EVALUATION OF POTENTIAL CYTOTOXIC EFFECTS OF HERBAL EXTRACTS

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EVALUACIJA BILJNIH EKSTRAKATA SA POTENCIJALNIM CITOTOKSIČNIM EFEKTOM

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Abstract

Herbal medicines have played an important role in treating different diseases since ancient times. Bioactive components of medicinal plants are a good starting point for discovering new drugs such as chemotherapeutics. Currently, there are four classes of plant-derived chemotherapeutic drugs used in clinical practice. However, to discover new potential cytotoxic molecules, the research effort on herbal extracts has not diminished. The aim of this review was to evaluate the chemical constituents of plants that possess cytotoxicity, the signalling pathways responsible for this effect, and the influence of solvent polarity on potential cytotoxic effect and to present the cytotoxic activity of selected herbal extracts. The polyphenolic, anthraquinon, diterpneoid, triterpenoid, flavonoid, betulinic acid and berberine content contributes to cytotoxicity of herbal extracts. The inhibitory effect on cancer cells viability could be a consequence of the non-apoptotic processes, such as cell cycle arrestment, and the apoptotic process in tumour cells through different signalling pathways. The influence of solvent polarity on potential cytotoxic effect of herbal extracts should not be ignored. In general, the best cytotoxic activity was found in nonpolar and moderately polar herbal extracts. The herbal extract with IC_{50} below 30 μg/ml could be considered a very strong cytotoxic agent. Considering that many antitumor drugs have been discovered from natural products, further research on plants and plant-derived chemicals may result in the discovery of potent anticancer agents.

Keywords: *cytotoxicity; herbal extracts; herbal chemical constituents, apoptosis.*

SAŽETAK

Biljni lekovi imaju značajnu ulogu u lečenju različitih bolesti od davnina. Boaktivna jedinjenja lekovitih biljaka mogu biti dobra osnova za otkriće novih lekova kao što su hemoterapeutici. Trenutno se u kliničkoj praksi koriste četiri klase hemoterapetika biljnog porekla, ali istraživanje biljnih ekstrakata sa ciljem otkrića novih potencijalno citotoksičnih molekula ne prestaje. Cilj ovog preglednog rada je bio procena hemijskih jedinjenja koja pokazuju citotoksični efekat, signalnih puteva odgovornih za ovakav efekat, uticaja polarnosti rastvarača na potencijalnu citotoksičnost i prikaz citotoksične aktivnosti odabranih biljnih ekstrakata. Sadržaj polifenolnih jedinjenja, antrahinona, diterpenoida, triterpenoida, flavonoida, betulinske kiseline i berberina doprinosi citotoksičnom efektu biljnih ekstrakata. Inhibicija vijabilnosti ćelija kancera može biti posledica neapoptotskih procesa, kao što je prekid diferencijacije ćelija i apoptotskog procesa posredstvom različitih signalnih puteva. Ne treba zanemariti ni uticaj polarnosti rastvarača na potencijalni citotoksični efekat biljnih ekstrakata, ali generalno se smatra da najbolju citotoksičnu aktivnost poseduju nepolarni i umereno polarni ekstrakti. Biljni ekstrakti čija je IC_{50} vrednost ispod 30 μg/ml se mogu smatrati snažnim citotoksičnim agensom. Imajuću u vidu da su mnogi antitumorski lekovi dobijeni iz prorodnih proizvoda, dalja istraživanja biljaka i njihovih hemijskih jedinjenja mogu rezultovati otkrićem potentnih antitumorskih agenasa.

Ključne reči: *citotoksičnost, biljni ekstrakti, hemijski sastav biljaka, apoptoza*

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INTRODUCTION

For centuries, herbal medicines played a very important role in treating different diseases as a part of the Indian ayurveda, the traditional Chinese and Greek medicines (1). Today, there are more than 20 000 plant species that are used in traditional medicines (2). Bioactive components of medicinal plants are a good starting point for discovering new drugs (3). For example, cardiac glycosides obtained from *Digitalis lanata* and *Digitalis purpurea* (*Plantaginaceae*) have been successfully used for treating heart failure and certain cardiac arrhythmia with no alternative among synthetic drugs (1). According to ethnomedicinal uses, some plant compounds are used in therapies for different widespread diseases, including cancer (4). Currently, there are four classes of plant-derived chemotherapeutic drugs that are used in clinical practice. These are vinca alkaloids (vinblastine, vincristine and vindesine), epipodophyllotoxins (etoposide and teniposide), taxanes (paclitaxel and docetaxel) and the camptothecin derivatives (camptotecin and irinotecan) (5). According to the American National Cancer Institute, the upper limit for the IC_{50} value, which indicates the promising plant material for further cytotoxic agent purification, is 30 μg/ml (6).

