**CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE**

**FACULTY OF TROPICAL AGRISCIENCES**

# Assessment of *in vitro* antioxidant and anti-proliferative properties of medicinal and edible plants

DOCTORAL THESIS

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***Certification***

I declare that given Ph.D. thesis is solely my own work unless otherwise referenced or acknowledged.

Prague on .....

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***Ing. Jan Tauchen***

He who fights with monsters should look to it that he himself does not become a monster. And when you gaze long into an abyss the abyss also gazes into you.

Friedrich Wilhelm Nietzsche

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***Summary***

Identification and characterization of plant-based products with antioxidant and anti-proliferative effects has received much interest over the past few years as possible therapeutic mean for treatment of diseases likely to be associated to oxidative stress (such as atherosclerosis, diabetes, and cancer). This study provides characterization of *in vitro* antioxidant and/or anti-proliferative potential and phytochemical profile of (i) 39 wine samples of underutilized Georgian grapevine cultivars, (ii) extracts of 22 samples of medicinal plants from Ethiopia and (iii) 23 samples of edible and medicinal plants from Peruvian Amazon. For this purpose, 2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH), oxygen radical absorbance capacity (ORAC) assay, total phenolic content (TPC), and cell viability assay based on metabolization of tetrazolium bromide (MTT) to formazan, together with methods based on high performance liquid chromatography (HPLC-ultra violet/visible spectrometry and HPLC-tandem mass spectrometry) were used. Georgian red wines (particularly Saperavi cultivars) exhibited higher antioxidant capacity (average DPPH and ORAC values at 5.1 and = 10.6 g TE/L wine, respectively) in comparison to Central and West European cultivars [Pinot Noir (DPPH = 3.1 and ORAC = 9.4 g TE/L wine), Cabernet Sauvignon (DPPH = 3.0 and ORAC = 7.3 g TE/L wine) and Cabernet Moravia (DPPH = 2.0 and ORAC = 8.5 g TE/L wine)]. Georgian wines contained significantly greater concentrations of quercetin (14.44 – 1.07 µg/mL), kaempferol (1.68 – 0.03 µg/mL) and syringic acid (12.59 – 4.72 µg/mL), whereas possessed lower quantities of resveratrol (5.11 – 0.32 µg/mL) in comparison to Central and West European wines. Amongst edible and medicinal plants from Ethiopia and Peruvian Amazon, only *Dodonaea angustifolia* ($IC_{50}$ for DPPH = 22.2 µg/mL, ORAC = 767.6 µg TE/mg extract; $IC_{50}$ for Hep-G2 = 120.0 µg/mL), *Rumex nepalensis* ($IC_{50}$ for DPPH = 5.7 µg/mL, ORAC = 1061.4 µg TE/mg extract; $IC_{50}$ for Hep-G2 = 50.5 µg/mL), *Inga edulis* (DPPH and ORAC = 337.0 and 795.7 µg TE/mg extract; $IC_{50}$ for Hep-G2 and HT-29 = 36.3 and 57.9 µg/mL) and *Oenocarpus bataua* (DPPH and ORAC = 903.8 and 1024.4 µg TE/mg extract; $IC_{50}$ for Hep-G2 and HT-29 = 102.6 and 38.8 µg/mL) have demonstrated combinatory antioxidant/anti-proliferative efficacy. Selective anti-proliferative activity was observed for *Verbascum sinaiticum* ($IC_{50}$ for Hep-G2 = 80.6 µg/mL) and *Annona montana* ($IC_{50}$ for Hep-G2 and HT-29 = 2.7 and 9.0 µg/mL, respectively). Above-mentioned plant material showed only weak or non-toxic effects towards normal cell line. Despite the fact that extracts of *Jasminum abyssinicum* ($IC_{50}$ for DPPH = 26.3 µg/mL, ORAC = 1023.7 µg TE/mg extract), *Rumex nepalensis* ($IC_{50}$ for DPPH = 5.7 µg/mL, ORAC = 1061.4 µg TE/mg extract), *Mauritia flexuosa* (DPPH and

ORAC = 1062.9 and 645.9 μg TE/mg extract), *Myrciaria dubia* (DPPH and ORAC = 641.9 and 642.6 μg TE/mg extract) and *Theobroma grandiflorum* (DPPH and ORAC = 714.8 and 821.9 μg TE/mg extract) have exhibited considerable antioxidant effect, these species were found to possess moderate to low anti-proliferative potential or have shown to be toxic to normal cells line. In all cases it was detected that phenolic compounds content correlated strongly with antioxidant activity, however weakly with anti-proliferative effect. Results suggest above-mentioned species as prospective materials for further development of novel plant-based agents effective against oxidative stress related diseases. However, it is necessary to perform further research which would be focused on detailed characterization of their chemical composition, pharmacological effects and toxicological safety, in order to verify their possible practical use.

***Keywords:*** antioxidant; anticarcinogenic; phenolic compounds; plant extracts; wine

***Shrnutí***

Identifikace a charakterizace rostlinných produktů s antioxidačními a anti-proliferačními účinky, jakožto potencionálního terapeutického prostředku pro léčbu nemocí spojených s oxidačním stresem (jako např. arterioskleróza, diabetes nebo rakovina), je neustále středem vědeckého zájmu. Tato studie se zaobírá charakterizací *in vitro* antioxidační a anti-proliferační aktivity a chemického složení 39 vzorků méně využívaných tradičních gruzínských odrůd vín, extraktů z 23 vzorků etiopských léčivých rostlin a 22 vzorků z jedlých a léčivých rostlin z peruánské Amazonie. Pro stanovení biologických účinků byly použity metody založené na měření inhibice radikálu 2,2-difenyl-1-pikrylhydrazyl (DPPH), kapacity absorpce kyslíkových radikálu (ORAC), celkového obsahu fenolických látek (TPC), a životaschopnosti buněčných linií pomocí metody založené na metabolické přeměně tetrazolium bromidu (MTT) na formazan. Chemické složení bylo stanoveno metodami založenými na vysokotlaké kapalinové chromatografii (HPLC-ultrafialovo-viditelné spektroskopii a HPLC-tandemové hmotnostní spektrometrii). V rámci experimentů s gruzínskými víny bylo prokázáno, že vína červená (zejména odrůdy Saperavi) vykázala silnější antioxidační účinek (průměrné DPPH a ORAC hondoty na 5.1 a 10.6 g TE/L vína) ve srovnání s běžnými středo- a západo-evropskými víny [Pinot Noir (DPPH = 3.1 a ORAC = 9.4 g TE/L vína), Cabernet Sauvignon (DPPH = 3.0 a ORAC = 7.3 g TE/L vína) a Cabernet Moravia (DPPH = 2.0 a ORAC = 8.5 g TE/L vína)]. Gruzínská vína byla oproti středo- a západo-evropským vínům výrazně bohatší na kvercetin (14.44 – 1.07 μg/mL), kaempferol (1.68 – 0.03 μg/mL) a syringovou kyselinu (12.59 – 4.72 μg/mL), avšak chudší na resveratrolu (5.11 – 0.32 μg/mL). Mezi jedlými a léčivými rostlinami z Etiopie a Peru jedině extrakty *Dodonaea angustifolia* ($IC_{50}$ pro DPPH = 22.2 μg/mL, ORAC = 767.6 μg TE/mg extrakt; $IC_{50}$ pro Hep-G2 = 120.0 μg/mL); *Rumex nepalensis* ($IC_{50}$ pro DPPH = 5.7 μg/mL, ORAC = 1061.4 μg TE/mg extrakt; $IC_{50}$ pro Hep-G2 = 50.5 μg/mL); *Inga edulis* (DPPH a ORAC = 337.0 a 795.7 μg TE/mg extrakt; $IC_{50}$ pro Hep-G2 and HT-29 = 36.3 a 57.9 μg/mL) a *Oenocarpus bataua* (DPPH a ORAC = 903.8 a 1024.4 μg TE/mg extrakt; $IC_{50}$ pro Hep-G2 a HT-29 = 102.6 a 38.8 μg/mL) projevily kombinovaný antioxidační/anti-proliferační účinek. Selektivní anti-proliferační aktivitu prokázaly pouze extrakty *Verbascum sinaiticum* ($IC_{50}$ pro Hep-G2 = 80.6 μg/mL) a *Annona montana* ($IC_{50}$ pro Hep-G2 a HT-29 = 2.7 a 9.0 μg/mL). Výše zmíněný rostlinný materiál vykázal pouze slabou nebo žádnou toxicitu vůči zdravé buněčné linii. Přestože extrakty z *Jasminum abyssinicum* ($IC_{50}$ pro DPPH = 26.3 μg/mL, ORAC = 1023.7 μg TE/mg extrakt), *Rumex nepalensis* ($IC_{50}$ pro DPPH = 5.7 μg/mL, ORAC = 1061.4 μg TE/mg extrakt), *Mauritia flexuosa* (DPPH a

ORAC = 1062.9 a 645.9 μg TE/mg extrakt), *Myrciaria dubia* (DPPH a ORAC = 641.9 a 642.6 μg TE/mg extrakt) a *Theobroma grandiflorum* (DPPH a ORAC = 714.8 a 821.9 μg TE/mg extrakt) projevily významný antioxidační účinek, tyto druhy taktéž prokázaly mírnou až velice nízkou anti-proliferační aktivitu nebo byly toxické pro zdravou buněčnou linii. Ve všech dílčích studiích obsah fenolických látek silně koreloval s antioxidační aktivitou, avšak slabě s anti-proliferačním efektem. Výsledky naznačují, že výše zmíněné druhy by mohly být perspektivní pro vývoj nových rostlinných přípravků, efektivních proti onemocněním souvisejícím s oxidačním stresem. Před jejich možným uvedením do praktického využití však bude nezbytný další výzkum zaměřený na podrobnější stanovení jejich chemického složení, farmakologických účinků a toxikologické bezpečnosti.

***Klíčová slova:*** antioxidant; cytostatikum; fenolické látky; rostlinné extrakty; víno

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## *List of Abbreviations*

| | |
|---|---|
| 5-FU | 5-fluorouracil |
| 8OHdG | 8-hydroxy-2'-deoxyguanosine |
| AAPH | 2,2′-Azobis(2-methylpropionamidine) dihydrochloride |
| Aβ | β-amyloid peptides |
| AGEs | Advanced glycation end-products |
| AIDS | Acquired immunodeficiency syndrome |
| ALS | Amyotrophic lateral sclerosis |
| ARDS | Acute respiratory distress syndrome |
| ATP | Adenosine triphosphate |
| BDNF | Brain-derived neurotrophic factor |
| BHA | Butylated hydroxyanisole |
| BHT | Butylated hydroxytoluene |
| CdK | Cyclin-dependent protein kinases |
| CLL | Chronic lymphotic leukaemia |
| CNS | Central nervous system |
| DFO | Desferrioxamine |
| DNA | Deoxyribonucleic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| FA | Friedreich's ataxia |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| GSH | Glutathione |
| HBED | *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid |
| HD | Huntington's disease |
| HDL | High-density lipoproteins |
| HIV | Human immunodeficiency virus |
| HO | Haem oxygenase |
| HOCl | Hydrochlorous acid |
| HPLC | High performance liquid chromatography |
| L1 | 1,2-dimethyl-3-hydroxypyridin-4-one |
| LAP | Lipoic acid plus |
| LDL | Low-density lipoproteins |
| MAO | Monoamine oxidase |
| MOG | Myelin oligodendrocyte glycoprotein |
| MPP+ | 1-methyl-4-phenylpyridinium |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |

| | |
|---|---|
| MS | Mass spectrometry |
| MTX | Methotrexate |
| NADH | Reduced nikotinamidadenindinukleotid |
| NADPH | Nikotinamidadenindinukleotid phosphate |
| NF-$\kappa$B | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| iNOS | Inducible nitrite oxide synthase |
| NOX | NADPH oxidase |
| ORAC | Oxygen radical absorbance capacity |
| $ONOO^-$ | Peroxynitrite radical |
| OTC | L-2-oxathiazolidine-4-carboxylate |
| PBN | Phenyl-*tert*-butylnitronine |
| PIH | Pyridoxal isonicotinoyl hydrazone |
| RCS | Reactive chlorine species |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| SOR | Superoxide reductase |
| STAZN | Stilbazulenyl nitrone |
| TBHQ | *Tert*-butylhydroquinone |
| TNF-α | Tumour necrosis factor alpha |
| TPC | Total phenolic content |
| TPP | triphenylphosphonium |
| VLDL | Very low-density lipoproteins |
| U.S. FDA | Food and drug administration of the United States of America |
| UV/Vis | Ultra-violet/visible light |

*List of original publications*

**I.** **Tauchen J**, Maršík P, Kvasnicová M, Maghradze D, Kokoška L, Vaněk T, Landa P. In vitro antioxidant activity and phenolic composition of Georgian, Central and West European wines. Journal of Food Composition and Analysis. 2015;41: 113-121.

**II.** **Tauchen J**, Bortl L, Huml L, Mikšátková P, Doskočil I, Maršík P, Villegas PPP, Flores YB, Van Damme P, Lojka B, Havlík J, Lapčík O, Kokoška L. Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon. Manuscript accepted (23. 3. 2016) to Revista Brasileira de Farmacognosia.

**III.** **Tauchen J**, Doskočil I, Caffi C, Lulekal E, Maršík P, Havlík J, Van Damme P, Kokoška L. In vitro antioxidant and anti-proliferative activity of Ethiopian medicinal plant extracts. Industrial Crops and Products. 2015;74: 671–679.

These publications are referenced in the text in form of Roman numerals (bold).

Publications are listed in Appendices A-C.

# 1 Introduction

It is widely believed that free radicals and oxidative stress are responsible for primary onset and secondary pathology in various diseases including atherosclerosis, rheumatoid arthritis, cancer, cystic fibrosis, diabetes, cardiovascular, inflammatory and neurodegenerative (e.g. Alzheimer's disease) disorders. Hence, identification and characterization of products which exhibit antioxidant and/or anti-proliferative activity and might also possess possible therapeutic benefit in above-mentioned diseases has received much interest over the past years [1].

Nowadays, antioxidant activity is primarily examined in common dietary plants such as fruits and vegetables. However, recent studies indicate that other categories of plant material, such as medicinal plants, also possess significant antioxidant efficacy [2]. Health beneficiary effect of plants in oxidative stress-related diseases are mainly attributed to certain types of phenolic compounds, however other plant-derived constituents possess this biological activity as well [3].

It has previously been proposed that progression of cancer might be strongly related to oxidative stress. For this reason, validation of antioxidant effect of tested plant material is nowadays routinely supplemented with analysis of anti-proliferative activity against various types of carcinoma cell lines [4]. Variety of plant-based constituents (such as paclitaxel, podophyllotoxin, and vincristine) has demonstrated significant anti-carcinomatous effect and are used in oncological praxis. However, majority of these also possess adverse toxicity to normal cells [5]. Recent studies have shown that several dietary phenolic compounds (e.g. flavonoids) exerted anti-proliferative activity as well [6]. Despite the fact that medicinal plants are regarded as the main sources of antineoplastic agents, there is now an increased interest in research of edible plants' anti-proliferative effects [7].

Even though plants are generally considered as very important factor for food and health security (mainly in third world countries), their health beneficial properties are not fully verified by modern scientific methods (including antioxidant and anticancer activity). Moreover, with contemporary trend in destruction and ongoing loss of natural habitats by human activities and genetic narrowing of several culturally used crops (e.g. grapevine), there is serious risk of plant diversity being irreversibly lost with interconnected valuable scientific information [8–10]. Despite the well-documented traditional use in treatment of diseases related to the oxidative stress, to my best knowledge, many plants worldwide remains to be examined for antioxidant and/or anti-proliferative efficacy [11–13]. Additionally, phytochemical

profile of compounds likely to be responsible for biological activities of majority of these plants was not properly established [14].

Proceeding from these facts, this dissertation thesis provides detailed information on *in vitro* antioxidant and/or anti-proliferative potential of underutilized plant material with special focus on grapevines traditionally used in Georgia, extracts of medicinal plants from Ethiopia and extracts of edible and medicinal plants from Peruvian Amazon. Additionally total phenolic content and other phytochemical analyses were performed [i.e. high-performance liquid chromatography (HPLC) hyphenated with ultra-violet/visible light (UV/Vis) or tandem mass spectrometry (MS/MS) detector] with the aim to determine the relationship between biological activity and contained compounds.

# 2 Theoretical background

## 2.1 Free radicals and antioxidants

Term free radical is applied to any atom or molecule which has one or more unpaired valence electron or has open electron shell. This characteristic makes free radicals highly chemically reactive to other organic compounds, often resulting in chain reaction. Free radicals are also called reactive species and are further divided due the prevalent presence of oxygen (reactive oxygen species – ROS), nitrogen (reactive nitrogen species – RNS) or other atoms (such as carbon, chloride, sulphur) [1,15]. Amongst biologically important free radicals belongs for example hydroxyl radical, carbonate radical, superoxide radical, peroxyl and alkoxyl radicals, nitric oxide, peroxynitrite ($ONOO^-$), hydrogen peroxide, sulphur radicals, hydrochlorous acid (HOCl) etc. [16–20,1]. Singlet oxygen and ozone are also sometimes recognized as free radicals [21,22]. Metals, such as copper, zinc, vanadium, chromium, molybdenum and others are also defined as oxidizing agents. Free radicals and other oxidizing agents are mainly involved in electron transfer reactions referred as oxidation processes [23–26].

It is well known, that oxidation reactions play key role in many biologically important processes, including oxidative phosphorylation (in mitochondria), cell signalling, phagocytosis, formation of enzymes and other important molecules with biological activity (e.g. leukotrienes, prostaglandins, pyruvate, thyroid hormones). This fact is supported by presence of various oxidizing enzymes in biological systems (e.g. cyclooxygenase, lipoxygenase, peroxidase, NADPH oxidase and xanthine oxidase) catalysing free radicals production and realization of above-mentioned biological activities [1,15]. However, free radicals are also involved in degradation of several biologically important molecules, for example lipids, proteins, DNA, and other biomolecules, resulting in development of serious diseases (such as cancer, diabetes, myocardial infarction, stroke and other disorders) [23,27–29]. On the basis of facts noted above, free radicals are important for many vital reactions in biological systems. However, due to their adverse damaging activity, free radical formation should be kept in balance. This function is ascribed to chemical structures called antioxidants [1].

Antioxidants are both low- and high-molecular weight compounds, which are capable of complete inhibition or partial retardation of oxidation processes by one (or more) mechanisms. This includes chelation of metal ions and free radical scavenging ability. In former mechanism of action, antioxidant structures are able to quench metal ions to its own skeleton

and therefore, metal ions are unable to produce radical species. In latter mechanism, antioxidants serve as electron or hydrogen donors to free radicals in order to stabilize them [3]. Although antioxidants loose one or more electrons or hydrogen, by particular chemical nature, they continue to be stable and do not become free radicals themselves [15]. It is important to say that biological systems are capable to produce endogenous antioxidant agents. Such antioxidants are further recognized as either low-molecular [e.g. ascorbic acid (vitamin C), α-keto acids, bilirubin, coenzyme Q, glutathione, lipoic acid, melanins, melatonin, vitamin E, trehalose, and uric acid] [30–38] or high-molecular: [e.g. catalase, histidine-containing dipeptides, glutathione peroxidase and other peroxidases, peroxiredoxins and other sulphur based antioxidants, protein-disulphide isomerase, various superoxide dismutases (SOD), superoxide reductases (SOR), thioredoxin and others]. Nevertheless some low-molecular-mass antioxidant agents could be also acquired in diet, especially plant-based [39–44,1].

## 2.2 Oxidative stress and human health

It is widely believed that free radicals and their reactive species are involved in human diseases [45]. Oxidative stress have been implicated in over 150 health disorders [46] and some specific diseases are primarily caused by oxidative stress [1]. However, in most cases, oxidative stress is rather consequence raised by particular conditions of the disease (secondary progress). Various studies have reported that administration of large doses of antioxidants have demonstrated little or no preventative or therapeutic effect in human diseases related to oxidative stress. Hence, many authors have raised doubts about effectivity of antioxidants in clinical practice [47]. With aim to summarize critically present data on medicinal potential of antioxidants, only diseases where these compounds have demonstrated positive or limited therapeutic benefit are discussed in this section. Relationship between oxidative damage and human diseases (even those not included in this section), as well as suggested therapeutic benefit of antioxidants is given in Table 1.

### 2.2.1 Atherosclerosis

Atherosclerosis is a disease characterized by local thickening of the vessel wall which is a result of invasion and accumulation of fatty streak-derived plague. Increased incidence of plagues in arteries causes limited blood flow to the region of specific organs such as heart or brain. This secondary condition of atherosclerosis develops into diseases known as heart attack (myocardial infarction) and stroke (localized cerebral ischemia) [48]. Specific inflammation, hypertension and other factors are widely recognized as important initiators of atherosclerosis [49]. However, it is believed that impairment of oxidized low-density lipoproteins

(LDL) into vessel wall is a key contribution factor in progress of this disease. LDL are oxidized by endogenously produced factors including metals ions, reactive oxygen species (ROS), reactive nitrogen species (RNS; e.g. $ONOO^-$), reactive chlorine species (RCS; e.g. HOCl), lipoxygenases and others [50,51]. Distraction of atherosclerosis development is thus seen in possible avoidance of the LDL peroxidation by antioxidants.

### 2.2.2 Diabetes mellitus

Diabetes is characterized by elevated blood and urinary glucose levels caused either via defective production of insulin by pancreatic islet cells (type 1) or by faulty response (resistance) of target cells to insulin (type 2). Former type is most often seen in adults as a result of obesity, lack of exercise and poor diet, whereas latter type is appearing in child age and it is believed to be triggered by genetic predisposition [1]. However, there are indications that oxidative stress can contribute to development of diabetes (reactive species-dependent destruction of islet β-cells) [52]. Complication of diabetes is further associated with tissue damage caused by extend exposure to glucose together with plasma lipids such as LDL, very low-density lipoproteins (VLDL) and lipoprotein (a) [53]. Increased levels of glucose further leads to disruption of mitochondrial function via overloaded generation of reduced nikotinamidadenindinukleotid (NADH) [54], and degradation of proteins via reaction with $-NH_2$ groups and formation of advanced glycation end-products (AGEs). Both of these chemical reactions contributes significantly to increased production of ROS, and therefore to oxidative stress in this disease [1].

### 2.2.3 Cystic fibrosis

Disease is caused by protein abnormality which is encoded by gene on chromosome 7 (autosomal recessive genetic disorder) [55]. Damaged protein couples ATP hydrolysis with $Cl^-$ transport across epithelial surfaces. This consequence further results in uneven distribution of water and electrolytes to various tissue systems such as respiratory tract, but also organs such as liver, bile ducts, pancreas, gastrointestinal tract and skin [1]. Oxidative damage can contribute (both positively and negatively) to injury linked with this disease, however significance is still uncertain [56]. Additionally, it was observed that patients suffering from cystic fibrosis have lowered plasma levels of β-carotene (~ 0.04 μM; normal levels should be around 0.2 μM), lutein, zeaxanthin, lycopene, α-carotene and others [1].

### 2.2.4 Inflammatory bowel disease

Reactive species are believed to be involved in tissue injury of Crohn's disease and ulcerative colitis. Former is a recurrent inflammation and ulceration of whole digestive tract, while latter affects colon and rectum only. In both conditions, autoantibodies to bowel components can be found in plasma [1]. Tissue injury in this disease involves extensive recruitment and activation of neutrophils, monocytes, lymphocytes, production of cytokines (e.g. TNF-α), eicosanoids, proteinases and of course reactive species (such as HOCl and $ONOO^-$) [57]. Several studies point that oxidative stress can contribute to development of this disease on mouse models together with progress of cancer (induced by reactive species–mediated DNA damage) [58,59]. However, other studies produce contradictory results, even indicating beneficiary effect of reactive species (e.g. depression of phagocyte recruitment) [57].

### 2.2.5 Cancer

Tumour may be defined as abnormal mass of tissue with exceeded rate of growth and uncoordinated communication with other cells presented in organism. Tumours are classified according to rate of growth (benign or malignant). Benign forms remain at the site of origin, which later usually become enclosed in layer of fibrous material formed by surrounding tissue. As contrast, malignant forms are very fatal; they are able to travel from their primary site trough bloodstream and lymphatic system to form secondary tumours (metastases). Pathogenicity (e.g. rates of growth and metastasis) is related to type of tumour – i.e. skin cancer rarely metastases, whereas melanoma frequently does. Malignant tumours usually lose their resemblance to their tissue of origin [1,60].

The complex process of conversion of a normal cell into the malignant state (cancerogenesis) is chiefly initiated by DNA alteration. Modified DNA is successfully replicated and further cell proliferation allows mutation to be incorporated into DNA of future cells (amitosis). Additional changes and proliferation of DNA (accumulation of defect DNA) leads to progress of cancer [61]. Carcinogenesis is triggered by genetic predisposition as well as by environmental factors, including gene defects (e.g. oncoviruses, faulty tumour-suppressor genes activity etc.) [62,63]. It was previously suggested by battery of studies that oxidative stress and reactive species are intimately involved in carcinogenesis [64]. It was also proposed that malignant tumours alone are capable of ROS production and that this might contribute to additional development of secondary tumours [65]. Despite the existence reports on reactive species impact on cancer development and ongoing pathology, it was previously demonstrated that reactive species in small quantities showed beneficiary effect [66,67].

Finally, treatment of cancer also covers administration of chemotherapeutics which are used as agents suppressing growth of malignant cells (generally) via interference with cell division. Although chemotherapeutics are designed to provide as little damage as possible to normal cells, they possess considerable side effects, among others, also provided by oxidative stress (e.g. doxorubicin [68], bleomycin [69]). Moreover, pathogenicity of tumours is even stressed out by fact that certain types of cancer cell lines possess resistance (intrinsic or acquired) to commonly used anticancer agents (e.g. methotrexate-resistant breast cancer lines) [70–72].

### 2.2.6 Exposure to ionizing radiation

Modern management of various types of cancer also includes treatment with radiation therapy (ionizing radiation) which possess serious negative impact on human health. Ionizing radiation [γ-and X-rays, high-energy electrons (β-particles) and neutrons, and $He^{2+}$ particles] is characterized as radiation at specific wavelength, which carries enough energy capable of liberating electrons from molecules and transmits them into state of ions. Significant part of the initial damage done to cells by ionizing radiation is due to increased occurrence of hydroxyl radical. DNA is particularly important target, suffering double- and single-stranded breaks, deoxyribose damage and base modification [1]. Double-strand breaks are especially important damaging agents because their repair can be error prone [73]. Subjects which have experienced even relatively small dosage of ionizing radiation are more prone to sterility, specific forms of cancer and cataract [74,1]. In patients undergoing radiotherapy, DNA damage is evidenced by previous reports of increased levels of 8-hydroxy-2'-deoxyguanosine (8OHdG; oxidation product of DNA) in urine [75]. Other mechanism involve increased availability of transition metal ions in plasma as a result of mitochondrial damage caused by radiation [76]. Exposure to ionizing radiation is also secondarily accompanied by significant inflammatory response (e.g. elevated cytokine production induced by reactive species) [77,78].

### 2.2.7 Stroke/CNS injury

Traumatic injury to the brain or spinal cord involves both direct tissue damage and secondary damage involving many of the events relevant to stroke [79]. Blood vessels which normally support the brain are ruptured and brain suffers from hypoxia. Bloodleak and oedema raise intracranial pressure and further decrease blood flow. Vasospasm that can occur several days after intracranial bleeding seems to involve oxidative damage (production of hydroxyl radical and increased rate of lipid peroxidation) [80,81]. Moreover, during the first 5 days after intracranial bleeding, erythrocytes in cerebrospinal fluid slowly haemolyse, majori-

ty of haemoglobin is further converted to bilirubin by haem oxygenase (HO). However part undergoes non-enzymatic oxidation and degradation [82]. Upon degradation, a potential pro-oxidant agent is released: iron. Moreover, soon after trauma, inflammatory/immune responses begin, involving production of cytokines, activation of transcription factors (e.g. NF-$\kappa$B) and microglia and phagocyte recruitment [1].

### 2.2.8 Parkinson's disease

Disease is characterized by rhythmic tremor in hand or foot and as the disease develops, patients have increasing problems in movement control; movement is slow (bradykinesia), tremor becomes more prominent and initiation of movement is difficult (akinesia). Disease is also accompanied by muscle rigidity. Particularly neurons in locus coeruleus and substancia nigra are affected in Parkinson's disease. Former brain region is responsible for production of noradrenalin; in developed stage of Parkinson's disease levels of this compound are significantly lowered [83,84]. Latter section is rich in granules containing black pigment neuromelanin [85]. Like other melanins, neuromelanin is redox-active and is able to chelate metal ions. In addition, Parkinson's disease is connected with appearance of Lewy bodies in residual neurons of substancia nigra and several other brain regions. These filamentous electron-dense inclusion bodies are composed of abnormal aggregated proteins, namely α-synuclein, ubiquitin and neurofilaments. All of these proteins are prone to oxidation or nitration [86].

Almost all Parkinson's disease cases are sporadic (appearing without obvious genetic cause). However rare inherited variants were previously identified which also appeared in juvenile (before age of 20) and early onset (before age of 40-50) patients [87,88]. To date it is unclear which factors trigger sporadic form of Parkinson's disease; however it is assumed, that toxins play important role. Compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [89], 2,5-hexanedione [90], deguelin, rotenone (both found in *Derris elliptica*) [91] and annonacin (presented in *Annona* species) [92,93] were reported to be significant inhibitors of mitochondrial complex I, resulting in mitochondrial defect and ongoing oxidative stress. Oxidized metabolites of dopamine by monoamine oxidase (MAO), have potential neurotoxic effects *in vivo* too [94].

### 2.2.9 Alzheimer's disease

In this disease, several brain regions involved in learning and memory, such as basal forebrain and hippocampus, suffer synaptic damage and eventual neuronal loss. This issue results in impairment of cognitive functions, including memory, ability to deal with daily life,

performance of sensimotor and social functions, language communication and control of emotional reactions. It is widely accepted, that genetics play key role in progression of Alzheimer's disease [95]. However, other factors also influence development of this disease, including poor diet (low dietary intake of folic acid), high level of plasma cholesterol (i.e. atherosclerosis) or homocysteine, low intellectual activity and repeated minor brain traumas [96].

Alzheimer's disease is characterized by presence of so called senile plague – i.e. extracellular localized areas of degenerating and frequently swollen axons, neuritis and glia surrounding a core of aggregated β-amyloid peptides (Aβ). Senile plagues are to some extent found in normally aging brain. However in Alzheimer's disease brain, levels of soluble and precipitable Aβ are at least 5 and 100 times higher in comparison to controls, respectively [97]. Aβ were accused to be principal cause of secondary neurodegeneration in Alzheimer's disease [98]. It was found out by several studies that Aβ peptides are able to produce reactive species. Resulting oxidative stress causes mitochondrial dysfunction that leads to neurotoxicity and damage to neuronal synapses [99–102]. High levels of 8OHdG and other DNA base oxidation products (both nuclear and mitochondrial) in Alzheimer's disease patients [103] point towards significant contribution of oxidative stress to this disease pathology (apparently stronger than for any other neurodegenerative disease) [1].

### 2.2.10 Amyotrophic lateral sclerosis

Disease is characterized by degeneration of motor neurons in the motor cortex, brainstem and spinal cord. Mean age of onset of amyotrophic lateral sclerosis (ALS) is 57 years; it begins as painless muscle weakness and impaired muscle tone, leading to atrophy. Problems with speech and swallowing follow. Disease is chronic and progression is rapid, often leading to death within few years of its appearance, usually of pneumonia or respiratory failure secondary to muscle paralysis [86,98,104]. Incidence of ALS cases is of 90% sporadic and of 10% inherited. It was demonstrated, that increased oxidative stress occurs in ALS (increased levels of DNA oxidation products etc.), however its importance to disease pathology is unclear [105].

### 2.2.11 Other neurodegenerative diseases

Friedreich's ataxia (FA) is the most common type of hereditary ataxia (impaired movement due to loss of motor co-ordination). Onset is usually after age of 15. Disease primarily affects neurons with long axons, which die back from periphery. FA is caused by mutations in gene on chromosome 9 that encodes protein frataxin involved in mitochondrial iron

metabolism. Low frataxin levels delay production of Fe/S clusters in aconitase and in complexes I, II and III. It has also been speculated (but not proven) that low frataxin levels are indirectly involved in free radical production reactions. Apart from brain, heart is also rich in frataxin and premature death of FA patients often involves cardiac problems [1].

Huntington's disease (HD) is inherited disease characterized by psychiatric disorders, dementia and itchings, writhings and other involuntary movements. It is caused by selective degeneration of striatal neurons, accompanied by astrocytosis, leading to atrophy of caudate nucleus and putamen. Disease does not usually appear before age of 30 and can last for 20 years or more with progressively worsening symptoms [106,107]. HD is caused by defect in gene on chromosome 4 encoding protein huntingtin. Its function in human body is unclear, although huntingtin was estimated to be involved in transport of brain-derived neurotrophic factor (BDNF) along microtubules [108]. Degenerated huntingtin possess neurotoxic properties by higher tendency to aggregate and form inclusions bodies containing ubiquitin and proteasome and impairing proteasomase activity [109].

**Table 1** Importance of oxidative stress in human diseases (adopted from **[1]**)

| **Condition** | **Is oxidative stress primary cause?** | **If oxidative stress is secondary does it contribute significantly to disease pathology?** | **Is there evidence that antioxidants have or will have therapeutic benefit?** |
|---|---|---|---|
| Atherosclerosis | In specific conditions[a] | Uncertain | Limited |
| Hypertension | In specific conditions | In specific conditions | Limited |
| Diabetes | Possibly | Yes | Limited |
| Rheumatoid arthritis | No[b] | Uncertain[c] | No |
| Autoimmune diseases | Probably no[b] | Uncertain[c] | Limited |
| Inflammatory bowel disease | Probably no[b] | Uncertain[c] | Limited |
| Acute respiratory distress syndrome (ARDS) | Generally no[d] | Possibly | No |
| Cystic fibrosis | No | Possibly | Limited |
| Cancer | In specific cases[e] | Probably yes | Limited[f] |
| Radiation-induced damage | Yes | Yes[g] | Yes |
| Stroke/Traumatic CNS injury | No | In specific conditions | Limited |
| Parkinson's disease | Possibly | Probably yes | Limited |

**Table 1** (*continued*)

| Condition | Is oxidative stress primary cause? | If oxidative stress is secondary does it contribute significantly to disease pathology? | Is there evidence that antioxidants have or will have therapeutic benefit? |
|---|---|---|---|
| Alzheimer's disease | Probably no | Probably yes | Limited |
| Amyotrophic lateral sclerosis (ALS) | No | Uncertain | Limited |
| Neuronal ceroid lipofuscinoses | No | N/A[h] | N/A[h] |
| Multiple sclerosis | No | Possibly | No[i] |
| Drug side-effects | In few cases | In few cases | Limited |
| Huntington's disease | No | Possibly | Limited |
| Friedreich's ataxia | No | Probably yes | Limited |
| HIV infection/AIDS | No | Possibly | No |

[a] reactive species can damage vascular endothelium to start the process; [b] however, oxidatively-modified proteins/DNA can be antigenic; [c] phagocyte ROS production may be of protective character; [d] but may contribute to phagocyte recruitment; [e] chronic inflammation-related, may contribute to mechanism of carcinogen action; [f] protective against chemotherapeutics side effects; [g] secondary production of reactive species via inflammation; [h] no data are available; [i] several studies declare dual beneficial/harmful effects of plant-based antioxidants

## 2.3 Medicinal and pharmaceutical preparations

### 2.3.1 Drugs with antioxidant action

Antioxidants have evoked great interest as possible therapeutic agents. This may involve usage of naturally occurring antioxidants (with or without structural modifications) or completely synthetic molecules [110]. Health beneficiary effect of certain drugs already clinically used (which were not primarily designed as antioxidants) may be partially or completely explained by their antioxidant mechanism [111]. Examples can be seen in aminosalycilates in treatment of inflammatory bowel disease [112] or various antibiotics (e.g. neuroprotective effect of some tetracyclines in stroke, ALS or Huntington's disease) [113]. Primarily, any antioxidant proposed for therapeutic use must be non-toxic. However, other important questions should be addressed as well – (i) what biomolecule(s) is the antioxidant designed to protect, (ii) what is the mechanism of action of proposed antioxidant, (iii) degree of damage of antioxidant-derived radicals and (iv) possibility of interference of antioxidants with beneficial effect of reactive species. For reason to declare that therapeutic benefit of antioxidant molecule in particular disease is clearly related to antioxidant activity, clinical trials of antioxidant drug are nowadays accompanied by measurements of oxidative damage biomarkers [1]. Further section provides summary of therapeutic antioxidants which have undergone clinical trials and demonstrated therapeutic benefit in oxidative stress-related diseases.

#### *2.3.1.1 Superoxide dismutases and catalases*

Antioxidant enzymes available for therapeutic use include recombinant human SODs (CuZnSOD, MnSOD, and EC-SOD) and their conjugates [114]. Studies done on animal models demonstrated reduction of ischaemia-reperfusion injury upon intravenous application of PEG-SOD and PEG-catalase [115]. Another study confirmed anti-cancerogenous effect of galactosylated or PEG-modified catalase by ability to decrease metastasis in mice [116]. However, in other clinical trials, antioxidant enzymes were found ineffective in treatment of several ailments such as chronic anti-inflammatory or autoimmune diseases [1].

#### *2.3.1.2 SOD/catalase mimetics*

SOD/catalase mimetics are low molecular weight compounds mostly containing transition metal ions. Research is nowadays mainly focused on iron or manganese porphyrins such as FeTMPyP, AEOL 10150 and other manganese complexes, e.g. EUK-8, EUK-134 or M40403. Unlike, enzymatic antioxidants, SOD-mimetics are more prone to undergo redox reactions. On the other hand, these compounds have advantage of easier penetration into host

cells in comparison to SOD enzymes [117]. SOD-mimetics have shown protection ability against oxidative damage in wide range of cell and animal model systems, even though they possess negative side effects as well [118]. Despite the existence of beneficial effects of SOD-mimetics in animal models, these compounds are not used in clinical praxis yet [1].

