

## Antiproliferative and apoptotic effects of *Lamium purpureum* L. extract on prostate cancer cells

Irem Atlıhan<sup>1,2</sup>, Ayca Güner<sup>1</sup>, Feyza Şule Aslan<sup>3</sup>, Ali Şen<sup>4</sup>, Oya Orun<sup>2</sup> & Pinar Mega Tiber<sup>2\*</sup>

<sup>1</sup>Department of Biophysics, Institute of Health Sciences, Marmara University, Istanbul, Turkey

<sup>2</sup>Department of Biophysics, Faculty of Medicine, Marmara University, Istanbul, Turkey

<sup>3</sup>Faculty of Medicine, Marmara University, Istanbul, Turkey

<sup>4</sup>Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

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The use of herbal agents in cancer treatment is an important concept. It is thought that the Lamiaceae family may have anticancer effects due to its polyphenol metabolites. The anticancer effect of the *Lamium* genus belonging to this family has been examined in some studies. However, there are few studies on the *Lamium purpureum* (purple dead-nettle) species. In this study, the *Lamium purpureum* extract/fractions were prepared in different solvents, and their effects on proliferation and apoptosis in the LNCaP prostate cancer cell line were examined *in vitro*. This was investigated using the CCK-8 test, JC-1 mitochondrial membrane potential assay, caspase 3/7 enzymatic activity test, and caspase-7 protein level by Western blot. It has been determined that *Lamium purpureum* ethanol extract suppresses cell proliferation with increasing concentration and may induce apoptosis *via* caspase-7. It is the first study to investigate the anticancer effect of *Lamium purpureum* on LNCaP cells.

**Keywords:** Cancer, LNCaP, Apoptosis, Purple dead-nettle

Around the world, cancer is the primary cause of mortality and a serious health issue<sup>1</sup>. After lung cancer in men, prostate cancer is the second most common cancer globally<sup>2</sup>. Prostate cancer cases that are older than 85 years have a higher risk of mortality<sup>3</sup>. The possible therapies available for prostate cancer are chemotherapy, radiation therapy, hormone therapy, and surgery<sup>4</sup>. These therapies have several disadvantages, complications, and side effects at present<sup>5-8</sup>. Finding medicines made from plants that are less expensive, have fewer side effects, and may have therapeutic effects is crucial given the high expense of treatment and adverse effects of chemical drugs<sup>9</sup>. The anticancer effects of some plants are already known<sup>10-14</sup>.

The mint family (Lamiaceae) is a common species of flowering plants. The leaves and flowers of plants in this family contain essential oils<sup>15</sup>. In Lamiaceae, polyphenols constitute the largest group of secondary metabolites. Through apoptosis mediated by many pathways, polyphenols are known to trigger the death of cancer cells<sup>16-18</sup>. The genus *Lamium* is a member of

the Lamiaceae family. There are over 40 species of this genus in Africa, Asia, and Europe<sup>19</sup>. In Turkey, the genus *Lamium* is represented by 26 taxa (14 species), 5 of which are endemic<sup>20</sup>. The antioxidant and cytotoxic activity of *Lamium garganicum* L. subsp. *pictum* species against *Bacillus cereus* bacteria has been previously demonstrated<sup>21</sup>. In a study, chloroform and methanol extracts of the *Lamium album* showed anticancer effects against lung cancer *in vitro* and *in vivo*<sup>22</sup>. A Lamiaceae species called *Cedronella canariensis* has also demonstrated inhibitory effects against melanoma, colon, and breast cancer in previous studies<sup>23</sup>. Apoptosis in leukemia cancer was enhanced by Lamiaceae metabolites in a study conducted in 2022<sup>24</sup>. In another study, phenolics of Lamiaceae were evaluated to be beneficial in terms of cytotoxicity in breast cancer<sup>25</sup>. It is conceivable that agents used against breast cancer may also be beneficial for prostate cancer. Because significant biological similarities have been identified between prostate and breast cancer<sup>26</sup>. Also, it has been reported that *Lamium album*, *Lamium maculatum*, and *Lamium purpureum* are used to cure constipation in Anatolia<sup>27</sup>.

