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Comparative Fingerprint of Aromatic Herbs and Yeast Alcoholic Extracts used as Ingredients for Promen, a Prostate Preventive Nutraceutical

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Abstract. The aim of this study was to characterize and identify different bioactive compounds in plant sources and yeast powders to obtain an original nutraceutical (Promen) which has beneficial effects in prostate disease prevention.

Seven plant and fruit sources, namely nettle (*Urtica dioica*), green tea (*Camellia sinensis*), fluff with small flowers (*Epilobium parviplorum*), tomato (*Solanum licopersicum*), sea buckthorn (*Hippophae rhamnoides*), pumpkin (*Cucurbita maxima*), sunflower (*Helianthus annus*) and lyophilized beer yeast (*Saccharomyces cerevisiae*) were investigated. Methanolic extracts were prepared using 15% plant concentration and the purified fractions were analyzed using high throughput techniques like UV-VIS spectroscopy, high performance liquid chromatography coupled with photodiode array detection (HPLC-DAD) and mass spectrometry LC-QTOF -MS.

The majority of the investigated plants were rich in phenolic derivatives, polyphenols (flavonoid glucosides), while yeast was rich in aminoacids, peptides and vitamins B. The major compounds identified were: Juglone, Resveratrol, Quercetin, Epigallocatechin, Gallocatechin, Biochanin A, Isorhamnetin 3-O-glucoside 7-O-rhamnoside, Quercetin 3-O-galactoside 7-O-rhamnoside, Kaempferol 3,7-O-diglucoside and *p*-Coumaroylquinic acid.

The specific biomarkers were identified for both plant extracts used as ingredients to obtain an nutraceutical Promen.

Combined UV-Vis spectroscopy, HPLC-PDA chromatography and LC-MS spectrometry are recommended as accurate, sensible and reliable tools to investigate the plants and nutraceutical fingerprints and to predict the relation between ingredients composition and their health effects.

Keywords: aromatic plants, yeast, prostate protection, UV-VIS spectroscopy, LC-MS spectrometry**.**

INTRODUCTION

The management of prostate cancer and its increasing incidence need novel preventive approaches like chemoprevention, by the administration of synthetic compounds or, better, using natural formula (Adhami et *al.*, 2007).The prostate diseases generally are detected in men in their fifties or older. The prostate cancer evolution implies a considerable period of time, lifestyle changes or use of dietary or chemo preventive agents that might delay the development or onset of clinically detectable disease (Thompson *et al.*, 2013).

The natural dietary supplements based on herbs or derived from plants or other natural nutritional agents can be used for this purpose, the epidemiologic, clinical, or basic science revealed their efficiency for prostate cancer prevention (Marshall, 2012). Moreover, recent studies showed that medicinal plants have beneficial effects on health promotion, out of side effects, as compared with synthetic drugs (Katz, 2007).

Herbs are known for their antioxidant activity and immunomodulatory leading to anticarcinogenic effect. The main antioxidants with anticancer activity found in plants and vegetables include vitamins, carotenoids, flavonoids, polyphenols, enzymes, minerals, saponins, lignin (Behara and Dash, 2012). The main herbs and fruits known for the antoxidant and anticarcinogenic effects are nettle (*Urtica dioica*), green tea (*Camellia sinensis*) and fluff with small flowers (*Epilobium parviplorum*), tomato (*Solanum licopersicum*), sea buckthorn (*Hippophae rhamnoides*), pumpkin (*Cucurbita maxima*) sunflower (*Helianthus annus*) and lyophilized beer yeast (*Saccharomyces cerevisiae*).

The sea buckthorn berries have a good chemoprotective effect (Upendra *et al.*, 2008) on prostate diseases because of its high content in bioactive compounds like lycopene, vitamins , β-caroten and phenolic acids and flavonoids (Novruzov, 2005).

Nettle consumption reduces the risk of prostate disease due to its antimicrobial, anti-inflammatory and anti-tumor activity because it contains polyphenols, tannins, triterpenes and beta-sitosterol (Lowe and Patel, 2008). Green tea is known to have chemoprotective effects due to its high content in catechins and tannins, with antioxidant action against prostate cancer risk (Oliveira *et al.*, 2013). Tomatoes chemoprotective effect in prostate diseases is due to their high content in carotenoids (especially lycopene and β-carotene) and minerals like selenium (Behara, 2012; Kujawski, 2009).