#### *2.3.1.3 Spin traps/nitroxides*

Idea of using spin traps as therapeutic antioxidants arisen from studies showing that α-phenyl-*tert*-butylnitronine (PBN) protects rats against death from shock induced by gut-ischaemia reperfusion or endotoxin injection [119]. Nevertheless, *in vitro* antioxidant characterization demonstrated PBN to be poor chain-braking antioxidant and inhibitor of lipid peroxidation [120]. Therefore, there is suggestion that observed therapeutic beneficial is not associated to antioxidant efficacy, but rather to another biological activity. Several derivatives of PBN have been developed as potential therapeutic agents, such as CPI-142 or NXY-059. Latter derivative, also known as Cerovive®, demonstrated significant improvements in primate stroke models and is nowadays under clinical trials in humans [1], Unlike previously mentioned compounds, LPBNAH [121], stilbazulenyl nitrone (STAZN) [122], OXANO and TEMPO [123] exerted significant antioxidant potential *in vitro*. Another examples of related compounds with considerable antioxidant efficacy would be hydroxylamines (e.g. *N*-*tert*-butyl hydroxylamine, derivative formed during spontaneous decomposition of PBN) or hydroxamates (e.g. *N*-methylhexanoylhydroxamate) [124,125]. Above-mentioned nitroxides have radioprotective effects and can decrease tissue damage in some animal model system of shock, ischaemia-reperfusion and inflammation [126].

#### *2.3.1.4 Ascorbic acid, α-tocopherol and their derivatives*

α-tocopherol (vitamin E) have been found to possess therapeutic benefits in premature babies or other patients suffering from α-tocopherol deficiency [127] or inborn lack of glutathione synthetase [or glucose-6-phosphate dehydrogenase (G6PDH)] [128]. However, attempts to use α-tocopherol in treatment other diseases such as diabetes, anthracycline-induced cardiotoxicity, cancer, Huntington's and Parkinson's disease, were found disappointing, although limited benefit was detected in Alzheimer's disease [129]. Low efficacy is explained by fact that oxidative damage has faster rate than distribution of α-tocopherol into tissue systems. Second reason is that oxidative damage frequently occurs by mechanisms other than lipid peroxidation, where α-tocopherol seems to be ineffective [1].

Several structural analogues of α-tocopherol, some with improved antioxidant activity have been described. However, for most of these compounds, few human studies have been

reported. One of α-tocopherol derivatives is BO-653 which demonstrated anti-atherosclerotic effects in animals [130]. Troglitazone, an antidiabetic agent with antioxidant properties is being used on patients, although relatively serious side effects have been recorded [131]. Raxofelast have been reported to improve endothelial dysfunction in diabetes. α-tocopherol succinate has been claimed to possess anticancer effect [132].

Various esters of ascorbic acid, for example ascorbyl plamitate and 2-octadecylascorbate, have been synthetized as lipophilic versions of ascorbate. These have been used as food preservatives and tested as antioxidants in some animal models, but had not attracted much interest as for therapeutic use. EPC-K1 is a combined phosphate ester of vitamin E and C that has been reported to exert protective effect in rat stroke model [133].

#### *2.3.1.5 Other chain breaking antioxidants*

Probucol have demonstrated cardioprotective [134] and anti-atherosclerotic effects *in vivo* as well as significant antioxidant properties *in vitro* [135]. Succinate ester of probucol, AGI-1067 is in clinical trials as anti-atherosclerotic agent and other derivatives are being considered for use in treating transplant rejection and rheumatoid arthritis [136]. Probucol has some side effects though: it tends to decrease plasma high-density lipoprotein (HDL) levels. However, its analogue AGI-1067 seems to lack this negative characteristic [137].

Coenzyme Q has anti-atherosclerotic effects as well. Moreover, it was discovered that this compound also possess mild benefit in human neurodegenerative diseases [138]. Modified coenzyme Q, idebenone has also been used in treatment of various neurodegenerative diseases [139]. By contrast, phenolic antioxidant OPC-14117, although showing limited benefit in animal models with neurodegenerative disease, did not help patients with Huntington's disease [110]. Compound BN82451 increased survival in transgenic mouse model of this disease, but unfortunately, it appears that so far it was not tested in human clinical trials [140].

Chain-breaking antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used in the food industry to prevent rancidity. To present-day, these compounds have not been explored as possible therapeutic agents, also partly due to supposed toxicity [3]. Propofol, active ingredient of the anaesthetic diprivan, resembles BHT and it is thought that propofol has antioxidant activities *in vivo* during anaesthesia [141].

Therapeutic potential of plant-based phenolic compounds is also under investigation; much work has been done on mixtures of *G. biloba* extract (beneficiary effects are mainly attributed to contained flavonoids myricetin and quercetin) [98]. Another thoroughly studied formula is daflon, which is micronized and purified flavonoid mixture of diosmin (90%) and

hesperidin (10%). Daflon increases venous tone and resistance in small blood vessels, for which is recommended in treatment of chronic venous insufficiency, venous ulcers and haemorrhoidal diseases [142]. Other flavonoids and their analogues are being assayed as anti-inflammatory and anticancer agents and for the treatment of vascular diseases. Since these structures are capable of inhibition of protein glycation and aldose reductase activity as well as exhibiting antioxidant activity, flavonoids are nowadays especially examined as potential therapeutic agent in diabetes [143]. Derivatives of curcumin are at present moment surveyed as potential therapeutics in patients suffering from Alzheimer's disease (decrease of IP levels and neuronal damage [144], reduced plague development [145]). Similarly, green tea extracts or epigallocatechin gallate (EGCG) injected into mice diminished damage caused by administration of 1-methyl-4-phenylpyridinium ($MPP^+$; triggering factor in various neurodegenerative diseases) [146].

#### *2.3.1.6 Thiol compounds*

Glutathione (GSH) is often suggested as possibly therapeutically useful. Areas of interest include preservation of organs for transplantation [147] and protection against tissue damage by cytotoxic agents such as aflatoxin B1 [5]. Aerosolized GSH solution has been suggested as mean for lowering lung damage by reactive species (e.g. in cystic fibrosis). However, when tested in asthma patients, solution induced bronchoconstriction [148]. Derivatives based on methyl, isopropyl and ethyl monoesters, which are more readily distributed to tissue systems and possess better efficacy, have been described [149]. L-2-oxathiazolidine-4-carboxylate (OTC) is hydrolysed to cysteine *in vivo* (by enzyme 5-oxoprolinase), and was reported to stimulate GSH synthesis in biological systems. [150]. This compound improved allergen-induced airway injury in mouse model of asthma [151].

*N*-acetylcysteine is used as antioxidant in many laboratory experiments and is effective in treating paracetamol over-dosage [152]. It has been widely used in human subjects for treatment of various diseases such as ARDS [153] or cardiovascular problems [154]. Although *N*-acetylcysteine was found to possess minimum toxicity, various studies had doubt about its therapeutic benefit via antioxidant mechanism [155]. *N*-acetylcysteine amide, which is able to cross blood-brain barrier, showed protective effects against myelin oligodendrocyte glycoprotein (MOG) induced autoimmune encephalomyelitis and towards MPTP (induction factor for Parkinson's disease) toxicity in rats [156].

2.3.1.6.1 Other thiols

Mercaptoethylguanidine is a powerful scavenger of $ONOO^-$ [157] and inhibitor of inducible nitrite oxide synthase (iNOS) [158]. Compound was found to have beneficial effects in various animal models of inflammatory conditions [159]. Lipoic acid can act as antioxidant *in vitro* [160] and has been used in treatment of diabetes [37]. In rat model, lipoic acid prevented falls in GSH levels with age [161]. Several structurally modified forms of lipoic acid with improved antioxidant activity have been described, some of which can cross blood-brain barrier [162]. Many thiols have been tested for their ability to protect cells and animals against ionizing radiation, including GSH, cysteine, bucillamine, cysteamine, dimercaprol, penicillamine, mesna (sodium salt of 2-mercaptoethanesulphonic acid) or amifostine [163].

### *2.3.1.7 Mitochondrially-targeted antioxidants*

Since mitochondria are major sources of reactive oxygen species *in vivo* and damage to them contributes to ageing and development of certain diseases, antioxidants selectively targeted to these organelles have been assumed to be especially beneficial. One approach was developed as linking antioxidant (such as thiol, α-tocopherol, coenzyme Q or spin trap) to the phosphorus of lipophilic compound triphenylphosphonium (TPP) [164]. Example of such hyphenated compound is $MitoQ_{10}$ (Coenzyme Q–TPP) [165]. Mitochondrially targeted antioxidants are able to enter mitochondria where they accumulate and exert protective antioxidant effect. Additionally, it was proposed that these compounds can enter all tissues, even brain (apparently are able to cross blood-brain barrier) [166]. Indeed, $MitoQ_{10}$ were found more effective in comparison to untargeted antioxidants, such as Coenzyme Q or TPP alone, in treatment of cardiac ischaemia-reperfusion injury [167]. Due to above-mentioned characteristics, mitochondrially-targeted antioxidants have been found to be exceptionally efficient in wide range of conditions, including Alzheimer's [168] and Parkinson's disease [169], hypertension [165] or diabetes [170]. Despite the existence of records on therapeutic benefit in numerous diseases, mitochondrially-targeted antioxidant are still not used in clinical praxis [1].

### *2.3.1.8 Iron chelators*

Several chelating agents have been used in attempts to inhibit iron- or copper- dependent oxidative damage. It is widely known, that antioxidant activity of chelators can be explained by multiple mechanisms, i.e. by scavenging free radicals. The first chelating agent reported to decrease the superoxide-dependent hydroxyl radical generation *in vitro* was DETAPAC [171]. However, other agents with better efficacy were described, among them for

example phytic acid or inositol hexaphosphate. These two chemicals have been used as food preservatives and were described as protective mean in colon, by binding iron in faeces [172]. Various plant phenolic compounds are able to bind transition-metals as well, and indeed, this may be the mechanism by which flavonoid derivatives (such as rutilemone) diminishes cardiotoxicity of doxorubicin in mice [173].

One of the very efficient and widely used iron chelator in praxis is desferrioxamine (DFO) produced by *Streptomyces pilosus*. Compound used for prevention and treatment of iron overload in patients who have ingested toxic oral doses of iron salts or who require multiple blood transfusions (i.e. thalassaemia, a form of inherited autosomal recessive blood disorder characterized by abnormal formation of haemoglobin, resulting in improper oxygen transport and destruction of red blood cells) [174,175]. DFO has been added to some organ preservation solutions, especially for heart transplantation [176]. Additionally, this compound have demonstrated anti-proliferative activity at therapeutically relevant concentrations [177]. High-molecular-mass forms of DFO have been developed, including cellulose, dextran or hydroxyethyl conjugates. Such derivatives were reported to exert lowered iron binding-activity, but on the other hand, toxicity of high doses were decreased as well [1]. DFO derivatives were reported to be protective in cases of septic shock and to delay onset of diabetes in animal models [178].

Hydroxypyridones were introduced in attempt to overcome major disadvantages of DFO in treating thalassaemia. Among chemicals substantially tested as alternatives to DFO, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid (HBED) [179], pyridoxal isonicotinoyl hydrazone (PIH) [180], desferrithiocin and 2,3-dihydroxybenzoate [181] have shown promising results. However most attention was focused on the 3-hydroxypyrid-4-ones. For example 3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one (L1) has undergone clinical trials in humans. Sadly, it appears more toxic than DFO, although still clinically useful [182,183].

Few orally active tridentate ligands have been described. One is ICL670 (Exjade®) which was tested in thalassaemia and its use has been approved by the Food and Drug Administration of the United States of America (U.S. FDA) [184]. Some authors have described so called “oxidative stress-activatable” iron chelators; i.e. compounds able to chelate transition metals at site of oxidative stress and not in the body generally (example of such compound is OR10141) [185]. Another interesting compound is lipoic acid plus (LAP; dithiolan-3-yl-pentanoic acid (2-dimethylamino-ethyl)-amide), derivative of lipoamide designed to accumulate within lysosomes. This compound is more efficient than DFO in cell protection against hydrogen peroxide [186]. Antioxidant derived from serine and vitamin $B_6$, *N*-(4-

pyridoxylmethylene)-L-serine, was found to protect mouse skin against damage by UV light, presumably by chelating iron [187]. Transition metal chelating ability was "added" to another sort of drugs, such as MAO inhibitors. Examples of this kind are compounds M10 and M30, which have been suggested for use in Parkinson's disease. Both of these compounds were claimed to exert protective action against MPTP neurotoxicity [1,188].

**Table 2** List of antioxidants available for therapeutic use

| **Category of compound** | **Example** |
|---|---|
| Naturally occurring | antioxidant enzymes (CuZnSOD, MnSOD, EC-SOD, SOR); peptides, proteins and other amino acids derivatives (histidine containing-dipeptides, transferrin, lactoferrin, adenosine, pyrodixamine, cysteine, glutathione); terpenoids (tocopherols, tocotrienols, coenzyme Q, lipoic acid, carotenoids); flavonoids and other phenolic compounds (resveratrol); antibiotics; hormones (melatonin, coelenterazine); carbohydrates (vitamin C); siderophores (desferrioxamine, rhodotorulic acid) |
| Synthetic | thiols (mercaptopropionylglycine, *N*-acetylcysteine); metal ion chelators (ICRF-187, Exjade®, hydroxypyridones); fullerenes; xanthine oxidase inhibitors; inhibitors of superoxide generation by phagocytes, inhibitors of phagocyte adhesion; lipid-soluble chain-breaking antioxidants; glutathione donors; SOD/Catalase mimetics; derivatives of vitamin E or C; coelenterazine derivatives; modified antibiotics |
| Compounds used in clinical praxis possessing antioxidant activity *in vivo*[a] | penicillamine[b], bucillamine[b]; aminosalicylates[b]; apomorphine[b]; selegiline; flupirtine; omeprazole; 4-hydroxytamoxifen[b]; ACE inhibitors (quinapril; ramipril; captopril) or angiotensin II receptor antagonists (losartan); ketoconazole; tetracyclines; probucol; propofol; various β-blockers (carvedilol, metoprolol)[b,c]; several $Ca^{2+}$ channel blockers[b,c]; cimetidine; phenylbutazone[b]; nitecapone[b]; entacapone[b]; idebenone, troglitazone[b]; tacrolimus |

[a] Agents primarily not developed as antioxidants, however exhibiting significant antioxidant effect; [b] Compounds reacting with reactive species resulting in formation of antioxidant-based radicals inducing further damage; [c] Several β-blockers and $Ca^{2+}$ blockers inhibits peroxidation *in vitro*, however it is uncertain if they do so *in vivo* at therapeutic levels normally achieved.

### 2.3.2 Anticancer drugs

Anticancer drugs embody a diverse array of chemical structures which are characterized as compounds possessing ability to supress cell growth and vitality of tumour cells. This biological activity is achieved by various mechanisms of actions. It is estimated, that there are over 50 clinically approved anticancer drugs. In addition there are many new anticancer drugs in various stages of preclinical and clinical development. There is nowadays excess need for novel and more effective antineoplastic agents. This is caused by increased incidence of cancer amongst human population as well as rapid spread of resistance of cancer cells towards commonly used chemotherapeutics (this fact is especially true for paclitaxel). Moreover, despite the fact that commonly used chemotherapeutics are designed to provide as little damage as possible to human health, these possess considerable adverse effects [5,189,190]. Examples of some commonly used anticancer agents (either used in natural, semi-synthetic or complete synthetic form), as well as their mode of action are discussed below.

#### *2.3.2.1 Antimetabolites*

Antimetabolites are one of the oldest families of anticancer drugs whose mechanism of action is based on the interaction with essential biosynthesis pathways, such as disruption of synthesis of nucleic acids via incorporation to cell components (e.g. 5-fluorouracil, mercaptopurine) or interference with enzymatic processes of metabolism (for example methotrexate). Most of these compounds are used in treatment of various types of leukaemia [190].

##### 2.3.2.1.1 Pyrimidine analogues

5-fluorouracil (5-FU) is a widely used cytotoxic agent for the treatment of breast tumours and cancers of the gastrointestinal tract, including advanced colorectal cancer [191]. It has also been given topically for treatment of certain types of skin cancers. Main side effects of this drug include myelosuppression and mucositis [192]. Tegafur and capecitabine are metabolised to 5-FU and are given orally for metastatic colorectal cancer. Major side effects of these two analogues are very similar to 5-FU [193]. Cytarabine is still one of the most effective single agents available in oncological praxis for treating acute myeloblastic leukaemia, although myelosuppression is a major side effect [194]. Gemcitabine is a more recently introduced compound of the antimetabolites and it is used intravenously in association with cisplatin for metastatic non-small cell lung, pancreatic, and bladder cancers [195]. It is generally well tolerated but prolonged administration can cause gastrointestinal disturbances, renal impairment, pulmonary toxicity, and influenza-like symptoms [196,197]. Another example of

pyrimidine analogues is azacitidine, in oncological praxis mainly used in treatment of myelodysplastic syndromes [198].

2.3.2.1.2 Purine analogues

Azathioprine was primarily developed as immunosuppressant agent and was administered to patients undergoing organ transplantation [199]. Compound was also used in treatment of various immune system disorders such as rheumatoid arthritis [200] and Crohn's disease [201]. Subsequently it was discovered that azathioprine is also useful antileukaemic drug. Mercaptopurine (6-methylmercaptopurine), a metabolization product of azathioprine displaying similar immunosuppressive activity as azathioprine, is almost exclusively used as maintenance therapy for acute leukaemia [202]. It has also demonstrated some *in vitro* effectiveness against *Mycobacterium paratuberculosis* [203]. Another purine analogue, thioguanine, is used orally to induce remission in acute myeloid leukaemia. Despite their considerable efficacy, all of these compounds possess similar side effects, including myelosuppression [204].

Cladribine is given by intravenous infusion for the first-line treatment of hairy cell leukaemia [205] and the second-line treatment of chronic lymphocytic leukaemia (CLL) in patients who have failed on standard regimens of alkylating agents [206]. Fludarabine, analogue of cladribine, is sometimes used in combinations with other chemotherapeutics (e.g. cyclophosphamide) in first-line treatment of CLL [207]. Another derivative, clofarabine is approved for treating refractory acute lymphoblastic leukaemia in children after failure of at least two other types of chemotherapeutical treatment [208]. As in case of other purine antimetabolites, usefulness is limited by bone marrow suppression [209].

2.3.2.1.3 Other antimetabolites

Methotrexate (MTX) is used as maintenance therapy for childhood acute lymphoblastic leukaemia, choriocarcinoma, non-Hodgkin's lymphoma, and several solid tumours [210]. It is also administered for the treatment of autoimmune diseases like psoriasis [211], rheumatoid arthritis [145], and lupus [212]. Side effects include myelosuppression, mucositis, and gastrointestinal ulceration with potential damage to kidneys and liver that may require careful monitoring [189]. Raltitrexed (Tomudex®) is given intravenously for palliation of advanced colorectal cancer in cases where 5-FU cannot be used. Compound is also sometimes used in combination with other chemotherapeutics (such as oxaliplatin, cisplatin, irinotecan or various anthracyclines) [213]. It is generally well tolerated, but can cause myelosuppression and gastrointestinal toxicity [189,214]. Another intravenously administered antimetabolite is

pentostatin, which regarded as is highly efficient mean for maintenance of hairy cell leukaemia [215]. However, drug is able to induce prolonged remissions [216]. Hydroxycarbamide, also called hydroxyurea, is an antineoplastic drug used mainly in myeloid leukaemia, often in combination with other drugs [217]. It can also be used for the treatment of melanoma and to reduce the rate of painful attacks in sickle-cell disease [190,218].

#### *2.3.2.2 DNA interactive agents*

DNA interactive agents are regarded as one of the largest and most important anticancer drug families. Members of this class of antineoplastic drugs are characterized by their ability to form adducts or otherwise interact with DNA molecule, thus preventing replication of target cell and inducing cell death [189].

##### 2.3.2.2.1 Alkylating agents

Dacarbazine is employed as single agent to treat metastatic melanoma and in combination with other drugs for soft tissue sarcomas [219]. Similarly as for antimetabolites, the predominant side effects are myelosuppression and intense nausea and vomiting [220]. Temozolomide is a more-recently introduced compound for the second-line treatment of brain cancers. Drug is structurally similar to dacarbazine and its main advantage is its good oral bioavailability and distribution properties with penetration into the central nervous system [189,221]. Procarbazine has previously demonstrated significant activity in lymphomas and carcinomas of the bronchus and in brain tumours [222,223]. However, its toxic effects include nausea, myelosuppression, and a hypersensitivity rash that disables supplementary use of the drug [224]. Ecteinascidin-743 is a novel DNA-binding agent derived from the marine tunicate *Ecteinascidia turbinata*. This compound was found to be significantly active towards various carcinomas, including melanoma, breast, ovarian, colon, renal, non-small cell lung and prostate cancers *in vitro*, which was subsequently confirmed by various clinical trials [225,226].

##### 2.3.2.2.2 Cross-linking agents

Platinum complexes belong to one of the most widely used class of anticancer drugs and these have previously exhibited pronounced activity in treatment of various types of cancers. Cisplatin was the first platinum complex used with a noticeable activity in testicular and ovarian cancers. Related derivatives carboplatin and oxaliplatin were developed later to reduce the problematic side effects of cisplatin (such as nephrotoxicity, ototoxicity, and peripheral neuropathy). At present, carboplatin is used in treatment of advanced ovarian and lung

cancers, while oxaliplatin is licensed for the treatment of metastatic colorectal cancer in combination with other chemotherapeutics, such as 5-FU, folinic acid or paclitaxel [190,227].

Nitrogen mustards are class of cytotoxic chemotherapy drugs chemically resembling mustard gas which was used in First World War as warfare agent [228]. Representatives of nitrogen mustard-derived compounds used as chemotherapeutical agents include cyclophosphamide, ifosfamide, melphalan, chlorambucil, chlormethine, estramustine. Cyclophosphamide remains one of the most successful and widely utilized antineoplastic drugs mainly due to its broad spectrum of clinical activity (carcinomas of the bronchus, breast, ovary, and various sarcomas, chronic lymphocytic leukaemia, and lymphomas) as well as bioavailability. Moreover, it is also a potent immunosuppressive agent and the most commonly used drug in blood and marrow transplantation. Due to these properties, cyclophosphamide is also sometimes used in treatment of certain autoimmune disorders [189,229]. Careful monitoring of cyclophosphamide administration is needed though, as it has some very serious and life-threatening adverse effects [230]. Its derivative, ifosfamide, has similar anticancer activity as well as spectrum, however needs to be administered intravenously. This compound is often used in conjunction with mesna (section 2.3.1.6.1) to avoid internal bleeding [231]. In comparison to ifosfamide, its analogue trofosfamide is orally active and generally well-tolerated [232]. Chlormethine (also known as mechlorethamine) is used for the treatment of Hodgkin's disease. Due to its chemical reactivity, it must be freshly prepared prior to administration and subsequently delivered via a fast-running intravenous infusion [190,233]. Estramustine phosphate is a conjugate consisting of chlormethine chemically linked to an oestrogen moiety. It is usually orally administered to patients with metastatic prostate cancer [234]. Chlorambucil is useful in the treatment of ovarian cancer, Hodgkin's disease, non-Hodgkin's lymphomas, and chronic lymphocytic leukaemia. Its lower chemical reactivity allows oral dosing. The most commonly occurring side effect of this drug is bone marrow suppression. Additionally, like many other cross-linking agents, chlorambucil has been associated with the development of other forms of cancer [189,190]. Melphalan, analogue of chlorambucil, is indicated for the treatment of myeloma, solid tumours (e.g., breast and ovarian) and lymphomas. As well as chlorambucil, this compound is orally active. Side effects are also very similar [235,236].

Nitrosourea is class of compounds where basic skeleton is composed of nitroso (R-NO) group and urea. Characteristic representatives of this class of antineoplastic agents are: lomustine, carmustine and fotemustine, to name a few [189,190]. Lomustine is characterized by relatively high degree of lipophilicity in comparison to other chemotherapeutics. Since lomustine is able to cross blood-brain barrier, it is considered as a very valuable anticancer

agent. Compound is administered orally and it is mainly prescribed for patients suffering from certain solid tumours (especially of brain) and Hodgkin's disease [237,238]. Activity and toxicity profile of carmustine is comparable to lomustine [239]. Fotemustine is approved for use in the treatment of metastasising melanoma, although some adverse effects have been observed [240].

Thiotepa (*N*,*N'*,*N''*-triethylenethiophosphoramide) is long used as an effective anticancer drug and appears to be one of the most effective chemotherapeutic agent when used in high dose regimens. Its main indications are the treatment of bladder or ovarian cancers, breast cancer and malignant effusions [241,242]. Main toxic effect of this compound is myelosuppression and bone marrow depression [243]. Treosulfan, related compound to thiotepa, is mainly used to treat ovarian cancer and it has similar major side effects to nitrogen mustards [244]. Busulfan is used for the treatment of chronic myeloid leukaemia and as part of conditioning regimens for patients undergoing bone marrow transplantation. Unfortunately, it can cause excessive myelosuppression, resulting in irreversible bone marrow aplasia and requires careful monitoring [245,246]. Mitomycin-C is a member of a group of naturally occurring antitumor antibiotics produced by *Streptomyces caespitosus* [247]. Mitomycin needs to be administered intravenously and is indicated for patients suffering from upper gastrointestinal and breast cancers. Moreover, administration via bladder instillation allows treatment of superficial bladder tumours. Adverse events of this compound include toxicity to bone marrow [248].

2.3.2.2.3 Intercalating agents

A mechanism of anticancer activity of so called intercalating agents is explained by their ability to attach themselves between the two strands of DNA. These compounds interfere with replication of the host cell. Typical members of this class are anthracyclines, characterized by presence of planar anthraquinone nucleus attached to an amino-containing sugar [249]. Despite the fact that these compounds exert antibacterial activity, high toxicity limits their use to anticancer therapy [250]. Doxorubicin, daunorubicin, and aclarubicin are natural products extracted from *Streptomyces peucetiusor* or *S. coeruleorubicus*, while epirubicin and idarubicin are semisynthetic analogues. Doxorubicin is widely used as an anticancer drug because of its broad spectrum of activity (acute leukaemia, lymphomas, and a variety of solid tumours). It is administered by intravenous injection and is largely excreted in the bile. Adverse effects include nausea, vomiting, myelosuppression, mucositis, hair loss and cardiotoxicity. Daunorubicin displays significant activity mainly towards acute lymphocytic and myelo-

cytic leukaemia. However, since it has a much less favourable therapeutic index (severe cardiotoxicity) than doxorubicin, daunorubicin is no longer used therapeutically to any extent. Aclarubicin is used as a second-line treatment for acute non-lymphocytic leukaemia. Epirubicin is similar in terms of efficacy for treatment of breast cancer, and produces lower side-effects in comparison to doxorubicin. Idarubicin is mainly used in advanced breast cancer where first-line chemotherapy has failed. Compound is also used in treatment of acute non-lymphocytic leukaemia. As well as epirubicin, idarubicin show increased activity with less cardiotoxicity than their precursor. The principal disadvantage of all of these agents is their severe cardiotoxicity [5,190]. Other examples of intercalating agents are mitoxantrone and actinomycin-D (dactinomycin). Mitoxantrone, synthetic analogue of anthracycline antibiotics, has reduced toxicity compared with doxorubicin and is indicated in cases of metastatic breast cancer, adult non-lymphocytic leukaemia and non-Hodgkin's lymphoma [5]. In addition, it is currently proving useful in multiple sclerosis treatment, where it can reduce the frequency of relapses [251]. Actinomycin-D, polypeptide antibiotic isolated from *Streptomyces parvullus*, is mainly used to treat paediatric cancers, some testicular sarcomas and AIDS-related Kaposi's sarcoma [252]. The side effects of mitoxantrone and actinomycin-D are similar to those of doxorubicin except that the cardiac toxicity is less prominent. However, cardiac examinations and monitoring are still recommended when a certain cumulative dose has been reached [190].

#### 2.3.2.2.4 Topoisomerase inhibitors

Topoisomerases are responsible for cleavage and resealing of the DNA strands during the replication process, and are broadly classified as type I or II according to their ability to cleave one or both strands. Inhibition of both enzymes leads to apoptosis of affected cell [253]. Typical representatives of topo-I and II inhibitors would be alkaloids camptothecin and ellipticine, respectively. Camptothecin is quinoline-based alkaloid with a unique five-ring system and is commercially extracted from the bark of trees belonging to genera *Camptotheca* and *Nothapodytes* (section 2.4.2). In clinical trials, camptothecin showed broad-spectrum anticancer activity, but on the other hand severe toxicity (such as myelosuppression, gastrointestinal disturbances including delayed diarrhoea, asthenia, alopecia, and anorexia) and poor solubility. This lead to development of semi-synthetic derivatives such as topotecan and irinotecan, which are now available for the treatment of ovarian and colorectal cancers, respectively [254–256]. Ellipticine, a terpenoid indole alkaloid, is isolated from bark of *Ochrosia* spp. (section 2.4.2). Clinical trials with ellipticine and 9-methoxyellipticine showed these two

compounds to be potent inhibitors of several cancerous disorders, but preclinical toxicology indicated a number of side-effects, including haemolysis and cardiovascular effects. Again, these compounds had problems with water solubility. Semi-synthetic analogue elliptinium acetate had demonstrated more promising results and it has been suggested to be used clinically in treatment of breast and renal cell cancers [257,258]. Amsacrine, another topoisomerase II inhibitor, has an acridine-based structure. Clinically, amsacrine has an activity and toxicity profile similar to doxorubicin. It is administered intravenously for the treatment of advanced ovarian carcinomas, myelogenous leukaemia, and lymphomas. Its side effects include myelosuppression and mucositis [259,260].

2.3.2.2.5 DNA cleaving agents

Typical example of DNA cleaving agents is bleomycin. Bleomycin is a mixture of glycopeptide antibiotics isolated from cultures of *Streptomyces verticillus*, and is used in oncological praxis for its anticancer activity. The major components of the mixture are bleomycin $A_2$ and bleomycin $B_2$. Exact mode of action of this compound is still not fully revealed. However, it is suggested that bleomycin possess significant intercalation and chelation activity (usually towards iron and oxygen ions), which are necessary for the DNA degradation reaction. Bleomycin is used alone, or in combination with other anticancer drugs, to treat squamous cell carcinomas of various organs, lymphomas, and some solid tumours. It is unusual amongst antitumor antibiotics in producing very little bone-marrow suppression, making it particularly useful in combination therapies with other drugs which do cause this response. However, there is some lung toxicity associated with bleomycin treatment [5,190].

***2.3.2.3 Antitubilin agents***

Antimitotics act by disruption of the normal mitotic spindle apparatus during mitosis, either through (i) microtubule stabilization or (ii) prevention of microtubule polymerization, resulting in condition where cells fail to complete normal mitosis [261]. Examples of former mode of action would be paclitaxel and jatrophine 1, while of latter combretastatin A-4, colchicine, maytansine, podophyllotoxin and vinca alkaloids. It is interesting to note, that compounds with antimitotic activity are almost exclusively acquired from plants [5,262].

2.3.2.3.1 Taxanes

Paclitaxel (Taxol®) is a highly complex tetracyclic diterpene found in the needles and bark of *Taxus brevifolia* and other related species (section 2.4.2). Pure paclitaxel was isolated in 1966 and its structure published in 1971. However, it did not appear in clinical praxis until

the 1990s [263]. Paclitaxel is used clinically in the management of ovarian and breast cancers, non-small-cell lung cancer, small-cell lung cancer, and cancers of the head and neck. Paclitaxel has disadvantage in its low water solubility, and lack of activity in some cancers with resistance. For this reason, side-chain analogues of paclitaxel with better activity as well as solubility were manufactured [264,265]. One of this compound is docetaxel. This drug displays similar therapeutic and toxicological properties as paclitaxel; it is mainly licensed for initial treatment of advanced breast cancer in combination with doxorubicin or alone when adjuvant cytotoxic chemotherapy has failed [266].

2.3.2.3.2 Vinca alkaloids

Vincristine and vinblastine are constituents isolated from *Catharanthus roseus* (section 2.4.2) and have proved to be extremely valuable drugs in the treatment of Hodgkin's disease and childhood leukaemia, respectively. Isolation and structural identification were reported in the 1960s [267]. Sadly, both compounds needs to be administered intravenously and possess some serious side effects (e.g. neurotoxicity, myelosuppression, hair loss). However new orally active derivatives with broader anticancer activity, together with lower neurotoxic side-effects have been developed [268,269]. Vindesin has been introduced for the treatment of acute lymphoid leukaemia in children [270]. Vinorelbine is a newer semi-synthetic modification obtained from anhydrovinblastine. In this structure, the indole $C_2N$ bridge in the catharanthine-derived unit has been shortened by one carbon. Vinorelbine is used for the treatment of advanced breast cancer, small-cell lung cancer and non-small-cell lung cancer [5,252].

2.3.2.3.3 Other antitubulin agents

Colchicine, obtained from *Colchicum autumnale* have shown to be very potent antimitotic drug, however due to its severe toxicity is unsuitable for clinical use as chemotherapeutic agent [271]. However, it still used as alternative treatment for gout in patients who are intolerant to commonly administered non-steroidal anti-inflammatory drugs (NSAIDs) [272]. Less-toxic variants of colchicine such as *N*-acetylcolchinol are under active development as potent anticancer drug [5]. Rhizomes of *Podophyllum* species (section 2.4.2) are source of phenolic compound podophyllotoxin with antitumor activity. Due to its adverse side-effects this compound is not used in medicine. However semi-synthetic derivatives (such as etoposide and teniposide) have been developed, which provided more promising results [273,274]. Etoposide is a very effective anticancer agent, and is used in the treatment of small-cell lung cancer, testicular cancer, and lymphomas, usually in combination therapies with other anticancer

drugs. It may be given orally or intravenously [275]. Teniposide has similar anticancer properties and, though not as widely used as etoposide, has value in paediatric neuroblastoma [262]. Another compound of medicinal interest as potential antineoplastic drug is combretastatin A-4, produced by bark of *Combretum* spp. (Section 2.4.2). This compound, which structurally resembles colchicine, is s currently showing considerable cytotoxic potential in clinical trials against several types of cancer [261,262]. Attracting considerable interest at the present time are the epothilones, a group of macrolides produced by cultures of the myxobacterium *Sorangium cellulosum* [278]. Epothilones display marked antitumor properties with a mode of action paralleling to that of the highly successful anticancer drug taxol (stabilization of microtubules) [279]. However, the epothilones have a much higher potency (2,000–5,000 times) and are active against cell lines which are resistant to taxol and other drugs [280]. There appears to be considerable potential for developing the epothilones or its analogues into valuable anticancer drugs, and several derivatives are undergoing clinical trials. These include epothilone B (patupilone) and its allyl analogue sagopilone. Lactam derivative ixabepilone has recently been approved for drug use in the treatment of breast cancer [5,190].

#### *2.3.2.4 Cdk inhibitors*

With the identification of an increasing number of molecular targets associated with particular cancers, high throughput screening of compounds against a range of such targets now forms the basis of anti-cancer drug discovery. Examples are the cyclin-dependent kinases (Cdks), which, together with their cyclin partners, play a key role in the regulation of cell cycle progression, and inhibition of their activity delays or arrests progression at specific stages of the cell cycle. There are over 2,000 kinases so far identified from genomic studies [281]. Anticancer agents with this mode of action are called Cdk inhibitors. Few plant-based compounds (and their analogues) with this activity have been suggested suitable candidates for preclinical development as anticancer agents. Flavopiridol, compound synthetized from flavonoid rohitukine, have demonstrated effect against a series of breast and lung carcinoma cell lines [282]. Another synthetic molecule based on natural model is roscovitine, derived from purine alkaloid olomucine, exhibited anticancer activity towards non-small cell lung cancer and various types of leukaemia [283]. β-lapachone was found out to possess significant activity against a range of tumour cell lines, including breast, leukaemia, prostate, together with several multidrug-resistant lines [284]. Indirubins are chief constituents presented in various herbal preparation used in traditional Chinese medicine and have shown to be effective to-

wards chronic myelogenous leukaemia [281]. Despite their significant activity, none of these compounds are used in oncological praxis yet [262].

## 2.4 Plants as a sources of potential therapeutic agents

Plants have a long history of use in the treatment of many human diseases [285]. Botanicals are considered as valuable source of wide variety of secondary metabolites which have been found to possess many biological activities affecting human health. Verpoorte [286] have earlier estimated that from approximately 250,000 – 500,000 known higher plant species, only 6% were tested for at least one biological activity and just 15% has been studied phytochemically. As contrast, it is expected that 25 to 50% of commonly used pharmaceutical preparations are derived from plants [287]. Hence, there is still considerable interest in identification and characterization of plant-derived components which would serve as leads for discovery of novel compounds with pharmacologically-important activity. This is also true for compounds with antioxidant and anti-proliferative effect. Especially when we take into consideration the ever-increasing endeavour in improvement of human health and nutrition, aggravated incidence of oxidative stress-related diseases amongst human population and resistance of cancer cells to commonly used chemotherapeutics [14,288]. This section summarizes chemistry and botanical sources of currently used antioxidants and anti-proliferative constituents as well as discovery of novel plant-based compounds and their possible use in medicinal practice.

### 2.4.1 Compounds with antioxidant activity

Despite the fact that various types of secondary metabolites such as alkaloids (caffeine [289]), diterpenoids (ginkgolides [290]), steroids (glycyrrhizic acid), carotenoids (canthaxanthin [291]), and others contributes to overall antioxidant potential of plants, the phenolic compounds are considered as the main class of low-molecular chemicals responsible for this biological activity [292–296].

