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Composition and antimicrobial activity of the essential oil of the leaves of black currant (*Ribes nigrum* L.) cultivar Čačanska crna

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Abstract: The essential oil from the leaves of the Serbian black currant cultivar Čačanska crna, obtained by hydrodistillation, was analyzed by gas chromatography-flame ionization detection and GC–mass spectrometry. The most abundant volatile compounds were Δ^3-carene (18.7 %), β-caryophyllene (17.7 %), sabinene (11.6 %), *cis*-β-ocimene (10.6 %) and α-terpinolene (10.6 %). The antimicrobial activity of the oil was evaluated against 14 micro-organisms, including two clinical isolated strains, using the broth microdilution method. The most susceptible micro-organisms were *Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, Candida albicans* and *Trichophyton mentagrophytes* isolates. Furthermore, the flavonol aglycones in the leaves after acid hydrolysis were qualitatively and quantitatively analysed by HPLC, and quercetin was found to be the dominant compound (84 mg/g dw).

Keywords: black currant leaves; Čačanska crna; essential oil; antimicrobial activity.

INTRODUCTION

Black currant, *Ribes nigrum* L. (Grossulariaceae), is a woody shrub spontaneously growing in central and eastern Europe, while in temperate regions it is mostly cultivated.[1] In the flora of Serbia, only four species of the genus *Ribes* are native, namely *Ribes grossularia* L., *R. alpinum* L., *R. petraeum* Wulfen and *R. multiflorum* Kit. *R. nigrum* is one of a few species from this genus that has been introduced in the country. Black currant cultivar Čačanska crna from Serbia was obtained in 1980 by open pollination of the cultivar Malling Jet, and was selected in 1984. It has now started to be grown experimentally in an area certified for organic production.

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The most important product of black currant is berries, due to their high levels of anthocyanins, flavonols, ellagitannins, and vitamin C. The leaves and buds are also important raw materials for the food and cosmetics industries.[2–4] Black urrant leaves are used in European folk medicine to treat rheumatism, arthritis and respiratory problems.[1] The chemical constituents of leaves are flavonoids, especially derivatives of kaempferol and quercetin, myricetin and isorhamnetin glycosides, and proanthocyanidins. The essential oil of black currant leaves was studied previously and differences in the monoterpene hydrocarbon profile between different cultivars were reported.[5,6]

As the black currant cultivar Čačanska crna has not hitherto been investigated, the aim of the present study was to analyze the composition of the essential leaf oil by the gas chromatography–mass spectrometry (GC/MS) method and to define its chemotype according to the percentages of the main constituents. Furthermore, the antimicrobial activity of the oil was assayed using the broth microdilution method. In order to obtain a more detailed chemical profile of this cultivar, the flavonol aglycones in the leaves after acid hydrolysis were analyzed qualitatively and quantitatively using high performance liquid chromatography (HPLC).

EXPERIMENTAL

Plant material

Leaves of black currant cultivar Čačanska crna were collected in June 2008 from the experimental field certified for organic production on the mountain Kopaonik (locality Lukovska Banja, 1000 m). The leaves were air-dried for five days and used for chemical analyses.

Essential oil isolation

The volatile oil of the *Ribes nigrum* leaves was obtained by hydrodistillation for four hours of 100 g of air-dried sample using a Clevenger-type apparatus.[7] The yield (%) of the oils was calculated based on the moisture-free mass. The oil was subjected to qualitative and quantitative analysis by GC and the antimicrobial activity of the oil was tested.