Studies on the *Lamium purpureum* (*L. purpureum*) species are not common<sup>28</sup>. Nonetheless, some

\*Correspondence:

Phone: +90 530 701 25 21

E-mail: pinar.tiber@marmara.edu.tr

research has shown that the *L. purpureum* species may have strong antioxidant properties<sup>29</sup>. The Fig. 1 shows the appearance of *L. purpureum*, which is widespread in Turkey. Additionally, one of the plants used in Turkey for the traditional treatment of prostate diseases is *L. purpureum*<sup>30</sup>. The aerial parts of this species are also used in traditional folk medicine against prostate cancer<sup>31</sup>.

This study aimed to scientifically demonstrate the effect of *L. purpureum*, traditionally used against prostate cancer, in LNCaP prostate cancer cells. For this purpose, ethanol extract (LPE) was prepared from the aerial part of *L. purpureum* with 96% ethanol solvent, and then n-hexane (LPH), aqueous ethanol (LPAE), chloroform (LPC), and ethyl acetate (LPEA) fractions were prepared from LPE with n-hexane, aqueous ethanol, chloroform and ethyl acetate solvents, respectively. For the reason that it has been found that solvents affect the activity of *Lamiaceae* plants<sup>32</sup>. Fundamental research on prostate cancer commonly uses PC-3, DU-145, and LNCaP cell lines<sup>33</sup>. The LNCaP cell line is one of the best models to use for prostate cancer studies<sup>34</sup>. The cytotoxic effects of LPH, LPAE, LPC, LPEA, and LPE on the LNCaP prostate cancer cell line were determined and IC<sub>50</sub> values were calculated within this research. Apoptosis indicators such as mitochondrial membrane potential (MMP), caspase 3/7 enzymatic activity, and caspase-7 protein level were all assessed.



Fig. 1 — *L. purpureum* plant collected in Turkey.

## Materials and Methods

### Plant material and extraction

The flowering above-ground parts of *L. purpureum* were collected by Dr. Ali Şen from Başbüyük district of Maltepe province on 30.03.2022. The identification of the plant was made by Dr. İsmail Şenkardeş and a few specimens of the plant are kept in the herbarium of Marmara University Faculty of Pharmacy under the number 22580. The plant was dried in an airy and shady environment, powdered and weighed 21.72 g. This prog was transferred into a flask and macerated with 125 mL 96% ethanol for 24 h. After filtration, 96% ethanol was again added to the residue and this process was repeated three times. The combined filtrates were evaporated to dryness in a rotary evaporator at a temperature not exceeding 40°C. After calculating the yield of the obtained ethanol extract (LPE) and taking an amount for activity, the remaining extract was dissolved with 50% ethanol, transferred to a separatory funnel and fractionated with hexane (3×50 mL), chloroform (3×50 mL) and ethyl acetate (3×50 mL), respectively. The solvents of the obtained fractions were evaporated to dryness (only hexane extract was oily) in a rotary evaporator to obtain hexane (LPH), chloroform (LPC), ethyl acetate (LPEA) and aqueous ethanol (LPAE) fractions. After calculating the yields of the fractions obtained, the extracts and fractions were stored at +4°C until analysis.

### Cell culture

LNCaP prostate cancer cells (ATCC CRL-1740) were incubated in RPMI-1640 (Gibco, 31870-025) medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin in a 37°C environment with 5% CO<sub>2</sub>. The culture medium was changed three times a week.

