Physics is an ever young science, Varna, October, 27 – 29, 2017
Физиката – вечно млада наука, Варна, 27 – 29 октомври 2017 г.

IN VITRO EVALUATION OF THE ANTIOXIDANT PROPERTIES OF OLIVE LEAF EXTRACTS – CAPSULES VERSUS POWDER

Hugo Saint-James, Gergana Bekova, Zhanina Guberkova, Nadya Hristova-Avakumova, Liliya Atanasova, Svobodan Alexandrov, Trayko Traykov, Vera Hadjimitova
Medical University of Sofia (Bulgaria)

Abstract. The olive leaf extract has been known for its healing abilities since ancient times, when it was used to brew tea and to treat multiple conditions. In modern times it is advertised as a natural source of antioxidants which have a positive effect on human health. The extract is offered on the market as a food supplement by many different manufacturers and can be found in either capsulated or bulk form. The purpose of this experiment is to compare the antioxidant effect of the extract in relation to the form in which it is. The effect was tested in two separate systems for evaluation of total antioxidant activity - ABTS and DPPH. It was established that the extract marketed in capsulated form has better antioxidant properties than the bulk form. The tests in biologically relevant systems showed that while both forms have strong antioxidant abilities, the capsulated form achieves the same biological effect in smaller doses of the extract. The carried out experiments in all tested systems proved that the antioxidant effect of the capsulated form is stronger than the bulk one. The difference in antioxidant activity is most likely due to the protective properties of the capsule.

Keywords: antioxidant; olive leaves extract; free radicals; chemiluminescence

Introduction. The olive trees have been cultivated for thousands of years. Nowadays this long-lived species is being spread worldwide due to the fact its edible fruits and products represent an indispensable part of the Mediterranean diet. During the years, olive trees were a symbol of friendship and peace, associated with social and religious ceremonies. Numerous performed investigations have proved that olive products are powerful tools with a valuable effect on human health (Soni et al, 2006; El & Karakaya, 2009).

According to the pharmaceutical companies and the leading supplement manufacturers olive leaf extracts possess blood pressure-lowering effects and strengthen and support the cardiovascular and immune system as well as reduce the cellular levels of free radicals. One of the reasons for these proven health benefits is the high content of compounds possessing antioxidant properties like polyphenols which have the capability to influence the oxidative stress processes *in vivo* (Soni et al, 2006). The performed experiments concerning the evaluation of the antioxidant properties of different plant extracts using the ORAC methods revealed that the olive leaves extract have better antioxidant potential compared to green tea and grape seed extracts[1)].

The numerous performed experiments have helped to detect the main constituents responsible for the antioxidant properties. Their differential antioxidant activities have been defined by the extent of their abilities to scavenge free radicals and reactive oxygen species. Among them are different types of polyphenols. Oleuropeinand hydroxytyrosol have, for a long time, been recognized as the most potent antioxidants (Benavente-García et al., 2000). The described in the literatures experiments investigating the biological activity of extracts rich of oleuropeinand hydroxytyrosol have proved that they possess antidiabetic and antimicrobial potency in animal experimental models. The observed pharmacological properties correlate with amelioration of the antioxidant status in the tested animals (Jemai et al., 2008; 2009; Pereira et al., 2007). Several studied have proved an antitumor effect in reduction of the progression of hormone related cancers (Boss et al. 2016; Samet et al. 2014).

Due to the increase in interest in the past decades in the possible medical application of compounds derives from natural sources and the attention paid to olive leaves tea used for medical purposes numerous supplements including olive leaves extracts have been developed (liquid or solid extracts). The term “olive leaves” refers to a mixture of leaves and branches from both the pruning of the olive trees and the harvesting and cleaning of olives (El & Karakaya, 2009). The facts that these are considered as a cheap raw material, are byproducts obtained during olive oil production and contain 100 times more polyphenols compared to olive oil has made them a preferred source of natural bioantioxidants. The standardization of the available on the market olive leaf extract powder is based on their oleuropein content which is used as a marker for the strength of the nutritional product.

The aim of the present investigation is to perform a comparative evaluation of the antioxidant potency of Olive leaf extracts containing the same amount of oleuropeinbut in different dosage forms - capsules and powder. The tested products are available as supplements on free market[2)].

Material and methods. The *in vitro* evaluation of the antioxidant properties was performed using chemiluminescent and spectrophotometric model systems. The chemical and solvents used in the performed experiments were of analytical grade and were purchased from Sigma-Aldrich.