GC and GC/MS analysis

The analysis was realized by gas chromatography with flame ionization detection (GC/FID) and mass spectrometric detection (GC/MS). In the first instance, a model HP-5890 Series II gas chromatograph equipped with a split-splitless injector, an HP-5 capillary column (25 m× ×0.32 mm, film thickness 0.52 μm) and a flame ionization detector (FID) was employed. Hydrogen was used as the carrier gas (1 mL/min). The injector was heated at 250 °C, the detector at 300 °C, while the column temperature was linearly programmed from 40–260 °C (4 °/min). The GC/MS analysis was performed under almost the same analytical conditions, using an HP G 1800C Series II GCD analytical system, equipped with an HP-5MS column (30 m×0.25 mm×0.25 μm). Helium was used as carrier gas (0.9 mL/min). The transfer line (MSD) was heated at 260 °C. The EI mass spectra (70 eV) were acquired in the scan mode in the *m*/*z* range 40–400. In each case, 1 μL of sample solution in ethanol (20 μL/2 mL) was injected in the split mode (1:30). Identification of constituents was realized by matching their mass spectra and retention indices with those obtained from authentic samples and/or NIST/ /Wiley spectra libraries, using different types of search (PBM/NIST/AMDIS) and available li-

terature data.[8,9] The percentage compositions were obtained from electronic integration measurements using flame ionization detection.

Determination of antimicrobial activity

Microbial strains. The antimicrobial activity was tested against Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Enterobacter cloacae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *P. tolaasii* (NCTC 387), *Proteus mirabilis* (ATCC 14273), Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Streptococcus faecalis* (ATCC 12952), *Bacillus subtilis* (ATCC 6051), *Micrococcus luteus* (ATCC 10240), *M. flavus* (ATCC 14452) and *Listeria monocytogenes* (ATCC 15313), the human pathogen yeast *Candida albicans*, as well as clinical isolated dermatomycetes *Trichophyton mentagrophytes* and *Epidermophyton floccosum*. The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research, Belgrade, Serbia.

Broth microdilution method. In vitro antimicrobial studies were performed according to the broth microdilution method. For antimicrobial testing, the oil was diluted 1:1 in DMSO. For each experiment, a control disk with pure solvent was used as the blind control. The minimum inhibitory concentrations (*MIC*) values of the essential oil were determined using the broth microdilution method in 96-hole plates.[10] Serial dilutions of stock solutions of the oil in broth medium (Muller–Hinton broth for bacteria and Sabouraud broth for yeast) were prepared in a microtiter plate (96 wells). The microbial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/ml. The microplates were incubated at 48 °C for 24 h. The *MIC* values were determined as the lowest concentration of the oil that visibly inhibited the growth of each organism in the microwells. The standard antibiotic streptomycin (1 mg/mL DMSO) was used to control the sensitivity of the tested bacteria, whereas nystatin (1 mg/mL DMSO) was used as the control against the yeasts.

The mycelial growth test with malt agar (MA) was used to investigate the antifungal activity of the essential oil. The essential oil was added into MA and poured into Petri dishes. The microplates were incubated for 72 h at 28 °C. Commercial fungicides, miconazole for *T. mentagrophytes* and bifonazole for *E. floccosum*, were used as positive controls. Each assay was repeated three times, independently.

Determination of total phenolics and tannin contents

The total phenolics were estimated by the Folin–Ciocalteu method with slight modifications.[11] Two hundred microliters of extract (20 mg/10 mL MeOH) were added to 1 mL of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 μl of sodium carbonate (75 g/L) were added. After 2 h incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0–100 mg/L) was used for the plotting of a standard curve. The results are expressed as milligrams of gallic acid equivalents per gram of dry weight of plant extract (mg GAE/g dw). The measurements were realized in triplicate and the mean values were calculated.

The tannin content in the extract was determined quantitatively by its adsorption on standard hide powder.[12] This method is an indirect determination. The tannin content is equivalent to the difference between the total polyphenol content and the polyphenol content that remained after the tannins had been adsorbed by the hide powder.

HPLC analysis of flavonoid aglycones

Three flavonol aglycones (quercetin, myricetin and kaempferol) were analyzed after extraction and acid hydrolysis of the flavonol glycosides. Leaf samples (2 g) were extracted with methanol (20 mL) and 5 % sulphuric acid (20 mL) on a boiling water bath for 2 h. The extract

was filtered, neutralized with 10 % $NaHCO_3$ to pH 7 and evaporated under vacuum to 1/3 volume. After re-extraction with diethyl ether (3×50 mL), the ether extract was evaporated and dissolved in methanol (5 mL).

