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POTENTIAL ROLE OF *AGRIMONIA EUPATORIA* L. EXTRACT IN CELL PROTECTION AGAINST TOXICITY INDUCED BY BISPHENOL A

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ABSTRACT

The aim of this study is to reveal the potentially protective role of ethanolic extract of agrimony (*Agrimonia eupatoria* L.) against the cytotoxic effect of bisphenol A (BPA) *in vitro*, using an intestinal porcine epithelial cell line (IPEC-1). The cells were exposed to different concentrations of BPA: 12.5, 25, 50, 100, and 200 µg.ml^{-1} alone and in combination with agrimony extract (250 µg.ml^{-1}). The proliferative cell response was monitored for 72 h by a xCELLigence system or real-time cell analyser (RTCA), recorded as the cell index (CI) and expressed as a proliferative activity (% PA) compared to the control cells without treatment. The metabolic activity was measured by a MTS colorimetric test, performed after 48 h of treatment with the tested substances. The cytotoxic effect on cells exposed to BPA alone, in comparison to the control cells without treatment, was observed in both assays ($P < 0.0001$). It was confirmed that BPA reduces both the metabolic activity and the proliferation of cells. After the cell treatment with agrimony, the metabolic activity had increased to reach over the control (101.52 %), while reducing the proliferation of the cells. The protective role of agrimony against cytotoxicity caused by BPA was observed after cell treatment with agrimony in combination with lower concentrations of BPA (12.5; 25 and 50 µg.ml^{-1}). The slight improvement in the adherence was observed in cells treated with these combinations, in comparison to the cells treated with BPA alone. On the other hand, the metabolic activity was slightly improved in cells treated with a combination of agrimony and BPA at higher concentrations (50 a 100 µg.ml^{-1}). This supported our assumption that agrimony can protect a model organism against cytotoxicity caused by BPA.

Key words: agrimony; bisphenol A; cytotoxicity; intestinal cell line; MTS assay; xCELLigence

INTRODUCTION

Environmental pollution adversely affects human health and the stability of an ecosystem. Various pollutants produced by human industrial activity (automobile exhaust, heavy metals, radioactive compounds, etc.) remain in nature and pose a great threat to the life on Earth [13]. One of the important chemical components used in the manufacturing of plastic materials is bisphenol A (BPA), commonly referred to as 2,2-(4,4-dihydroxydiphenol) propane, which is present in different ecological media, such as water, sediment, soil, biomass and air [20]. BPA is a chemical substance obtained by condensation of phenol with acetone in the presence of a strongly acidic ion-exchange resin, in the gel form, as a catalyst [11]. It was first synthesised in 1905. Since the 1960s BPA is the main ingredient used for the production of a variety of polymers such as polycarbonate plastics, epoxy resins, or thermal papers and is therefore found in a wide range of consumer products, including plastics and food packaging, medical equipment (for dialysis and blood oxygenation), bottles for feeding infants, and kitchen dishes [11]. The polycarbonates have wide utilization for their advantageous properties, such as: durability, light weight, high tensile strength, high modulus of elasticity, high melting point and high vitrification temperature [2, 5]. BPA was considered as neutral to human health for many years, but its detection in the natural environment, in drinking water and food products (since 1990) induced the interest of many researchers and the negative effects of this compound on human health was established. Many authors have studied the migration of BPA and its derivatives into food from polymer packaging in which it was stored, especially at elevated temperatures (e. g. microwave heating or other thermal process) [11]. In 1996 BPA was classified by the European Commission as a substance of external origin with a harmful effect on human health. It was confirmed that BPA had estrogenic properties and an agonistic effect toward the estrogenic receptors. In recent studies, BPA has been characterised as an endocrine disruptor, which disturbed the hormonal balance in humans and animals [3, 7]. The effects of exposure to BPA can be particularly harmful to foetus, infants and young children, because of a lack of feedback regulating the activity, synthesis and elimination of hormones [11]. It has also been reported that the cytotoxicity of BPA may not be linked to endocrine disruptors, but induced by the production of reactive oxygen species that cause oxidative stress leading to cell damage.

