HPLC Analysis of Phytoesters in Prunus spinosa L. Extracts and Their Antiproliferative Activity on Prostate Cancer Cell Lines


Abstract

Sloe (Prunus spinosa L.) extracts are a good source of natural bioactive compounds, including phytosterols. Phytoesters are known to be applied in the treatment of various prostate diseases. The in vitro antiproliferative activity of sloe ethanolic extracts (flower, leaf, and fruit), collected from three areas in Bosnia and Herzegovina, were investigated against human prostate cancer cell lines PC-3 and DU145 using MTT assay. β-sitosterol, campesterol and stigmasterol were quantified by HPLC-PDA analysis using Symmetry C18 chromatographic column. The results of the analysis proved the presence of phytoesters, mostly β-sitosterol in all extracts. All extracts possess antiproliferative activity. The highest activity against PC-3 and DU145 was gathered from leaf extracts obtained by different extraction methods (microwave-assisted extraction and ultrasound-assisted extraction). To the best of our knowledge, no other studies have presented results on antiproliferative activity of ethanol sloe extracts. Based on these results, further investigation should be recommended on other cancer cell lines as well.

Keywords

HPLC, phytoesters, sloe, cytotoxicity, PC-3, DU145

1 Introduction

Prunus spinosa L. (blackthorn or sloe), which belongs to the family Rosaceae, is a perennial plant that grows as a shrub on slopes of wild uncultivated areas in various regions in Bosnia and Herzegovina. Sloe fruits, flowers, and leaves are commonly used in food industry and phytotherapy due to their long-term use in traditional medicine.1-3 There is a wide variety of plant-derived bioactive molecules often called phytochemicals, that have been increasingly consumed by humans given their renowned health benefits.4-6 Phenolic compounds, organic acids, carotenoids, alka-
loids, as well as sterols are the most common bioactive components present in plant material. Broadly, plant ster-
ols are functional ingredients solely obtained from plant resources.7

The rapid growth of chronic diseases, including cancer, is one of the most difficult issues for public health systems in underdeveloped and developing countries due to its growing prevalence, mortality rate, and high treatment costs.8 It remains not only a cause of tremendous damage to health, but also the second leading cause of morbidity worldwide. In the past decades, much attention has been focused on prostate cancer.9,10 Based on several reports, prostate can-
cer was the most diagnosed cancer in the United States of America, Europe and Oceania.11-13 In fact, prostate cancer is the second most deadly malignancy after skin cancer among men.14 The treatment of choice by professionals for many malignant tumours is chemotherapy, despite the fact that this treatment damages perfectly healthy cells, and thus dramatically reduces the quality of the patient’s life. Additionally, cancer cells might develop a multidrug-resistant phenotype in prolonged treatment.15

Based on published data from animal studies, breast and prostate cancers could be treated alternatively by phytosterols, particularly β-sitosterol.16-19 Phytoesters are present in all plant foods, unrefined plant oils, vegetables, nuts, and olive oils, cereals, fruits, and berries.9 More than 200 types of phytoesters have been found in various plant spe-
cies. The human organism is not able to synthesise phytoesters, and they can only be obtained from the diet.20 In vitro studies have found that β-sitosterol and campesterol inhibit PC-3 cell growth by 70 % and 14 %, respectively, while cholesterol supplementation increased growth by 18 % compared with controls. It has been confirmed that consumption of phytoesters as part of a healthy diet and lifestyle, are directly related to its ability to help maintain or reduce the low-density lipoprotein cholesterol level.21 Phytoesters or plant sterols are a group of biologically active steroid alcohols (sterols) structurally similar to cholesterol with minor modifications. Modifications include changes on a side chain on the C17 steroid backbone, and the ad-
dition of a double bond and/or a methyl or ethyl group. Phytosterols exist in several forms in plants, including β-sitosterol, campesterol, stigmasterol (Fig. 1).22,23