The HPLC analyses were performed using an Agilent series 1200 RR instrument with a DAD detector, on a reverse phase Lichrospher RP-18 analytical column, 250×4 mm i.d., particle size 5 μm (Agilent). Mobile phase A (H_2O containing 1 vol % 0.03 M H_3PO_4) and mobile phase B (MeCN); the elution was performed according to the following scheme: 90 % A 0–5 min, 90–80 % A 5–10 min, 80 % A 10–20 min, 80–40 % A 20–30 min, 40–0% A 30––35 min. Detection was performed at 260 and 340 nm. Standards quercetin, myricetin and kaempferol were obtained from Fluka, Germany. The amounts of the compounds were calculated using calibration curves. All experiments were repeated three times. The results are presented as milligrams per gram of dry weight.

RESULTS AND DISCUSSION

Chemical composition of essential oil

The light yellow oil was obtained in 0.12 % yield from the leaves of black currant cultivar Čačanska crna. The results of GC and GC/MS analysis are summarized in Table I. Of the 62 detected compounds, 59 were identified, representing 99.6 % of the total oil composition. According to literature data, black currant buds and leaves essential oils mainly consist of aliphatic and oxygenated mono- and sesquiterpenes.[3,5,6] Hydrocarbon terpenes were the most abundant components in the Čačanska crna cultivar oil (93 %), while oxygenated terpenes constituted 4.7 % to the total oil. A similar ratio of these two main fractions was reported previously in steam distillates of black currant buds.[13] The major constituents in the oil in the present study were Δ^3-carene (18.7 %), β-caryophyllene (17.7 %), sabinene (11.6 %), *cis*-β-ocimene (10.6 %) and α-terpinolene (10.6 %). Previous chemotaxonomic studies defined Δ^3-carene, sabinene and terpinolene as distinguishing components in black currant cultivars.[3,14] All of these hydrocarbon monoterpenes were also present in high amounts in Čačanska crna, as reported in Ben Lomond cultivar leaf oil.[5] In the other earlier studied cultivars, such as Ben Alder, Ben Connan, Ben Tirran and Wellington, the level of sabinene was considerably lower than the amounts of Δ^3-carene and terpinolene. Therefore, these cultivars might represent another chemotype, according to the main essential oil components.[5,6]

TABLE I. Constituents of black currant leaf essential oil from the cultivar Čačanska crna (KIE: RRI experimentally determined (calibrated AMDIS); KIL: RRI, literature values[8])

Constituents	KIE	KIL	Content, mass %
trans-2-Hexenal	859	846	0.10
p-Xylene	870	864	0.06
α-Thujene	920	924	0.20
α-Pinene	926	932	0.98
α-Fenchene	939	945	0.08
Camphene	940	946	0.12

TABLE I. Continued

Constituents	KIE	KIL	Content, mass %
Sabinene	969	969	11.63
1-Octen-3-ol	977	974	0.67
β-Myrcene	987	988	1.83
Δ^2-Carene	995	1001	0.21
α-Phellandrene	999	1002	0.24
Δ^3-Carene	1007	1008	18.67
α-Terpinene	1111	1014	0.51
p-Cymene	1017	1020	0.06
o-Cymene	1019	1022	0.26
β-Phellandrene	1023	1025	2.06
cis-*β*-Ocimene	1036	1032	10.64
trans-*β*-Ocimene	1046	1044	6.94
γ-Terpinene	1054	1054	0.34
cis-Sabinene hydrate	1064	1065	0.05
α-Terpinolene	1084	1086	10.58
Undecane	1095	1100	0.05
allo-Ocimene	1125	1128	0.09
trans,*trans*-2,6-dimethyl-1,3,5,7-Octatetraene	1131	1134	0.05
trans-epoxy-Ocimene	1142	1137	0.09
Terpinen-4-ol	1173	1174	0.17
α-Terpineol	1188	1186	0.05
Methyl salicylate	1190	1190	0.07
Nerol	1227	1227	0.07
Geraniol	1253	1249	0.46
Bornyl acetate	1281	1287	0.19
δ-Elemene	1332	1335	0.04
α-Terpenyl acetate	1346	1346	0.07
Citronellyl acetate	1351	1350	0.12
β-Bourbonene	1379	1387	0.05
β-Elemene	1387	1389	0.50
β-Caryophyllene	1418	1417	17.67
γ-Elemene	1429	1434	0.18
α-Humulene	1449	1452	2.43
Alloaromadendrene	1455	1458	0.07
γ-Muurolene	1472	1478	0.14
Germacrene D	1477	1484	4.28
β-Selinene	1481	1489	0.07
Bicyclogermacrene	1491	1500	0.80
α-Muurolene	1499	1500	0.42
trans,*trans*- α-Farnesene	1504	1505	0.19
δ-Cadinene	1518	1522	0.42
Hedycaryol	1545	1546	0.05
Germacrene B	1550	1559	0.62
Germacrene D-4-ol	1570	1574	0.92
Caryophyllene oxide	1577	1582	1.23
Humulene epoxide II	1603	1608	0.20