### Cell viability and cytotoxicity

96-well plates were seeded with  $5 \times 10^3$  cells/well. After an overnight incubation, the culture medium was replaced. A control group (0 µg/mL) and an experimental group containing *L. purpureum* extracts at different concentrations (10, 25, 50, 75, 100 µg/mL) were applied with three replicates each. After 24 and 48 h, the culture medium in the wells was aspirated<sup>35</sup>. Subsequently, 100 µL of fresh culture medium and 10 µL of CCK-8 solution (Cell Counting Kit-8, KTC011001, Abbkine) were added to each well. After four hours, absorbance values at 450 nm were

measured using a microplate reader (Synergy H1, Biotek Instruments Inc., USA).

#### JC-1 mitochondrial membrane potential test

96-well black opaque plates were seeded with  $5 \times 10^4$  cells/well. After an overnight incubation, the culture medium was replaced. The experimental group was treated with different concentrations (10, 25, 50, 75, 100  $\mu\text{g/mL}$ ) of *L. purpureum* extracts for 48 h. After 48 h, JC-1 was applied according to the manufacturer's instructions (JC-1 Mitochondrial Membrane Potential Assay Kit, 10009172, Cayman Chemical). Fluorescence values were measured using a microplate reader, with excitation and emission wavelengths set at 535 and 595 nm for healthy cells, and 485 and 535 nm for apoptotic cells<sup>36</sup>.

#### Caspase 3/7 enzymatic activity

$5 \times 10^4$  cells/well were seeded into 96-well black opaque plates. After an overnight incubation, the culture medium was replaced. For the 48 h treatment with different concentrations (10, 25, 50  $\mu\text{g/mL}$ ) of *L. purpureum* extracts, the working solution for Caspase 3/7 was prepared according to the manufacturer's protocol. Then, 100  $\mu\text{L}$  of the working solution was added to each well. After incubating at room temperature for 1 h, fluorescence values were measured with excitation and emission wavelengths at 535 nm and 620 nm, respectively (Cell Meter Caspase 3/7/8 Activity Apoptosis Assay Kit, 22820, AAT Bioquest, Sunnyvale, USA).

#### Western blot

Cell lysis, electrophoresis and transfer to membrane were performed as in our previous study<sup>37</sup>. Primary antibodies used were  $\beta$ -actin (Novus Biologicals, NB600-501) and Caspase-7 (Novus Biologicals, NBP3-14866). HRP chemiluminescent substrates were used for the detection of the bands (WesternBright ECL HRP Substrate, K-12045-050, Advansta). Imaging (Celvin S, Biostep) and quantification (ImageJ, version 1.54)<sup>38</sup> of the protein bands were performed.

#### Statistical analysis

GraphPad Prism software (version 5.01, GraphPad Software, CA, USA) was used for statistical analysis of the obtained data. One-way ANOVA test followed by Dunnett's multiple comparison test was performed. A significance level of  $P < 0.05$  was considered statistically significant.

## Results

This study aimed to evaluate cytotoxic effects of *L. purpureum* extract/fractions on the LNCaP cell line. By quantifying the yields of these extracts and assessing their biological activity, we sought to identify potential therapeutic candidates for further development in cancer treatment. The following results detail the findings related to cell proliferation and cytotoxicity, mitochondrial depolarization, caspase activity, and protein expression levels.

#### Cell proliferation and cytotoxicity

The yields of LPE and its fractions LPH, LPC, LPEA and LPAE were calculated on 100 g of dry plant powder and were found to be 6.68, 2.42, 0.59, 0.57, 3.13%, respectively. Among the fractions, the highest yield was found in LPAE while the lowest yield was found in LPEA. IC50 values obtained from the results of the CCK-8 test are shown in Table 1. These values are crucial to assess the cytotoxic potential of each fraction against the LNCaP cell line.

#### Depolarization of mitochondrial membrane potential

Depending on the obtained IC50 values, 48 h incubation of LPH, LPAE, LPC, LPEA and LPE in the LNCaP cell line was selected for the JC-1 mitochondrial membrane potential test. The depolarisation of the mitochondrial membrane potential was assessed by comparing the green and red fluorescence values acquired (Fig. 2). For LPH and LPC, no significant increase in fluorescence ratio was observed in the experimental group compared to the control group. In contrast, a significant increase in the fluorescence ratio was observed in the LPAE and LPE groups across all tested concentrations (10, 25, 50, 75, 100  $\mu\text{g/mL}$ ). This mitochondrial depolarization might be a hallmark of early apoptotic events, warranting further investigation into the underlying mechanisms.