The extraction and isolation of phytosterols from different plant material is still complicated and time-consuming. Selecting the most appropriate method through optimising the extraction conditions can reduce solvent consumption (e.g., microwave-assisted extraction, ultrasonic-assisted extraction). Some non-conventional methods, such as supercritical fluid chromatography, using CO₂, could be applied without the use of hazardous organic solvents. Furthermore, the use of non-conventional methods yielded high amounts of phytosterols from different plant materials.24 Until recently, GC and HPLC were the most commonly used methods for the analysis of phytosterols. Although GC is the best choice for identifying phytosterols in samples, HPLC can also be considered to determine and isolate individual phytosterols. The increasing demand for these bioactive compounds as a supplement in foods may lead to further innovations in extraction, isolation, and analytical methods that are more efficient, rapid, less costly, and environment-friendly.25

The main goal of this study was to present the characterisation of the major phytosterol constituents of the sloe flower, leaf, and fruit ethanol extracts by HPLC-PDA. The antiproliferative activity of those extracts against human prostate cancer cells PC-3 (androgen-sensitive prostate cancer cells) and Du145 (androgen-insensitive prostate cancer cells) was also evaluated.

To avoid influence of extraction solvent, extracts were intentionally prepared with 97 % ethanol. According to the literature, the use of more concentrated solutions will result in almost immediate coagulation of surface on cell wall proteins, and prevent passage of the alcohol into the cell.26

No bacterial growth inhibition zone was observed around the wells containing 97 % ethanol, as in the case with the use of 70 % concentration of alcohol, which is the most effective at killing pathogens.27

2 Experimental

2.1 Chemicals and reagents

All the solvents, reagents, and standards used were of analytical grade. Ethanol (97 %) was obtained from Merck (Darmstadt, Germany). Standard campesterol (65 %), stigmasterol (95 %) and β-sitosterol (≥ 90 %) were purchased from Sigma-Aldrich, Chemie GmbH, Germany. Acetonitrile and methanol were of HPLC grade. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI-1640, DMEM, FBS, L-glutamine, HEPES were products of Sigma-Aldrich, St. Louis, MO.

2.2 Plant material

Wild growing sloe flowers and leaves were collected during April and May, while fruits were collected in October 2020 from three locations in Bosnia and Herzegovina (Borče, Vareš and Trnovo). Ethanol extracts were prepared from finely chopped plant material, which was air-dried at room temperature for one week using microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). Microwave reactor Merck, MW 500 MAE was used for the experiments. Samples of flowers, leaves, and fruits (1.000±0.001 g) were mixed with 20 ml of 97 % ethanol. The duration of extraction was one minute at the temperature of 60 °C. Ultrasonic homogeniser Iskra (UZ 4R) was used for UAE. Extraction temperature was kept constant at 30 ± 1 °C using water bath. Fine powdered plant material
(2.000 ± 0.001) g was extracted with 20 ml of 97 % ethanol by ultrasound extraction for 20 min. Obtained extracts were centrifuged at 4000 rpm for 10 min and supernatant was collected. MAE and UAE extracts in triplicate were stored in glass vials at 4°C to be used for quantification of phytosterols and determination of antiproliferative activity.

2.3 HPLC analysis of phytosterols

The ethanol extracts were analysed using HPLC system (Agilent 1200 series) equipped with Photodiode-Array Detection (PDA), pump and autosampler. Chromatographic separation was achieved using Symmetry C18, Waters, 150 × 4.6 mm, 5 μm column. The conditions were based on the previously published method applied for the determination of phytosterols.28

Flow rate was 1 ml min⁻¹ with oven set at 25 °C, and injection volume of 20 μl. Mobile phase 15 % ethanol: 85 % acetonitrile (v/v) was filtered through a 0.45-μm hydrophilic polypropylene membrane filter prior to HPLC injection. The chromatograms were obtained at 198 nm and each analysis lasted 50 min. Identification and quantification of chromatographic peaks were confirmed by the comparison of retention time (tR) of components and phytosterol standards (β-sitosterol, campesterol, and stigmasterol). Calibration curves were constructed utilising five concentrations of three sterol standards in range of 1.7–130 μg ml⁻¹, and used for determination of phytosterols content in investigated extracts. All analyses were done in triplicate.