Chemotherapy is routinely used for cancer treatment, but the toxicity of chemotherapeutics on healthy cells of the human body is obvious (5). This is the reason for discovering new, natural origin, substances with potential cytostatic effect and less toxic side effects on the healthy cells (2). Therefore, the main advantage of herbal extracts compared to synthetic drugs is their lower toxicity. For example, it is well known that long-term anthracycline (doxorubicin) therapy causes severe complications such as cardiotoxicity and acute myelocytic leukaemia (7). However, repetitive treatment with natural anthracycline (aloin) did not produce oxidative stress-induced cardiotoxicity in the animal model (8). Another important problem related to chemotherapeutic treatment is its multi-drug resistance, which leads to the decreased effectiveness of anti-cancer drugs. Several plant derivatives from the flavonoid class demonstrated a synergistic effect with anti-cancer drugs and increased the accumulation of drug in resistant cancer cells (9). A number of studies indicated that quercetin and its derivatives act synergistically with doxorubicin and daunorubicin to overcome multi-drug resistance in cancer patients (9, 10). Nevertheless, research effort on herbal extracts to discover new bioactive molecules with potential cytotoxic effect has not diminished (11).

The aim of this review was to evaluate both the chemical constituents of plants that possess cytotoxicity and the signalling pathways responsible for this effect. The solvent polarity influence on the potential cytotoxic effect of herbal extracts was taken into account. Additionally, the cytotoxic activity of selected herbal extracts was discussed.

Chemical constituents that exhibit cytotoxicity

There is a wide range of phytochemical compound classes that may contribute to cytotoxic effect of the plant. Some of the most common classes are anthraquinons, polyphenolic compounds and triterpenoid saponins. The high content of polyphenolic compounds and anthraquinons is responsible for the antioxidant activity, which is associated with stronger cytotoxic activity (6, 12). Several mechanisms have been involved in the anticancer activity of anthraquinons. These include the intercalation of DNA, inhibition of DNA topoisomerase II, production of free radicals and subsequent cleavage of DNA (13, 14). Esmat et al. researched the cytotoxic effect mechanism of the constituent (anthraquinone – aloin) in different aloe varieties on human breast cancer cell lines. Aloin showed dose-dependent cytotoxic action by reducing the proportion of cells that undergo mitosis by inducing apoptosis, inhibiting topoII**α** protein expression and downregulating cyclin B1 protein expression in breast cancer cells without erbB-2 and topoII**α** coamplification. TopoII**α** is primarily produced in the late S phase and during the G2M phase of the cell cycle. Meanwhile, cyclin B1 is important in the G2M progression because its reduced expression results in the accumulation of inactive Cdk1-cyclin B1 kinase complex (15). It is concluded that anthraquinone (aloin) induced the apoptosis and cell cycle arrest at the G2M phase (7).

The antioxidant activity of polyphenolic compounds is partly responsible for the correlation between the increased polyphenol intake and the reduced cancer risk. Polyphenols have the ability to down-regulate oxidative and inflammatory signal cascades by inhibiting transcription factors, such as the nuclear factor-kappaB (NF-kB) and the activator protein-1 (AP-1), which are responsible for the expression of the reactive oxygen species (ROS)-induced inflammatory enzyme cascade (16 - 18). The natural polyphenolic compounds, such as curcumin, genistein, resveratrol and catechins, can inhibit the number of growth factors involved in carcinogenesis. Additionally, phenolics have the ability to arrest cells in the S/G2 transition phase, increase the number of cells in the G1/S phase and induce apoptosis. Apoptosis is, probably, the consequence of decreased expression of the major anti-apoptotic oncogene (Bcl-2) (19). Phenolic suppression of angiogenesis is the result of matrixmetalloproteinase (MMP) attenuation and inhibition of vascular endothelial growth factor (VEGF) and their Src kinases (16). Additionally, phenolic compounds can influence the reduced metastatic process by attenuating the expression of vascular adhesion molecules (19). Flavonoid (apigenin) induces apoptosis by increasing the phosphorylation of Her2/neu via the PI3/Akt kinase pathway. The subsequent proteosomal degradation in breast cancer cells that over-express this receptor/transcription factor suppresses tumour growth (20). The possible attenuation mechanism of tumour metastasis is down-expression of the circulating soluble adhesion molecules (ICAM-1, E-selectin and E-cadherin) (19).