Herbs play an important role in the protection against oxidative stress. *Agrimonia eupatoria* L. is a herb of the Rosaceae family, which is used in traditional (folk) medicine for its beneficial effects. Common agrimony is a perennial herbaceous plant with small yellow star-shaped flowers, a short rhizome and a hard, hairy stem inhabiting pasturelands across Europe. The historical documents about the beneficial effects of agrimony date back as far as the 4th to 5th centuries and this herb was mentioned in the Old English Herbarium from the 10th century [4, 18, 19]. Agrimony contains polyphenol-enriched fractions (tannins, phenolic acids, flavonoids and terpenoids), which has a very important role in antioxidative properties [12]. Its water extracts (infusions and decoctions) or hydroalcoholic extracts (tinctures) has also been used in traditional medicine to treat lungs, inflammation, liver diseases, cholecystitis, cholestasis, intestinal or bladder atony, pyelonefrititis, bleeding disorders, skin defects, and inflammatory of oral mucosa [8].

The aim of our study was to evaluate the potential protective effects of *Agrimonia eupatoria* L. ethanolic extracts against harmful cytotoxic effects of BPA using a model porcine intestinal epithelial cell line (IPEC-1).

MATERIAL AND METHODS

Plant extracts

Lyophilised ethanol extract *Agrimonia eupatoria* L. (Calendula, Nová Ľubovňa, Slovakia) was diluted shortly before the experiment with sterile water in order to reach a final concentration of 250 $\mu g.ml^{-1}$. This concentration was selected based on preliminary study (data not shown) in which the effects of different concentrations (0.01—1000 $\mu g.ml^{-1}$) were evaluated.

Bisphenol A—solution

Bisphenol A purchased from Sigma Aldrich (Germany) was diluted with sterile water and tested at the final concentrations of: 12.5, 25, 50, 100, and 200 $\mu g.ml^{-1}$.

Cell cultivation

For this experiment porcine intestinal epithelial cells (IPEC-1, CVCL 2245) were used. The cells were cultivat-

ed in Earl's Minimal Essential Medium (EMEM; Lonza, Valais, Switzerland) supplemented with 10 % (v/v) foetal bovine serum (FBS; Lonza, Valais, Switzerland), 1 % L-glutamine, 0.1 % gentamicin and 1 % penstrepten. The cells were grown in a humidified atmosphere of 37 °C and 5 % CO_2, subcultured each for 3—4 days and were regularly checked for the absence of mycoplasma contamination.

xCELLigence system

In the experiment, the real-time cell analyser xCELLigence system (RTCA; ACEA Biosciences Inc., San Diego, CA, USA) was used to monitor the changes in cell proliferation or adherence. This system allows label-free monitoring of cell behaviour (adhesion, proliferation, growth and morphology) throughout the treatment. It is based on measurement of impedance on gold electrodes at the bottom of microplate wells. The more cells are attached the higher the impedance is recorded. The values are expressed as dimensionless cell index (CI) and recorded in curves each hour throughout the experiment [6, 15, 16].

For our experiment, the cells were seeded at 5×10^3 cells per 16-well E-plate (Acea Bioscience, San Diego, CA, USA). After 22 hours (cells were still in a log phase) BPA at concentrations of 12.50 $\mu g.ml^{-1}$—200 $\mu g.ml^{-1}$ alone, and in combination with agrimony extract (at concentration 250 $g.ml^{-1}$) were added to the cells. The cell response was monitored for 48 h. The cells without treatment served as the control and their activity was considered as 100 %. Change in the proliferative activity (PA) was calculated using the following formula:

$$\% PA = CIsample \times 100/CIcontrol$$

MTS assay

For the measurements of changes in the metabolic activity of the cells MTS colorimetric assay (CellTiter 96®AqueousOne Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was performed. This method is based on the viable cells ability to reduce MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium) compound to form a coloured formazan product that is soluble in cell culture media. The cells were seeded into two 96-well plates (Greiner-Bio-One, Kremsmünster, Austria) in an amount of 8.6×10^3 cells per well in a 100 µl medium. After 22 h cultivation, the cells were treated the same way as in RTCA and incubated for 48 hours. Then 2 µl of the MTS solution was added to each well. The absorbance was measured at 490 nm after 4 h incubation using a microplate reader (Synergy HT; Biotek, Winooski, VT, USA). The absorbance of the control cells was considered as 100 %.

Statistical analysis

The data were statistically evaluated by the GraphPad Prism 8.3.1 software (USA), using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test. The results are presented as the means ± SD. The significance level was set to $P < 0.05$. All experiments were conducted in triplicate.

