

ANTIOXIDATIVE PROPERTIES OF *Plantago lanceolata* L. EXTRACTS EVALUATED BY CHEMILUMINESCENCE METHOD

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Abstract

Plantago lanceolata L. is used as astringent, anti-inflammatory, immunoregulatory, antispasmodic, expectorant, emollient, topical anodyne, antibacterial for cuts, bruises and abscesses. These pharmacological properties are due to its complex chemical composition: iridoid glycosides, polysaccharides (plantaglucide, glucomannon), terpenoids, coumarins, lignans, caffeic acid derivatives, polysaccharides, flavonoids, flavone glycosides and other constituents, like allantoin. In this paper, antioxidant activity, qualitative and quantitative determination of some active components (flavonoids, polyphenols, and polyphenols carboxylic acids) are evaluated, taking into account the biological properties. Antioxidant properties were evaluated by chemiluminescence (CL) technique in system generator based at 5-amino-2,3-dihydrophthalazine-1,4-dione and hydrogen peroxide, in alkaline medium and DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The total flavonoids, polyphenols content and polyphenol carboxylic acids was spectrophotometrically determined according to Romanian Pharmacopoeia (FR). In addition, the qualitative biochemical fingerprint was investigated by mass spectrometry LC-ESI-MS. The mass spectral data and amount of active components (flavonoids, polyphenols, polyphenols carboxylic acids) demonstrated the chemical structural complexity. The results of the chemiluminescence in vitro tests revealed that the *Plantago lanceolata* L. extracts exhibit high antioxidant activity (84-96%), being in accordance with the value provided by DPPH free radical scavenging assay. Also is established a dependence directly proportional to the content of polyphenolic compounds found in herbal extracts with level of antioxidant activity.

Key words: antioxidant, chemiluminescence, polyphenols, *Plantago lanceolata* L.

INTRODUCTION

Herbal antioxidants can protect the human body from destructive effect of oxidative stress, whose potential cause in diseases such as: atherosclerosis, ischemic heart diseases, diabetes mellitus, cancer, neurodegenerative diseases and others, is well known and already proven (Pryor, 1991; Young, 2001).

These arguments justify entirely the continuous growth of the scientific interest in natural antioxidants, increasingly more use in obtaining new pharmaceutical and dermatocosmetics products.

Among the medicinal species of therapeutic interest is included *Plantago lanceolata* L. which is a species of the genus *Plantago* in the *Plantaginaceae* botanical family originated

from Europe, widely spread throughout the world. *Plantago lanceolata* L. is a common perennial weed of arable fields and grassland abundant throughout Europe, North and Central Asia.

It is native in grassy places on neutral or basic soils (Bond et al., 2007).

The complex chemical composition of *Plantago lanceolata* L. species is represented by a series of categories of substances, among which the main components are: iridoid glycosides, polysaccharides (plantaglucide, glucomannon), flavonoids and flavone glycosides, phenolic carboxylic acids, alkaloids (indicain, plantagonin), terpenoids (loliolide, ursolic acid, oleanolic acid (Figure 1a) and other constituents, like allantoin (Figure 1b) (Beara et al., 2012).

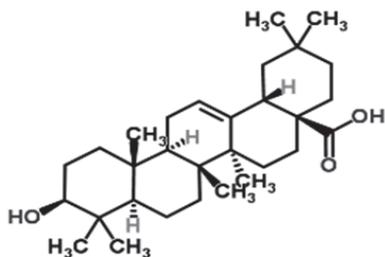


Figure 1a. Chemical structure a) oleanolic acid

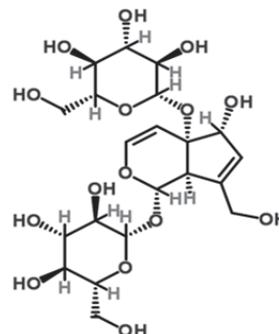


Figure 2e. Chemical structure melittoside

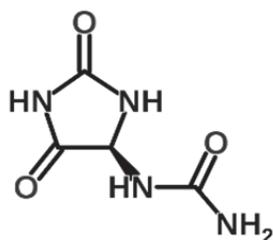


Figure 1b. Chemical structure b) allantoin

Iridoid glycosides are representative compounds class that include: aucubin (Figure 2a), and catapol (Figure 2b), as the main compounds, as well as asperuloside, globularin, gardoside (Figure 2c), geniposidic acid (Figure 2d), mayoroside, melittoside (Figure 2e) and desacetylasperuloside acid methylester.