Phenolics are generally defined as any organic compound whose basic skeleton is consisted of one or more aromatic ring (simple phenols or polyphenols, respectively) which is substituted by at least one hydroxy group. Presented hydroxy groups may occurs either in free form (these phenolics are sometimes referred to as aglycons) or are engaged in another function: ether, ester or glycosides [297–299]. Phenolic compounds are almost exclusively produced by plants, fungi and microorganisms. Animals are unable to synthetize these constituents [300]. There are few exception however – i.e. terpenoid quinones (such as ubiquinones or plastoquinones) [5]. All other phenolic compounds found in animal tissue probably originate

from dietary intake [300]. Although above-mentioned definition of phenolic compounds could be applied for wide range of plant-based secondary metabolites, these compounds are additionally more strictly characterized by their specific biosynthetic pathway. Two main pathways of synthesis of phenolic compounds are widely accepted:

1. Shikimate pathway (also known as phenylpropanoids pathway). Aromatic amino acids, such as L-tyrosine and L-phenylalanine, are important products/intermediates of this synthesis pathway [301–303].

2. Polyketide pathway, based on sequential building of the phenolic ring from two carbon units originating in decarboxylation of malonyl-coenzym A [304–307].

Moreover, some phenolic compounds are pure products of above-mentioned pathways (e.g. coumarins and lignans) while others are of mixed origin (e.g. flavonoids). Despite the fact that phenolic compounds may be categorised according to various schemes (such as number of carbon atoms in skeleton or type of basic skeleton) [308,309], following classification based on biosynthesis pathway is nowadays widely accepted [5]:

1. derivatives of cinnamic acid (e.g. coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, sinapic acid)

2. derivatives of benzoic acid (e.g. salicylic acid, vanillic acid, vanillin)

3. phenylpropenes (e.g. anethole, cinnamaldehyde, cinnamyl acetate, estragole, eugenol, myristicin, elemicin)

4. lignans and lignins (e.g. pinoresinol, podophyllotoxin)

5. coumarins (aesculin, scopoletin, umbeliferone, coumarin)

6. styrylpyrones and diarylheptanoids (e.g. yangonin, curcumin)

7. flavonoids and stilbenes (e.g. epigallocatechin gallate, myricetin, naringenin, quercetin, resveratrol)

8. flavonolignans (e.g. taxifolin, silybin)

9. isoflavonoids (e.g. daidzein, genistein, formononetin, biochanin A)

10. terpenoid quinones (e.g. α-tocopherol)

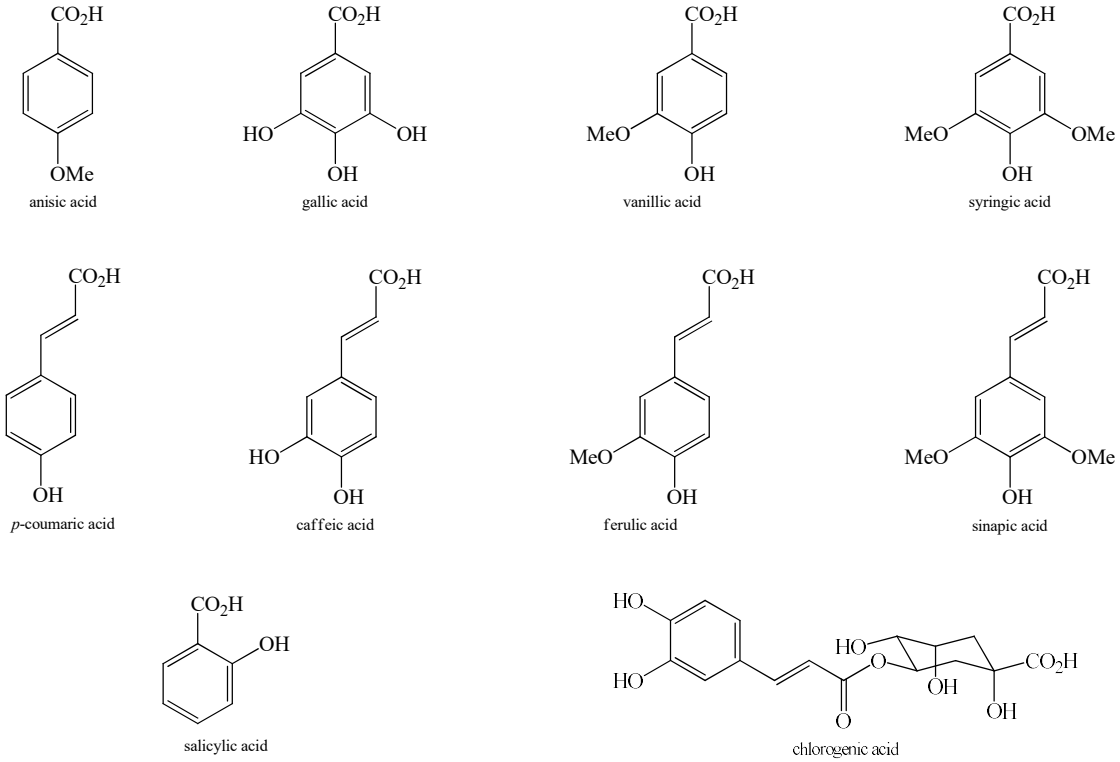


**Figure 1** Phenolic acids

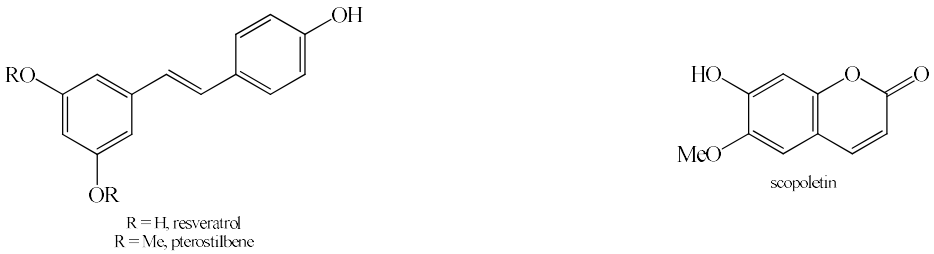


**Figure 2** Examples of stilbenoid and coumarin structures

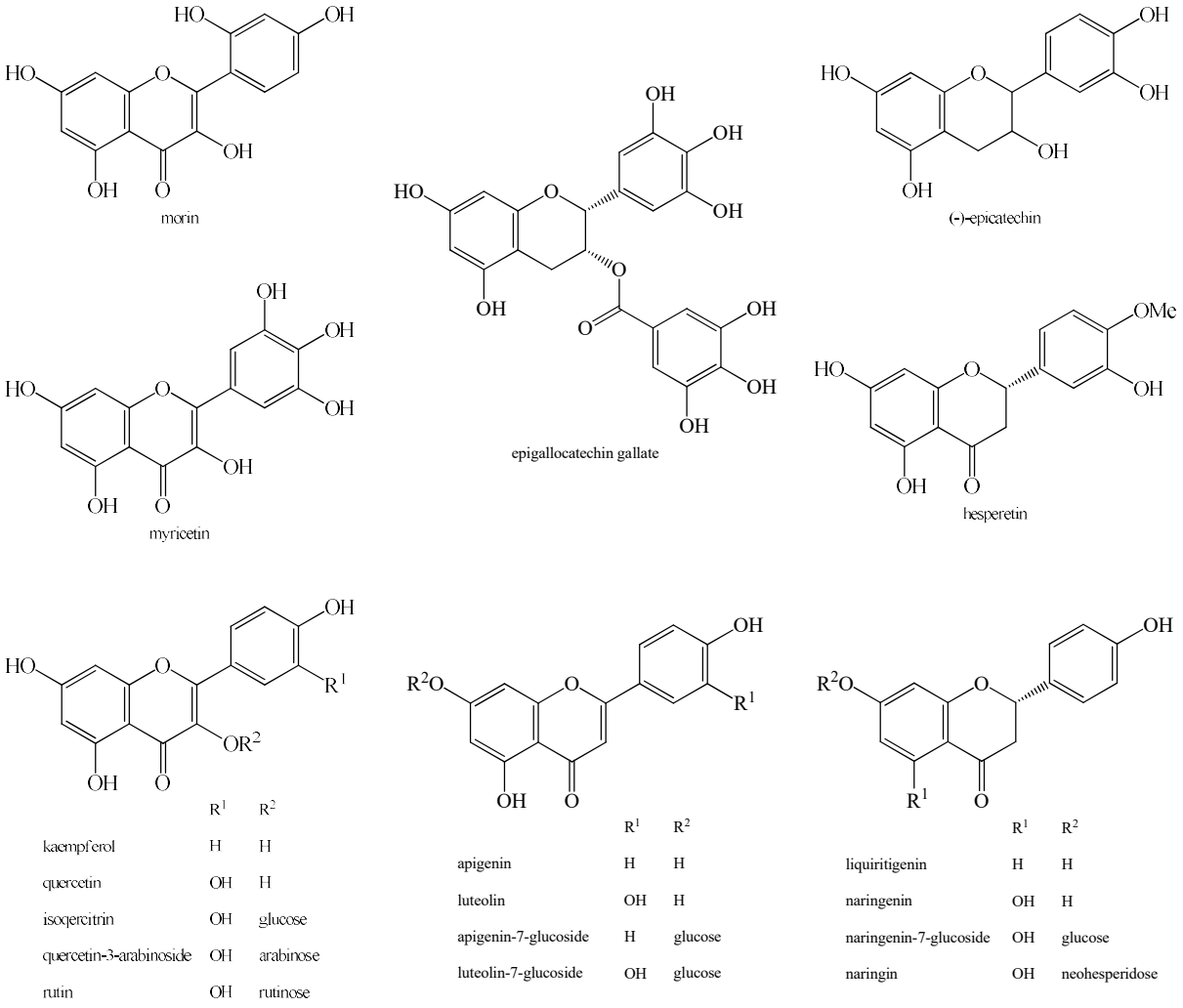


**Figure 3** Examples of free and glycosylated flavonoids

Several authors agree that phenolic compounds are distributed virtually in all vascular plants (both dicotyledons and monocotyledons). It is estimated, phenolic compounds encompasses more than 10,000 chemical structures, with hundreds of new constituents being discovered every year [310,297]. Generally, phenolics are found in dietary plants, such as fruits, vegetables, legumes, nuts, oilseeds, and cereals [311]. However more recent studies are showing that certain medicinal plants may also be regarded as valuable source of these chemical structures [312,2]. Additionally, distribution of phenolic compounds is not plant-part specific; phenolics are found in leaves, flowers, seeds, stems, roots, and other botanical parts. Some typical examples of phenolic compounds and their distribution in both edible and medicinal plants is given in Table 3.

**Table 3** Distribution of phenolic compounds in plant kingdom

| **Compound** | **Sources** | |
|---|---|---|
| | **Dietary plants** | **Medicinal Plants** |
| **Benzoic acid derivatives** | | |
| anisic acid | *Fagopyrum esculentum* (Polygonaceae) [313] | *Pimpinella anisum* (Apiaceae) [314] |
| gallic acid | *Ribes nigrum* (Grossulariaceae) [315] | *Salvia officinalis* (Lamiaceae) [316] |
| salicylic acid | *Vaccinium oxycoccos* (Ericaceae) [317] | *Salix* species (Salicaceae) [318] |
| syringic acid | *Euterpe oleracea* (Arecaceae) [319] | *Rosmarinus officinalis* (Lamiaceae) [320] |
| vanillic acid | *Vanilla planiflora* (Orchideaceae) [5] | *Aristolochia* species (Aristolochiaceae) [321] |
| **Cinnamic acid derivatives** | | |
| caffeic acid | *Spinacia oleracea* (Chenopodiaceae) [311] | *Echinacea purpurea* (Compositae) [322] |
| ferulic acid | *Beta vulgaris* (Amaranthaceae) [323] | *Allium sativum* (Amaryllidaceae) [324] |
| chlorogenic acid | *Ipomoea batatas* (Convolvulaceae) [325] | *Coffea arabica* (Rubiaceae) [311] |
| *p*-coumaric acid | *Solanum lycopersicum* (Solanaceae) [311] | *Cuminum cyminum* (Apiaceae) [326] |
| sinapic acid | *Myristica fragrans* (Myristicaceae) [327] | *Borago officinalis* (*Boraginaceae*) [328] |
| **Stilbenes** | | |
| resveratrol | *Vitis vinifera* (Vitaceae) [5] | *Polygonum cuspidatum* (Polygonaceae) [5] |
| pterostilbene | *Vaccinium myrtillus* (Ericaceae) [329] | *Pterocarpus marsupium* (Leguminosae) [330] |

**Table 3** (*continued*)

| **Compound** | **Sources** | |
|---|---|---|
| | **Dietary plants** | **Medicinal plants** |
| **Flavanols** | | |
| epicatechin | *Theobroma cacao* (Malvaceae) [331] | *Hypericum perforatum* (Hypericaceae) [332] |
| **Flavanones** | | |
| hesperetin | *Citrus aurantium* (Rutaceae) [333] | *Cyclopia genistoides* (Leguminosae) [334] |
| liquiritigenin | *Cicer arietinum* (Leguminosae) [335] | *Glycyrrhiza glabra* (Leguminosae) [336] |
| naringenin | *Citrus paradisi* (Rutaceae) [337] | *Satureja hortensis* (Lamiaceae) [338] |
| **Flavonols** | | |
| kaempferol | *Armoracia rusticana* (Brassicaceae) [339] | *Syzygium aromaticum* (Myrtaceae) [340] |
| morin | *Psidium guajava* (Myrtaceae) [341] | *Morus alba* (Moraceae) [342] |
| quercetin | *Olea europaea* (Oleaceae) [343] | *Ginkgo biloba* (Ginkgoaceae) [344] |
| myricetin | *Vitis vinifera* (Vitaceae) [345] | *Myrica rubra* (Myricaceae) [346] |
| **Flavones** | | |
| apigenin | *Apium graveolens* (Apiaceae) [347] | *Achillea millefolium* (Compositae) [348] |
| luteolin | *Capsicum annuum* (Solanaceae) [349] | *Matricaria chamomilla* (Compositae) [350] |
| **Condensed tannins** | | |
| epigallocatechin gallate | *Camellia sinensis* (Theaceae) [351] | *Cinnamomum verum* (Lauraceae) [352] |

### 2.4.2 Compounds with anti-proliferative activity

Anti-proliferative efficacy of plant species is mainly credited to alkaloids, certain phenolic compounds and various terpenoids. Sources of plant-based anticancer agents (or their semisynthetic analogues) which have been suggested for clinical use or are already used in oncological praxis is given in Table 4.

In comparison to antioxidant compounds, plant-based anti-proliferative constituents are more restricted to specific families in plant kingdom. Despite the fact that camptothecin (pyrroloquinoline alkaloid) is chiefly obtained from *Camptotheca acuminata* belonging to Cornaceae family, there are other sources of this alkaloid as well, including Icacinaceae (*Nothapodytes nimmoniana*, *Merilliodendron megacarpum*, *Pyrenacantha klaineana*), Rubiaceae (*Ophiorrhiza pumila*) and Apocynaceae (*Ervatmia heyneana*) families. Other members of Apocynaceae family are important sources of anticancer agents as well. Two extremely valuable terpenoid indole alkaloids with anticancer activity, vincristine and vinblastin, are produced by *Catharanthus roseus*. Bark of *Ochrosia elliptica* and other related *Ochrosia* species are sources of another indole alkaloids ellipticine and its derivative 9-methoxyellipticine. Taxaceae family is also regarded as significant source of plant-based anticancer agents – i.e. diterpenoid paclitaxel along with several key precursors (baccatins), extracted from the leaves and bark of *Taxus brevifolia*, and alkaloid homoharringtonine found in the *Cephalotaxus harringtonii.* Podophyllotoxin and other related lignans (phenolics) are found in the roots of *Podophyllm peltatum* and *P. hexandrum* belonging to Berberidaceae family. Another phenolic compound with significant anticancer activity, combretastatin A-4 is isolated from bark of *Combretum caffrum* (Combretaceae) [252,5].

List of few examples of other plant-based compounds which have evoked interest as potential anticancer agents and are in clinical or pre-clinical development is given below: flavonoid rohitukine (*Dysoxylum gotadhora*, Meliaceae) [282], quinone β-lapachone (*Tabebuia* spp., Bignoniaceae) [284], polyketide maytansine (*Gymnosporia serrata*, Celastraceae) [353], sesquiterpene lactone thapsigargin (*Thapsia garganica*, Apiaceae) [354], diterpenoid bruceantine (*Brucea antidysenterica*, Simaroubaceae) [355], diterpenoic jatrophane esters (*Euphorbia semiperfoliata*, Euphorbiaceae) [356], triterpene betulinic acid (*Betula* spp., Betulaceae) [357], bisindolic alkaloids indirubins (*Indigofera tinctoria*, Fabaceae) [281], tropane alkaloids pervilleines (*Erythroxylum pervillei*, Erythroxylaceae) [358], purine alkaloid olomucine (*Raphanus raphanistrum*, Brassicaceae) [359], protoberberine alkaloids nitidine (*Zanthoxylum* spp., Rutaceae) [360]. This list is encompasses only few examples of plant-derived anti-proliferative compounds and serves only as illustrational. Approximately 3,000 plant species

are till present moment reported to be traditionally used in the treatment of cancer and are waiting to be properly screened for active constituents [262]. New plant-based antineoplastic agents based on selective activity against cancer-related molecular targets are discovered from these plants each year. Some of these are regarded as promising candidates for use in clinical praxis.

**Table 4** Overview of clinically used plant-based anticancer agents

| **Compound** | | **Source** | |
|---|---|---|---|
| | **Mode of anti-proliferative action** | **Plant** | **Family** |
| **Alkaloids** | | | |
| camptothecin[a] | topoisomerase I inhibition | *Camptotheca acuminata* | Cornaceae |
| ellipticine[a] | topoisomerase II inhibition | *Ochrosia elliptica* | Apocynaceae |
| colchicin[a] | antimitotic | *Colchicum autumnale* | Colchicaceae |
| homoharringtonine | Cdk inhibiton | *Cephalotaxus harringtonii* | Taxaceae |
| vinca alkaloids | antimitotic | *Catharanthus roseus* | Apocynaceae |
| **Phenolic compounds** | | | |
| combretastatin A-4 | antimitotic | *Combretum caffrum* | Combretaceae |
| podophyllotoxin[a] | antimitotic | *Podophyllum peltatum* | Berberidaceae |
| **Terpenoids** | | | |
| paclitaxel | antimitotic | *Taxus brevifolia* | Taxaceae |

[a] due to severe toxicity is not used in clinical praxis, however semi-synthetic derivatives with lowered adverse-effects were developed or are subjected to development

### 2.4.3 Compounds with combined antioxidant/anti-proliferative effect

Clinically used antioxidants as well as chemotherapeutics should be designed to provide as little damage to human health. Hovewer, the majority of these possess serious adverse and in some cases even life-threatening effects (as noted in previous sections). Additionally, there are more and more indications, that development of cancer might be strongly connected to oxidative stress [1]. For these reason there is nowadays interest in search for compounds which would potentially possess combined antioxidant and anticancer activity, providing pre-

ventive as well as therapeutic benefit in oxidative stress-related diseases, particular cancer [361,362]. More recently, phenolic compounds (mainly flavonoids and their derivatives) have evoked interest as promising compounds with above-mentioned combinatorial biological activity [6].

Antioxidant mechanism of phenolic compounds is relatively well-understood – i.e. mainly scavenging of free radicals or sequestration of metal ions (e.g. section 2.1.) [363]. However, the mechanism underlying anti-proliferative action of phenolic compounds (especially flavonoids) is not completely understood and still remains to be properly clarified. Tangeretin and nobiletin, flavonoids produced by citrus species, are among phenolics regarded as one of the most effective inhibitors of cancer cell growth *in vitro* and *in vivo* [364]. In one study where these flavonoids were tested towards several human breast cancer cell lines, cytostatic activity by induction of cell cycle arrest have been observed [365]. It was suggested that this effect is mediated by ability of tangeretin and nobiletin to influence expression of various cell cycle-controlling proteins and enzymes [366].

Various studies described other mechanism of anti-proliferative activity of phenolic compounds – i.e. through interference with apoptosis by their ability to promote formation of various apoptosis-inducing proteins and enzymes (such as caspase-3). This effect was for example described for apigenin and kaempferol. Moreover, increased levels of antioxidant compounds (e.g. catalase and SOD) were detected in treated cancer cells, suggesting that apigenin and kaempferol-mediated apoptosis might be facilitated by pro-oxidant activity of these flavonoids (generation of reactive species) [367,368]. Some compounds, such as quercetin, were even reported to possess both mechanisms of above-mentioned anti-proliferative activity (through cell-cycle arrest and induction of apoptosis), e.g. quercetin [369]. Antitumor activity of various phenolic compounds was also evidenced in clinical trials [370]. As contrast to antimitotics and topoisomerase inhibitors (section 2.3.2), above-mentioned compounds were claimed to be ineffective towards non-cancer cells, representing less-toxic alternative to commonly used chemotherapeutics. With regard to given information, it seems that anti-proliferative potential is not mediated via antioxidant-related manner. Though various studies declare partial contribution [371,372].

In view of above-mentioned information, it seems that phenolic compounds are nowadays regarded as important class of chemicals that would potentially serve as leads for discovery of novel compounds with sole or combined antioxidant and anticancer effect. Further sections will be focused on brief overview of traditional and modern use of edible and medic-

inal plants in connection with prevention and/or therapeutic benefit towards oxidative stress-related diseases. Special emphasis will be placed on reference of phenolic compounds.

## 2.5 Plant foods with specified health-promoting properties

It is widely believed, that nutrition is closely related to human health and that poor diet may play crucial role in progress of various chronic diseases. In the last few decades, scientific disciplines such as food science and preventive medicine had undergone great progress. This has led to development of foods, which, apart from their nutritional value, contain components and/or artificially added ingredients with specific medical (preventive or therapeutic) or other physiological benefit [373]. Several types of modified food products are given below:

1. Fortified foods and beverages. These were especially developed to lower micronutrients deficiencies within population (e.g. fortification of food with niacin) [374].

2. Medical food. Specifically formulated foods to treat diseases with distinctive nutritional needs (inability to metabolize a common molecule such as lactose or food containing slowly digestible carbohydrates in diabetes patients) [375].

3. Dietary supplements. Chiefly characterized as liquid or encapsulated product containing food derived ingredients (often used as complex mixtures such as herbs and other botanicals however may occur as sole components, including enzymes, vitamins, minerals etc.) [376].

4. Foods containing food additives. Constituents added to food products with aim to reduce natural decomposition. Few of these have also been suggested as agents with therapeutic benefit in various diseases [377]. However, this option is nowadays acknowledged only at theoretical/experimental level.

Definition of functional food and other modified food products are occasionally not clearly stated, which is also apparent from fact that above-mentioned terms are overlapped in some cases. As functional food is also considered as food product in which functional component have been lost due to processing and was afterwards artificially added back [378].

Despite the existence of relatively diverse types of functional foods, special emphasis was also placed to development of modified foods which could be helpful in prevention and treatment of diseases related to oxidative stress. Examples of these would be fruits, vegetables and other plant products which possess natural antioxidant potential and/or are subsequently

enriched with antioxidant molecules (plant-based compounds or their derivatives) [379]. These include primarily enriched foods with antioxidants or foods designed to treat other diseases (such as certain types of malnutrition) which later on demonstrated mechanism via antioxidant effect [380]. It is accepted that anticancer properties of plant foods is only restricted to preventive (not therapeutic) potential [381]. Few foodstuffs that are suggested to be helpful in oxidative stress-related diseases are listed below.

It have been previously revealed that tomatoes (*Solanum lycopersicum*) and watermelons (*Citrullus lanatus* var. *lanatus*) are relatively rich on triterpenoid lycopene. Consumption of these two plant species have been found to be associated with lowered risk of prostate cancer both *in vitro* and *in vivo* [382,383]. Broccoli (*B. oleracea* var. *italica*) had demonstrated relatively high levels of lutein [384] (another compound belonging to chemical class of triterpenoids) and there are reports of regular intake of this vegetable to decrease cognitive impairment in Alzheimer disease's patients [385]. Generally, various species of citruses (*Citrus* spp.) are regarded as rich source of flavonoids, namely naringin and quercetin. One study shows that administration of citrus fruits to patients suffering from lung cancer brought clinical benefit [386]. It was numerously proved that various anthocyanins (such as cyanidin, peonidin) are responsible for colouration of cranberries (*Vaccinium macrocarpon*) [387]. Cranberries and its products (e.g. jams, juices) were found to significantly reduce proinflammatory cytokines and alleviated oxidative stress-dependent mitochondrial dysfunction in human model [388]. Various foodstuffs containing isoflavonoids, such as soybean (*Glycine max*), were observed to reduce levels of LDL and total cholesterol in randomized controlled trials [389]. Soy extracts are sold as dietary supplement in some countries [390]. Stilbene resveratrol, which is mainly found in grapevine (*V. vinifera*) is also nowadays available as dietary supplement [5]. In pre-clinical findings it was discovered that resveratrol possess protective effect against various types of cancer and in cardiovascular disease [391]. Several plant-derived compounds, such as isoquercitrin (flavonoid found in *Mangifera indica* and *Rheum nobile*) [392] or bixin (apocarotenoid from *Bixa orellana*) [393], are added to certain foodstuff as colouring agent. Former compound was effective in experimental treatment of liver cancer [394], while latter demonstrated positive results in animal model of diabetes [395]. Hence, various food additives, apart from their preservative function, also might exhibit health beneficiary properties. Phytosterol-enriched foods and dietary supplements have been marketed for decades (e.g. sitosterol, stigmasterol, ergosterol) and vegetables are considered as rich source of these compounds. These include *Solanum surattense*, specific species of potatoes containing high levels of β-sitosterol, which demonstrated significant antidiabetic effect

in animal model [396]. Additionally, phytosterols are also regarded as cholesterol lowering agents [397], however their beneficial impact in cardiovascular problems was never proved [398]. Food products with artificially added folic acid (e.g. grains or flour) were developed primarily to be consumed by pregnant woman, in order to reduce neural tube defects in newborns [399]. However, clinical benefit of folic acid-fortified food was also evidenced in other conditions such as prevention of age-related macular degeneration, especially in patients with increased risk of cardiovascular disease [400]. Other groups of chemicals, such as thiols, isothiocyanates, fatty acids, polyols of various vitamins or phenolic compounds are regarded as functional constituents as well.

## 2.6 Traditional uses of plants

Plant products have always played an important role in human live. At present, there is considerable interest in detailed identification and characterization of antioxidant and anti-proliferative potential of plant-based natural products and their possible use in various branches of industry (including the use as foods and pharmaceutics). For this reason, many ethnobotanists gather information on plant material in which traditional use could be explained by antioxidant and anti-proliferative mechanism [401,402]. Various ethnomedicinal indications for antioxidant and/or anti-proliferative activity are generally accepted, which serves as selection criteria for plant material collection. For antioxidant activity reference is made to conditions such as arthritis, diabetes, cardiovascular, inflammatory and neurodegenerative disorders, rheumatism, snakebite and wound-healing properties [1,403]. In case of anticancer effect, following examples of descriptions are taken into consideration: abscesses, calluses, cancer, corns, hard swellings, polyps, stomach-ache, tumours, and warts [285,262]. It should be noted that many of the traditional claims for efficacy should be viewed with some scepticism because anticancer and (especially) antioxidant activity, is likely to be poorly defined in terms of folklore and traditional medicine. Few cases of traditional use of plants in conditions or diseases likely to be related to oxidative stress are listed below.

*Curcuma longa*, plant traditionally used in Indian continent as mean for food colorant and as natural remedy (by Ayurvedic medicine) in treatment of various illnesses, including those of the skin, pulmonary, and gastrointestinal systems, aches, pains, sprains, tumours, wounds and liver disorders [404]. These activities were credited to phenolic compound curcumin. Lower incidence of neurodegenerative diseases in Indian continent is explained by long tradition of consumption of foods containing *C. longa* (such as curry spice) by local population. Indeed, curcumin was found to prevent and delay brain pathology in Alzheimer's

disease [405]. Consistent with claims to be traditionally used in treatment of tumours, curcumin have demonstrated anticancer activity in animal models [406]. Extract of the leaves of *Ginkgo biloba* has been used to improve memory for thousands of years. This tree was found to produce ginkgolides, which exhibits antioxidant and neuroprotective activities [407]. Since this discovery, many other plants traditionally used to treat brain disorders (such as Alzheimer's and other neurodegenerative diseases) have been described [408]. Ginger (*Zingiber officinale*) was considered by indigenous Asian and Arabic cultures as important component of diet, however was used as medicinal plant, especially in treatment of arthritis [409]. Beneficial effect of ginger extract in this disease was later on affirmed by clinical studies [410]. Kampo medicines are traditionally practiced in Japan, above all also used in treatment of certain types of cancer [411,412]. Extracts used in Kampo are composed of multiple herbs and it was found out to contain a complex mixture of phenolic compounds, but aslo glycyrrhizic acid, the chief sweet-tasting constituent of *Glycyrrhiza glabra* root [413]. This compound has evoked interest as possible anticancer drug [414]. Extracts of propolis, plant-originated resinous substance used by bees in hive construction, have often been used in herbal medicine in treatment of different disorders, including cancer [415]. Propolis contains high levels of phenolic and other plant-derived compounds (such as caffeic acid phenethyl ether), which are believed to be responsible for its health beneficiary properties [416]. Roots of *Polygonum cuspidatum*, used extensively in traditional Chinese medicine to treat cancer [321], are rich in stilbene resveratrol [5]. Various fruit-producing plants, including *Euterpe oleracea*, were reported to be traditionally used by indigenous cultures in South America to treat diabetes, but also cancer and cardiovascular problems. These therapeutic effect have been attributed to phenolic compounds and vitamin C [417]. In Madagascar, *Catharanthus roseus* was primarily used in management of diabetes [418]. Apart from antimitotic alkaloids (see section 2.4.2), this plant was demonstrated to be rich in phenolic compounds, namely derivatives of 2,3-dihydroxybenzoate and phenylpropanoids [419], which are believed to be responsible for observed antidiabetic effect.

Even though plant-based natural products with high levels of phenolic compounds are generally considered as safe and to possess health-promoting properties, few have been reported to possess adverse toxicological effects. This include nordihydroguaiaretic acid isolated from *Larrea divaricata* [420], extract of *Cratoxylum cochinchinense* [421] and also various flavonoids (e.g. hesperitin) [422].

## 2.7 Underutilized crops and wild plants

Despite the fact that there are many records throughout ethnobotanical studies aimed on traditional usage of plants in treatment of various illnesses connected to oxidative stress, relatively large proportion of these stays scientifically undiscovered (especially true for plant material originated in third world countries). Antioxidant and anti-proliferative assays were primarily examined in relatively well-known plant material (e.g. spices; as noted above). However, there is nowadays increased interest in characterization of above-mentioned biological activities together with analysis of related secondary metabolites in plant-material generally recognized as underutilized or wild. Underutilized crops are described as plant species with strong linkage to cultural heritage of their places of origin and which have been traditionally used for various purposes (drink, food, medicinal etc.); however their importance and public awareness over time have been reduced due to biological and/or socioeconomic reasons [423]. Special emphasis is nowadays placed on examining following categories of underutilized crops and wild plants: (i) food-derived beverages, (ii) edible plants and (iii) medicinal plants [424,425]. Brief summary of these categories and their examples is given below.

### 2.7.1 Wine from Georgia

Grapevine and its products are considered to be one of the richest natural sources of antioxidant compounds such as phenolic acids, stilbenes, flavonoids, and anthocyanidins [426]. Accordingly, the moderate wine consumption is nowadays recognized as risk-reducing factor in several human diseases related to oxidative-stress such as cancer, type 2 diabetes, inflammation and especially myocardial infarction [427–430]. Paradoxical epidemiological phenomena, also known as “French paradox”, was observed among French population, who had relatively low incidence of coronary heart disease, while consuming diet rich on saturated fats (such as cheese). It was suggested that adverse side-effects of inappropriate food intake is compensated by consumption of relatively large amounts of wine [431,432]. It is widely believed, that these health-promoting properties of wine are ascribed to antioxidant potential of above-mentioned compounds. Composition of health-beneficiary chemicals (e.g. phenolics) presented in wine varies markedly depending mainly on the grape cultivar. However other factors, such as soil, nutrition, climatic conditions, weather, winemaking procedure, conditions of maturation and storage, are also considered to be important [433–435]. Approximately 1,400 cultivars are registered and used for commercial production of wine [436]. Large proportion of these (mainly the lesser known cultivars) remains undiscovered by means of

detailed phytochemical composition, biological activities and herewith connected health-promoting properties.

Wine grapes are chiefly grown between the 30$^{th}$ and the 50$^{th}$ degree of latitude, in both the Northern and Southern hemispheres. France, Italy, Spain, USA, Argentina, China Australia, Chile, South Africa and Germany are considered as top 10 wine producing countries in the world. Georgia is listed on position 22 among top 40 wine producing countries [437]. Yet, Georgia, which is considered to be cradle of the world's winemaking [438], is one of the countries where many underutilized (or less known) local grapevine varieties are cultivated. It is believed that viticulture and winemaking first began in the South Caucasus region around 6,000 years ago, and this fact is supported by several archaeological findings [439]. At present, the Georgian vine gene pool contains up to 525 underutilezed white and red varieties (e.g. Aladasturi, Budeshuri Titseli, Chinuri, Dzvelshavi Obchuri, Goruli Mtsvane, Mtsvane Kakhuri, Ojaleshi, Shavkapito, Tavkveri, Usakhelouri) [10]. Moreover, some of the Georgian wines are reported to be prepared by so called Kakhetian method. This winemaking technique, which is believed to be elaborated in Kakhetian region, is characterized by long period (up to 5 month) of maceration and fermentation of must with usage of 100% of grape pomace (mixture of skin, seeds, stems) in clay vessel called "kvevri" buried under ground. Despite the above-mentioned information, to the best of my knowledge, only scarce data about phytochemical profile and antioxidant activity of Georgian wines, especially prepared by Kakhetian methods, is available [440]. Accessible studies done on traditional grape cultivars originated in Georgia are given below.

Chkhikvishvili et al. [441] compared antioxidant activity and the content of *trans*- and *cis*-resveratrol in Georgian (from cultivars Saperavi, Kakhuri Tsarchinebuli), European, and South and North American red wines. A comparison among several red and white wines produced by Kakhetian and commonly used "European method" in total content of phenolics, catechins, proanthocyanidins, anthocyanins and antioxidant activity was published by Shalashvili et al. [442]. Recently, another study done by Shalashvili et al. [12] was aimed at investigation of the content of some flavonoids, phenolic acids and resveratrol in Georgian wines prepared from local cultivars Saperavi (red) and Rkatsiteli (white). The content of catechins, hydroxycinnamic acids, volatile compounds and their glycosides in wines prepared by Kakhetian and European wine making processes (juice fermented without stems for 7–30 days; white wines fermented without the presence of pomace) has been compared by two studies of Mikiashvili et al. [443,444]. The content of total catechins, proanthocyanidins, flavanols, and some phenolic acids in wines produced from white cultivars Kakhuri Mtsvivani,

Rkatsiteli, Kakhuri Mtsvane, and Khikhvi prepared by Kakhetian and European wine making method was investigated by Glonti [445]. Glonti and Glonti [446] also published extensive study where total phenolics, sulphur, various volatile compounds, amino acids, and minerals were quantified in wines fermented in kvevri and by standard process.

### 2.7.2 Edible and medicinal plants from Peruvian Amazon

As indicated above, common dietary plants (such as fruits, vegetables, nuts) are recognized as main source of antioxidant compounds, and indeed, antioxidant potential is primarily examined in these categories of plant material [3]. However, recent studies indicate that other categories of plants (e.g. medicinal plants) possess this biological activity as well [312,2]. On the other hand, medicinal plants are considered as valuable source of pharmacologically active substituents, among other those that exert antineoplastic activity. Previously it was proposed that progression and secondary complications of cancer is strongly related to oxidative stress. Anticancer activity of medicinal plants is chiefly credited to alkaloids, diterpenoids but also various phenolic compounds (section 2.4.2) [262]. Despite the beneficial effect in cancer treatment, majority of these compounds also provide damage to normal cells. More recent studies are showing that dietary phenolics (e.g. flavonoids) may exert anti-proliferative effect as well [6], and also less side-effects in comparison to commonly used plant-based chemotherapeutics. Even though medicinal plants are regarded as the main sources of antineoplastic agents, there is now an increased interest in research of edible plants' anti-proliferative effect [7]. For reasons given-above, newly introduced edible and medicinal plants are nowadays routinely supplemented by validation of antioxidant effect as well as analysis of anti-proliferative activity against various types of carcinoma cell lines [4].

Even though wild plants used for dietary and medicinal purposes are generally considered as very important factor for maintaining food and health security (mainly in third world countries), till present-day health-promoting properties of majority of these plants have not been properly verified via modern scientific methods [447]. Moreover, with current trends in destruction and ever-increasing loss of natural habitats by human activities (such as agriculture practices, logging), there is serious risk of plant diversity being irreversibly lost with interconnected valuable scientific information. One of the world's regions where the above-mentioned phenomena occur quite intensively is, among others, Peruvian Amazon [448]. In the same time, this area is considered as genetic centre for huge array of plant species and as a biodiversity hotspot. Since many unexplored areas in Amazon still exists, it is nearly impossible to determine exact plant diversity of this region. However, it is estimated by various au-

thors that Amazon Basin shelters more than 50,000 species of higher plants [449]. Among commercially and pharmaceutically interesting plants with edible and medicinal purpose, Amazon basin encompasses species such as brazil nut (*Bertholletia excels*), cassava (*Manihot esculenta*), cocoa (*Theobroma cacao*), crown cinchona (*Cinchona officinalis*), ipecac (*Carapichea ipecacuanha*), maize (*Zea mays*), pineapple (*Ananas comosus*), and papaya (*Carica papaya*) to name a few [450]. Despite the fact that traditional use of edible and medicinal plants from Peruvian Amazon in treatment of diseases related to oxidative stress (such as cancer, diabetes, chronic inflammatory, cardiovascular and neurodegenerative diseases) is relatively well documented, as far as I know, in only a very small proportion of these plants antioxidant and anti-proliferative profiles were assessed [451].

