Reactive Oxygen Species Scavenging Activity and Hepatoprotective Effects of a Polyphenolic Extract Obtained from *Cuscuta Europaea*

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The study intended to investigate reactive oxygen species scavenging ability and the hepatoprotective effect of a polyphenolic extract obtained from Cuscuta europaea in N-acetyl-p-aminophenol - induced liver injuries in rats. Chemiluminescence investigations showed that polyphenols extracted from Cuscuta europaea have a strong capacity to scavenge reactive oxygen species H_2O_2, $^1O_2$, •OH, O_2•> , and RCOO•. In vivo studies demonstrated that polyphenols extracted from Cuscuta europaea have a remarkable hepatoprotective potential. Thus, the administration of Cuscuta europaea polyphenols in liver injuries induced by N-acetyl-p-aminophenol in rats determined significant improvements in the levels of serum enzymes - markers of liver cytolysis, increased activity of the antioxidant enzymes, increased levels of liver glutathione and decreased levels of compounds that react with thiobarbituric acid in liver.

Keywords: Cuscuta europaea; polyphenols, reactive oxygen species, hepatoprotective activity, antioxidant activity

Reactive oxygen species, collectively known as ROS, is a term used to describe a variety of molecules and free radicals such as •OH, $^1O_2$, H_2O_2, O_2•> , RCOO•, which are derived from molecular oxygen and are in a more reactive state than this one, with high pro-oxidant potential. ROS represent a double face medal and they can act either positively or negatively on cell functioning, depending on the intensity and duration of the oxidative stress produced on a cell [1]. ROS are generated as by-products in normal metabolism of oxygen and have an important role in cell signaling, apoptosis, and redox-homeostasis. Cells are provided with numerous enzymes and endogenous small molecular weight antioxidants (such as glutathione) to strictly control the intracellular ROS level and to maintain the balance between oxidant and antioxidant molecules [1, 2]. An imbalance between the generation of ROS and the antioxidant defense of the cell can cause extensive cellular damages because ROS affect major cellular components, such as lipids (including those in cell membranes), proteins and nucleic acids.

ROS take an important part in the induction and the progression of various liver diseases with different etiologies [1-8]. Many studies conducted on patients with viral hepatitis, alcoholism or chemical intoxications showed a correlation between liver damage and the increase in pro-oxidant cellular markers of lipid oxidation, such as malondialdehyde and 4-hydroxynonenal, associated with the decrease of reduced glutathione (GSH), vitamin E, vitamin C and selenium [9].

Reactive oxygen species exert an important role in the development of hepatotoxicity caused by N-acetyl-*p*-aminophenol (APAP) [10, 11]. The initial step of this substance toxicity is the formation of N-acetyl-p-benzoquinone imine (NAPQI) metabolite, under the influence of cytochrome P450 (CYP) [12].

Cuscuta europaea is a parasitic plant commonly known as European dodder. These plants twirl around the stems of their hosts and probe into their vascular systems, stealing water and nutrients. The leaves of dodder are virtually non-existent, reduced to just tiny scales, and many species, including this one, do not engage in photosynthesis and once they are wrapped around their host and soaking up its nutrients, dodder even loses its roots. Dodder may be the most reviled parasitic plant as it can infest a large variety of hosts, including both agricultural plants and horticulture species.

In Romania, *Cuscuta europaea* is used in popular medicine in the treatment of liver diseases induced by viruses with hepatic tropism, chronic alcoholism, intoxications, etc.

This study aim was to investigate ROS scavenging ability and the hepatoprotective effect of a polyphenolic extract obtained from *Cuscuta europaea* in case of APAP-induced liver injuries in rats.

Experimental part

Plant material

In this study, it was used *Cuscuta europaea* harvested in August from an alfalfa crop located in Southern Romania, Ilfov County.

Preparation of crude polyphenolic extract

Plants were dried at room temperature in low light; 10 g of dried plants were ground into a fine powder and extracted with 60% ethanol at 60°C for 3 h.

Phytochemical analysis

Determination of total phenolic content

The total polyphenols content was determined using an assay based on reaction with Folin-Ciocalteu reagent [13] and it was expressed as mg of gallic acid equivalents/mL extract (mg GAE/mL).

Determination of total flavonoid content

Quantitative determination of flavonoids was made by a colorimetric assay [14]. Total flavonoid content of crude extract was expressed as μg of + (-) catechin equivalents/mL extract (μg CAT/mL).