Fluff with small flowers is active in preventing and treating prostate adenoma and cancer due to its high content in polyphenols (Awad and Fink, 2000). Pumpkin and sunflower seeds are important for prevention of prostate diseases because their high content of phytosterols and minerals (Hernández, 2012; Alfawaz, 2004).

Lyophilized beer yeast (*Saccharomyces cerevisiae)* has chemoprotective effects on prostate diseases due to its high content of bioactive compounds like vitamins, mineral and aminoacids (Blagovi *et al*,. 2001).

The UV-Vis spectroscopy is a simple, cheap and easy-to-use technique to identify and quantify the main phytochemicals, discriminating between the lipophilic and hydrophilic phytochemicals, in relation to the polarity of the extraction solvent (Zavoi *et al.*, 2011). Other advanced techniques for evaluation of an herbal product by its metabolomic fingerprinting can be HPLC with UV or diode-array detection (DAD) (Kammerer, 2004; Tang, 2008) as well with mass spectrometry detection.

The aim of this study was to investigate a number of medicinal plants with known beneficial effects, as ingredients for an original formula named PROMEN, a nutraceutical which can be recommended in the prevention and treatment of prostate cancer. The specific chemical biomarkers for individual plants and their possible recognition in the final product were identified by UV-Vis, HPLC-DAD and LC QTOF-MS analysis.

MATERIALS AND METHODS

Plant ingredients and PROMEN preparation

Seven types of medicinal plants and fruits from wild flora of different areas of Transylvania were numbered as follows: 1- nettle, 2- green tea, 3- fluff with small flowers, 4- tomato, 5- sea buckthorn ,6- pumpkin, 7- sunflower, 8- lyophilized beer yeast and 9- final product obtained Promen. The collected plants were dried, ground and stored in a cool place (10-15^{0}C) with low humidity. Promen product was achieved by mixing different proportions of the seven plants were dried and ground. The weight ration was: 0.5:0.5:0.5:2:0.5:1:1:4.

Extraction of bioactive compounds

Aliquots of 1.5 g of each plant powder (1-8) and Promen (9) were extracted in 8.5 ml solvent (methanol 96% in water, acidulated with 1% hydrochloric acid. After sonication for 30 min, centrifugation and filtration, the clear extracts were kept in deep freezer until analysis.

UV-Vis spectra

The UV-Vis spectra were recorded for each extract using a Jasco V 530 Spectrophotometer. There were identified the maxima wavelengths specific for phenolic acids (220-280 nm), flavonoids (330-360 nm) and/or quinones (398-420 nm).

Total polyphenols content

Total polyphenols content of methanolic extracts 1-9 was determined using Folin Ciocalteu method. 2.350 ml distilled water, 0.05 ml sample, 0.150 ml Folin-Ciocalteu reagent and 0.450 ml Na2CO3 were added to the pleasant work; For the 0.05 ml blank sample were replaced with 0.05 ml 40% ethanol; After 2 hours in the dark plates were read multidetector spectrometer working at Biotek.

Extraction efficiency

To compare the yields of extraction the Extraction Factors (EF) of phenolic acids (EF-FA), flavonoids (EF-F) and quinones (EF-Q) from each plant and Promen were calculated according to the formula: EF = A (λ_{max}) x D, where A ($_{\lambda_{max}}$) represents the absorption values recorded for each λ_{max} identified in the UV-Vis spectra and D represents the dilution factor. The λ_{max} values used for EF-FA, EF-F and EF-Q were 280, 330 and 410 nm, respectively.

HPLC-DAD AND LC–ESI(+)QTOF-MS analysis

All plant extracts and Promen extract were diluted (1:1) with methanol and aliquots of 5 µl of each sample were subjected to two types of chromatography, HPLC coupled with photodiode array detection (HPLC-DAD) and LC-ESI (+) QTOF-MS analysis, both using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system Dionex and MS detection by a Bruker Daltonics MaXis Impact device.

