Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 25, issue 1, 2015, pp.41-46

PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY AND EFFECT ON COLLAGEN SYNTHESIS OF A TRADITIONAL WOUND HEALING POLYHERBAL FORMULA

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ABSTRACT. The aim of this study was to investigate the ethanol extracts of four medicinal plants, *Achillea millefolium* L., *Hyssopus officinalis* L., *Equisetum arvense* L. and *Echinacea purpurea* L. and their polyherbal formula, used in traditional medicine for wound healing. The study analyzed their total phenolics content using Folin-Ciocalteu method and identified the main constituents by HPLC. Their antioxidant activity was evaluated by DPPH and ABTS assays and the formula's capacity to enhance collagen synthesis in L929 fibroblast cell culture was determined by Sircol assay. The results showed that the polyherbal extract had phenolic constituents with pharmacological properties: chlorogenic acid, caffeic acid, luteolin and apigenin. It was showed that the polyherbal formula presented higher antioxidant activity than plant extracts and induced a stimulation of collagen synthesis by fibroblasts, which could contribute to wound strength. In conclusion, the proposed polyherbal formula demonstrated high potential as therapeutic agent in wound healing.

Keywords: polyherbal formula, wound healing, antioxidant activity, collagen, medicinal plants

INTRODUCTION:

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the endogenous antioxidant system. The human body cells are equipped with multiple mechanisms to fight against ROS and to maintain the cellular redox homeostasis (Bergendi *et al.*, 1999). When the antioxidant protection mechanism became unbalanced, the exogenous antioxidants, such as those from plants, can help reducing the oxidative damage. The phenolic compounds (phenolic acids, flavonoids, flavanols, anthocyanins, etc.) from medicinal plants have been reported to be potent free radical scavengers (Mathew *et al.*, 2006). The antioxidant properties of phenolic compounds have been substantiated by their high reactivity and potential to chelate metal ions (Rice-Evans *et al.*, 1997).

In acute and chronic wounds, the expression of enzymatic antioxidants increased, while their activity decreased, due to high oxidative stress (James *et al.*, 2001). Besides, several studies reported that depletion of non-enzymatic antioxidants was more pronounced in chronic wounds than in acute wounds (Shukla *et al.*, 1999; Steiling *et al.*, 1999). Addition of substances with antioxidant effect was proved to be important in the successful treatment of skin wounds (Houghton *et al.*, 2005).

Healing of wounds involves the activity of an intricate network of blood cells, cytokines and growth factors, resulting in restoration of normal skin tissue condition (Clark, 1991). The interest in evaluating the utility of plant extracts for wound healing has been increased during the last decade. The importance of plant secondary metabolites as potential agents that interfered with various wound repair stages has been demonstrated, both *in vitro* and *in vivo* (Parasanta *et al.*, 2013; Tsala *et al.*, 2013).

Traditional medicine often used multiple herb formulae for a wide range of treatments. In skin wound healing, four medicinal herbs, *Achillea millefolium* L. (Compositae), *Hyssopus officinalis* L. (Labiatae), *Equisetum arvense* L. (Equisetaceae) and *Echinacea purpurea* L. (Compositae) were used either alone or in combination with other herbs. These plants contributed to wound healing and tissue regeneration by multiple mechanisms, which still need assessment and validation by scientific studies.

The present study aimed to evaluate, for the first time, their combination in a particular polyherbal formula. Its assessment consisted of the identification and quantification of polyphenolic compounds and the determination of total antioxidant activity. In order to support the use of this four-herb formula as a new, natural product for skin wound healing, it was also investigated its *in vitro* effect on collagen secretion by L929 fibroblast cells in culture.