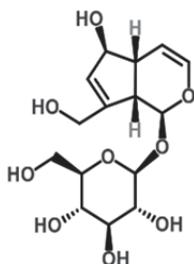


Figure 2a. Chemical structure Aucubin

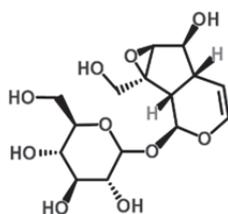


Figure 2b. Chemical structure Catapol

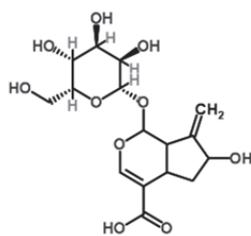


Figure 2c. Chemical structure Gardoside

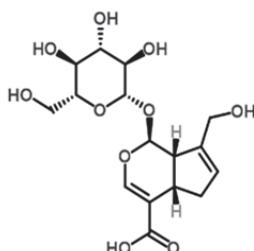


Figure 2d. Chemical structure Geniposidic acid

The antioxidant properties of herbal products are mainly attributed to phenolic compounds such as flavonoids and polyphenolic derivatives (cinnamic acid, p-coumaric acid, syringic acid, vanillic, salicylic acid), compounds that are found in the leaves of *Plantago lanceolata* L. (Beara et al., 2012).

Among these we could mention apigenin, balcain and luteolin as well as their derivatives with the main compounds apigenin-6,8-di-C-glucoside and luteolin-7-O-glucuronide, luteolin-7-O-glucoside and 7-O-glucuronide-3'-glucoside, in addition to the 7-O-glucuronyl-glycosides of apigenin and luteolin, apigenin-7-O-glucoside and 7-O-glucuronide (Kuhn et al., 2000). Among phenolic carboxylic acids could be mentioned: p-hydroxybenzoic acid, protocatechic, gentisic, chlorogenic and neochlorogenic acid and others. Inorganic constituents include 1% salicylic acid and mineral salts high in zinc and potassium (Wichtl, 2004).

Due to the above mentioned chemical composition, *Plantago lanceolata* L. has multiple and complex pharmacodynamic actions. The extracts obtained from the leaves of this plant, *Plantago lanceolata* L. are used as vasoconstrictor, antacid, diuretic, emollient, topical anodyne, connective tissue tonic, astringent, anti-inflammatory (Samuelsen, 2000), antispasmodic (Fleer, 2007) antihemorrhagic, expectorant, demulcent, antibacterial (Andary et al., 1982), immunoregulatory (Huang et al., 2009) mild laxative (Duke, 1992) hepatoprotector (Chang, 1984; Yang, 1983), promotes uric acid excretion, and strong superoxide anion scavenger and antioxidant (Zhou et al., 1991; Heimler et al., 2007; Gálvez et al., 2003). The liquid extract and the pressed juice of fresh plantain herb possess proven bacteriostatic and bactericidal effects due to the iridoid glycoside

(especially aucubin compound) and tannin content. The mucilage from the leaves has a soothing and anti-inflammatory effect on the lower respiratory tract. The exact mechanism is not clear (Stewart, 1996). Due to the complexity of herbal extracts composition, separating each compound with potential antioxidant properties and studying it individually is hard and inefficient. In addition, there might be synergistic interactions among the antioxidant compounds, important aspect mentioned in numerous scientific papers (Huang et al., 2005). Several studies develop methods for investigating the antioxidant properties, such as: ferric reducing antioxidant power (FRAP) assay, Trolox equivalent antioxidant capacity (TEAC), DPPH (diphenyl-p-picrylhydrazyl radical), oxygen radical absorbance capacity (ORAC), chemiluminescence (CL), to name a few (Miser-Salihoglu et al., 2013). These methods are being applied by the food, pharmaceutical and cosmetic industries.

In this paper we focused on investigating the antioxidant activity by CL method and checked by DPPH technique. The fundamental chemistry of CL assays is based on the reaction of radical oxidants with marker compounds to produce excited state species that emit chemiluminescence (chemically induced light). Any compounds that react with the initiating radicals inhibit the light production. CL is light produced by a chemical reaction.

Chemiluminescence assays, covering a great variety of applications in human and veterinary medicine, forensic medicine, agriculture and food industry. The use of CL as a detection principle permits antioxidant capacity determination of various compounds at low concentrations. Moreover, CL emission intensities are sensitive in the first place to the generator system of chemiluminescence as well as to a variety of environmental factors such as temperature, solvent, ionic strength, pH, and other species presented in the system (Baeyens et al., 1998).

