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Application of olive pomace extract in the synthesis of selenium nanoparticles - antioxidative and antifungal properties and effects

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PRIMJENA EKSTRAKTA KOMINE MASLINE U SINTEZI SELENOVIH NANOČESTICA – ANTIOKSIDATIVNA I ANTIFUNGALNA SVOJSTVA I UČINCI

Emerik Galić

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Kratki sažetak doktorske disertacije:
Ekstrakt komine masline (EKM) bogat polifenolima korišten je za funkcionalizaciju površine nanočestica selena (SeNPs) u novorazvijenom postupku zelene sinteze. Funkcionalizacija SeNPs je rezultirala smanjenjem veličine nanočestica i povećanjem njihove stabilnosti, te je doprinijela značajnom povećanju direktne antioksidacijske i redukcijske učinkovitosti. Funkcionalizirane nanočestice (fSeNPs) nisu pokazale značajan toksični učinak u kontekstu negativnih utjecaja na oksidacijski status HepG2 i Caco-2 staničnih linija. SeNPs su pokazale protektivan učinak na stanice tretirane prooksidansom tert-butil hidroperoksidom, a utjecaj funkcionalizacije ovisio je o tipu stanične linije i primijenjenoj metodologiji. fSeNPs su pokazale izraženiji antifungalni učinak u usporedbi s SeNPs. Rezultati ovog istraživanja pokazuju da se ekstrakt komine masline može koristiti u sintezi SeNPs radi poboljšanja njihovih fizikalno-kemijskih karakteristika i bioloških učinaka.

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APPLICATION OF OLIVE POMACE EXTRACT IN THE SYNTHESIS OF SELENIUM NANOPARTICLES - ANTIOXIDATIVE AND ANTIFUNGAL PROPERTIES AND EFFECTS

Emerik Galić

Thesis performed at: Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek and Faculty of Pharmacy and Biochemistry, University of Zagreb
Supervisors: Ph.D. Tomislav Vinković, Full Professor; Ph.D. Dubravka Vitali Čepo, Full Professor

Short abstract:
Polyphenolic rich olive pomace extract (OPE) was used to functionalize the surface of selenium nanoparticles (SeNPs) in a novel green synthesis process. The size, stability and direct antiradical- and reductive potential of functionalized nanoparticles (fSeNPs) were positively affected by functionalization. fSeNPs did not show a pronounced toxic effect in HepG2 and Caco-2 cells in the context of the impairment of the oxidative status of the cells. SeNPs had a pronounced protective effect on cells treated with prooxidant tert-butyl hydroperoxide (tBOOH) and the effect of functionalization on antioxidant potency depended on the cell type and applied methodological approach. fSeNPs had a significantly stronger antifungal effect compared to non-functionalized SeNPs. This study indicates that OPE can be used in nanoparticles synthesis to improve their physico-chemical characteristics as well as their biological activity.

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CONTENTS

1. INTRODUCTION

1.1. Selenium in nutrition and health

Selenium (Se) is a non-metallic element located in the 16th group and 4th period of the periodic table. It belongs to the same group as the oxygen and sulphur since it has six valence electrons in its outer shell. Because of this, Se and sulphur have similar chemical properties such as atomic diameter, electronegativity and ionization potential. However, Se compounds tend to be more nucleophilic, resulting in their essential functions in the biological systems (1). Se concentrations in the soils are variable and dependent on geographical region. Some areas in the world are poor in Se, which consequently leads to a decrease in its food chain content (2). Seis present in the soil in the form of inorganic salts - sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4), which have different abilities to be accumulated in plants. Sodium selenite has low bioavailability, while in contrast, sodium selenate is highly available and accumulated in plants. Se bioavailability is therefore dependent on various factors such as its concentration and formulation in the soil, pH, redox status and the abundance of sulphur anions in the soil (3). Plants use sulphate transporters to absorb Se from the soil, which can be transformed via plant metabolism from inorganic salts into organic selenocompounds (4,5). Seis available in foods in inorganic and organic forms, the latter mentioned having a higher bioavailability. The absorption of selenocompounds occurs in the small intestine. Organic forms and sodium selenate are absorbed via active transport, while sodium selenate passes the intestinal barrier by passive diffusion (6). Furthermore, inorganic Se shows adverse effects in significantly lower concentrations, indicating its higher toxicity and decreased biocompatibility compared to organic Se forms (7). In humans, Seis mostly present in the form of amino-acids selenocysteine (Se-Cys) and selenomethionine (Se-Met). The synthesis of Se-Cys is homeostatically regulated since it is incorporated in the active sites of selenoenzymes. It utilizes specific tRNA carrier and UGA codon in protein translation. Therefore, it is considered as the 21st essential amino acid (8). Conversely, Se-Met is incorporated non-specifically during protein synthesis at the same sights as methionine

(9). There are multiple types of selenoproteins in the body with diverse functions, from regulation of redox homeostasis to thyroid function. Selenoenzymes are a specific group of selenoproteins that can be divided into four distinct types - glutathione peroxidases (GPx), thioredoxin reductases (TrxR), methionine sulfoxide reductases (MSR) and iodothyronine deiodinases (DIO). GPx are crucial in protection against free radicals generated as a consequence of cellular oxidative metabolism or external stress. As their name suggests, they use glutathione as a cofactor for the reduction of hydrogen peroxide and other similar substrates like lipo-peroxides and tertbutyl peroxides. GPx are expressed in many tissue types, such as the epithelium of the gastrointestinal tract, kidneys, lungs, olfactory system, as well as testes and liver. Certain GPx members are ubiquitous, while others are tissue-specific, indicating their specialized function. For example, GPx2 is highly expressed in intestinal crypts implying its role in cellular proliferation (10). TrxR utilizes thioredoxin to catalyse the reduction of disulphide bonds of cellular proteins. Its activity modulates the DNA affinity for certain transcription factors, therefore influencing gene expression (11). MSR specifically reduce methionine sulfoxide to methionine, preventing impairment in the function of cellular proteins (12). Lastly, DIO play a critical role in the regulation of thyroid hormones. They are expressed in a range of tissues, mainly in the thyroid gland, kidneys, liver, skeletal muscles, etc. The dysregulation of thyroid hormones leads to severe metabolic disruptions, emphasizing the importance of DIO in organism homeostasis (13). Functions of other selenoproteins range from Se transportation, calcium metabolism to phospholipid synthesis. It is important to note that selenoproteins are sensitive to insufficient Se levels in the organism, which is therefore incorporated into selenoproteins hierarchically, according to the importance of their function (10).

The recommended daily intake (RDI) for adults is 70 µg/day according to European Food Safety Authority (14) and 55 µg per day according to Harvard school of public health (15). It is estimated that between 500 million and 1 billion people have insufficient Se intake (16). The soils in many geographical areas are poor in Se, for example, in Eastern and Northern Europe, especially Finland, parts of Western Europe and the United States of America. Also, Se deficiency in the soil is prevalent in some regions of China, Siberia and New Zealand (16). Se deficiency has been linked to various pathological states from

thyroid, immunological, neurological, skeletal and cardiovascular impairments to increased risk of cancer, viral infections, fertility and cognitive decline (17). Recent research implicated Se deficiency to the degradation of low-density lipoprotein (LDL) and blood cholesterol regulation (18). Keshans disease, cardiomyopathy described in the endemic regions of China, is considered to be caused by a combination of Se deficiency and viral infection (19). However, optimal Se status has many benefits. It reduces the overall oxidative stress in the organism and the susceptibility to viral infections. It was also noticed to lower the risk of cardiovascular diseases, certain types of cancer, enhance cognitive health and positively impact reproductive health (17). Different strategies have been undertaken to achieve the optimal status of Se in the population. One approach consisted of using inorganic Se as fertilizers to ensure a sufficient level of Se in the plants. This approach was proven successful in Finland, which had one of the lowest Se levels in the human population in the world (20). On the other hand, Se can be directly supplemented with food supplements rich in Se. Both inorganic - selenite and selenate and organic forms - SeMet, SeCys and selenomethylselenocysteine (SeMSeCys) are commonly used in food supplementation. However, inorganic forms are characterized by a low rate of bioavailability, narrow therapeutic window and increased toxicity compared to organic forms (21). Therefore, organic forms should be the preferred choice for nutritional supplementation. Moreover, there are differences among the commonly used organic forms of Se. For example, Se rich yeast, a food supplement containing Se mostly in the form of SeMet was evidenced to have improved bioavailability and ability to accumulate in the tissues (22). On the other hand, SeMet is metabolized to selenols and selenolates that are redox reactive, and can give rise to superoxide formation. This free radical reacts with thiols which can in turn lead to impairments in redox equilibrium, oxidative and endoplasmic reticulum (ER) stress as well as defects in cell signalling and protein structure and function (23). Supplementation with SeCys is less investigated. However, since the synthesis of SeCys in the organism is finely regulated, it may not be the best option to disrupt the physiologically required levels of the compound with additional intake. SeMSeCys is a Se form commonly found in plants. It is metabolized in the body to methylselenol (CH3SeH), which was proven to reduce the incidence of certain types of cancers such as mammary cancer, skin cancer and prostate cancer. However,

there have been different results when it was supplemented directly (using the synthetical form) and via SeMSeCys rich plant foods, the latter having beneficial effects against cancer, while the synthetic form did not show similar effects (22). A relatively newer approach is the application of nano-selenium in elemental Se (0) form which is described by excellent bioavailability and reduced toxicity. Moreover, in the nanoparticle form, additional nutritionally valuable compounds can be attached to the surface of the particle, enabling both the delivery of Se and other health promoting compounds. This approach can lead to sufficient supplementation of Se along with other beneficial effects on human health, depending on the identity of selected compounds.

On the other hand, excessive doses lead to acute Se poisoning (selenosis), which includes symptoms such as hair and nail loss, diarrhoea, vomiting, nausea and pain. In contrast, overly excessive doses result in death (24). However, cases of Se poisoning are rear.

1.2. Nanomaterials

According to The International Organization for Standardization (ISO), the nanomaterial is defined as “material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale” (25). Of particular interest are nanoparticles (NPs) which have the size of all three dimensions in the nanoscale. It is possible to differentiate NPs of natural origin and engendered nanoparticles generated by human activity. Engendered NPs, which will be further discussed, are characterized by unique physicochemical characteristics, which are significantly affected by the exact conditions of the synthesis process. These include size, shape, surface area, surface charge and chemical composition (26). Unique characteristics make the nanomaterials very reactive, which is the reason why their application is being studied in many scientific fields, including biomedicine and nutrition (27). The application of nanotechnology could lead to many potential benefits. Firstly, NPs have been extensively studied in precision medicine for targeted delivery. The drug or bioactive compound can be attached to the nanoparticles surface, along with the targeting ligand for specific recognition of targeted tissues. This methodology leads to the precise delivery of selected compounds which

results in improved efficiency as well as a reduction in potential adverse effects (28). Secondly, nanoparticle carrier can enhance the stability of drugs or nutraceuticals. Oral delivery is one of the most common ways of drug administration and nutraceuticals are almost exclusively applied *per os*. The nutraceutical has to remain in its desired form to reach the desired sight of action. Its metabolism begins in the saliva and continues to the stomach. Here, the conditions include low pH (1-2) and gastric mucus and enzymes which can result in instability or breakdown of the nutraceutical. Further down the GI tract, the environment is characterized by intestinal mucus, enzymes, interactions with microbiota, and other foods. All of the described influences the stability of the applied nutraceutical. It has been shown that nanoparticle carrier can protect the bioactive component of the nutraceutical by increasing its gastrointestinal stability and, consequently, bioavailability (29). The compound absorption occurs in the small intestine, and the critical part includes the crossing of the intestinal barrier. Many active compounds have a low absorption rate, resulting in decreased therapeutic efficiency. NPs generally enter the cells using active transport and can carry the desired compound inside the cells in a “trojan horse” manner (30). Generally, the absorption occurs via a transcellular pathway, described by direct passage through the cells, or via a paracellular pathway, which involves the absorption through the intracellular space. In the transcellular pathway, the nutraceutical will be subjected to cellular metabolism and possibly, the cellular efflux pumps, further reducing its bioavailability. The important thing to note is that absorption does not necessarily imply that the compound will be distributed to the desired site since the first pass metabolism in the liver can further significantly reduce the bioavailable fraction of the nutraceutical (31). Therefore, it should be emphasized that the usage of protective agents in nutraceutical development is necessary and one of the most promising could be nano-nutraceuticals. For example, polyphenolic compounds have various beneficial effects on human health but are characterized by low bioavailability (32). Their application is limited due to low water solubility, intestinal absorption, sensitivity to environmental changes (temperature, pH, light) and interactions with other foods in the GI tract (33). Polyphenols do not utilize any specific transporter, therefore the absorption in the intestinal epithelium occurs via passive diffusion. Since the average molecular weight of polyphenolic molecules is relatively high, the diffusion rate is low. Moreover, upon entering the cells, they are

eliminated via the activity of efflux pumps. Phase II metabolism in the cells also results in efflux pump activation, additionally reducing the amount of available polyphenolics (34). There is evidence that nano-systems can significantly improve the bioavailability of polyphenols. One such example is the application of liposomes, nanocarriers usually consisting of a hydrophilic core surrounded by an outer amphiphilic phospholipid layer. It was reported that liposomal delivery improved the bioavailability of polyphenol curcumin (35) as well as resveratrol (36). Protein based nanoparticles have also been shown to impact the delivery of polyphenolic compounds. It was evidenced that β-casein nanoparticles increased the water solubility rate and antioxidative activity of curcumin (37). Furthermore, gelatine nanoparticles were used as delivery vehicles of epigallocatechin gallate (EGCG), curcumin, tannic acid and catechin, leading to a gradual release of polyphenolics while at same time preserving their biological activity (38). Polymeric nanoparticles have also been demonstrated to improve the properties of polyphenolics. For example, polylactic acid (PLA) and polylactic acid-glycolic acid copolymer (PLGA) based nanoparticles have improved the water solubility and stability of polyphenols and resulted in a controlled release as well as improvement in their biological activity (39, 40). Metallic nanoparticles that are extensively investigated as potential drug delivery vehicles are of particular interest. Gold nanoparticles (AuNPs) can act as polyphenol carriers, as demonstrated in several studies. Curcumin bound to AuNPs was evidenced to have improved action against breast and colon cancer cells compared to free curcumin, while at the same time not being toxic to normal kidney cells (41,42). Similarly, a stronger pro-apoptotic effect in prostate cancer cells and breast cancer cells was evidenced for EGCG loaded AuNPs compared to free EGCG (43). Metallic nanoparticles are interesting for the delivery of bioactive compounds due to the ability to control their characteristics by precising the synthesis conditions. Certain types of metallic NPs possess biological activity “per se”, such as AgNPs and SeNPs, therefore their conjugation to the selected bioactive compounds can result in synergistic effects for human health.

Unfortunately, the application of NPs is also characterized by certain disadvantages. The toxicity of nanoparticles is still under-investigated and it is known that NPs interact with biological macromolecules such as proteins, lipids and, DNA which results in the

impairment of their function. Moreover, the NPs can disrupt the integrity of biological membranes, influencing membrane permeability and transport (44). As mentioned before, NPs internalize in the cells which has been linked to oxidative stress, apoptosis, necrosis and DNA damage (45). The other concern is the overall stability of nano-systems. Certain types are sensitive to changes in temperature and light and their stability also diminishes overtime. Therefore, NPs should be thoroughly characterized in the context of size, shape, zeta potential, surface properties and chemical composition (46,47) before any application can be achieved. If the NPs are intended for application in biomedicine, its assessment should be additionally performed on relevant biological models, such as cell models, organoid based systems and/or in vivo models.

1.3. Selenium nanoparticles (SeNPs)

Selenium nanoparticles (SeNPs) are relatively novel formulations in which Se is present in elemental Se (0) form. Generally, the synthesis of SeNPs based on chemical reduction consists of several crucial steps. The first step, the reduction of the precursor, includes the usage of reduction agents in the generation of elemental Se. It is followed by nucleation, where elemental Se forms seeds that further grow into nanoparticles. The stabilization involves the usage of surface coating agents to prevent the aggregation of SeNPs. Various coating agents have been used in SeNPs synthesis, such as surfactants, polymers, sugars, acids, etc. By changing the conditions of the nanoparticle synthesis, such as reaction time, temperature, the concentration of reactants, selection of precursors, reduction agents and surface stabilizers, it is possible to obtain NPs of different characteristics, enabling the versatility of their application (48,49). The synthesis of SeNPs can be divided into two main approaches - chemical and biological. Chemical synthesis involves the utilization of standardized chemical agents in each step of nanoparticle synthesis. The reduction of precursor can be achieved among others, with ascorbic acid, glutathione, potassium borohydride, mercaptoethanol, etc. Similarly, surface stabilization can be achieved by utilizing various coating agents (49). There are many methods and protocols for the chemical synthesis of SeNPs and new ones are still

being developed. However, they are characterized by high cost, harsh reducing agents and the usage of other toxic chemicals which can be damaging for the environment (49). Therefore, more attention has recently been given to the development of biological methods of synthesis in which bacteria, fungi and plants are utilized to generate the NPs. So called biogenic NPs are characterized by improved biocompatibility and reduced environmental impact (50). So far, there have been many types of research on different types of biologically synthesized NPs with admirable biological activity. One of the "greener" approaches of nanoparticle synthesis includes the usage of plant derived extracts in precursor reduction, surface stabilization and functionalization (51,52). They are easily obtained from various sources and are rich in health-promoting bioactive compounds, while at the same time being environmentally friendly. So far, there have been reported successful generation of SeNPs using extracts derived from common grapes, ginger, pepper and many more (53–58). The NPs in the studies were characterized by satisfactory physico-chemical characteristics and had additional beneficial properties, such as antioxidative, antitumor or antimicrobial activity, probably due to the presence of health-promoting surface stabilizers originating from plant extracts used in the synthesis process.

Namely, among the most interesting features of the NPs is the ability for surface functionalization. It implies decorating NP's surface with selected bioactive compounds. Surface functionalization can significantly change the physico-chemical characteristics of NPs, as well as their biological activity and, as mentioned before, it can improve the bioavailability of molecules used for surface functionalization. It was evidenced that SeNPs functionalized using 5-fluoroacil effectively internalized in the cells and inhibited the growth of human cancer cell lines (59). Similarly, in vivo study on SeNPs functionalized with doxorubicin, a common anticancer drug, has shown enhanced antiproliferative and apoptotic effects against cancer cells (60). Food components have also been used for SeNPs functionalization. For example, betacyanin improved the antitumor activity of SeNPs towards HepG2 cells (61) and curcumin loaded SeNPs showed similar effects (62). An interesting study was performed by Lai et al. (2020) who used tea-derived polyphenols for SeNPs' surface decoration and reported that such nano-systems had a protective effect against oxidative stress and cisplatin induced kidney injury (63). Another

example of successful synthesis describes polyethylene glycol (PEG) stabilized SeNPs functionalized with a valuable carotenoid crocin (64) characterized by potent antitumor activity in mice model. These studies provide evidence of the possibilities and benefits of SeNPs surface functionalization. Generally, SeNPs are characterized by strong biological activity ranging from antitumor, anti-inflammatory, antioxidative to antibacterial and antifungal activity. A variety of cancerous cell lines have been utilized to study the effects of SeNPs. For example, human hepatocellular carcinoma (HepG2)(65), colorectal adenocarcinoma (Caco-2)(66), colon carcinoma (HT-29)(67), osteosarcoma (MG63)(68), as well as melanoma (A375)(69) and breast cancer cells (MCF-7) (70) were shown to be sensitive to the treatment with SeNPs. There is a great deal of research reporting the antioxidative activity of SeNPs. In one study, pectin stabilized SeNPs were characterized by strong radical scavenging potential (71). Moreover, chitosan stabilized Se significantly reduced oxidative stress parameters in experimental animals (72). Zhai et al. (2017) reported that chitosan stabilized SeNPs were potent in radical scavenging activity (73). It is evident that using the plant extracts in SeNPs' synthesis can further improve their antioxidative properties (74). Overall, the antioxidative potential depends on the type of synthesis and physico-chemical characteristics of SeNPs, with special consideration to their surface decoration. Another interesting feature of SeNPs is their chemo preventive effect. It was evidenced that they could reduce the adverse effects of common tumor therapeutic cisplatin (63). Moreover, reduction in the inflammation induced in mice with acute colitis was also evidenced (75), while another study reported an anti-inflammatory effect in gamma irradiated rats (76). Furthermore, another field of possible application of SeNPs is microbiology. One of the most pressing issues in modern medicine are the multiple resistant microbes. Much effort is being put in the production of novel antimicrobials and nanotechnology could offer new solutions. It has been shown that SeNPs possess strong antimicrobial activity against several clinically relevant bacteria, such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Escherichia* and antifungal activity against *Rhizopus stolonifer*, *Aspergillus brasiliensis* and *Fusarium anthophilum* (77). Fungal pathogens also present a major problem in today's agriculture and their management is largely reliant on the use of pesticides to ensure sufficient product yield. Unfortunately, it has a negative impact on environmental

health since pesticides contaminate water and soil, which further negatively effects the food chain and the overall biosphere. Also, the usage of pesticides has a negative impact on human health. It is known to cause impairments in the neurological, endocrine, immunological system and was linked with cancer. Therefore, the shift has turned to the development of “green” pesticides to circumvent the mentioned problems. Nanotechnology has been used in the field to offer novel solutions (78,79) and SeNPs have also been investigated in this regard. So far, it has been evidenced that SeNPs are potent growth inhibitors of phytopathogenic fungi *Alternaria solani* (80). Another *in vitro* study reported antifungal effects against *Pyricularia grisea* and the ability to inhibit the infection of chili and tomato leaves by *Colletotrichum capsica* and *Alternaria solani* (81). A recent study by Vrandečić et al. (2020) reported the antifungal activity of SeNPs coated with different surface stabilizers against *Macrophomina phaseolina*, *Sclerotinia sclerotiorum* and *Diaporthe longicolla* (82). Therefore, SeNPs show a promising potential for application in the field due to their strong antifungal activity and reduced environmental and health impact.