Brief summary of ethnomedicinal data as well as research done on these species with relevance to antioxidant and anti-proliferative activity is given below. *Annona montana* is an edible fruit and medicinal plant traditionally used mainly in treatment of cancer [451]. There are numerous records on anti-proliferative efficacy of various Annonaceous species (such as *A. montana*, *A. squamosa* or *A. reticulata*), and sometimes are referred to as cancer-killers [452]. Acetogenins are believed to be chiefly responsible for anti-proliferative effect of *Annona* species [453]. However, up till now the cytotoxicity and the respective compounds for this biological activity were never described for *A. montana*. *Inga edulis* is widely grown by indigenous Amazonian inhabitants for various purposes, including as dietary and medicinal plant. Records in literature indicates that *I. edulis* has been especially used in treatment of rheumatoid arthritis [454]. Pompeu et al. [455] have earlier assessed anti-proliferative effect of *Inga edulis* towards various carcinoma cell lines including multidrug resistant variants. Only moderate to weak activity was observed though. Souza et al. [456] detected gallic acid, catechin, epicatechin and derivatives of myricetin and quercetin in leaves of in *I. edulis*. *Oenocarpus bataua* is considered locally as highly nutritious fruit, and is widely consumed throughout the Amazon basin [457]. Moreover it has been widely used by Amazonian healers in treatment of cancer [458]. Rezaire et al. [459] have assayed *O. bataua* for antioxidant activity and analysed 11 phenolic compounds (derivatives of chlorogenic acid, syringic acid, resveratrol and various anthocyanidins) in fruit. Records on anti-proliferative activity of this palm species are unavailable.

### 2.7.3 Wild medicinal plants from Ethiopia

Similarly as in Peruvian Amazon, at this moment majority of plant species originated in Ethiopia have not been subjected to modern scientific evaluation of their proposed biologi-

cal activities. In the same time, local flora is in danger of being irretrievably lost by present-day tendencies in natural habitat devastation, mainly achieved by above-mentioned human activities [9,460]. Moreover, Ethiopia is also recognized as genetic centre for wide range of plant species. It is predictable, that Ethiopian flora covers approximately up to 8,000 vascular plant species [461]. Medicinal plants with commercial and pharmaceutical interest originated in Ethiopia are for example Abyssinian hard wheat (*Triticum aethiopicum*), castor (*Ricinus communis*), coffee (*Coffea arabica*), grain sorghum (*Sorgum bicolor*), cowpea (*Vigna unguiculata*), niger seed (*Guizotia abyssinica*), okra (*Abelmoschus esculentus*), sesame (*Sesamum indicum*) [462]. Indeed, as in case of plants originated in Peruvian Amazon, many of these plants from Ethiopia were comprehensively documented to be traditionally used in treatment of various conditions (these are mentioned above) likely to be mediated by oxidative stress. Yet, to my very best knowledge, only a very small percentage of these species were assayed for their antioxidant and anti-proliferative properties [463].

Although research with regard to antioxidant and anti-proliferative activity of Ethiopian medicinal plants (including *Dodonaea angustifolia*, *Rumex nepalensis* and *Verbascum sinaiticum*) is very limited (in some cases unavailable), there are at least some records on phytochemical analyses. Brief summary of ethnomedicinal information together with available phytochemical data of these species is given below. *D. angustifolia* was previously reported to be traditionally used as medicine in treatment of lymphatic swelling [464]. Omosa et al. [465] investigated leaf extract of *D. angustifolia* for presence of secondary compounds and discovered various chemicals belonging to neo-clerodane diterpenoids. Some members of this chemical class have previously demonstrated antioxidant and anti-proliferative effect [466–468]. Traditional medicinal knowledge describes *R. nepalensis* to be used in treatment of rheumatism [469] and stomach-ache [470]. Various derivatives of anthraquinones were previously isolated and analysed from *R. nepalensis* [471]. These have subsequently demonstrated antioxidant and anti-proliferative effect [472,473]. *V. sinaiticum* has relatively long tradition in treatment of cancer [474]. This plant have also been reported to be used by African medicinemans in treatment of various conditions referred to as stomach-ache [475]. *V. sinaiticum* was previously described to possess significant anti-proliferative activity against towards various carcinoma cells (Hep-2, MCF-7, and Vero lines) [476] and moderate antioxidant activity in DPPH assay [477]. Furthermore, various compounds such as two flavonolignans hydrocarpin and sinaiticin, and two flavones chrysoeriol and luteolin were identified in *V. sinaiticum*. All constituents exhibited dose-dependent cytotoxicity when tested against P-388 (lymphoma) cells [478].

# 3 Hypothesis

Since human population is nowadays facing to an increased incidence of oxidative-stress related diseases with additional rapid spread of resistance of cancer cells towards commonly used chemotherapeutics, there is an urgent need of new effective agents. One option to overcome above-mentioned phenomena is seen in use of novel plant-derived preparations (including food products) or sole compounds with combinatorial antioxidant/anti-proliferative efficacy, which would serve either as prevention or as active therapeutics. Selection of plant material (underutilized Georgian wine cultivars, extracts of edible and medicinal plants from Peruvian Amazon and extracts of Ethiopian medicinal plants) tested in this study was based on their previous report on traditional usage for treatment of diseases likely to be associated with oxidative stress. Even though, some of these plants have been indicted to possess significant antioxidant and/or anti-proliferative potential, to our best knowledge, (i) many of these were not tested with ORAC assay (which is considered as the most biologically relevant method to measure *in vitro* antioxidant activity), (ii) assayed towards specific combination of carcinoma/normal cell lines and (iii) detailed phytochemical profile was not established. Therefore, there is a high probability that systematic screening of these plants can lead to discovery of extracts and novel compounds with potent antioxidant/anticancer activity.

# 4 Objectives

Objective of this study was to investigate antioxidant and anti-proliferative effects and chemical composition of wines, food and medicinal plants from Georgia, Ethiopia and Peru.

The specific aims of this study were:

1. To determine *in vitro* antioxidant activity with use of specific combination of assays based on measurement of different antioxidant mechanisms, in order to ensure the maximal biological relevancy of gained results.

2. To assess *in vitro* anti-proliferative effect where special emphasis was payed to detect selective cytotoxic activity towards carcinoma and normal cell lines.

3. To identify relationship between tested biological activity and chemical composition with use of analytical techniques and statistical methods.

# 5 Materials and methods

## 5.1 Plant material

Based on previously reported data on traditional use for treatment of diseases likely to be associated with oxidative stress, following plant material was selected for further testing: (i) 39 wine samples of underutilized Georgian grapevine cultivars, (ii) 23 samples of edible and medicinal plants from Peruvian Amazon and (iii) extracts of 22 samples of Ethiopian medicinal plants. Detailed description of selection, collection/purchasing and other essential information (e.g. ethnomedicinal data, voucher specimen numbers) of each category of collected plant material is properly given in **I**, **II** and **III.** Map of the study area is given in Figure 4.

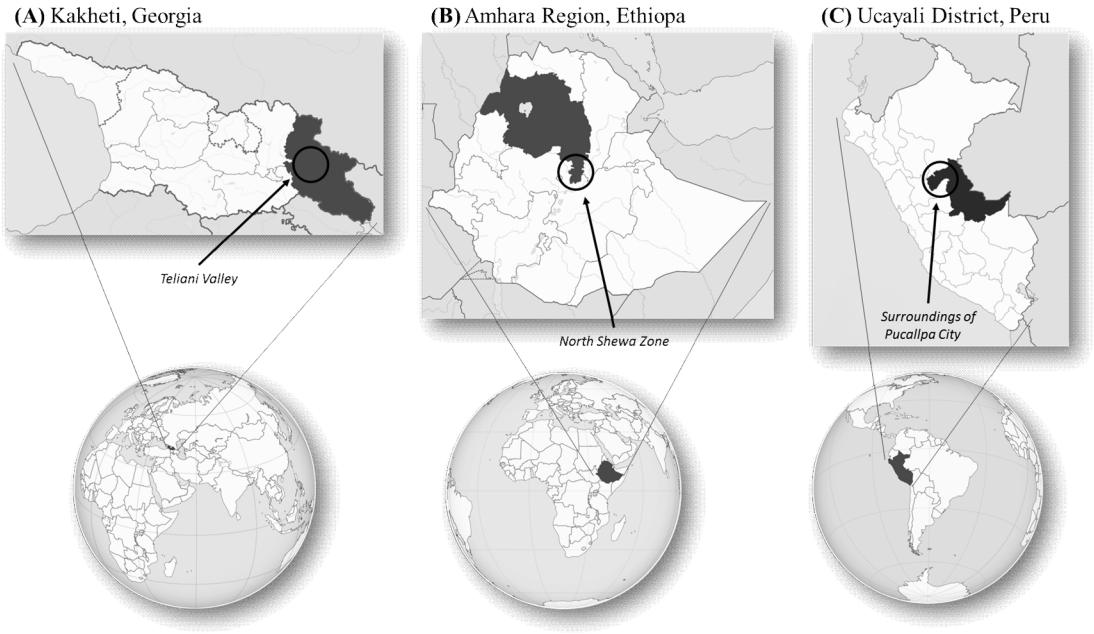


**Figure 4** Collection sites of tested plant material (adopted from **[479]**)

## 5.2 Sample preparation

Bottled samples of wines were tested unprocessed. Ethiopian medicinal plants and edible and medicinal plants from Peruvian amazon were dried and extracted. Exact process of preparation is described in **II** and **III**, respectively.

## 5.3 Reagents, solvents and standards

Complete list of reagents, solvents, standards and other chemicals used in this study is given in **I**, **II** and **III**.

## 5.4 Cell culture

Human liver carcinoma cell line (Hep-G2), human colon carcinoma cell line (HT-29) and normal cell line (MRC-5) were used in this study for assessment of cell viability assay. Proper description of maintenance of cell cultures is described in **II** and **III**.

## 5.5 Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging [480], oxygen radical absorbance capacity (ORAC) [481] and total phenolic content (TPC) [482] assays have been used for determination of antioxidant properties of samples tested. Specific conditions of each assay are properly described in **I**, **II** and **III**.

## 5.6 Total sulphite content assay

In order to evaluate influence of sulphites added to wine on samples' antioxidant efficacy (via free radical scavenging ability), wines were submitted to analysis of total sulphite content. For this purpose, commercially available kit EnzytecTM Colour $SO_2$-Total kit (R-BiopharmAG, Darmstadt, Germany) was used. Detailed description is given in **I**.

## 5.7 Cell viability assay

Determination of anti-proliferative effect of samples were performed using modified MTT (tetrazolium bromide) cell proliferation assay [483]. Method is properly stated in **II** and **III**.

## 5.8 High performance liquid chromatography

For better understanding of relationship between tested biological activity and chemical composition, analytical techniques based on high performance liquid chromatography (HPLC) hyphenated to ultraviolet/visible light (UV/Vis) or tandem mass spectrometer (MS/MS) detectors were used. HPLC methods are described in detail in **I** and **III.** Structures of some analysed phenolic compounds tested in this study are given in Figures 1 – 3.

## 5.9 Statistical analysis

Gained results were analysed using various statistical tests. Detailed description of statistical analyses is given in each original publication (**I**, **II** and **III**).

# 6 Results and discussion

In this study, the *in vitro* antioxidant and/or anti-proliferative activity and chemical composition of wines, food and medicinal plants from Georgia, Ethiopia and Peru were examined. The results showed that Georgian wines possessed significantly higher antioxidant potential in DPPH ($p$ = 0.01), ORAC ($p$ = 0.05) and TPC ($p$ = 0.01) assays in comparison to Central- and West-European wines. The highest antioxidant effect was recorded in red wines, particularly Saperavi cultivars (range of DPPH, ORAC and TPC values: 8.59 – 2.08 g TE/L wine; 12.14 – 7.29 g TE/L wine and 4.46 – 1.59 g GAE/L wine, respectively), followed by European cultivars (Pinot Noir and Cabernet Sauvignon). Similar results were also observed elsewhere [441]. White wines were significantly less effective in antioxidant assays than the red wines. Only exception was sample RK 4 (cuvee of Rkatsiteli), which was fermented by Kakhetian method. Wine RK 4 exhibited antioxidant efficacy comparable to red wines (DPPH, ORAC and TPC values 2.90 g TE/ L wine; 4.01 g TE/ L wine and 1.88 g GAE/ L wine, respectively). Stronger antioxidant effect of white wines prepared by traditional Kakhetian technology (in comparison to wines prepared by European method) was also recorded by Shalashvili et al. [442]. Results of HPLC-UV/Vis analysis revealed that Georgian wine samples contained significantly higher concentrations of quercetin ($p$ = 0.01), kaempferol ($p$ = 0.05), and syringic acid ($p$ = 0.05), while West and Central European samples were considerably richer in *trans*-resveratrol ($p$ = 0.01). The highest quantities of quercetin, kaempferol and syringic acid were recorded in wines prepared from Saperavi cultivar (quercetin = 14.44 – 1.07 µg/mL; kaempferol = 1.68 – 0.03 µg/mL and syringic acid 12.59 – 4.72 µg/mL), whereas Pinot Noir was the richest sample in resveratrol content, followed by representatives of Cabernet Sauvignon (concentration range 8.71 – 2.43 and 7.41 – 1.13 µg/mL, respectively). These results are in accordance with several comparative studies dealing with differences in phenolic composition across wine cultivars, vintage and production area [484–486]. None statistical differences between white wines grouped according to their origin were revealed. In view of these results, it is suggested that that oenological techniques and cultivar have higher impact than climatic factors on the content of phenolic compounds and therefore antioxidant potential red and white of wines, which is in agreement with studies of Goldberg et al. [487,488]. Additionally, results from correlation demonstrated that content of sulphites had negligible impact on the antioxidant capacity (in ORAC and DPPH models) of tested wines which is in the agreement with study of Zúñiga et al. [489].

Combined antioxidant/anti-proliferative effect was observed for extracts of *D. angustifolia*, *R. nepalensis* (Ethiopian medicinal plants), *I. edulis* and *O. bataua* (edible and medicinal plants from the Peruvian Amazon). Values for antioxidant and anti-proliferative activity were recorded as follows: *D. angustifolia* ($IC_{50}$ for DPPH = 22.2 µg/mL, ORAC = 767.6 µg TE/mg extract, TPC = 531.7 µg GAE/mg extract; $IC_{50}$ for Hep-G2 = 120.0 µg/mL); *R. nepalensis* ($IC_{50}$ for DPPH = 5.7 µg/mL, ORAC = 1061.4 µg TE/mg extract, TPC = 1101.5 µg GAE/mg extract; $IC_{50}$ for Hep-G2 = 50.5 µg/mL); *I. edulis* (DPPH and ORAC = 337.0 and 795.7 µg TE/mg extract, TPC = 262.3 µg GAE/mg extract; $IC_{50}$ for Hep-G2 and HT-29 = 36.3 and 57.9 µg/mL) and *O. bataua* (DPPH and ORAC = 903.8 and 1024.4 µg TE/mg extract, TPC = 672.3 µg GAE/mg extract; $IC_{50}$ for Hep-G2 and HT-29 = 102.6 and 38.8 µg/mL). Despite the fact that extracts of *Jasminum abyssinicum* ($IC_{50}$ for DPPH = 26.3 µg/mL, ORAC = 1023.7 µg TE/mg extract), *Rubus steudneri* ($IC_{50}$ for DPPH = 5.8 µg/mL, ORAC = 1019.2 µg TE/mg extract), *Mauritia flexuosa* (DPPH and ORAC = 1062.9 and 645.9 µg TE/mg extract), *Myrciaria dubia* (DPPH and ORAC = 641.9 and 642.6 µg TE/mg extract) and *Theobroma grandiflorum* (DPPH and ORAC = 714.8 and 821.9 µg TE/mg extract) have exhibited considerable antioxidant effect, these species were found to possess moderate to low anti-proliferative potential or have shown to be toxic to normal cells line. Antioxidant profiles of *D. angustifolia*, *R. nepalensis*, *I. edulis* and *O. bataua* have previously been reported [490,472,491,459], whereas these results are with correspondence with our study. Except of *I. edulis*, this is the first report on anti-proliferative activity of above-mentioned plant species. Despite the fact that *I. edulis* have earlier demonstrated relatively low cytotoxic efficacy towards various carcinoma cell lines including multidrug resistant variants [455], in our study leaves of *I. edulis* exhibited moderate anti-proliferative activity against Hep-G2 and HT-29 cells. Based on the literature reports on secondary metabolites so far detected in these plant species [472,459,473,465,492–494] together with results in this study from statistical analysis (correlation between TPC and antioxidant and anti-proliferative activity) and results from HPLC-MS/MS analysis, it is suggested that phenolic compounds and their derivatives might completely or partially be responsible for observed combinatory antioxidant/anti-proliferative effect.

Among Ethiopian medicinal plants and edible and medicinal plants from the Peruvian Amazon, *V. sinaiticum* and *A. montana* demonstrated significant anti-proliferative potential. Values for anti-proliferative efficacy were recorded as follows: *V. sinaiticum* ($IC_{50}$ for Hep-G2 = 80.6 µg/mL) and *A. montana* ($IC_{50}$ for Hep-G2 and HT-29 = 2.7 and 9.0 µg/mL, respectively). Moreover, these plant extracts also proved to be nontoxic to normal MRC-5 cells

($IC_{50}$'s for both samples were detected at levels higher than 500 µg/mL). Anti-proliferative efficacy of *V. sinaiticum* is relatively well established and our results are in accordance with cytotoxic properties of this plant observed in other studies [476]. Despite the existence of records on anti-proliferative efficacy of various Annonaceous species (such as *A. muricata*, *A. squamosa* or *A. reticulata*) [452], up till now the cytotoxicity and the respective compounds for this biological activity were never described for *A. montana*. Even though extracts of *V. sinaiticum* and *A. montana* demonstrated considerable selective cytotoxic effect, their antioxidant potential was recognized as moderate to low. With regard to results observed in this study as well as information from the literature [453,478,495], phenolic compounds are considered to be only partially responsible for anti-proliferative efficacy of these plant species. Results and discussion for Georgian, Central- and West-European wines, Ethiopian medicinal plants and edible and medicinal plants from the Peruvian Amazon are described in detail and summarized in **I**, **II** and **III**, respectively.

# 7 Conclusion

In summary, the current study proved *in vitro* antioxidant and anti-proliferative activity of various underutilized wines, food and medicinal plants of Georgian, Ethiopian, and Peruvian origin. Especially antioxidant potential of Georgian grapevine varieties Saperavi and Rkatsiteli, combinatory antioxidant/anti-proliferative efficacy of extracts of *Dodonaea angustifolia*, *Rumex nepalensis*, *Inga edulis* and *Oenocarpus bataua*, and selective anti-proliferative effect of *Verbascum sinaiticum* and *Annona montana* extracts deserve deeper research attention. Despite the fact that other plant species demonstrated substantial activity, such as *Jasminum abyssinicum* and *Rubus steudneri* (Ethiopian medicinal plants), *Mauritia flexuosa*, *Myrciaria dubia*, and *Theobroma grandiflorum* (edible and medicinal plants from Peruvian Amazon), some of these have also exerted toxicity to normal cells and/or significant efficacy in only one of the performed assays. Results indicate that phenolic compounds might be fully responsible for observed combinatory antioxidant/anti-proliferative effect, whilst only partially in case of selective anti-proliferative activity. Above-mentioned plant species might potentially serve as prospective material for further development of plant-derived antioxidant and/or anti-proliferative agents. However, further research which would be focused on detailed characterization of their chemical composition, pharmacological effects and toxicological safety is required, in order to verify possible practical use of these plant species.

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# 9 Appendices

## Appendix A:

**Tauchen J**, Maršík P, Kvasnicová M, Maghradze D, Kokoška L, Vaněk T, Landa P. In vitro antioxidant activity and phenolic composition of Georgian, Central and West European wines. Journal of Food Composition and Analysis. 2015;41: 113-121.

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Original Research Article

# In vitro antioxidant activity and phenolic composition of Georgian, Central and West European wines

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ABSTRACT

Georgia is a traditional country of viticulture and winemaking but only a few studies have been focused on wines originating from this area. In this study, we compared antioxidant effect, total sulfite content and concentration of 14 phenolic compounds of some native Georgian red and white wines with wines commonly produced in Central and Western Europe. Georgian red wines exhibited higher antioxidant capacity in DPPH, ORAC and total phenolic content assay. Further, Georgian red wines were richer in quercetin, kaempferol and syringic acid content, while the concentration of *trans*-resveratrol was lower than in Central and West European red wines. While differences among red wines from different origins and cultivars were observed, winemaking technology was the most important factor in the case of white wines. Kakhetian method increased antioxidant effect and levels of some phenolic compounds in comparison with white wines prepared by common European method. Our findings suggest that Georgian wines deserve further attention because of their high content of phenolics and high antioxidant capacity.



## 1. Introduction

Antioxidant activity of natural products has received much interest over the past few years, both in public and scientific community. Generally, it is believed that consumption of plant phenolics decreases the risk of occurrence of diseases related to oxidative stress (Georgiev et al., 2014). This biological effect is explained by the ability of these compounds to act as antioxidant agents; i.e. act as regulators of unwanted damaging oxidative processes (Liang et al., 2014; Gris et al., 2013). Therefore, the identification of foodstuff rich in phenolic compounds is still important.

Grapevine and its products are considered to be one of the richest natural sources of phenolic compounds such as phenolic acids, stilbenes, flavonoids, and anthocyanidins (Teixeira et al., 2014). Accordingly, the moderate wine consumption is nowadays

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recognized as risk-reducing factor in several human diseases related to oxidative-stress such as cancer, type 2 diabetes, inflammation and myocardial infarction (Fagherazzi et al., 2014; Kutil et al., 2014; Rossi et al., 2014; Russo et al., 2014). Composition of health-promoting chemicals (e.g. phenolics) presented in wine is influenced by many factors such as winemaking procedure, conditions of maturation and storage, grape cultivar, soil, nutrition, climatic conditions and weather (Singleton, 1982; de Pascali et al., 2014; King et al., 2014; Ivanova-Petropulos et al., 2015). Large proportion of nearly 1400 cultivars used for commercial production of wine (Robinson et al., 2012), stays still undiscovered by means of phytochemical composition, biological activities and herewith connected health-promoting properties.

Georgia, which is considered to be cradle of the world's winemaking (This et al., 2006), is one of the countries where many local grapevine varieties are cultivated. It is believed that viticulture and winemaking first began in the South Caucasus region around 6000 years ago, and this fact is supported by several archeological findings (Imazio et al., 2013). At present, the Georgian vine gene pool contains up to 525 white and red varieties (Maghradze et al., 2012). However, to the best of our knowledge, only scarce information about phytochemical profile and antioxidant activity of Georgian wines is available. Chkhikvishvili et al. (2008) compared antioxidant activity and the content of *trans*- and *cis*-resveratrol in Georgian (from cultivars Saperavi, Kakhuri Tsarchinebuli), European, and South and North American red wines. A comparison among several red and white wines produced by Kakhetian and European method in total content of phenolics, catechins, proanthocyanidins, anthocyanins and antioxidant activity was published by Shalashvili et al. (2007). Recently Shalashvili et al. (2012) investigated the content of some flavonoids, phenolic acids and resveratrol in Georgian wines prepared from local cultivars Saperavi (red) and Rkatsiteli (white). The content of catechins, hydroxycinnamic acids, volatile compounds and their glycosides in wines prepared by Kakhetian (juice fermented with skins, seeds, and bunch stems in clay vessel [kvevri] dug into the ground for three to five months) and European wine making processes (juice fermented without stems for 7–30 days; white wines fermented without the presence of pomace) has been compared by Mikiashvili et al. (2010a,b). The content of total catechins, proanthocyanidins, flavanols, and some phenolic acids in wines produced from white cultivars Kakhuri Mtsvivani, Rkatsiteli, Kakhuri Mtsvane, and Khikhvi prepared by Kakhetian and European wine making method was investigated by Glonti (2010a). Glonti and Glonti (2013) also published extensive study where total phenolics, sulfur, various volatile compounds, amino acids, and minerals were quantified in wines fermented in kvevri and by standard process.

In our study, we investigated antioxidant activity (using ORAC and DPPH method), total phenolic content, total sulfite content and quantified 14 phenolic compounds (by HPLC-UV/Vis method) including phenolic acids, flavonoids and stilbenes in Georgian red (Alexandrouli, Saperavi and cuvee of Saperavi and Saperavi Budeshuriseburi) and white wines (Rkatsiteli, and cuvee of Rkatsiteli and other local cultivars). Central and West European red (Cabernet Moravia, Cabernet Sauvignon, Pinot Noir) and white wines (Chardonnay, Sauvignon Blanc) were assayed with the aim to compare the differences between regions and cultivars.

## 2. Materials and methods

### 2.1. Wines

Red and white wine samples were acquired from different regions of Georgia, Czech Republic, France, Italy, and Austria, and were provided by local producers or purchased from supermarkets or wine stores. A total of 26 red wines of the cultivars Pinot Noir ($n = 5$), Cabernet Sauvignon ($n = 7$), Cabernet Moravia ($n = 2$), Seperavi ($n = 9$), cuvee of Saperavi and Saperavi Budeshuriseburi ($n = 2$), and Alexandrouli ($n = 1$) and 13 white wine samples of the cultivars Chardonnay ($n = 6$), Sauvignon Blanc ($n = 3$), Rkatsiteli ($n = 2$), and cuvee of Rkatsiteli and other local cultivars ($n = 2$) were assayed. Detailed information about tested wines (cultivar, vintage, producer, origin and wine type) is given in Table 1.

### 2.2. Winemaking methods

The majority of red and white wines included in this study were made based on the common "European methods". However, white wine samples RK 1 and RK 4 from Georgia, were made by the Kakhetian method, which is one of method elaborated in Georgia. This style of wine is based on long period (up to 5 month) maceration and fermentation of must with usage of 100% of grape pomace (skin, seeds, stems) in clay vessel called "kvevri" buried under ground (Gagunashvili, 2006; Glonti, 2010a,b).

### 2.3. Reagents, solvents and standards

2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, purity 97.0%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-methylphenol (BHT, ≥99.0%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox, 97.0%), analytical grade quality solvents, Folin-Ciocalteu reagent, fluorescein sodium salt (FL, 93.3%), fluorosalicylic acid (97.0%), standards of phenolic acids: 3,4-dihydroxybenzoic (purity ≥ 97%.0), caffeic (≥98%.0), chlorogenic (94.9%), gallic (97.9%), ferulic (99.0%), *p*-coumaric (≥98.0%), syringic (≥95.0%) and vanillic acid (≥97.0%); flavonoids: epigallocatechin gallate (≥95.0%), kaempferol (≥97.0%), myricetin (≥96.0%), quercetin (≥95.0%) and rutin (≥95.0%), and stilbene resveratrol (99.0%) were obtained from Sigma–Aldrich (Prague, Czech Republic). Inorganic salts of p.a. grade used for buffer preparation and total phenolic content assessment were purchased from Lach-ner (Brno, Czech Republic).

### 2.4. Antioxidant activity

#### 2.4.1. DPPH radical-scavenging assay

The antioxidant effect of wine and positive control (trolox) was tested using DPPH assay according to the slightly modified method previously described by Sharma and Bhat (2009). Two-fold serial dilution of each sample was prepared in absolute methanol (175 μL) in 96-well microtiter plate. Subsequently, 25 μL of freshly prepared 0.4 mM DPPH solution in methanol was added to each well (final volume 200 μL), creating a range of concentration 50–0.78125 μL $mL^{-1}$ for red wines and 500–7.8125 μL $mL^{-1}$ for white wines. Range of concentration for trolox (used as a reference antioxidant) was settled at 80–1.25 μg $mL^{-1}$ and calibration curve was thereafter established. Mixture was kept in dark at room temperature for 30 min. Absorbance was measured at 517 nm using Infinite 200 reader (Tecan, Männedorf, Switzerland). Results were expressed as trolox equivalents (g TE $L^{-1}$ wine).

#### 2.4.2. Oxygen radical absorbance capacity (ORAC) assay

Slightly modified method previously described by Ou et al. (2001) was used. Firstly, outer wells of black 96-well microtiter plates were filled with 200 μL of distilled water, in order to provide better thermal mass stability. Stock solution of AAPH radical and FL were prepared in 75 mM phosphate buffer (pH 7.0). Each sample (25 μL) was diluted in 150 μL FL (48 nM) and incubated in 37 °C for 10 min. Reaction was started by application of 25 μL AAPH (153 mM) yielding final volume of 200 μL and final dilution at 1:4000 for red wines and 1:800 for white wines (in phosphate

**Table 1**
Information on variety, producer, origin and type of studied wines.

| Wine code | Cultivar and vintage | Producer | Origin | Wine type |
|---|---|---|---|---|
| PN 1 | Pinot Noir, 2008 | VINIUM Velké Popovice | Moravia, Czech Republic | Red dry |
| PN 2 | Pinot noir, 2010 | Augustinian cellar Neoklas | Moravia, Czech Republic | Red dry |
| PN 3 | Pinot noir, 2007 | Gsellmann & Hans | Burgenland, Austria | Red dry |
| PN 4 | Pinot noir, 2008 | Virely Arcelain | Bourgogne, France | Red dry |
| PN 5 | Pinot noir, 2007 | André Goichot | Bourgogne, France | Red dry |
| CS 1 | Cabernet Sauvignon, 2010 | Famiglia Cielo | Venetia, Italy | Red dry |
| CS 2 | Cabernet Sauvignon, 2010 | Folonari | Venetia, Italy | Red dry |
| CS 3 | Cabernet Sauvignon, 2010 | Brise de France | N/A[a], France | Red dry |
| CS 4 | Cabernet Sauvignon, 2011 | Jean D?Aosque | Languedoc, France | Red dry |
| CS 5 | Cabernet Sauvignon, N/A[a] | Víno Mikulov | Moravia, Czech Republic | Red dry |
| CS 6 | Cabernet Sauvignon, 2008 | Templar cellars Čejkovice | Moravia, Czech Republic | Red dry |
| CS 7 | Cabernet Sauvignon, 2005 | Teliani valley PLC | Teliany, Georgia | Red dry |
| CM 1 | Cabernet Moravia, N/A[a] | Templar cellars Čejkovice | Moravia, Czech Republic | Red dry |
| CM 2 | Cabernet Moravia, 2007 | Vinselekt Michlovský | Moravia, Czech Republic | Red dry |
| SA 1 | Saperavi, 2008 | P.E. Givi Nikolashvilli | Gurjaani, Georgia | Red dry |
| SA 2 | Saperavi, 2007 | Teliani valley PLC | Tsinandali, Gerogia | Red dry (aged in oak, unfiltered) |
| SA 3 | Saperavi, 2005 | Teliani valley PLC | Napareuli, Georgia | Red dry |
| SA 4 | Saperavi, 2006 | Teliani valley PLC | Mukuzani, Georgia | Red dry |
| SA 5 | Saperavi, 2004 | Teliani valley PLC | Kidzmarauli, Georgia | Red semi-sweet |
| SA 6 | Saperavi, 2006 | Geovan Wine | Kakheti, Georgia | Red dry |
| SA 7 | Saperavi, 2010 | Tbilvino | Kakheti, Georgia | Red dry |
| SA 8 | Saperavi, 2009 | Kindzmarauli Marani | Kakheti, Georgia | Red dry |
| SA 9 | Saperavi, 2007 | Kindzmarauli Marani | Kakheti, Georgia | Red dry (barrel aged) |
| SA 10 | Kvareli (Saperavi + Saperavi Budeshuriseburi), 2005 | Kindzmarauli Marani | Kakheti, Georgia | Red dry |
| SA 11 | Saperavi (95%) + Saperavi Budeshuriseburi (5%), N/A[a] | Kindzmarauli Marani | Kakheti, Georgia | Red dry (fermented 25–30 days) |
| AL 1 | Alexandrouli, 2007 | Chrebalo Wine Factory | Ambrolauri district, Georgia | Red dry |
| CH 1 | Chardonnay, 2011 | VINIUM Velké Pavlovice | Moravia, Czech Republic | White dry |
| CH 2 | Chardonnay, N/A[a] | Chateau Valtice | Moravia, Czech Republic | White dry |
| CH 3 | Chardonnay, 2010 | Folonari | Venetia, Italy | White dry |
| CH 4 | Chardonnay, 2010 | Delibori | Venetia, Italy | White dry |
| CH 5 | Chardonnay, 2011 | Joseph Castalan | Pays d?oc, France | White dry |
| CH 6 | Chardonnay, 2010 | André Goichot | Bourgogne, France | White dry |
| SB 1 | Sauvignon Blanc, 2010 | Famiglia Cielo | Venetia, Italy | White dry |
| SB 2 | Sauvignon Blanc, 2011 | Brise de France | N/A[a], France | White dry |
| SB 3 | Sauvignon Blanc, 2008 | VINIUM Velké Pavlovice | Moravia, Czech Republic | White dry |
| RK 1 | Rkatsiteli, 2010 | Institute of Horticulture, Viticulture and Oenology (IHVO) | Kakheti, Georgia | White dry (fermented by Kakhetian meathod) |
| RK 2 | Rkatsiteli, 2009 | Tbilvino | Kakheti, Georgia | White |
| RK 3 | Rkatsiteli + Mtsvane, 2008 | Geovan Wine | Kakheti, Gerogia | White dry |
| RK 4 | Rkatsiteli (50%) + Mtsvane Kakhuri (20%) + Khikhvi (15%) + Kisi (15%), N/A[a] | Kindzmarauli Marani | Kakheti, Georgia | Amber (fermented by Kakhetian method) |

[a] Information not available.

buffer). Trolox was tested at range of concentration 4–0.5 μg $mL^{-1}$ and calibration curve was thereafter established. Fluorescence changes were measured in one minute intervals for 120 min with emission and absorbance wavelengths were set at 494 nm and 518 nm, respectively. Antioxidant capacity was calculated as area under the calibration curve as proposed by (Cao and Prior, 1998). Results were expressed as trolox equivalents (g TE $L^{-1}$ wine).

### 2.4.3. Total phenolic content (TPC) assay

Total phenolic compounds were measured using modified method previously described by Singleton et al. (1998). Sample in volume of 100 μL (dilution: red wine:water 1:19; white wine:-water 1:1) was added to 96-well microtiter plate. Range of concentration of gallic acid (used as a reference compound) was settled at 16.7–0.008 μg $mL^{-1}$. Thereafter, 25 μL of pure Folin-Ciocalteu reagent was added. Plate was inserted in orbital shaker at 100 rpm for 10 min. Reaction was started by addition of 75 μL 20% $Na_2CO_3$. Mixture was kept in dark at 37 °C for 2 h and then absorbance was measured at 700 nm. Results were expressed as gallic acid equivalents (g GAE $L^{-1}$ wine).

### 2.4.4. Total sulfite content assay

Commercially available kit EnzytecTM Color $SO_2$-Total kit (R-Biopharm AG, Darmstadt, Germany) was used for the quantification of total sulfites in wine samples. The test was performed according to manufacturer's instructions with slight modifications. Briefly, sample solutions were prepared by mixing 950 μL of buffer with 50 μL of each sample. Sample solution (200 μL) was added to 96-well microtiter plate and measured using Infinite 200 reader with absorbance set at 340 nm. Thereafter, 40 μL of chromogen was added and 200 μL of this solution was transferred to new 96-well microtiter plate and measured at 340 nm again. Calibration curve was constructed by using calibrator ($SO_2$) in concentration range between 300 and 0 mg $L^{-1}$. Difference in optical densities ($\Delta A$) was calculated as $\Delta A = (A2 - \mathrm{Rf} \times A1)_{\text{sample or calibrator}} - (A2 - \mathrm{Rf} \times A1)_{\text{blank}}$, where $A2$ is the absorbance value acquired from the second measurement and $A1$ absorbance value of samples before application of the chromogen. Rf refer to diffraction factor which was settled at 0.952 according to manufacturer's instructions. Total sulfite content was further calculated according to following equation: $C_{\text{sample}} = C_{\text{calibrator}}/\Delta A_{\text{calibrator}} \times \Delta A_{\text{sample}}$. Results were expressed as mg $SO_2$ $mL^{-1}$ wine.

## 2.5. High performance liquid chromatography (HPLC) analysis

### 2.5.1. Extraction procedure

Each wine sample (4.75 mL) was treated by addition of 150 μL BHT (200 μg dissolved in 1 mL of methanol) to prevent oxidation

of phenolic compounds. As inner standard 100 μL of fluorosalicylic acid (200 μg dissolved in 1 mL of methanol) was used. Each sample was acidified to pH 2.8 by adding 10 μL of 35% HCl. Remaining alcohol (ethanol and methanol) presented in each wine was further evaporated by inserting sample to refrigerated CentriVap concentrator (Labconco, Kansas City, MO, USA) for 2 h under 50 °C. Extraction procedure was performed by liquid–liquid extraction using 5 mL of diethylether. Wine:diethylether solution was vortexed for 60 s. Separation of the ether layer was facilitated by centrifugation at 3500 rpm for 5 min. Extraction procedure was repeated three times and provided about 15 mL of ether extract which was further evaporated to dryness on rotary evaporator R210 (Büchi, Flawil, Switzerland). Residue was dissolved in 500 μL acetonitrile:water (50:50) solution and poured into HPLC vial glass for further analysis.