The chemiluminescent evaluation was performed by determining the luminol-dependent chemiluminescence in a system of NaOCl generated hypochlorite (Assay I) and horse-radish peroxidase – H_2O_2 containing system (Assay II) (Hadjimitova et al., 2002). The experiments were performed using an LKB 1251 luminometer (BioOrbit, Turku, Finland) connected with an AT-type computer via serial interface. The chemiluminescent response was determined via calculating the area under the obtained chemiluminescent light curves (CL - integral). The CL ratio in the presence and in the absence (control sample) of the tested compound was termed chemiluminescence scavenging index (CL-SI).

$$CL - SI, \% = \frac{CL\ integral_{probe}}{CL\ integral_{control}}.100\%$$

Assay I - luminol-dependent CL in a system of NaOCl-generated hypochlorite

The experiments were performed using one milliliter samples of 50 mmol/l PBS buffer, pH 7.4, containing 0.1 mM luminol, 0.06 mM NaOCl and either of the studied olive extracts or buffer for control. The chemiluminescence was registered for 1 min. every 50 milliseconds after the addition of NaOCl.

Assay II - luminol-dependent CL in a system HRP-H_2O_2

The assay was carried out using one ml samples of 50 mmol/l PBS buffer, pH 7.4, contains 0.1 mM luminol, 50 μg/ml horseradish peroxidase (HRP), 1mM H_2O_2 and the tested olive extracts or buffer for the controls. The reaction was started by adding of 50 μl H_2O_2 (1 mmol/l). The CL was measured for 30 min.

The spectrophotometric evaluation of the antioxidant properties was performed using Shimadzu UV 500 Spectrophotometer for the absorption measurements. The studied properties were evaluated in two systems containing stable free radicals (ABTS and DPPH) and one comprising the estimation effect against UV induced free radical damage of biologically relevant molecule (deoxyribose).

Assay III – ABTS radicals scavenging activity

The procedure followed the method as described by Re et al. (1999). The $ABTS^{\bullet+}$ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) working solution was

prepared by the mixing of 14 mM ABTS stock solutions with potassium persulfate 2.45 mM (final concentration). The $ABTS^{\bullet+}$ was mixed with PBS to obtain final solution with absorbance 0.70 ± 0.01 units at 734 nm. The reduction in absorbance of 2 ml of $ABTS^{\bullet+}$ after adding the extracts was recorded at 734 nm exactly 60 min after the mixing. Fresh $ABTS^{\bullet+}$solution was prepared for each assay.

Assay IV – DPPH radicals scavenging activity

The DPPH (1,1-diphenyl-2-picrul-hydrazil) free radical-scavenging activity was determined according to Goupy et al., (2003) $DPPH^{\bullet}$ solution in ethanol was prepared with initial optical absorbance of 1.0 at 518 nm. Two mL of $DPPH^{\bullet}$ solution was allowed to react with the extracts. The probes were left to stay at room temperature with no exposure to light. After 1 hour of incubation, the absorbance of the solution was measured at 518 nm.

The antioxidant activity was calculated by the formula:

$$AOA\% = \frac{Abs_{control} - Abs_{probe}}{Abs_{control}}.100\%$$

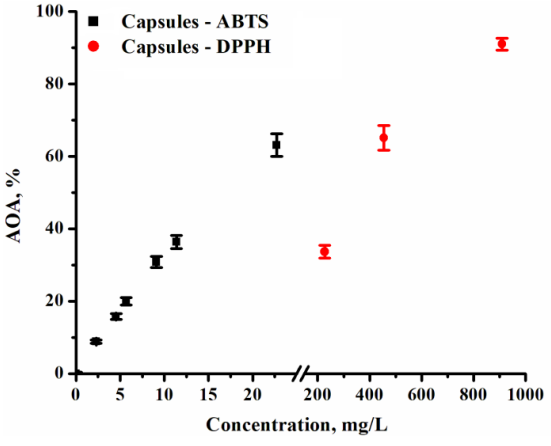
The concentration that corresponds to 50% decreasing of radicals was termed C-50.