1.4. Food waste and olive pomace

Food waste represents a major ecological and economic challenge and strong effort is being put into its reduction or reusage. Many novel application areas have emerged recently in the context of circular economy and zero-waste principle. Food waste represents a source of highly valuable components such as dietary fibre, antioxidants, essential fatty acids, antimicrobials and minerals which can be utilized to obtain new added-value products (83,84). So far there have been attempts to use it as animal feed and crop fertilizers. The ability to extract valuable compounds from agro and food waste, makes them an interesting source of bioactive compounds for application in human nutrition, pharmacy, cosmetics and medicine (85). For example, waste remaining after the processing of tomatoes - seed, skin and pulp are rich in pectin and carotenoids such as lycopene and β- carotene (86). Pectin is known to be used in the food industry as an emulsifier and stabilizer, while the mentioned carotenoids have significant beneficial

effects on human health. The appropriate extraction technique as well as nutraceutical formulation could result in the development of an eco-friendly nutraceuticals for human nutrition, while at the same time providing solutions for the reduction of agro- and food waste. Furthermore, grape pomace remaining after wine production is rich in polyphenolic compounds anthocyanins, flavanols, phenolic acids, and resveratrol with strong antioxidant activity that have a perspective to be used in pharmaceutical and cosmetic products as well as in nutrition (87). Biologically active peptides that could be utilized as antimicrobial agents and in disease management, can be extracted from various sources of waste such as microalgae, soybean, olive waste, chicken feathers, fish waste and egg proteins (88). One of the most interesting sources of bioactive compounds is olive pomace, waste remaining after olive oil production which presents an ecological hazard since it can be toxic to the soil and the biosphere (89,90). Olive pomace is a mixture of various compounds such as metals and sugars, and is particularly rich in polyphenols, mainly hydroxytyrosol, tyrosol and oleuropein (91,92). Polyphenols are known to benefit human health (92,93). It was reported that olive polyphenols have significant radical scavenging activity, and can protect the cells against oxidative stress. They were also implicated in the regulation of NRf2 transcription factor, crucial in cellular antioxidative defence (94). They can also contribute to mitochondrial biogenesis (95). Additionally, olive polyphenols have been recognized as metal chelators that can increase cellular protection against heavy metal toxicity (96). Another beneficial effect describes the ability to reduce the expression of pro-inflammatory cytokines, prostaglandins and nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2), matrix metallopeptidase-9, all of which are important contributors in inflammation (97). Moreover, it was demonstrated that olive polyphenols have a pronounced antitumor effect in vitro against different cancerous cell lines, namely Caco-2 and HCT116 (98). Various reports suggest their cardioprotective effects which are evidenced in decreased blood pressure, reduction in cardiac hypertrophy, and improvements in the levels of blood cholesterol and angiotensin II (99). Next,, they possess admirable antimicrobial effects and could be used as biopesticides (100,101). Inhibitory action was demonstrated against plant pathogens *Fusarium oxysporum*, *Pythium spp*., *Sclerotinia sclerotiorum*, *Verticillium dahlia*, and *Botrytis cinerea* (102).

1.5. Goals and hypotheses

The main goal of this study was to evaluate the possibility of utilization of polyphenolic rich olive pomace extract (OPE) for the synthesis of functionalized SeNPs to achieve improved physico-chemical characteristics and improved biological activity, compared to SeNPs obtained by using conventional chemical synthesis.

Specific goals of investigation were focused on:

- Evaluation of utilization of OPE for the reduction of inorganic Se precursor - sodium selenite, surface stabilization and functionalization and its impact on the average size, size distribution, shape and zeta potential of SeNPs;
- Evaluation of the cytotoxicity of SeNPs and assessing the impact of functionalization
- Evaluation of the antioxidative activity of SeNPs and assessing the impact of functionalization
- Evaluation of the antifungal activity of SeNPs and assessing the impact of functionalization

The hypotheses were defined as follows:

- OPE can be utilized for the reduction, stabilization and functionalization of SeNPs
- Polyphenolic compounds of OPE will improve physico-chemical characteristics of SeNPs compared to conventionally synthesized SeNPs
- SeNPs obtained using OPE will have decreased cytotoxic effects compared to conventionally synthesized SeNPs
- SeNPs obtained using OPE will have improved antioxidative activity compared to conventionally synthesized SeNPs
- SeNPs obtained using OPE will have improved antifungal activity compared to conventionally synthesized SeNPs

2. MATERIALS AND METHODS

2.1. Preparation of olive pomace extract (OPE)

The extraction of OPE was performed according to Radić et al. (2020) (103). Briefly, fresh olive pomace remained after olive oil production was obtained from the mill and dried at 60°C for 24 h in a thermostat (INKO, Zagreb, Croatia). The pits were then removed by sieving the mass through 0.8 mm diameter (Prüfseib DIN 4118, Kassel, Germany). The next step included the removal of the lipids with petroleum ether (Carlo Erba reagents V7L559307L, Barcelona, Spain) for 2h using Soxhlet extraction (INKO SK6ESS, Zagreb, Croatia). 8 g of olive pomace was extracted with 400 mL of 20% EtOH at 700 W of microwave power in high-performance microwave digestion unit (Milestone 1200 mega, Sorisole, Italy) for 10 min. The extract was filtered through a standard filter paper, and EtOH was evaporated at 60°C using rotavapor (Büchi Vacuum controller V-800, heating bath B-490, Vac V-500, Flawil, Switzerland). The extract was frozen overnight and lyophilized for 48h the following day using Alpha 1-4 LOC-1 (Martin Christ Gefriertrocknungsanlagen GmbH, Osterodeam Harz, Germany) equipped with a vacuum pump (Leybold Trivac D 2,5 E, Cologne, Germany) to obtain a dry powder. The filtration step in the preparation of OPE is depicted in Figure 1.

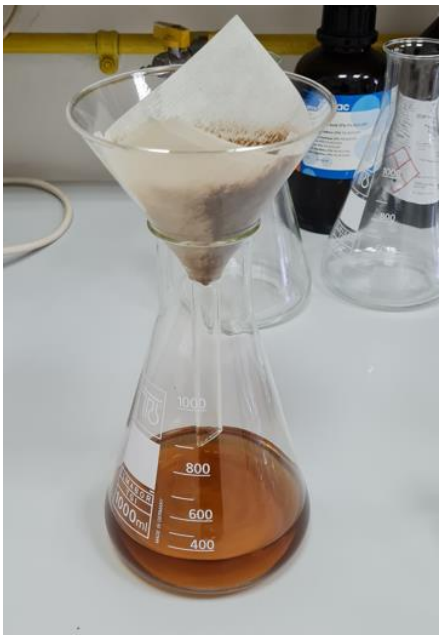

Figure 1. Preparation of olive pomace extract (Original photography)

2.2. Determination of polyphenolic compounds

The contents of tyrosol, hydroxytyrosol, and oleuropein in OPE were determined as reported by Tsarbopoulos et al. (2003), using Waters 2695 HPLC system (Waters Milford, MA, USA) coupled with a 2475 multi-fluorescence detector (FLD) (104). The dry extract was dissolved in deionized ultrapure water (UPW) and filtered through 0.45 µm filters (polyethersulfone membrane, Macherey-Nagel, Düren, Germany). The C18 reversed-phase column (250 × 4.6mm, 5 µm, Agilent Zorbax Eclipse plus, Agilent Technologies, Santa Clara, USA) was used with 0.05 mol/L ammonium acetate buffer (pH 5.0) and acetonitrile as a mobile phase. The flow rate was set to 1 ml/min, and the injection volume was 20 µL. The analysis was performed at 25°C and the fluorescence was measured at 280/625 nm. The identification was carried out by comparing the peaks with those obtained for the pure standards, while the quantification was obtained by external standard calibration.

2.3. Total reducing capacity

Total reducing capacity was determined using Folin-Ciocalteu (FC) reagent (105). The method is based on the measurement of the ability of the analyte to reduce the phosphomolybdic/phosphotungstic acid complexes in the Folin-Ciocalteu reagent to chromophores with a peak absorbance at 765 nm. The reaction takes place in an alkaline environment. The experiment included mixing 50 µL of 10 % water solution of FC reagent and 20 µL of analyte (OPE) in a 96-well plate. Negative control wells contained FC reagent and an equal volume of UPW, as for the analyte. The plate was shaken for 1 min and incubated for 5 min, at 37°C. The alkaline conditions were achieved by adding 160 µL of 0.7 M Sodium carbonate to each well. The plate was put on a shaker for 1 min and incubated for 30 min at 37°C. The absorbance was measured at 750 nm using Victor X3 (Perkin Elmer, Waltham, MA, USA). A standard curve of gallic acid was obtained and the results were expressed as gallic acid equivalents in mg/g.

2.4. Radical scavenging activity

Radical scavenging activity was evaluated by performing Trolox equivalent antioxidant capacity (TEAC) assay (106). The basis of the assay is the measurement of the decrease in absorbance of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, as the result of the radical scavenging activity of the analyte. The first step included mixing 7 mM solution of ABTS reactant and 1.63 mM potassium persulfate in a 1:1 ratio. The solution was incubated in the dark for 12 to 16h at room temperature (RT) to generate the $ABTS^+$ radical. Next, the $ABTS^+$ was diluted accordingly, to achieve an absorbance value of around 0.7. The experiment was performed by adding 200 µL of $ABTS^+$ and 20 µL of the analyte (OPE) in the 96 well plates. Negative control wells contained $ABTS^+$ radical and UPW. The plate was put on a shaker for 1 min, followed by 1.5 min incubation at 30°C. The absorbance was read at 750 nm using Victor X3 (Perkin Elmer, Waltham, MA, USA). A standard curve of Trolox® was obtained and the results were expressed as Trolox® equivalents in mg/g.

2.5. Synthesis and purification of SeNPs

The development of the SeNPs synthesis procedure included the evaluation of the utilization of OPE as the sole reducing agent for sodium selenite and its impact on surface stabilization and surface functionalization of SeNPs. The optimization process included variations in the concentrations and volumes of reactants, the time and the reaction temperature. Since using OPE as a reducing agent did not result in the generation of SeNPs (preliminary data), L-ascorbic acid was introduced into the synthesis process as the food-grade reductant. In this case, OPE was filtered through 0.45 μM filters (Syringe filters PES, Agilent Technologies, Santa Clara, USA) prior to its usage in the synthesis. However, obtained nanoparticles showed very wide size distribution ranges, high PI and were prone to agglomeration (unpublished data), indicating that OPE doesn't possess adequate stabilization properties and cannot be used as the coating agent. This is why two different surface coatings were used - polyvinylpyrrolidone (PVP) or polysorbate 20 (PS) and were thereafter used in the synthesis process, to obtain stable SeNPs of different physicochemical characteristics. Both coatings are food-grade and have been used previously for the stabilization of SeNPs (107-110). The final compositions of reaction mixtures used for obtaining samples used in this study, identified after the optimization process, are presented in Table 1, and the synthesis procedure is shown in Figure 2.

Figure 2. Synthesis of selenium nanoparticles (Original photography)

Table 1. Reaction mixtures for the synthesis of SeNPs and final samples used in this investigation. (PVPSeNPs – selenium nanoparticles stabilized with PVP; fPVPSeNPs - selenium nanoparticles stabilized with PVP and functionalized with OPE; PSSeNPs – selenium nanoparticles stabilized with polysorbate; fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with OPE).

Final samples	ASC (0.1M)	PVP (1 % m/V)	PS (10% m/m)	OPE (1 % m/V)	Na_2SeO_3 (0.1M)	UPW	Total volume (mL)
	mL						
PVPSeNPs	3.3	3	0	0	0.33	23.37	30
fPVPSeNPs	3.3	3	0	5	0.33	18.37	30
PSSeNPs	1.7	0	0.08	0	0.35	27.87	30
fPSSeNPs	1.7	0	0.08	5	0.35	22.87	30

All reactants were prepared in UPW. First, the UPW, PVP or PS were added to an Erlenmeyer flask and were mixed for 15 min on a magnetic stirrer at 300 rpm, at RT. Next, L-ascorbic acid was added to all samples and OPE to the fSeNPs flasks. The final step included adding sodium selenite drop by drop, and the reaction took place for 20 min. Every synthesis procedure was performed in duplicates.

The optimization of SeNPs purification was performed on PVPSeNPs and fPVPSeNPs (in duplicates) and the process consisted of evaluating three different approaches - centrifugation, centrifugation using Amicon®Ultra-15 10K cuvettes (Merck, Kenilworth, NJ, USA) and dialysis using dialysis tubing cellulose (Sigma-Aldrich, St. Louis, MO, USA; MWC 14,000 Da). Centrifugation was performed at 10000 g for 15 min (Biofuge stratos, Heraeus, Hanau, Germany) using 50 mL Falcon tubes. The supernatants were removed, the pellet was dispersed in 10 ml of UPW and used for further characterization. Centrifugation using Amicon® cuvettes consisted of the addition of 15 mL of the reaction mixture to the upper chamber of the cuvettes and centrifugation at 4000 g for 20 min (Megafuge 1.0, Heraeus, Hanau, Germany). The remaining fraction of the upper chamber was resuspended in 10 mL of UPW and centrifugation was repeated. For the dialysis procedure, 30 mL of the reaction mixture was transferred to dialysis tubes which were then put into UPW. The dialysis of SeNPs is presented in Figure 3. The outer phase was exchanged after 1 h, 5 h, 23 h and 31 h, and its conductivity was measured to evaluate the efficacy of the procedure. To evaluate the impact of the procedures on the purity and physico-chemical characteristics of SeNPs, their size, zeta potential and antioxidant capacity were measured. The optimal procedure was selected for the purification of all types of SeNPs that were used further in the study.

Figure 3. Purification of selenium nanoparticles using dialysis procedure (Original photography)

2.6. Total content of Se in SeNPs

Prior to analysis, the samples were diluted in UPW in 50 ml Falcon tubes. The content of Se was determined using Inductively Coupled Plasma Optic Emission Spectrometer (ICP-OES; Perkin Elmer Optima 5300 DV Waltham, MA, USA) at the Central analytical unit of the Faculty of Agrobiotechnical Sciences Osijek, J.J. Strossmayer University of Osijek. The analysis was performed in duplicates.

2.7. Nanoparticle visualization

Transmission electron microscopy was used to visualize the SeNPs and evaluate their shape. A small volume of SeNPs suspension was put on a Formvar® coated copper grid (SPI Supplies, West Chester, PA, USA). The grid was incubated for 24 h at RT to allow the samples to dry. Visualization was performed on TEM 902A, (Carl Zeiss Meditec Ag, Jena, Germany) that operated in a bright-field mode. The acceleration voltage was set to

80 kV. Canon PowerShot S50 camera (Canon, Tokyo, Japan) was used to obtain the micrographs.

2.8. Size determination

Nanoparticles size in suspension was determined by dynamic light scattering (DLS). It is a commonly used technique for the measurement of hydrodynamic diameter (dH), i.e., it considers the aqueous layer around the nanoparticles and gives a more realistic estimate of the size of nanoparticles in liquid media. NPs in dispersion are characterized by Brownian motion. When a laser beam hits the NPs, they tend to scatter the light beam depending on its size - stronger scattering will be observed by smaller particles due to their higher rate of Brownian motion. Therefore, the scattering light can be correlated to particle size by using the Stokes-Einstein equation. Another important parameter of the technique is the polydispersity index (PDI), which gives information about the uniformity of size distribution of NPs. High PDI implies that the system is heterogenous meaning that the size distribution is not uniform. In order to ensure a reliable estimate of dH, the distribution of particle size in the system should be homogenous (111). The size of SeNPs was determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The NPs were analysed using a 532 nm green laser, and the scattered light was detected at an angle of 173°. The measurements were performed on RT. dH was calculated as an average value of 10 measurements, while the size distribution was quantified by applying the size-volume distribution function. Data analysis was performed using Zetasizer software 6.32 (Malvern Instruments, Malvern, UK).

2.9. Determination of zeta potential

Electrophoretic light scattering was utilized to measure zeta potential, a parameter describing the particle surface charge and its stability. The charged particles in dispersion are surrounded by a compact layer of oppositely charged ions, known as the Stern layer

followed by a more loosely bound diffuse layer. Together they make up an electrical double layer (DL) around the particle. The difference in electrical potential between the DL and the surrounding fluid is called the zeta potential (112). By measuring electrophoretic mobility and applying the Henry equation with the Smoluchowski approximation, zeta potential can be calculated. It is dependent on the particle surface charge, as well as the particle concentration, pH, dielectric constant and ionic strength of the solvent. Zeta potential describes the electrostatic stabilization of the NPs. Higher absolute values imply increased stabilization (113,114). The samples were diluted accordingly and pipetted in an ELS cuvette (Malvern Instruments, Malvern followed by measurements on Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Zeta potential was calculated as an average of 5 measurements. Data analysis was performed using Zetasizer software 6.32 (Malvern Instruments, Malvern, UK).

2.10. Cell culture studies

Cell lines used in this study were human hepatocellular carcinoma (HegG2) and human colorectal adenocarcinoma cell line (Caco-2) obtained from the American Type Culture Collection ATCC (Manassas VA, USA). The HepG2 cells are commonly used in pharmaceutical and toxicological studies. They are characterized by epithelial-like morphology and can be easily cultured in vitro (115). They also present a reliable model for the evaluation of cellular antioxidative response (116). Caco-2 cells differentiate to enterocytes upon reaching confluence. They are routinely used as a model of the intestinal barrier in drug permeability studies (117). However, they are also used to evaluate the toxicity of novel compounds, especially when considering the local effects of drugs or nutraceuticals following oral administration (118).

Figure 4. Experiments performed in the cell culture hood (Original photography)

HepG2 cells were cultured in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS; Capricorn Scientific, Ebsdorfergrund, Germany), 1 % Antibiotic/Antimycotic (A/A; Sigma-Aldrich, St. Louis, MO, USA), 1 % Non-essential amino acid (NEAA; Capricorn Scientific, Ebsdorfergrund, Germany) and 4 mM L-Glutamine (Sigma-Aldrich, St. Louis, MO, USA). Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20 % FBS, 1 % A/A, 1 % NEAA and 4 µM L-Glutamine. The cells were maintained in cell culture flasks at 37°C and 5 % CO2. Upon reaching confluence, they were detached from the flask surface using 1x trypsin-EDTA Capricorn Scientific (Ebsdorfergrund, Germany), seeded in 96-well plates in a concentration of 20 000 cells/well and incubated and incubate for 48 h, before they were used for the experiments. All experiments were performed in quadruplicates. One of the experiments in cell culture is presented in Figure 4.

2.10.1. Cytotoxicity evaluation

2.10.1.1. Cell viability determination

Cell viability was investigated by performing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this assay, the reagent penetrates inside the cellular mitochondria where it is reduced to formazan product and its absorbance can easily be measured at 530 nm. The formazan will only be generated by the activity of mitochondrial enzymes of viable cells (119). After the 48-h incubation, 90 µL of fresh media was added to each well. The cells were treated with 10 µL of SeNPs or sodium selenite in different concentrations (diluted in UPW). The negative controls were incubated with 10 µL of UPW, and positive controls were incubated with 10 µL of tert-butyl hydroperoxide in UPW (tBOOH; Sigma-Aldrich, St. Louis, MO, USA), making the final concentration in the well 500 µM. The plate was incubated for 24 h in a cell culture incubator. The next day, the cells were washed two times with 200 µL of PBS to avoid potential interferences of SeNPs with the assay. 30 µL of MTT reagent (Carbosynth Limited, Compton, UK) was added to the wells and the plates were incubated for 3h in an incubator. Next, 170 µL of dimethyl sulfoxide (DMSO; Kemika, Zagreb, Croatia) was added and the plate was shaken for 45 min, followed by the measurement of absorbance at 530 nm using a plate reader (Victor X3, PerkinElmer, Waltham, MA, USA).

2.10.1.2. Reactive oxygen species (ROS) determination

To evaluate short-term disruptions in cellular redox equilibrium, a dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was carried out, which measures the relative content of reactive oxygen species (ROS) in the cells. The nonfluorescent form of the dye (DCFH-DA) permeates the cellular membrane to the cytoplasm where it is deacetylated to DCFH. DCFH is unable to cross the cell membrane, therefore it remains inside the cell. In the next step, the dye reacts with ROS within the cell, resulting in the fluorescent form

(DCF) and its fluorescence can be measured (120). The fluorescence intensity is therefore correlated to the content of intracellular ROS. The cells were seeded in a black 96-well plate suitable for fluorescence-based assays. The culture media was removed and the cells were incubated with 100 µL of 25 µM of DCFH-DA in PBS, for 45 min. The dye was then removed and the cells were treated with 100 µL of SeNPs or sodium selenite in different concentrations (prepared in PBS) for 3h. The negative controls were incubated with 100 µL of PBS, and positive controls were incubated with 100 µL of 100 µM tBOOH prepared in 1x PBS. The fluorescence was then measured at 485/535 nm using Victor X3 (Perkin Elmer, Waltham, MA, USA).

2.10.1.3. Reduced glutathione (GSH) determination

The changes in GSH levels were evaluated by performing a moncohlorobimane (mBCl) assay. It is a fluorescence-based assay that includes the penetration of a nonfluorescent form of dye across the cell membrane followed by reduction to fluorescent form in a reaction with GSH (121). Briefly, 90 µL of fresh media was added to the well, followed by the treatment with 10 µL of different concentrations of SeNPs or sodium selenite prepared in UPW. The negative controls were incubated with 10 µL of UPW, while the positive controls were treated with 10 µL of tBOOH in UPW (final concentration in the well 100 µM). The cells were incubated for 3h. tBOOH was then removed and the cells were washed one time with PBS. The cells were incubated with 100 µL of 40 µM mBCl reagent (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, after which the fluorescence was measured at 355/460 nm using Victor X3 (Perkin Elmer, Waltham, MA, USA).