RESULTS

Real-time monitoring of cell proliferation using the xCELLigence system

The cell response to the exposition to the tested substances was monitored in real time using the xCELLigence system (RTCA). We observed significant changes in proliferative activity (PA) expressed as CI values of the treated cells in comparison to the control cells without treatment ($P < 0.05$). Figure 1 illustrates CI changes in wells containing IPEC-1 cells and BPA of different concentrations, in comparison to control cells without treatment. The lowest CI values were recorded at the highest concentrations 100 $\mu g.ml^{-1}$ and 200 $\mu g.ml^{-1}$. Within the first 2 hours after treatment with BPA, CI values drop from 1.0 (set as normalised cell index) to less than 0.1, indicating that these concentrations are the most effective. Cells treated with BPA at concentrations 12.5, 25 and 50 $\mu g.ml^{-1}$ show significantly lower CI values ($P < 0.05$) compared to the control cells.

The following figures (Figs. 2, 3, 4, 5 and 6) illustrate changes in the CI after treatment with agrimony extracts and BPA alone and in mutual combinations. The most significant change can be seen in Figure 2 in which the growth of CI or cell proliferation treated with the combination of BPA at 12.5 $\mu g.ml^{-1}$ and agrimony is higher in comparison with cells treated with BPA alone. Similarly, the concentrations 25 and 50 $\mu g.ml^{-1}$ in combination with agrimony caused slight improvement in CI (Figs. 3 and 4). On the other hand, the highest tested concentrations 100 and 200 $\mu g.ml^{-1}$ caused a fast decline in the adherence im-

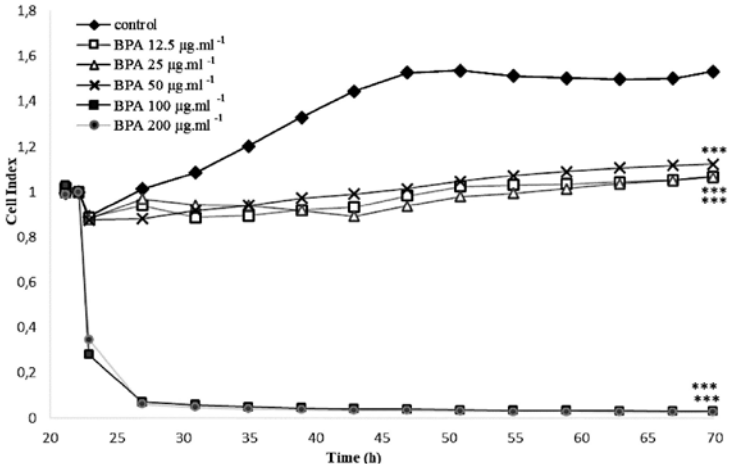


Fig. 1. Real-time monitoring of proliferation of cells treated with different concentrations of bisphenol A (12.5—200 µg.ml^{-1}) compared to control without treatment. Statistically different to control *—P < 0.0001**

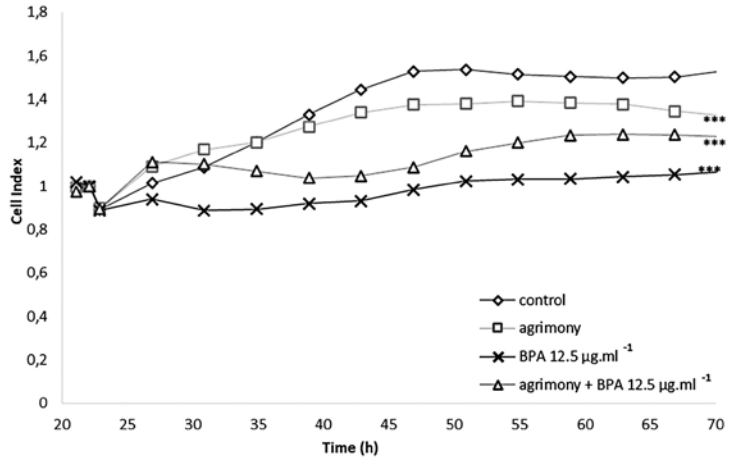


Fig. 2. Real-time monitoring of proliferation of cells after treatment with bisphenol A at 12.5 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