MATERIALS AND METHODS:

Materials

The plants *Equisetum arvense* L., *Achillea millefolium* L., *Hyssopus officinalis* L and *Echinacea purpurea* L. were collected from Neamt and Suceava counties, located in the North of Romania. The plant material was authenticated by prof. dr. Nicolae Stefan (Botany Department, Faculty of Biology, "Alexandru Ioan Cuza" University, Iasi). Voucher specimens were deposited at the Herbarium of Iasi Botanical Garden, Romania. HPLC-grade gallic acid, chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, rutoside, myricetin, luteolin, quercetin, apigenin, acetonitrile and methanol were purchased from Sigma-Aldrich

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Article published: February 2015

(Germany). Butylated hydroxytoluene (BHT), Folin-Ciocalteu's phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and all other chemicals and solvents of analytical grade were purchased from Sigma-Aldrich (Germany). The fibroblast cell line NCTC clone L-929 was purchased from the European Collection of Cell Cultures (ECACC), minimum essential medium Eagle (MEM) from Sigma-Aldrich (Germany) and fetal calf serum (FCS) from Biochrom AG (Germany). Sircol collagen assay kit was purchased from Biocolor Ltd. (Newtownabbey, UK).

Extraction procedures

The aerial parts of each plant were air dried, in the dark and minced using a blender. In order to obtain the polyherbal extract, dried herbs were mixed as follows: 4 g *Equisetum arvense*, 3 g *Achillea millefolium*, 2.5 g *Echinacea purpurea*, 0.5 g *Hyssopus officinalis*. The mixture (10 g) was extracted in 100 mL ethanol (70 %, v/v), at room temperature, in the dark, for 10 days. Then, the polyherbal extract was separated from the residue by filtration through Whatman No.1 filter paper and concentrated under vacuum, at 40 °C using a rotary evaporator (VVMicro, Heidolph, Germany. For cell culture experiments, the solid residue of the polyherbal extract, resulted after concentration under vacuum, was weighed, dissolved in distilled water and sterilized by filtration through 0.2 µm membrane. On the experiment day, several extract dilutions were prepared in the culture medium. Individual plant ethanol extracts were prepared in the same conditions, in order to be used as controls.

Total phenolics content assay

Total phenolics content of the herb extracts was determined using a modified Folin-Ciocalteu method (Singleton *et al.*, 1999). Briefly, 2.5 mL herb extract was mixed with 2.5 mL Folin-Ciocalteu reagent and, after 5 min, 2 mL sodium carbonate (12%, w/w) were added. The mixture was allowed to stand at room temperature, for 15 min. The optical density (OD) of the resulting blue complex was measured at 731 nm using an UV-Vis spectrophotometer (Jasco V-650, Japan). Total phenolic content was calculated from the linear equation of the calibration curve obtained for chlorogenic acid. The results were expressed as mg chlorogenic acid equivalents (ChAE)/g dry extract.

DPPH free radical scavenging activity assay

The method is based on scavenging DPPH stable radical in the presence of hydrogen donor antioxidant, along with color turn from purple to yellow. We measured the free radical scavenging activity of each extract using the method of Hatano *et al.* (1988) with some modifications. Briefly, different herb extract concentrations (10, 25, 50, 100, 250, 500 µg/mL) were added to DPPH methanol solution (0.25 mM) and each mixture was incubated in the dark, for 30 min. The OD was measured at 517 nm against the blank (DPPH methanol solution), using an UV/VIS spectrophotometer (Jasco V650, Japan). The inhibition percentage was calculated using the following formula:

$$\text{Inhibition (\%)} = (OD_{blank}-OD_{sample}) / OD_{blank} \times 100 \quad (1)$$

The sample concentration that inhibited 50% of DPPH free radicals (IC_{50}, µg/mL) was calculated from the graph plotting inhibition percentage against extract concentration by linear regression analysis. BHT was used as positive control.

ABTS radical cation scavenging assay

The method is based on the capacity of a sample to scavenge the ABTS radical cation ($ABTS^{\bullet+}$), compared to Trolox as standard antioxidant. We determined the antioxidant activity of each extract according to the method of Rice-Evans and Miller (1994). Briefly, 2.5 mL ABTS stock solution (7 mM) in potassium persulfate (2.45 mM) was mixed with 0.1 mL sample (herb extract) or standard (Trolox) and 0.4 mL ethanol, and the mixture was allowed to stand at room temperature, for 3 min. Then, the OD was recorded at 731 nm against the blank, containing all reagents except the tested extract, at an UV/VIS spectrophotometer (Jasco V 650). The results were expressed as Trolox equivalents antioxidant capacity (TEAC) calculated using the formula:

$$\text{TEAC (µM Trolox equivalents/g dry weight)} = C_{Trolox} \times f \times (OD_{sample} - OD_{blank}) / (OD_{Trolox} - OD_{blank}) \quad (2)$$

where C_{trolox} is Trolox concentration and f is the sample dilution factor.