2.4 Cell lines

Sloe extracts were treated according to target cell lines, human prostate cancer cells PC-3 and DU145. The analysis was performed in referent laboratory at the Institute of Oncology and Radiology of Serbia in Belgrade. PC-3 and DU145 cancer cell lines were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C. Media were supplemented with 10 % heat-inactivated at 56 °C foetal bovine serum (FBS), 3 mmol l⁻¹ L-glutamine, 100 μg ml⁻¹ streptomycin, 100 IU ml⁻¹ penicillin, and 25 mmol l⁻¹ HEPES adjusted to pH 7.2 by bicarbonate solution. The cells were grown at 37 °C in an atmosphere of 5 % CO₂ and humidified air.

2.5 Treatment of cell lines

In vitro assay for the cytotoxic activity of investigated extracts was performed. Ethanol extracts, prepared by different extraction methods, were evaporated to a dry residue, and then a stock solution was made by dissolving dry residue of the extract in DMSO to concentration of 50 mg ml⁻¹. This stock solution (50 mg ml⁻¹) of every extract was dissolved in corresponding nutrient medium to obtain required working concentrations of 1000, 500, 250, 125, and 62.5 μg ml⁻¹. Target cells PC-3 (5000 cells per well) and DU145 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after the cell adhesion, 50 μl of five different concentrations of tested extracts were added to the wells, except for the control wells, where only nutrient medium was added. All investigated concentrations were set up in triplicate. Nutrient medium was used as a blank. The cultures were incubated for 72 h.

2.6 MTT cytotoxicity assay

The effect of the investigated extracts on the viability of the target cell lines (PC-3 and DU145) was determined by the microculture tetrazolium test (MTT) 72 h after addition of the compounds according to Mosmann29 with modification by Ohno and Abe.30 This assay is based on metabolic reduction of colourless tetrazolium salt by the mitochondrial enzyme activity in viable cells, to formazan salt (purple) which can be quantified spectrophotometrically. The cells were incubated for 72 h in a CO₂ incubator at 37 °C. After incubation, 20 ml of MTT solution (5 mg ml⁻¹ phosphate-buffered saline) was added to each well. Sample was incubated for a further 4 h at 37 °C in a humidified atmosphere of 95 % air/5 % CO₂ (v/v). Then, 100 μl of 100 g l⁻¹ sodium dodecyl sulphate was added to dissolve the insoluble product formazan resulting from conversion of the MTT dye by viable cells. The absorbance (A) was measured 24 h later at 570 nm. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader. Cell viability (%) was determined according to Eq. (1) and expressed via the IC₅₀ value. IC₅₀ is defined as the concentration of an agent that inhibits cell survival by 50 %, and was calculated compared to a vehicle-treated control. All experiments were done in triplicate.

\[
\text{cell viability (\%) = } \left( \frac{A_t - A_b}{A_v - A_b} \right) \times 100
\]

where \( A_v, A_b, \) and \( A_t \) represent absorbance of tested extracts, blank, and control, respectively.

3 Results and discussion

3.1 Content of phytosterols in sloe extracts determined by HPLC

The contents of three major phytosterols, namely, β-sitosterol, campesterol, and stigmasterol in analysed sloe extracts are summarised in Table 1. All three of phytosterols were detected separately. Great differences in the contents of investigated phytosterols were observed in various sloe extracts. The sum of determined phytosterols content was highest in MAE flower extract (21.296 μg ml⁻¹) from Tomvo, and the lowest in MAE fruit extract (0.908 μg ml⁻¹) from Vareš. β-sitosterol was the dominant sterol, and it was determined in all investigated extracts, while stigmasterol and campesterol were present at low concentrations in all samples. Campesterol was quantified in all flower and leaf extracts. High content of campesterol was determined in two flower extracts obtained by UAE (Borije 2.801 μg ml⁻¹ and Tomvo 2.856 μg ml⁻¹). Stigmasterol was not present in all analysed extracts. The best results for stigmasterol,
regardless of the method, were obtained for flower extracts in the range 0.223–4.201 µg ml\(^{-1}\). The lowest content of stigmasterol was found in MAE fruit extract from Trnovo (0.027 µg ml\(^{-1}\)). Finally, stigmasterol was only detected in one leaf extract obtained by UAE method from sloe sample collected from Borije. As expected from the previously published literature data, different distribution of phytosterols in different plant parts (flowers, leaves, fruits) has been proven. The influence on the content of tested phytosterols in the extracts was expected depending on the place of collection of plant material, as well as the method of sample preparation, which was not the case in present study.