2.10.2. Antioxidative activity evaluation

The evaluation of the antioxidative activity of SeNPs and sodium selenite in cellular models consisted of modified protocols of the assays described for cytotoxicity determination. The non-toxic concentrations of the tested compounds were selected

based on the results from the viability assay described previously (concentrations below IC_{20} were selected).

2.10.2.1. Cell viability determination

For the measurement of cell viability, the culture media was removed, and the cells were incubated with 90 µL of fresh media and 10 µL of SeNPs or sodium selenite (at two concentration levels). The negative and positive controls were incubated with 10 µL of UPW. The plate was incubated for 24 h. The next day, the test cells and the positive controls were treated with 20 µL of tBOOH prepared in PBS, so the final concentration of tBOOH in the wells was 300 µM. The negative controls were incubated with an equal volume of PBS. The treatment was removed after 5h and the cells were washed one time with PBS, after which 30 µL of MTT reagent (Carbosynth Limited, Compton, UK) was added to the wells and the plate was incubated for 3h. Next, 170 µL of dimethyl sulfoxide (DMSO; Kemika, Zagreb, Croatia) was added and the plate was shaken for 45 min, followed by the measurement of absorbance at 530 nm using plate reader (Victor X3, PerkinElmer, Waltham, MA, USA).

2.10.2.2. ROS determination

For ROS determination the cells were incubated with 90 µL of fresh media and treated with 10 µL of SeNPs or sodium selenite (at two concentration levels) for 24h. Both the negative and positive controls were treated with 10 µL of UPW instead. On the next day, the treatment solutions were removed and the cells were incubated with 100 µL of 25 µM of DCFH-DA in PBS, for 45 min. The excess dye was removed, and the test cells and positive controls were incubated with 100 µL of 100 µM tBOOH prepared in PBS. The negative controls were incubated with an equal volume of PBS. After 3 h incubation, the fluorescence was measured at 485/535 nm, using the plate reader Victor X3 (Perkin Elmer, Waltham, MA, USA).

2.10.2.3. GSH determination

For GSH determination the cells were provided with 90 µL of fresh media and treated with 10 µL of SeNPs or sodium selenite (at two concentration levels) for 24h after which the treatment solutions were removed and the cells were washed one time with PBS. Next, the test cells and positive controls were treated with 100 µL of 100 µM tBOOH prepared in PBS, while the negative controls were incubated with 100 µL of PBS. The plates were incubated for 3h followed by the measurement of fluorescence at 355/460 nm using plate reader Victor X3 (Perkin Elmer, Waltham, MA, USA).

2.11. Determination of antifungal activity

Fungal cultures of *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea*, *Macrophomina phaseolina (Tassi) Goid* and *Fusarium graminearum* were cultured on Potato Dextrose Agar (PDA; BD Difco™, Franklin lakes, USA). The fungal cultures were obtained from the Faculty of Agrobiotechnical Sciences Osijek collection. The fungi were grown in 10 ml of agar media in Petri dishes for 1 week, followed by a transfer on the medium containing previously described SeNPs (Table 1) or sodium selenite, at three concentration levels (20, 50 and 100 ppm). The negative controls were grown on a normal agar medium. The dishes were incubated at 22 ± 1 °C in a growth chamber, with 12 h interchanging cycles of light/dark regime. The diameters of the colonies were measured after 168 hours as the final measurement and are shown in the section Results. The experiment was carried out in the Laboratory of phytomedicine at the Faculty of Agrobiotechnical Sciences Osijek.

2.12. Data analysis

The results are expressed as average values of replicates with standard deviations (the experiments were performed in quadruplicates if not stated otherwise in the description of the particular method). The differences between groups were tested using one-way

analysis of variance (ANOVA) with the post hoc Tukey test of multiple comparison. The differences were considered significant if $p < 0.05$. The differences were compared relative to control cells and/or between different test groups. The groups were formed according to coating applied and/or functionalization of SeNPs primarily to test the effects of SeNPs functionalization. Inhibitory concentration 20 (IC_{20}) values were determined using nonlinear logistic regression. The data were processed using Microsoft Office Excel (Redmond, Washington, United States) and GraphPad®Prism 6 Software (San Diego, CA, USA).

3. RESULTS

3.1. OPE characterization

The average values for TEAC and FC were 30.82±0.89 mg TE/g and 40.48±0.14 mg GA/g, respectively. The content of polyphenols was previously analysed at the Department of Food chemistry at Faculty of Pharmacy and Biochemistry, University of Zagreb. The highest content was determined for HTS with 71.7±1.21 µg/100 mg of extract, followed by OLE with 31.9±0.79 µg/100 mg. The content of TS was shown to be the lowest of the three evaluated compounds with a concentration of 23.5±0.48 µg/100 mg (data previously published in Radić et. al 2020 (103).

3.2. Synthesis and purification of SeNPs

The synthesis of SeNPs using only OPE as a reduction agent did not result in the formation of nanoparticles. The procedure that included the reduction of selenite using L-ascorbic acid as a reduction agent and raw OPE as surface stabilizer resulted in the formation of particles with dH > 1000 nm. Purification of OPE using 0.45 µm filters reduced the size of nanoparticles; however, the system was heterogeneous consisting of particles of different sizes. The next approach that was evaluated was the usage of L-ask as a reducing agent with synthetic coatings (PVP or PS) and OPE for surface stabilization and functionalization, respectively. The obtained nano-systems are presented in Figure 5. This procedure resulted in the generation of SeNPs with narrow particle size distribution and satisfactory PI. The reaction mixtures for obtaining the final samples are presented in Table 1. of Materials and methods section. Results concerning SeNPs purification are presented in Figure 6.

Figure 5. Different types of synthesized selenium nanoparticles (Original photography)

The main goal of optimizing SeNPs purification was to achieve efficient removal of remaining reagents used in the SeNPs synthesis process and obtain purified SeNPs, but without impairing their physicochemical properties such as size, PI or zeta potential. The main parameter chosen as an indicator of a successful separation process was the radical scavenging capacity of PVPSeNPs (since PVPSeNPs did not show significant direct antiradical capacity against ABTS radical and activity, and in case it was detected, it would originate from the remaining ascorbic acid used in the synthesis process). Results concerning TEAC assay are shown in Figure 6A and show that the antiradical activity of analysed samples was the highest in samples purified by Amicon-assisted centrifugation (PVPSeNPs) or simple centrifugation (fPVPSeNPs) indicating inadequate purification. Figures 6B and 6C show the impact of different purification methods on the average dH and zeta potential of analysed samples. It is crucial to purify the NPs of the remaining reactants prior to investigation concerning their biological activity. Three different purification procedures were evaluated - using conventional centrifugation, centrifugation using Amicon filters and dialysis. The average dH of obtained PVPSeNPs was the highest in the samples purified by centrifugation using Amicon filters (253.5±0.5; 261.9±10.8) while in the case of fPVPSeNPs, the highest average values of dH were determined for dialysed samples (109.1±6.3; 106.1±4.9). Zeta potential was highest after centrifugation

for both PVPSeNPs and fPVPSeNPs with values of -16.0±0.4; -10.6±0.1 and -22.9±0.3; -19.7±0.5, respectively.

Based on obtained results for dialysis of PVPSeNPs, this procedure was chosen as the optimal way of separating newly synthetized nanoparticles from the remaining reactants in the reaction mixture. To additionally optimize the dialysis, process the conductivity of the outer phase was measured, and the dialysis process was conducted until the conductivity of the outer phase reached values <2 µS/cm. As presented in Figure 7., the conductivity of the outer phase (UPW) before the start of dialysis was 0.99 µS/cm. During the dialysis procedure, the conductivity was highest at the 2nd outer phase on the 5th hour (28.6 µS/cm). At the end of the procedure, in the 4th outer phase, the values were <2 µS/cm.

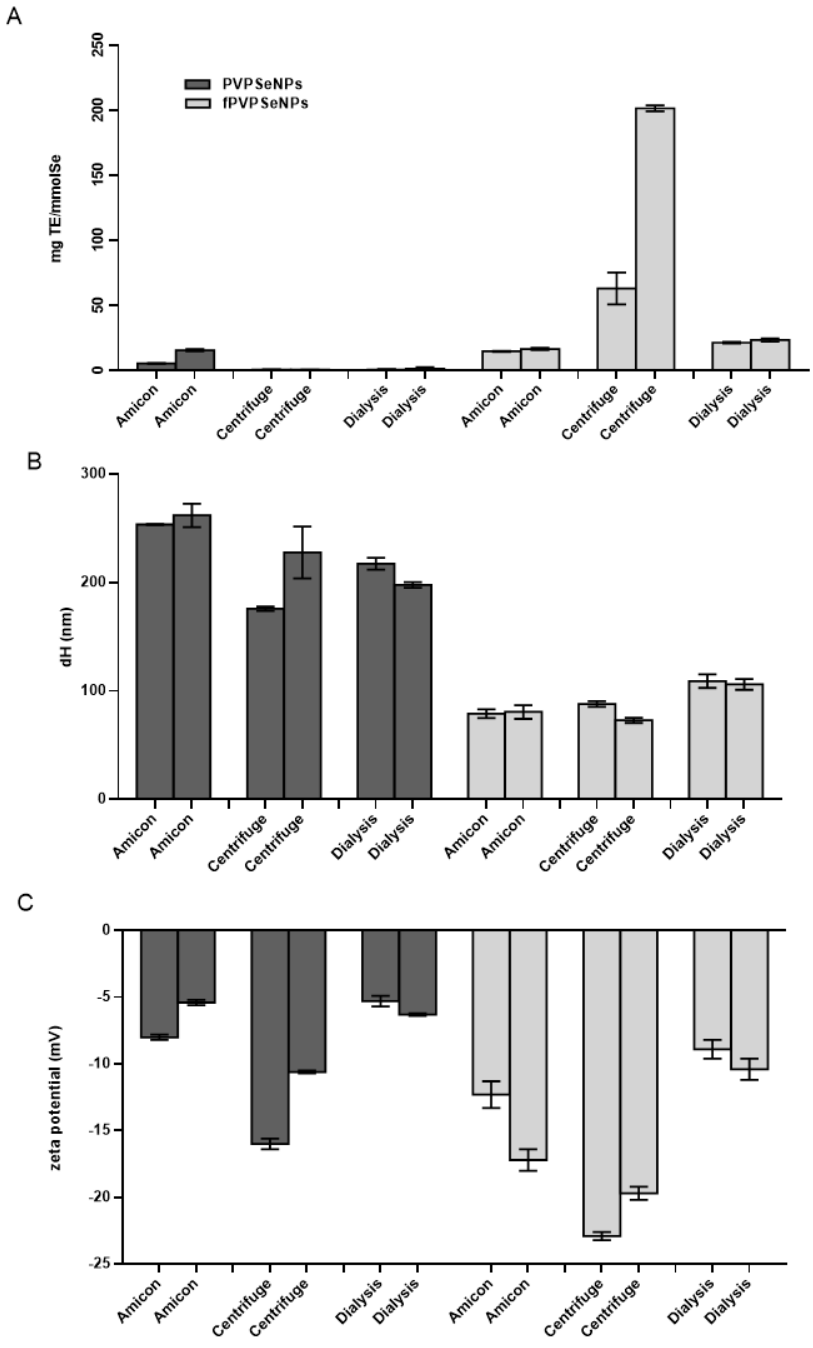

Figure 6. Impact of different purification procedures on (A) Trolox equivalent capacity, (B) size and (C) zeta potential of PVPSeNPs and fPVPSeNPs. (PVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; dH - hydrodynamic diameter).

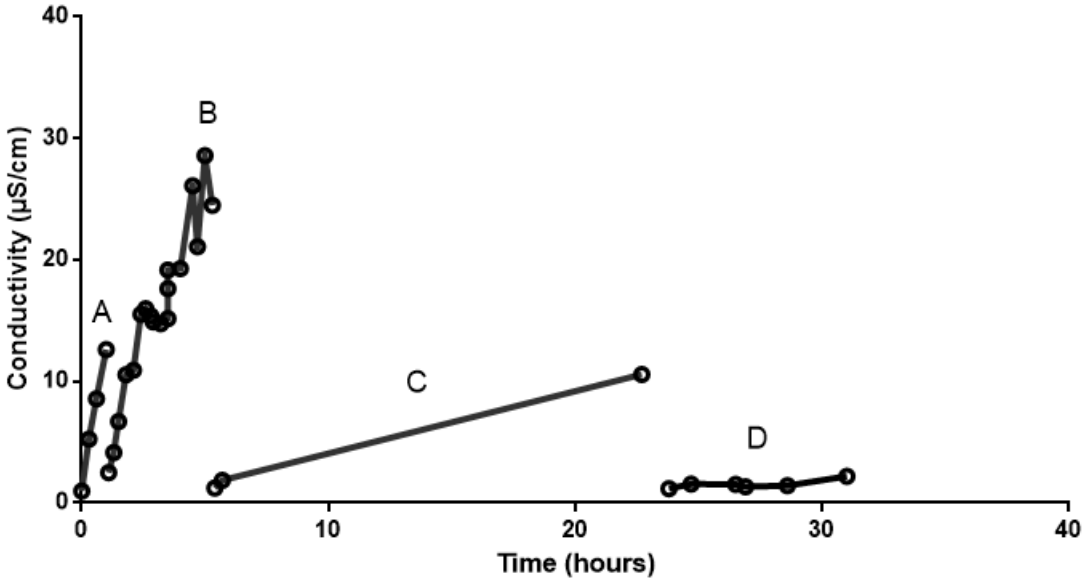

Figure 7. The conductivity of outer phases in different time periods during dialysis procedure of PVPSeNPs. 1st outer phase (A), 2nd outer phase (B), 3rd outer phase (C), 4th outer phase (D).

3.3. Nanoparticle characterization

Table 2. Average dH, zeta potential, polydispersity index and pH of SeNPs.

Sample	Average dH (nm)	Zeta potential (mV)	Polydispersity index	pH
PVPSeNPs	181.7 ± 0.58[a]	-5.9 ± 0.48[a]	0.11±0.02[a]	8.16
fPVPSeNPs	107.6 ± 0.36[b]	-10.3 ± 0.99[b]	0.18±0.03[b]	7.88
PSSeNPs	74.9 ± 0.29[c]	-33.0 ± 1.22[c]	0.12±0.01[a]	7.94
fPSSeNPs	56.0 ± 0.45[d]	-30.0 ± 1.05[d]	0.12±0.01[a]	7.61

PVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs - selenium nanoparticles stabilized with polysorbate; fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract). Data marked with different letters are significantly different ($p < 0.05$) according to ANOVA and Tukey's post hoc test.

The main physicochemical characteristics (average dH, zeta potential and PI) of SeNPs synthetized and purified under optimized conditions are presented in Table 2. Among the evaluated SeNPs, the highest value of dH was evidenced for PVPSeNPs (181.7 ± 0.58). Functionalization using OPE significantly reduced the dH of fPVPSeNPs (107.6 ± 0.36). Similar was observed in the case of PSSeNPs and fPSSeNPs, with dH values of 74.9 ± 0.29 and 56.0 ± 0.45, respectively. Zeta potential was positively affected by modification with OPE in the case of PVPSeNPs (-5.9 ± 0.48), and fPVPSeNPs (-10.3 ± 0.99). When observing PSSeNPs and fPSSeNPs, it is visible that OPE slightly reduced the absolute value of zeta potential (-33.0 ± 1.22 and-30.0 ± 1.05). PI values of the SeNPs were around 0.1, while pH ranged from 7.61 to 8.16. All types of SeNPs were spherical in shape, as presented in Figure 8.

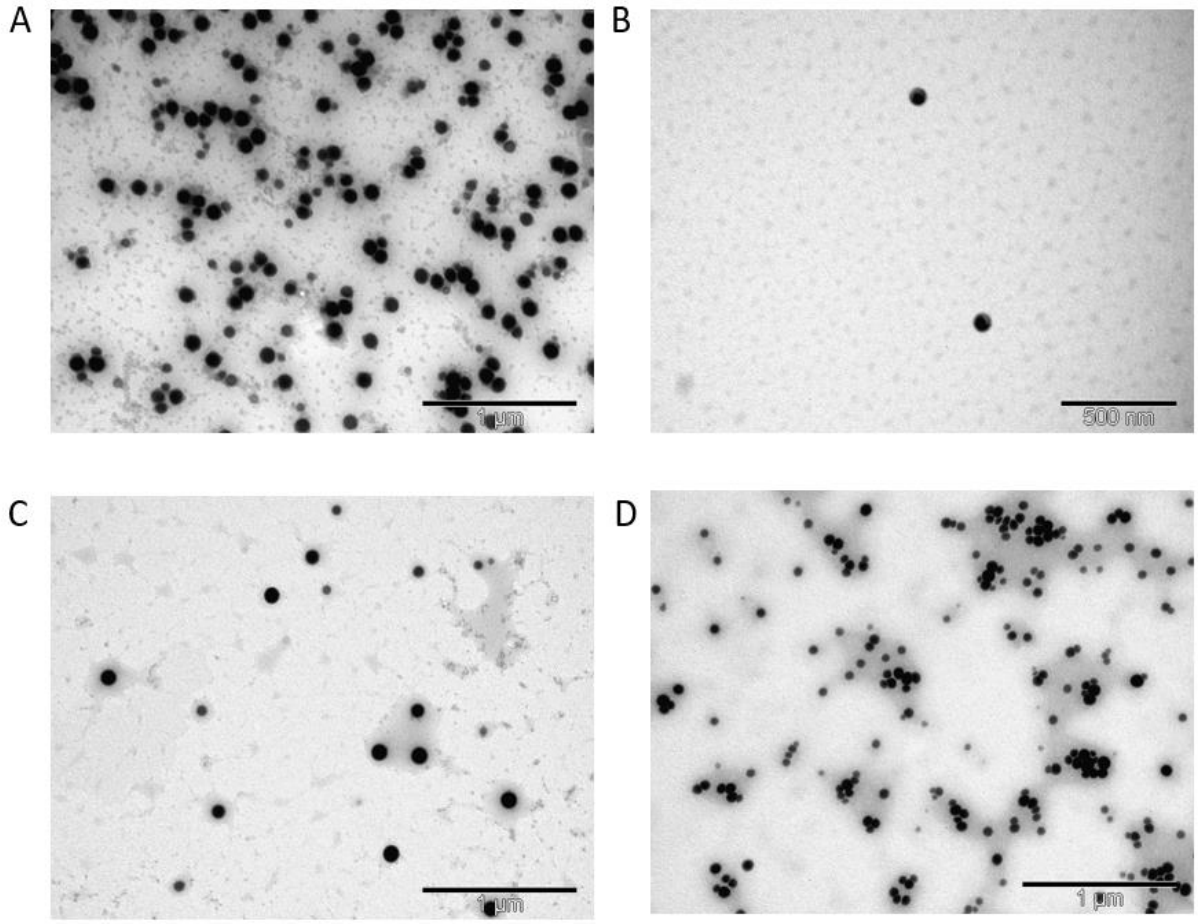

Figure 8. Visualization of SeNPs using transmission electron microscopy (TEM). (A) PVPSeNPs, (B) fPVPSeNPs; (C) PSSeNPs, (D) fPSSeNPs. Scale bar was set at 500 nm or 1000 nm. (PVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs — selenium nanoparticles stabilized with polysorbate; fPSSeNPs — selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

Results presented in Figure 9. show that fPVPSeNPs and fPSSeNPs had significantly higher antiradical capacity (24.4 ± 1.84 and 30.9 ± 2.47 mg TE/mmol Se) in comparison with PVPSeNPs and PSSeNPs (0,6 ± 0,48 and 7,30 ± 1,15 mg TE/mmol Se), respectively. A similar trend was noticed for total reducing capacity presented in Figure 10., where the values fPVPSeNPs and fPSSeNPs (50 ± 3.16 and 53.6 ± 3.22 mg GAE/mmol Se) were

significantly higher compared to PVPSeNPs and PSSeNPs (14.9 ± 0.48 and 21.7 ± 0.68 mg GAE/mmol Se).