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Reactive oxygen species scavenging activity. Chemiluminescence screening

In order to evaluate the optimal dilution of polyphenols for which antioxidant activity is maximum, it was tested the chemiluminescence quenching by crude polyphenolic extract and diluted with 60% ethanol in ratio of 1:100, 1:1000 and 1:10000 (v/v). Hydrogen peroxide scavenging activity. H_2O_2 scavenging activity was assayed in luminol – hydrogen peroxide system ($LH_2 - H_2O_2$) [15]. Singlet oxygen scavenging activity. Singlet oxygen ($^1O_2$) scavenging activity was determined in luminol – [$^1O_2$] system ($LH_2 - {}^1O_2$) [16], with the only difference that instead of lactoperoxidase it was used horseradish peroxidase. Hydroxyl radical scavenging activity. For hydroxyl radical scavenging effect assays, •OH was generated by a Fenton-type reaction system [17]. Superoxide anion scavenging activity. $O_2^{\bullet -}$ was generated from a pyrogallol autooxidation system [18]. Determination of peroxil radical scavenging activity. Radical peroxil was determined using the method proposed [16].

For the evaluation of chemiluminescence emission kinetics, chemiluminescence measurements were performed at room temperature for 170 s (2 min and 50 s), in test tubes (Φ12 x 75 mm), using a Berthold FB 12 luminometer. Chemiluminescence emission was counted at every 5 s. The intensity of chemiluminescence is given as the relative light units/second (RLU/s). Five measurements were made and an average value was calculated, obtaining a maximum 10% relative scattering of the results from the mean value. In the presence of an antioxidant compound (which consumes free radicals), the chemiluminescence intensity decreases, while the effect of a pro-oxidant compound (which increases the concentration of free radicals) leads to an increase of chemiluminescence intensity, comparatively with the reference system [19].

The percentage of quenching effect against the reactive oxygen species was calculated using the following equation:

$$Q\% = \frac{I_0 - I}{I_0} x100 \qquad (1)$$

where Q represents the quenching effect, while I_0 and I represent chemiluminescence intensity measured for the reference system and for *Cuscuta europaea* extract, respectively. The concentrations of the generated free radicals could not be estimated.

Animals

Female Wistar rats, weighting 150–170 g were used for the study. The animals were housed in cages and maintained in controlled temperature (22 ±2°C) and light cycle (12 h light and 12 h dark), with free access to food and water.

Inducing of liver injuries in rats by N-acetyl-p-aminophenol and Cuscuta europaea extract administration

The rats were randomly divided into 4 groups of 10 animals each, as follows:

- *Group 1*: Normal control rats (N rats) – represented by untreated individuals.

- *Group 2*: *Cuscuta europaea* control rats (CE rats) – represented by individuals which were orally administered *Cuscuta europaea* crude polyphenolic extract diluted with water in ratio of 1:100 (v/v) as their sole source of drinking water, for 21 days.

- *Group 3*: N-acetyl-p-aminophenol rats (APAP rats) – represented by individuals intoxicated with N-acetyl-*p*-aminophenol in a dose of 2 g/kg, orally, for 7 days (days 8-14 of the experiment).

- *Group 4*: N-acetyl-p-aminophenol – *Cuscuta europaea* rats (APAP-CE rats) – represented by individuals which were orally administered *Cuscuta europaea* crude polyphenolic extract diluted with water in ratio of 1:100 (v/v) as their sole source of drinking water 7 days before, 7 days during the administration of N-acetyl-p-aminophenol (in the same doses as in Group 3), and 7 days after N-acetyl-*p*-aminophenol administration.

Hepatoprotective activity

At the end of the experiment, animals were light ether anesthetized and sacrificed by cervical dislocation. Blood samples were collected in order to assess the activity of enzymes markers of hepatic cytolysis. The liver was sampled immediately, washed in ice-cold isotonic saline solution and blotted between two filter papers. A 10% (w/v) liver homogenates was prepared in ice-cold 0.1M potassium phosphate buffer, *p*H 7.5. Homogenates were used to estimate antioxidative enzymes, reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).

Enzymes markers of cytolysis activity

The activity of serum aspartate transaminase (AST), glutamate transaminase (ALT) and alkaline phosphatase (ALP) was assayed using kits supplied by Randox (UK), according to the standard procedures described by the manufacturer.