### 2.5.2. HPLC-UV/Vis

Apparatus consisted of autosampler Midas (Spark, Emmen, Netherlands), thermostat (Midas, Spark, Emmen, Netherlands) and pump (Q-Grad, Watrex, Prague, Czech Republic). System was coupled on-line to UV6000 LP detector (SpectraSystem, ThermoFinnigan, Waltham, MA, USA) and was controlled by software Clarity (DataApex, Prague, Czech Republic) and EZ-Chrom Elite (ThermoFinnigan, Waltham, MA, USA). For identification of simple phenolic acids, stilbenes and flavonoids, Kinetex (2.6 μm) PFP, 100 A (150 × 4.6 mm) column was used (Phenomenex, Torrance, CA, USA). Gradient elution was carried out employing mobile phase A (water with 0.5% acetic acid) and B (acetonitrile with 0.5% acetic acid) as follows: 0 min, 96:4 (A:B); 10 min, 85:15; 14 min, 79:21; 25 min, 78:22, 34 min, 59:41; 38 min, 0:100; 48 min, 0:100; 51 min, 96:4; 61 min, 96:4. Injection volume was settled at 10 μL, flow rate at 1 mL/min and thermostat temperature at 33 °C.

### 2.5.3. Quantitative analysis

UV absorption was monitored at wavelengths between 194 and 500 nm. Quantification was done under 260 and 300 nm (chromatogram of wine sample SA 8 and chromatogram of standard solution is shown in Fig. 1). Evaluation of acquired data was performed in software Clarity (DataApex, Prague, Czech Republic) and EZ-Chrom Elite (ThermoFinnigan, Waltham, MA, USA). Standard calibration curves were obtained in a concentration range of 100–0.2 μg $mL^{-1}$ with nine concentrations levels (100, 50, 20, 10, 5, 2, 1, 0.5, 0.2 μg $mL^{-1}$). UV peak areas of the external standards (at each concentration) were plotted against the corresponding standard concentrations (μg $mL^{-1}$) using weighed linear regression to generate standard curve. Retentions times and linear equations for each standard are given in Supplementary Table S1. Amount of compounds were finally expressed as μg $mL^{-1}$ wine.

### 2.5.4. Method validation

Linearity of calibration based on regression analysis was obtained at all measured concentration levels. The coefficients of determination ($R^2$) for particular compounds are shown in Supplementary Table S1.

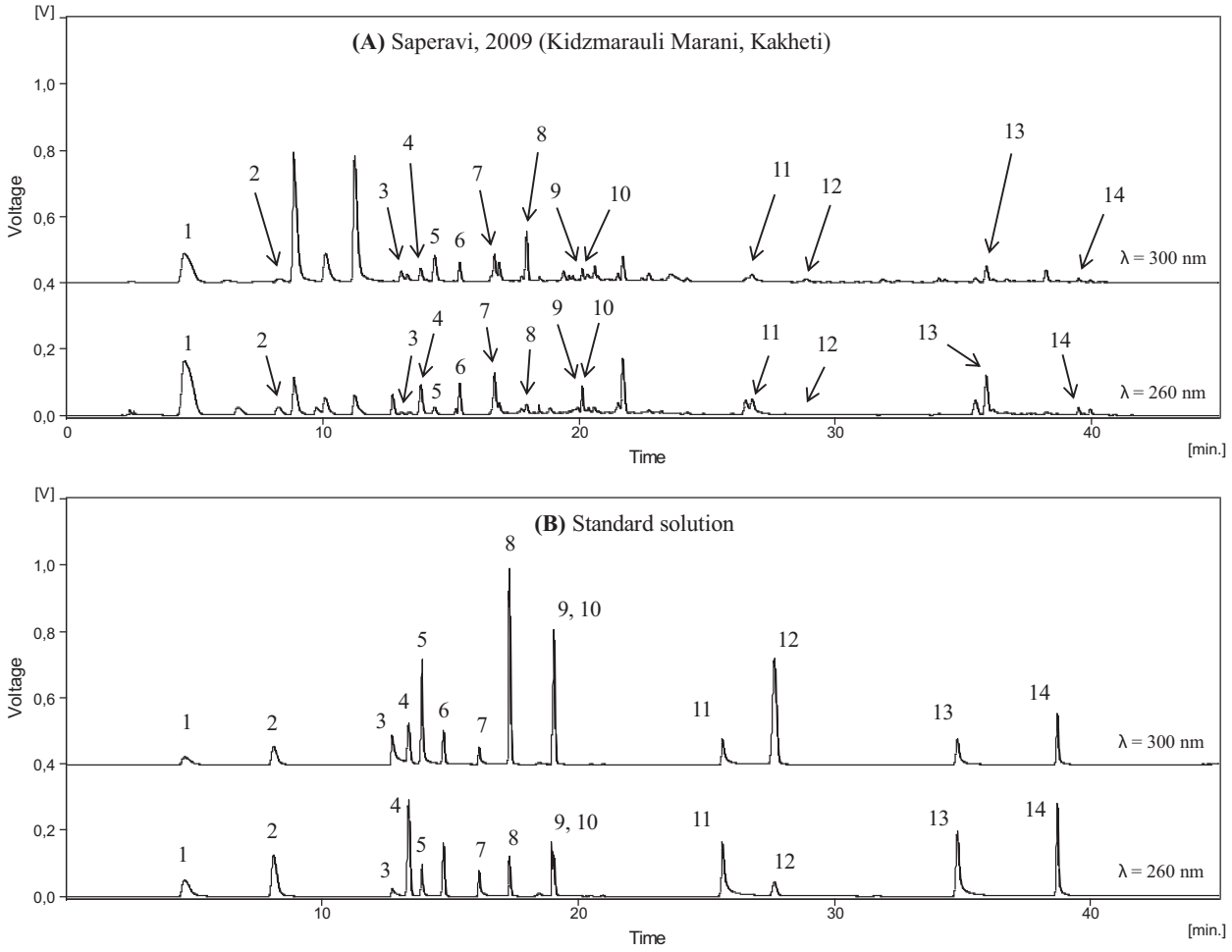


**Fig. 1.** Chromatogram of wine sample SA 8 (A) in comparison to standard solution (B) at concentration of 50 μg $mL^{-1}$. 1 = gallic acid; 2 = 3,4-dihydroxybenzoic acid; 3 = chlorogenic acid; 4 = vanillic acid; 5 = caffeic acid; 6 = syringic acid; 7 = epigallocatechin gallate; 8 = *p*-coumaric acid; 9 = rutin; 10 = ferulic acid; 11 = myricetin; 12 = resveratrol; 13 = quercetin; 14 = kaempferol.

Limit of detection (LOD) as well as limit of quantification (LOQ) were calculated from the standard additional curves as a 1:3 and 1:10 signal-to-noise ratio, respectively. For the measured compounds, LOD and LOQ ranged between 0.010–0.398 and 0.012–0.790 μg $mL^{-1}$, respectively (for details see Supplementary Table S1).

The precision of the HPLC measurement expressed as relative standard deviation (RSD, %) was evaluated by five replicate injections of standard solution and randomly selected wine sample during the same day. The precision for the standards and samples ranged between 2.1–9.7% and 1.3–14.7%, respectively. The detailed data for all analyzed compounds are listed in Supplementary Table S1.

Accuracy of measurement was determined as the average deviation between computed and obtained concentrations of standards in spiked samples. The recovery for the measured compounds varied from 2.6 to 10.9% (for details see Supplementary Table S1).

Repeatability of the extraction method was determined as recovery of spiked standards solved in freshly prepared wine samples. Recoveries of gallic acid, 3,4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, epigallocatechin gallate, *p*-coumaric, rutin, ferulic acid, myricetin, resveratrol, quercetin and kaempferol were determined at 50 μg $mL^{-1}$. The recovery was expressed as percentage of corresponding determined concentration of pure standards. The value was computed as an average of five technical replicates. Recovery values of analyzed compounds ranged from 82.7 to 105.7% (Supplementary Table S2).

### 2.6. Statistical analysis

In vitro antioxidant experiments were performed in three separate experiments, each in duplicate. Results were expressed as mean values with standard deviations (mean ± SD). Extraction and HPLC/UV-Vis analyses were performed two times for each sample. HPLC data (concentration of studied compounds) were expressed as mean.

For further statistical analyses, wine samples were grouped according to color (red and white) and further into three groups according to their production area: Georgia, Central Europe and Western Europe. Georgian group consisted, up to one exception (CS 7 – Cabernet Sauvignon), of Georgian autochthonous cultivars Saperavi, Alexandrouli, and Saperavi Budeshuriseburi only (present only in the cuvee with Saperavi) in the case of red wines and Rkatsiteli, Mtsvane, Khikhvi, and Kisi (last three cultivars present only in the cuvee with Rkatsiteli). Central European group consisted from Cabernet Sauvignon, Pinot Noir, Cabernet Moravia cultivars from Czech Republic and Austria in the case of red wines and Chardonnay and Sauvignon Blanc from Czech Republic in the case of white wines. West European group consisted from Cabernet Sauvignon and Pinot Noir cultivars from France and Italy in the case of red wines and Chardonnay and Sauvignon Blanc from France and Italy in the case of white wines.

Values recorded in DPPH, ORAC, TPC, and $SO_2$ assay and concentrations of 14 compounds were analyzed using one way analysis of variance (ANOVA) followed by Tukey's test in order to ascertain possible significant differences between groups. Linear correlation coefficients (*r*) between total sulfite content and antioxidant assays DPPH and ORAC were established using Pearson product moment correlation. For all statistical tests STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA) software was used.

## 3. Results and discussion

### 3.1. Antioxidant activity

Georgian red wines possessed significantly higher antioxidant activity (complete results for each sample are summarized in Table 2; average values for samples separated according to geographical regions are shown in Table 3) than West and Central European samples in DPPH ($p = 0.01$), ORAC ($p = 0.05$) as well as in TPC ($p = 0.01$) assay. The highest antioxidant effect in all three assays was recorded in wines from Georgian cultivar Saperavi and cuvee Saperavi + Saperavi Budeshuriseburi (range of DPPH, ORAC and TPC values: 8.59–2.08 g TE $L^{-1}$ wine; 12.14–7.29 g TE $L^{-1}$ wine and 4.46–1.59 g GAE $L^{-1}$ wine, respectively), followed by Pinot Noir (4.22–1.88 g TE $L^{-1}$ wine; 10.49–8.59 g TE $L^{-1}$ wine and 3.31–1.56 g GAE $L^{-1}$ wine) and Cabernet Sauvignon (4.58–1.97 g TE $L^{-1}$ wine; 8.42–6.70 g TE $L^{-1}$ wine and 2.49–1.11 g GAE $L^{-1}$ wine). In contrast to our results, Chkhikvishvili et al. (2008) recorded

**Table 2**
Antioxidant activity measured by DPPH and ORAC, total phenolic content (TPC) and total sulfite content of tested wines (mean ± standard deviation).

| Wine code | DPPH [g TE[a] $L^{-1}$ wine] | ORAC [g TE $L^{-1}$ wine] | TPC [g GAE[b] $L^{-1}$ wine] | Total $SO_2$ [mg $SO_2$ $L^{-1}$ wine] |
|---|---|---|---|---|
| PN 1 | 3.06 ± 0.41 | 8.59 ± 1.01 | 1.56 ± 0.02 | 24.63 ± 2.14 |
| PN 2 | 3.12 ± 0.34 | 10.49 ± 1.55 | 2.08 ± 0.03 | 56.73 ± 4.44 |
| PN 3 | 1.88 ± 0.24 | 8.67 ± 1.55 | 1.57 ± 0.05 | 11.35 ± 1.14 |
| PN 4 | 4.22 ± 0.38 | 10.01 ± 1.48 | 3.31 ± 0.13 | 2.44 ± 0.33 |
| PN 5 | 3.38 ± 0.46 | 9.19 ± 1.73 | 2.46 ± 0.04 | 73.18 ± 4.09 |
| CS 1 | 2.37 ± 0.26 | 6.93 ± 1.45 | 1.55 ± 0.04 | 59.60 ± 4.47 |
| CS 2 | 2.95 ± 0.39 | 6.70 ± 1.37 | 1.89 ± 0.05 | 58.43 ± 3.99 |
| CS 3 | 3.31 ± 0.41 | 7.11 ± 1.33 | 2.03 ± 0.07 | 59.60 ± 4.47 |
| CS 4 | 2.82 ± 0.29 | 7.09 ± 1.1 | 1.86 ± 0.06 | 36.06 ± 4.52 |
| CS 5 | 2.78 ± 0.22 | 7.61 ± 1.54 | 1.69 ± 0.04 | 25.23 ± 0.29 |
| CS 6 | 1.97 ± 0.42 | 7.02 ± 0.76 | 1.11 ± 0.01 | 93.85 ± 4.49 |
| CS 7 | 4.58 ± 0.33 | 8.42 ± 1.39 | 2.49 ± 0.07 | 18.17 ± 4.13 |
| CM 1 | 1.97 ± 0.31 | 8.29 ± 0.91 | 1.40 ± 0.04 | 34.65 ± 3.80 |
| CM 2 | 2.12 ± 0.20 | 8.77 ± 1.63 | 1.50 ± 0.03 | 32.46 ± 1.73 |
| SA 1 | 5.89 ± 0.63 | 10.39 ± 1.31 | 2.78 ± 0.08 | 18.22 ± 1.59 |
| SA 2 | 5.27 ± 0.46 | 10.81 ± 1.68 | 2.74 ± 0.07 | 10.79 ± 0.37 |
| SA 3 | 5.03 ± 0.72 | 11.16 ± 1.75 | 2.42 ± 0.05 | 8.38 ± 0.49 |
| SA 4 | 4.79 ± 0.32 | 10.56 ± 2.11 | 2.61 ± 0.08 | 9.13 ± 2.06 |
| SA 5 | 2.08 ± 0.14 | 7.29 ± 0.81 | 1.59 ± 0.03 | 60.69 ± 2.94 |
| SA 6 | 4.50 ± 0.43 | 9.99 ± 2.14 | 2.67 ± 0.07 | 25.59 ± 5.34 |
| SA 7 | 4.70 ± 0.34 | 10.92 ± 2.00 | 3.05 ± 0.07 | 26.68 ± 2.77 |
| SA 8 | 8.59 ± 0.50 | 11.37 ± 2.01 | 4.46 ± 0.18 | 39.45 ± 3.80 |
| SA 9 | 5.21 ± 0.45 | 9.82 ± 1.48 | 3.22 ± 0.13 | 21.36 ± 2.38 |
| SA 10 | 5.01 ± 0.47 | 11.71 ± 1.65 | 3.87 ± 0.10 | 29.28 ± 1.89 |
| SA 11 | 5.43 ± 0.48 | 12.14 ± 2.15 | 3.39 ± 0.08 | 20.95 ± 5.78 |
| AL 1 | 1.91 ± 0.18 | 5.87 ± 0.66 | 1.36 ± 0.04 | 0.00 |
| CH 1 | 0.10 ± 0.02 | 0.48 ± 0.07 | 0.21 ± 0.01 | 103.61 ± 6.98 |
| CH 2 | 0.24 ± 0.03 | 1.12 ± 0.19 | 0.29 ± 0.01 | 91.81 ± 2.80 |
| CH 3 | 0.14 ± 0.02 | 0.84 ± 0.12 | 0.22 ± 0.01 | 98.24 ± 2.54 |
| CH 4 | 0.13 ± 0.01 | 0.85 ± 0.14 | 0.22 ± 0.001 | 91.09 ± 2.26 |
| CH 5 | 0.34 ± 0.04 | 1.12 ± 0.21 | 0.28 ± 0.01 | 121.57 ± 9.47 |
| CH 6 | 0.12 ± 0.01 | 0.73 ± 0.05 | 0.23 ± 0.01 | 108.2 ± 3.54 |
| SB 1 | 0.12 ± 0.01 | 0.74 ± 0.14 | 0.22 ± 0.01 | 92.94 ± 2.88 |
| SB 2 | 0.15 ± 0.01 | 1.04 ± 0.2 | 0.23 ± 0.01 | 115.14 ± 2.34 |
| SB 3 | 0.06 ± 0.01 | 1.27 ± 0.11 | 0.20 ± 0.01 | 202.19 ± 18.68 |
| RK 1 | 0.51 ± 0.04 | 1.67 ± 0.25 | 0.40 ± 0.01 | 2.66 ± 0.10 |
| RK 2 | 0.17 ± 0.02 | 0.99 ± 0.13 | 0.24 ± 0.01 | 86.96 ± 2.15 |
| RK 3 | 0.29 ± 0.02 | 1.64 ± 0.24 | 0.29 ± 0.01 | 95.39 ± 2.65 |
| RK 4 | 2.90 ± 0.27 | 4.01 ± 0.70 | 1.88 ± 0.04 | 38.21 ± 4.48 |

[a] Trolox equivalency.
[b] Gallic acid equivalency.

**Table 3**
Antioxidant activity of red wine samples grouped according to their origin (mean ± SD).

| Region | DPPH (g TE/L wine)** | ORAC (g TE/L wine) * | TPC (g GAE/L wine)** |
|---|---|---|---|
| Georgia ($n = 13$) | 4.84 ± 1.64[a] | 10.04 ± 1.82[a] | 2.82 ± 0.83[a] |
| Western Europe ($n = 6$) | 3.18 ± 0.63[ab] | 7.84 ± 1.40[b] | 2.18 ± 0.62[ab] |
| Central Europe ($n = 7$) | 2.42 ± 0.55[b] | 8.50 ± 1.09[ab] | 1.56 ± 0.29[b] |

Different letters within each column showed significant differences (ANOVA with Tukey post hoc test; * $p \leq 0.05$, ** $p \leq 0.01$).

comparable or lower antioxidant activity of Georgian Saperavi wines prepared by European method in comparison with French wine. However, in mentioned study was only one sample of French wine for comparison. Higher antioxidant effect of Pinot Noir than antioxidant activity of Cabernet Sauvignon wines is in the agreement with the study of Hosu et al. (2011) and Landrault et al. (2001). Other representatives of red wines (Alexandrouli, Cabernet Moravia) were regarded as samples with moderate antioxidant efficacy (values ranging as follows: DPPH = 2.13–1.92 g TE $L^{-1}$ wine; ORAC = 8.77–5.87 g TE $L^{-1}$ wine and TPC = 1.50–1.36 g GAE $L^{-1}$ wine).

Comparison of antioxidant activity among regions in the frame of one cultivar (Cabernet Sauvignon and Pinot Noir) indicates that red wines produced in Western Europe were more effective than those from Central Europe. The activity of Cabernet Sauvignon from Western Europe ranged from 3.31 to 2.37 TE $L^{-1}$ while samples from Central Europe ranged from 2.79 to 1.97 TE $L^{-1}$. Similarly, the activity of West European Pinot Noir ranged from 4.22 to 3.39 TE $L^{-1}$ while Central European samples ranged from 3.12 to 1.87 TE $L^{-1}$ in DPPH. However, for statistical analyses and reliable conclusions more samples for each cultivar and geographical region would be needed.

White wines were significantly less effective in antioxidant assays than the red wines. Only exception was sample RK 4 (cuvee of Rkatsiteli), which was fermented by Kakhetian method. Wine RK 4 exhibited antioxidant efficacy comparable to some red wines (DPPH, ORAC and TPC values 2.90 g TE $L^{-1}$ wine; 4.01 g TE $L^{-1}$ wine and 1.88 g GAE $L^{-1}$ wine, respectively). Also another representative of white wine (RK 1) prepared by Kakhetian method showed higher antioxidant effect in comparison to the rest of tested white wines. However, its antioxidant activity (values ranging as follows: DPPH = 0.51 g TE $L^{-1}$ wine; ORAC = 1.67 g TE $L^{-1}$ wine and TPC = 0.40 g GAE $L^{-1}$ wine) was not as strong as in case of RK 4 sample. Positive effect of Kakhetian technology (more than two times better than in case of European method) on antioxidant activity of white wines was recorded also by Shalashvili et al. (2007). This could be explained by the higher content of phenolics in wines fermented with pomace. Rest of tested white wines (Chardonnay, Sauvignon Blanc and Rkatsiteli made by common method used in Europe) were regarded as weak antioxidants (range of values for DPPH, ORAC and TPC were 0.34–0.07 g TE $L^{-1}$ wine; 1.64–0.48 g TE $L^{-1}$ wine and 0.29–0.20 g GAE $L^{-1}$ wine, respectively) without significant differences between regions of origin.

### 3.2. Total sulfite content

In the framework of this study the content of total sulfite was measured in wine samples. Generally, white wines contained higher quantities of mg $SO_2$ $L^{-1}$ (sulfite content in all samples is shown in Table 2). Two Sauvignon Blanc SB 3 (202.19 mg $SO_2$ $L^{-1}$ wine) and SB 2 (115.14 mg $SO_2$ $L^{-1}$ wine) samples and one Chardonnay sample CH 5 (121.57 mg $SO_2$ $L^{-1}$ wine) were most abundant samples on total $SO_2$ level. Sulfites in red wines ranged from 73.18 to 0 mg $SO_2$ $L^{-1}$ wine. Wines with strongest antioxidant activity (SA 8, SA 10 and SA 11) contained low sulfite levels 39.45, 29.28 and 20.95 mg $SO_2$ $L^{-1}$ wine, respectively. Total sulfite content in tested wines was similar to values recorded by Zúñiga et al. (2014) and Comuzzo et al. (2013). We also ascertained if artificially added sulfites could possibly alter the antioxidant potential of wine in ORAC and DPPH assay. $SO_2$ possessed $EC_{50}$ = 289 mg $L^{-1}$ in DPPH assay. Since the highest concentration of $SO_2$ in wine samples was 202 mg $L^{-1}$ and highest tested concentration of white wine was 0.5 mL $mL^{-1}$, the maximal concentration of $SO_2$ in sample was 101 mg $L^{-1}$. It means that in the sulfite most rich sample $SO_2$ level does not reach its $EC_{50}$. In the case of red wines the highest tested concentration was 0.05 mL $mL^{-1}$ which means that concentration and impact of $SO_2$ was negligible. In ORAC assay the effect of $SO_2$ was even lower. While 1 mL of weakest white wine was equal to activity of 0.48 mg trolox, the activity of 200 μg $SO_2$ (contained in 1 mL of most $SO_2$ rich wine) was equal only to 1.5 μg of trolox in ORAC assay. According to recorded results, no positive correlation between $SO_2$ and antioxidant potential ($r = -0.77$ for ORAC and $r = -0.82$ for DPPH) was revealed. These results demonstrate that content of sulfites had negligible impact on the antioxidant capacity of wines tested using ORAC and DPPH assays which is in the agreement with study of Zúñiga et al. (2014). In white wines, three times higher average concentration of total sulfites was observed in comparison to red wines (97.0 and 32.5 mg $SO_2$ $L^{-1}$ for white and red wines, respectively). Since white wines do not contain as much quantities of phenolic compounds as red wines, higher concentrations of sulfites must be added in order to prevent degradation (Comuzzo and Zironi, 2013).

### 3.3. HPLC analysis

Individual concentrations of 14 phenolic compounds presented in each wine sample were quantified by HPLC-UV/Vis analysis with the aim to find differences between wines produced in Georgia, Central and Western Europe (concentrations of compounds in all wine samples is shown in Table 4). Order of mean content of presented compounds in all investigated samples were as follows: gallic acid > epigallocatechin gallate > caffeic acid > *p*-coumaric acid > myricetin > syringic acid > vanillic acid > 3,4-dihydroxybenzoic acid > quercetin > chlorogenic acid > resveratrol > rutin > ferulic acid > kaempferol.

Georgian wine samples contained significantly higher concentrations of quercetin ($p = 0.01$), kaempferol ($p = 0.05$), and syringic acid ($p = 0.05$), while content of *trans*-resveratrol was considerably lower ($p = 0.01$) in Georgian wines than in West and Central European samples (Table 5). The highest quantities of quercetin, kaempferol and syringic acid were recorded in wines prepared from Saperavi cultivar (quercetin = 14.44–1.07 μg $mL^{-1}$; kaempferol = 1.68–0.03 μg $mL^{-1}$ and syringic acid 12.59–4.72 μg $mL^{-1}$), followed by Pinot Noir (quercetin = 9.64–0.00 μg $mL^{-1}$; kaempferol = 0.60–0.00 μg $mL^{-1}$ and syringic acid = 12.02–5.17 μg $mL^{-1}$) and Cabernet Sauvignon cultivars (quercetin = 8.51–0.00 μg $mL^{-1}$; kaempferol = 0.63–0.00 μg $mL^{-1}$ and syringic acid = 8.00–4.28 μg $mL^{-1}$). On the contrary, Pinot Noir was the richest samples in resveratrol content, followed by representatives of Cabernet Sauvignon (concentration range 8.71–2.43 and 7.41–1.13 μg $mL^{-1}$, respectively). In contrast to our results, McDonald et al. (1998) recorded higher content of quercetin in Cabernet Sauvignon (2.6–4.6 μg $mL^{-1}$) than in Pinot Noir (0.9–2.6 μg $mL^{-1}$) wines from France. However, our results are in the agreement with the study of Nikfardjam et al. (2006) where Hungarian Pinot Noir wine samples contained higher concentrations of quercetin and resveratrol (7.5 and 3.2 μg $mL^{-1}$, respectively) than Cabernet Sauvignon samples (5.5 and 2.8 μg $mL^{-1}$, respectively). Our data recorded for *trans*-resveratrol in Czech Pinot Noir samples (2.43 and 8.71 μg $mL^{-1}$) are similar or slightly higher than those of Kolouchova-Hanzlikova et al. (2004) which were in range from 1.322 to 6.253 μg $mL^{-1}$. Wines belonging to Saperavi cultivar contained only moderate to low concentration of resveratrol, ranging from 5.11 to 0.32 μg $mL^{-1}$. In comparison, Chkhikvishvili et al. (2008) recorded values in range 0.69–1.17 μg $mL^{-1}$ for Saperavi wines.

Although the differences were observed in red wines grouped according to geographical origin, it seems possible that cultivar played also important role because Georgian group included only two wines from different cultivars than Saperavi (one Cabernet Sauvignon and one Alexandrouli sample). Several comparative studies dealing with differences in phenolic composition across wine cultivars, vintage, and production area revealed elevated

**Table 4**
Concentration of phenolic compounds in studied wines (expressed as mean of two measurements).

| Wine code | Compound[a]/(μg mL$^{-1}$) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3,4-DB | CAF | COUM | CHL | FER | GAL | SYR | VAN | RESV | EGCG | KAEM | MYR | QUER | RUT |
| PN 1 | 7.15 | 13.84 | 9.32 | 2.11 | 1.45 | 117.84 | 9.16 | 7.06 | 8.71 | 52.25 | ND[b,f] | 5.95 | 0.97 | ND[b,i] |
| PN 2 | 1.43 | 10.39 | 3.27 | ND[b,c] | 1.53 | 64.08 | 5.17 | 5.06 | 2.43 | 36.31 | ND[b,f] | 3.34 | ND[b,h] | 0.89 |
| PN 3 | 10.62 | 6.77 | 4.45 | 3.77 | 0.58 | 38.00 | 12.02 | 9.12 | 7.66 | 19.89 | ND[b,f] | 6.94 | 0.32 | ND[b,i] |
| PN 4 | 2.60 | 26.40 | 4.65 | 2.53 | 1.33 | 104.65 | 5.69 | 5.17 | 3.50 | 58.87 | 0.47 | 3.17 | 7.95 | 3.26 |
| PN 5 | 3.19 | 15.46 | 5.40 | 3.98 | ND[b,d] | 99.59 | 5.48 | 4.51 | 5.75 | 54.83 | 0.60 | 6.66 | 9.64 | 1.99 |
| CS 1 | 4.40 | 5.69 | 5.04 | 2.43 | ND[b,d] | 70.48 | 5.33 | 3.73 | 1.95 | 31.97 | 0.17 | 5.31 | 1.12 | 1.52 |
| CS 2 | 5.40 | 3.62 | 2.92 | 1.98 | 0.77 | 85.44 | 5.46 | 3.12 | 1.13 | 49.16 | 0.07 | 7.46 | 3.48 | 1.50 |
| CS 3 | 3.25 | 9.15 | 6.18 | 2.24 | 1.31 | 43.77 | 5.54 | 2.58 | 2.05 | 24.13 | 0.22 | 14.50 | 8.51 | 4.46 |
| CS 4 | 6.27 | 8.39 | 7.45 | 1.93 | 0.65 | 51.32 | 5.57 | 3.07 | 2.93 | 26.80 | ND[b,f] | 11.34 | 0.58 | 2.94 |
| CS 5 | 3.49 | 27.05 | 14.06 | 1.38 | 1.31 | 63.09 | 6.67 | 4.88 | 7.41 | 30.36 | ND[b,f] | 7.64 | 0.45 | ND[b,i] |
| CS 6 | 5.23 | 6.31 | 7.34 | 2.97 | 1.06 | 47.38 | 4.28 | 3.39 | 2.50 | 19.42 | ND[b,f] | 4.04 | ND[b,h] | ND[b,i] |
| CS 7 | 4.28 | 12.55 | 7.87 | 1.91 | 0.59 | 96.74 | 8.00 | 3.82 | 1.17 | 50.19 | 0.63 | 9.21 | 7.62 | ND[b,i] |
| CM 1 | 0.68 | 11.02 | 11.14 | 1.67 | 1.12 | 24.79 | 8.57 | 5.64 | 4.50 | 17.89 | ND[b,f] | 8.90 | 0.69 | ND[b,i] |
| CM 2 | 1.80 | 6.42 | 9.23 | 1.90 | 0.36 | 39.65 | 8.85 | 4.14 | 3.35 | 14.21 | ND[b,f] | 7.09 | 1.58 | ND[b,i] |
| SA 1 | 6.87 | 15.51 | 13.89 | 2.01 | 1.74 | 85.72 | 8.42 | 8.52 | 3.79 | 44.59 | 0.36 | 13.39 | 12.57 | 2.73 |
| SA 2 | 3.25 | 8.10 | 15.93 | 2.04 | 1.28 | 61.81 | 9.09 | 4.81 | 5.11 | 30.57 | 1.19 | 18.67 | 14.44 | 2.61 |
| SA 3 | 3.28 | 4.98 | 9.56 | 2.20 | 0.88 | 74.03 | 8.44 | 3.97 | 1.36 | 32.59 | 0.54 | 11.27 | 8.71 | ND[b,i] |
| SA 4 | 1.86 | 19.29 | 16.53 | 2.01 | 1.77 | 43.27 | 8.14 | 4.46 | 3.29 | 27.40 | 0.80 | 15.30 | 9.70 | 2.45 |
| SA 5 | 3.75 | 7.59 | 10.94 | 2.41 | 0.76 | 34.68 | 6.12 | 3.16 | 0.34 | 15.04 | 0.03 | 7.04 | 3.48 | ND[b,i] |
| SA 6 | 3.62 | 6.32 | 14.40 | 3.04 | 1.01 | 52.00 | 12.37 | 5.11 | 1.14 | 29.12 | 0.38 | 10.94 | 6.19 | 2.96 |
| SA 7 | 2.13 | 3.94 | 5.24 | 3.92 | 0.52 | 41.50 | 4.72 | 2.83 | 1.24 | 24.12 | 1.68 | 14.46 | 12.18 | 2.37 |
| SA 8 | 1.86 | 3.15 | 2.79 | 3.34 | 0.29 | 43.65 | 4.73 | 3.06 | 0.32 | 26.91 | 0.76 | 9.92 | 7.56 | 4.13 |
| SA 9 | 9.06 | 7.99 | 9.08 | 2.82 | 0.65 | 101.38 | 12.59 | 6.89 | 0.85 | 52.60 | 0.27 | 8.79 | 3.53 | ND[b,i] |
| SA 10 | 7.15 | 6.63 | 12.70 | 3.49 | 1.79 | 91.70 | 12.59 | 9.63 | 1.12 | 56.28 | 0.24 | 3.98 | 1.28 | ND[b,i] |
| SA 11 | 6.72 | 4.81 | 3.62 | 1.79 | 0.45 | 97.57 | 10.24 | 8.05 | 0.43 | 53.18 | 0.22 | 2.63 | 1.07 | 1.82 |
| AL 1 | 8.93 | 9.10 | 4.57 | 1.40 | 0.95 | 60.40 | 11.53 | 8.52 | 0.13 | 26.94 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 1 | 0.32 | 1.11 | 0.84 | 0.79 | 0.32 | 4.10 | 0.10 | 0.15 | ND[b,e] | 0.61 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 2 | 1.00 | 2.77 | 0.99 | 1.82 | 0.17 | 7.22 | 0.25 | 0.25 | ND[b,e] | 2.24 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 3 | 1.21 | 2.40 | 1.13 | 1.50 | 0.37 | 8.24 | 0.40 | 0.37 | ND[b,e] | 1.93 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 4 | 1.28 | 3.38 | 1.35 | 1.57 | 0.48 | 15.66 | 0.25 | 0.62 | ND[b,e] | 3.74 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 5 | 1.44 | 8.24 | 3.47 | 1.55 | 0.52 | 3.23 | 0.37 | 0.33 | ND[b,e] | 1.23 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| CH 6 | 1.59 | 6.77 | 2.43 | 1.64 | 0.32 | 2.11 | 0.25 | 0.23 | ND[b,e] | 0.99 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| SB 1 | 0.81 | 1.41 | 0.76 | 1.94 | 0.25 | 15.02 | 0.27 | 0.33 | ND[b,e] | 3.38 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| SB 2 | 0.94 | 4.09 | 2.43 | 1.47 | 0.46 | 1.99 | 0.17 | 0.20 | ND[b,e] | 1.02 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| SB 3 | 1.06 | 1.33 | 1.76 | 1.35 | 0.30 | 5.77 | 0.22 | 0.28 | ND[b,e] | 1.36 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| RK 1 | 1.29 | 7.41 | 0.84 | 3.22 | 0.21 | 9.93 | 0.21 | 0.35 | 0.32 | 1.68 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| RK 2 | 0.76 | 5.25 | 0.96 | 2.84 | 0.30 | 7.14 | 0.16 | 0.18 | ND[b,e] | 1.39 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| RK 3 | 2.70 | 7.34 | 3.32 | 2.49 | 0.57 | 10.31 | 0.51 | 0.55 | ND[b,e] | 2.00 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |
| RK 4 | 3.21 | 0.91 | 0.73 | 2.04 | 0.17 | 38.48 | 0.25 | 0.46 | ND[b,e] | 22.91 | ND[b,f] | ND[b,g] | ND[b,h] | ND[b,i] |

[a] 3,4-DB = 3,4-hydroxybenzoic acid; CAF = caffeic acid; COUM = *p*-coumaric acid; CHL = chlorogenic acid; FER = ferulic acid; GAL = gallic acid; SYR = syringic acid; VAN = vanillic acid; RESV = resveratrol; EGCG = epigallocatechin gallate; KAEM = kaempferol; MYR = myricetin; QUER= quercetin; RUT = rutin.
[b] Compound not detected.
[c] Limit of detection (LOD) = 0.398 μg mL$^{-1}$.
[d] LOD = 0.070 μg mL$^{-1}$.
[e] LOD = 0.072 μg mL$^{-1}$.
[f] LOD = 0.010 μg mL$^{-1}$.
[g] LOD = 0.220 μg mL$^{-1}$
[h] LOD = 0.074 μg mL$^{-1}$.
[i] LOD = 0.042 μg mL$^{-1}$.

content of flavonoids (especially quercetin, myricetin, and kaempferol), whereas decreased levels of *trans*-resveratrol in local grape varieties in comparison to commonly cultivated (Pinot noir, Cabernet Sauvignon) (McDonald et al., 1998; Landrault et al., 2001; Atanacković et al., 2012).

Cabernet Moravia were relatively rich in resveratrol and syringic acid content (concentration ranging between 4.50–3.35 μg mL$^{-1}$ and 8.85–8.57 μg mL$^{-1}$, respectively). Nevertheless, representatives of Cabernet Moravia had low quantities of quercetin (range of concentrations were 1.58–0.69 μg mL$^{-1}$). Kaempferol was not detected in Cabernet Moravia samples. Cabernet Sauvignon and Pinot Noir samples from Western Europe contained higher levels of kaempferol, quercetin and rutin then those from Central Europe. For example, quercetin concentrations reached from 0.58 to 9.64 μg mL$^{-1}$ in West European samples while in Central European samples quercetin reached from 0.00 to 0.97 μg mL$^{-1}$. Also Goldberg et al. (1998) observed higher levels of quercetin in samples from warmer climates.

**Table 5**
Content of syringic acid, *trans*-resveratrol, quercetin, and kaempferol in red wine samples grouped according to their origin (mean ± SD).

| Region | Syringic acid* | Resveratrol** | Quercetin** | Kaempferol* |
|---|---|---|---|---|
| Georgia ($n$ = 13) | 9.00 ± 2.77[a] | 1.56 ± 1.53[a] | 7.36 ± 4.43[a] | 0.59 ± 0.47[a] |
| Western Europe ($n$ = 6) | 5.51 ± 0.12[b] | 2.88 ± 1.63[ab] | 5.21 ± 3.98[ab] | 0.31 ± 0.22[ab] |
| Central Europe ($n$ = 7) | 7.82 ± 2.64[ab] | 5.22 ± 2.65[b] | 0.80 ± 0.50[b] | 0.00 ± 0.00[b] |

Different letters within each column showed significant differences (ANOVA with Tukey post hoc test; * $p \leq 0.05$, ** $p \leq 0.01$).