The phytosterol standards were fully separated with high peak resolution. Chromatogram in coordinates Retention time-Line intensity/(minutes on x-axes)-(mAU on y-axes) is shown in Fig. 2. Campesterol appeared first at 15.43 min, followed by stigmasterol at 16.13, and β-sitosterol at 17.41 min. The same chromatographic conditions were applied for the quantitative determination of selected phytosterols in sloe extracts (flower, leaf, and fruit) compared to phytosterol standards.

### Table 1 – Content of phytosterols in flower, leaf, and fruit extracts of sloe

<table>
<thead>
<tr>
<th>Samples (method)</th>
<th>β-sitosterol /µg ml(^{-1})</th>
<th>Campesterol /µg ml(^{-1})</th>
<th>Stigmasterol /µg ml(^{-1})</th>
<th>Sum of determined phytosterols /µg ml(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flower extracts</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Borije (MAE)</td>
<td>15.121 ± 0.002</td>
<td>1.871 ± 0.001</td>
<td>4.201 ± 0.000*</td>
<td>21.193</td>
</tr>
<tr>
<td>Trnovo (MAE)</td>
<td>18.978 ± 0.001</td>
<td>2.095 ± 0.000*</td>
<td>0.223 ± 0.000*</td>
<td>21.296</td>
</tr>
<tr>
<td>Vareš (MAE)</td>
<td>7.450 ± 0.000*</td>
<td>1.944 ± 0.000*</td>
<td>0.646 ± 0.000*</td>
<td>10.040</td>
</tr>
<tr>
<td>Borije (UAE)</td>
<td>13.607 ± 0.000*</td>
<td>2.801 ± 0.000*</td>
<td>4.154 ± 0.000*</td>
<td>20.562</td>
</tr>
<tr>
<td>Trnovo (UAE)</td>
<td>11.283 ± 0.000*</td>
<td>2.856 ± 0.001*</td>
<td>ND</td>
<td>14.139</td>
</tr>
<tr>
<td>Vareš (UAE)</td>
<td>13.778 ± 0.001</td>
<td>2.145 ± 0.000*</td>
<td>0.563 ± 0.000*</td>
<td>16.486</td>
</tr>
<tr>
<td><strong>Leaf extracts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borije (MAE)</td>
<td>7.981 ± 0.000*</td>
<td>2.263 ± 0.000*</td>
<td>ND</td>
<td>10.244</td>
</tr>
<tr>
<td>Trnovo (MAE)</td>
<td>6.420 ± 0.000*</td>
<td>1.895 ± 0.000*</td>
<td>ND</td>
<td>8.315</td>
</tr>
<tr>
<td>Vareš (MAE)</td>
<td>6.281 ± 0.000*</td>
<td>2.482 ± 0.000*</td>
<td>ND</td>
<td>8.763</td>
</tr>
<tr>
<td>Borije (UAE)</td>
<td>6.173 ± 0.000*</td>
<td>1.912 ± 0.000*</td>
<td>0.694 ± 0.000*</td>
<td>8.779</td>
</tr>
<tr>
<td>Trnovo (UAE)</td>
<td>5.130 ± 0.000*</td>
<td>2.075 ± 0.000*</td>
<td>ND</td>
<td>7.205</td>
</tr>
<tr>
<td>Vareš (UAE)</td>
<td>9.268 ± 0.000*</td>
<td>2.692 ± 0.000*</td>
<td>ND</td>
<td>11.960</td>
</tr>
<tr>
<td><strong>Fruit extracts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borije (MAE)</td>
<td>1.392 ± 0.000*</td>
<td>1.860 ± 0.000*</td>
<td>ND</td>
<td>3.252</td>
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<tr>
<td>Trnovo (MAE)</td>
<td>1.627 ± 0.000*</td>
<td>ND</td>
<td>0.027 ± 0.000*</td>
<td>1.654</td>
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<tr>
<td>Vareš (MAE)</td>
<td>0.908 ± 0.000*</td>
<td>ND</td>
<td>ND</td>
<td>0.908</td>
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<tr>
<td>Borije (UAE)</td>
<td>3.557 ± 0.000*</td>
<td>2.472 ± 0.000*</td>
<td>0.745 ± 0.000*</td>
<td>6.774</td>
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<tr>
<td>Trnovo (UAE)</td>
<td>2.483 ± 0.000*</td>
<td>1.899 ± 0.000*</td>
<td>ND</td>
<td>4.382</td>
</tr>
<tr>
<td>Vareš (UAE)</td>
<td>2.079 ± 0.000*</td>
<td>1.888 ± 0.000*</td>
<td>ND</td>
<td>3.967</td>
</tr>
</tbody>
</table>