In this work the main goals consisted in the following: (1) to determine the qualitative and quantitative biochemical profile; (2) to determine the antioxidant activities of various extracts of *Plantago lanceolata* L. by chemiluminescence method; (3) to compare the antioxi-

dant activity of several pure phenolic compounds namely quercetin, apigenin, rutin, ferulic acid, caffeic acid, chlorogenic acid and allantoin (widely distributed in *Plantago lanceolata* L.); (4) to compare the antioxidant activity by two methods: chemiluminescence and DPPH technique.

MATERIALS AND METHODS

Plant materials. The *Plantago lanceolata* L. are commercial samples, obtained from FARES: S.C. Romania.

Chemicals. Aluminium chloride, Sodium acetate, Folin-Ciocalteu phenol reagent, Arnou reagent, ethanol, methanol, acetic acid, ethyl acetate, acetone, rutin, quercetin, apigenin, chlorogenic acid, caffeic acid, ferulic acid, gallic acid, allantoin were purchased from Sigma-Aldrich and ultrapure water (Millipore water system).

Reagents for antioxidant activity/radical scavenging activity determination: luminol (5-amino-2,3-dihydro-1,4-phthalazinedione-H₂O₂) in buffer TRIS-HCl, at pH 8.6 (chemiluminescence methods) and DPPH (2,2-diphenyl-1-picrylhydrazyl).

Equipments. Soxhlet system for crude extracts, *Spectrophotometer* UV-Vis, Jasco V-570, for DPPH method and quantitative determination of flavonoids, polyphenols, polyphenol-carboxylic acids. Centrifuge EBA 20 Hettich-Germany. Digital Rotary Evaporator RE100-Pro LCD (Dragon Laboratory Instruments Limited), Chemiluminometer (Sirius Luminometer Berthelot - GmbH Germany): for *antioxidant activity* measurements by chemiluminescence technique (CL).

The selective herbal extracts from *Plantago lanceolata* L. specie

Plant leaves were extracted with 50% (w/v) ethanol. A mass of 100 g of dry herb was added to 500 ml of solvent and was stirred up in a glass flask tightly closed for 45 minutes. Then, 1000 ml of solvent was added and stirred up for another 45 min and finally Soxhlet extraction was carried out. After the extraction procedure achieved in a Soxhlet installation (4 extractive cycles), the vegetal material used was removed, and the combined filtrates (crude extract PL1) were processed by vacuum concentration

(Digital Rotary Evaporator RE100-Pro LCD, equipped with a constant temperature water bath) until obtaining a residue which was passed through successive precipitations with different solvents. The processing for obtaining selective extracts of *Plantago lanceolata* L. was carried out further by applying technological operations of centrifugation, filtering at low pressure and purification. The technological methods of extraction and separation have led to the obtaining of three selective herbal extracts (PL2, PL3, PL4) with aspect of fine powder, green brown color, non-hygroscopic.

Samples (PL2, PL3, PL4) have been obtained by variation of the operational parameters, specific to each stage mentioned above, in accordance with Table 1.

Table 1. Processing the extract of *Plantago lanceolata* L.

| Samples | PL2 | PL3 | PL4 |
|-----------------------------------|-------------------|---------------|---------|
| Temperature of concentration (°C) | 35 | 50 | 65 |
| Speed of concentration (rpm) | 100 | 300 | 600 |
| Solvent of precipitation | Acidified Ethanol | Ethyl acetate | Acetone |
| Ratio of precipitation (w/v) | 1: 20 | 1:5 | 1:10 |
| Time of centrifugation (min) | 30 | 40 | 20 |
| Speed of centrifugation (rpm) | 6000 | 3000 | 6000 |

Qualitative analysis (LC-ESI-MS analysis). Qualitative information was gathered using a mass spectrometer instrument LCMS Shimadzu 2010 EV, consisting of a single quadrupole analyser and a diode array detector, operated in the positive electrospray mode and by direct infusion of sample. Ion source parameters were as follows: capillary voltage 1.5 kV, interface voltage 2.5 kV, source temperature 120°C, desolvation temperature 250°C, nitrogen gas flow 1.5 l/min and the sample injection flow rate of 10 µl/min. The acquisition was made by single ion monitoring, from 200 to 700 m/z (of ions of interest: 271, 288, 347, 355, 363, 433, 449, m/z). Sample solutions were filtered with a 0.45-µm (pore size) disposable syringe filter (Sigma-Aldrich).