#### Change in enzymatic activity of caspase-3/7

To explore the involvement of apoptosis in the observed cytotoxic effects, the enzymatic activity of

Table 1 — IC50 values (mean $\pm$ SD) calculated after 24 and 48 h of incubation of *L. purpureum* extracts in the LNCaP cell line

<i>L. purpureum</i> extract/fraction	IC50 ( $\mu\text{g/mL}$ )	IC50 ( $\mu\text{g/mL}$ )
	24 h	48 h
LPH	20.33 $\pm$ 0.10	19.93 $\pm$ 0.27
LPAE	86.66 $\pm$ 0.06	48.81 $\pm$ 0.07
LPC	78.07 $\pm$ 0.08	35.05 $\pm$ 0.05
LPEA	70.41 $\pm$ 0.11	56.92 $\pm$ 0.14
LPE	27.18 $\pm$ 0.12	15.35 $\pm$ 0.18

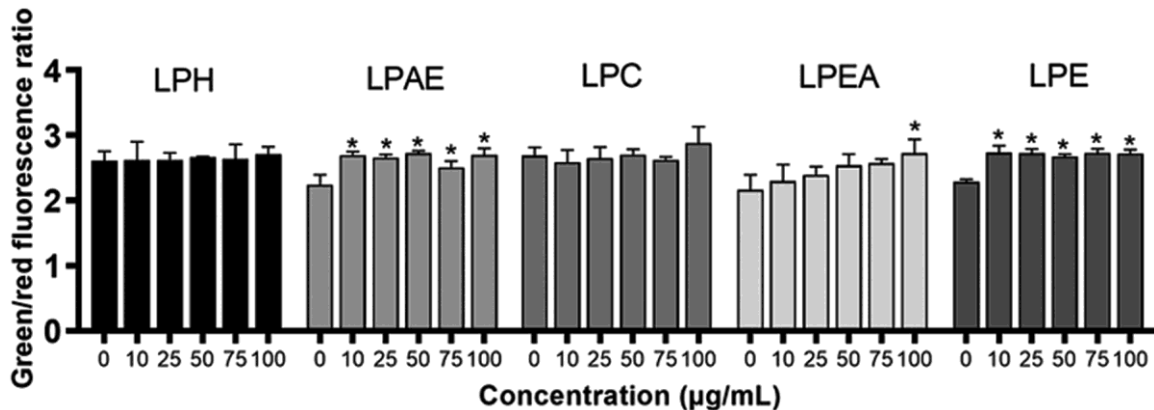


Fig. 2 — Green/red fluorescence ratios obtained from the JC-1 test as a result of treatment of the LNCaP cell line with LPH, LPAE, LPC, LPEA, and LPE for 48 h (n = 3). \* $P < 0.05$

caspase-3/7 was assessed for LPH, LPAE, and LPE for 48 h incubation period. Caspase-3 and caspase-7 are critical executors of apoptosis, and their activation indicates commitment to the apoptotic pathway. As shown in Fig. 3, an increase in caspase-3/7 activity was noted for LPAE and LPE. However, these increases were not statistically significant.

#### Increase of caspase-7 protein level

LPE was selected for the detection of caspase-7 protein expression level according to the results of caspase 3/7 enzymatic activity test. Western blot analysis was conducted for the determination of caspase-7 level (Fig. 4). The results demonstrated a clear dose-dependent increase in caspase-7 levels, indicating that higher concentrations of LPE correlate with increased expression of this apoptotic marker. This suggests that LPE not only influences enzymatic activity but also promotes the expression of proteins integral to the apoptotic pathway. Overall, these results highlight the potential of *L. purpureum* extracts to induce cytotoxicity and initiate apoptotic mechanisms in prostate cancer cells, laying the groundwork for further investigations into their therapeutic applications.