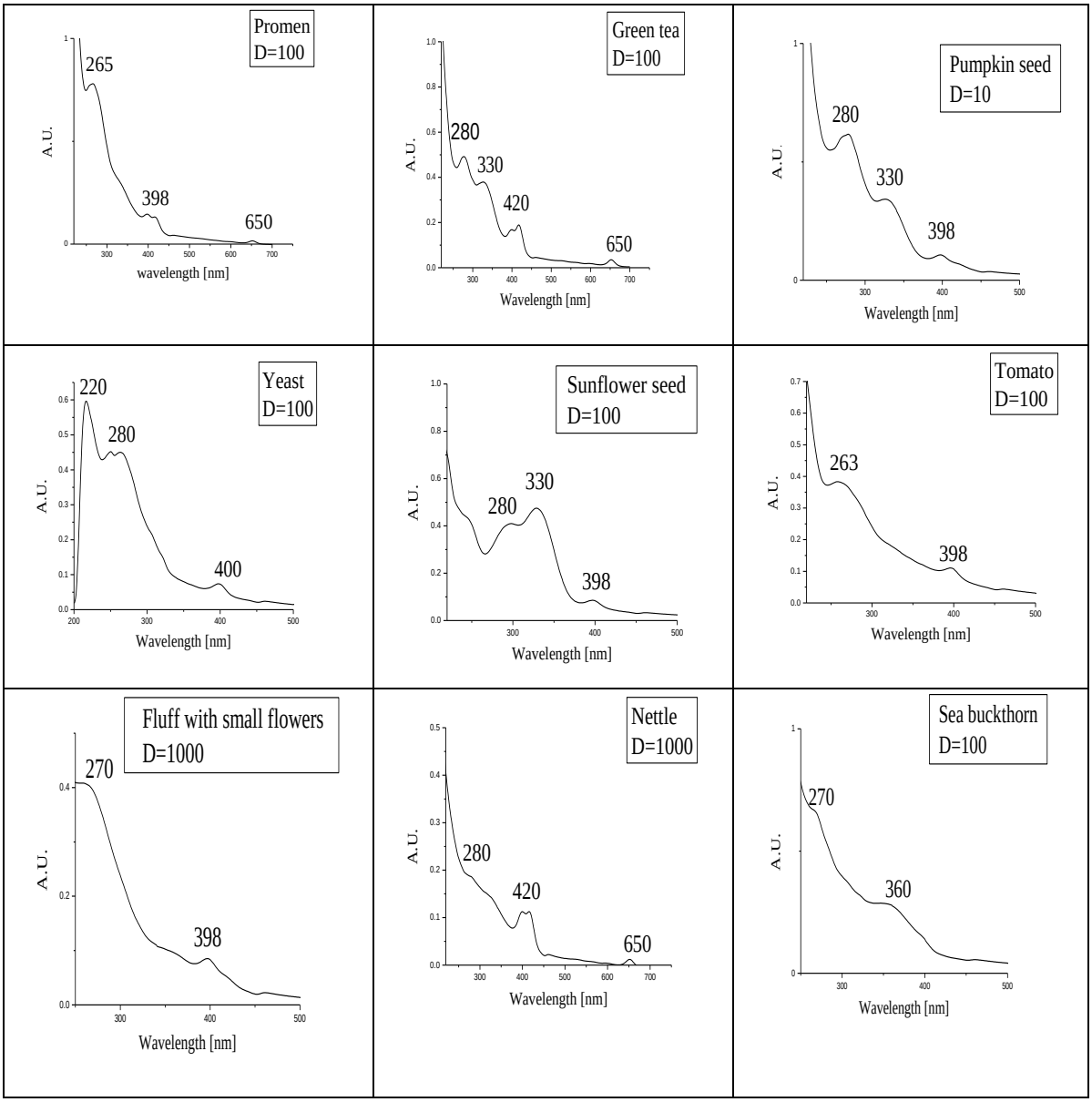
The plant metabolites were separated on the Thermo Scientific Acclaim C_{18} column (3µm, 2.1 X 50 mm) at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 $mL \cdot min^{-1}$. The gradient elution initial conditions were 1% B with linear gradient to 15% B from 0 to 3 min, followed by linear gradient to 50% B at 6 min, linear gradient to 95% B at 9 min, isocratic on 95% B for 6 min and then returned to initial conditions at 15 min and kept isocractic on 1%B for 5 min. The DAD detector was set at 270 nm. The separated molecules were introduced directly into the mass spectrometer by electrospray. The mass range was set between 50-1000m/z, using a nebulising gas pressure set at 2 bar, the drying gas flow at 8 L/min, the drying gas temp at 180 ºC. Before each separation run, a calibrant solution of sodium formate was injected. The control of the instrument and the data processing were done using TofControl 3.2 and Data Analysis 4.1 (Bruker Daltonics), respectively.