* – Standard deviation less than 0.001; ND – not detected

Edible fruits, especially berry fruits, are among the best sources of dietary compounds, and regular intake is associated with the prevention and delayed development of oxidative stress and chronic human disorders. Fruit can be
consumed fresh or processed into specialised products, including standardised extracts, which show the strongest biological effects due to the increased content of bioactive constituents. Ethnopharmacological sources suggest that consumption of sloe can control systemic and local inflammations, especially in the digestive tract, but also in the urinary tract and cardiovascular system.31

3.2 Investigation of in vitro antiproliferative activity

Experiments on the in vitro antiproliferative activity of sloe extracts were carried out against target malignant cells of prostate cancer, PC-3 and DU145. The final concentrations of the extracts applied to the target cells were: 1000, 500, 250, 125, and 62.5 µg ml⁻¹. The results of these experiments are shown in Table 2. All analyses were carried out in triplicate, and results are expressed as mean value ± standard deviation.

<table>
<thead>
<tr>
<th>Sample location (method)</th>
<th>IC₅₀/µg·ml⁻¹</th>
<th>PC-3</th>
<th>DU145</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flower extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borije (MAE)</td>
<td>766.86 ± 75.71</td>
<td>760.15 ± 92.94</td>
<td></td>
</tr>
<tr>
<td>Trnovo (MAE)</td>
<td>423.88 ± 33.76</td>
<td>377.12 ± 3.16</td>
<td></td>
</tr>
<tr>
<td>Vareš (MAE)</td>
<td>679.74 ± 9.73</td>
<td>924.34 ± 12.36</td>
<td></td>
</tr>
<tr>
<td>Borije (UAE)</td>
<td>930.00 ± 30.00</td>
<td>840.00 ± 50.00</td>
<td></td>
</tr>
<tr>
<td>Trnovo (UAE)</td>
<td>681.64 ± 11.55</td>
<td>438.32 ± 54.19</td>
<td></td>
</tr>
<tr>
<td>Vareš (UAE)</td>
<td>684.84 ± 4.78</td>
<td>464.88 ± 1.80</td>
<td></td>
</tr>
</tbody>
</table>

| Leaf extracts            |              |      |       |
| Borije (MAE)             | 220.00 ± 0.63  | 231.89 ± 15.18 |      |
| Trnovo (MAE)             | 394.50 ± 16.36 | 405.93 ± 55.73 |      |
| Vareš (MAE)              | 304.80 ± 23.48 | 346.17 ± 6.70  |      |
| Borije (UAE)             | 386.95 ± 36.11 | 216.91 ± 28.16 |      |
| Trnovo (UAE)             | 370.45 ± 10.54 | 444.58 ± 9.36  |      |
| Vareš (UAE)              | 409.46 ± 78.76 | 361.21 ± 12.87 |      |