Antioxidant enzymes activity and the levels of non-enzymatic compounds - markers of oxidative stress

The activity of glutathione-S-transferase (GST) was assayed spectrophotometrically by monitoring the conjugation of 1-chloro-2,4-dinitro benzene (CDNB) with glutathione (GSH) at λ_{max}=340nm at 37°C [8]. Glutathione peroxidase (GSH-Px) was determined in liver homogenate using a method based on oxidation of reduced nicotinamide adenine dinucletide phosphate (NADPH) using hydrogen peroxide as the substrate [20]. Catalase (CAT) activity was measured using H_2O_2 as substrate for this enzyme [21]. For evaluation of superoxide dismutase (SOD) activity in liver homogenate, superoxide anion was generated by pyrogallol autoxidation [22]. Glutathione (GSH) was measured spectrophotometrically using 5',5'dithiobis (2-nitrobenzoic acid), that was converted to 2-nitro-S-mercaptobenzoic acid [23]. The concentration of thiobarbituric acid reactive substances (TBARS), index for lipid peroxidation, was investigated spectrophotometrically [24], the result being expressed as nmol malondialdehyde (MDA)/mg protein (nmol MDA/mg). Total protein content was determined using the method proposed [25].

Results and discussions

Reactive oxygen species scavenging activity

Polyphenols extracted from *Cuscuta europaea* possess a strong antioxidant activity due to the ability of these compounds to scavenge reactive oxygen species. The concentration in polyphenols and flavonoids determined for the crude extract was 3.16 mg GAE/mL and 2035.16 μg CAT/mL, respectively. In general, the ability of the extracts to scavenge reactive oxygen species was dependent on the concentration of polyphenols (fig. 1-5).

Calculation of quenching effects 5 s after the start of each reaction showed a remarkable antioxidant activity

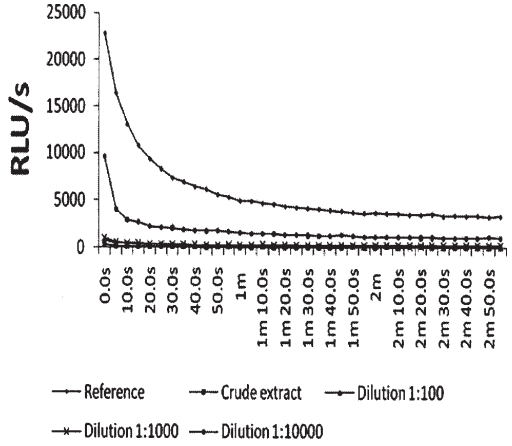

Fig. 1. The effect of *Cuscuta europaea* polyphenolic extracts on chemiluminescence emission kinetics produced by LH_2 - H_2O_2 system in Tris-HCl buffer *p*H 8.5

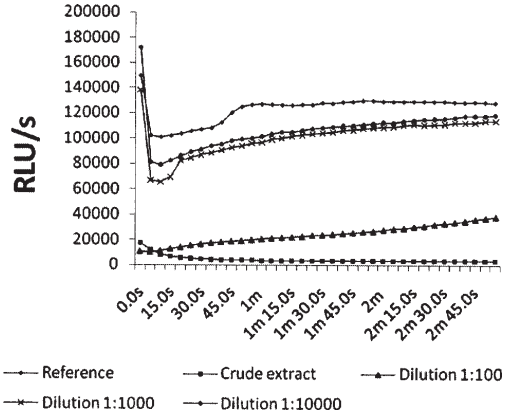

Fig. 2. The effect of *Cuscuta europaea* polyphenolic extracts on chemiluminescence emission kinetics produced by LH_2 - $^1O_2$ system in acetate buffer *p*H 4.5

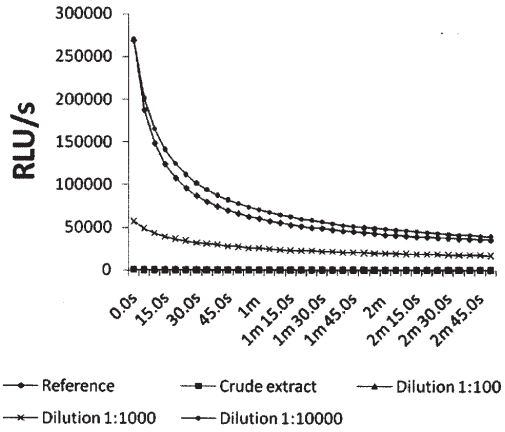

Fig. 3. The effect of *Cuscuta europaea* polyphenolic extracts on chemiluminescence emission kinetics produced by LH_2 – •OH system in PBS pH 7.4