The cytotoxic effect of most of the researched triterpenoid saponins was based on their ability to stimulate apoptotic process in tumour cells, usually through its intrinsic pathway. In addition, the non-apoptotic processes, such as cell cycle arrestment, autophagic cell death stimulation, metastasis inhibition and cytoskeleton disintegration, were involved in the cytotoxic activity (21). The cytotoxic activity of triterpenoid saponins isolated from different plant species has been reported for various cell lines, which include human cervix (HeLa), human hepatocyte (Hep-G2), fibrosarcoma (HT1080) and human promyelocytic leukaemia (HL-60) cell lines (22). There are several possible apoptosis mechanisms. One of them is the caspase-3-mediated apoptotic pathway of triterpenoid saponins isolated from *Anemone flaccida* and confirmed on the HeLa cell line (23). The cell death induced by the plant extracts via the classic caspase-3-dependent apoptosis follows one substrate of caspase 3, the poly (ADP-ribose) polymerase (PARP) protein that undergoes cleavage after receiving the apoptotic signal (24). Additionally, the triterpenoid saponin class showed antitumor activities via the COX-2/PGE2 signalling pathway. Flaccidoside II, isolated from *Anemone flaccida,* has the ability to suppress COX-2 on both the mRNA and protein levels as well as on the PGE_2 synthesis level (the product of COX-2). Considering that the increased levels of COX-2 and its product PGE_2 are identified in cancer cells as markers of tumour angiogenesis, the inhibition of the COX-2/PGE2 pathway may be one of the possible apoptotic mechanisms of triterpenoid saponins (23, 25). Triterpenoid saponin, tubeimoside 1, which was isolated from *Bolbostemma paniculatum* and applied to HeLa cells, induced the depletion of mitochondrial transmembrane potential and caused the activation of caspase-dependent apoptotic cell death (21). Additionally, the increased expression of GADD153/CHOP transcription factor, which is associated with growth arrest and apoptosis in the event of prolonged endoplasmic reticulum (ER) stress, was noticed (26). The saponin-enriched fraction, isolated from *Bupleurum kaoi*, showed cytotoxic activity on human non-small cell lung cancer (A549) via the extrinsic apoptosis pathway. The enhancement in Fas and its two ligand forms, membrane-bound Fas ligand and soluble Fas ligand, was observed (27).

Betulinic acid, an antitumor agent, is a natural pentacyclic triterpenoid, which activates the mitochondria-mediated intrinsic apoptosis pathway (9). It was detected that betulinic acid has the ability to induce mitochondrial outer membrane permeabilization as the initial step in the activation of the mitochondrial pathway (28). In intact cells, betulinic acid caused the release of apoptogenic factor, cytochrome c, in a caspase-independent and permeability transition pore-dependent manner. It was reported that betulinic acid treatment caused ROS generation in different cancer cells that were involved in initiating the mitochondrial membrane permeabilization (29, 30). This is associated with the activation of pro-apoptotic p38 and stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) without change in the phosphorylation of extracellular signal-regulated kinases (ERK). Therefore, ROS act upstream of the ERK in the betulinic acid signalling pathway (28). Additionally, betulinic acid was marked as the activator of NF-κB transcription factor and the regulator of stress-induced transcriptional activation in numerous cancer cell lines (31).

Berberine, which is extracted from roots, rhizomes, stems and bark of different medicinal plants, has the ability to inhibit cellular growth in melanoma, hepatocellular carcinoma, breast, prostate, colon cancers and leukaemia (32 - 34). It was suggested that the death receptor pathway may be involved in the apoptotic pathway induced by berberine because it influenced the up-regulated mRNA and/or protein expressions of Fas, FasL, TNF-alpha, caspase-3 and down-regulation of pro-caspase-3. Additionally, there is evidence that berberine-induced apoptosis was linked with up-regulated expression of the tumour suppressor gene p53 and prohibitin (PHB) and decreased vimentin expression (35). The possible mechanisms of berberine-induced breast cancer cell toxicity and apoptosis are presented in Image 1.