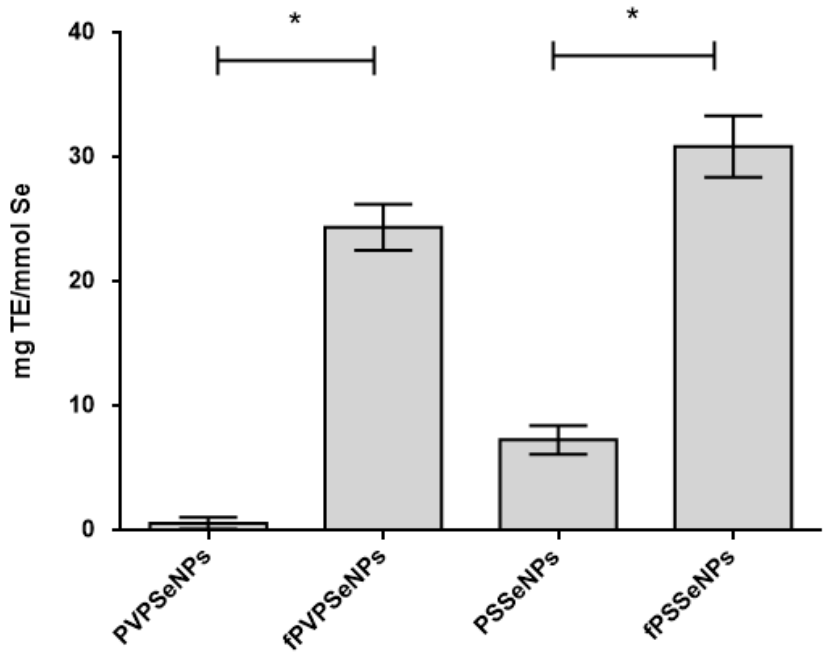

Figure 9. Determination of SeNPs antioxidant capacity. The results are presented as mg of equivalents of Trolox (TE) per mmol of selenium. The asterisk (*) indicates statistically significant differences between functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (PVPSeNPs- selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs- selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs-selenium nanoparticles stabilized with polysorbate; fPSSeNPs- selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

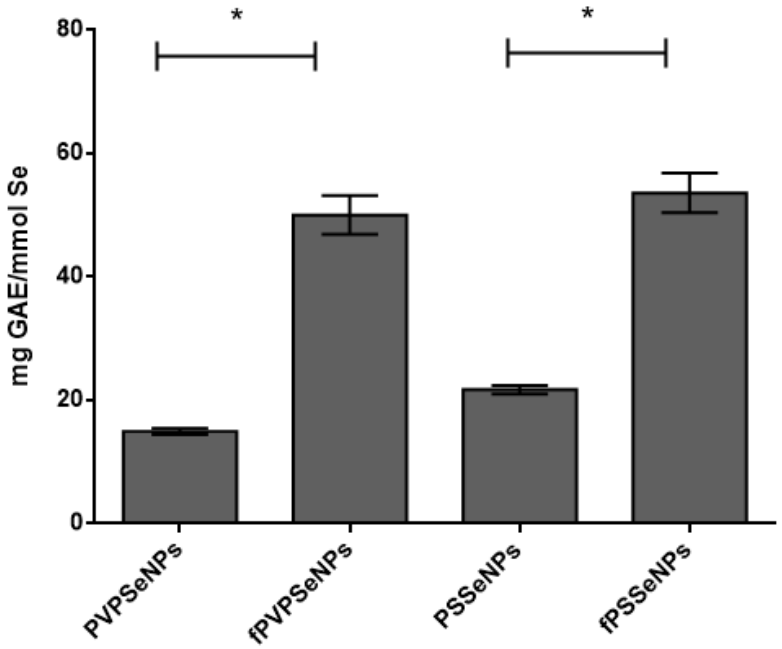

Figure 10. Determination of total reducing capacity of SeNPs. The results are presented as mg of gallic acid (GAE) per mmol of Se. The asterisk (*) indicates statistically significant differences between functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (PVPSeNPs- selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs-selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs-selenium nanoparticles stabilized with polysorbate; fPSSeNPs- selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

3.4. Cytotoxicity of SeNPs

As presented in Figure 11. and Figure 12., it was not evidenced that the cells treated with SeNPs or selenite had significantly higher ROS levels than negative controls. The results concerning GSH presented in Figure 13. and Figure 14. show that the HepG2 cells treated with selenite had significantly reduced GSH content which was around 15.43 ± 5.84 % of the levels in negative controls. Similar but less prominent was evidenced in Caco-2 cells, where GSH content in the cells treated with selenite was 62.65 ± 3.99 % of the levels in the negative controls. Obtained IC_{20} values are presented in Table 3. The highest values

were determined for PVPSeNPs in both HepG2 and Caco-2 cells (3.65 mg/L and 4.3 mg/L). The lowest value in HepG2 cells was shown for PSSeNPs (0.48 mg/L) and fPSSeNPs (1.13mg/L). Similarly, was seen in Caco-2 cells, where the mentioned SeNPs also had the lowest IC_{20} values (PSSeNPs (0.87mg/L and fPSSeNPs 0.41 mg/L). Selenite was characterized by low IC_{20} in HepG2 cells with a value of 0.18 mg/L, while in Caco-2 cells, the value was 2.29 mg/L. The concentrations lower than IC_{20} were selected for antioxidative assays.

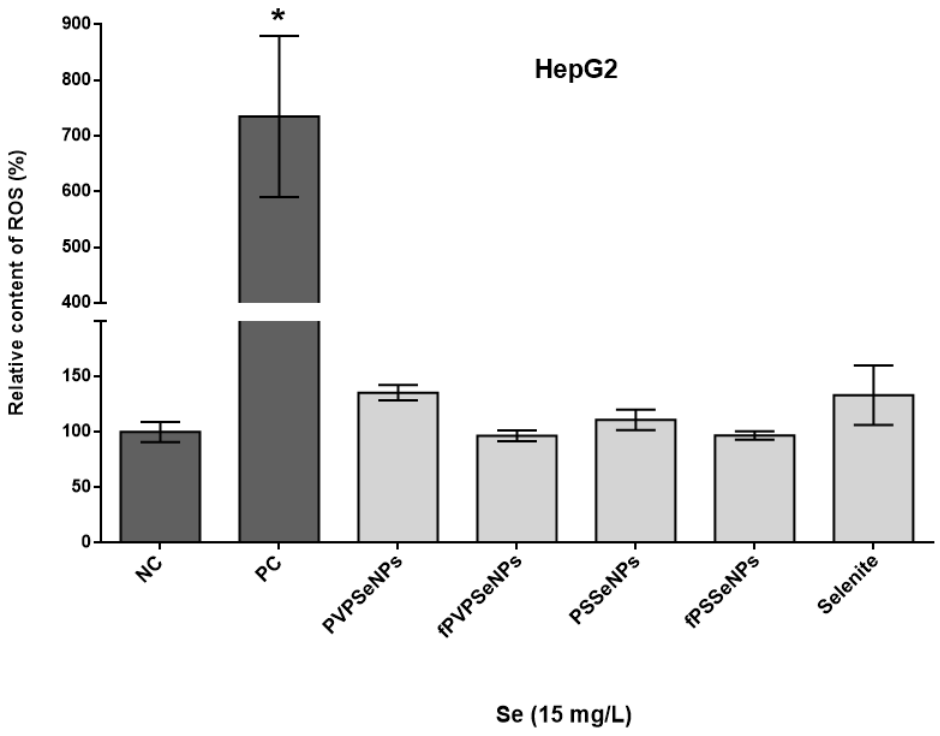

Figure 11. The relative content of ROS determined by DCFH-DA assay in HepG2 cells treated with SeNPs or sodium selenite. The asterisk (*) indicates statistically significant differences compared to the negative control ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVPSeNPs— selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs— selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs— selenium nanoparticles stabilized with polysorbate; fPSSeNPs— selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

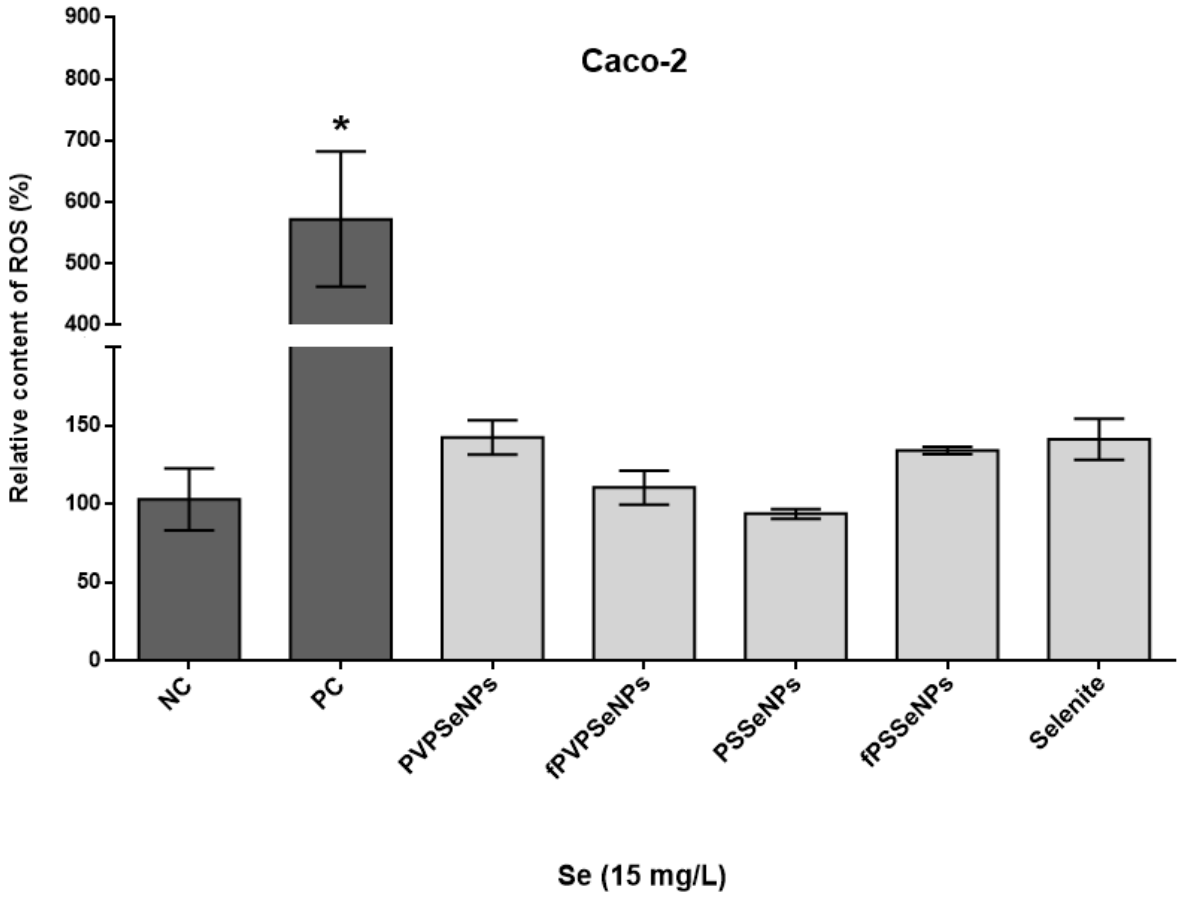

Figure 12. The relative content of ROS determined by DCFH-DA assay in Caco-2 cells treated with SeNPs or sodium selenite. The asterisk (*) indicates statistically significant differences compared to the negative control ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs — selenium nanoparticles stabilized with polysorbate; fPSSeNPs — selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

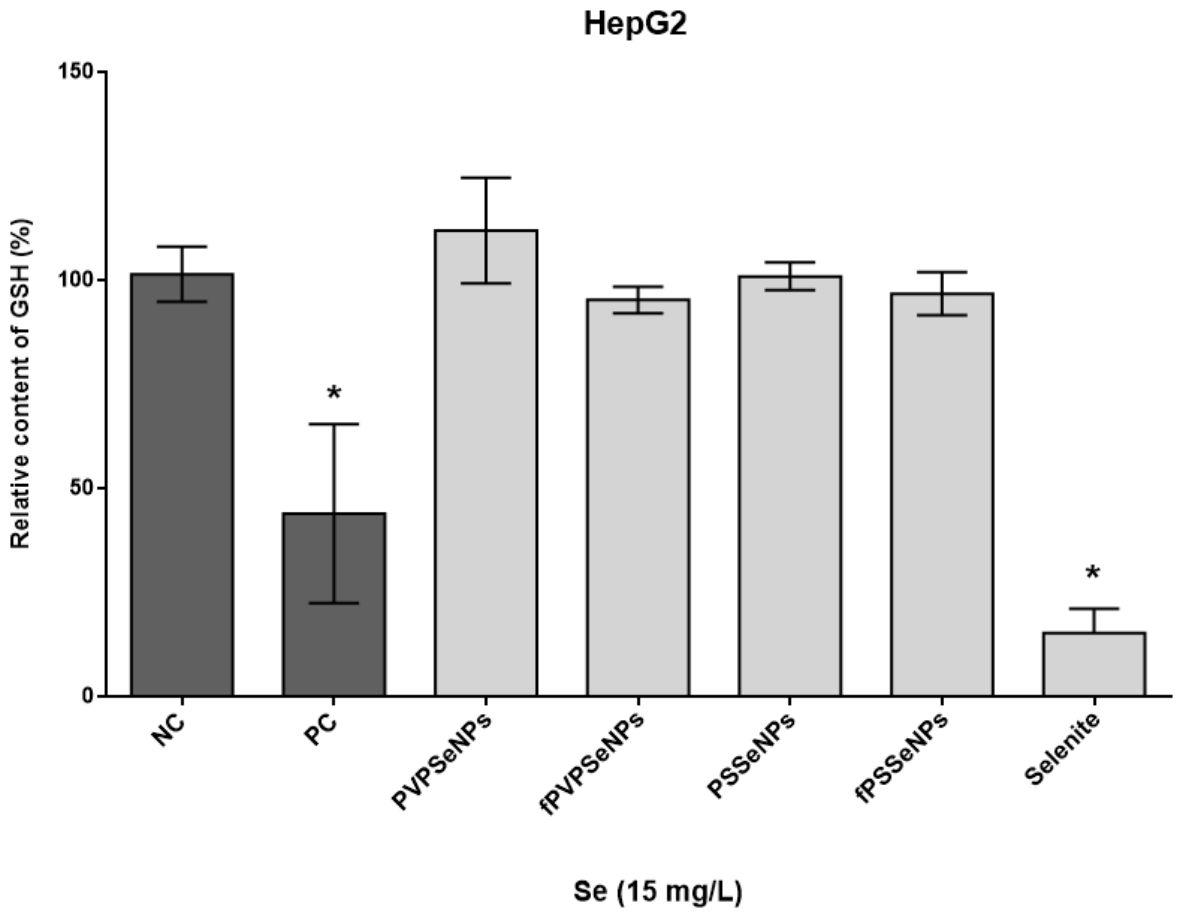

Figure 13. The relative content of GSH determined by mCBL assay in HepG2 cells treated with SeNPs or sodium selenite. The asterisk (*) indicates statistically significant differences compared to the negative control ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs — selenium nanoparticles stabilized with polysorbate; fPSSeNPs — selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

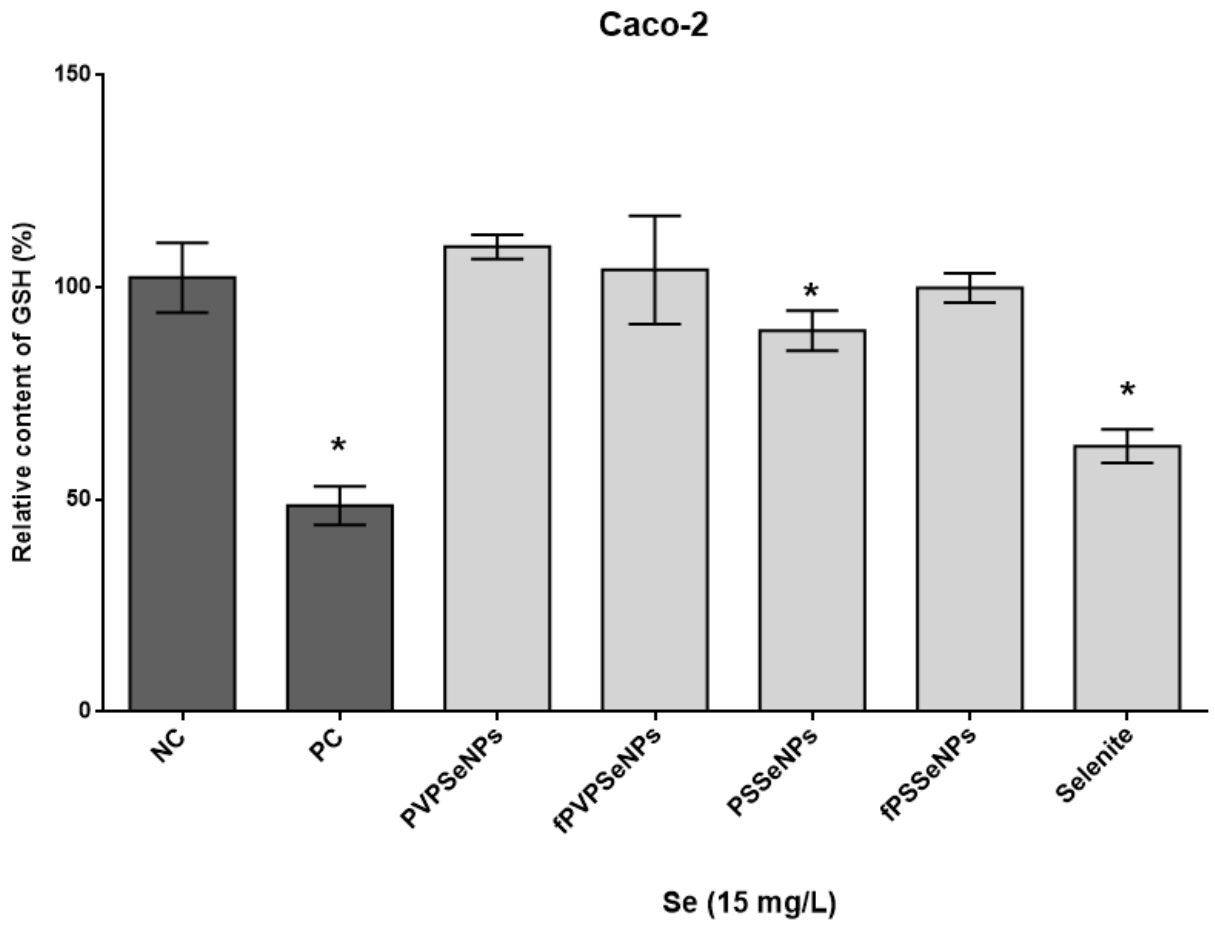

Figure 14. The relative content of GSH determined by mCBL assay in Caco-2 cells treated with SeNPs or sodium selenite. The asterisk (*) indicates statistically significant differences compared to the negative control ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs — selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs — selenium nanoparticles stabilized with polysorbate; fPSSeNPs — selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

Table 3. IC_{20} values of SeNPs and sodium selenite in HepG2 and Caco-2 cell lines.

IC20 (mg/L)		
Samples	**HepG2**	**Caco-2**
PVPSeNPs	3.65	4.30
fPVPSeNPs	1.16	1.26
PSSeNPs	0.48	0.87
fPSSeNPs	1.13	0.41
Selenite	0.18	2.29

(IC_{20} - Inhibitory concentration 20; PVPSeNPs— selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVPSeNPs— selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PSSeNPs— selenium nanoparticles stabilized with polysorbate; fPSSeNPs— selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

3.5. Cellular antioxidative activity of SeNPs

As presented in Figure 15., HepG2 cells treated with SeNPs had significantly higher viability compared to the positive control, especially when treated with lower concentrations of SeNPs. The positive control cells treated only with tBOOH were characterized by low viability (22.05±7.84 %). The highest viability was evident in the case of fPSSeNPs (98.025±9.13 %). Moreover, fPSSeNPs showed the most pronounced difference between the two concentration levels - the lower tested concertation (0.04 mg/L) having a significant protective effect compared to the higher concentration (0.4 mg/L) that did not show any protective effect in terms of cell viability (98.025±9.13 % and 19,35±2,54 %). The protective effect was evidenced for all types of nanoparticles at the lower concertation level. However, the viability of the cells treated with selenite was also significantly higher compared to the positive control, independent of the concentration applied (102.47±8.52 and 107.7±0.71 %). The average cell viability was comparable to the viability of negative control cells. Results for Caco-2 cells presented in Figure 16.,

show that they had a different response compared to HepG2 cells. The viability of positive control cells was 66.66±8.94 % of the negative controls. Of all the cell groups treated with SeNPs, the highest viability was evidenced for treatment with the lower concentration of PSSeNPs (103.05±17.36 %). However, there was a significant difference between concentration levels when observing PS SeNPs, the cell viability in the case of higher tested concentration was not changed compared to positive control. Conversely, the cells treated with PVP SeNPs, fPVPSeNPs and fPSSeNPs had increased viability compared to positive control in both tested concentrations. Again, the viability of the cells treated with sodium selenate was not dependent on the applied concentration (111.21±17.67 and 103.46±17.78 %) and was comparable to the viability of negative control cells.