RESULTS AND DISCUSSIONS

1. Extracts fingerprint by UV-VIS analysis and extraction efficiency

Tab.1

Comparative UV-VIS fingerptints of methanolic sample extracts



The Tab. 1 shows the comparative UV spectral fingerprints (200-700 nm) of the nutraceutical product Promen and its plant ingredients.

Previously we made the spectra of all ingredients, the green tea, pumpkin seed, sunflower seed and yeast extracts showed three main peaks, at 280, 330 and 400 nm while the Promen, tomato, fluff with small flowers, nettle and sea buckthorn had only two absorption regions at 280 and 400 nm.

The evaluation of Extraction efficiency according to the formula presented above (see materials and methods) sowed that generally, the phenolic acids were extracted better

than flavonoids, being maximal for fluff with small flowers and approximately 10 times smaller, compared with nettle. The nonpolar components from pumpkin seeds, sunflower seed, sea buckthorn and tomatoes were not extracted in the hydrophilic solvent, explaining the low concentrations of FA, F and Q released (for abbreviations see materials and methods). Other explanation could be that the other plants used as ingredients with high content in polar compounds contribute to the extraction efficiency in limited amount due to their 5% contribution in the final product.

2. Total polyphenols concentration

The total polyphenol content identified in ingredient plants and Promen are shown in Tab. 1, being expressed in gallic acid equivalents per 100 ml extract.

Tab. 2.

The mean values of polyphenol content for each plant and Promen

Samples	Total polypheols [mg GAE/ 100 ml extract]
Promen	**76.611**
Yeast	8.843
Tomato	15.381
Pumpkin seed	11.342
Sunflower seed	39.386
Nettle	55.347
Fluff with small flowers	191.210
Green tea	57.846
Sea buckthorn	40.225

The fluff with small flowers had the highest polyphenol content being aproximatively 2.5 times higher than Promen while all the other extracts had lower phenolic concentrations.

3. HPLC-DAD AND LC–ESI(+)QTOF-MS analysis

Fig. 1A represents a generic HPLC-DAD chromatogram of Promen and Fig. 1B represents the TIC (Total Ion chromatograms) obtained for the same final product.

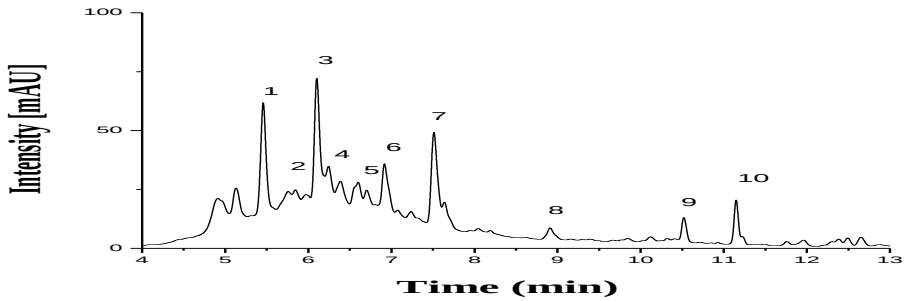


Fig. 1. A- HPLC-DAD chromatogram and compounds identified in Promen extract between 5.5-11 min.

Tab. 3.

Main compounds identifed in Promen extract between 5.5-11

Peak number	Retention time (min)	Peak number	Retention time (min)
1	5.5	6	6.9
2	5.8	7	7.5
3	6.1	8	8.9
4	6.2	9	10.5
5	6.6	10	11.1

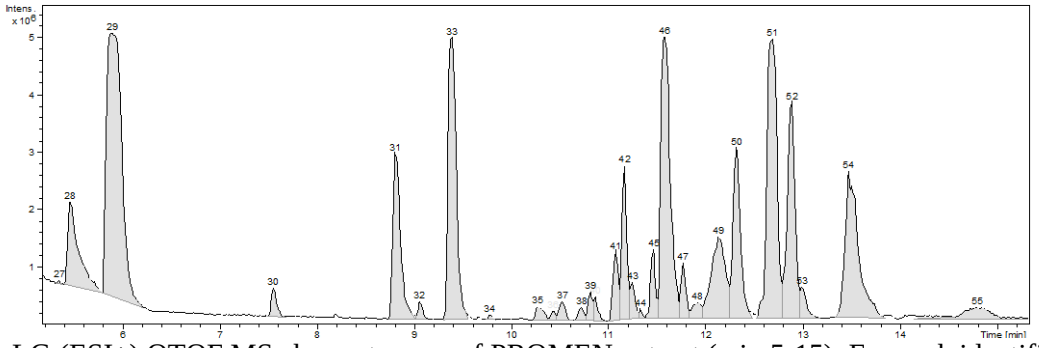


Fig. 1B. LC-(ESI+) QTOF MS chromatogram of PROMEN extract (min 5-15). For peak identification see Tab. 4