None statistical differences between white wines grouped according to their origin were revealed. Quercetin and kaempferol were under detection limits in representatives of white wines. Resveratrol was only detected in sample RK 1 (0.32 $\mu g\ mL^{-1}$). In comparison to other white wines, RK 4 differed in high gallic acid and epigallocatechin gallate content (38.48 and 22.91 $\mu g\ mL^{-1}$, respectively). Both RK 1 and RK 4 wine samples were prepared by Kakhetian method. Higher content (5.82 $\mu g\ mL^{-1}$) of resveratrol in Saperavi wine prepared by Kakhetian method than in Saperavi wines prepared by European method (0.69–1.17 $\mu g\ mL^{-1}$) was recorded also by Chkhikvishvili et al. (2008). Also the higher content of epigallocatechin gallate is in concordance with study of Shalashvili et al. (2007) where the white wines manufactured by Kakhetian method where significantly richer in total catechines. Further, our observations are in the agreement with conclusions made by Goldberg et al. (1999) that enological techniques have higher impact than climatic factors on the content of phenolic compounds in the case of white wine.

## 4. Conclusion

In summary, Georgian red wines showed higher antioxidant potential than red wines from Central and Western Europe. Red wines prepared from Georgian native cultivar Saperavi and cuvee Saperavi + Saperavi Budeshuriseburi showed highest antioxidant effect followed by Pinot Noir and Cabernet Sauvignon. Further, Georgian red wines differed from Central and West European red wines by higher content of quercetin, kaempferol, and syringic acid and by lower content of *trans*-resveratrol. No statistical differences among cultivars and regions were revealed in the case of white wines. However, white wines prepared by Kakhetian winemaking method possessed increased antioxidant activity and contained higher amounts of phenolic compounds. Our results indicate that antioxidant effect and content of phenolics in red wines was influenced by geographical origin and by cultivar while wine-making technology was the main factor influencing antioxidant activity and concentration of phenolic compounds in studied white wines. This study showed interesting potential of Georgian wines and therefore we suggest further detailed research of grapevine cultivars originated from Caucasus region because it could bring interesting results regarding chemical composition and biological activity connected with health-promoting effects.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfca.2014.12.029.

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## Appendix B:

**Tauchen J**, Bortl L, Huml L, Mikšátková P, Doskočil I, Maršík P, Villegas PPP, Flores YB, Van Damme P, Lojka B, Havlík J, Lapčík O, Kokoška L. Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon. Manuscript accepted (23. 3. 2016) to Revista Brasileira de Farmacognosia.

Date: Mar 23, 2016
To: "Ladislav Kokoska" kokoska@ftz.czu.cz
cc: sgleitao@pharma.ufrj.br;sgleitao@superig.com.br;revista@sbfgnosia.org.br
From: "Revista Brasileira de Farmacognosia" esubmissionsupport@elsevier.com
Subject: Your Submission - BJP-D-15-00415R2

Ms. Ref. No.: BJP-D-15-00415R2
Title: Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon
Revista Brasileira de Farmacognosia - Brazilian Journal of Pharmacognosy

Dear Prof. Kokoska,

I am pleased to inform you that your paper "Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon" has been accepted for publication in Revista Brasileira de Farmacognosia - Brazilian Journal of Pharmacognosy.

Below are comments from the editor and reviewers.

Thank you for submitting your work to Revista Brasileira de Farmacognosia - Brazilian Journal of Pharmacognosy.

Yours sincerely,

Suzana Guimaraes Leitao, Ph.D.
(Associate editor)
Revista Brasileira de Farmacognosia - Brazilian Journal of Pharmacognosy

Comments from the editors and reviewers:

The answers can be acceptable and corrected manuscript is now suitable for publication.

**********

Elsevier Editorial System(tm) for Revista Brasileira de Farmacognosia – Brazilian Journal of Pharmacognosy
Manuscript Draft

Manuscript Number: BJP-D-15-00415R2

Title: Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon

Article Type: Original Article

Keywords: antioxidant, anticarcinogenic, phenolic compounds, plant extracts

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First Author: Jan Tauchen

Order of Authors: Jan Tauchen; Ludvik Bortl; Lukas Huml; Petra Miksatkova; Ivo Doskocil; Petr Marsik; Pablo Villegas; Ymber B Flores; Patrick Van Damme; Bohdan Lojka; Jaroslav Havlik; Oldrich Lapcik; Ladislav Kokoska

Abstract: Among 23 extracts of medicinal and edible plants tested, Mauritia flexuosa showed significant antioxidant ability (DPPH and ORAC = 1062.9 and 645.9 ± 51.4 µg TE/ mg extract, respectively), while Annona montana demonstrated the most promising anti-proliferative effect (IC50 for Hep-G2 and HT-29 = 2.7 and 9.0 µg/ml, respectively). However, combinatory antioxidant/anti-proliferative effect was only detected in Oenocarpus bataua (DPPH = 903.8 and ORAC = 1024 µg TE/ mg extract; IC50 for Hep-G2 and HT-29 at 102.6 and 38.8 µg/ml, respectively) and Inga edulis (DPPH = 337.0 and ORAC = 795.7 µg TE/ mg extract; IC50 for Hep-G2 and HT-29 at 36.3 and 57.9 µg/ml, respectively). Phenolic content was positively correlated with antioxidant potential, however not with anti-proliferative effect. None of these extracts possessed toxicity towards normal foetal lung cells, suggesting their possible use in development of novel plant-based agents with preventive and/or therapeutic action against oxidative stress-related diseases.

Suggested Reviewers:

Opposed Reviewers:

Response to Reviewers: ITEMIZED RESPONSE TO THE REVIEWER'S COMMENTS
Ms. Ref. No.: BJP-D-15-00415
Authors: Jan Tauchen, Ludvik Bortl, Lukas Huml, Petra Miksatkova, Ivo Doskocil, Petr Marsik, Pablo Pedro Panduro Villegas, Ymber Bendezu Flores, Patrick Van Damme, Bohdan Lojka, Jaroslav Havlik, Oldrich Lapcik, Ladislav Kokoska
Title: Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon

Editor's comments

Query: DPPH and ORAC tests are said to have been modified - which were those changes? Inform the concentrations of the stock solutions. Which are the concentrations of the samples on the serial dilution? No blank, no control?
Response: Following changes were made in the text:

Modifications performed in DPPH and ORAC methods were described:
Lines 124 – 125: Concentrations and volumes of samples, standard and reagent were adjusted in order to be used in a microplate format.
Lines 136 – 138: Outer wells of black absorbance 96-wellmicrotiter plates were filled with 200 µl of distilled water, in order to provide better thermal mass stability, as suggested by Held (2005).

Concentrations of the stock solutions were added:
Line 139: Stock solutions of AAPH radical (153 mM) and FL (540 µM) were prepared in 75 mM phosphate buffer (pH 7.0).

Final concentrations at which extracts were tested were added:
Lines 125 - 127: Two-fold serial dilution of each sample (final concentration range: 1.25 – 5120 µg/ml) was prepared in absolute methanol (175 µl) in 96-well microtiter plates.
Lines 140 - 141: Afterwards, 25 µl of each sample at final concentration range of 6.4 – 32 µg/ml were diluted in 150 µl FL (54 nM) and incubated at 37°C for 10 min.

Positive and negative controls are mentioned:
Lines 131 – 132: Trolox (at concentrations 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 254 and 512 µg/mL) was used as positive control and methanol as blank.
Lines 142 - 143: Standard calibration curves of positive control Trolox were acquired at five concentration levels (0.5, 1, 2, 4, 8 µg/ml).
Line 143: The 75 mM phosphate buffer was used as a blank.

Query: Which were the concentrations used to build the calibration curve and express the results as Trolox equivalents? revise trolox x Trolox
Response: Concentrations of Trolox were added to DPPH and ORAC methodologies at lines 131 – 132 and 142 – 143, respectively, as mentioned in the previous paragraph.
Moreover, Trolox now appears in the whole manuscript with upper case character at the beginning of the word: e.g. Lines 106, 131, 132, 142, 146, 208, and 213.

Query: r.149 Which were the concentrations used to build the calibration curve and express the results galic acid equivalents? How did you prepare the sample for TPC determination?
Response: Following sentences were added:
Lines 153 – 155: Nine concentration levels of gallic acid (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/ml) were used to create the standard calibration curve.
Lines 148 – 149: Firstly, each sample (diluted in water; final concentration ranging from 16 to 80 µg/ml)

Query: Characterization of phenolic compounds by UHPLC-MS/MS - authors inform that eleven concentration levels ranging from 0.1 to 1000 ng/ml were used to generate calibration curves. Specify which were these concentrations.

Response: Concentrations of standards used to generate calibration curves for UHPLC-MS/MS analysis were added to the text:
Lines 183 – 185: Peak areas of standards (eleven concentration levels ranging from 0.1 to 1000 ng/ml – i.e. 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 500 and 1000 ng/ml).

Query: Check the conditions of the gradient elution - 15 min, 60:40, 19 min, 60:40 ??? Is that correct?
Response: Yes, the given gradient is correct. Four minutes of starting mobile phases are used to prepare the conditions for the next analysis. For better understanding, "to reach starting conditions" was positioned at the end of the sentence at lines 175 – 177.

Query: Which was the collision gas? Cone voltage?
Response: Nozzle voltage and type of collision gas was added to the text at line 181 (nozzle voltage 2.0 kV and 1.8 kV) and line 182 (Nitrogen was used as collision gas), respectively.

Query: Linear correlation coefficients should read r2, not r
Response: The symbol for correlation coefficient was rewritten from r to r2 (line 192).

Query: check the spelling of isoquercetrin (not isoqercetrin) along the text.
Response: The misspelled word isoqercitrin was corrected to isoquercitrin throughout the text: e.g. lines 250, 252 and in Table 6.

Other changes:
The following reference was added to the list of references:
Held, P., 2005. Performing oxygen radical absorbance capacity assays with synergyTM HT: ORAC antioxidant tests. Application Note. Biotek Instruments, Winooski, USA.

Combined antioxidant and anti-proliferative activity

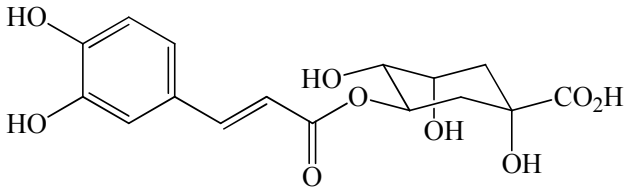
*Inga edulis* Mart.

*Oenocarpus bataua* Mart.

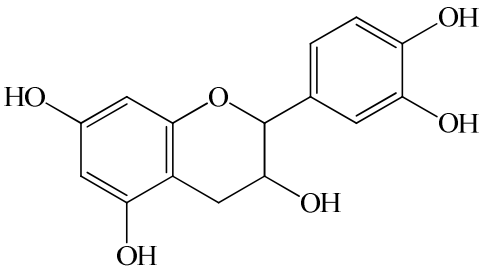
Selective anti-proliferative effect

*Annona montana* Macfad.

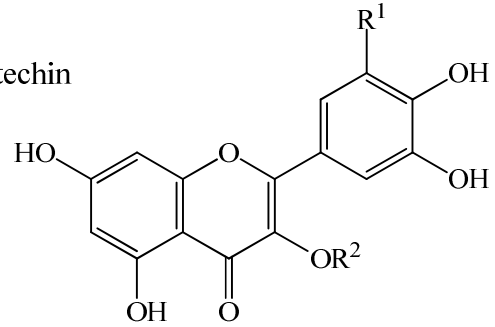
UHPLC-ESI-MS/MS analysis

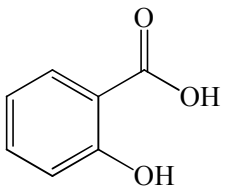



chlorogenic acid




(-)-epicatechin




salicylic acid

| | $R^1$ | $R^2$ |
|---|---|---|
| myricetin | OH | H |
| isoquercitrin | H | glucose |
| quercetin-3-arabinoside | H | arabinose |
| rutin | H | rutinose |

# Phenolic composition, antioxidant and anti-proliferative activities of edible and medicinal plants from the Peruvian Amazon

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**Running head:** Chemistry and biological activity of Peruvian plants

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**Abstract**

Among 23 extracts of medicinal and edible plants tested, *Mauritia flexuosa* showed significant antioxidant ability (DPPH and ORAC = 1062.9 and 645.9 ± 51.4 µg TE/ mg extract, respectively), while *Annona montana* demonstrated the most promising anti-proliferative effect ($IC_{50}$ for Hep-G2 and HT-29 = 2.7 and 9.0 µg/ml, respectively). However, combinatory antioxidant/anti-proliferative effect was only detected in *Oenocarpus bataua* (DPPH = 903.8 and ORAC = 1024 µg TE/ mg extract; $IC_{50}$ for Hep-G2 and HT-29 at 102.6 and 38.8 µg/ml, respectively) and *Inga edulis* (DPPH = 337.0 and ORAC = 795.7 µg TE/ mg extract; $IC_{50}$ for Hep-G2 and HT-29 at 36.3 and 57.9 µg/ml, respectively). Phenolic content was positively correlated with antioxidant potential, however not with anti-proliferative effect. None of these extracts possessed toxicity towards normal foetal lung cells, suggesting their possible use in development of novel plant-based agents with preventive and/or therapeutic action against oxidative stress-related diseases.

**Keywords:** antioxidant, anticarcinogenic, phenolic compounds, plant extracts

Conflict of interest: The authors declare no conflicts of interest.

## 1 Introduction

It is widely accepted that oxidative stress is involved in the development and/or secondary pathology of various human diseases (Halliwell and Gutteridge, 2007). Several studies show evidence that regular consumption of plant foods is associated with lowered risk of incidence of these (Alasalvar and Shahidi, 2013). It is believed that health beneficial effect of plants foodstuffs can mainly be credited to number of phenolic compounds and their ability to promote antioxidant effect (Brewer, 2011). Currently, antioxidant activity is primarily examined in common food plants such as fruits and vegetables. However, recent studies indicate that other plant categories, such as medicinal plants, also possess significant antioxidant efficacy (Jaberian et al., 2013).

Previously it was proposed that progression of cancer is strongly related to oxidative stress. Thus, validation of antioxidant effect of tested plant material is nowadays routinely supplemented with analysis of anti-proliferative activity against various types of carcinoma cell lines (Loizzo et al., 2014; da Costa et al., 2015). In case of phenylporpanoids, the compounds toxic to normal cells (e.g. podophyllotoxin) may be responsible for this anti-carcinomatous effect (Dewick, 2009). However, more recent studies are showing that dietary phenolics (e.g. flavonoids) may exert anti-proliferative effect as well (Ferry et al., 1996; Anter et al., 2011). Despite the fact that medicinal plants are regarded as the main sources of antineoplastic agents, there is now an increased interest in research of edible plants' anti-proliferative effects (De la Rosa et al., 2014).

Even though plants are generally considered as very important factor for maintaining food and health security (mainly in third world countries), health-promoting properties of majority of these plants have not been properly verified via modern scientific methods. Despite the well-documented traditional use of plants from that region for treatment of diseases related to oxidative stress such as cancer, diabetes, cardiovascular, inflammatory and

neurodegenerative diseases (Duke and Vásquez, 1994; Duke et al., 2009), to our best knowledge, only a very small proportion of edible and medicinal plants from the Peruvian Amazon have ever been assessed for their combinatory antioxidant/anti-proliferative properties (Neri-Numa et al., 2013). In addition, for a majority of these plants, the phytochemical profile was never fully characterized (Newman and Cragg, 2012).

Proceeding from these facts, this study provides detailed information on *in vitro* antioxidant and anti-proliferative potential of 23 methanol extracts from 12 Peruvian medicinal and edible plant species which were additionally analysed by UHPLC-MS/MS with the aim to determine the relationship between biological activity and phenolic compound content.

## 2 Materials and methods

### 2.1 Plant material

Selection of plant material was based on previously reported data on traditional use for treatment of diseases likely to be associated with oxidative stress (Table 1). Plants were collected from farms in areas surrounding Pucallpa city in the Peruvian Amazon, between March and June 2013. Voucher specimens were authenticated by Ymber Bendezu Flores and deposited at herbarium of IVITA-Pucallpa, Universidad Nacional Mayor de San Marcos (UNMSM).

### 2.2 Sample preparation

Fresh plant samples were frozen and lyophilized in Free-Zone 1 freeze dry system (Labconco, Kansas City, USA). Samples were finely grounded in IKA A 11 electric mill (IKA Werke GMBH&Co.KG, Staufen, Germany). Subsequently, 2 g of plant material were extracted in a Soxhlet-like IKA 50 extractor (IKA Werke GMBH&Co.KG, Staufen, Germany) in 70% ethanol in a 1/20 (w/v) proportion during three 7-mins cycles at 130°C followed by cooling to 50°C. Extracts were subsequently filtered through a Teflon (PTFE)

97 syringe filter (17 x 0.45 mm) and evaporated to dryness using a rotary evaporator R-3000
98 (Büchi, Flawil, Switzerland) *in vacuo* at 40°C. Dry residues were dissolved in 80% methanol
99 to create 50 mg/ml stock solutions and subsequently stored at -20°C. Extracts for UHPLC-
100 MS/MS analysis were evaporated to dryness and re-dissolved at a concentration of 0.4 g dry
101 weight per ml.

102 **2.3 Chemicals and reagents**

103 The following chemicals and reagents, purchased from Sigma-Aldrich (Prague, Czech
104 Republic), were used in this study: 2,2′-azobis(2-methylpropionamidine) dihydrochloride
105 (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiazolyl blue tetrazolium bromide (MTT),
106 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Dulbecco's modified
107 Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), fluorescein (FL),
108 Folin-Ciocalteu reagent, Griess reagent and penicillin–streptomycin solution. Analytical
109 standards (given in Table 2) were purchased from Indofine Chemical Company
110 (Hillsborough, USA) or Sigma-Aldrich. Formic acid, methanol and water of HPLC-grade
111 were purchased from Merck (Darmstadt, Germany); ethanol and dimethyl sulfoxide (DMSO)
112 from Penta (Prague, Czech Republic).

113 **2.4 Cell culture**

114 Liver carcinoma cell line Hep-G2 and normal foetal lung cells MRC-5 (ATCC,
115 Rockville, USA) were maintained in EMEM supplemented with foetal bovine serum (10%),
116 penicillin–streptomycin solution (1%), non-essential amino acids (1%) and glutamine (4 mM
117 and 2 mM for Hep-G2 and MRC-5, respectively). Colon carcinoma cell line HT-29 (ATCC,
118 Rockville, USA) was maintained in DMEM solution and otherwise were treated identically as
119 Hep-G2 and MRC-5. Cultures were incubated in 5% $CO_2$ atmosphere at 37°C using MCO
120 170AIC-PE $CO_2$ incubator (Panasonic Corporation, Osaka, Japan).

## 2.5 *In vitro* antioxidant activity

### *2.5.1 DPPH radical-scavenging assay*

Slightly modified method described by Sharma and Bhat (2009) was used for evaluation of samples' ability to inhibit DPPH radical. Concentrations and volumes of samples, standard and reagent were adjusted in order to be used in a microplate format. Two-fold serial dilution of each sample (final concentration range: 1.25 – 5120 µg/ml) was prepared in absolute methanol (175 µl) in 96-well microtiter plates. Subsequently, 25 µl of freshly prepared 1 mM DPPH in methanol was added to each well in order to start the radical-antioxidant reaction. Mixture was kept in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using Infinite 200 reader (Tecan, Männedorf, Switzerland). Trolox (at concentrations 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 254 and 512 µg/mL) was used as a positive control and methanol as a blank. Results were expressed as Trolox equivalents (µg TE/ mg extract).

### *2.5.2 Oxygen radical absorbance capacity (ORAC) assay*

Adjusted ORAC method was used for determination of samples' ability to protect FL from AAPH-induced damage (Cao and Prior, 1998; Ou et al., 2001). Outer wells of black absorbance 96-wellmicrotiter plates were filled with 200 µl of distilled water, in order to provide better thermal mass stability, as suggested by Held (2005). Stock solutions of AAPH radical (153 mM) and FL (540 µM) were prepared in 75 mM phosphate buffer (pH 7.0). Afterwards, 25 µl of each sample at final concentration range of 6.4 – 32 µg/ml were diluted in 150 µl FL (54 nM) and incubated at 37°C for 10 min. Reaction was started by adding 25 µl AAPH Standard calibration curves of positive control Trolox were acquired at five concentration levels (0.5, 1, 2, 4, 8 µg/ml). The 75 mM phosphate buffer was used as a blank. Fluorescence changes were measured in 1-min intervals for 120 min using an Infinite 200

reader with emission and absorbance wavelengths set at 494 nm and 518 nm, respectively. Results were expressed as Trolox equivalents (µg TE/ mg extract).

### *2.5.3 Total phenolic content (TPC)*

TPC was measured using the method developed by Singleton et al. (1998). Firstly, each sample (diluted in water; final concentration ranging from 16 to 80 µg/ml) with a volume of 100 µl was added to 96-well microtiter plates. Thereafter, 25 µl of pure Folin-Ciocalteu reagent was added. Plate was inserted in an orbital shaker at 40 rpm for 10 min. Reaction was started by adding 75 µl of 12% $Na_2CO_3$ (w/v). Mixture was kept in dark at 37°C for 2 h. Absorbance was measured at 700 nm (Infinite 200 reader). Nine concentration levels of gallic acid (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/ml) were used to create the standard calibration curve. Results were expressed as gallic acid equivalents (µg GAE/ mg extract).

## 2.6 Cell viability assay

Modified method based on metabolization of MTT to blue formazan by mitochondrial dehydrogenases in living cells previously described by Mosmann (1983) was used to test cell viability. Cells were pre-incubated (24 h) in a 96-well plate at a density of $2.5\times10^3$ cells per well and afterwards treated with two-fold serial dilutions of plant extracts in range of 0.24 – 500 µg/ml for 72 h. After addition of MTT reagent (1 mg/ml) in EMEM or DMEM solution, plates were incubated for an additional 2 h. Media were then removed, and the intracellular formazan product was dissolved in 100 µl of DMSO. Absorbance was measured at 555 nm (Infinite 200 reader) and percentage of viability calculated when compared to untreated control. Results were expressed as 50% inhibitory concentration ($IC_{50}$) in µg/ml.

## 2.7 Characterization of phenolic compounds by UHPLC-MS/MS

UHPLC-MS/MS analysis of 30 phenolic acids, flavonoids and related compounds was carried out using previously developed and validated method (Prokudina et al., 2012). Instrument was composed of Agilent 1290 Infinity instrument (Agilent, Santa Clara, USA)

equipped with a binary pump (G4220B), autosampler (G4226A), autosampler thermostat (G1330B), column compartment thermostat (G1316C), coupled to an Agilent triple quadrupole mass spectrometer (6460A) with a Jet Stream ESI ion source. A Kinetex PFP column (2.6 µm, 100 A, 150.0 x 3.0 mm) from Phenomenex (Torrance, USA) was used for the chromatographic separation of extracts. Column temperature was set at 35°C and injection volume at 3 µl. Gradient elution was carried out employing mobile phase A (10 mM formic acid) and B (100% methanol) as follows: 0 min, 60:40 (A:B); 10 min, 0:100; 14 min, 0:100; 15 min, 60:40, 19 min, 60:40 to reach starting conditions. Flow rate was set at 0.3 ml/min. The MS/MS apparatus was operating in positive and negative mode in the same analysis. Conditions of Jet Stream Ion Source were: drying gas temperature 290°C; drying gas flow 4 l/min; sheath gas temperature 380°C; sheath gas flow 10 l/min; nebulizer pressure 35 psi; nozzle voltage 2.0 kV and 1.8 kV; and capillary voltage was set at 3.5 and 5.0 kV in positive and negative acquisitions, respectively. Nitrogen was used as collision gas. Multiple reaction monitoring (MRM) mode was used for the detection. Peak areas of standards (eleven concentration levels ranging from 0.1 to 1000 ng/ml – i.e. 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 500 and 1000 ng/ml) were plotted against the corresponding response using weighed linear regression to generate calibration curves. Specific parameters of MS/MS method are given in Table 2. Agilent Mass Hunter (Agilent, Santa Clara, USA) was used for data acquisition and quantification of samples.

### 2.8 Statistical analysis

All *in vitro* assays were performed in three separated experiments, each in duplicate. UHPLC-MS/MS data were acquired in two separate experimental measurements. Results were expressed as mean values with standard deviations. Linear correlation coefficients ($r^2$) were established using Pearson product moment correlation between TPC and (i) antioxidant assay (plotted against DPPH and ORAC values) and (ii) anti-proliferative assay (plotted

against $IC_{50}$ values for Hep-G2 and HT-29). Statistical analysis was performed in STATISTICA 7.1 (StatSoft Inc., Tulsa, USA) software.

## 3 Results

Six plant extracts out of total 23 tested, namely leaves of *Annona montana, Inga edulis*, *Myrciaria dubia* and *Theobroma grandiflorum*; exocarp of *Mauritia flexuosa* and fruit without seed of *Oenocarpus bataua* showed significant antioxidant and/or anti-proliferative activity (Table 3). None of the tested plants exhibited toxicity to normal cells. Gallic, chlorogenic, salicylic and vanillic acids, (-)-epicatechin, myricetin, quercetin and its derivatives (isoquercitrin, quercetin-3-arabinoside and rutin) were the most predominant constituents in all analysed extracts. Complete results for antioxidant efficacy and cytotoxicity are given in Table 3, whereas for UHPLC-MS/MS analysis in Tables 4 – 6.

### 3.1 Antioxidant activity

In DPPH assay, *M. flexuosa* (exocarp) extract possessed higher antioxidant potential than positive control Trolox (1062.9 μg TE/mg). The promising antioxidant efficacy was also detected for *O. bataua* fruit, *T. grandiflorum* leaves, *M. dubia* leaves and pericarp (903.8, 714.8, 641.9, and 440.9 μg TE/mg, respectively). Other extracts showed only weak to moderate free radical scavenging ability (range 0.2 - 337.0 μg TE/mg). In ORAC assay, *O. bataua* fruits showed highest antioxidant activity (1024.4 μg TE/mg), being stronger than Trolox. Leaf extracts of *T. grandiflorum*, *I. edulis* and *M. dubia*; extracts of *M. flexuosa* exocarp and *I. edulis* pericarp, also showed promising results with μg TE/mg values at 821.9, 795.7, 642.6, 645.9 and 645.7, respectively. The rest of the tested plants showed weak to moderate efficacy (from 10.7 to 613.3 μg TE/mg). Highest content of phenolic compounds (TPC assay) was observed in *O. bataua* fruit, *M. flexuosa* (exocarp), *T. grandiflorum* (leaves) and *M. dubia* (leaves and pericarp) with values at 672.3, 461.5, 400.6 and 342.0 μg GAE/mg, respectively (Table 3). The rest of plant extracts tested exhibited only low to moderate

quantities of phenolic compounds (range 3.6 - 266.4 μg GAE/mg). Strong correlation was found between TPC and both antioxidant assays used: DPPH (r = 0.946) and ORAC (r = 0.899).

### 3.2 Cell viability assay

*A. montana* (leaves) demonstrated to be the plant extract with the most-promising anti-proliferative effect to Hep-G2 cell line ($IC_{50}$ = 2.7 μg/ml), followed by extracts of *I. edulis* (leaves), *O. bataua* (fruit), *M. dubia* (pericarp, leaves), *T. grandiflorum* (leaves) and *I. edulis* (seed) ($IC_{50}$'s at 36.3, 102.6, 124.0, 149.5, 140.4 and 179.1 μg/ml, respectively). The other samples exhibited very low anti-proliferative activity to carcinoma cells with $IC_{50}$ values higher than 500 μg/ml. In tests performed on HT-29 cell line, leaves of *A. montana* proved again to be the most-effective plant extract, with $IC_{50}$ value at 9.0 μg/ml, followed by extracts of *O. bataua* fruit, leaves of *Bertholletia excelsa*, *T. grandiflorum*, *I. edulis*, *Theobroma cacao* and pericarp of *T. grandiflorum*: $IC_{50}$'s at 38.8, 41.3, 46.5, 57.9, 82.6, and 83.9 μg/ml, respectively. The other plants possessed $IC_{50}$ values in a range of 137.6 - 294.0 μg/ml or exhibited non-toxic effect ($IC_{50}$ > 500 μg/ml). Toxicity assessment on normal MRC-5 cells revealed all plant extracts to be non-toxic ($IC_{50}$ > 500 μg/ml) (Table 3). Weak correlation was found between phenolic content and cell viability assays, whereas correlation coefficients of TPC vs. $IC_{50}$'s for Hep-G2 and HT-29 were 0.050 and 0.230, respectively.

### 3.3 UHPLC-MS/MS analysis

With regard to quantity of phenolic compounds identified by UHPLC-MS/MS in individual species, the highest amount was evidenced in *M. flexuosa* (exocarp) and *Solanum sessiliflorum* with values of 0.003% of dry weight. Noticeable results were also observed for *M. flexuosa* (mesocarp), pericarps of *T. bicolor* and *T. grandiflorum*, *O. bataua* (fruit without seed), and leaves of *I. edulis*, whose phenolic compound content in dry weight was detected at

0.002%. Remaining species had 0.001% or lower percentages of phenolic compounds on a dry weight basis.

Predominant constituents identified in *M. flexuosa* (exocarp) and *S. sessiliflorum*, which are expressed as percentage of phenolic compounds quantity, were chlorogenic acid, rutin and isoquercitrin (36%, 23%, 23.0% for *M. flexuosa* and 50%, 12%, 16.0% for *S. sessiliflorum*, respectively). Similar to the exocarp of *M. flexuosa*, its mesocarp predominantly contained chlorogenic acid, rutin and isoquercitrin, although in slightly different ratios (48%, 19% and 27%, respectively); (-)-epicatechin (31%) and chlorogenic acid (27%) were regarded as principal constituents in pericarp of *T. bicolor*, while isoquercitrin (42%), quercetin-3-*O*-arabinoside (22%), (-)-epicatechin (15%) and vanillic acid (14%) were most in evidence in pericarp of *T. grandiflorum*. Fruit without seed of *O. bataua* showed relatively high levels of epicatechin (45%), chlorogenic acid (12%) and isoquercitrin (11%). Leaves of *I. edulis* were shown to be mostly composed of myricetin (21%), isoquercitrin (21%) and salicylic acid (13%) (Tables 4 – 6).

## 4 Discussion

In this study, we investigated potential of Peruvian edible and medicinal plants for elimination of oxidative stress-related diseases using innovative approach based on determination of their combinatory antioxidant and anti-proliferative effects (Tauchen et al., 2015). As a result of our experiments, *O. bataua* and *I. edulis* possessed the best antioxidant/anti-proliferative properties. Although previous studies on chemistry of *O. bataua* have suggested high contents of anthocyanins (Rezaire et al., 2014), a compounds known to produce antioxidant and anticancer activity (Prior and Wu, 2006; Wang and Stoner, 2008), this is the first report on combined antioxidant and anti-proliferative effects of this plant. In contrast to earlier demonstrated relatively low cytotoxic efficacy of *I. edulis* towards various carcinoma cell lines (UACC-62, MCF-7, 786-O, NCI-460, PCO-3, OVCAR-03, HT-29 and

K-562) including multidrug-resistant variants (NCI-ADR) (Pompeu et al., 2012), we recorded moderate anti-proliferative activity against Hep-G2 and HT-29 cells of this plant. Differences between results of these experiments can be caused by dissimilar response of cancer cells to active compounds present in *I. edulis* as it has previously been observed for various classes of natural compounds (Sak, 2014). Since the kojic acid, recently found in leaves of *I. edulis* (Tchuenmogne et al., 2013), have exerted significant antioxidant as well as anti-proliferative activities (Novotny et al., 1999; Kusumawati and Indrayanto, 2013) it might considerably contribute to combined biological effect of the plant.

The most-interesting results regarding selectivity of anti-proliferative effect towards carcinoma and normal cells were observed for *A. montana*. Despite the existence of records on anti-proliferative efficacy of various Annonaceous species (such as *A. muricata*, *A. squamosa* or *A. reticulata*) (Barbalho et al., 2012), the cytotoxicity have not previously been recorded for *A. montana*. Acetogenins are regarded as being chiefly responsible for prominent anticancer effect of Annonaceous species (Smith et al., 2014). Hence, supposedly these constituents are also responsible for the cytotoxic effect of *A. montana* observed in this study. Contrary to the fact that our results suggests *A. montana* extract to be safe, a study of Potts et al. (2012) describes present acetogenins (e.g. annonaine) as the induction factor for neurotoxicity. Additional studies regarding toxicological profile of this plant and its constituents are thus required. Low correlation between TPC and anti-proliferative activity in the rest of tested plant extracts, as well as similar findings in literature (Yang et al., 2009), suggest only partial responsibility of phenolic compounds for anticancer effect.

Among the plant species tested in this study, *I. edulis*, *M. dubia*, *M. flexuosa*, *O. bataua* and *T. grandiflorum* have been found to be the most effective antioxidants. Despite the existence of previous records on antioxidant effect of these species (Souza et al., 2008; Fracassetti et al., 2013; Koolen et al., 2013; Pugliese et al., 2013; Rezaire et al., 2014), to our

best knowledge, majority of these were not using ORAC assay, regarded as one of the most biological relevant methods to determine antioxidant activity *in vitro* (MacDonald-Wicks et al., 2006). Our results from phytochemical and statistical analyses suggested phenolics to be major constituents responsible for the observed antioxidant effect of all five above-mentioned species that is corresponding with earlier published studies (De Sousa Dias et al., 2010; Fracassetti et al., 2013; Pugliese et al., 2013; Bataglion et al., 2014; Rezaire et al., 2014).

## 5 Conclusion

The current study provides novel information on *in vitro* antioxidant activity and/or anti-proliferative activity of six plant species, namely *A. montana*, *I. edulis*, *M. dubia*, *M. flexuosa*, *O. bataua* and *T. grandiflorum.* None of the tested extracts exerted significant toxicity towards normal MRC-5 cells, pointing their relative safety. We conclude that the above-noted plant extracts could serve as prospective material for further development of novel plant-based antioxidant and/or anti-proliferative agents. Particularly *O. bataua* and *I. edulis*, the only extracts exhibiting combinatory antioxidant and anti-proliferative efficacy in this study, deserve deeper research attention. Detailed analysis of their chemical composition and *in vivo* antioxidant/anti-proliferative activity should be carried out in order to verify their potential practical use.

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**Authors' contribution**

LB, PPPV and YBF collected the plant samples and organized the botanical identification and confection of herbarium specimens. JT performed the antioxidant assays and drafted the paper. ID organized the anti-proliferative test. LH and PMik did the UHPLC-MS/MS analysis. PMar provided statistical analysis of gained data. PVD, BL, JH and OL contributed to critical reading of the manuscript. LK designed the study, supervised the laboratory work and revised the final version of the paper. All the authors have read the final manuscript and approved the submission.