Taxane, a naturally occuring diterpneoid, exhibits an antiproliferative activity against breast cancer cells (36). There is no well-defined apoptotic pathway of taxanes. However, one important characteristic is the down-regulation of Bcl-2 and/or up-regulation of p53 and p21/WAF-1 (37). Some compounds that were later developed into full anticancer agents originated from natural sources and are lignans (38). Some of the lignan derivatives have reached phases I and II of clinical trials as anti-cancer agents and include GP-11, NK-611, TOP-53, NPF and GL-331 (39). The new native lignan, vitexin 6, activates the Jun N-terminal kinase (JNK) pathway and leads to the inhibition of cancer cell proliferation. Vitexin 6 treatment increases JNK phosphorylation, which is followed by the upregulation of P-Bcl-2 and P-C-Jun expression (40).

Methods for evaluating the cytotoxic effect

There are several *in vitro* and *in vivo* methods described in the literature that are used for evaluating cytotoxic activity. The most frequently used methods are the neutral red method, the Brine shrimp lethality assay, and the MTT and XTT assays (41 – 44).

The influence of solvent polarity on potential cytotoxic effect of herbal extracts

Herbal extracts could be obtained using extraction with polar or nonpolar solvents. The most commonly used polar solvents are water, ethanol, methanol, and the water/ethanol or water/methanol mixtures in different ratios. The nonpolar solvents used in herbal extraction are acetone, chloroform, methylene chloride and petrol ether (45). The type of extracted constituent depends on solvent polarity and, thus, contributes to different cytotoxicity.