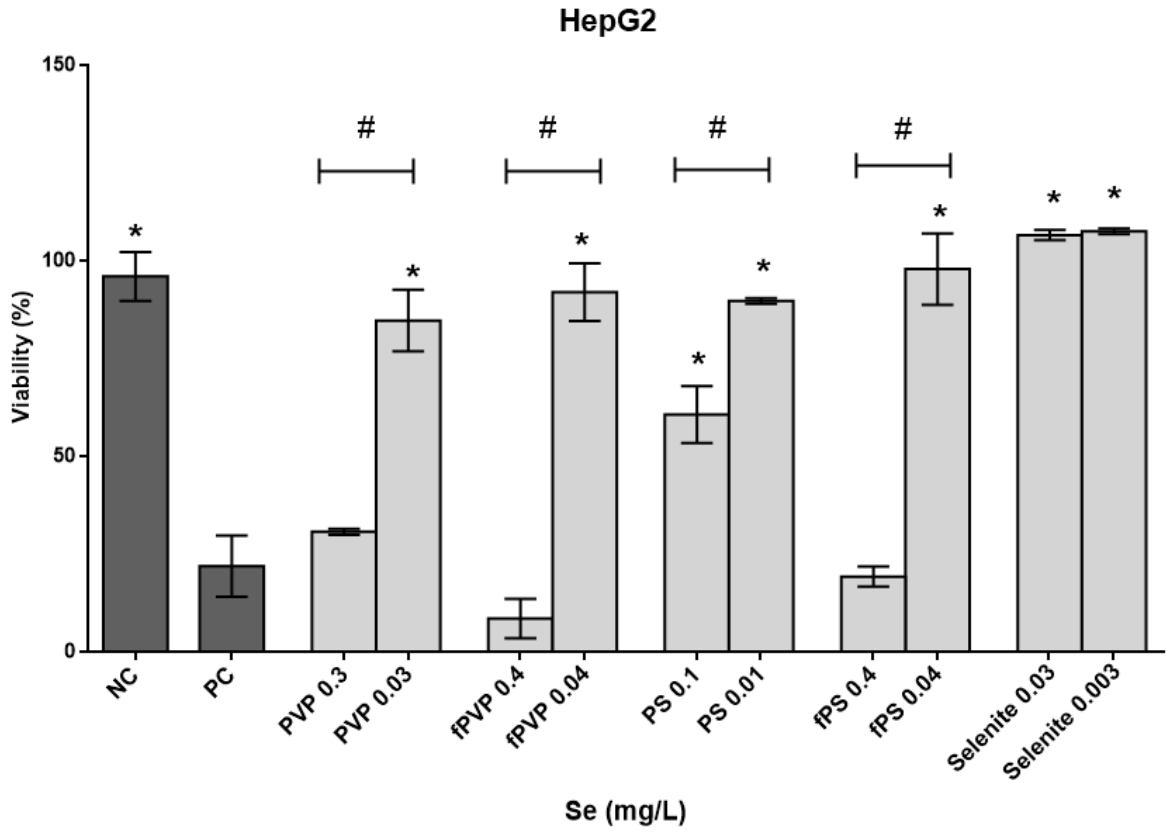

Figure 15. Cell viability determined using an MTT assay of HepG2 cells pre-treated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 300 µM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$) and hashtag (#) indicates statistically significant differences between different applied concentration of the same type of SeNPs ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

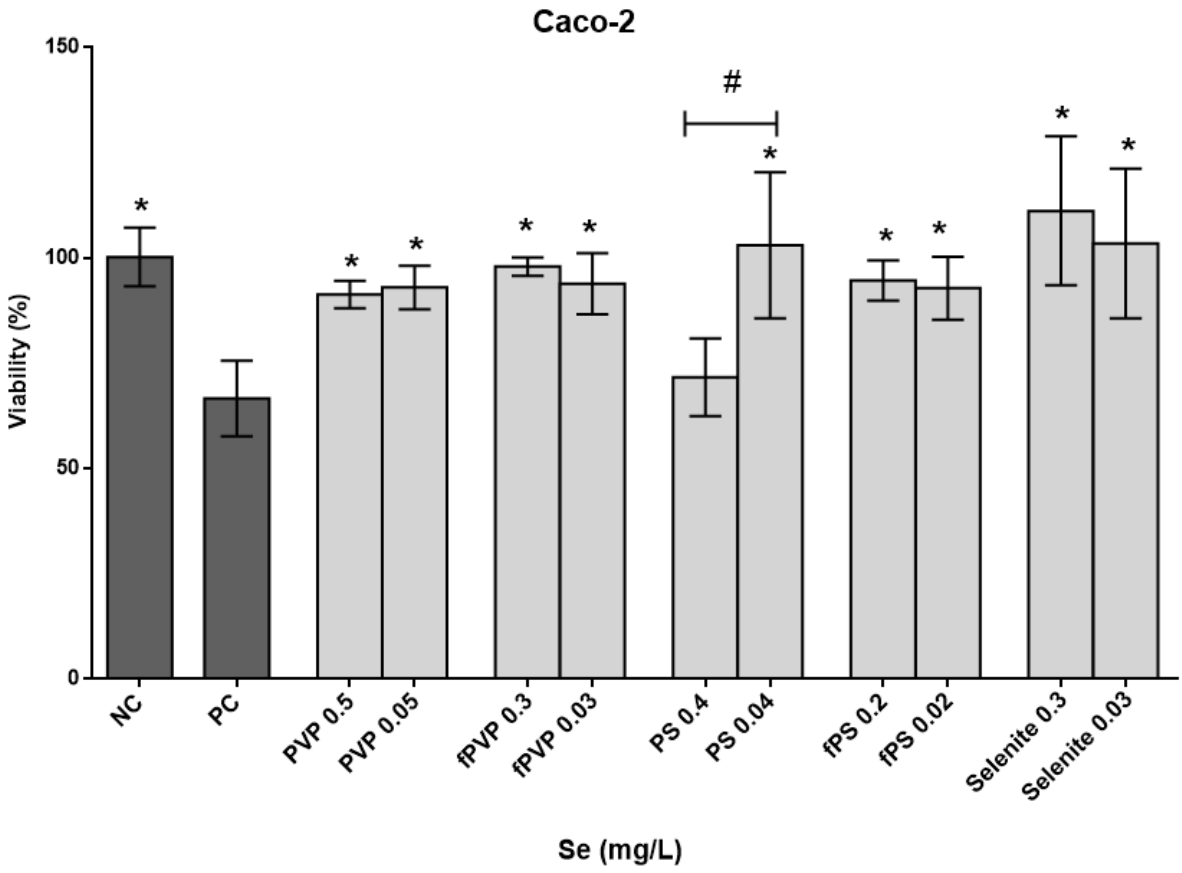

Figure 16. Cell viability determined using an MTT assay of Caco2 cells pre-treated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 300 μM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$) and hashtag (#) indicates statistically significant differences between different applied concentration of the same type of SeNPs ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

The results concerning protective antioxidative activity in the context of ROS content are presented in Figures 17. and 18. In HepG2 cells, the positive control showed elevated levels of ROS (246.75±52.53 %). All evaluated SeNPs, except for fPVPSeNPs showed

protective activity at the lower concentration level. The protective effect was not evidenced at the higher concentration levels. The lowest content of ROS was observed in the case of fPSSeNPs (137.51±14.54 %).

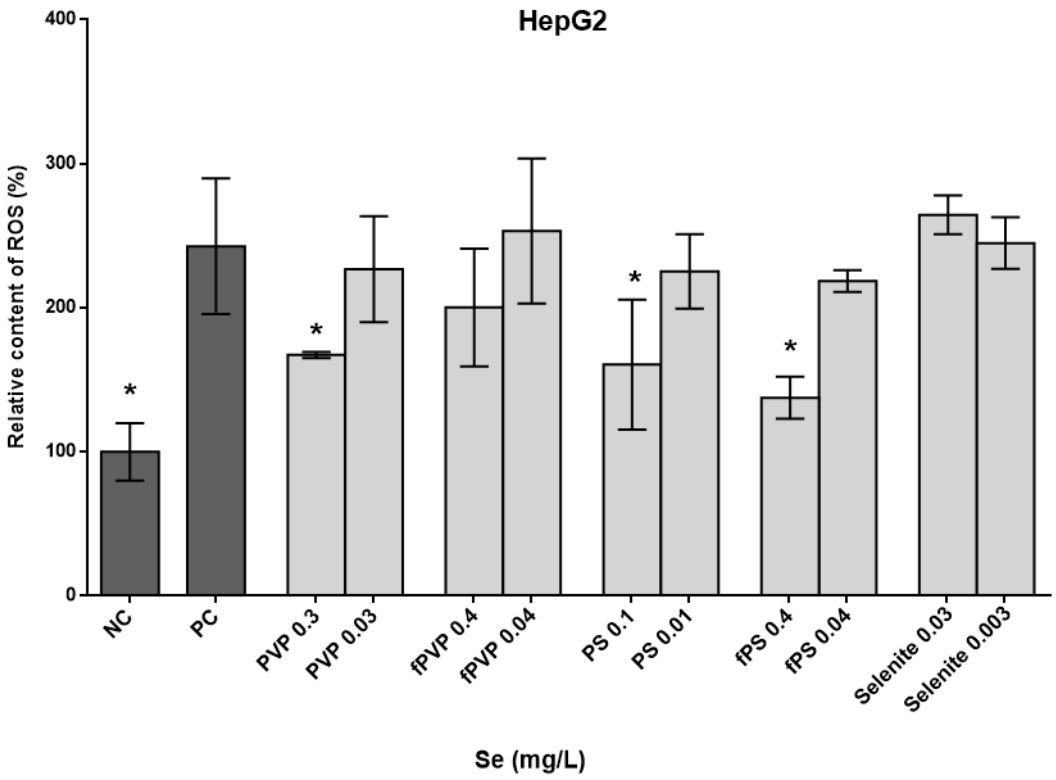

Figure 17. The relative content of ROS determined using DCFH-DA assay in HepG2 cells pre-treated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 100 µM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

Furthermore, ROS content in the cells treated with sodium selenite was not significantly different from positive control cells. Results for Caco-2 cells showed that SeNPs and

selenite had a protective effect at the lower concentration level, except for fPVPSeNPs. The lowest ROS content was determined in the case of PSSeNPs (148.27±22.76 %), followed by fPSSeNPs (163.74±25.66 %) and PVPSeNPs. (165.62±17.56 %).

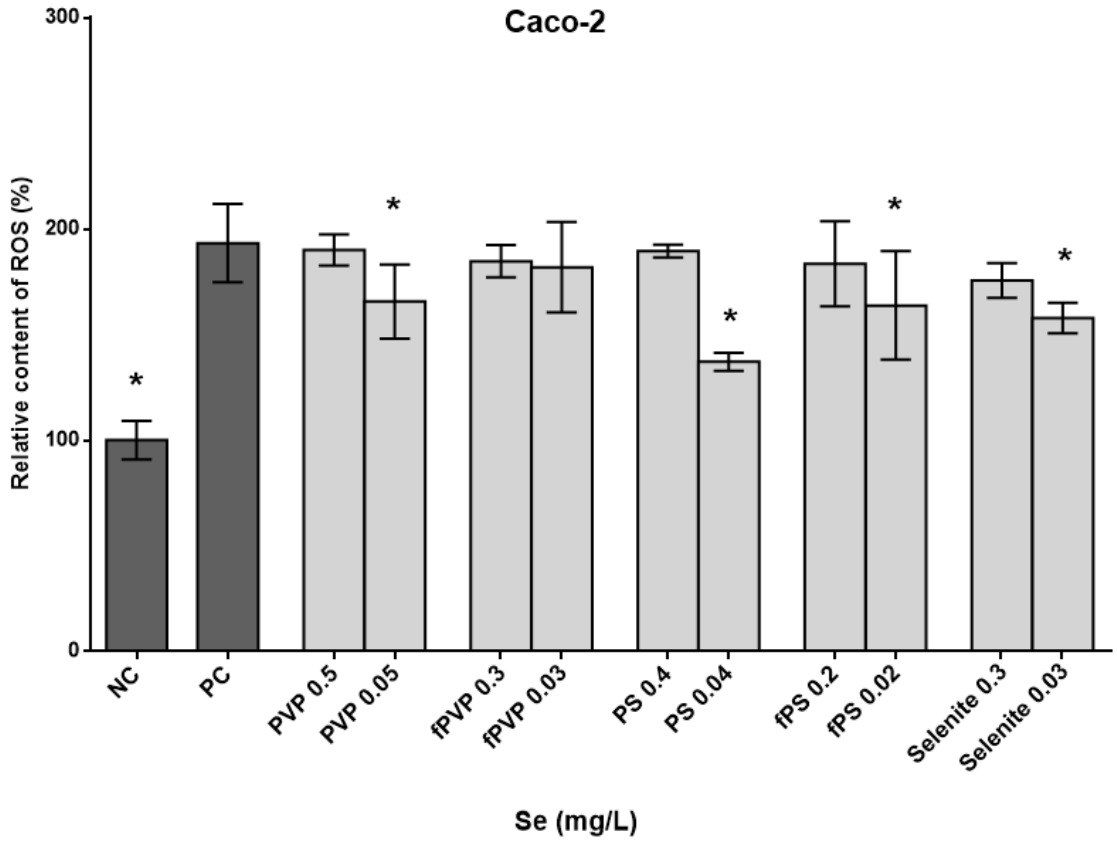

Figure 18. The relative content of ROS determined using DCFH-DA assay in Caco2 cells pre-treated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 100 µM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

Figure 19. represents results obtained for the impact of SeNPs of GSH content of HepG2 and Figure 20. on the Caco-2 cell line.

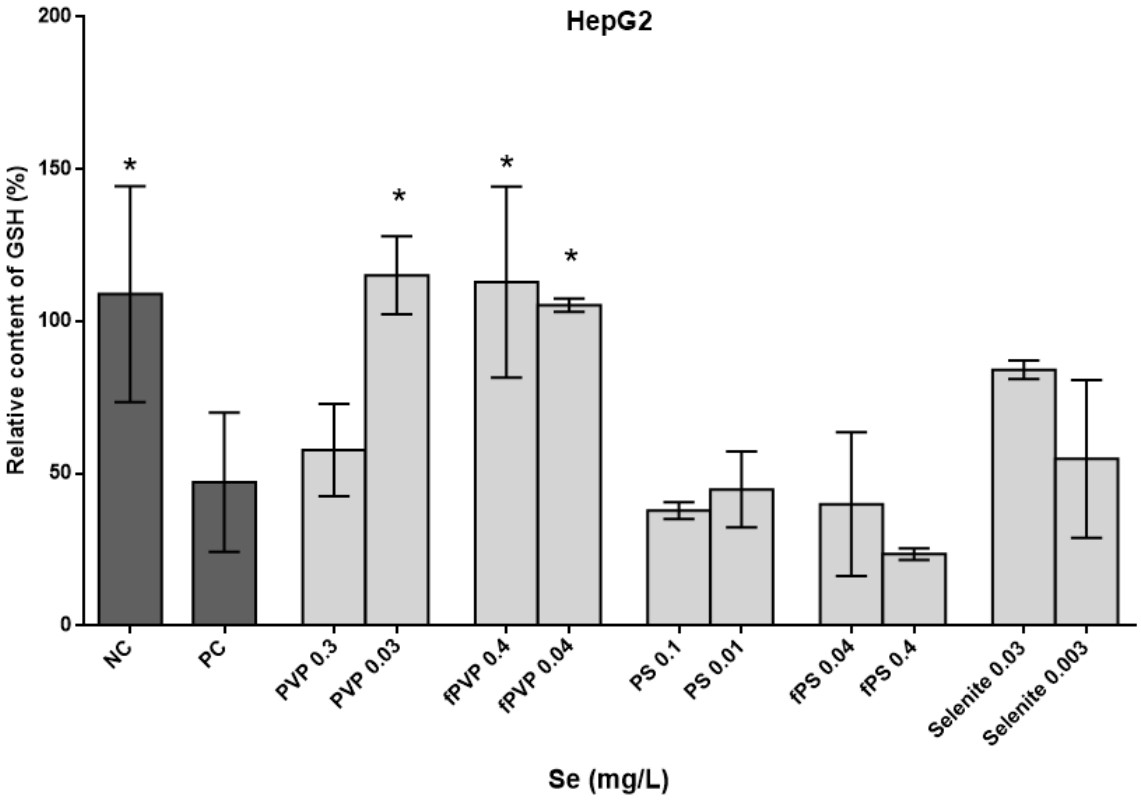

Figure 19. The relative content of GSH determined using mCBL assay in HepG2 cells pre-treated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 100 µM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

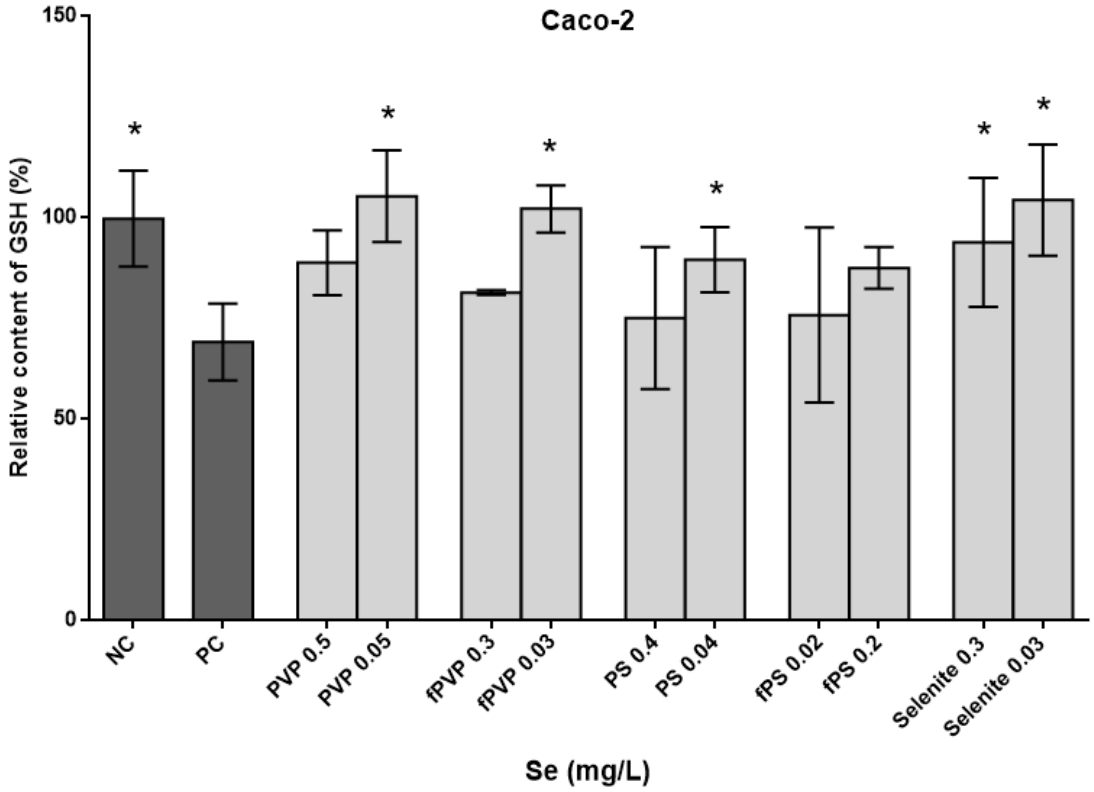

Figure 20. The relative content of GSH determined using mCBL assay in (A) HepG2 and (B) Caco2 cells pretreated with SeNPs or selenite followed by treatment with tBOOH. The negative control cells were non-treated cells and positive control cells were treated with 100 µM of tBOOH. The asterisk (*) indicates statistically significant differences compared to positive control cells ($p < 0.05$). (NC - negative control cells; PC - positive control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

HepG2 cells treated with lower concentrations of PVPSeNPs (115.16±12.85 %) and both concentration levels of fPVPSeNPs (112.9±31.41 and 105,33±2.21 %) had a significantly higher content of GSH compared to positive control cells (47.17±22.84). Cells treated with PSSeNPs, fPSSeNPs or selenite did not show significant differences in GSH content compared to the positive control. Results obtained in Caco-2 cells show that the cells

treated with lower concentrations of PVPSeNPs (105.375±11.38 %), fPVPSeNPs (102.22±5.86 %) and PS SeNPs (89.6±8.08 %) had a significantly higher content of GSH compared to positive controls (68.12±11.46 %). The increased GSH content was observed in the cells treated with selenite, at both concentration levels (93.9±16.01 % and 104.4±13.79 %). However, the cells treated with fPSSeNPs did not show significant differences in the content of GSH compared to positive control cells.

3.6. Antifungal activity of SeNPs

Results concerning the antifungal activity of investigated SeNPs and sodium selenite are presented in Figures 21-30. All measurements were carried out after 168 hours after the treatment with SeNPs.

The strongest antifungal activity against *S. sclerotiorum* was evidenced for fPSSeNPs (100 mg/L) with a colony diameter of 43.25±5.85 mm, compared to 90 nm diameter (the maximum diameter in the petri dish) in negative controls (Figure 21). fPSSeNPs were the only among investigated Se formulations that showed significant activity at the concentration of 50 mg/L (colony diameter: 79.75±4.99 mm). The colonies treated with 100 mg/L of sodium selenite also had reduced colony diameter (55±8.04 mm).

In the case of *B. cinerea*, the strongest imbibition was evidenced in the treatment with 100 mg/L of sodium selenite (20.75±4.99 mm) compared to the maximum colony diameter of 90 mm in negative controls (Figure 22). fPVPSeNPs, PSSeNPs and fPSSeNPs also showed significant activity at the mentioned concentration. However, at the lowest evaluated concentration (20 mg/L) only colonies treated with fPSSeNPs had significantly reduced average diameter (49.5±11.21).

M. phaseolina was sensitive to treatments with functionalized SeNPs at concentrations as low as 20 mg/L, with average colony diameter treated with fPVPSeNPs and fPSSeNPs reduced to 70±7.34 nm and 27.25±6.55 nm, respectively. Again, the average diameter of negative controls was 90 mm. The strongest inhibition was evidenced for the treatment with PSSeNPs and fPSSeNPs at the 100 mg/L concentration level (Figure 23). The average diameters were 16±2.94 mm and 16±2.44 mm, respectively.