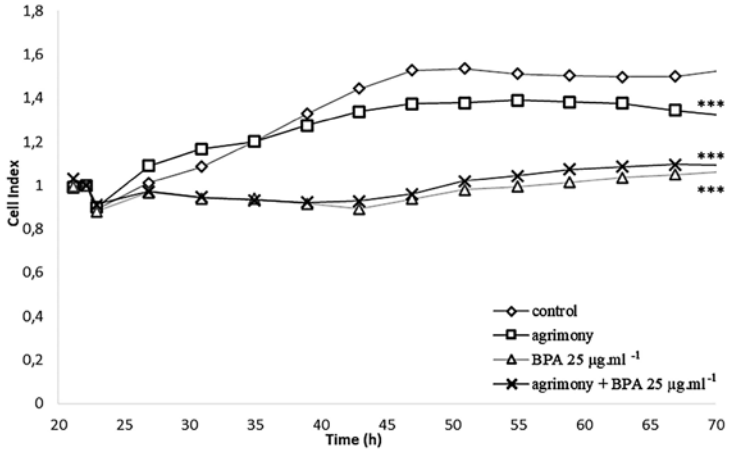


Fig. 3. Real-time monitoring of cell proliferation after treatment with bisphenol A at 25 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

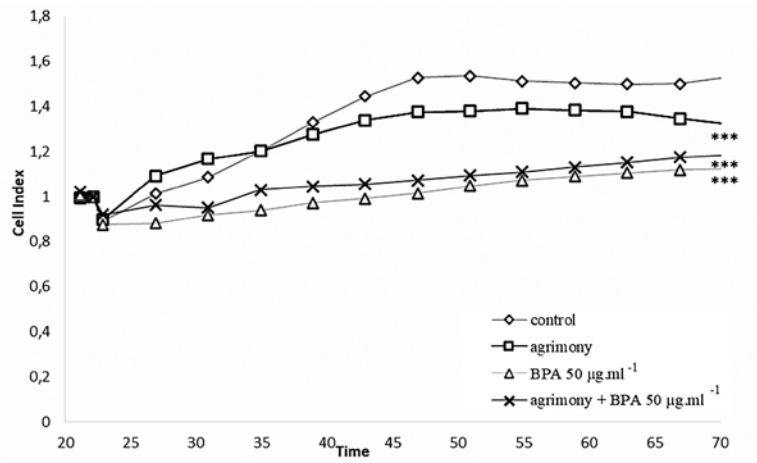


Fig. 4. Real-time monitoring of cell proliferation after treatment with bisphenol A at 50 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

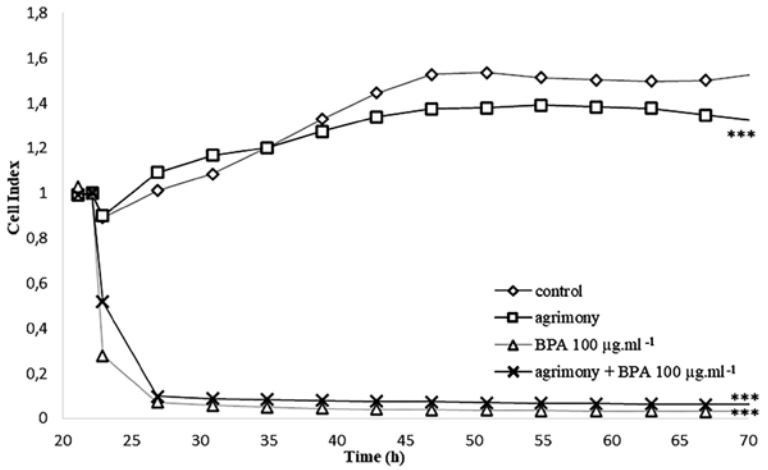


Fig. 5. Real-time monitoring of cell proliferation after treatment with bisphenol A at 100 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control * —P < 0.0001**

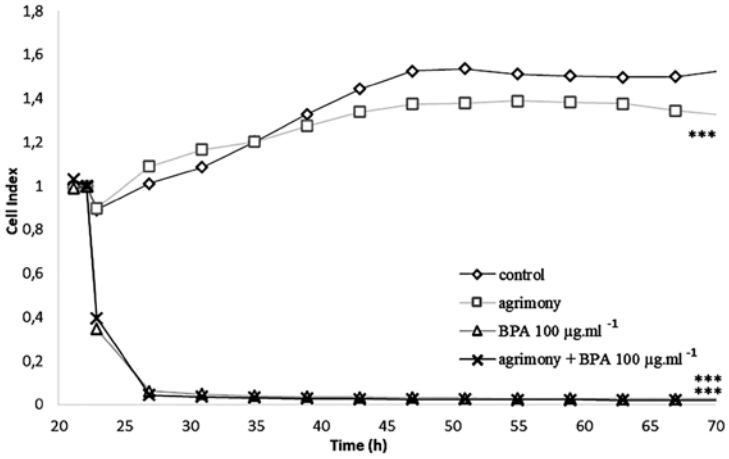


Fig. 6. Real-time monitoring of cell proliferation after treatment with bisphenol A at 200 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