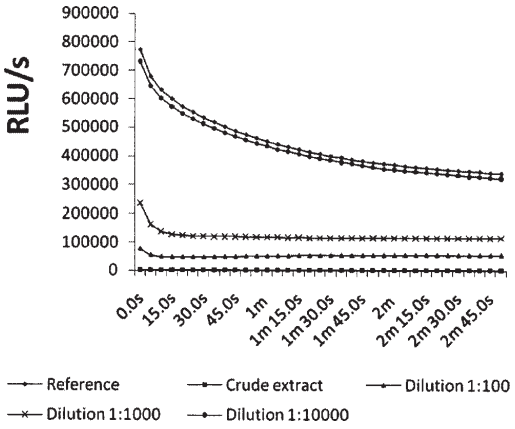

Fig. 4. The effect of *Cuscuta europaea* polyphenolic extracts on chemiluminescence emission kinetics produced by LH_2 – O_2•- system, in carbonic acid/buffer saline solution (CBSS) pH 10.2

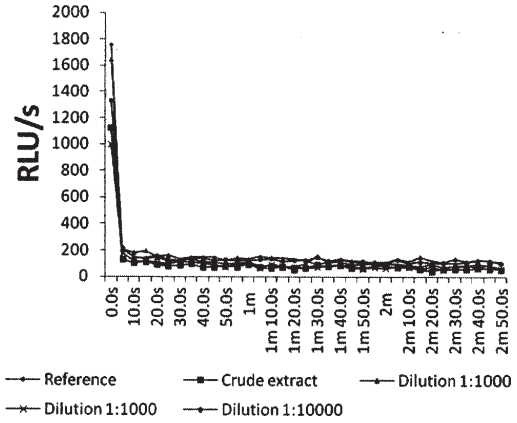

Fig. 5. The effect of *Cuscuta europaea* polyphenolic extracts on chemiluminescence emission kinetics produced by LH_2–R–COO• system in *n*-BuOH

Type of extract	H_2O_2	$^1O_2$	•OH	O_2•$^-$	RCOO•
Crude polyphenolic extract	99.13	88.21	99.84	99.81	35.97
Crude polyphenolic extract diluted 1:100(v/v)	97.17	90.19	99.42	90.09	58.71
Crude polyphenolic extract diluted 1:1000(v/v)	96.74	34.51	73.91	69.40	43.04
Crude polyphenolic extract diluted 1:10000(v/v)	75.57	20.61	- 7.42	5.28	23.95

Table 1
PERCENTAGE OF QUENCHING EFFECT (Q%) AGAINST REACTIVE OXYGEN SPECIES, 5 S AFTER THE BEGINNING OF THE REACTION, OF *CUSCUTA EUROPAEA* POLYPHENOLIC EXTRACTS

Enzyme	N rats	CE rats	APAP rats	APAP-CE rats
ALT (U/L)	77.9 ± 4.8	81.1± 5.1	635.8 ± 22.6	421.5 ± 18.3
AST (U/L)	22.1± 3.1	19.8 ± 1.9	385.7 ± 17.6	162.9 ± 7.0
ALP (U/L)	75.3 ± 4.3	70.1± 3.7	311.2 ± 16.8	251.3 ± 10.21

Table 2
INFLUENCE OF *CUSCUTA EUROPAEA* POLYPHENOLIC EXTRACT ON THE ACTIVITY OF ENZYMES MARKERS FOR HEPATOCELLULAR CYTOLYSIS

Parameter	N rats	CE rats	APAP rats	APAP-CE rats
GST (nmols/min/mg protein)	235.3 ± 15.2	244.9 ± 13.9	182.8 ± 16.2	205.5 ± 13.7
GPx (U/mg protein)	17.8 ± 3.8	18.2 ± 2.9	10.7 ± 2.1	12.5 ± 1.8
CAT (nmols/mg protein)	59.3 ± 4.1	63.1 ± 4.9	37.6 ± 4.6	44.5 ± 5.3
SOD (U/mg protein)	73.6 ± 5.7	74.1± 5.5	58.3 ± 5.1	72.9 ± 6.1
GSH (nmols/mg protein)	27.2 ± 4.2	28.1± 3.9	19.5 ± 2.8	22.8 ± 2.8
TBARS (nmols/mg protein)	2.11 ± 0.27	1.90 ± 0.11	24.38 ± 1.91	10.17 ± 1.80

Table 3
INFLUENCE OF ORAL ADMINISTRATION OF *CUSCUTA EUROPAEA* POLYPHENOLIC EXTRACTS ON OXIDATIVE STRESS MARKERS

for crude polyphenolic extract and for the dilution 1:100 (v/v) (table 1).