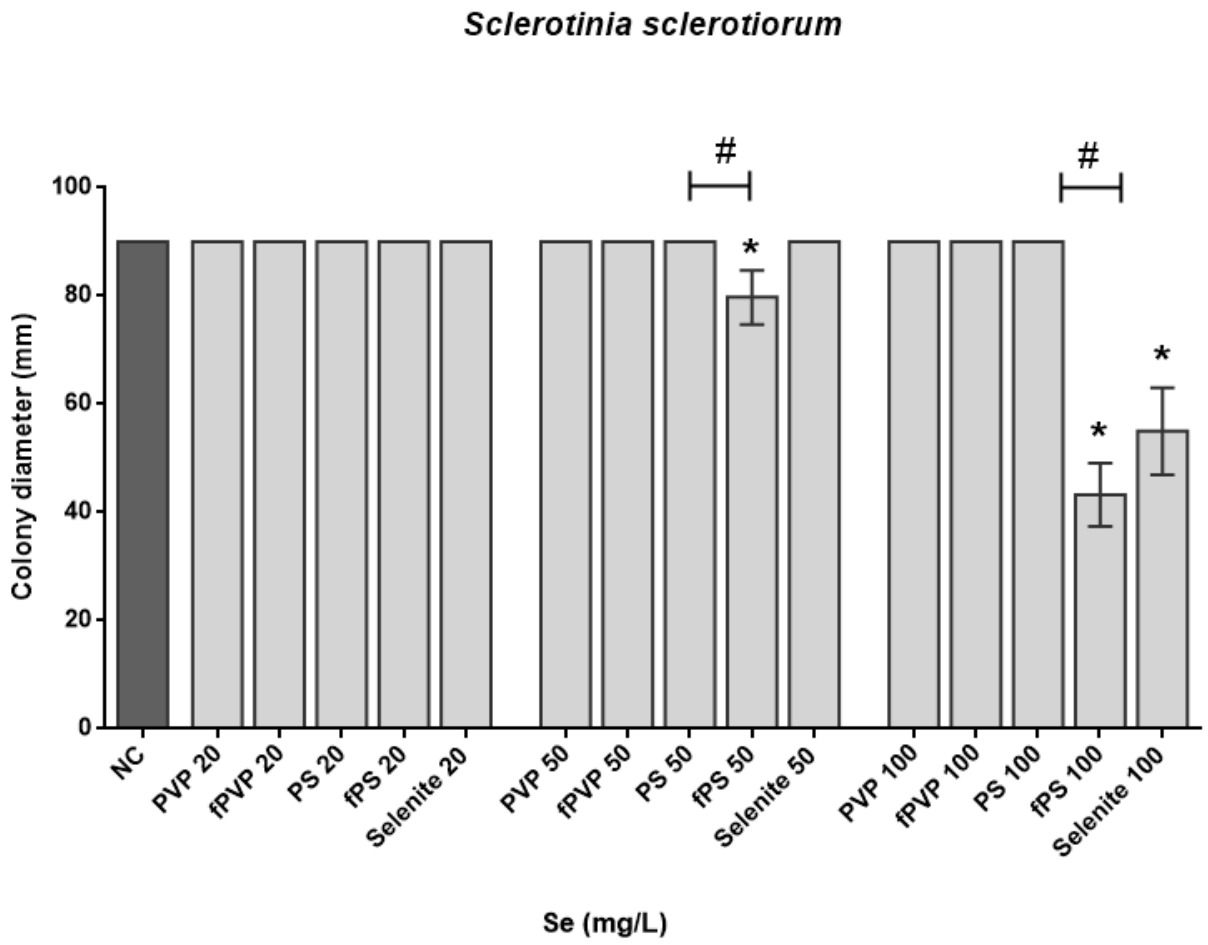

Figure 21. Colony diameters of fungal species *Sclerotinia sclerotiorum* (Lib.) de Bary treated with SeNPs or sodium selenite. The negative controls were non-treated fungi. The asterisk (*) indicates statistically significant differences compared to negative controls ($p < 0.05$) and hashtag (#) indicates statistically significant differences between the same applied concentrations of functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (NC - negative control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

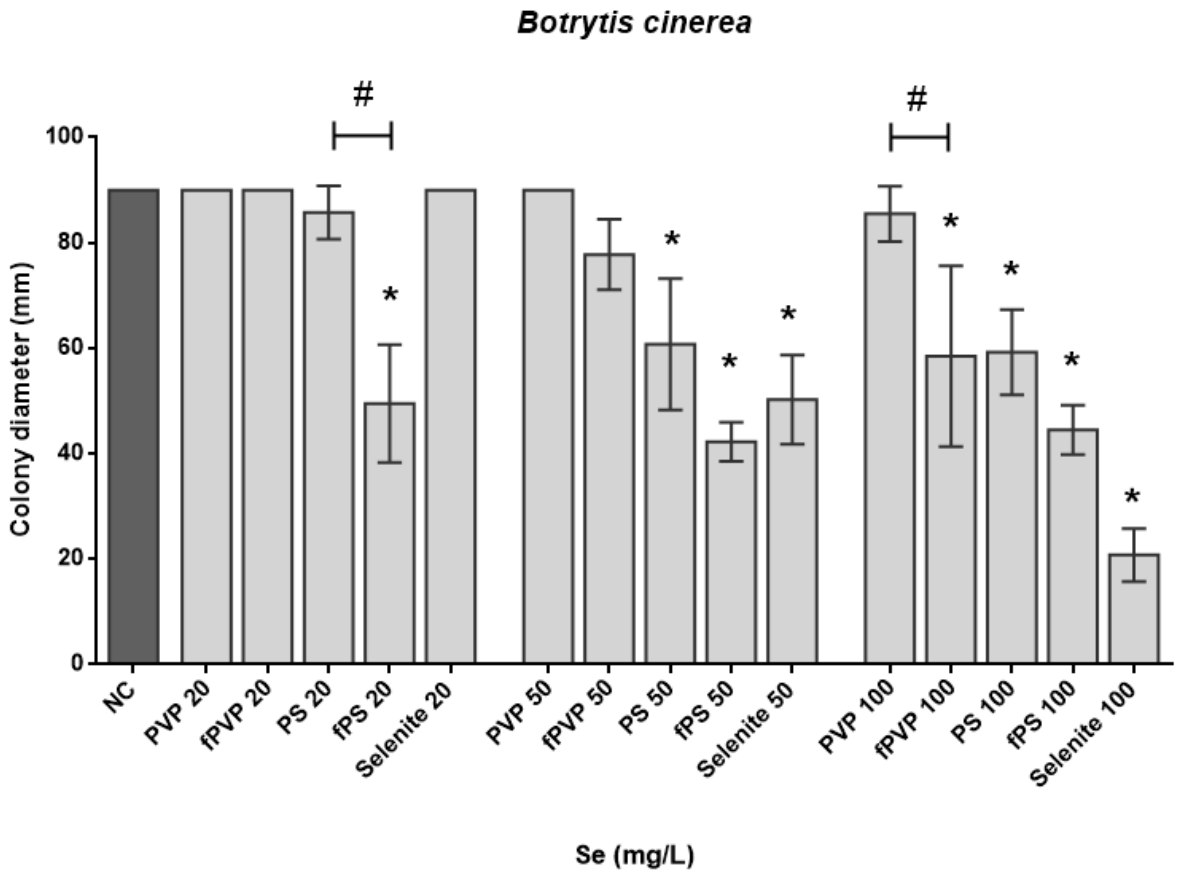

Figure 22. Colony diameters of fungal species *Botrytis cinerea* treated with SeNPs or sodium selenite. The negative controls were non-treated fungi. The asterisk (*) indicates statistically significant differences compared to negative controls ($p < 0.05$) and hashtag (#) indicates statistically significant differences between the same applied concentrations of functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (NC - negative control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

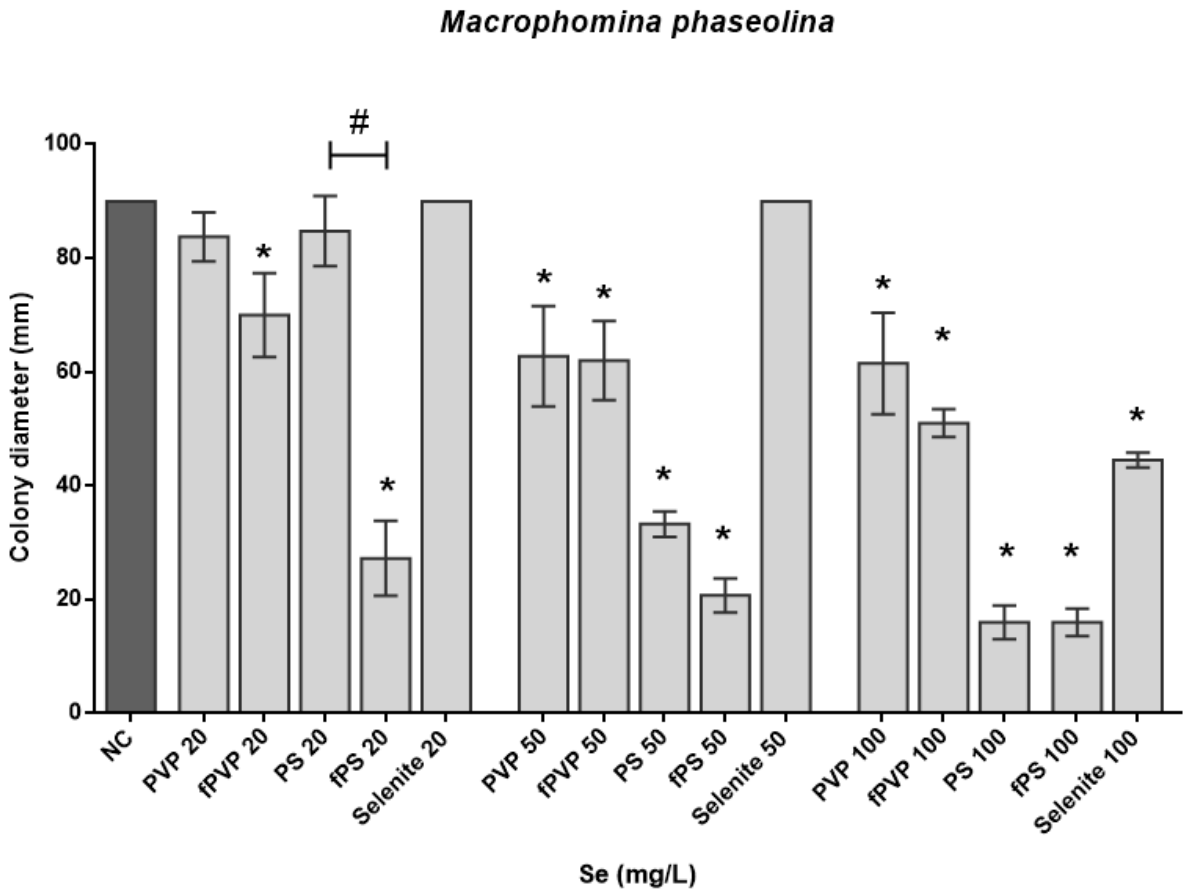

Figure 23. Colony diameters of fungal species *Macrophomina phaseolina* (Tassi) Goid treated with SeNPs or sodium selenite. The negative controls were non-treated fungi. The asterisk (*) indicates statistically significant differences compared to negative controls ($p < 0.05$) and hashtag (#) indicates statistically significant differences between the same applied concentrations of functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (NC - negative control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

The last evaluated fungal species was *F. graminearum* (Figure 24). The inhibition of growth was evidenced in the case of treatment with fPVPSeNPs in all tested

concentrations, with the strongest reduction compared to the negative control (90 mm) for treatment at the concentration level of 100 mg/L (53.75±2,21 mm).

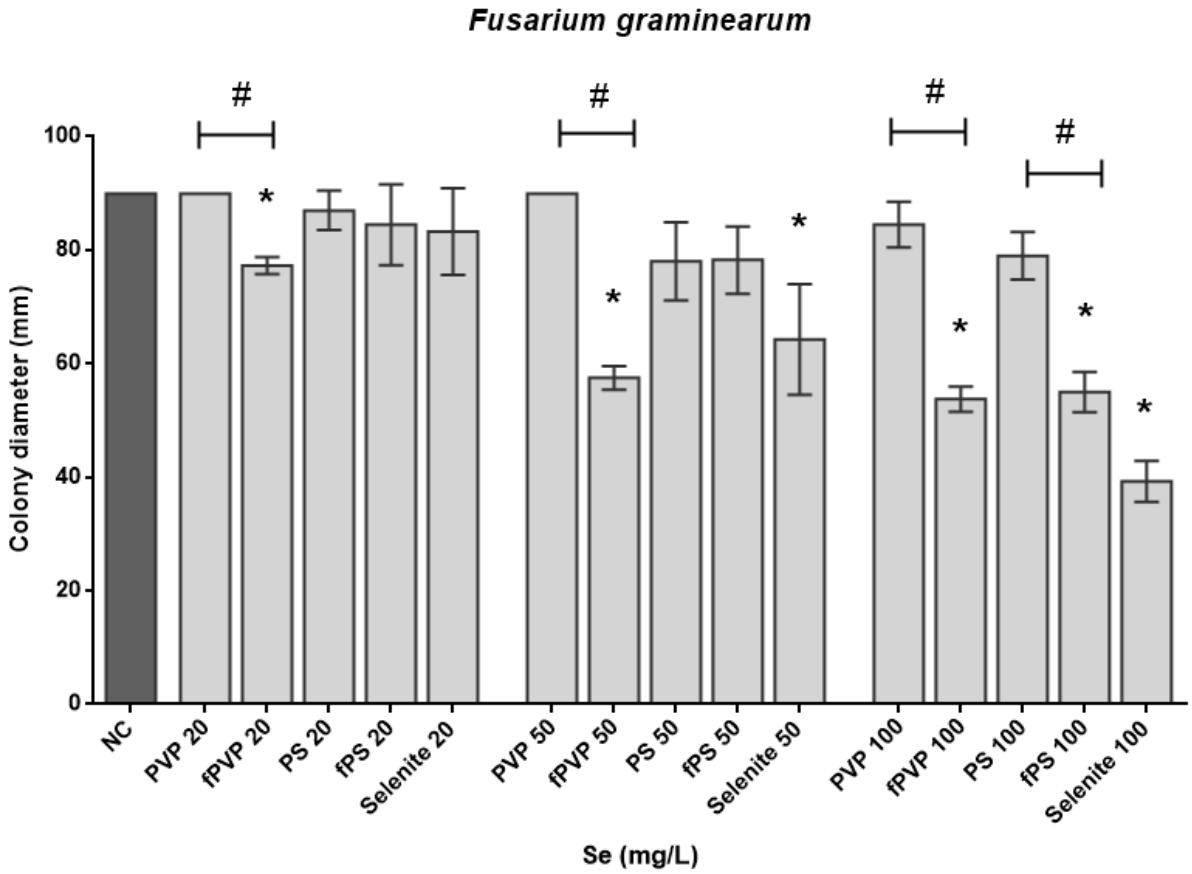

Figure 24. Colony diameters of fungal species *Fusarium graminearum* treated with SeNPs or sodium selenite. The negative controls were non-treated fungi. The asterisk (*) indicates statistically significant differences compared to negative controls ($p < 0.05$) and hashtag (#) indicates statistically significant differences between the same applied concentrations of functionalized SeNPs and their respective nonfunctionalized controls ($p < 0.05$). (NC - negative control cells; PVP - selenium nanoparticles stabilized with polyvinylpyrrolidone; fPVP - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract; PS - selenium nanoparticles stabilized with polysorbate; fPS - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract).

Additionally, reduction in colony diameter was determined for fungi treated with fPSSeNPs (55±3.55) and sodium selenite (39.25 ±3.592), at the same concentration level (100 mg/L).

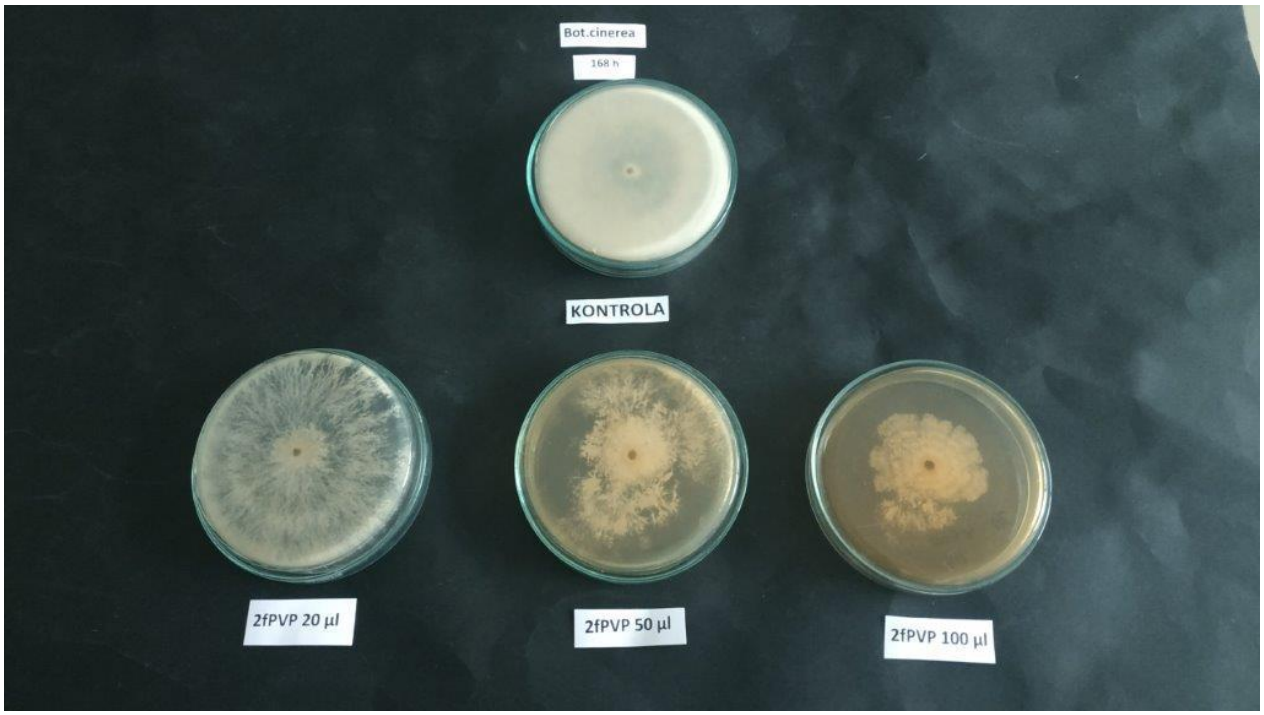

Figure 25. Representation of *in vitro* determination of the antifungal activity of fPVPSeNPs after 168 h treatment, against fungal species *Botrytis cinerea.* The negative controls were non-treated fungi. (fPVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract) (Original photography).

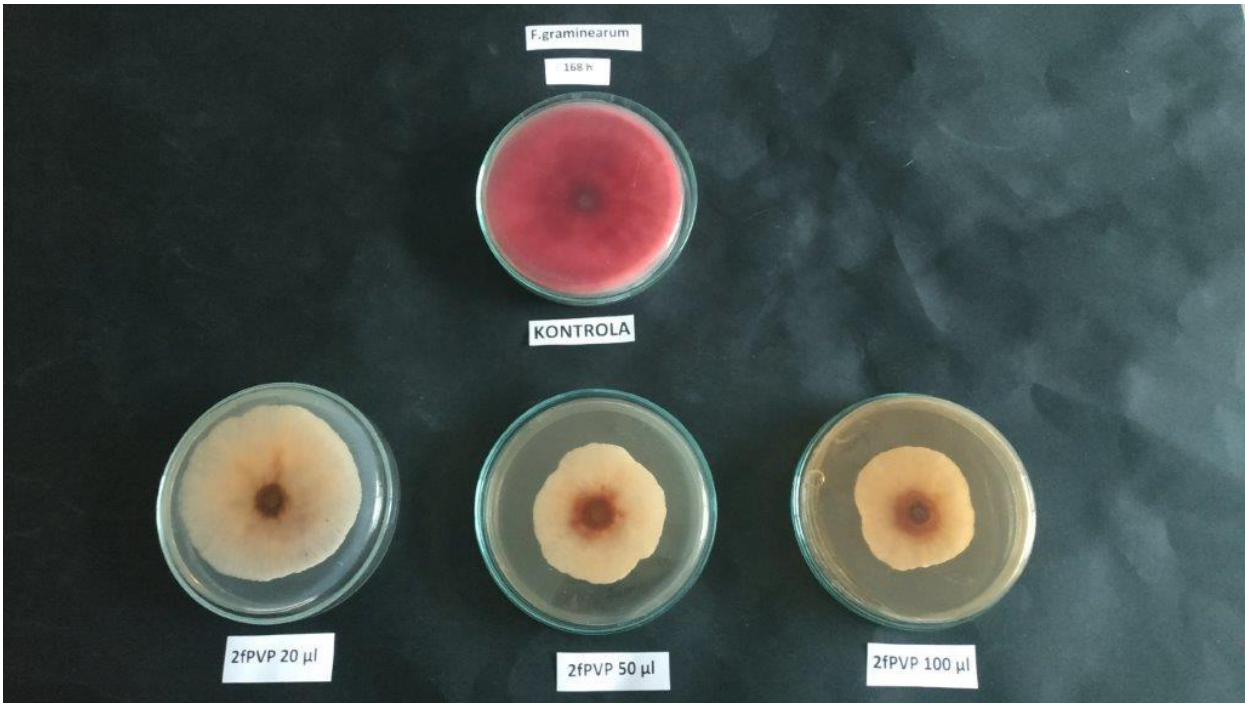

Figure 26. Representation of *in vitro* determination of the antifungal activity of fPVPSeNPs after 168 h treatment, against fungal species *Fusarium graminearum.* The negative controls were non-treated fungi. (fPVPSeNPs - selenium nanoparticles stabilized with polyvinylpyrrolidone and functionalized with olive pomace extract) (Original photography).