TABLE I. Continued

Constituents	KIE	KIL	Content, mass %
Helifolen-12-al	1623	1338	0.06
τ-Cadinol	1635	1338	0.31
α-Cadinol	1649	1652	0.41
14-Hydroxy-*cis*-caryophyllene	1666	1666	0.13
Eudesma-4(15),7-dien-1*β*-ol	1680	1687	0.12
Amorpha-4,9-dien-2-ol	1712	1725	0.04
Phytol	2107	2112	0.53
Total			99.62

The sesquiterpene fraction constituted 31.3 % of the oil of Čačanska crna leaves. Some sesquiterpenes could be considered as black currant flavour contributors.[13] Among them, germacrene B and bicyclogermacrene have not been previously detected in the leaves of black currant.

Antimicrobial activity

In this study, the antimicrobial activity of the oil was evaluated *in vitro* against Gram-positive and Gram-negative bacterial strains, human pathogen yeast *Candida albicans*, as well as two clinical isolated micromycetes, using the broth microdilution method. The results of antimicrobial activity are given in Table II. The black currant leaf oil showed antimicrobial activity with *MIC* values ranging from 1.0–27.0 μL/mL. The most sensitive was the *Trichophyton mentagrophytes* isolate (*MIC* = 1.0 μL/mL), followed by the bacteria *Escherichia coli*, *Streptococcus faecalis* and *Staphylococcus aureus* and the yeast *C. albicans* (*MIC* = 2.7 μL/mL). *Listeria monocytogenes* showed high resistance to the tested oil.

TABLE II. Minimal inhibitory concentrations (*MIC* / μL mL^{-1}) of black currant cultivar Čačanska crna leaf essential oil

Tested microorganisms	Leaf oil	Control[a]
Escherichia coli	2.7	5.2
Salmonella typhimurium	13.5	38
Streptococcus faecalis	2.7	27
Staphylococcus aureus	2.7	5.2
Pseudomonas aeruginosa	13.5	16
Pseudomonas tolaasii	16.2	27
Proteus mirabilis	13.5	5.2
Bacillus subtilis	5.4	5.2
Micrococcus luteus	13.5	16
Micrococcus flavus	16.2	5.2
Listeria monocytogenes	27.0	16
Candida albicans	2.7	5.2[b]
Trichophyton mentagrophytes	1.0	1.0[c]
Epidermophyton floccosum	3.0	3.0[d]

[a]Streptomycin; [b]nystatin; [c]miconazole; [d]bifonazole

As suggested in a previous investigation, the activity of the oils is related to the respective composition of the essential oils.[15] The obtained inhibitory effect of the essential oil in the present study might have been due to the activity of its main constituents against particular bacteria or yeasts. Previous studies indicated that Δ^3-carene showed high activities against *E. coli*, *Micrococcus luteus*, *S. aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, while β-caryophyllene exerted a strong activity against *P. aeruginosa* and a moderate activity against *E. coli*.[15,16] In addition, it was possible that components present in lower amounts in the oil might be involved in some type of synergism with the other active compounds.[17] The mechanism of the antimicrobial action of terpenes is not fully understood but it is speculated to involve membrane disruption by the lipophilic compounds.[15]