Quantitative analysis: the total flavonoids and polyphenols content, polyphenolcarboxylic acids and the specific physical-chemical indicators were performed according to the FR

X (Romanian Pharmacopoeia, 1993; Ciulei et al., 1995).

Antioxidant activity

CL method. The antioxidant activity (AA%) of samples (PL1, PL2, PL3, PL4) has been determined and compared with standards: quercetin, apigenin, rutin, ferulic acid, caffeic acid, chlorogenic acid and allantoin (Sigma-Aldrich).

Chemiluminescence *in vitro* tests (CL), was applied using luminol - H₂O₂ as generator system, in an alkaline buffer solution TRIS-HCl, pH=8.7 by using Sirius Luminometer Berthelot-GmbH Germany. 5-amino-2,3-dihydro-1,4-phthalazinedione has the role of amplifier of light in the chemiluminescence system for to increase the detection sensitivity of reactive oxygen species. The antioxidant activity of samples was calculated by using the relation (Iftimie et al., 2004; Barbinta Patrascu et al., 2008; Barbinta Patrascu et al., 2013):

$$AA \% = \frac{I_0 - I}{I_0} \cdot 100$$

where: I₀=the maximum CL for standard at t=5 s; I=the maximum CL for sample at t=5 s. The CL assay was repeated three times.

DPPH Radical Scavenging Activity

The free radical scavenging activity (SR%) was quantitatively tested using 2,2'-diphenyl-1-picrylhydrazyl according to the modified method of Brand-Williams et al. (Brand-Williams et al., 1995). A DPPH solution (80 µM) was freshly prepared in 95% methanol.

A volume of 250 µl of this solution was allowed to react with 35 µl sample and the absorbance was measured at 515 nm. The radical scavenging activity (SR%) was calculated as follows:

$$SR\% = 100 \left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}} \right)$$

The DPPH• assay was repeated three times.

RESULTS AND DISCUSSIONS

Qualitative analysis

In mass spectrum of *Plantago lanceolata* L. crude extract PL1 carried out by direct injection in ESI positive mode were detected peaks molecular correspond to isomers having empirical formulae and molecular weight of

$C_{15}H_{10}O_5$ (M=270), $C_{15}H_{10}O_6$ (M=286),
 $C_{15}H_{22}O_9$ (M=346), $C_{16}H_{18}O_9$ (M=354),

$C_{15}H_{22}O_{10}$ (M=362), $C_{21}H_{20}O_{10}$ (M=432),
 $C_{21}H_{20}O_{11}$ (M=448).

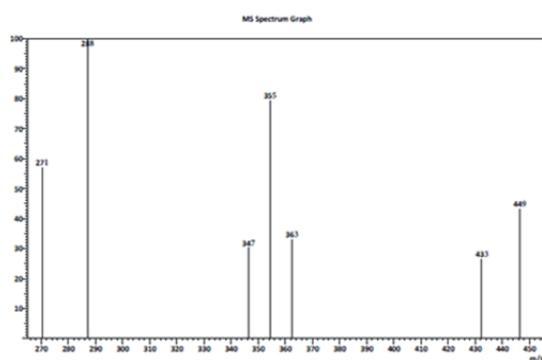


Figure 3. MS fingerprint for crude extract PL1 (SIM mode by direct injection)

Table 2. Chemical compounds identified in crude extract of *Plantago lanceolata* L. - SIM mode

| Compounds | Molecular formula | Molecular weight $g \cdot mol^{-1}$ | [M+H] ⁺ m/z |
|---------------------------------------|----------------------|-------------------------------------|------------------------|
| Apigenin | $C_{15}H_{10}O_5$ | 270.237 | 271 |
| Luteolin | $C_{15}H_{10}O_6$ | 286.236 | 288 |
| Aucubin | $C_{15}H_{22}O_9$ | 346.330 | 347 |
| Chlorogenic acid | $C_{16}H_{18}O_9$ | 354.310 | 355 |
| Catalpol | $C_{15}H_{22}O_{10}$ | 362.329 | 363 |
| 7,3',4'-Trihydroxyflavone 7-glucoside | $C_{21}H_{20}O_{10}$ | 432.380 | 433 |
| Luteolin-7-O- β -glucoside | $C_{21}H_{20}O_{11}$ | 448.376 | 449 |

The figure 3 and table 2 show, the compounds identified by ESI⁺/MS in the positive single ion monitoring (SIM). Also in previous studies,

Gálvez et al. have identified luteolin-7-O- β -glucoside as major flavonoid present in most of the *Plantago* species (Gálvez M., 2003).