HPLC analysis

The separation, identification and quantification of phenolic compounds from polyherbal extract were performed by HPLC, using an Agilent 1200 system (Agilent, USA) equipped with diode array detector and Eclipse XDB-C18 (150 x 4.6 mm i.d.; 5 µm particles) chromatographic column, after injection of 10 µl sample. The mobile phase used for phenolics separation was a mixture of phase A (2 mM sodium acetate buffer, pH 3.05) and phase B (acetonitrile), in linear gradient mode, as follows: 0-30 min, 2-20% B in A; 30-40 min, 20-30% B in A; 40-50 min, 30% B in A; 50-60 min, 30%-2% B in A. The flow rate was 1 mL/min. Chromatograms were recorded at a wavelength of 260 nm for phenolic acids and 320 nm for flavonoids. The constituents present in the polyherbal extract were identified by comparing the recorded UV profile and their retention times with those obtained for a mixture of known standards of phenolic acids (gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid) and flavonoids (rutoside, myricetin, luteolin, quercetin, apigenin). In order to calculate the content of each polyphenol identified in the polyherbal extract, calibration curves for standards were built as five-point plots, in the range of 0.976 – 15.625 µg/mL.

Soluble collagen assay

Mouse fibroblasts cell culture (NCTC cell line clone L929) was used to study the effect of polyherbal extract on collagen secretion. Cells were seeded in the wells of 24-well culture plate, at a density of $5x10^4$ cells/well in MEM supplemented with10% FCS.

After 24 h of incubation in standard conditions, the cells adhered to plastic and the culture medium was changed with MEM supplemented with 5% FCS, containing different concentrations of polyherbal extract (35-140 µg/mL). The plates were incubated at 37 ºC, in humidified 5% CO_2 air atmosphere, for 48 h and 72 h, respectively. The control group consisted of untreated cells cultivated in MEM with 5% FCS. Collagen secretion in the culture medium was determined using Sircol collagen assay kit, according to manufacturer's instructions. Briefly, the harvested culture media were centrifuged at 1,500 rpm, for 4 min and, then, 100 µl supernatant was mixed with 1 mL Sircol dye, for 30 min. The mixture was centrifuged at 10,000 rpm, for 5 min to precipitate the collagen-dye complex. Then, the pellets were dissolved in 1 mL alkali reagent and vortexed. The OD of the solution was read at 540 nm using Sunrise microplate reader (Tecan, Austria).

Statistical analysis

All chemical analyses were run in triplicate and three cell culture independent experiments were performed in three replicates. Data were reported as mean ± standard deviation (SD). Pair comparison of control and each sample was carried out by *t*-test. Significant statistical differences were considered at $p < 0.05$.

RESULTS AND DISCUSSIONS

Total phenolics content and antioxidant activity

The results of total phenolics content in *E. arvense*, *H. officinalis*, *A. millefolium*, *E. purpurea* ethanolic extracts and their polyherbal formula extract are showed in Fig. 1. The amount of total phenolics in plant extracts varied from 8.95 mg ChAE/g dry extract for *E. purpurea*, to 12.43 mg ChAE/g dry extract for *A. millefolium*. Significant ($p < 0.05$) higher phenolic compounds level was detected in the polyherbal extract (14.42 mg ChAE/g dry extract), compared to each plant extract.