#### Discussion

An important source of therapeutic agents is the plant kingdom<sup>39</sup>. Some species of the *Lamium* genus have been studied to assess their antioxidant/anticancer properties<sup>21-25</sup>. However, studies on the *L. purpureum* species are very few. In this study, the effects of extract/fractions obtained from the *L. purpureum* plant on cell proliferation and apoptosis in prostate cancer were examined. Some species from Lamiaceae family have previously showed cytotoxic effects in MCF-7 breast cancer cells<sup>25</sup> and glioblastoma cells<sup>40</sup>.

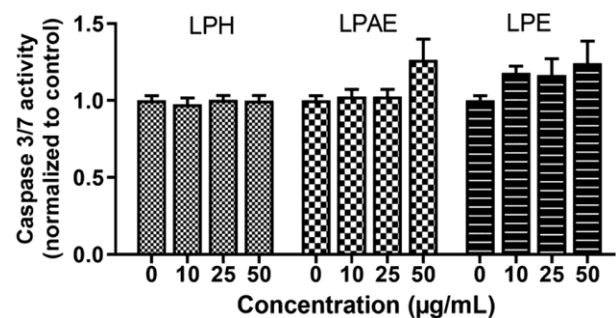


Fig. 3 — Red fluorescence obtained from the caspase 3/7 activity test as a result of treatment of the LNCaP cell line with LPH, LPAE, and LPE for 48 h (n = 3). \* $P < 0.05$

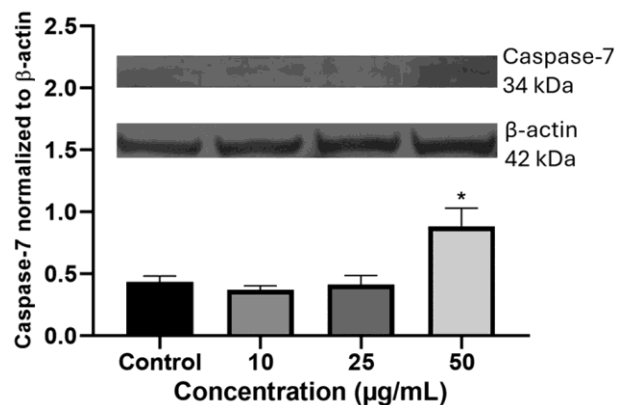


Fig. 4 — Western blot analysis for caspase-7 protein expression after LPE treatment of LNCaP cell line for 48 h (n=2). Bands are normalized to beta-actin. \* $P < 0.05$

Even some of the plants in this family have been shown to have therapeutic benefits for lung inflammation caused by coronaviruses<sup>41</sup>. Also, the cytotoxic and apoptotic effect of *L. purpureum* on melanoma cells has been previously demonstrated<sup>42</sup>. Although the antioxidant and anti-inflammatory effect of the Lamiaceae family has been demonstrated

many times, research on its potential anticancer properties is scarce.

According to our CCK-8 test results, the IC50 values of *L. purpureum* extract/fractions in LNCaP prostate cancer cells were determined. In particular, the IC50 value of 15.35 µg/mL for 48 h of LPE application shows that this extract has an inhibitory effect on cell viability and proliferation when applied at low concentrations. This reveals that LPE has potentially cytotoxic and antiproliferative properties. In a previous study<sup>43</sup>, an extract from Lamiaceae family showed a cytotoxic effect on MCF-7 breast cancer cells with an IC50 value of 18.15 µg/mL (48 h). This IC50 value determined for MCF-7 and the IC50 value we determined for LNCaP in our study are very close to each other. This may suggest that prostate and breast cancer cell lines have common target molecules affected by agents from the Lamiaceae family.