| Fruit extracts           |              |      |       |
| Borije (MAE)             | 637.67 ± 6.57  | > 1000.00 |      |
| Trnovo (MAE)             | 870.99 ± 83.65 | 385.39 ± 30.39 |      |
| Vareš (MAE)              | 807.29 ± 39.46 | 953.52 ± 65.73 |      |
| Borije (UAE)             | 866.54 ± 44.51 | 681.62 ± 7.19  |      |
| Trnovo (UAE)             | 950.12 ± 70.55 | 948.68 ± 20.59 |      |
| Vareš (UAE)              | 860.06 ± 5.11  | 686.33 ± 7.14  |      |

The obtained results show that the tested extracts have dose-dependent antiproliferative activity against the in vitro tested malignant cells. Better cytotoxicity showed the sloe extracts obtained by MAE compared to UAE. The results that stand out the most are those of leaf extracts with IC₅₀ values ranging from 220.00 to 409.46 µg·ml⁻¹ for PC-3 malignant cells, and from 216.91 to 444.58 µg·ml⁻¹ for DU145 cells, depending on the location and type of extraction (Table 2). It is clear that leaf extracts have more significant and up to three times higher inhibitory activity compared to flower or fruit extracts. These results are in correlation with the traditional use of sloe and the beneficial effect of sloe leaf tea on the treatment of various diseases, including the prostate.32 In relation to the sensitivity of malignant cell lines to the cytotoxic effect of the extracts, it should be noted that DU145 cells were visibly more sensitive to the cytotoxicity of the extracts compared to the sensitivity of PC-3 cells. Recent studies of berry fruits, such as cherries or blueberries, have confirmed their exceptional effectiveness against malignant cancer cells and gastrointestinal inflammation.33,34

4 Conclusion

The search for new drugs to treat various cancers is one of the most important challenges of modern scientific research. In the presented study, total and individual content of phytosterols (β-sitosterol, campesterol and stigmasterol) in sloe flower, leaf, and fruit extracts obtained by MAE and UAE was estimated. In vitro antiproliferative activity evaluation suggested that even low amounts of β-sitosterol, campesterol, and stigmasterol present in extracts may be responsible for the inhibitory growth effect against investigated prostate cancer cell lines. All tested extracts contained β-sitosterol as well as stigmasterol and campesterol to a greater or lesser extent. In addition, it could be concluded that extraction methods, as well as origin of plant material from different areas in Bosnia and Herzegovina, had not significantly affected the results of the analysis. Comparative analysis of the inhibitory effect of individual parts of sloe provides insight into the overall antiproliferative activity and content of β-sitosterol and accompanying phytosterols in this plant species. Consequently, this research might fill a gap in understanding the effectiveness of phytosterols extracted from sloe in relieving benign prostatic hyperplasia. Furthermore, due to absence of research on phytosterols and their antiproliferative activity in sloe ethanol extracts, the paper has presented preliminary data that is particularly interesting given the proven medicinal properties of these plants, their use in phytotherapy, and the confirmed usage in traditional medicine. To the best of our knowledge, to date, published studies have not examined the presence of phytosterols in ethanol sloe extracts or their antiproliferative activity against prostate cancer cell (PC-3 and DU145), thus rendering this study useful in understanding this correlation.

ACKNOWLEDGEMENTS

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List of abbreviations and symbols

PC-3 – androgen-sensitive prostate cancer cells
Du145 – androgen-insensitive prostate cancer cells
GC – gas chromatography
HPLC – high performance liquid chromatography
PDA – photodiode-array detection
MAE – microwave-assisted extraction
UAE – ultrasound-assisted extraction
t_R – retention time
IC_{50} – half of the maximal inhibitory concentration

References

Literatura


SAŽETAK
HPLC analiza fitosterola u ekstraktima Prunus spinosa L. i njihovo antiproliferativno djelovanje na stanične linije raka prostate

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Ključne riječi
HPLC, fitosteroli, trnina, citotoksičnost, PC-3, DU145

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