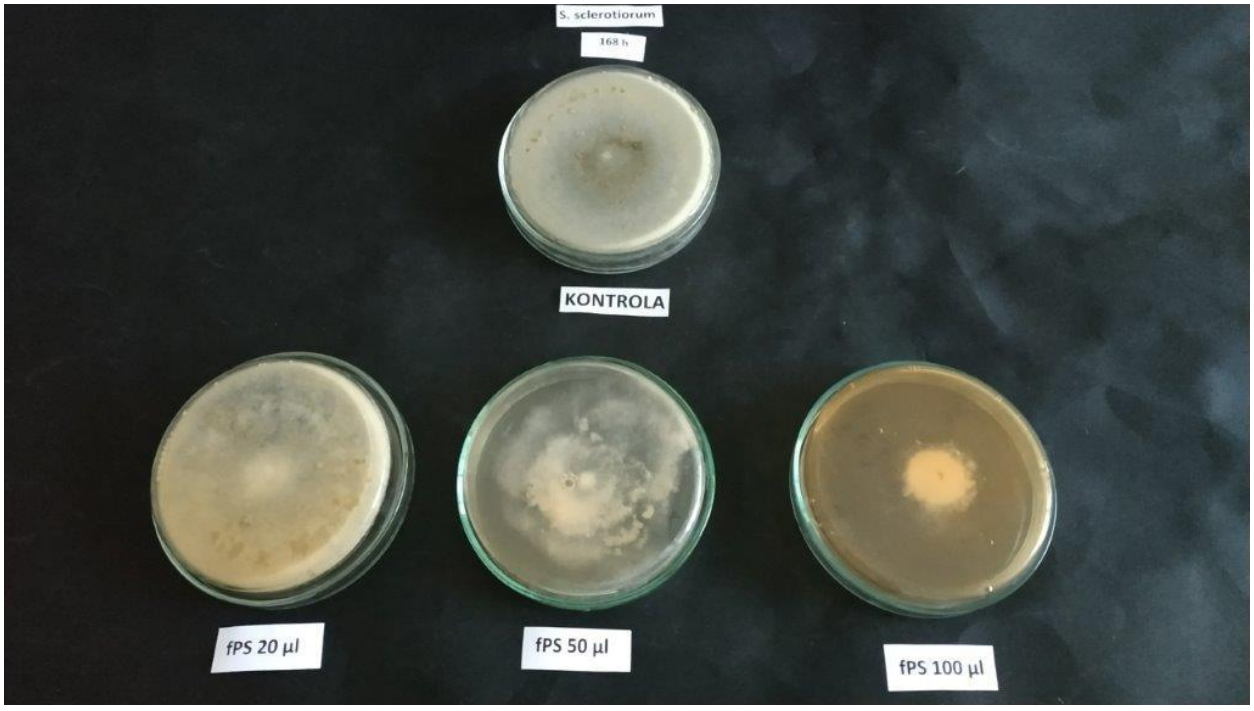

Figure 27. Representation of *in vitro* determination of the antifungal activity of fPSSeNPs after 168 h treatment, against fungal species *Sclerotinia sclerotiorum* (Lib.) de Bary. The negative controls were non-treated fungi. (fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract) (Original photography).

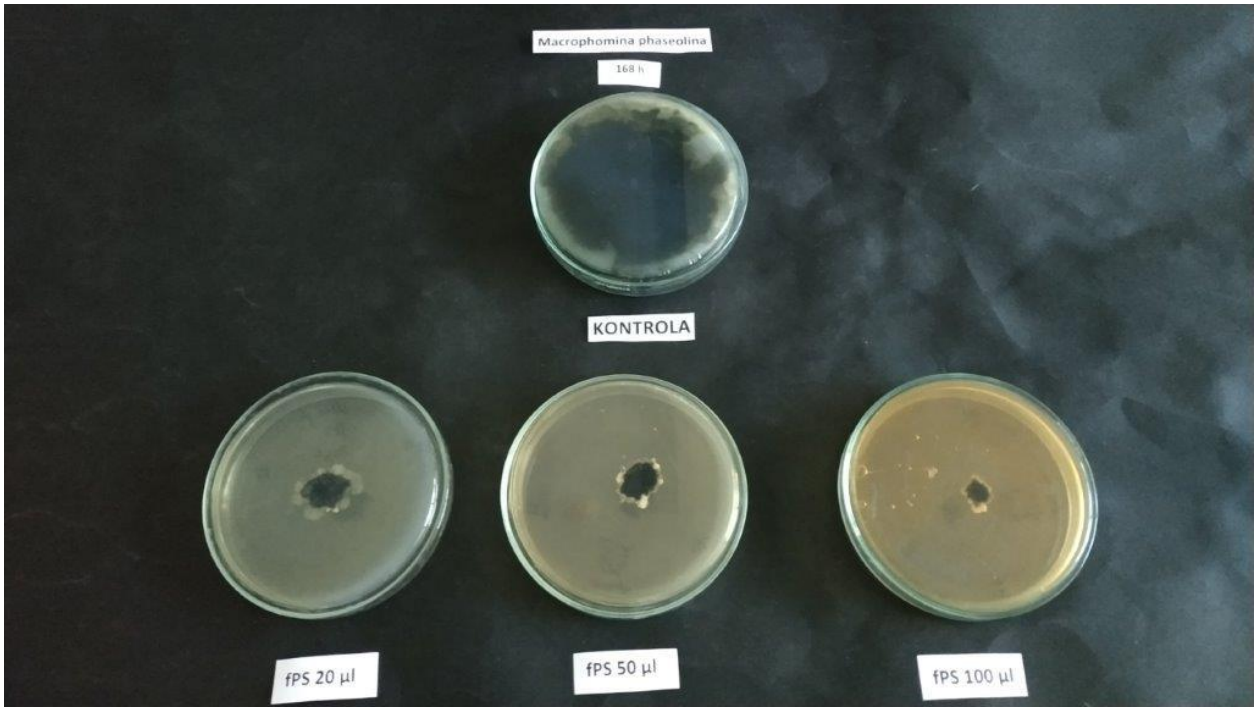

Figure 28. Representation of *in vitro* determination of the antifungal activity of fPSSeNPs after 168 h treatment, against fungal species *Macrophomina phaseolina* (Tassi) Goid. The negative controls were non-treated fungi. (fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract) (Original photography).

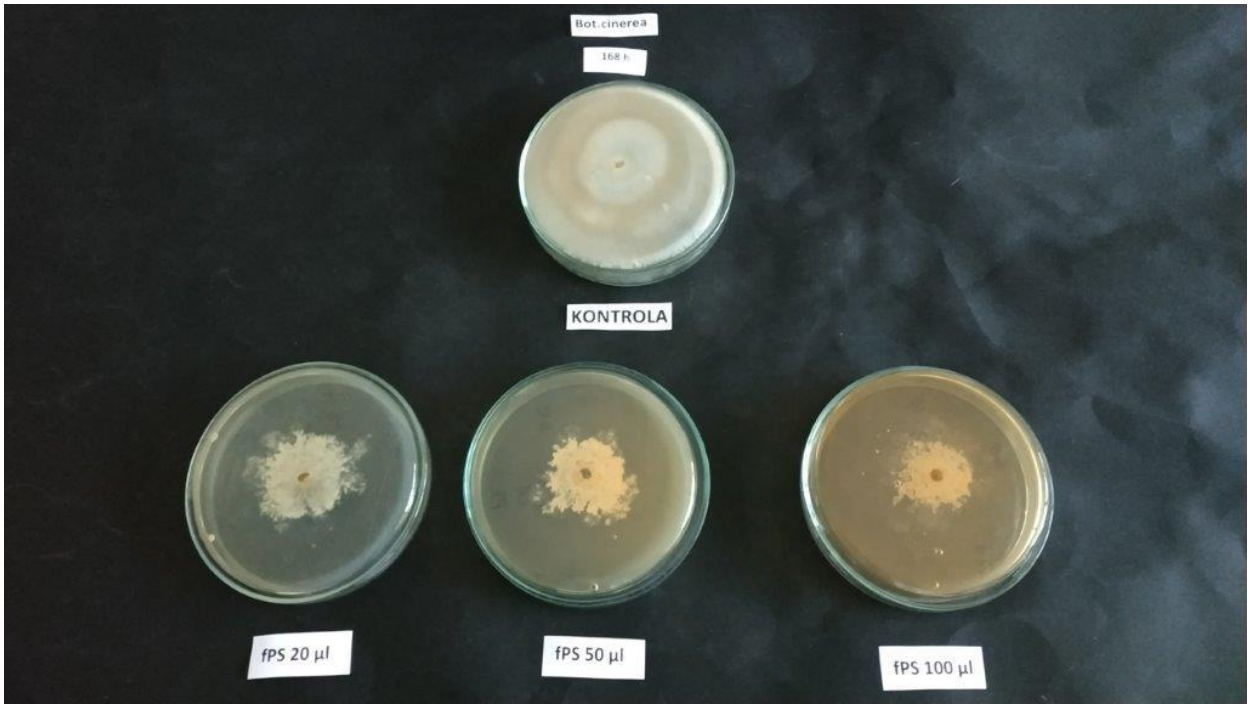

Figure 29. Representation of *in vitro* determination of the antifungal activity of fPSSeNPs after 168 h treatment, against fungal species *Botrytis cinerea.* The negative controls were non-treated fungi. (fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract) (Original photography).

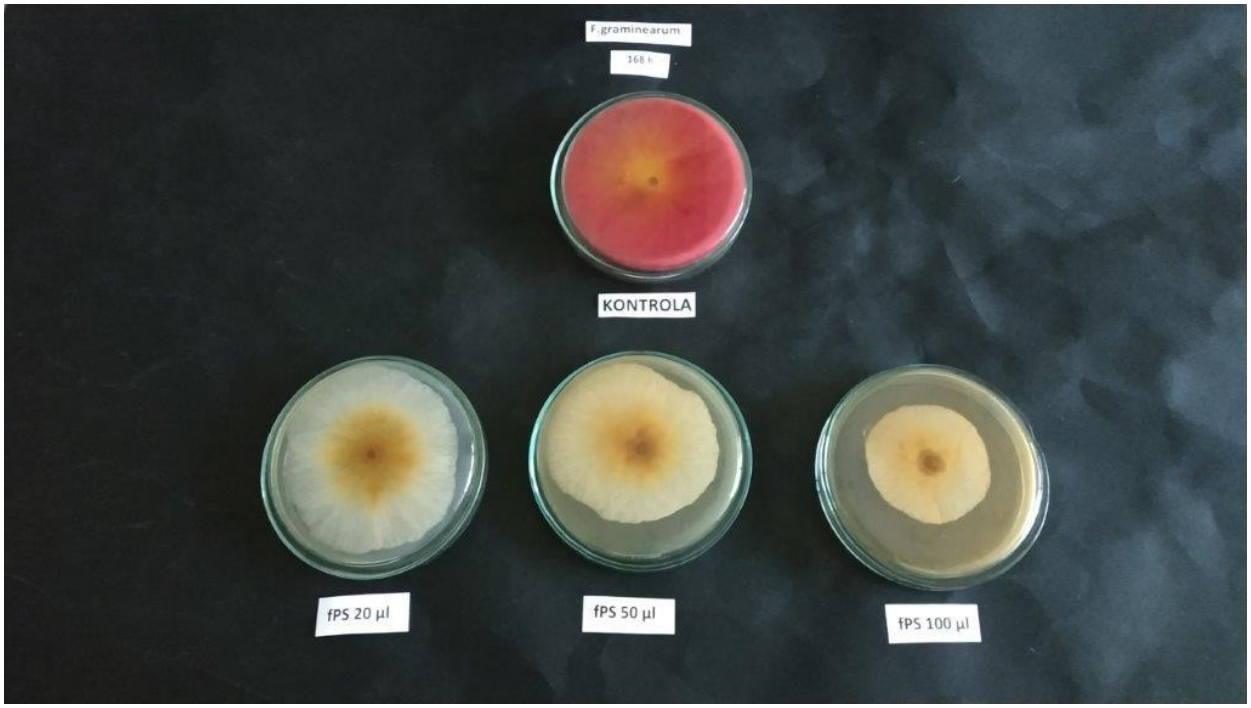

Figure 30. Representation of *in vitro* determination of the antifungal activity of fPSSeNPs after 168 h treatment, against fungal species *Fusarium graminearum.* The negative controls were non-treated fungi. (fPSSeNPs - selenium nanoparticles stabilized with polysorbate and functionalized with olive pomace extract) (Original photography).

4. DISCUSSION

Olive pomace (OP) served as the raw material for obtaining OPE by applying previously optimized microwave assisted extraction (103). OPE was rich in polyphenolic compounds; HTS and OLE were the most represented, while the content of TS was lower. Previous research on the chemical composition of OPE determined a relatively higher content of OLE compared to HTS or TS in raw material (122,123). It is safe to assume that the type of extraction and the choice of solvent significantly impact the chemical composition of OPE (124). Additionally, the chemical composition of OPE can vary in relation to the variable composition of the raw material used in the extraction process which is to a great extent dependent on the olive cultivar, collection season, processing conditions as well as type of extraction used for olive oil production (125,126). As presented, OPE was characterized by high antiradical activity and reducing capacity, consistent with previous investigations showing its applicability as an antioxidant in chemical, biological and complex food systems (127). Due to the mentioned characteristics, it was hypothesized that OPE could be successfully used as the reductant in the phytochemical synthesis of SeNPs, and additionally ensure stabilization and/or functionalization of the nanoparticles surface. Utilization of plant extracts in the phytochemical synthesis of metallic nanoparticles has numerous advantages: they are more eco-friendly when compared to the other methods, cheap and result in the formation of nanoparticles that are usually free from contaminants and are characterized by well-defined morphology and size (128). However, the utilization of OPE did not reduce selenite to elemental Se (0). This was contrary to our expectations; however available literature data on the phytochemical synthesis of metal nanoparticles are mostly focused on Au-, Ag-, Cu- and metal-oxides nanoparticles whereas data on the successful application in the synthesis of SeNPs are scarcer (129). Olive pomace has never been investigated for the phytochemical synthesis of nanoparticles.

Consequently, as previously explained, OPE was combined with L-Ask to ensure adequate sodium selenite reduction. Since it is known that polyphenolic compounds are characterized by π-π interaction; are able to form H-bonds and act as metal chelators

(130,131) it implied possible usage of OPE as a nanoparticle surface stabilizer. The synthesis resulted in the formation of unstable particles prone to agglomeration, with the dH> 1000 nm. Therefore, surface stabilization was achieved by using synthetic coatings (PS or PVP) and OPE was used for NP surface functionalization.

The effect of OPE functionalization was assessed by comparing the physico-chemical characteristics of functionalized nanoparticles- size, shape, zeta potential and direct antioxidant capacity, with their respective non-functionalized pairs. In the case of PVPSeNPs and fPVPSeNPs, it was determined that the functionalization with OPE resulted in decreased dH, as well as lower average values of zeta potential, indicating improved electrostatic stabilization. Overall, PSSeNPs were better stabilized by electrostatic interactions compared to PVPSeNPs, which can be seen from the results obtained for zeta potential. It was observed that fPVPSeNPs had an improved stabilization compared to its non-functionalized control - PVPSeNPs. However, PVPSeNPs are additionally stabilized by steric interactions which cannot be assessed by measuring the zeta potential (132).

A comparison of PSSeNPs and fPSSeNPs, showed smaller dH of functionalized nanoparticles, indicating that the utilization of OPE in the synthesis can result in SeNPs of smaller diameter. Size is one of the key determinants of the biological activity of nanoparticles. It is known that smaller NPs have a larger surface area and are more reactive when interacting with biological systems. Unfortunately, the smaller size can also result in increased toxicity of nano-systems due to its higher rate of cellular internalization (133). On the other hand, larger NPs have reduced toxicity but also reduced beneficial biological activity. Biological barriers in the GI tract, skin, as well as blood-brain barrier and blood-testicular barrier prevent the absorption of NPs to the desired site of action, and more potent absorptive ability was evidenced for NPs of smaller diameter (134). Furthermore, the cellular uptake is largely dependent on nanoparticle size. For example, NPs sized below 200 nm are able to cross the intestinal mucus layer (135). Generally, a decrease in the average diameter of the NPs leads to a higher rate of cellular internalization (136). The size of SeNPs in this work is below 200 nm, which implies their ability to penetrate across intestinal mucus, which can increase their bioavailability.

Obtained values of PI indicate a relatively unimodal particle size distribution in the system. All evaluated SeNPs had a spherical shape that was not affected by OPE functionalization. It is known that different synthesis strategies and conditions can lead the o production of various types of SeNPs, such as spheres, rods, trigonal butterfly-shaped etc. (52). However, the sphere is among the most common shapes of NPs (52,56,109,110, 137). Shape and size are one of critical determinants of nanoparticle interactions with biological systems. A recent study performed by Barbir et al. (2021) demonstrated that the interactions of silver nanoparticles and model protein transferrin were dependent on the size, shape and surface characteristics of the NPs (138). PH of analysed nano-systems indicated slightly alkaline conditions regardless of the synthetic stabilizer or functionalization type. Regarding nutraceutical development, the acidic pH corresponding to the GI tract's physiological conditions would be preferable since the changes in surrounding pH may destabilize the nano-systems (109).

The radical scavenging potential of functionalized SeNPs (fSeNPs) was significantly higher than their corresponding non-functionalized controls. Similar can be observed in the case of total reducing capacity. These results indicate the successful binding of antioxidants from OPE to SeNPs' surface. Since the control SeNPs did not show direct antioxidant activity, fSeNPs can be considered as added-value products. These results are consistent with available literature data reporting on the successful utilization of plant bioactive compounds in SeNPs synthesis to improve their antioxidative properties. One such study demonstrated that glucan and rosmarinic acid stabilized SeNPs improved their antiradical activity (139). Furthermore, SeNPs synthesized using *Embilica officinalis* were effective at free radical scavenging (58), which was also demonstrated in the case of SeNPs generated using *Withania somnifera* extract (140). Surface decoration of SeNPs with pectin resulted in vigorous scavenging activity (141) and a recent study from Golub et al. 2023 demonstrated that pectin and OPE functionalized SeNPs had significant antiradical and reductive activity (142). In this work, pectins were extracted from mandarin pomace, highly valuable agro-waste which is, however, not being exploited. Moreover, polyphenols from olive pomace were evaluated as antioxidants in relevant food models. This approach is an example of how to use agro waste for green, eco-friendly synthesis of valuable nutraceuticals. Another interesting study by Liu et al. (2021) analysed the

polysaccharide derived from the fungi *Oudemansiella radicata* to synthesize SeNPs with improved radical scavenging activity compared to control SeNPs synthesized without the polysaccharide (74).

An important step in the evaluation of the biological activity of novel compounds is their toxicity assessment. Toxic effects are one of the major issues to the broader application of nanoparticles in the pharmacy and food industry, therefore it is imperative to perform a risk assessment of novel products prior to any type of application. Unfortunately, many nano-based products are being used in the industry, even though their toxic potential was not investigated, which poses a significant risk to the safety of consumers (143). As mentioned earlier, oxidative stress is one of the potential hazards associated with the usage of nanoparticles. The excessive generation of free radicals damages biological macromolecules (DNA, proteins, lipids), leading to mitochondrial impairments and cell death. Furthermore it is known that both short-term and prolonged exposure to oxidative stress inducers leads to severe pathological states such as neurodegenerative disease, cardiovascular and many more. (143,144). In this work, two cell lines were selected to evaluate the biological effects of SeNPs. HepG2 cells represent a reliable model to study the toxicological and antioxidative effects of nanoparticles, due to their stable expression of antioxidative enzymes. Caco-2 cells were used to assess the local effects of SeNPs on the intestines, since they are intended to be taken up by oral administration. The cells were treated with a relatively high concentration of SeNPs (above IC_{20}) for 3 h, to evaluate their impact on the short-term redox equilibrium of the cells. Therefore, the relative content of ROS was determined since they are one of the key players in cellular redox physiology (145). Moreover, GSH content was investigated because it is one of the first non-enzymatic lines of defence against oxidation (146). The changes in the content of ROS in the cells treated with SeNPs or selenite were not evidenced, which implies low short-term toxicity of the tested Se formulations, which is in accordance with one of our previous works (110).

GSH content remained unchanged in the HepG2 cells treated with SeNPs, while in the case of Caco-2 cells, only cells treated with PSSeNPs had a reduced level of GSH. Interestingly, the same was not observed in the case of fPSSeNPs indicating that surface

modification of SeNPs using OPE may modulate their prooxidative effects in Caco-2 cells. A previous study performed by Zheng et al. (2012) reported elevated levels of superoxide radicals in HepG2 cells treated with SeNPs (147). Moreover, a study by Guo et al. (2020) investigated *in vivo* effects of the SeNPs and reported a reduction in the levels of antioxidants - GSH, SOD, GPx and catalase (148). Based on available data it can be concluded that the toxic/prooxidative impact of SeNPs is variable and highly dependent on the model system tested as well as on the physicochemical characteristics of the investigated SeNPs. The fact that Caco-2 cells treated with PSSeNPs had reduced content of GSH compared to negative control cells could be explained by their relatively small dH, making them more potent for cellular internalization (44). Furthermore, in a complex environment such as cell culture media and the interior of the cells, it is expected that the SeNPs will be subjected to destabilization and a certain amount of coating can be released from the surface. The polysorbate (Tween 20) is a surfactant that can act disruptively on the cellular membranes and consequently increase their permeability (149,150). Altogether, it may lead to prooxidative effects evidenced in the reduction of GSH. On the other hand, the reduction in GSH content was evidenced in both cell lines treated with sodium selenite, implying increased toxicity of inorganic Se forms. It can be assumed that the GHS was depleted as a response to selenite treatment to protect the cells from oxidative stress induction (which consequently prevented ROS formation). The results are similar to those obtained by previous studies that also reported GSH content disruptions in the HepG2 cells treated with sodium selenite (151).

As stated previously, the functionalization of the SeNPs surface with OPE resulted in the formation of nanosystems with considerable direct radical scavenging and reducing capacity. However, to fully assess its antioxidant potential, it was necessary to evaluate it in biological models. In order to manifest their antioxidative activity the NPs have to be actively internalized in the cells. The activity could then by directly achieved by scavenging the free radicals, or indirectly by modulating the gene expression of cellular antioxidants. Therefore, the observed direct radical scavenging and reducing capacity determined using simple chemical models (TEAC, FC) does not necessarily correlate with

antioxidative activity asses using cell models. Furthermore, the surface of SeNPs is modified by polyphenolic compounds, which can lead to their increased bioavailability. Polyphenols can also act as direct antioxidants as well as regulators of the expression of enzymes involved in the cellular antioxidative response. The described results imply that cellular effects are more complex since many factors determine the outcomes of the treatment.