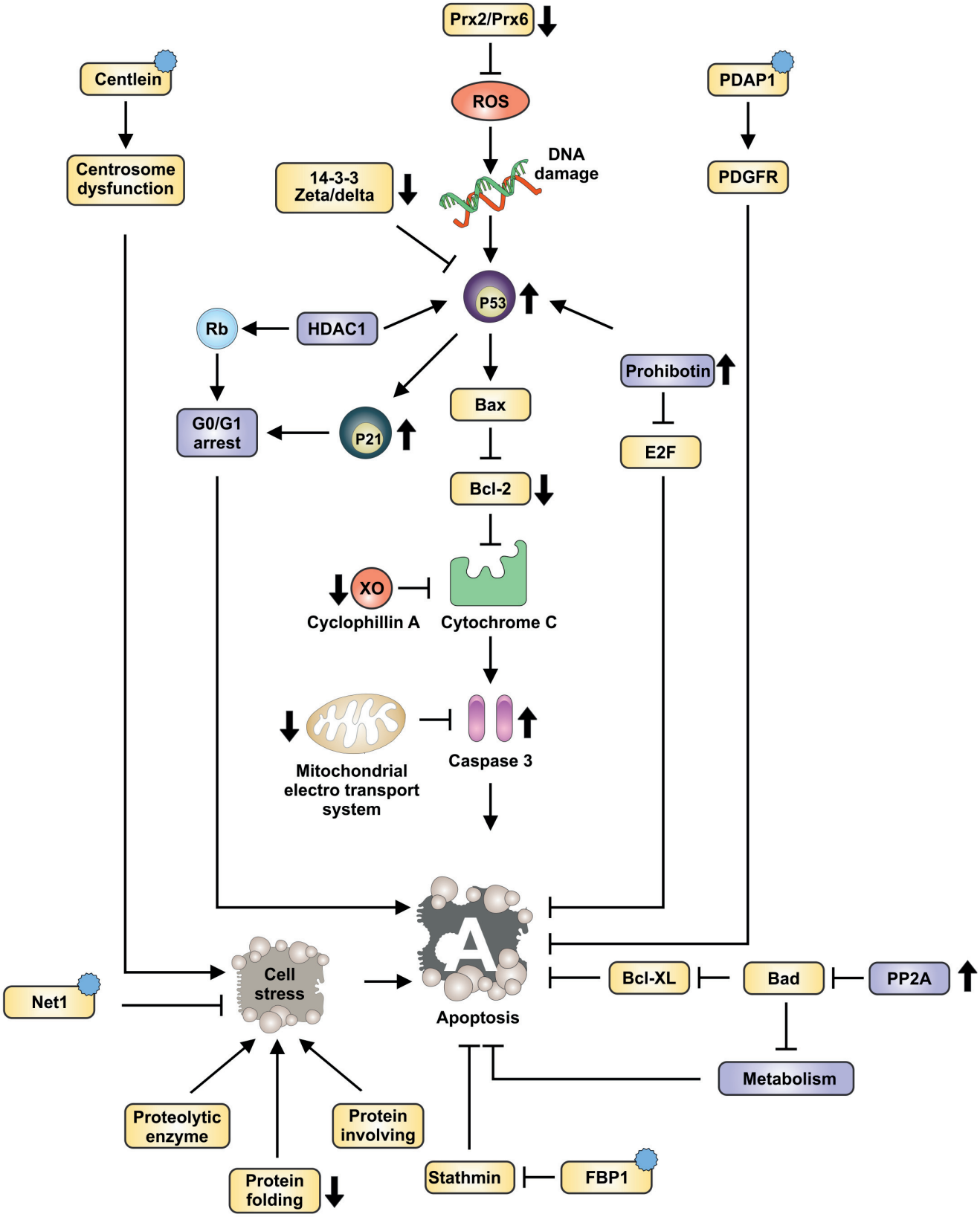

Image 1 - Mechanisms of berberine-induced breast cancer cell toxicity and apoptosis.

Even traditional herbal medicines are usually prepared as infusions or decoctions (water as an extraction solvent), and most of the aqueous extracts demonstrate a low cytotoxic activity (46, 47). The effect is usually observed by applying the extract in concentration above 250 μg/ml (46). However, components from the nonpolar extract possessed more clastogenic activity, which can be specifically targeted to destroy cancer cells (48). Additionally, the best cytotoxic activity was found in nonpolar and moderately polar herbal extracts (49). Data indicate that the majority of potent cytotoxic phytochemical compound classes can be extracted with nonpolar solvents. Triterpenes can be extracted with ether, petroleum ether and hexane, while flavonoids are usually detected in the chloroform and methanol extracts (50). Therefore, extraction with a nonpolar organic solvent is more appropriate for achieving a better cytotoxic effect.

Cytotoxic activity of the selected herbal extracts

Determination of cytotoxic activity of herbal extracts has become a prevalent research topic with numerous publications. A significant number of publications has been published in the last five years (2010 - 2014). The list of the investigated plant species for potential cytotoxic activity during this period is extensive: *Urtica dioica*, *Cucurbita pepo*, *Potentilla reptans*, *Struchium sparganophora*, *Sideritis scardica*, *Moringa oleifera*, *Cynometra cauliflora*, *Onosma paniculatum*, *Biophytum sensitivum*, *Ocimum viride*, *Terminalia arjuna*, *Garcinia indica*, *Acacia nilotica* and others (51, 2, 46, 38, 52 - 55, 11, 56-59). The cytotoxic effect of the extracts prepared from the selected medicinal plants will be discussed in this section.

Urticae folium (*Urtica dioica* L., *Urticaceae*) – the hydro-alcoholic extract (50:50) – was evaluated on HepG2 cells using the MTT assay in the presence of vinblastine as a positive control. The obtained EC_{50} value was 0.76±0.13 mg/mL. The authors concluded that the *Urticae folium* extract did not show a significant cytotoxic effect (51). This can be attributed to the extraction with a mixture of polar solvents and the absence of potent cytotoxic phytochemicals.

Solani fructus (*Solanum nigrum* L., *Solanaceae*) - hydro-alcoholic extracts with concentrations of 5, 25, 50, 100, 150 μg/ml were investigated on the HepG2 and CT26 (human colon) cancer cell lines in the presence of cisplatin and *Taxus baccata* extract as the positive control substances. The experiment was performed using the colonogenic assay. The IC_{50} values were 56.4±9.3 and 77.6±1.2 μg/ml for the HepG2 and CT26 cell lines, respectively. The *Solanum nigrum* extract could be a promising cytotoxic agent because of the obtained IC_{50} values that are similar and for the *Taxus baccata* extract, which is considered to be a common natural anticancer product (2). The cytotoxic activity of glycoprotein isolated from *Solanum nigrum* supports the fact that a single active compound usually possess a stronger activity compared with the entire extract, which is a mixture of all present compounds, the active and ballast material. Glycoprotein showed a significant cytotoxic and apoptotic effect at a concentration of 40 μg/ml after only 4 hours. It is suggested that the *Solanum nigrum* glycoprotein induces apoptosis via the mitochondrial apoptotic signal pathway. It caused the release of cytochrome c, stimulation of caspase-8, -9 and -3 activities, and the cleavage of poly(ADP-ribose)polymerase in HCT-116 cells (60). The cytotoxic activity of *Solanum nigrum* could be a consequence of the anthocyanidin presence in the fruits.

