibition of a head and neck squamous cell carcinoma cell line treated with the plant’s essential oil. 33 The present study also showed the antitumor and antitoxic effects of essential oils of *T. vulgaris* on MCF-7 cells.

Our observation also indicated the cytotoxicity of Taxol and essential oils of *T. vulgaris*, *R. officinalis*, and *L. x intermedia* on MCF-7 cell line. Furthermore, the results of MTT assay in this study demonstrated that Taxol increased the cytotoxic effects of essential oils after 24, 48, and 72 hours of incubation. Assessment of morphological changes indicates that some changes, such as nuclear disintegration, formation of apoptotic bodies, and membrane blebbing, observed in the cells treated with Taxol and essential oils are indicative of apoptosis. However, based solely on these results, we cannot demonstrate the occurrence of apoptosis and this requires specific tests such as TUNEL assay or flow cytometry-based assays.

Eventually, it can be concluded that effective compounds in essential oils of *T. vulgaris*, *R. officinalis*, and *L. x intermedia* induce cell death in MCF-7 cancer cells. Literature review and the results of various studies, as well as our results, show that these three plants of Lamiaceae family have biological and pharmacological potential, and it seems that isolating effective components of these plants as well as determining their structure and exact mechanisms of action should be the topics for future research.

**Acknowledgement**

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**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial relationships that could be construed as a potential conflict of interest.

**References:**


apoptosis through both the mitochondrial apoptotic pathway and death receptor pathway in human colon adenocarcinoma COLO 205 cells. *Food and Chemical Toxicology.* 2011;49(2):485-93.