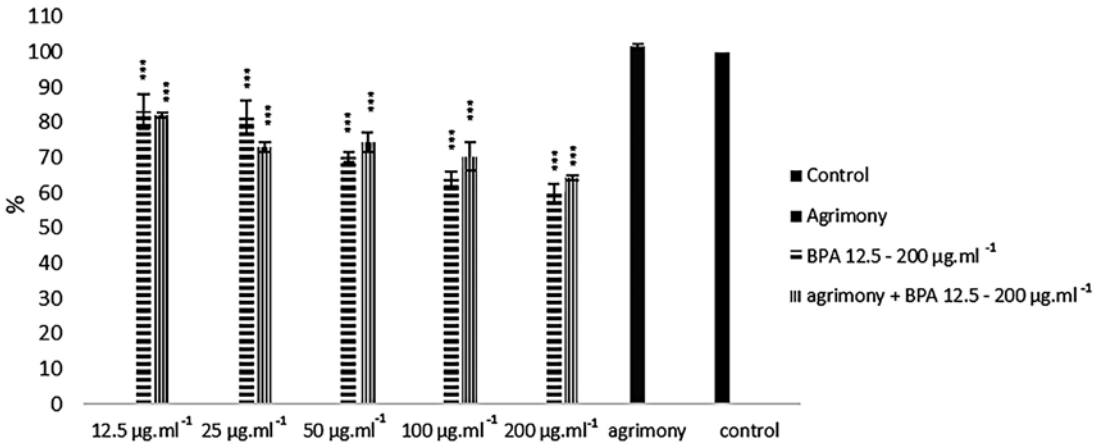


Fig. 7. Change in metabolic activity of cells treated with bisphenol A, agrimony and their mutual combinations. *—P < 0.0001 significantly different to control**

Tab. 1. Comparison of metabolic activity and cell adherence [%]

	% MA	± SD	% PA	± SD
BPA 12.5 µg.ml^{-1}	83.22***	4.84	66.83***	1.23
BPA 25 µg.ml^{-1}	81.38***	4.97	66.27***	1.79
BPA 50 µg.ml^{-1}	70.34***	1.38	73.28***	2.52
BPA 100 µg.ml^{-1}	63.91***	2.11	1.98***	0.26
BPA 200 µg.ml^{-1}	59.77***	2.87	2.43***	3.15
BPA 12.5 µg.ml^{-1} + agrimony	82.07***	0.69	80.14***	2.02
BPA 25 µg.ml^{-1} + agrimony	73.10***	1.38	71.42***	1.95
BPA 50 µg.ml^{-1} + agrimony	74.48***	2.76	77.33***	0.94
BPA 100 µg.ml^{-1} + agrimony	70.34***	4.14	4.08***	0.27
BPA 200 µg.ml^{-1} + agrimony	64.14***	0.69	1.30***	0.26
Agrimony (250 µg.ml^{-1})	101.52	1.90	75.13***	3.62
Control	**100.00**	**0.20**	**100.00**	**0.50**

BPA—bisphenol A at concentrations 12.5—200 µg.ml^{-1}; % MA—change in metabolic activity; % PA—change in proliferative activity; significant difference compared to control cells ***—P < 0.001

mediately after addition to cells and the protective effect of agrimony was not observed (Figs. 5 and 6).

MTS results

The metabolic activity (MA) of the IPEC-1 cells was measured 48 hours after the treatment with the tested substances using the MTS test. The results are recorded in Fig. 7. Cells treated with agrimony showed metabolic activity comparable with the control cells (101.5%; P > 0.05). A negative effect of BPA was observed in a dose-dependent manner. Interestingly, there was an improvement in values of MA when treated with combina-

tions of agrimony and higher concentrations of BPA (50; 100 and 200 μg.ml^{-1}) in comparison to MA of cells treated with corresponding concentrations of BPA alone.

Comparison of RTCA and MTS results

The results of changes in the metabolic activity (MA) and the proliferative activity (PA), measured 48 hours after addition of tested substances, expressed in percentage are summarized in Table 1. All the recorded values after cell treatments were significantly different to values observed in control cells ($P < 0.05$). Improvement in PA was recorded after the treatment with agrimony in combination with BPA at 12.5, 25, 50 μg.ml^{-1} (compared to cells treated with BPA at corresponding concentrations alone) and MA was slightly but not significantly improved after the treatment with agrimony in combination with BPA 50, 100 and 200 μg.ml^{-1} in comparison to cells treated with BPA alone.