Quantitative analysis

Table 3. Physico-chemical indicators and chemical composition from samples (PL1, PL2, PL3, PL4).

| Sample code | Ash, % | Humidity, % | Flavonoids, mass % (as rutin) | Polyphenols, mass % (as gallic acid) | Polyphenolcarboxylic acids, mass % (as caffeic acid) |
|-------------|------------------|------------------|-------------------------------|--------------------------------------|--|
| PL1 | - | - | 0.63 ± 0.027 | 0.89 ± 0.006 | 0.70 ± 0.002 |
| PL2 | 1.83 ± 0.007 | 2.45 ± 0.041 | 1.15 ± 0.004 | 1.73 ± 0.021 | 2.11 ± 0.057 |
| PL3 | 2.51 ± 0.002 | 3.01 ± 0.021 | 2.31 ± 0.007 | 2.18 ± 0.011 | 2.47 ± 0.006 |
| PL4 | 2.59 ± 0.007 | 4.98 ± 0.004 | 3.01 ± 0.008 | 2.59 ± 0.001 | 3.22 ± 0.003 |

All test analyses were run in triplicate. Data are presented as mean \pm standard deviation (SD)

It could be noticed a difference between the content of flavonoids was expressed in terms of rutin equivalent (1.15-3.01%), polyphenols was expressed in terms of gallic acid equivalent (1.73-2.59%) and polyphenol carboxylic acids was expressed in terms of caffeic acid equivalent (2.11-3.22%) for samples analyzed, which is owed to different technological process.

The amount of polyphenols in the crude extract is compatible with results obtained in other studies (Heimler, 2007; Vârban, 2012).

After processing, active fractions enriched in polyphenols, polyphenolcarboxylic acids and flavonoids compounds could be obtained.

The amount of active principles of polyphenolic type, is variable and depends obviously on the processing of crude extract. It

may be noted that the operational parameters, appropriate to each stage of processing, influence the content of active ingredients extracted. Therefore at high temperatures, there is a risk of destructing flavonoid active principles. All processes applied (Soxhlet extraction, concentration, centrifugation) were conducted with the main objective to avoid thermal degradation.

Antioxidant activity

The antioxidant activity of the samples PL1, PL2, PL3, PL4 has been evaluated by CL method in generator system based on 5-amino-2,3-dihydrophthalazine-1,4-dione and hydrogen peroxide, in an alkaline medium.

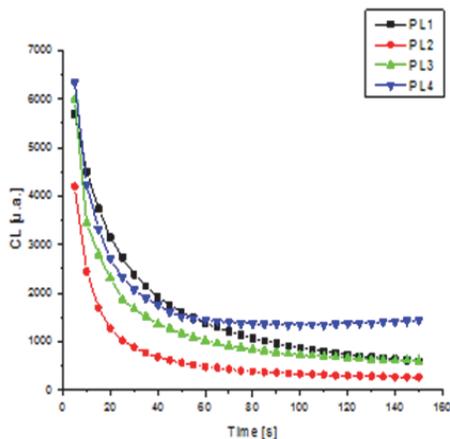


Figure 4. CL evolution in time of the samples PL1, PL2, PL3, PL4

Table 4. Chemiluminescence and kinetic characteristics

| Samples | PL1 | PL2 | PL3 | PL4 |
|-----------------------|--------|--------|--------|--------|
| ki (s ⁻¹) | 0.123 | 0.079 | 0.052 | 0.073 |
| vi (s ⁻¹) | 658.00 | 254.50 | 540.50 | 229.00 |
| AA (%) | 44.75 | 58.93 | 62.14 | 63.91 |

*k: ratio constant; vi: ratio of reaction, AA (%) antioxidant activity by CL, indexed from the chemiluminescence curves

The samples show average values and a wide range between 44.75%-63.91% evaluated by CL (PL1<PL2<PL3<PL4, respectively PL1=44.75%, PL2=58.93%, PL3=62.14%, PL4=63.91%). Considering that DPPH is a robust method for evaluating the antioxidant

activity, this work carried out research for checking the value obtained by CL method for PL1, PL2, PL3, PL3, PL4 samples and pure standards. It could be detected a correlation between the content of the antioxidant activity and the polyphenolic compounds. Therefore, an increase in flavonoid content by 2.38% (mass % as rutin), in polyphenols by 1.7% (mass % as gallic acid) and polyphenol carboxylic acids by 2.52% (mass % as caffeic acid) caused an increase in antioxidant activity by approximately 21%. The phenomenon of correlating the content of flavonoidic active principles with antioxidant effect is manifested in both CL and DPPH assessment methods.