Analysis of the phenolic compounds

The amounts of total phenolics and tannins were 40.1±2.1 mg GAE/g dw and 2.1 %, respectively. The flavonoid compounds were analysed in a methanol extract of black currant cultivar Čačanska crna leaves after acid hydrolysis using HPLC (Fig. 1). Myricetin, quercetin and kaempferol were detected, of which quercetin was the most abundant (84±2.4 mg/g dw), followed by kaempferol (43.6±1.6 mg/g dw) and myricetin (9.5±0.4 mg/g dw). These flavonols were previously reported to be dominant in buds and leaves of other black currant cultivars, such as Goliath and Noir de Bourgogne, and the ratios between these aglycones were different in the various tested cultivars.[18] The significant amounts of flavonol agly-

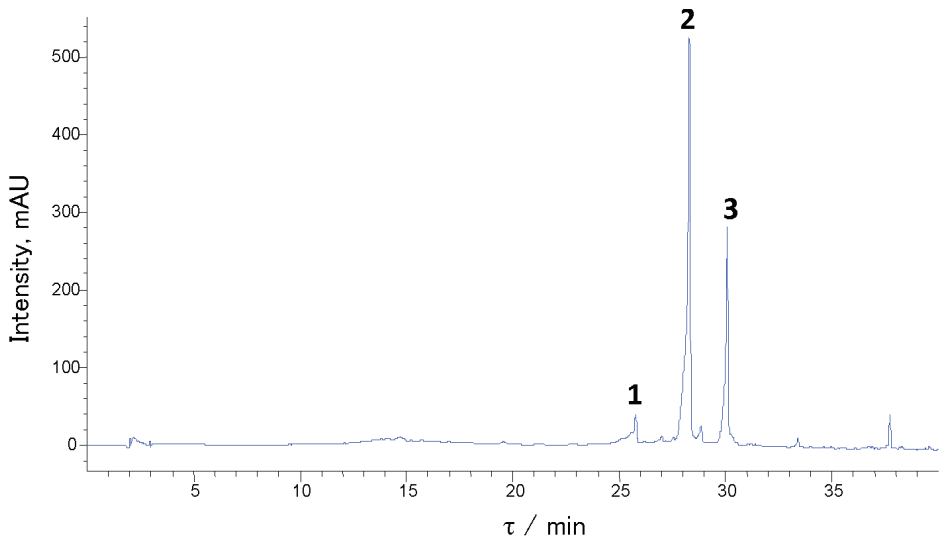

Fig. 1. HPLC chromatogram of the methanol extract of black currant cultivar Čačanska crna leaves; **1** – myricetin; **2** – quercetin; **3** – kaempferol.

cones, known to possess the anti-oxidative activity, confirms the usage of leaves of black currant in traditional medicine. Recent studies have indicated that the incidence of rheumatoid arthritis is partially related to damage of the anti-oxidative system.[19,20]

CONCLUSIONS

The essential leaf oil from black currant cultivar Čačanska crna contains a very complex mixture of terpene compounds, with Δ^3-carene, β-caryophyllene, sabinene, *cis*-β-ocimene and α-terpinolene as the major compounds. According to these results, this black currant cultivar might belong to the same chemotype as the Ben Lomond cultivar. Since the leaf oil showed a wide range of antimicrobial effect, its use in the treatment of various bacterial and fungal infections could be beneficial. These inhibitory effects are also interesting in relation to the prevention of contamination in many food products caused by micro-organisms such as *Staphylococcus* spp., *Salmonella* spp., *Bacillus* spp., *Pseudomonas fluorescens* and *Clostridium botulinum*.[17]

The leaves of the black currant cultivar Čačanska crna are also a rich source of natural antioxidants such as polyphenols. The intake of flavonoids and other antioxidant compounds from food is associated with reduced risk of coronary heart disease, stroke and cancer. In view of this, black currant leaves extracts could be of interest for further investigations.