DISCUSSION

BPA is known to act as an endocrine-disrupting chemical, by mimicking estrogen and compatibility binding to its receptors in the body. It has also been discovered that BPA has several other negative effects on the organism [10]. The cytotoxicity of BPA may be mediated by an increase of reactive oxygen species, thus BPA could directly cause oxidative stress through their release, causing cell damage [19]. The reason for its wide use is based on chemical properties, low adsorption of moisture, and thermal stability [14]. The main exposition is through oral intake with food and water (it represents more than 90 %) [9]. BPA is only one of many substances known to have negative effects on an organism, therefore it is relevant to find the way how to protect live organism and reduce negative impact of toxicants.

Plants containing phytochemicals, which are the biologically active part of the plants, have several positive health-bringing effects. For the plant itself, phytochemicals are produced as part of their defences against environmental stress [17]. *Agrimonia eupatoria* L. is a plant that is well known for its positive effects on numerous health problems. In early times before modern medicine, this plant was used to treat: eye-infections, gastrointestinal problems, and liver- and kidney-related problems. Recent studies have shown that this plant also potentially acts with: inherited anti-tumour, anti-mutagenic, hepatoprotective, anti-viral, anti-oxidant, and anti-inflammatory qualities. All of these properties are related to its: chemical composition, agrimony is rich in flavonoids, aromatic-rings, triterpenes, tannins, coumarin, glycosides, and vitamins B and K [1]. It makes this plant attractive in research and searching for the new ways of utilization of these positive and protective abilities.

This study focused on revealing the potential role of medicinal plant agrimony in protection against the toxic effect caused by BPA. For the experiment, porcine intestinal epithelial cells IPEC-1 were employed and exposed to agrimony at 250 μg.ml^{-1} and BPA at concentrations between 12.5—200 μg.ml^{-1} alone and in their mutual combinations. After 48 h exposition we could observe a decrease in the proliferative activity of cells that were monitored in real-time by RTCA. The two highest concentrations (100 and 200μg.ml^{-1}) caused a decrease in the CI immediately after addition to cells that indicated the highest toxicity. Loss of adherence may lead to cell death of adherent cells. On the other hand, lower concentrations (12.5—50 μg.ml^{-1}) did not cause complete loss of cell adherence and the CI did not drop which indicated inhibition of cell proliferation. The potential protective role of agrimony was observed after the treatment with combination of agrimony and BPA at lower concentrations (12.5—50 μg.ml^{-1}) due to which we could observe an increase in the PA in comparison to cells treated with BPA alone.

RTCA is useful in *in vitro* studies, such as toxicology studies, cell function, and potential drug effects. Since the RTCA alone will not give any results about the changes inside the cell, this experiment was performed simultaneously with the MTS test that measure the metabolic activity (MA) of cells. The addition of the tested substances caused a significant decrease in MA except for agrimony ($P > 0.05$). An interesting finding was observed after the addition of higher concentrations of BPA (50—200 μg.ml^{-1}) in combination with agrimony that caused a slight increase in the metabolic activity in comparison to cells exposed to BPA alone. This indicated a potential protective role of agrimony against the cytotoxic effect of BPA. BPA is only one of many potentially harmful substances in the environment. Medicinal plants could perhaps aid to eliminate such negative effects and additional experiments are desirable to establish the true role of these plants.

CONCLUSIONS

The positive effects of medicinal plants (phytochemicals) have been known for a long time, and the plants are still in popular use today. In the form of tea or other supplements we can make use of beneficial effects of these plants with minimal negative side effects. This is important not only in treating diseases, but also in preventing them. Agrimony is known to contain several phytochemicals belonging to the group of polyphenols that are known for their antioxidant effect and ability to protect cells from oxidative stress. On the other hand, BPA cytotoxicity may be induced by oxidative stress caused by the release of reactive oxygen species. This could explain why agrimony could be beneficial in protection against toxic effects of BPA.

Our study revealed a potential cytoprotective effect of ethanolic extract of *Agrimonia eupatoria* L. against the environmental toxin BPA at different concentrations by using porcine intestinal epithelial cells as a model organism. Improvement in the proliferative activity of cells treated with a combination of the agrimony and BPA was recorded at lower concentrations in comparison to cells treated with BPA alone. Interestingly, the metabolic activity was higher after the treatment with a combination of agrimony and a higher concentrations of BPA. This could be ascribed to different principles of the methods used. However, further studies are needed, especially *in vivo*, to confirm the beneficial effects of the agrimony and its bioactive compounds.

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