In figure 5 is showed the evaluation of antioxidant activity for samples (PL1 crude extracts of *Plantago lanceolata* L. and selective herbal extracts PL2, PL3, PL4 obtained by processing, as described in paragraph Materials and methods-table 1) as well as of pure standards antioxidant phytochemicals components, as well as plant extracts.

There was found a correlation between the antioxidant activities of the herbal extracts determined by both CL and DPPH methods.

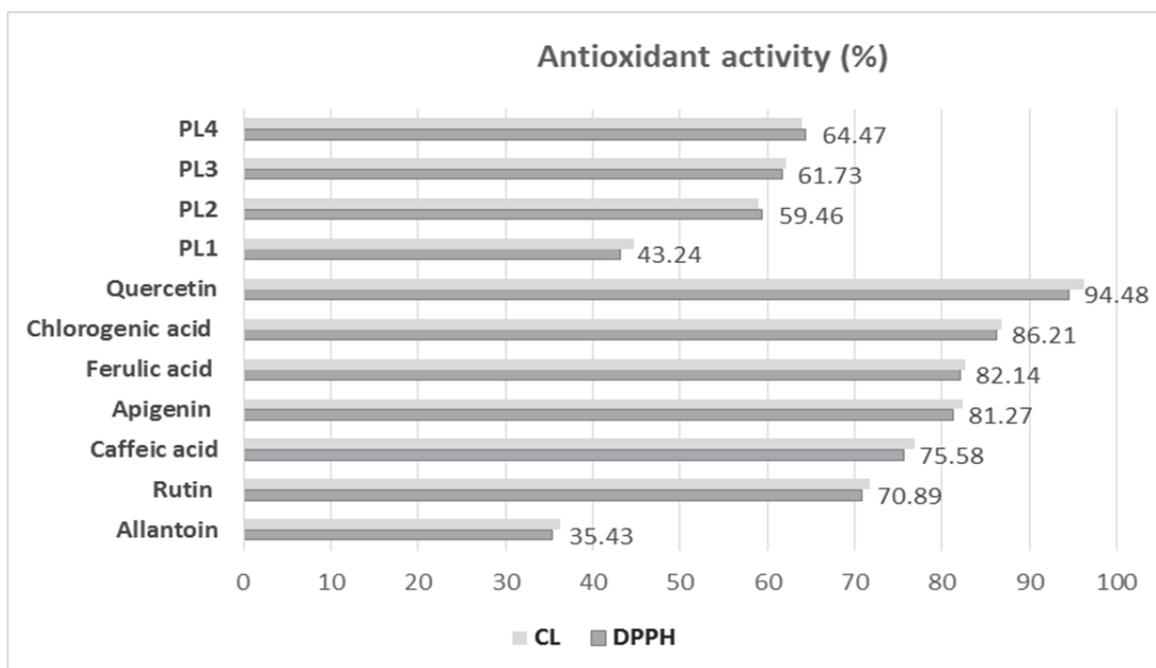


Figure 5. Evaluation of antioxidant activity by CL and DPPH method

CONCLUSIONS

By extractive process and processing of the crude vegetal extract from *Plantago lanceolata* L. species a number of three selective extracts were obtained.

Evaluation of the antioxidant effect by chemiluminescence in 5-amino-2,3-dihydro-1,4-phthalazinedione-H₂O₂ generator system have been revealed significant values for the analyzed samples (selective extracts and pure standards) confirmed by DPPH technique.

From the study achieved, it can be ascertained that the values of antioxidant activity obtained by chemiluminescence are sensibly similar to values obtained through the investigation with DPPH technique and this aspects has also been noted in other previous studies (Nichita et al., 2015).

Antioxidant properties investigations by (CL method and DPPH) on herbal extracts emphasised the presence and contribution of some phenolic and flavonoid structures, also confirmed by qualitative (LC-ESI-MS) and quantitative determination of the flavonoids, of the polyphenols and polyphenolcarboxylic acids.

CL method demonstrated the effectiveness and rapidity of evaluating the effect of the antioxidant phytochemicals components, as well as plant extracts.

There was found a correlation between the antioxidant activities of the herbal extracts determined by both CL and DPPH methods.

ACKNOWLEDGEMENTS

This paper was supported by the Romanian National Authority for Scientific Research, Project PN II PCCA No. 113/2012 and PN II PCCA No. 210/2014.

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