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**Table 1.** Ethnobotanical data of tested plant species

| Botanical name [voucher specimen] | Family | Vernacular name[a] | Part(s) tested | Way of consumption | Traditional medicinal use[b] | Reference[d] |
|---|---|---|---|---|---|---|
| *Annona montana* Macfad. [LB0037] | Annonaceae | *Guanabana* | leaf | Infusion/decoction | Cancer | (Duke and Vásquez, 1994; Barbalho et al., 2012) |
| *Bertholletia excelsa* Bonpl. [LB0120] | Lecythidaceae | *Castaña* | leaf | Infusion/decoction | Cancer | (Duke et al., 2009) |
| *Bunchosia armeniaca* (Cav.) DC. [LB0044] | Malpighiaceae | *Ciruela (china)* | seed, pericarp | Fruit eaten fresh or in processed form | n/a[c] | (Lim, 2012) |
| *Genipa americana* L. [LB0032] | Rubiaceae | *Huito, Lana* | whole fruit | Fruit eaten fresh or in processed form | Cancer | (Duke et al., 2009) |
| *Inga edulis* Mart. [LB0013] | Fabaceae | *Guaba* | leaf, pericarp, aril, seed | Pulp eaten fresh or used for flavouring; leaves used as infusion | Rheumatoid arthritis | (Lim, 2012) |
| *Mauritia flexuosa* L. f. [LB0084] | Arecaceae | *Aguaje* | exocarp, mesocarp | Processed into juices | Neurodegenerative diseases | (Duke et al., 2009) |
| *Myrciaria dubia* (Kunth) McVaugh [LB0095] | Myrtaceae | *Camu camu* | leaf, pericarp | Processed into juices; leaves used as infusion | Neurodegenerative diseases | (Duke et al., 2009) |
| *Oenocarpus bataua* Mart. [LB0123] | Arecaceae | *Ungurahui* | exocarp + mesocarp | Fruit eaten fresh or in processed form | Cancer | (Sosnowska and Balslev, 2009) |
| *Solanum sessiliflorum* Dunal [LB0046] | Solanaceae | *Cocona* | whole fruit | Fruit eaten fresh or cooked | Diabetes | (Duke and Vásquez, 1994; Lim, 2012) |
| *Theobroma bicolor* Humb. & Bonpl. [LB0073] | Malvaceae | *Macambo* | pericarp, aril + seed | Pulp is eaten fresh, seeds are consumed roasted | Cardiovascular diseases, cancer, diabetes | (Lim, 2012) |
| *Theobroma cacao* L. [LB0016] | Malvaceae | *Cacao* | leaf, pericarp, aril + seed | Pulp is eaten fresh, seeds are consumed roasted; leaves used as infusion/decoction | Cardiovascular diseases, cancer, diabetes | (Lim, 2012) |
| *Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum. [LB0052] | Malvaceae | *Copoazú* | leaf, pericarp, aril | Pulp is eaten fresh, seeds are consumed roasted; leaves used as infusion/decoction | Cardiovascular diseases, cancer, diabetes | (Lim, 2012) |

[a] vernacular names apply in the area of collection (Ucayali region, Peruvian Amazon); [b] only diseases or conditions likely to be associated to oxidative stress are recorded; [c] to our best knowledge, documentation on traditional use as remedy in the Amazon region is not available; [d] references are related to plant parts tested in this study

**Table 2.** Transitions and MS/MS parameters of analysed compounds

| Compound | Ionization mode | Retention time (min)[a] | Fragmentor (V) | Precursor ion (m/z) | Product (m/z) | | | | LOD (ng/ml)[c] | LOQ (ng/ml)[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Quantification transition | E (eV)[b] | Confirmation transition | E (eV)[b] | | |
| anisic acid | ESI + | 5.35 (0.5) | 72 | 153.06 | 77.2 | 5 | 109.1 | 9 | 3.6 | 11.9 |
| apigenin | ESI - | 7.98 (0.6) | 108 | 269.04 | 117.0 | 3 | 151.1 | 7 | 0.1 | 0.3 |
| apigenin-7-glucoside | ESI + | 5.54 (0.5) | 109 | 433.12 | 271.0 | 3 | 153.0 | 60 | 0.2 | 0.8 |
| caffeic acid | ESI - | 3.60 (0.4) | 81 | 179.00 | 89.0 | 30 | 135.1 | 3 | 2.3 | 7.7 |
| chlorogenic acid | ESI - | 3.01(1.0) | 81 | 353.09 | 191.1 | 9 | * | * | 0.6 | 1.9 |
| *p*-coumaric acid | ESI + | 4.37 (0.5) | 60 | 165.05 | 147.1 | 9 | * | * | 0.9 | 3.0 |
| (-)- epicatechin | ESI - | 3.10 (0.5) | 111 | 289.07 | 109 | 3 | 245.1 | 5 | 0.7 | 2.4 |
| ferulic acid | ESI + | 4.65 (0.5) | 63 | 195.07 | 145.0 | 3 | 177.0 | 5 | 1.0 | 3.2 |
| flavone | ESI + | 8.80 (0.5) | 119 | 223.10 | 77.2 | 1 | 121.1 | 25 | 0.2 | 0.8 |
| gallic acid | ESI - | 2.43 (0.6) | 75 | 169.01 | 124.9 | 9 | 169.0 | 5 | 0.7 | 2.2 |
| hesperetin | ESI - | 7.30 (0.5) | 108 | 301.07 | 164.0 | 17 | 286.0 | 9 | 0.1 | 0.3 |
| isoquercitrin | ESI - | 4.87 (0.5) | 150 | 463.09 | 300.3 | 18 | 271.0 | 42 | 0.4 | 1.2 |
| kaempferol | EIS + | 7.55 (0.6) | 161 | 287.06 | 153.0 | 1 | 69.1 | 3 | 1.1 | 3.6 |
| luteolin | ESI - | 7.23 (0.8) | 128 | 285.04 | 133.0 | 33 | 151.0 | 0 | 0.4 | 1.4 |
| luteolin-7-glucoside | ESI - | 4.92 (0.6) | 151 | 447.09 | 285.0 | 25 | 133.0 | 0 | 1.3 | 4.4 |
| morin | ESI + | 6.22 (0.8) | 141 | 303.05 | 152.9 | 0 | 69.1 | 4 | 22.0 | 73.2 |
| myricetin | ESI - | 5.72 (0.8) | 113 | 317.03 | 151.0 | 7 | 137.0 | 1 | 30.3 | 101.0 |
| naringenin | ESI - | 7.05 (0.5) | 93 | 271.06 | 119.0 | 1 | 151.0 | 9 | 0.1 | 0.1 |
| naringenin-7-glucoside | ESI - | 4.76 (0.5) | 117 | 433.11 | 271.1 | 0 | 119.0 | 0 | 0.1 | 0.4 |
| naringin | ESI - | 4.57 (0.5) | 166 | 579.17 | 271.1 | 9 | 151.0 | 9 | 3.0 | 10.0 |
| pterostilbene | ESI - | 9.07 (0.5) | 102 | 255.10 | 240.1 | 3 | 197.1 | 5 | 0.3 | 1.0 |
| quercetin | ESI - | 6.70 (0.9) | 106 | 301.03 | 151.0 | 3 | 121.1 | 4 | 1.3 | 4.2 |
| quercetin-3-arabinoside | ESI - | 5.18 (0.5) | 114 | 433.07 | 300.0 | 7 | 271.0 | 7 | 0.3 | 0.9 |
| resveratrol | ESI - | 5.25 (0.5) | 102 | 227.10 | 143.0 | 5 | 185.1 | 3 | 0.1 | 0.2 |
| rutin | ESI - | 4.69 (0.5) | 163 | 609.14 | 271.0 | 1 | 300.0 | 5 | 0.3 | 0.9 |
| salicylic acid | ESI - | 5.22 (0.6) | 72 | 137.02 | 93.1 | 3 | 65.1 | 9 | 0.5 | 1.8 |
| scopoletin | ESI - | 4.84 (0.5) | 81 | 191.03 | 176.0 | 0 | 104.0 | 2 | 0.4 | 1.2 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| sinapic acid | ESI - | 4.80 (0.4) | 81 | 223.06 | 208.1 | 9 | 149.0 | 7 | 0.1 | 0.2 |
| syringic acid | ESI + | 3.99 (0.5) | 60 | 199.06 | 140.1 | 3 | 77.2 | 5 | 0.5 | 1.7 |
| vanillic acid | ESI + | 3.79 (0.5) | 78 | 169.05 | 65.2 | 2 | 125.1 | 5 | 1.4 | 4.6 |

[a] retention time window (minutes) is given in brackets; [b] collision energy; [c] limits of detection (signal-to-noise ratio of 3); [d] limits of quantification (signal-to-noise ratio of 10); [*] Only one transition was used for detection.

**Table 3.** Total phenolic content, antioxidant and anti-proliferative activity of tested plant extracts

| Species | Plant part(s)[a] | Antioxidant assay / Mean ± SD[b] | | | Cell type / Mean $IC_{50}$ ± SD[b] | | |
|---|---|---|---|---|---|---|---|
| | | DPPH[c] | ORAC[c] | TPC[d] | Hep-G2[e] | HT-29[e] | MRC-5[e] |
| ***A. montana*** | **L** | 186.9 ± 16.7 | 608.3 ± 18.8 | 196.8 ± 10.7 | 2.7 ± 0.2 | 9.0 ± 1.3 | > 500 |
| ***B. excelsa*** | **L** | 258.8 ± 6.4 | 613.3 ± 26.8 | 266.4 ± 14.1 | > 500 | 41.3 ± 3.4 | > 500 |
| ***B. armeniaca*** | **P** | 1.5 ± 0.1 | 10.7 ± 1.0 | 6.5 ± 0.7 | > 500 | > 500 | > 500 |
| | **S** | > 0.2 | 27.3 ± 2.6 | 3.6 ± 0.7 | > 500 | > 500 | > 500 |
| ***G. americana*** | **FW** | 20.6 ± 4.7 | 113.9 ± 4.7 | 28.0 ± 2.2 | > 500 | > 500 | > 500 |
| ***I. edulis*** | **A** | 21.2 ± 3.2 | 69.9 ± 3.9 | 20.8 ± 1.8 | > 500 | > 500 | > 500 |
| | **L** | 337.0 ± 26.3 | 795.7 ± 25.4 | 262.3 ± 11.8 | 36.3 ± 15.7 | 57.9 ± 2.1 | > 500 |
| | **P** | 288.0 ± 8.8 | 645.7 ± 33.9 | 207.2 ± 13.8 | > 500 | 190.9 ± 1.1 | > 500 |
| | **S** | 7.9 ± 0.5 | 51.5 ± 2.8 | 17.2 ± 3.3 | 179.1 ± 13.7 | 148.5 ± 41.7 | > 500 |
| ***M. flexuosa*** | **E** | 1062.9 ± 163.9 | 645.9 ± 51.4 | 461.5 ± 32.5 | > 500 | > 500 | > 500 |
| | **M** | 130.8 ± 15.4 | 244.5 ± 7.5 | 87.0 ± 3.9 | > 500 | 262.6 ± 2.2 | > 500 |
| ***M. dubia*** | **L** | 641.9 ± 127.9 | 642.6 ± 32.7 | 342.0 ± 18.7 | 149.5 ± 23.8 | > 500 | > 500 |
| | **P** | 440.9 ± 62.7 | 333.0 ± 21.6 | 275.8 ± 13.2 | 124.0 ± 12.3 | > 500 | > 500 |
| ***O. bataua*** | **FO** | 903.8 ± 158.1 | 1024.4 ± 69.3 | 672.3 ± 46.9 | 102.6 ± 4.2 | 38.8 ± 5.4 | > 500 |
| ***S. sessiliflorum*** | **FW** | 8.8 ± 1.2 | 88.9 ± 6.0 | 18.1 ± 2.0 | > 500 | > 500 | > 500 |
| ***T. bicolor*** | **A+S** | 107.4 ± 13.6 | 243.0 ± 20.7 | 102.9 ± 4.3 | > 500 | 294.0 ± 34.9 | > 500 |
| | **P** | 152.4 ± 3.8 | 217.9 ± 16.8 | 104.9 ± 4.7 | 388.5 ± 22.2 | 156.8 ± 11.4 | > 500 |
| ***T. cacao*** | **A+S** | 329.9 ± 59.5 | 587.3 ± 48.8 | 217.2 ± 5.5 | 407.8 ± 4.6 | 137.6 ± 12.0 | > 500 |
| | **L** | 152.2 ± 7.5 | 542.7 ± 23.1 | 149.8 ± 4.4 | > 500 | 82.6 ± 5.5 | > 500 |
| | **P** | 51.6 ± 6.1 | 179.7 ± 12.3 | 49.4 ± 3.6 | > 500 | > 500 | > 500 |
| ***T. grandiflorum*** | **A** | 25.9 ± 4.2 | 145.5 ± 9.5 | 57.6 ± 3.2 | > 500 | > 500 | > 500 |
| | **L** | 714.8 ± 111.3 | 821.9 ± 65.6 | 400.6 ± 25.9 | 140.4 ± 3.0 | 46.5 ± 0.2 | > 500 |
| | **P** | 188.2 ± 10.9 | 434.9 ± 38.8 | 163.0 ± 8.1 | 218.6 ± 26.2 | 83.9 ± 0.7 | > 500 |

[a] abbreviation refers to plant part(s): A = aril, E = exocarp, FO = fruit without seed, FW = whole fruit, M = mesocarp, L = leaves, P = pericarp, S = seed; [b] standard deviation; [c] µg TE/mg extract; [d] µg GAE/mg extract; [e] µg/ml.

**Table 4.** Concentrations of phenolic acids in tested plant extracts

| Species | Plant part(s)[a] | Compound (ng/g DW)[bc] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | anisic a. | caffeic a. | chlorogenic a. | *p*-coumaric a. | ferulic a. | gallic a. | salicylic a. | sinapic a. | syringic a. | vanillic a. |
| ***A. montana*** | **L** | 116.3 ± 3.0 | 115.7 ± 4.3 | 267.1 ± 9.6 | 153.4 ± 4.2 | 81.8 ± 2.4 | 253.2 ± 1.4 | 64.3 ± 0.6 | 17.7 ± 0.5 | 94.2 ± 2.7 | 317.7 ± 7.2 |
| ***B. excelsa*** | **L** | 35.3 ± 0.7 | 25.5 ± 0.4 | 45.6 ± 2.3 | 391.5 ± 7.0 | 84.5 ± 1.5 | 3929.8 ± 25.4 | 665.9 ± 0.1 | 35.4 ± 1.1 | 123.9 ± 1.0 | 183.6 ± 5.4 |
| ***B. armeniaca*** | **P** | ND | <LOQ | 65.4 ± 3.1 | 48.1 ± 0.3 | 10.6 ± 0.0 | ND | 37.1 ± 0.4 | 1.6 ± 0.0 | 43.9 ± 1.0 | 37.2 ± 0.5 |
| | **S** | ND | ND | <LOQ | 25.3 ± 0.6 | 22.7 ± 0.3 | ND | 16.2 ± 0.3 | ND | ND | ND |
| ***G. americana*** | **FW** | 197.5 ± 2.4 | 46.9 ± 1.1 | ND | 56.1 ± 1.8 | 591.2 ± 5.4 | ND | 104.2 ± 0.1 | 155.6 ± 3.4 | 87.7 ± 2.2 | 6642.9 ± 86.1 |
| ***I. edulis*** | **A** | ND | <LOQ | 16.8 ± 0.9 | 33.6 ± 0.7 | 19.5 ± 0.5 | 647.5 ± 11.1 | 681.7 ± 5.2 | 26.8 ± 0.6 | 18.6 ± 0.4 | 43.9 ± 1.1 |
| | **L** | ND | 46.7 ± 2.0 | ND | 272.8 ± 0.4 | 32.1 ± 0.4 | 829.5 ± 3.5 | 2158.9 ± 2.5 | 17.8 ± 0.6 | 107.1 ± 2.2 | 1270.1 ± 29.6 |
| | **P** | ND | <LOQ | 5.7 ± 0.3 | 60.4 ± 1.4 | 15.0 ± 0.1 | 789.0 ± 19,3 | 1985.2 ± 4.1 | 1.0 ± 0.0 | 47.9 ± 1.9 | 456.7 ± 10.3 |
| | **S** | ND | <LOQ | <LOQ | 42.0 ± 1.2 | 277.8 ± 8.6 | 62.4 ± 1.1 | 43.1 ± 0.9 | 66.1 ± 0.4 | 16.9 ± 0.5 | 87.1 ± 2.1 |
| ***M. flexuosa*** | **E** | <LOQ | 162.7 ± 4.9 | 11767.9 ± 75.0 | 52.3 ± 0.5 | 98.9 ± 2.9 | 159.0 ± 0.9 | 13.9 ± 0.1 | 188.0 ± 1.4 | 177.3 ± 6.4 | 390.5 ± 7.9 |
| | **M** | ND | 53.8 ± 1.8 | 10354.6 ± 73.5 | 58.8 ± 1.6 | 93.4 ± 3.5 | 61.7 ± 1.0 | 16.5 ± 0.1 | 347.3 ± 3.4 | 48.6 ± 1.5 | 115.1 ± 2.1 |
| ***M. dubia*** | **L** | 37.6 ± 0.3 | <LOQ | 66.3 ± 0.1 | 159.9 ± 5.8 | <LOQ | 4087.7 ± 10.1 | 111.8 ± 0.8 | 5.2 ± 0.1 | 82.9 ± 1.2 | 108.0 ± 1.4 |
| | **P** | <LOQ | <LOQ | 15.3 ± 0.4 | 165.2 ± 5.1 | 19.0 ± 0.1 | 163.8 ± 4.1 | 51.3 ± 1.3 | ND | 10.7 ± 0.1 | 38.3 ± 3.1 |
| ***O. bataua*** | **FO** | ND | 256.3 ± 8.1 | 2324.7 ± 45.2 | 501.8 ± 14.7 | 351.6 ± 1.2 | 15.4 ± 0.4 | 39.6 ± 0.1 | 52.2 ± 0.5 | 704.2 ± 4.1 | 980.1 ± 24.0 |
| ***S. sessiliflorum*** | **FW** | ND | 235.4 ± 5.3 | 15066.5 ± 106.2 | 295.0 ± 7.2 | 99.6 ± 1.1 | ND | 432.8 ± 3.6 | 103.0 ± 2.6 | 24.1 ± 0.3 | 59.8 ± 1.2 |
| ***T. bicolor*** | **A+S** | 49.1 ± 0.9 | 23.8 ± 0.3 | ND | 47.9 ± 1.0 | 34.7 ± 0.6 | ND | 153.8 ± 4.3 | 134.7 ± 3.0 | 166.7 ± 4.5 | 261.7 ± 6.8 |
| | **P** | 36.6 ± 0.5 | 59.1 ± 0.7 | 5318.4 ± 29.1 | 100.0 ± 3.3 | 178.1 ± 2.3 | 15.3 ± 1.0 | 51.9 ± 1.2 | 90.9 ± 1.5 | 165.7 ± 3.8 | 1066.3 ± 31.7 |
| ***T. cacao*** | **A+S** | 20.9 ± 0.8 | 42.6 ± 1.0 | <LOQ | 22.6 ± 0.3 | 8.5 ± 0.0 | <LOQ | 13.5 ± 0.1 | 1.0 ± 0.0 | 7.2 ± 0.1 | 121.1 ± 2.0 |
| | **L** | ND | 180.9 ± 4.9 | 6678.4 ± 38.5 | 748.0 ± 15.7 | 198.1 ± 5.1 | 6.1 ± 0.3 | 350.6 ± 1.3 | 274.7 ± 8.2 | 170.1 ± 3.8 | 593.6 ± 8.5 |
| | **P** | ND | 66.3 ± 2.0 | <LOQ | 29.5 ± 1.2 | 620.0± 11.7 | <LOQ | 53.8 ± 0.2 | 87.3 ± 2.7 | 54.7 ± 1.5 | 307.6 ± 5.9 |
| ***T. grandiflorum*** | **A** | 20.2 ± 0.7 | <LOQ | 9.5 ± 0.8 | 112.0 ± 2.6 | 52.5 ± 1.0 | 8.4 ± 0.9 | 146.3. ± 1.5 | 3.7 ± 0.1 | 240.5 ± 7.9 | 1179.8 ± 25.5 |
| | **L** | <LOQ | 27.9 ± 0.7 | 8.7 ± 0.4 | 142.6 ± 3.3 | 54.7 ± 0.5 | 28.2 ± 1.0 | 1853.9 ± 1.8 | 102.7 ± 2.3 | 379.9 ± 6.4 | 948.1 ± 9.8 |
| | **P** | ND | <LOQ | 16.9 ± 0.8 | 148.8 ± 3.9 | 76.8 ± 0.2 | 6.7 ± 0.2 | 121.6 ± 0.4 | 13.1 ± 0.2 | 497.5 ± 0.7 | 2723.8 ± 36.9 |

[a] abbreviation refers to plant part(s): A = aril, E = exocarp, FO = fruit without seed, FW = whole fruit, M = mesocarp, L = leaves, P = pericarp, S = seed; [b] ND = compound not detected; [c] <LOQ = compound presented in sample under limit of quantification.

**Table 5.** Concentrations of flavonoids in tested plant extracts

| Species | Plant part(s) | Compound (ng/g DW)[bc] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | apigenin | (-)-epicatechin | flavone | hesperetin | kaempferol | luteolin | morin | myricetin | naringenin | quercetin |
| ***A. montana*** | **L** | ND | 602.2 ± 4.9 | ND | ND | 54.8 ± 1.1 | <LOQ | ND | 473.4 ± 0.7 | <LOQ | 55.3 ± 0.6 |
| ***B. excelsa*** | **L** | <LOQ | 137.1 ± 3.9 | ND | ND | 271.9 ± 7.7 | <LOQ | <LOQ | 500.0 ± 2.3 | 5.7 ± 0.0 | 293.3 ± 0.3 |
| ***B. armeniaca*** | **P** | ND | 11.5 ± 0.4 | ND | 2.0 ± 0.1 | <LOQ | <LOQ | ND | ND | 1.6 ± 0.0 | 12.8 ± 0.2 |
| | **S** | ND | 9.8 ± 0.3 | ND | ND | ND | ND | ND | ND | ND | ND |
| ***G. americana*** | **FW** | <LOQ | 126.3 ± 3.8 | ND | 1.5 ± 0.0 | 34.3 ± 0.1 | <LOQ | ND | ND | 1.6 ± 0.1 | 29.2 ± 0.8 |
| ***I. edulis*** | **A** | 32.6 ± 0.4 | 1284.7 ± 24.9 | ND | ND | <LOQ | 167.6 ± 1.9 | ND | ND | 0.6 ± 0.0 | 127.9 ± 2.5 |
| | **L** | 18.4 ± 0.0 | 298.3 ± 9.9 | ND | ND | 32.2 ± 0.6 | 692.1 ± 19.3 | ND | 3593.1 ± 29.5 | 1.2 ± 0.0 | 934.0 ± 8.5 |
| | **P** | 7.7 ± 0.2 | 2229.3 ± 22.4 | ND | 1.4 ± 0.0 | ND | 600.4 ± 5.2 | ND | ND | 1.6 ± 0.0 | 153.8 ± 1.6 |
| | **S** | 16.5 ± 0.1 | 14.1 ± 0.9 | ND | ND | 51.5 ± 1.4 | 281.5 ± 8.3 | ND | 569.7 ± 1.4 | 0.3 ± 0.0 | 134.4 ± 5.3 |
| ***M. flexuosa*** | **E** | 528.6 ± 0.4 | 228.8 ± 7.5 | ND | 8.7 ± 0.1 | 158.6 ± 3.7 | 477.8 ± 3.2 | 454.2 ± 1.2 | 471.2 ± 0.0 | 171.0 ± 2.6 | 252.6 ± 4.9 |
| | **M** | 15.0 ± 0.1 | 186.1 ± 6.7 | ND | <LOQ | ND | 5.5 ± 0.1 | ND | ND | 7.1 ± 0.1 | 32.7 ± 0.5 |
| ***M. dubia*** | **L** | ND | <LOQ | ND | ND | 247.2 ± 3.5 | ND | ND | 1147.8 ± 8.0 | 8.7 ± 0.3 | 375.9 ± 8.9 |
| | **P** | ND | ND | 6.0 ± 0.2 | ND | 27.7 ± 0.7 | ND | ND | 1010.4 ± 2.3 | 0.7 ± 0.0 | 161.9 ± 1.1 |
| ***O. bataua*** | **FO** | 54.4 ± 0.4 | 8628.5 ± 36.8 | ND | 2.4 ± 0.1 | 82.3 ± 2.7 | 30.9 ± 0.2 | ND | 473.7 ± 0.7 | 21.9 ± 0.1 | 687.2 ± 8.3 |
| ***S. sessiliflorum*** | **FW** | 7.1 ± 0.0 | ND | ND | ND | 302.0 ± 8.9 | 9.2 ± 0.1 | ND | ND | 1449.8 ± 17.0 | 124.0 ± 2.2 |
| ***T. bicolor*** | **A+S** | ND | 6495.1 ± 7.4 | ND | 0.7 ± 0.0 | 16.4 ± 0.3 | 7.3 ± 0.6 | ND | 6282.9 ± 38.8 | 4.2 ± 0.2 | 358.4 ± 3.5 |
| | **P** | 1.2 ± 0.0 | 6055.4 ± 46.3 | ND | 7.9 ± 0.0 | 60.1 ± 0.6 | 24.9 ± 0.5 | ND | 597.6 ± 4.5 | 440.8 ± 2.2 | 343.9 ± 4.0 |
| ***T. cacao*** | **A+S** | <LOQ | 6672.0 ± 51.1 | ND | ND | 9.4 ± 0.0 | 62.4 ± 0.8 | ND | <LOQ | 14.0 ± 0.3 | 948.0 ± 4.9 |
| | **L** | 29.5 ± 0.3 | 4128.6 ± 36.8 | ND | ND | <LOQ | 88.6 ± 0.5 | ND | ND | ND | 21.7 ± 0.1 |
| | **P** | 21.3 ± 0.1 | 1324.1 ± 58.5 | ND | <LOQ | <LOQ | 194.6 ± 2.2 | ND | ND | 5.5 ± 0.0 | 30.5 ± 0.1 |
| ***T. grandiflorum*** | **A** | 3.1 ± 0.2 | 3635.7 ± 23.0 | ND | ND | 33.7 ± 0.1 | 266.4 ± 7.7 | ND | 471.2 ± 0.1 | 7.7 ± 0.2 | 134.6 ± 3.3 |
| | **L** | 1.9 ± 0.0 | 1100.7 ± 15.0 | ND | <LOQ | 844.8 ± 0.8 | 128.7 ± 3.0 | ND | 474.7 ± 0.0 | 4.9 ± 0.1 | 1011.8 ± 1.7 |
| | **P** | <LOQ | 2905.7 ± 37.4 | ND | ND | 44.8 ± 0.1 | <LOQ | ND | ND | 8.4 ± 0.1 | 296.6 ± 0.0 |

[a] abbreviation refers to plant part(s): A = aril, E = exocarp, FO = fruit without seed, FW = whole fruit, M = mesocarp, L = leaves, P = pericarp, S = seed; [b] ND = compound not detected; [c] <LOQ = compound presented in sample under limit of quantification.

**Table 6.** Concentrations of flavonoid derivatives, stilbenes and other phenolic compounds in tested plant extracts

| **Species** | Plant part(s)[a] | **Compound (ng/g DW)[bc]** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | apigenin-7-glucoside | luteolin-7-glucoside | naringenin-7-glucoside | quercetin-3-arabinoside | naringin | isoquercitrin | rutin | pterostilbene | resveratrol | scopoletin |
| ***A. montana*** | **L** | 2.0 ± 0.0 | 22.2 ± 0.6 | 1.2 ± 0.1 | 2.6 ± 0.0 | ND | 1486.8 ± 21.0 | 8086.8 ± 92.4 | ND | ND | <LOQ |
| ***B. excelsa*** | **L** | 8.7 ± 0.1 | <LOQ | 2.5 ± 0.0 | 603.9 ± 5.7 | ND | 4156.2 ± 46.5 | 3007.1 ± 43.0 | ND | 1.9 ± 0.0 | ND |
| ***B. armeniaca*** | **P** | ND | ND | 11.0 ± 0.0 | 2.3 ± 0.0 | ND | 1112.7 ± 3.0 | 7024.2 ± 52.1 | 2.4 ± 0.0 | 0.6 ± 0.0 | <LOQ |
| | **S** | ND | ND | 2.5 ± 0.0 | <LOQ | ND | <LOQ | 8.1 ± 0.1 | ND | ND | ND |
| ***G. americana*** | **F** | <LOQ | ND | 1.5 ± 0.1 | 23.3 ± 0.1 | 28.1 ± 0.6 | 378.7 ± 2.0 | 3046.7 ± 0.3 | 2.5 ± 0.0 | <LOQ | <LOQ |
| ***I. edulis*** | **A** | 22.5 ± 0.4 | 49.6 ± 0.7 | 7.1 ± 0.1 | 2386.0 ± 37.2 | ND | 6241.1 ± 2.5 | 849.8 ± 4.5 | 2.5 ± 0.0 | ND | <LOQ |
| | **L** | 121.0 ± 1.5 | 416.5 ± 7.4 | 1.9 ± 0-1 | 1588.6 ± 28.0 | 36.8 ± 0.8 | 3478.6 ± 59.4 | 838.7 ± 6.1 | ND | 4.5 ± 0.1 | 6.4 ± 0.0 |
| | **P** | 35.2 ± 0.4 | 132.8 ± 2.2 | 7.9 ± 0.1 | 848.3 ± 9.4 | ND | 2892.0 ± 4.9 | 81.2 ± 0.8 | 2.4 ± 0.0 | <LOQ | 4.1 ± 0.1 |
| | **S** | 195.9 ± 2.8 | 310.0 ± 3.6 | 4.4 ± 0.1 | 20.2 ± 0.0 | ND | 458.2 ± 5.0 | 90.2 ± 1.4 | ND | ND | ND |
| ***M. flexuosa*** | **E** | 333.6 ± 9.5 | 501.5 ± 1.3 | 45.9 ± 0.9 | 70.0 ± 2.3 | 85.9 ± 2.5 | 7417.6 ± 22.2 | 7435.8 ± 70.8 | 184.3 ± 0.7 | 590.1 ± 13.8 | ND |
| | **M** | 20.8 ± 0.1 | 54.4 ± 0.1 | 30.3 ± 0.3 | 28.2 ± 0.6 | <LOQ | 5858.6 ± 80.0 | 3998.2 ± 106.2 | 5.8 ± 0.0 | 6.5 ± 0.1 | ND |
| ***M. dubia*** | **L** | <LOQ | ND | 11.2 ± 0.0 | 1392.0 ± 3.9 | ND | 3345.6 ± 2.5 | ND | ND | 1.9 ± 0.1 | ND |
| | **P** | ND | ND | 2.1 ± 0.1 | 124.0 ± 2.7 | ND | 170.3 ± 1.5 | ND | ND | <LOQ | ND |
| ***O. bataua*** | **FO** | 68.9 ± 0.4 | 14.2 ± 0.1 | 5.0 ± 0.0 | <LOQ | ND | 2128.0 ± 16.5 | 650.6 ± 4.7 | 39.1 ± 0.8 | 907.3 ± 15.3 | ND |
| ***S. sessiliflorum*** | **F** | 26.9 ± 0.1 | 35.0 ± 1.0 | 3186.4 ± 35.1 | 76.6 ± 2.9 | ND | 4823.8 ± 5.1 | 3659.3 ± 87.3 | ND | <LOQ | 21.1 ± 0.0 |
| ***T. bicolor*** | **A+** | ND | ND | 1.6 ± 0.1 | 54.0 ± 1.5 | ND | 695.5 ± 6.1 | 7.3 ± 0.2 | ND | 8.2 ± 0.1 | 84.3 ± 0.7 |
| | **P** | <LOQ | 12.3 ± 0.3 | 437. 3 ± 5.2 | 90.2 ± 1.5 | 198.2 ± 9.6 | 2064.9 ± 5.0 | 1936.1 ± 2.5 | 2.6 ± 0.8 | 7.5 ± 0.1 | 384.9 ± |
| ***T. cacao*** | **A+** | 3.3 ± 0.0 | 71.3 ± 2.6 | 15.4 ± 0.0 | 3269.1 ± 85.9 | <LOQ | 10259.3 ± 2.6 | 2.4 ± 0.1 | <LO | 1.5 ± 0.0 | <LOQ |
| | **L** | 747.9 ± 4.3 | 1239.8 ± 2.2 | 5.1 ± 0.2 | 281.5 ± 2.1 | <LOQ | 917.4 ± 6.6 | <LOQ | ND | <LOQ | 3.2 ± 0.0 |
| | **P** | 69.7 ± 0.6 | 295.6 ± 0.7 | 7.6 ± 0.1 | 298.9 ± 5.4 | <LOQ | 360.1 ± 7.5 | 2.9 ± 0.1 | ND | <LOQ | ND |
| ***T. grandiflorum*** | **A** | 10.2 ± 0.1 | 29.8 ± 0.6 | 2.7 ± 0.0 | 285.1 ± 3.2 | 40.8 ± 1.0 | 1753.9 ± 17.8 | 44.7 ± 0.7 | ND | 1.1 ± 0.0 | 35.3 ± 0.4 |
| | **L** | 2.5 ± 0.1 | <LOQ | <LOQ | 422.3 ± 6.4 | <LOQ | 2474.5 ± 13.1 | 255.4 ± 5.8 | ND | 1.6 ± 0.0 | 82.5 ± 0.9 |
| | **P** | ND | ND | 7.2 ± 0.1 | 4184.2 ± 17.0 | 114.1 ± 0.1 | 8149.4 ± 2.4 | 25.6 ± 0.9 | 2.6 ± 0.1 | 0.7 ± 0.0 | 46.5 ± 0.3 |

[a] abbreviation refers to plant part(s): A = aril, E = exocarp, FO = fruit without seed, FW = whole fruit, L = leaves, M = mesocarp, P = pericarp, S = seed; [b] ND = compound not detected; [c] <LOQ = compound presented in sample under limit of quantification.

## Appendix C:

**Tauchen J**, Doskočil I, Caffi C, Lulekal E, Maršík P, Havlík J, Van Damme P, Kokoška L. In vitro antioxidant and anti-proliferative activity of Ethiopian medicinal plant extracts. Industrial Crops and Products. 2015;74: 671–679.

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# *In vitro* antioxidant and anti-proliferative activity of Ethiopian medicinal plant extracts

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Medicinal plants

ABSTRACT

Identification and characterization of natural products with antioxidant and anti-proliferative activity has received much interest over the past few years. Ethiopia is one of the developing countries which have enormous diversity of plants and yet majority stays scientifically neglected and undiscovered. In this study, the ethanol extracts of 18 Ethiopian wild medicinal plants were investigated for their *in vitro* antioxidant and anti-proliferative potential. For this purpose DPPH (2,2-diphenyl-1-picrylhydrazyl), ORAC (oxygen radical absorbance capacity) and TPC (total phenolic content) assays together with MTT cell viability assay (performed on Hep-G2 and MRC-5) were used. Extracts of *Carissa spinarum*, *Dodonaea angustifolia*, *Jasminum abyssinicum*, *Rumex nepalensis*, *Rubus steudneri* and *Verbascum sinaiticum* exhibited the most significant results. However, it was discovered that *C. spinarum*, *J. abyssinicum* and *R. steudneri* possessed considerable toxicity against normal MRC-5 cell line. Only extracts of *D. angustifolia* and *R. nepalensis* demonstrated significant combinatory antioxidant/anti-proliferative effect, while *V. sinaiticum* showed best selective anti-proliferative activity. Since aforementioned extracts also exerted low or minimal toxicity to normal cells, we suggest these as prospective material for further development of novel plant-based agents effective against oxidative stress related diseases.



## 1. Introduction

Identification and characterization of natural products with antioxidant and anti-proliferative activity has received much interest over the past few years (Lo et al., 2002). Plants are nowadays recognized as a rich source of antioxidant compounds (*e.g.,* simple phenolics, anthocyanins, stilbenes, flavonoids), which are used primarily in food industry (Raza and John, 2008; Brewer, 2011). It is considered that consumption of plant-based antioxidants could be connected with lowered risk of occurrence of several human diseases related to the oxidative stress, including cancer (Halliwell and Gutteridge, 2007). Moreover, plants are also providers of anti-proliferative compounds (such as podophyllotoxin, paclitaxel or vincristine), which are able to actively inhibit growth of carcinoma cells (Dewick, 2009).

In general, common dietary plants, such as edible fruits and vegetables are primarily examined for their antioxidant activity. However, recent studies have indicated that other categories of plant material, such as medicinal plants (MPs), might possess stronger antioxidant potential than aforementioned food crops *in vitro* (Inayatullah et al., 2012; Jaberian et al., 2013). Since oxidative stress is possibly connected to development of cancer, the validation of antioxidant effect of tested material is nowadays routinely supplemented with assessment of anti-proliferative activity against various types of carcinoma cell lines (Ghali et al., 2013; De la Rosa et al., 2014; Loizzo et al., 2014). Hence, MPs are nowadays regarded as valuable material for development of food and pharmaceutical products, which are able to serve as preventive (antioxidant) and therapeutic (anti-proliferative) agents to several types of cancer and other diseases connected to oxidative stress

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(Rao, 2012). However, in contrast to common food plants that are generally considered as safe, the practical usability of MPs for industrial purposes hugely depends on possible risk to human health such as toxicity to vital organs. Therefore, toxicological profile of newly introduced plant material should be properly described (Doehmer et al., 2011).

MPs are generally considered as very important factor for food and health security in third world countries. However, these resources suffer from notable disregard from research and development plans (Addis et al., 2005) and moreover, are rapidly diminishing due to catastrophic destruction of natural habitats (Mengistu and Hager, 2008; Teklehaymanot and Giday, 2010). Hence economic value as well as scientific information of those plant resources is in danger of being irreversibly lost. Ethiopia is one of the developing countries where MPs are not subjected to research or development intention. In same time, this country is considered as a genetic centre for wide range of plant species and as a diversity hotspot. It is estimated, that local flora covers approximately up to 7000 vascular plant species (Hedberg et al., 2003). Despite the well-documented traditional use of medicinal plants in the region for treatment of diseases related to the oxidative stress (such as cancer, diabetes, chronic inflammatory diseases), to our best knowledge, only a very small proportion of MPs originated in Ethiopia were assessed for their antioxidant/anti-proliferative and toxicological properties (Rao et al., 2005; Eyob et al., 2008; Umer et al., 2010; Asamenew et al., 2011; Amoo et al., 2012).

In this article, we report a detailed investigation of the *in vitro* antioxidant and anti-proliferative potential of ethanol extracts of eighteen Ethiopian wild medicinal plants which are traditionally used for treatment of cancer and other diseases related to the oxidative stress. In addition, the phenolic content and toxicity of this extracts have been evaluated with aim to determine the relationship between their biological efficacy, chemical composition and safety.

## 2. Materials and methods

### 2.1. Plant material

Based on previously reported data on traditional medicinal usage for treatment of cancer and other oxidative stress-related diseases (Table 1), following plants and their botanical parts were selected for further testing: *Bersama abyssinica* (leaves and twigs), *Calpurnia aurea* (roots), *Carissa spinarum* (roots), *Clematis hirsuta* (leaves), *Clutia abyssinica* (root), *Croton macrostachyus* (leaves and twigs), *Cyathula cylindrica* (roots), *Dodonaea angustifolia* (leaves), *Embelia schimperi* (leaves and twigs; seeds/fruits), *Jasminum abyssinicum* (leaves), *Maesa lanceolata* (leaves and twigs), *Ocimum lamiifolium* (leaves), *Rubus steudneri* (leaves and twigs; roots), *Rumex nepalensis* (leaves and twigs; roots), *Thalictrum rhynchocarpum* (roots), *Verbascum sinaiticum* (leaves), *Vernonia amygdalina* (flowers; leaves and twigs) and *Zehneria scabra* (leaves and twigs). Plants were collected in Ankober District (172 km northeast of the capital Addis Ababa), North Shewa Zone, Amhara Region Ethiopia, between June 2009 and May 2011. Identification of specimens was performed both in the field and at the National Herbarium of Ethiopia (ETH) using taxonomic keys and floras (Hedberg and Edwards, 1989; Edwards et al., 2000; Hedberg et al., 2003) and by comparison with voucher reference herbarium specimens. The identified voucher specimens were deposited at the National Herbarium of Ethiopia. Detailed description of tested plants (ethnobotanical data and voucher specimen numbers) are provided elsewhere (Lulekal et al., 2014a,b).

### 2.2. Sample preparation

15 g of air-dried plant material of each species was finely ground using Grindomix GM100 apparatus (Retsch, Germany) and extracted at room temperature in 80% ethanol using a laboratory shaker for 24 h. Because of its high efficiency in extraction of wide range of biologically active secondary metabolites and due to fact that it is conventionally used in preparation of plant-based medicinal formulations (*e.g.*, tinctures), aqueous ethanol is generally recognized as universal solvent utilized in antioxidant and anti-proliferative assays of plant material (Schmidt et al., 2000; Huie, 2002). Extracts from each specimen were subsequently filtered and evaporated to dryness using a rotary evaporator R-200 (Büchi, Switzerland) *in vacuo* at 40 °C. Dry residues were then dissolved in 100% dimethyl sulfoxide (DMSO) to create a concentration of 51.2 mg/mL stock solution of each extract that was stored at −20 °C until tested.