Hepatoprotective activity

The administration of *Cuscuta europaea* polyphenols in a ratio of 3.16 x 10^{-2} mg GAE/mL drinking water showed an important hepatoprotective effect in rats. The activity of ALT, AST and ALP enzymes, biochemical markers of hepatocellular damage, showed significantly increased values in rats intoxicated with N-acetyl-p-aminophenol (APAP rats), compared to normal control rats (N rats) and *Cuscuta europaea* control rats (CE rats). The administration of *Cuscuta europaea* polyphenols before, during and after the intoxication with N-acetyl-p-aminophenol (APAP-CE rats), led to a decrease in the activity of enzymes markers of hepatocellular cytolysis comparatively with rats intoxicated with N-acetyl-*p*-aminophenol (APAP rats) (table 2).

The influence of *Cuscuta europaea* polyphenols on the activity of antioxidant enzymes (GST, GPx, CAT, SOD), GSH levels and TBARS in rats liver is presented in table 3.

N-acetyl-p-aminophenol - induced liver injuries in rats manifested by a decrease in the activity of investigated antioxidant enzymes, compared to normal control rats. Reduced activity of these enzymes can be attributed to an increased lipid peroxidation process in the cell membranes and inactivation of antioxidant enzymes due to ROS attack carried out on the functional labile groups (e.g. amino, sulfhydryl) present on polypeptide chains. It was observed that the polyphenols from *Cuscuta europaea* have a special ability for scavenging the •OH, considered the most aggressive free radical, with a very short *in vivo* half-life, of approximately 10^{-9} s [26]. Due to its high reactivity, this radical can easily snatch a hydrogen atom of different biomolecules (e.g. lipids, proteins, carbohydrates, nucleic acids), initiating radicals chain reactions very difficult to be controlled by the defense systems of the human and the animal organisms [27-29].

On the other hand, N-acetyl-p-aminophenol administration to rats determined the increase of TBARS levels by 1055.45% and the decrease of GSH levels by 28.31%, indicating a strong oxidative process both in unsaturated fatty acids and GSH. *Cuscuta europaea* polyphenolic extract administration to rats (CE rats) resulted in no significant changes in the activity of the studied antioxidant enzymes, but it determined a slight increase of GSH levels (3.31%) and decrease of TBARS levels (9.95%), which demonstrates that polyphenols extracted from *Cuscuta europaea* exerted an antioxidant effect in CE rats liver (group 2), compared to normal control rats (group 1). The effect of polyphenols extracted from *Cuscuta europaea* on APAP-CE rats (group 4), compared to APAP rats is also presented in table 3. In this group, polyphenols extracted from *Cuscuta europaea* determined an increase of the antioxidant enzymes activity levels and GSH level in liver. Increased antioxidant enzymes activity levels in APAP-CE rats group inhibited N-acetyl-*p*-aminophenol - induced hepatic injury and thereby the level of oxidative process of unsaturated fatty acids, aspect reflected by the decrease with 58.28% of the most important indicator for lipid peroxidation (TBARS).

These results demonstrate that polyphenols extracted from *Cuscuta europaea* are able to reduce hepatic injuries caused by *N*-acetyl-p-aminophenol, probably by scavenging reactive oxygen species and by enhancing the activities of endogenous antioxidants. The dosage used in this experiment did not produce any adverse effect in the experience animals; body weight and organ weights were unaltered and the antioxidative status (i.e., the balance between pro-oxidants and antioxidants) was improved in experimental rats.

Conclusions

Polyphenols extracted from *Cuscuta europaea* have a strong capacity to scavenge ROS and a remarkable hepatoprotective potential. Administration of polyphenols extracted from *Cuscuta europaea* in liver injuries N-acetyl-p-aminophenol-induced in rats determined significant improvements in the activity of serum enzymes - markers of liver cytolysis, increased activity of antioxidant enzymes, increased levels of reduced glutathione and decreased levels of the compounds that react with thiobarbituric acid in liver.

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