Concentrations of SeNPs used in the evaluation of antioxidant activity in biological models were lower than previously determined IC_{20}, and the evaluation was performed at two concentration levels. The obtained results indicate that the cells pre-treated with the combination of SeNPs and prooxidant showed significantly higher viability than positive control cells only treated with prooxidant. Interestingly, the protective effect was visible in all the treated cells, independently of the type of SeNPs which does not correspond to the direct antioxidative activity determined by TEAC and FC where only functionalized cells showed the ability to reduce Folin-Ciocalteu reagent or scavenge ABTS radical. It has to be noted that tBOOH is a different species of free radical, and it could possibly be directly inhibited by the SeNPs. Furthermore, the mentioned changes in the expression of cellular antioxidative enzymes could also contribute to its inhibition. It may be presumed that the observed was a combination of both direct scavenging of free radicals by SeNPs and the activity of cellular antioxidative enzymes.

Obtained results differed to a certain extent depending on the cell type investigated. In contrast to results obtained in Caco-2 cells, where the protective effect was not significantly dependent on the applied concentration of SeNPs, in HepG2 cells those treated with a lower concentration of SeNPs were more resilient to prooxidative cytotoxic effects of tBOOH. Similar was observed in one of the previous research performed by Cheng et al. (2017) (152). It can be seen that lowering the applied concentrations of SeNPs can have a beneficial effect on the cells in the context of their resilience to oxidative injury.

In Caco-2 cells, the impact of concentration was observed only in the case of PSSeNPs where the higher concentration applied resulted in a significant decrease in viability. This is in accordance with the described GSH depletion effects (Figure 14) and can again be

explained by lower dH, greater cellular permeability, and potential toxicity of Tween 20. Likewise, toxic effect was reduced by functionalization since fPSSeNPs were not toxic at the higher concentration level. The protective effects of SeNPs against oxidative injury were previously demonstrated by Xu et al. (2018) who also evaluated the outcomes of the treatment of colon epithelial cells with biogenically synthesized SeNPs against oxidative damage (153). The described implies that SeNPs could act beneficially on GI tract by preventing or decreasing oxidative stress induction.

Contrary to our expectations, both HepG2 and Caco-2 cells were also protected against tBOOH induced toxicity by the addition of sodium selenite too. The observed effect could be explained by the ability of sodium selenite to scavenge tBOOH radical; namely, as described in the protocol, the tBOOH has been added directly to the culture media containing SeNPs or sodium selenite, enabling therefore, their direct interaction. This observation is consistent with an interesting study performed by Thiry et al. (2013) that demonstrated that the majority of selenite added to the culture media is not absorbed by Caco-2 cells and is therefore available for reaction with tBOOH (154).

To further investigate the impact of SeNPs, another parameter evaluated in this work was the level of cellular ROS. As previously mentioned, the ROS content was assessed by measuring the fluorescence of fluorophore (DCF) which specifically reacts with ROS. The protocol included the removal of the pre-treatment solution which was followed by incubation of the cells with prooxidant (tBOOH). Therefore, any observed effect is exclusively the result of cellular response to prooxidant exposure. Thus, different results indicate that cellular response to oxidative stress has been affected by the exposure of the cell line to the tested compound during pre-treatment. Again, the observed response in both cell lines was concentration dependent. In the HepG2 case the cells treated with higher concentrations of SeNPs had lower content of ROS. It can be assumed that increased concentrations of SeNPs resulted in higher cellular uptake which in turn resulted in the protective effect. Treatment with selenite did not have any protective effect. Results for Caco-2 cells show that cells treated with SeNPs, again with the exception of fPVPSeNPs, had a reduced content of ROS, however in the case of lower concentration of nanoparticle treatment a protective effect can be explained by the surface morphology

of Caco-2 cells. This cell line differentiates to enterocytes characterized by microvilli (155) which increase the surface of the cells and lead to more efficient internalization of surrounding particles, which could be responsible for the demonstrated effect.

The final parameter evaluated with a broader goal to assess the antioxidative effect of SeNPs was the relative content of GSH. The GSH content in HepG2 cells treated with PVP SeNPs and fPVP SeNPs was significantly higher compared to positive controls. The same was not observed in the case of PS SeNPs, fPS SeNPs or selenite. However, no clear pattern could be demonstrated in the case of HepG2 cells. In Caco-2 cells, the results are in accordance with ROS content. Again, the cells treated with a lower concentration of SeNPs had an increased GSH content compared to cells treated with tBOOH. The mentioned was not evidenced in the case of fPS SeNPs. Taken together, the results on antioxidative effects are in accordance with one of the previous studies that demonstrated that ROS content was decreased in the cells incubated with SeNPs due to treatments with the prooxidant (73). Furthermore, Song et al. (2017) have concluded that the described effect can be attributed to differential expression of the Nrf2 transcription factor and its downstream genes (156).

The next goal of this study was to evaluate the antifungal activity of SeNPs. It was previously reported that both SeNPs and polyphenols possess antimicrobial properties. This work showed that SeNPs functionalized with extract rich in polyphenols brought significantly improved activity against common fungal plant pathogens. It is known that surface molecules significantly impact the biological activity of SeNPs. PSSeNPs had a more pronounced activity compared to PVPSeNPs, which can be explained by their smaller diameter and more potent penetration in the cells, as mentioned before. OPE functionalization improved the activity of chemically synthetized SeNPs in all evaluated species of fungi, which can be attributed to the antimicrobial properties of polyphenols (102). Furthermore, in one of the previous studies, biogenically synthetized SeNPs from *Lactobacillus acidophilus* ML14 were potent in inhibiting the growth of *Fusarium culmorum* and *Fusarium graminearum* (157), which is similar to the result obtained in this work. It can be speculated that SeNPs can be effective in the prevention of diseases caused by *Fusarium* species such as wheat crown and root rot disease. A study performed by

Fardsadegh et al. (2019) reported *in vitro* antimicrobial activity of SeNPs synthesized using *Aloe vera* leaf extract against *Penicillium digitatum*, fungal species responsible for spoilage of orange fruit (158). It is therefore evident that SeNPs have considerable antifungal activity and could be used in the future as biocompatible pesticides. It was expected that the functionalization of SeNPs with olive polyphenols would further improve the inhibitory effect on fungal growth. The effect was specifically linked to low molecular polyphenols such as HTS and TS (159), which were abundant in OPE used in this work. Previous research found that polyphenols derived from olives can be used against *Botrytis cinerea* mold in strawberries and red peppers (102). Another study also evidenced *in vitro* antifungal effects of olive polyphenols on *Sclerotinia sclerotiorum* (160). Therefore, the joint action of olive polyphenols and SeNPs resulted in considerable antifungal effects, which is in accordance with reporters from the literature. The next step in the research of SeNPs obtained in this work should be focused on the direct application of SeNPs on infected plants to verify their efficiency in natural environment. The usage of nanomaterials including SeNPs is in the rise all over the planet. However, even though SeNPs show increased biocompatibility, their effects on the ecological systems are poorly studied. They can be toxic to naturally occurring bacteria, fresh water crustaceans as well as marine diatoms, under in vitro condition (109,161). Se compounds, including the nanosized particles, can also distrust oxidative metabolism in fish (162). Therefore, more studies dealing with human risk assessment and ecotoxicological assessments are necessary (163). Furthermore, if SeNPs were to be used as pesticides in the future, great concern has to be taken to assess the amount of Se that will end up in the food products, since Se is biologically active and its effects are highly dependent on the applied concentrations.

5. CONCLUSION

In this work, the synthesis procedure of SeNPs using OPE was optimized. The functionalization of SeNPs with OPE resulted in the synthesis of nanoparticles of satisfactory physicochemical characteristics. The functionalization of SeNPs had several important effects as follows:

1. The functionalized SeNPs had significantly higher direct antiradical and reductive capacity compared to control SeNPs.
2. Treatment with SeNPs did not induce short term oxidative stress in the treated cells, with the exception of PSSeNPs.
3. Functionalization with OPE reduced the toxicological effect of PSSeNPs in the context of GSH depletion.
4. SeNPs had a protective effect against prooxidation in HepG2 and Caco-2 cells where the effects were not solely dependent on the functionalization of SeNPs, indicating the complexity of involved factors involved in the antioxidative response.
5. The antifungal activity of SeNPs was significantly improved by functionalization, evidenced in all fungal pathogens used in this study.

Protective effects of SeNPs in tested cells were contributed to both direct scavenging of prooxidants by SeNPs and an indirect effect via the expression of antioxidative enzymes. It was shown that ROS and GSH are important factors in determining the overall outcomes of treatment with SeNPs. Future research should be focused on assessing the long-term toxicity of SeNPs, which should be investigated using also *in vivo* models, as well as human subjects.

Considering the antifungal effects, SeNPs and different functionalization should be further investigated. However, according to the results of this research, SeNPs possess a great potential in plant protection leading to sustainable and environmentally friendly agricultural production.

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7. SUMMARY

Selenium (Se) is an essential micronutrient for humans and animals. It is an essential compound necessary for normal physiological processes in the human organism. Low concentrations of Se in the soil in some geographical regions lead to inadequate accumulation of Se in the food chain and consequently in human nutrition. Insufficient nutritional intake of Se is common and it has been linked to various pathological states, ranging from cancer and thyroid disease to immunological and cognitive decline. Se intake can be improved by using Se-rich food or supplementation. Due to the increased demand for supplementation and the narrow therapeutic range of Se in conventional formulations, novel formulations containing Se in nano form have been investigated and evidenced to exert improved biocompatibility and bioavailability compared to other Se forms.

Therefore, the aim of this work was to investigate the physico-chemical properties, toxicity and biological effects (antioxidative efficiency and antifungal properties) of novel selenium nanoparticles (SeNPs). For this purpose, the green procedure for obtaining SeNPs functionalized with olive pomace extract (OPE) rich in polyphenolic compounds has been developed. After the optimization of the synthesis process, the size, shape and zeta potential (as a measure of nanoparticle stability) of novel nanosystems were evaluated. The functionalization resulted in improved stabilization and reduced size of SeNPs. Moreover, functionalized SeNPs (fSeNPs) were characterized by stronger direct antiradical and reductive capacity which can probably be attributed to the activity of surface OPE-derived polyphenols. The toxicity and antioxidative activity of SeNPs and fSeNPs were investigated in HepG2 and Caco-2 cell lines. HepG2 cells represent a valuable model due to the stable expression of various antioxidative enzymes, while Caco-2 cells enable the assessment of the local effects of compounds intended for oral application. Functionalization using OPE prevented the increase of the content of ROS, an indicator of oxidative stress, in Caco-2 cells. The levels of GSH in both cell lines treated with SeNPS remained unchanged, indicating the low toxic effect of the tested nanoparticles. The cellular antioxidative activity was evaluated by investigating the

protective effects of SeNPs against prooxidant tert-butyl hydroperoxide (tBOOH). It was evidenced that HepG2 and Caco-2 cells treated with SeNPs had increased viability and reduced content of ROS in response to tBOOH, compared to cells not treated with SeNPs. The effects were dependent on the applied concentration of SeNPs as well as their identity. The protective activity was positively influenced by the surface functionalization of SeNPs and observed effects were attributed to the joint action of direct radical scavenging activity and effects on the cellular response (in the form of modulation of its antioxidative defense system). *In vitro*, antifungal effects of SeNPs were investigated against common plant pathogens *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea*, *Macrophomina phaseolina (Tassi) Goid* and *Fusarium graminearum.* Results showed that surface functionalization of SeNPs with OPE significantly enhanced the antifungal activity against all analyzed fungal species. The results imply that this type of SeNPs could be potentially used as a novel type of biopesticides. Future research should be focused on the evaluation of the biological effects of nanoparticles in relevant food models as well as in suitable *in vivo* models.

8. SAŽETAK

Uvod

Selen (Se) je esencijalni mikroelement nužan za normalnu funkciju organizma. Niske koncentracije Se u tlima rezultiraju nedovoljnom akumulacijom ovog nutrijenta u hranidbenom lancu i posljedično u ljudskoj prehrani. Nedostatak Se u prehrani je uobičajen i povezuje se s pojavnošću raznih bolesti kao što su bolesti štitne žlijezde, kardiovaskularnog i imunološkog sustava te neurodegeneracije. Potreba za nadomjeskom Selena je sve veća zbog čega se razvijaju novi oblici hrane i dodataka prehrani obogaćenih selenom. Konvencionalni oblici Se koji se primjenjuju kao dodaci prehrani imaju usku primjenu. Međutim, recentna istraživanja pokazuju da je Se u nano obliku karakteriziran povećanom biodostupnošću i biokompatibilnošću u usporedbi s drugim oblicima. Primjena nanočestica selena (SeNPs) se intenzivno istražuju u biomedicini, farmaciji i ljudskoj prehrani zahvaljujući mogućnosti funkcionalizacije njihove površine. Pokazano je da je vezanje bioaktivnih tvari na površinu nanočestice rezultiralo u njihovoj efikasnijoj isporuci te povećanoj biološkoj aktivnosti. Komina masline je otpad zaostao nakon proizvodnje maslinovog ulja te je izrazito o bogat polifenolnim spojevima koji mogu djelovati pozitivno na ljudsko zdravlje. Polifenoli, između ostalog imaju izraženo antioksidacijsko i antimikrobno djelovanje. Moguće je primjenom odgovarajućih tehnika ekstrahirati vrijedne polifenolne spojeve iz komine masline te ih koristiti u proizvodnji novih oblika nutraceutika s ciljem poboljšanja ljudskog zdravlja. Cilj ovog rada je bio istražiti fizikalno-kemijske karakteristike, toksičnost i biološku aktivnost (antioksidacijsku aktivnost i antifungalnu aktivnost) novih oblika SeNPs dobivenih korištenjem ekstrakta komine masline (EKM) bogatog polifenolima u svrhu funkcionalizacije površine nanočestica.

Materijali i metode

Nakon optimizacije sinteze i pročišćavanja novih oblika nanočestica, njihova veličina je istražena tehnikom dinamičkog raspršenja svijetlosti, dok je zeta potencijal (kao mjera stabilnosti) istražena tehnikom elektroforetskog raspršenja svijetlosti. U svrhu procjene oblika nanočestica, vizualizirane su tehnikom transmisijske elektronske mikroskopije. Direktni antioksidacijski kapacitet je istražen pomoću *Trolox Equivalent Antioxidant*

Capacity (TEAC*)* eseja, dok je ukupni redukcijski potencijal određen korištenjem Folin-Ciocalteu reagensa. Toksičnost i biološka aktivnost je istražena *in vitro* korištenjem dva stanična modela – HepG2 i Caco-2. HepG2 su stanična linija ljudskog hepatocelularnog karcinoma i predstavljaju pouzdan model za ovakav tip istraživanja, budući imaju stabilnu ekspresiju antioksidacijskih enzima. Caco-2, stanična linija ljudskog kolorektalnog adenokarcinoma predstavlja model tankog crijeva, prema tome je moguće istražiti lokalni utjecaj spojeva koji bi se primjenjivali oralnim putem. Procjena toksičnog učinka je bila fokusirana na određivanje sadržaja reaktivnih kisikovih vrsta (ROS) pomoću 2,7 - diklorofluorescein diacetat (DCFH-DA) eseja, te sadržaja reduciranog glutationa (GSH) pomoću monoklorobiman (mCBL) eseja. Antioksidacijski (protektivni) učinak je mjeren nakon tretmana stanica sa SeNPs i prooksidansom terc-butil hydroperoksidom (tBOOH). Određivana je vijabilnost stanica [3-(4,5-Dimetiltiazol-2-il)-2,5-Difeniltetrazolij Bromid] (MTT) esejem te sadržaj ROS-a i GSH prethodno spomenutim esejima. Antifungalna aktivnost SeNPs je istražena *in vitro* i to na biljnim patogenima kako slijedi: *Sclerotinia sclerotiorum* (Lib.) de Bary*, Botrytis cinerea, Macrophomina phaseolina* (Tassi) Goid i *Fusarium graminearum*, tako da je promjer kolonija (kao mjera rasta gljiva) mjeren 168 sati nakon tretmana sa SeNPs.

Rezultati

Pokazano je da je funkcionalizacija pozitivno utjecala na stabilnost nanočestica te na smanjenje njihove veličine i da nije utjecala na njihov oblik. SeNPs funkcionalizirane pomoću EKM (fSeNPs) su imale značajno pojačan antioksidacijski kapacitet i ukupni redukcijski potencijal. Nadalje, SeNPs testirane u ovom radu nisu pokazale značajnu toksičan učinak u kontekstu promjene sadržaja ROS-a i GSH, s izuzetkom Caco-2 stanica tretiranih s PSSeNPs. Također, SeNPs su imale slabiji toksičan učinak od anorganskog oblika Se - natrijevog selenita. Antioksidacijski (protektivni) učinak SeNPs na stanice tretirane prooksidansom terc-butil hidroperoksidom je pokazan za sve tipove nanočestica (funkcionalizirane i nefunckionalizirane). Dokazano je protektivno djelovanje na vijabilnost, sadržaj ROS-a i sadržaj GSH. Efekt je bio izraženiji u HepG2 stanica. Rezultati su pokazali da su fSeNPs imale značajno jači antifungalni učinak u usporedbi s nefunkcionaliziranim SeNPs, prema svim vrstama analiziranih gljiva. Efekt je bio vrlo

izražen u vrsta *Botrytis cinerea, Macrophomina phaseolina (Tassi) Goid* i *Fusarium graminearum,* a manje u vrste *Sclerotinia sclerotiorum (Lib.) de Bary.*

Rasprava

OPE bogat polifenolima je uspješno korišten u sintezi SeNPs radi poboljšanja njihovih fizikalno-kemijskih karakteristika i bioloških učinaka. Ovaj pristup može dovesti do poboljšanih oblika SeNPs, ali i povećanja nisko biodostupnih polifenola. Poboljšanje stabilnosti SeNPs je moguće pripisati dobroj stabilizaciji polifenolima budući su oni sposobni stvarati vodikove veze, kelirati metale te su karakterizirani π-π interakcijama. Veličine svih tipova nanočestica u ovom radu su bile manje od 200 nm, što omogućuje relativno dobru biodostupnost. Pojačan antioksidacijski i redukcijski kapacitet fSeNPs je vjerojatno rezultat aktivnosti polifenola vezanih na površinu nanočestica budući je poznato je da polifenoli imaju snažan antioksidacijski potencijal. Nadalje, SeNPs nisu pokazale značajan toksičan utjecaj na stanice. U slučaju tretmana Caco-2 stanica s SeNPs stabiliziranih polisorbatom 20 (PSSeNPs) je utvrđeno smanjenje sadržaja GSH, što nije primijećeno u slučaju funckionaliziranog oblika (fSeNPs), što može upućivati na to da polifenoli komine masline mogu pozitivno utjecati na biokompatibilnost SeNPs. Antioksidacijski učinak nanočestica na stanične modele je utvrđen za sve tipove nanočestica (funkcionalizirane i nefunckionalizirane). Moguće je pretpostaviti da je učinak bio kombinacija direktnog gašenja prooksidansa od strane SeNPs i pojačanog staničnog antioksidacijskog odgovora kao odgovor na tretman s SeNPs. Funkcionalizacija korištenjem OPE je značajno doprinijela antifungalnoj aktivnosti SeNPs. Spomenuti rezultati su bili očekivani budući da i SeNPs i polifenoli masline imaju izraženo antimikrobno djelovanje. Iz literature je poznato da je aktivnost polifenola masline je polifenoli niske molekularne mase, kao što su tirozol i hidroksitirozol za koje je utvrđeno da su prisutni u EKM korištenom u ovom radu, odgovorni za antimikrobni učinak. Moguće je pretpostaviti da su inhibicija rasta gljiva rezultat zajedničkog djelovanja samih SeNPs i površinskih polifenola. Opisano upućuje na moguće korištenje ovih tipova nanočestica kao novi oblik biopesticida.

Zaključci

EKM je korišten u sintezi SeNPs koji su imale zadovoljavajuće fizikalno-kemijske karakteristike i izražen antioksidacijski i redukcijski potencijal. Testirane SeNPs nisu imale značajan toksičan učinak. Antioksidacijski (protektivni) učinak na stanice je pokazan za sve tipove nanočestica korištenih u ovom radu. Funkcionalizacija je značajno pojačala antifungalni učinak SeNPs. Sljedeća istraživanja bi trebala biti usredotočena na procjenu toksičnosti i bioloških učinaka dugoročne izloženosti selenovim nanočesticama. U tu svrhu, u istraživanjima bi trebalo bi koristiti relevantne modele ishrane te prikladne *in vitro* i *in vivo* modele.

9. CURRICULUM VITAE

Emerik Galić was born in 1992. in Našice, Croatia. After finishing High school in Osijek, he enrolled in Bachelor program in biology at Department of Biology, Josip Juraj Strossmayer University of Osijek. He obtained his bachelor degree in 2016. and the same year he enrolled in master program of molecular biology at Faculty of Science, University of Zagreb. He obtained his mater degree in 2019. The same year he was employed as a research assistant at Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek. In 2020. He enrolled in University Postgraduate Interdisciplinary Study of Molecular Biosciences Josip Juraj Strossmayer University of Osijek. During his work, he was granted with STSM scholarship on two occasions, during which he was trained in the expertise of stem cell maintenance at The Institute of Biomedical Research of Bellvitge (IDIBELL) in Barcelona, in a total time of more than 2 months. He also received an Erasmus+ scholarship for 1- month training at Polytechnical University of Catalonia in Barcelona. He was also awarded with NAWA scholarship of Polish National Agency for Academic exchange, during which he was a guest researcher for 3 weeks at the University of Rzeszów, Poland. He also received training on cell culture bioreactors, imaging mass spectrometry and experimental animal maintenance. Emerik Galić collaborated with researchers from Institute for Medical Research and Occupational Health Zagreb, University Hospital Centre Osijek and Faculty of Pharmacy and Biochemistry, University of Zagreb which resulted in joint publications. He co-authored 8 scientific publications (WoS database) of which 2 publications as the first author.

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