### 2.3. Chemicals and reagents

2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), fluorescein sodium salt (FL), Eagle's minimum essential medium (EMEM), fetal bovine serum, glutamine, non-essential amino acids and penicillin-streptomycin solution were obtained at Sigma–Aldrich (Prague, Czech Republic). Analytical grade quality solvents and Folin-Ciocalteu reagent were acquired at Penta (Prague, Czech Republic).

### 2.4. Cell culture

Human liver carcinoma cell line Hep-G2 and normal human cells fetal lung MRC-5 (ATCC, Rockville, MD, USA) were maintained in EMEM supplemented with fetal bovine serum (10%), penicillin–streptomycin solution (1%), nonessential amino acids (1%) and glutamine (4 mM and 2 mM for Hep-G2 and MRC-5, respectively). Cell cultures were incubated in 5% $CO_2$ atmosphere at 37 °C.

### 2.5. Antioxidant activity and phenolic content

#### 2.5.1. DPPH radical-scavenging assay

Slightly modified method previously described by Sharma and Bhat (2009) was used. Initially, two-fold serial dilutions of each sample were prepared in methanol (175 μL) in 96-well microtiter plates. Subsequently, 25 μL of freshly prepared 1 mM DPPH in methanol solution was mixed with the sample in each well creating a range of concentrations from 2 to 5120 μg/mL (final volume of 200 μL) to start the radical-antioxidant reaction. The mixture was kept in the dark at laboratory temperature and absorbance was measured after 30 min at 517 nm using Infinite 200 reader (Tecan, Switzerland). Results were expressed as half maximal inhibitory concentration (μg/mL $IC_{50}$).

#### 2.5.2. Oxygen radical absorbance capacity (ORAC) assay

Slightly modified method previously described by Ou et al. (2001) was used. Firstly, outer wells of black absorbance 96-well microtiter plates were filled with 200 μL of distilled water, in order to provide better thermal mass stability. Prior to the study, stock solution of AAPH radical and FL were prepared in 75 mM phosphate buffer (pH 7.0). Subsequently, 25 μL of each sample was diluted in 150 μL FL (54 nM) and incubated in 37 °C for 10 min. Reaction was started by application of 25 μL AAPH (153 mM) yielding final volume of 200 μL in each well. Extracts were tested in range

**Table 1**
Ethnomedicinal data on plants tested.

| Scientific name | Family | Vernacular name (*Amharic*) | Ethnomedicinal uses |
|---|---|---|---|
| *Bersama abyssinica* Fresen. | Melianthaceae | *Azamir* | Treatment of diabetes (Keter and Mutiso, 2012) |
| *Calpurnia aurea* (Aiton) Benth. | Fabaceae | *Digita* | Treatment of diabetes (Giday et al., 2007; Suleman and Alemu, 2012), snakebite (Teklehaymanot et al., 2007) |
| *Carissa spinarum* L. | Apocynaceae | *Agam* | Antiarthritic activity (Hegde et al., 2010) |
| *Clematis hirsuta* Guill. & Perr. | Ranunculaceae | *Azo hareg* | Treatment of cataract (Giday et al., 2009), swelling (Teklehaymanot and Giday, 2007) |
| *Clutia abyssinica* Jaub. & Spach | Euphorbiaceae | *Fiyele fej* | Treatment of enlarged spleen (Ramathal and Ngassapa, 2001) |
| *Croton macrostachyus* Hochst. ex Delile | Euphorbiaceae | *Bisana* | Treatment of cancer (Ochwang'i et al., 2014), snakebite (Giday et al., 2009) |
| *Cyathula cylindrica* Moq. | Amaranthaceae | *Yedem abinet* | Treatment of snakebite (Lulekal et al., 2013) |
| *Dodonaea angustifolia* L. f. | Sapindaceae | *Kitkita* | Treatment lymphatic swelling (Mesfin et al., 2009) |
| *Embelia schimperi* Vatke | Primulaceae | *Inkoko* | Treatment of swelling (Lulekal et al., 2014a,b) |
| *Jasminum abyssinicum* Hochst. ex DC. | Oleaceae | *Abita* | Treatment of snakebite (Teklehaymanot et al., 2007) |
| *Maesa lanceolata* Forssk. | Primulaceae | *Kelewa* | Treatment of snakebite (Teklehaymanot et al., 2007) |
| *Ocimum lamiifolium* Hochst. ex Benth. | Lamiaceae | *Dama kessie* | Treatment of swelling (Lulekal et al., 2014a,b) |
| *Rubus steudneri* Schweinf. | Rosaceae | *Injori* | Treatment of stomach-ache (Giday et al., 2009) |
| *Rumex nepalensis* Spreng. | Polygonaceae | *Lut* | Treatment of rheumatism (Singh and Lal, 2008), stomach-ache (Giday et al., 2009) |
| *Thalictrum rhynchocarpum* Quart.-Dill. & A. Rich. | Ranunculaceae | *Sire bizu* | Treatment of cancer (Mugera, 1977) |
| *Verbascum sinaiticum* Benth. | Scrophulariaceae | *Yeahya joro* | Treatment of cancer (Teklehaymanot, 2009), stomach-ache (Teklehaymanot and Giday, 2007) |
| *Vernonia amygdalina* Delile | Asteraceae | *Girawa* | Treatment of cancer (Gresham et al., 2008; Kumar et al., 2011), swelling of thyroidal glands (Yirga, 2010), stomach-ache (Teklehaymanot et al., 2007) |
| *Zehneria scabra* (L. f.) Sond. | Cucurbitaceae | *Hareg iresa* | Treatment of stomach-ache (Teklehaymanot, 2009), unidentified swelling (Teklehaymanot et al., 2007) |

of 3.2–64 μg/mL (final concentration). Standard calibration curves of trolox were obtained in a concentration range of 0.5–8 μg/mL (final concentration) at five concentrations levels (0.5, 1, 2, 4, 8 μg/mL). Fluorescence changes were measured in 1-min intervals for 120 min with emission and absorbance wavelengths set at 485 nm and 520 nm, respectively. Quantification of the antioxidant capacity was calculated as area under the calibration curve as proposed by Cao and Prior (1998) and was expressed as trolox equivalents (μg TE/mg extract).

#### 2.5.3. Total phenolic content (TPC)

Total phenolic content was measured using modified method previously described by Singleton et al. (1998). Each sample in volume of 100 μL was added to 96-well microtiter plate creating range of concentrations from 21 to 341 μg/mL. Afterwards, 25 μL of pure Folin–Ciocalteu reagent was added. Plate was inserted in orbital shaker at 40 rpm for 10 min. Reaction was started by addition of 75 μL 12% $Na_2CO_3$ (w/v). Mixture was kept in dark at 37 °C for 2 h. Absorbance was measured at 700 nm. Nine concentration levels of gallic acid (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 μg/mL) were used to create standard calibration curves. Results were expressed as gallic acid equivalents (μg GAE/mg extracts).

### 2.6. Cell viability

Modified method based on metabolization of MTT to blue formazan by mitochondrial dehydrogenases in living cells previously described by Mosmann (1983) was used. Hep-G2 and MRC-5 cells were preincubated in a 96-well plate at a density of $2.5 \times 10^3$ cells per well for 24 h in a humidified atmosphere of 5% $CO_2$ in air at 37 °C. After 24 h, the cells were treated with plant extracts in twofold serial dilutions (range: 15.6–500 μg/mL) for 72 h. Thereafter, MTT reagent (1 mg/mL) in EMEM solution was added to each well and plates were incubated for additional 2 h at 37 °C. The media were then removed, and the intracellular formazan product was dissolved in 100 μL of DMSO. The absorbance was then measured at 495 nm and the percentage of viability was calculated when compared to untreated control. Results were expressed as half maximal inhibitory concentration of proliferation ($IC_{50}$) in μg/mL concentration.

### 2.7. Statistical analysis

All tests were performed as three independent experiments; each carried out in triplicate. Results were expressed as mean values with standard deviations (mean ± SD). Linear correlation coefficients ($r$) between total phenolic content and antioxidant assays (DPPH and ORAC) were established using Pearson product moment correlation. Data were log-transformed to fit the specific parameters of statistical test used. Statistical analysis was performed in STATISTICA 7.1 (StatSoft Inc., USA) software.

## 3. Results

In this study, 18 Ethiopian medicinal plants traditionally used for treatment of oxidative stress-related diseases were assayed for *in vitro* antioxidant and anti-proliferative activity. Four of these species exhibited significant antioxidant efficacy while nine species possessed significant anti-proliferative potential. However, majority of above mentioned efficient extracts exhibited toxicity to normal cells. With regard to all observed results two extracts exhibited combinatory antioxidant and anti-proliferative effect and one species showed pronounced selective anti-proliferative activity.

### 3.1. Antioxidant activity and content of phenolic compounds

Complete results for antioxidant activity of tested plants extracts are summarized in Table 2. In DPPH assay, the lowest $IC_{50}$ values were detected for *R. nepalensis* (roots), *R. steudneri* (roots) followed by *R. nepalensis* (leaves and twigs) at values 5.7; 5.8 and 10.7 μg/mL, respectively. The moderate effect was also detected for *D. angustifolia* (22.2 μg/mL), *B. abyssinica* (26.0 μg/mL) and for *J. abyssinicum* (26.3 μg/mL). $IC_{50}$ of rest of the plants were in range of 32.2–2668.3 μg/mL. None of the tested plants exceeded trolox

**Table 2**
Antioxidant activity and total phenolic content of tested plant extracts.

| Sample | Plant part(s)[a] | Antioxidant activity (mean value ± SD[b]) | | |
|---|---|---|---|---|
| | | DPPH ($IC_{50}$[c]; μg/mL) | ORAC (μg TE[d]/mg extract) | TPC (μg GAE[e]/mg extract) |
| *B. abyssinica* | LT | 26.0 ± 3.9 | 444.3 ± 18.5 | 286.4 ± 37.2 |
| *C. aurea* | R | 799.7 ± 70.7 | 331.6 ± 30.9 | 72.6 ± 17.1 |
| *C. spinarum* | R | 97.2 ± 4.9 | 363.9 ± 30.9 | 117.4 ± 22.6 |
| *C. hirsuta* | L | 67.0 ± 1.6 | 342.5 ± 19.1 | 133.9 ± 20.5 |
| *C. abyssinica* | R | 695.4 ± 109.9 | 221.6 ± 7.8 | 58.6 ± 8.0 |
| *C. macrostachyus* | LT | 1145.7 ± 161.2 | 130.8 ± 11.4 | 135.2 ± 38.9 |
| *C. cylindrica* | R | 2668.3 ± 212.7 | 37.8 ± 1.9 | 12.8 ± 2.0 |
| *D. angustifolia* | L | 22.2 ± 1.2 | 767.6 ± 47.5 | 531.7 ± 82.0 |
| *E. schimperi* | LT | 90.9 ± 9.3 | 210.1 ± 15.0 | 109.8 ± 10.4 |
| | S/F | 210.0 ± 21.6 | 107.3 ± 4.0 | 60.6 ± 6.9 |
| *J. abyssinicum* | L | 26.3 ± 6.5 | 1023.7 ± 49.5 | 401.3 ± 67.8 |
| *M. lanceolata* | LT | 41.3 ± 8.0 | 361.8 ± 17.2 | 153.5 ± 22.9 |
| *O. lamiifolium* | L | 45.9 ± 6.5 | 567.2 ± 28.3 | 156.8 ± 25.4 |
| *R. steudneri* | LT | 58.0 ± 6.4 | 1206.5 ± 72.8 | 829.5 ± 90.9 |
| | R | 5.8 ± 1.1 | 1019.2 ± 99.3 | 693.8 ± 113.8 |
| *R. nepalensis* | LT | 10.7 ± 1.7 | 371.1 ± 36.6 | 122.6 ± 12.4 |
| | R | 5.7 ± 0.9 | 1061.4 ± 106.4 | 1101.5 ± 157.1 |
| *T. rhynchocarpum* | R | 618.1 ± 107.8 | 96.3 ± 7.0 | 41.1 ± 6.3 |
| *V. sinaiticum* | L | 2015.2 ± 173.1 | 565.5 ± 24.3 | 22.2 ± 5.5 |
| *V. amygdalina* | F | 32.2 ± 7.1 | 665.9 ± 98.0 | 193.7 ± 27.4 |
| | LT | 39.6 ± 8.8 | 609.3 ± 38.2 | 191.8 ± 38.9 |
| *Z. scabra* | LT | 93.7 ± 14.5 | 372.9 ± 31.3 | 133.7 ± 10.8 |

[a] L = leaves, LT = leaves and twigs, F = flowers, R = roots, S/F = seeds/fruits.
[b] Standard deviation.
[c] Half maximal inhibitory concentration.
[d] Trolox equivalency.
[e] Gallic acid equivalency.

antioxidant efficiency. $IC_{50}$ for trolox was detected at concentration of 5.8 μg/mL.

The best results for ORAC assay were seen for *R. steudneri* (leaves and twigs), *R. nepalensis* (roots), *R. steudneri* (roots) and *J. abyssinicum* (1206.5; 1061.4; 1019.2 and 1023.7 μg TE/mg extract, respectively). In contrast to the results of the DPPH test, these four extracts demonstrated stronger antioxidant effect than trolox. The moderate results were only seen for *D. angustifolia* (767.6 μg TE/mg extract), whereas the rest of tested plants had ORAC values in range of 37.8–666.6 μg TE/mg extract.

The highest contents of phenolic compounds were determined for *R. nepalensis* (roots), followed by *R. steudneri* leaves and roots, and *D. angustifolia*, with values 1101.5; 829.5; 693.8 and 531.7 μg GAE/mg extract, respectively. The significant contents of phenolic compounds were also detected in *J. abyssinicum* (401.3 μg GAE/mg) and *B. abyssinica* (286.4 μg GAE/mg) extracts. Statistically significant correlation regarded as strong ($p < 0.001$) was found between TPC and both antioxidant assays used; DPPH ($r = 0.852$) and ORAC ($r = 0.798$) (Fig. 1).

### 3.2. Anti-proliferative activity

Complete results for anti-proliferative activity of tested plant extracts are given in Table 3. Significant anti-proliferative activity against Hep-G2 carcinoma cell lines was observed for *C. spinarum*, *M. lanceolata*, *B. abyssinica*, *E. schimperi* (leaves), *R. steudneri* (roots), *R. nepalensis* (roots), *Z. scabra* and *V. amygdalina* (flowers) whose $IC_{50}$ were detected at 32.8; 34.5; 35.4; 44.9; 45.5; 50.5; 57.1 and 57.8 μg/mL, respectively. Moderate cytotoxicity was detected for *V. sinaiticum* (80.6 μg/mL) and seeds of *E. schimperi* (81.8 μg/mL). Rest of the extracts showed weak anti-proliferative activity ($IC_{50}$ range from 103.5 to 267.6 μg/mL) or were ineffective ($IC_{50} > 500$ μg/mL). Weak positive correlation (statistically not significant at level $p = 0.05$) was detected between TPC and anti-proliferative effect towards Hep-G2 ($r = 0.389$).

Only extract of *V. sinaiticum* did not affect growth of MRC-5 cell line at the concentration 500 μg/mL. Low or moderate toxicity was observed for extracts of *C. cylindrica*, *C. abyssinica*, *C. aurea*, *D. angustifolia*, *C. hirsuta*, *V. amygdalina* (leaves), *R. nepalensis* (leaves), *R. steudneri* (leaves), *T. rhynchocarpum*, *R. nepalensis* (roots), *O. lamiifolium*, *J. abyssinicum* and *E. schimperi* (seeds) exhibiting $IC_{50}$ values in range of 119.7–426.4 μg/mL (Table 3). The remaining species demonstrated strong toxicity with $IC_{50}$ ranging from 10.9 to 97.4 μg/mL.

### 3.3. *Antioxidant and/or anti-proliferative effectivity* versus *toxicity*

Out of all tested plant species, combinatory antioxidant and anti-proliferative effect, together with relatively low toxicity to normal cells, was only detected for extracts of *D. angustifolia* and *R. nepalensis* (roots). By comparison of $IC_{50}$ (DPPH assay) and $IC_{50}$ (MTT assay), antioxidant potential of *D. angustifolia* and *R. nepalensis* (roots) was 5.4× and 10× stronger than observed anti-proliferative effect, respectively. Furthermore, $IC_{50}$ concentrations of both plants towards Hep-G2 were approximately two times lower than those for normal MRC-5 cells. Antioxidant effect of *D. angustifolia* and *R. nepalensis* (roots) is graphically illustrated in Fig. 2 (ORAC assay). Although *V. sinaiticum* did not prove to be considerable antioxidant agent, it exhibited fairly best selective cytoxicity towards carcinoma/normal cells of all extracts tested (*V. sinaiticum* extract showed approx. 25× higher $IC_{50}$ concentration in DPPH assay than in MTT anti-proliferative test). Anti-proliferative efficacy of *V. sinaiticum* extract is shown in Fig. 3.

## 4. Discussion

In accordance with our study, the antioxidant efficacy of *D. angustifolia* and *R. nepalensis* was previously described *in vitro* (Gautam et al., 2010; Anusuya et al., 2012). To our best knowledge this is the first report on characterization of antioxidant properties of *J. abyssinicum* and *R. steudneri*. Secoiridoid glycosides, which are presented in *J. abyssinicum* (Gallo et al., 2005), are well known to possess considerable antioxidant potential in assays per-

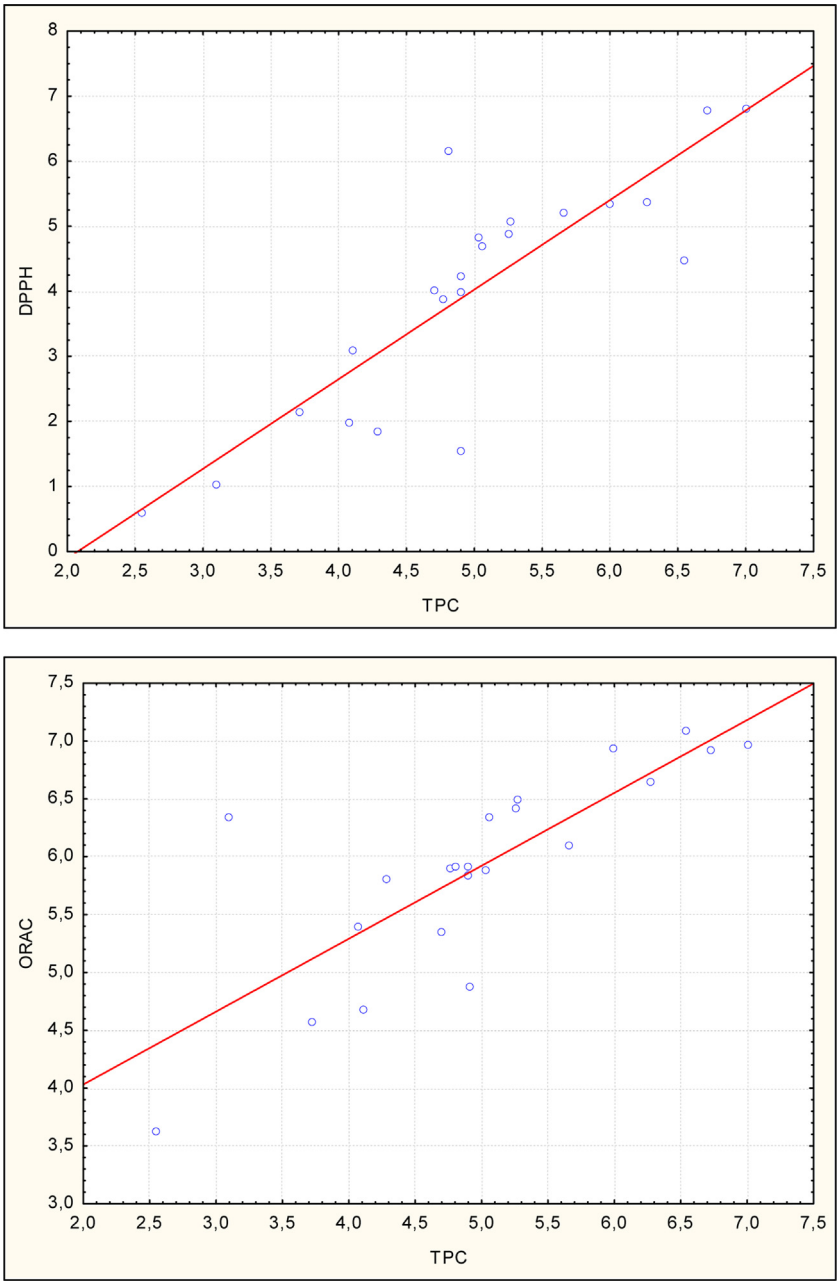


**Fig. 1.** Linear correlation of total phenolic content with DPPH and ORAC ($r = 0.852$ and $r = 0.798$, respectively). Log-transformed values were used in order to fit the specific parameters of Pearson correlation.

formed *in vitro* (Dinda et al., 2009; Bi et al., 2011). However, till present moment, specific secoiridoidal compounds presented in *J. abyssinicum* (namely craigosides) have not been verified for antioxidant efficacy. Phytochemical analyses of this plant are very limited; nevertheless other *Jasminum* species were reported to contain high levels of phenolic compounds (Sulaiman et al., 2013). Moderate content of phenolic compounds was also proved for *J. abyssinicum* in this study by TPC assay. Thus, above mentioned secoiridoid structures together with phenolics could be considered as major factor affecting antioxidant activity of *J. abyssinicum* extract. Similarly as in case of *J. abyssinicum*, phytochemical information of *R. steudneri* is unavailable. However related species, *R. ulmifolius* and *R. rigidus* which are believed to be native to different parts of Africa, were found to be rich in phenolic acids flavonoids, anthocyanins, diterpenoids, carotenoids and their glycosidal derivatives (Barros et al., 2010; Nguelefack et al., 2011; Lee et al., 2012). It could be assumed, that significant antioxidant potential of *R. steudneri* is attributed to high content of above mentioned group of chemicals. With regard to the discussion on phytochemicals responsible for antioxidant effects of above mentioned species, it is necessary to note that chemical composition and biological activity of plant extracts can significantly be affected by extraction technique and type of solvent used (Dai and Mumper, 2010). In comparison with plants used commonly as herbal medicines and spices such as Ceylon cinnamon tree (*Cinnamomum verum*) and Turmeric (*Curcuma longa*) which previously demonstrated strong antioxidant capacity in ORAC assay (1000–2000 μg TE/mg extract) (Wojcikowski et al., 2007; Dudonné et al., 2009), the extracts of *R. steudneri*, *R. nepalensis*, *J. abyssinicum* and *D. angustifolia* exhibited results similar to above mentioned plant species. Furthermore, strong correlation between TPC and antioxidant assays (DPPH and ORAC) was previously observed for *C. verum* and *C. longum* (Wojdyło et al., 2007; Dudonné et al., 2009)

**Table 3**
Anti-proliferative activity and toxicity of tested plant extracts.

| Sample | Plant part(s)[a] | $IC_{50}$[b] (mean ± SD[c])/Cell line | |
|---|---|---|---|
| | | HEP-G2[d] (μg/mL) | MRC-5[e] (μg/mL) |
| *B. abyssinica* | LT | 35.4 ± 8.8 | 10.9 ± 2.3 |
| *C. aurea* | R | >500 | 292.7 ± 37.6 |
| *C. spinarum* | R | 32.8 ± 5.7 | 97.4 ± 19.6 |
| *C. hirsuta* | L | >500 | 224.6 ± 1.8 |
| *C. abyssinica* | R | >500 | 355.0 ± 18.1 |
| *C. macrostachyus* | LT | 267.6 ± 1.0 | 27.4 ± 12.1 |
| *C. cylindrica* | R | >500 | 426.4 ± 1.9 |
| *D. angustifolia* | L | 120.0 ± 10.8 | 225.5 ± 17.7 |
| *E. schimperi* | LT | 44.9 ± 0.1 | 37.0 ± 2.6 |
| | S/F | 81.8 ± 2.3 | 119.7 ± 10.7 |
| *J. abyssinicum* | L | 217.5 ± 10.3 | 122.2 ± 1.8 |
| *M. lanceolata* | LT | 34.5 ± 6.3 | 30.1 ± 0.2 |
| *O. lamiifolium* | L | 115.4 ± 5.0 | 124.1 ± 12.2 |
| *R. steudneri* | LT | 103.5 ± 8.9 | 183.6 ± 41.2 |
| | R | 45.5 ± 6.9 | 38.4 ± 1.7 |
| R. nepalensis | LT | 237.4 ± 8.9 | 184.0 ± 14.0 |
| | R | 50.5 ± 4.3 | 132.7 ± 5.2 |
| *T. rhynchocarpum* | R | 142.6 ± 0.4 | 149.0 ± 21.7 |
| *V. sinaiticum* | L | 80.6 ± 46.1 | >500 |
| *V. amygdalina* | F | 57.8 ± 5.4 | 32.2 ± 10.1 |
| | LT | 191.5 ± 15.0 | 200.1 ± 30.8 |
| *Z. scabra* | LT | 57.1 ± 6.9 | 38.4 ± 1.7 |

[a] L = leaves, LT = leaves and twigs, F = flowers, R = roots, S/F = seeds/fruits.
[b] Half maximal inhibitory concentration of proliferation.
[c] Standard deviation.
[d] Human liver carcinoma cell line.
[e] Normal human fetal lung cell line.

as well as for samples tested in our study. These results suggest that phenolics are the main class of compounds responsible for antioxidant effect of tested plant extracts. This finding was also previously reported for many other plant species including those with industrial interest (Qader et al., 2011; Clarke et al., 2013).

Practical usability of plants as anti-proliferative agents hugely depends on their toxicity and it is requested that these compounds should exert selective cytotoxic effect towards carcinoma and normal cells (Lindholm et al., 2002). Anti-proliferative efficacy of *V. sinaiticum* is relatively well established and our results are in accordance with cytotoxic properties of *V. sinaiticum* observed in other studies (Talib and Mahasneh, 2010). As far as we know, this is the first report on anti-proliferative efficacy of *C. spinarum* plant extract, together with first report on selective cytotoxic effect of *V. sinaiticum* towards normal cells. *C. spinarum* was found to contain lignans, namely (−)-carinol, (−)-carissanol and (−)-nortrachelogenin, which exhibited cytotoxicity against various cancer and leukaemia cells (Sehar et al., 2011; Wangteeraprasert et al., 2012). These chemicals were therefore considered as major factor influencing anti-proliferative effect of this plant. However, *C. spinarum* also produces cardiac glycosides (including evomonoside), which previously demonstrated to be significant inhibitors of $Na^+/K^+$-ATPase, and therefore presents possible toxicological risk (Dewick, 2009; Schön et al., 1995). Most interesting results

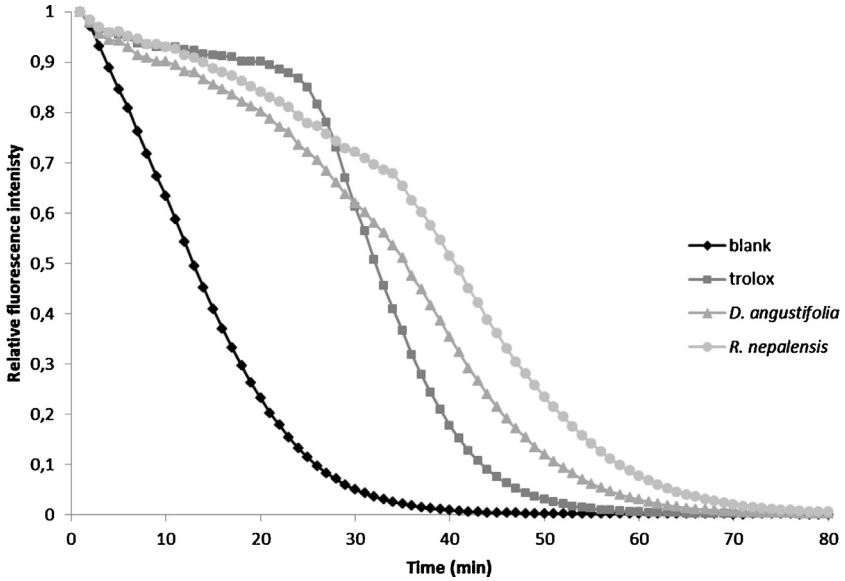


**Fig. 2.** Kinetic curves of fluorescein decay induced by AAPH radical in the presence of *Dodonaea angustifolia* and *Rumex nepalensis* (roots) extracts at concentration of 1 μg/mL in comparison to positive (trolox) and negative (blank) controls (ORAC assay).

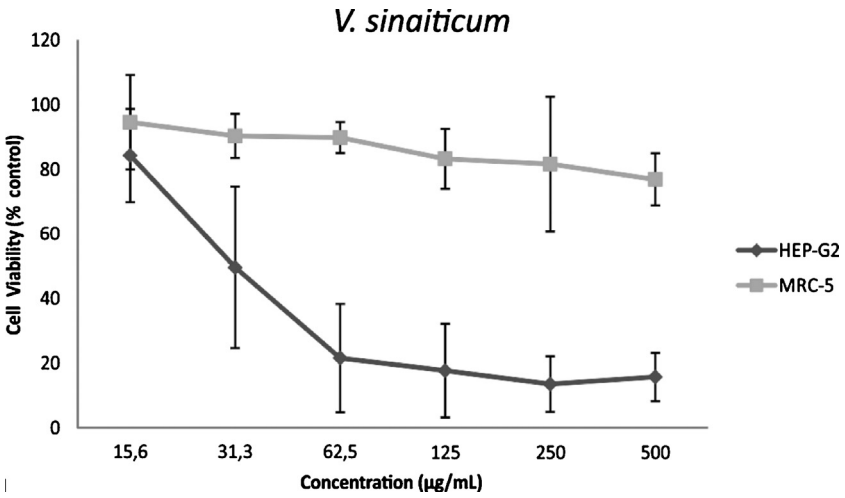


**Fig. 3.** Anti-proliferative effect of *Verbascum sinaiticum* extract towards hepatocellular carcinoma (Hep-G2) and normal (MRC-5) cells after 48 h exposure (MTT assay).

regarding selectivity in anti-proliferative effect towards carcinoma and normal cells was seen in *V. sinaiticum*. From compounds presented in *V. sinaiticum*, only luteolin, chrysoeriol, hydrocarpin and sinaiticin were tested for cytotoxic potential with positive effect against leukaemia P-388 cells (Afifi et al., 1993). Since these compounds belong to chemical classes of flavonoids and flavonolignans (Afifi et al., 1993; Mahmoud et al., 2007), they are regarded as non-toxic to its potential user. These constituents are therefore considered as contributory to observed selective anti-proliferative activity of *V. sinaiticum* against Hep-G2 and MRC-5 cell line. Previously, $IC_{50}$'s of podophyllotoxin, paclitaxel and vincristine (commonly utilized anti-cancer drugs) towards Hep-G2 carcinoma cells were assessed at concentrations around 1–2 ng/mL (Jin et al., 2010; Zhao et al., 2012; Hussain et al., 2014). As contrast to these compounds, extracts of *C. spinarum* and *V. sinaiticum* were significantly less effective anti-proliferative agents. On the other hand, in comparison to Garlic (*Allium sativum*) supplements, which are recommended to lower risk of certain types of cancer (Stan et al., 2008) and earlier demonstrated significant activity towards Hep-G2 carcinoma cell line ($IC_{50}$ at 330 μg/mL) (Siegers et al., 1999), extracts of *C. spinarum* and *V. sinaiticum* possessed considerably higher anti-proliferative efficacy. Even though sulphide compounds (*e.g.* allicin) are considered as chief constituents responsible for anticancer activity of garlic (Ariga and Seki, 2006), this plant was also found to possess moderate amounts of phenolic compounds (approx. 51 μg GAE/ mg extract) (Stratil et al., 2006); *i.e.* at levels comparable to *C. spinarum* and *V. sinaiticum* in our study. Additionally, garlic also previously showed mild antioxidant properties in ORAC assay (Javier Moreno et al., 2006; Lim, 2015). Our data from statistical analysis (correlation between TPC and $IC_{50}$ for Hep-G2 cells) suggest that phenolic compounds might be partially answerable for observed anti-proliferative effect of tested plant species, which is in correspondence with above mentioned data, as well as with other studies (Yang et al., 2009).

Previously several plant-based compounds have demonstrated combinatory antioxidant and anti-proliferative effect (Znati et al., 2014). Even though antioxidant potential of *D. angustifolia* and *R. nepalensis* was previously determined (as mentioned above), to our best knowledge this is the first report on anti-proliferative efficacy of these plants. Anthraquinones and naphthalene derivatives isolated from *R. nepalensis* have previously showed both significant antioxidant activity and anti-proliferative properties towards various carcinoma cell lines *in vitro* (Gautam et al., 2010; Liang et al., 2010). It is therefore assumable that these compounds are probably responsible for observed combinatory antioxidant and anti-proliferative effect. None of suggested active constituents were submitted to toxicity assessment; however, it could be suggested that these compounds possess selective anti-proliferative effect with mild toxicity. Clerodane diterpenoids, which are produced by *D. angustifolia* (Omosa et al., 2010, 2014), have previously demonstrated considerable antioxidant efficacy and anti-proliferative activity against several carcinoma and leukaemia cell lines *in vitro* (Huang et al., 2004; Van Nguyen et al., 2009; Liu et al., 2014). Some structures belonging to clerodane diterpenoids were also found to be toxic to normal cells (Ma et al., 2012). Additionally, nephrotoxicity of *D. angustifolia* being accompanied by avascular renal necrosis was previously described in mice and ruminants (Steenkamp and Stewart, 2005). According to these findings, above mentioned compounds contribute significantly to cytotoxic and possibly even toxic activity of *D. angustifolia* extract. Examination of plant material for combinatory antioxidant/anti-proliferative potential (extracts or sole plant-derived compounds) is at present moment recognized as a novel approach (Nagahama et al., 2011). In previous study, the ethanol extract of *Berberis vulgaris*, a well-known medicinal plant traditionally used in treatment of various illnesses likely associated to oxidative stress including cancer (Abd El-Wahab et al., 2013), have demonstrated significant anti-proliferative activity towards Hep-G2 cells ($IC_{50}$ 106 μg/mL), as well as considerable antioxidant effect in DPPH assay ($IC_{50}$ approx. at 25 μg/mL) (Hanachi et al., 2006). These results were relatively comparable to activities observed for *D. angustifolia* in this study. However, *R. nepalensis* exhibited stronger effect in both assays in comparison to *B. vulgaris*. With regard to above mentioned information, we suggest that *D. angustifolia* and *R. nepalensis* might possess compounds with combinational antioxidant/anti-proliferative properties.

## 5. Conclusion

In summary, the current study proved *in vitro* antioxidant and anti-proliferative activity of *D. angustifolia*, *R. nepalensis* and *V. sinaiticum* extracts. Therefore these plants seem to serve as prospective material for further development of novel plant-based antioxidant and/or anti-proliferative agents. Especially antioxidant potential of *R. nepalensis* and selective anti-proliferative effect of *V. sinaiticum* deserve deeper research attention. However, detailed analysis of their chemical composition and *in vivo* antioxidant/anti-

proliferative activity should be carried out in order to verify their possible practical use.

## Conflict of interest

All authors disclose that they have no financial and personal relationships with other people or organization that could inappropriately influence (bias) their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registration, and grants or other funding.

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## **Appendix D:** Photographic documentation

**Apx. D 1** Kvevri – clay vessel traditionally used in Georgia for fermentation of wine (Kakhetian winemaking mehod). Photograph by courtesy of David Maghradze.

**Apx. D 2** Example of wine cellar with holes especially designed for kvevri insertion. Grape pomace is mashed inside the kvevri and enclosed by wooden lids, which are subsequently sealed, typically with wax. Wine is usually fermented for 3 - 5 months. Photograph by courtesy of David Maghradze.


**Apx. D 3** Samples of Georgian, Central and West European wines ready to be assayed. Photograph by courtesy of Přemysl Landa.

**Apx. D 4** A woman selling plants used in traditional Ethiopian medicine. Gorabela medicinal plant market, Ankober District, Ethiopia. Photograph by courtesy of Ermias Lulekal**.**

**Apx. D 5** Back from the field. Herbarium specimens mounted on Donkey's back. Ankober District, Ethiopia. Photograph by courtesy of Ermias Lulekal.


**Apx. D 6** Herbarium specimens of Ethiopian medicinal plants. From left to right: *Rumex nepalensis*, *Dodonaea angustifolia* and *Verbascum sinaiticum*. Photograph by courtesy of Ermias Lulekal.

**Apx. D 7** Habitus of young individual of *Inga edulis* (Guaba) at surrounding farms of Pucallpa City, Ucayali District, Peru. Photograph by courtesy of Ludvík Bortl.

**Apx. D 8** Inflorescence of *I. edulis*. Photograph by courtesy of Ludvík Bortl.

**Apx. D 9** Young seedlings of palm *Oenocarpus bataua* (Ungurahui). Forest nursery of Universidad Nacional Intercultural de la Amazonia (UNIA), Pucallpa City. Photograph by courtesy of Ludvík Bortl.

**Apx. D 10** Habitus of mature individual of *O. bataua*. Photograph by courtesy of Ludvík Bortl.


**Apx. D 11** Fruit of *O. bataua* in detail. Photograph by courtesy of Ludvík Bortl.


**Apx. D 12** Local product of *O. bataua* – icecream made from fruit of this palm has been manufactured in Peru by Shambo® since 1963. Photograph by courtesy of Ludvík Bortl.


**Apx. D 13** Fruit of *Annona montana* (Guanabana). Photograph by courtesy of Ludvík Bortl.


**Apx. D 14** Cross-section of *A. montana* fruit. Photograph by courtesy of Ludvík Bortl.