Our MMP test results showed that mitochondrial depolarization increased in LNCaP prostate cancer cells after applying LPAE and LPE. This loss in MMP suggests that these extracts may induce apoptosis through the mitochondrial pathway. Depolarization in MMP plays a critical role in the initiation of intracellular apoptotic signals, and this finding supports the apoptosis-inducing effect of the extracts. However, the loss of MMP is not limited to apoptosis. Conditions such as cellular stress or oxidative damage can also cause changes in MMP<sup>44</sup>. Therefore, it is necessary to evaluate whether changes in MMP are associated with apoptosis together with other apoptotic indicators.

Targeting apoptosis in cancer treatment is a known approach<sup>45</sup>. Apoptosis is a programmed cell death that occurs *via* many mechanisms<sup>46</sup>. The two categories of apoptosis that are studied are intrinsic and extrinsic<sup>47</sup>. Caspases are especially involved in the process of intrinsic and extrinsic apoptosis. Caspase-9 is activated in intrinsic, caspase-8 in extrinsic, and caspase 3/7 in both apoptosis pathways<sup>48</sup>. Research suggests that inhibiting caspase-7 could provide a cancer therapeutic approach<sup>49</sup>. It has already been shown that *Glechoma hederacea* (Lamiaceae) effects the caspase-3 activity in the PC-12 cell line<sup>50</sup>. In addition to this, extracts of *Teucrium sandrasicum* from the Lamiaceae family also induced both MMP depolarization and caspase-9 activity in HeLa and MCF-7 cells<sup>51</sup>. In the present study, we found that *L. purpureum* extract-treated LNCaP cells had increased caspase-3/7 activity; however, this increase was not statistically significant. Caspase 3/7 are the

executing caspases of apoptosis, and their activation plays a vital role in cell death. These results suggest that the extracts may activate the caspase pathway, but this effect may not be strong enough at low concentrations.

It is known that especially caspase-7 is overexpressed in the caspase-related apoptotic pathway in LNCaP prostate cancer cells<sup>52,53</sup>. Our Western blot analysis showed that applying 50 µg/mL LPE increased the level of caspase-7. This finding suggests that at high concentrations, this extract can induce apoptosis by increasing the caspase-7 protein level. The increase in caspase-7 reveals that the apoptotic signal in the cell is strengthened and the molecular mechanisms that lead to cell death are activated.

In phytochemical studies conducted on the *L. purpureum*, it has been reported that this species contains phenolic compounds<sup>54</sup> and terpenic compounds (germacrene D, β-pinene, α-pinene, *etc.*)<sup>19</sup>. It has been suggested that some compounds in the phenolic compounds and terpenic classes have anticancer activity on LNCaP prostate cancer cells<sup>55-57</sup>. Therefore, compounds in this class may be responsible for the anticancer effect shown by *L. purpureum*, which carry compounds in this class.

The fact that apoptosis markers other than caspase activity were not examined is a limitation of this study. Also, the LNCaP cell line is androgen-dependent. Conducting the study with androgen-independent prostate cancer cells (PC-3) will support the results. Thus, we can better understand the effects of *L. purpureum* extracts on prostate cancer.

## Conclusion

In conclusion, this study shows that LPE, ethanol extract of the *L. purpureum*, may contribute to the induction of apoptosis in LNCaP prostate cancer cells. Tests have revealed that LPE reduces cell viability, increases MMP depolarization, and increases caspase-7 levels at high concentrations. These findings support the apoptosis-inducing potential of LPE and provide an important basis for future studies. However, the statistically non-significant increases observed in the caspase-3/7 activity test indicate that these mechanisms should be investigated in more detail. Additionally, the active components of LPE need to be determined. The antioxidant effect of *L. purpureum* on LNCaP cells can also be planned as a future study. Also, although further scientific studies are needed, as a preliminary study, these results scientifically support the traditional use of the plant against prostate cancer.

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## Conflict of interest

The authors have no conflicts of interest to declare.

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