The tentative assignment of compound identification was based on their retention times, the released ions of protonated molecules $[M + H]^+$ and literature data (Yanga et *al.*, 2009; http://www.phenol-explorer.eu).

Tab. 4

Main compounds identifed in Promen extract between 5.5-14.8 min. and tentavive structure assignment based on $[M+H]^+$

Peak number	Retention time (min)	Tentative structure assignment	$[M + H]^+$ *m/z*	Plant ingredient
28	5.5	Ferulic acid	195.08	Yeast, tomato
29	5.9	Juglone	175.06	Sunflower seed
30	7.6	Isorhamnetin	319.13	Sea buckthorn
31	8.8	Resveratrol	230.24	Sea buckthorn, green tea
33	9.4	Quercetin	302.30	Green tea, fluff with small flowers
34	9.8	5,3',4'-Trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4'-O-glucuronide	520.34	Sunflower seed
38	10.7	Caffeoylquinic acid	353.26	Yeast, tomato, pumpkin seed, sunflower seed, nettle
39	10.8	Gallic acid 3-O-gallate	324.29	Nettle, pumpkin seed, tomato
41	11.1	Feruloylquinic acid	365.26	Pumpkin seed
42	11.2	Epigallocatechin, Gallocatechin	306.24	Green tea, fluff with small flowers, nettle
43	11.2	Caffeoyl aspartic acid	295.26	Sea buckthorn, pumpkin seed, sunflower seed
44	11.3	Feruloyl tartaric acid	326.30	Sea buckthorn
45	11.5	Pinocembrin	256.26	Tomato, yeast
46	11.6	Biochanin A	284.28	Pumpkin seed
47	11.8	Apigenin 7-O-diglucuronide	621.27	Fluff with small flowers, nettle
50	12.3	Isorhamnetin 3-O-glucoside 7-O-rhamnoside	623.29	nettle, green tea
51	12.7	Quercetin 3-O-galactoside 7-O-rhamnoside	607.29	Nettle, green tea, fluff with small flowers
52	12.9	Kaempferol 3,7-O-diglucoside	607.29	Nettle, green tea, fluff with small flowers
53	13	Resveratrol 5-O-glucoside	391.28	Tomato, sea buckthorn
54	13.5	p-Coumaroylquinic acid	338.34	All plants
55	14.8	Luteolin 7-O-(2-apiosyl-6-malonyl)-glucoside	664.43	Yeast, sunflower seed

The majority of the investigated plants were rich in phenolic derivatives, polyphenols (flavonoid glucosides), while yeast was rich in aminoacids, peptides and vitamins B. The major compounds identified were: Juglone, Resveratrol, Quercetin, Epigallocatechin, Gallocatechin, Biochanin A, Isorhamnetin 3-O-glucoside 7-O-rhamnoside, Quercetin 3-O-galactoside 7-O-rhamnoside, Kaempferol 3,7-O-diglucoside and *p*-Coumaroylquinic acid.

CONCLUSION

The phenolic acids were extracted better than flavonoids, being maximal for fluff with small flowers. The nonpolar components from samples were not extracted in the hydrophilic solvent, explaining the low concentrations of FA, F and Q released.

The fluff with small flowers had the highest polyphenol content being aproximatively 2.5 times higher than Promen while all the other extracts had lower phenolic concentrations.

Using LC-QTOF-MS analysis 21 specific compounds were identified in the final product Promen and in the different plant extracts used as ingredients.

The main biomarkers which differentiate the individual plants were identified and will be further used to evaluate the quality of nutraceutical product and their synergistic effect against prostate metabolic dysfunction.

Combined UV-Vis and HPLC-PDA and LC-MS chromatography can be recommended as accurate, sensible and reliable tools to investigate the plants and nutraceuticals' fingerprints and to predict the relation between the ingredients' composition and effects.

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