Potentillae rhizome and *herba* (*Potentilla reptans* L. *Rosaceae*) – in a previous work of the author, the cytotoxic activity of aqueous extracts for the concentration range of 100 – 800 μg/ml was evaluated on a mouse breast cancer cell line (4T1) using the MTT assay. The authors performed investigations with aqueous extracts because of the plant preparation method in its ethnopharmacological use (61). The results showed the dose-dependent low cytotoxic effect of *Potentilla reptans* extracts, which was in agreement with the literature data for the aqueous extracts activity (46). Flavonoids and phenol carboxylic acids are identified in the aerial parts of *Potentilla reptans* (62). These classes of compounds have shown the cytotoxic effect, but the limited extraction with water could be the reason for weak cytotoxic activity.

Struchii folium (*Struchium sparganophora* L., *Asteraceae*) – the methanol, aqueous and chloroform extracts were investigated. The extracts were used in concentrations of 10, 20, 40 and 80 μg/ml. The method used for the experiment was the *in vivo* test of mortality rate of tadpoles during 24 h. The aqueous extract applied at low concentrations (10 μg/ml and 20 μg/ml) showed a high cytotoxic effect. The chloroform extract showed the same cytotoxic effect as the aqueous extract, but at a higher concentration (80 μg/ml) (38). For the *in vivo* cytotoxicity testing of this plant, the aqueous extract showed a significant activity. This could be explained by the content of protocatechuic acid, p-coumaric acid and caffeic acid, which, because of their relative polarities, could be found in the aqueous fraction of the plant (63). The apoptosis mechanism has not yet been investigated.

Sideriti herba (Sideritis scardica L. *Lamiaceae*) – diethyl ether, ethyl acetate and *n*-butanol extracts were prepared. The extracts were used at a concentration range of 0 – 100 μg/ml, and the evaluation was conducted on mouse melanoma (B16) and promyelocitic leukaemia (HL-60) cell lines. The evaluation method used was the MTT assay. The diethyl ether extract showed a significant dose-dependent cytotoxicity on both cell lines at a concentration of 100 μg/ml. The most cytotoxic compounds in the diethyl ether extract of *Sideriti herba* were phenolics: luteolin, luteolin-7-o-β-glycoside, apigenin and apigenin-7-o-β-glycoside. There were two possible mechanisms involved in the cytotoxic activity of the *Sideriti herba* extract. The used cell types significantly increased their ROS production as a result of the extract treatment. Therefore, induction of the oxidative

stress might be involved in the cytotoxic activity. Additionally, the cell cycle block in the S/G2 M phase was observed in B16 cells after the extract treatment (52).

Moringae folium (*Moringa oleifera* L. *Moringaceae*) – the plant extraction was performed with a mixture of methanol and water with an 80:20 ratio. The experiment was performed on the Hela cell line using the MTT assay. Doxorubicin was used as the positive control substance. The authors applied the extract at very high concentrations (100, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400 and 500 μg/ml). A significant and concentration-dependent cytotoxic activity was observed at concentrations above 260 μg/ml (53). A high cytotoxic concentration obtained for the *Moringae folium* extract indicates that this plant did not show a significant cytotoxic activity. However, there is evidence that the aqueous extract of *Moringae folium* inhibits the NF-κB signalling pathway and increases the efficacy of chemotherapy in human pancreatic cancer cells. The nuclear factor kappa B plays a significant role in the resistance of pancreatic cancer cells to the apoptosis-based chemotherapy. An application of the investigated extract to the pancreatic cancer cells (Panc-1) caused expression down-regulation of the key NF-κB signalling pathway proteins and allowed sensitization of pancreatic cancer cells to chemotherapy. Additionally, the combined therapy of *Moringa folium* extract with cisplatin demonstrated a synergistic effect (64).