Assay V – UV induced deoxyribose damage

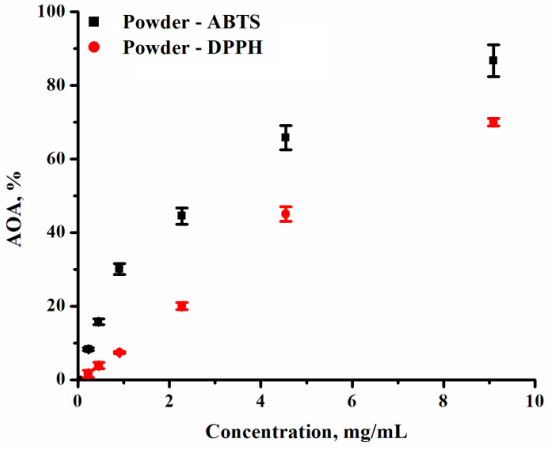
The experiment was conducted according to Halliwell et al. (1987) with some modifications. The investigated extracts and the 2' deoxyribose [0.6 mmol/L] were diluted in PBS buffer (K_2HPO_4/KH_2PO_4, pH 7.4). Control samples containing only deoxyribose were prepared. All test tubes were UV irradiated for 30 min (UV light 220-400 nm). To 1 ml of each irradiated sample solution were added 0.6 ml of 1% trichloroacetic acid and 0.6 ml of 0.6% thiobarbituric acid. The obtained mixture was heated for 20 min at 100˚C. The absorbance of the samples was measured at 532 nm.

Results. The first step of the evaluation of the antioxidant potential of the tested olive leaf extracts includes their testing in model systems which have been regularly used in the *in vitro* determination of the antioxidant capacity of natural product extracts of plants – the stable free radicals containing model systems. The combination of these results and those from the other chosen spectrophotometric and chemiluminescent results will present us with the possibility to reveal different features of the observed antioxidant properties (Benavente-García et al., 2000; Buyukbalci & El, 2008).

Free radical scavenging activity is the most important mechanism by which natural and synthetic compounds influence oxidative stress parameters and inhibit the free radical damages of biologically relevant molecules. For the determination of the total antioxidant capacity of the olive leaf extracts we have chosen two



(a)

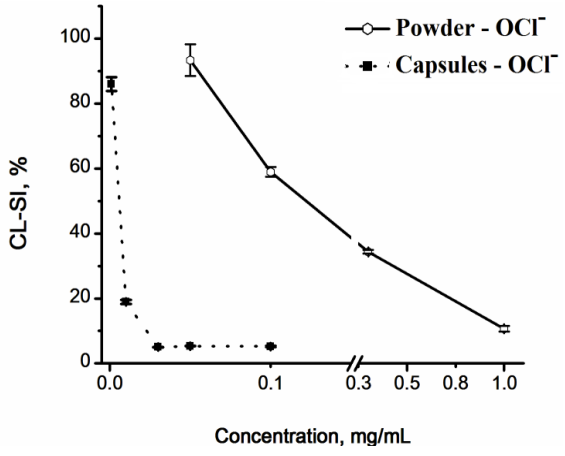


(b)

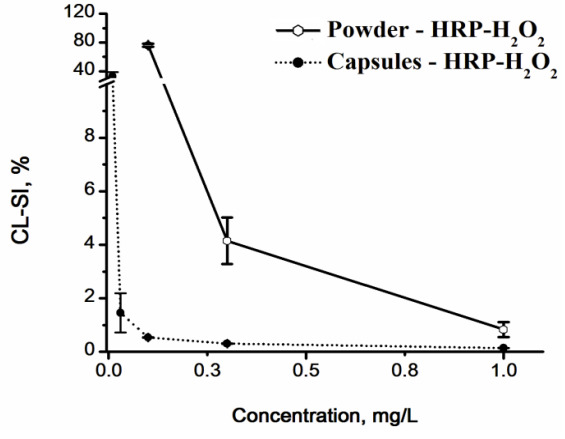
Figure 1. AOA of Olive leaf extracts – capsules (1a) and powder (1b) in the ABTS and DPPH model systems

stable free radicals – ABTS and DPPH. Both radicals have different mechanism of neutralizing. DPPH• absorbance decreases in presence of hydrogen-donating antioxidants due to the formation of the stable DPPH-H compound whilst ABTS is involved in an electron transfer processes.

The results from the experiments in the stable free radical containing model systems in the presence of Olive leaf extracts – capsules (1a) and powder (1b) –



(a)



(b)