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ИЗВОД

ХЕМИЈСКИ САСТАВ И АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКОГ УЉА ЛИСТА ЦРНЕ РИБИЗЛЕ (*Ribes nigrum* L.), СОРТА ЧАЧАНСКА ЦРНА

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Применом GC/MS методе анализирано је етарско уље изоловано хидродестилацијом из листа црне рибизле, сорта чачанска црна. Међу идентификованим компонентама, најзаступљеније су биле Δ^3-карен (18,7 %), β-кариофилен (17,7 %), сабинен (11,6 %), *cis*-β-оцимен (10,6 %) и α-терпинолен (10,6 %).Антимикробна активност уља је одређена микродилуционом методом на 14 сојева микроорганизама од којих су 2 клинички изолати. Најосетљивији микроорганизми били су *Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, Candida albicans,* и изолат *Trichophyton mentagrophytes.* Урађена је и квалитативна и квантитативна HPLC анализа хидролизованог екстракта листа којом је утврђено да је флавонол кверцетин доминантна компонента.

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REFERENCES

1. M. Wichtl, *Teedrogen*, Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1994, p. 421
2. M. Anttonen, R. Karjalainen, *J. Agric. Food Chem.* **54** (2006) 7530
3. A. Dvaranauskaite, P. Venskutonis, C. Raynaud, T. Talou, P. Viškelis, E. Dambrauskiene, *J. Agric. Food Chem.* **56** (2008) 3279
4. J. Piry, A. Pribela, J. Durčanska, P. Farkaš, *Food Chem*. **54** (1995) 73
5. R. Mariott, in *Flavors and Fragrances, a World Perspective*, B. M. Lawrence, B. D. Mookherjee, B. J. Willis, Eds., Elsevier, Amsterdam, 1988, p. 387
6. D. W. Griffiths, G. W. Robertson, A. N. E. Birch, R. M. Brennan, *Phytochem. Anal.* **10** (1999) 328
7. *Yugoslavian Pharmacopoeia Ph. Yug. IV*, National Institute for Health Protection, Belgrade, Serbia, 1984, p. 126
8. R. P. Adams, *Identification of Essential Oils Components by Gas Chromatography/Quadrupole Mass Spectroscopy*, 4th ed., Allured Publishing Corporation, Card Stream, IL, 2007
9. H. Detlev, *Mass Finder 4: GC/MS Visualisation, Interpretation, and Library Adminis-tration Mass Spectral Library: Terpenoids and Related Constituents of Essential Oils*, Hamburg, 2008–2009
10. National Committee for Clinical Laboratory Standards (NCCLS), *Methods for dilution antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, Approved Standard, 5th ed., NCCLS document, Wayne, PA, 2000, p. M7
11. P. G. Waterman, S. Mole, *Analysis of Phenolic Plant Metabolites*, Blackwell Scientific Publication, Oxford, 1994, p. 16
12. *European Pharmacopoeia 5.0*, Council of Europe, Strasbourg Cedex, 2005, 221
13. J. L. Le Quere, A. Latrasse, *J. Agric. Food Chem*. **38** (1990) 3
14. M. Kerslake, A. Latrasse, J. L. Le Quere, *J. Sci. Food Agric*. **47** (1989) 43
15. H. J. D. Dorman, S. G. Deans, *J. Appl. Microbiol.* **88** (2000) 308
16. L. Jirowetz, S. Bail, G. Buchbauer, Z. Denkova, A. Slavchev, A. Stoyanova, E. Schmidt, M. Geissler, *Sci. Pharm*. **74** (2006) 189
17. M. Marino, C. Bersani, G. Comi, *Int. J. Food Microbiol*. **67** (2001) 187
18. J. Tabart, C. Kevers, J. Pincemail, J. O. Defraigne, J. Dommes, *J Agric. Food Chem.* **54** (2006) 6271
19. A. Seven, S. Guezel, M. Aslan, V. Hamuryudan, *Clin. Biochem*. **41** (2008) 538
20. K. M. Surapneni, V. S. C. Gopan, *Ind. J. Clin. Biochem.* **23** (2008) 41.