Cynometri fructus (*Cynometra cauliflora* L. *Fabaceae*) – the methanol extract with a concentration range of 5 - 30 μg/ml was applied to the HL-60 cell line. The cytotoxicity was determined using the colorimetric MTT assay, in the presence of vincristine sulphate as the cytotoxic control compound. The concentration of the extract that reduced 50% of the cell population was 0.9 μg/ml, which indicated that the methanol extract of *Cynometri fructus* possessed a very strong cytotoxic activity towards the HL-60 cells (54). *Cynometra cauliflora* contains phenolic compounds, flavonoids, triterpenoids and exhibits an antioxidant activity. The cytotoxic effect mechanism was not investigated, but it could be attributed to some signalling pathways described for its main constituents (65).

Onsomae radix (*Onosma paniculatum* L. *Boraginaceae*) – the cytotoxic activity of petrol ether extract with a concentration of 10 μg/ml was investigated on the human CCRF-CEM leukaemia, human breast (MDA-MB-231), glioblastoma (U251) and colon cancer (HCT 116) cell lines. The experiments were conducted using the XTT assay in the presence of vinblastine as the positive control. The viability of all cells treated with the petrol ether extract of *Onsomae radix* was strongly inhibited with a low extract concentration (10 μg/ml). The main compounds that express cytotoxicity belong to the naphthoquinone derivatives (66). The caspase-3-dependent apoptosis was detected in melanoma cells in response to the *Onosma paniculatum* extract (55). This is in agreement with the apoptosis pathway results for other naphthoquinone derivatives (67).

Cynarae flos (*Cynara cardunculus*, L. *Asteraceae*) – the n-hexane extract with five different concentrations (0.1-1 mg/ml) was applied to colorectal cancer cells (DLD1), and the cytotoxic effect was determined using the MTT assay. The *Cynarae flos* extract showed a dose-dependent inhibitory effect on the viability of DLD1 cells. The authors confirmed that this extract inhibited cell proliferation and induced the apoptotic pathway on DLD1 cells. Furthermore, the pro-apoptotic (BAX) gene expression and a cell cycle inhibitor (p21) were induced, while the anti-apoptotic BCL-2 gene expression was reduced in the extract's presence. However, a BAX/BCL-2 ratio increment was detected, which implied that the *Cynarae flos* extract had the ability to induce the intrinsic apoptotic pathway (68). Additionally, the phytochemical composition, which includes polyphenolic compounds, inhibits angiogenesis related to cancer (69).

Dilleniae radix (*Dillenia suffruticosa*, L. *Dilleniaceae*) – the cytotoxic effect of dichloromethane extract at the maximum concentration of 100 μg/ml was applied to the breast cancer cell line (MCF-7). The experiment was conducted using the MTT assay in the presence of tamoxifen as the positive control. The extract showed the time- and dose-dependent strong cytotoxic activity with an IC_{50} value of 15.5 ± 0.5 μg/mL after 72 hours of exposure. This significant result indicates that the *Dilleniae radix* extract has a potential for developing a new cytotoxic agent. In addition, the authors explained how the investigated extract exhibited the cytotoxic effect. The dichloromethane extract of *Dilleniae radix* induced the G0/G1 and G2/M phase cell cycle arrest and apoptosis in caspase-3 deficient MCF-7 cells probably via the up-regulation of NF-κB, JNK1 and down-regulation of the anti-apoptotic genes AKT1 and ERK1 (70). Additionally, other studies confirmed the signalling pathway responsible for cytotoxicity of *Dillenia suffruticosa* extract on breast cancer cells (71, 72). Qualitative phytochemical screening of *Dillenia suffruticosa* extracts indicated the presence of saponins, triterpenes, sterols, and polyphenolic compounds, which contribute to the cytotoxic activities (72).

Euphorbiae herba (*Euphorbia platyphyllos* L. *Euphorbiaceae*) – the extracts were prepared using four solvents with different polarity and applied with a concentration range of 10 to 300 μg/ml on the MCF-7 cell line. The trypan blue exclusion method was used for cytotoxicity evaluation. Unusually, the water extracts obtained using two different methods showed the lowest IC_{50} among all tested extracts. The IC_{50} values for infusion and decoction of *Euphorbiae herba* were 38.29 ± 0.57 and 27.79 ± 0.58, respectively. Therefore, decoction with an IC_{50} value lower than 30 μg/ml could be the potential source for obtaining a potent cytotoxic agent. It was shown that all *Euphorbiae herba* extracts induce significant DNA damage in MCF-7 cells. The direct interaction between a DNA-reactive agent and DNA is one of several pathways that leads to primary DNA damage. The *Euphorbia platyphyllos* content of flavonoids, which induces DNA damage, could be the reason

for cytotoxic activity. Additionally, the presence of jatrophane diterpenes might contribute to cytotoxicity of *Euphorbia platyphyllos* extracts (73).