Figure 2. Olive leaf extracts – capsules and powder influence on HRP dependent elimination of H_2O_2 (2a) and evaluation of their capability to interact with hypochlorite (2b)

are presented on figure 1. The combination of both methods has been widely used in the evaluation of the TAOC of multicomponent systems due to the possibility to determine the activity of the hydrophilic and lipophilic fractions. The obtained results indicate that no matter what is the used dosage form of the olive leaf extract in the system where we are supposed to observe diminishment of the radicals due to

single electron transfer (SET), the measured TAOC is higher when compared to the one where the mechanism of neutralization of the stable radical includes hydrogen atom transfer (HAT). The capsule dosage form demonstrated better antioxidant potential – corresponding to two-fold higher activity compared to the powder form. In order to compare the two dosage forms in the stable free radicals containing model systems on the base of the concentration dependence of the AOA presented at Fig. 1 have been calculated the concentrations corresponding to 50% decrease of radicals (C-50). For the ABTS assay C-50 $_{powder}$ = 2.11 mg/mLandC-50$_{capsules}$ = 16,86mg/L and in the DPPH containing system - $C_{50capsules}$ = 0.449 mg/mL и $C_{50powder}$ = 6.15 mg/mL. The obtained C-50 values revealed the necessity of a bigger quantity extract per milliliter from the powder form in order to obtain the same effect corresponding to the samples containing the capsuled dosage form.

Despite the fact that the used stable free radicals (ABTS and DPPH) do not resemble any known biological radical, the obtained results have valuable pharmaceutical and clinical implications having in mind that information, cited in various literary sources states that the observed beneficial effects, associated with the supplementation with olive leaves extracts, correlate with the antioxidant potential of the tested dosage form and its impact on the antioxidant status of the tested model animals. We have performed additional experiments to evaluate the capability of the tested extract in both dosage forms to scavenge biologically relevant ROS using chemiluminescent methods and we have determined their potency in protecting the deoxyribose molecule during UV irradiation.

For the chemiluminescent investigation we have chosen ROS which are normally produced in living systems and which are implicated with pathophysiological conditions associated with oxidative stress generation. The chosen model systems give the opportunity to estimate the capability of the extracts to influence on HRP dependent elimination of H_2O_2 and to evaluate their ability to interact with hypochlorite.

At Fig. 2 are presented the results from the chemiluminescent evaluation of the antioxidant potential of both dosage forms of the olive leaves extracts. The obtained for the different tested concentration values of the CL-SI index are an indicator revealing the percentage of the scavenged in the systems radicals.

The olive leaf extracts have demonstrated better antioxidant effect in the chemiluminescent model system of HRP-dependent elimination of H_2O_2, compared to the hypochlorite containing one. Despite its poor reactivity hydrogen peroxide can easily promote the oxidative damage by producing hydroxyl radicals (in the presence of transition metal ions) or hypochlorite (in the presence of Cl^- and myeloperoxidase). The $HO^\bullet$ is the most reactive oxygen species and can cause severe oxidative damage of the basic cellular components – lipids, proteins and DNA - which are relevant to the process of carcinogenesis. On the other hand, the activation of phagocytizing cells during the inflammatory processes is associated with the production

of significant amounts of ROS and the neutrophils activation leads to release of hypochlorousacid in the living system. The latter is associated with endothelial dysfunction via vascular inflammation and initiates the formation of plaques in blood vessels. Due to this fact the evaluation of the capability of the tested extracts to eliminate this substrate for the production of these two most reactive free radicals is of crucial importance for the estimation of their possible beneficial effects of the tested supplements. On the basis of the presented at Fig. 3 results we can make the assumption that the tested extracts could have a beneficial effect in the mentioned pathophysiological conditions.

The results against the biologically relevant ROS from the chemiluminescnet experiments are in agreement with the data obtained during the TAOA evaluation – the powder dosage has lower antioxidant potential when compared to the capsulated one. The concentration of the extracts reducing by 50% the quantity of the hypochlorite in the system are respectively $C_{50capsules}$ = 5.83mg/ L and $C_{50powder}$ = 0.149mg/mL.

The deoxyribose method confirms the results obtained for the higher antioxidant activity of the extract in capsules. The antioxidant activity of the capsules forms by this method is about 15 % higher than the powder form.

Conclusion. In all used in vitro model systems, regardless of whether they contain stable free radicals or are biologically relevant, the tested leaves extracts demonstrated good antioxidant properties. The observed activity is due to the capacity of the compounds to scavenge stable free radicals and reactive oxygen species and does depend of the used dosage form. In all tested systems the capsulated olive leaves extract has presented itself as the more potent which could be attributed to the protection effect of the capsule leading to limited exposure to the conditions of the environment medium.

NOTES

1 https://www.swansonvitamins.com/swanson-superior-herbs-olive-leaf-extract-super-strength-750-mg-60-caps

2. http://www.clinicians.co.nz/orac/

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✉ **Prof. Trayko Traykov (corresponding author)**
Department of Medical Physics and Biophysics
Faculty of Medicine
Medical University of Sofia
Sofia, Bulgaria
E-mail: tttraykov@gmail.com