Flueggea herba (*Flueggea leucopyrus*, L. *Phyllanthaceae*) – the aqueous extract cytotoxic effect was evaluated on human endometrial carcinoma cells (AN3CA) using the MTT assay, in the presence of thymoquinone as the positive control substance. The decoction exhibited a significant dose-dependent cytotoxicity with low IC_{50} values of 22.09 and 14.60 μg/mL at 24 and 48 h post-incubation, respectively (74). However, this result could not be considered as favourable because the extract was administered at a high concentration (approximately 400 μg/mL). *Flueggea leucopyrus* contains polyphenols, flavonoids and terpenoids, which could influence the cytotoxic effect (75). The apoptosis of AN3CA cells was improved using enhanced DNA fragmentation and caspase 3 and 9 activities, as well as the moderately increased radical scavenging activity (74).

Hydrangeae folium, (*Hydrangea angustipetala*, L. *Saxifragaceae*) – the extracts were prepared using extraction with 70% acetone and 50% ethanol. The cytotoxic effect was observed on the human gastric carcinoma cell lines (AGS and SNU-1) using the MTT test and also *in vivo* on the mouse lymphoid macrophage (P-388D1) cells transplanted intraperitoneally into the CDF1 male mice. The MTT assay was performed in the presence of doxorubicin. The acetone extract showed higher cytotoxicity than the ethanol extract, with an IC_{50} of 21.43 μg/ml. However, the two isolated active compounds (febrifugine and trans-3-p-coumaroylquinic acid) from the ethanol extract showed much lower IC_{50} values and a strong cytotoxic effect. The cytotoxicity of *Hydrangea angustipetala* extracts could be explained through decrease in PARP and pro-caspase 3 of the AGS and SNU-1 cells. The *in vivo* experiment showed that the *Hydrangea angustipetala* extract prolonged the survival days of the P-388D1- CDF1 bearing mice (76).

Picralimae semen, (*Picralima nitida* L. *Apocynaceae*) - aqueous, methanol, ethylacetate and hexane extracts were evaluated for cytotoxic activity *in vivo*, using the brine shrimp lethality assay. The extracts were applied at concentrations of 10, 100 and 1000 μg/ml. The hexane extract showed the strongest cytotoxic activity and the lowest LC_{50} value. The content of saponins, tannins, flavonoids and anthraquinones could be responsible for the expressed cytotoxicity (77).

Anemone rhizome, (*Anemone flaccida*, L. Ranunculaceae) - the subject of this research was not to investigate the cytotoxic effect of *Anemone rhizome* extract, but of the isolated triterpenoid saponins using the MTT test. The isolated constituents were applied to the human hepatocellular liver carcinoma (HepG2) and human hepatoma (BEL-7402) cell lines at a concentration range of 2.5 to 40 μg/ml. All tested substances showed a strong cytotoxic activity, considering the fact that all obtained IC_{50} values were below 30 μg/ml. The mechanism responsible for the cytotoxic effect of *Anemone flaccida* was inhibition of the COX-2/PGE2 pathway because this is confirmed for its main constituents, triterpenoid saponins (23).

CONCLUSION

This paper presents a review of the importance of investigations of herbal extracts to obtain natural products that potentially have a cytotoxic effect. According to the American National Cancer Institute, the herbal extract with IC_{50} lower than 30 μg/ml could be considered as a very strong cytotoxic agent. The majority of extracts presented in this review showed a significant cytotoxic effect, but only some of them are interesting for future research for developing an anticancer agent. An appropriate solvent for plant extraction should be chosen according to the phytochemical composition. In general, the best cytotoxic activity was found in the nonpolar and moderately polar herbal extracts. It was confirmed that the phytochemical compound classes, which contribute to cytotoxicity, have the ability to induce apoptosis using different signalling pathways.

This research topic is not of significant interest in Serbia, although our country is a notable source of medicinal plants. *Potentilla reptans* L. and *Sideritis scardica* L. that grow in Serbia are the only two species investigated for cytotoxicity by the domestic scientists. In generally, more research on plants and plant-derived chemicals is necessary to discover potent anticancer agents.

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