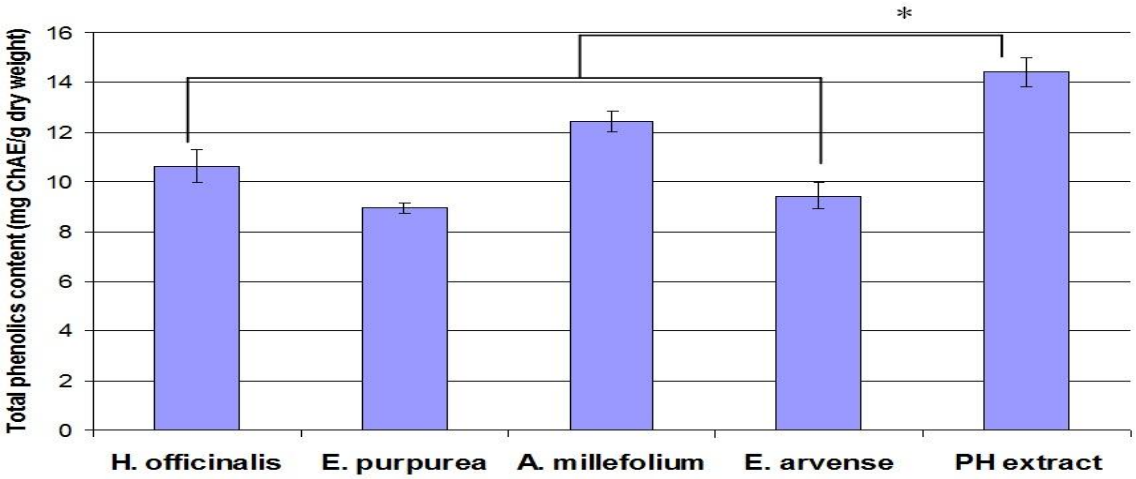

Fig. 1 Total phenolics content in plant extracts and polyherbal (PH) extract. *p < 0.05, compared to each plant extract

In order to determine the antioxidant activity of the polyherbal extract, in comparison to individual plant extracts, two complementary test systems have been applied, DPPH and ABTS assays. The results of DPPH assay showed the extract concentration that resulted in 50% DPPH free radical inhibition (IC_{50}) (Fig. 2).

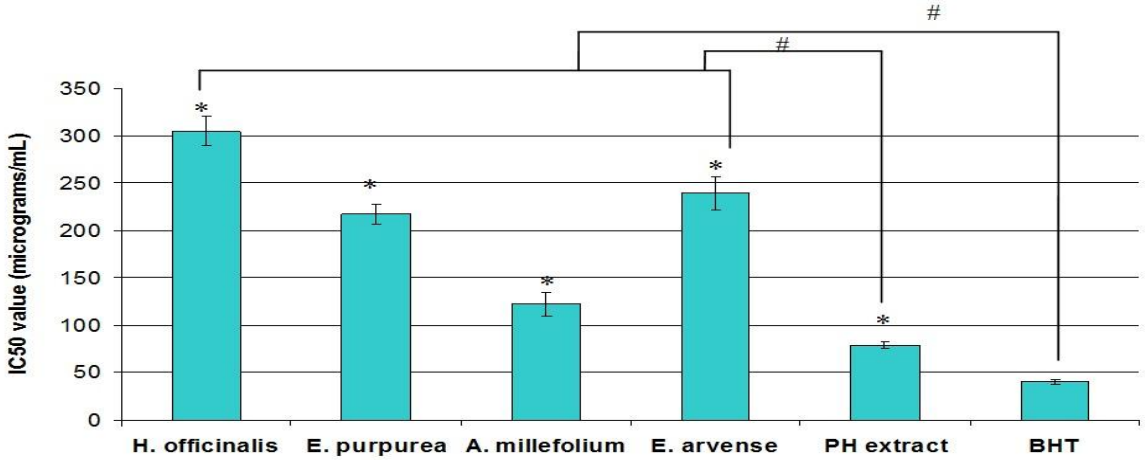

Fig. 2 Radical scavenging activities of plant extracts and polyherbal (PH) extract on DPPH radical. Results were expressed as IC50 (mean ± SD). BHT was used as standard reference. *p<0.05, compared to BHT; #p<0.05, compared to plant extracts.

IC_{50} values decreased in the folowing order: *H. officinalis* >*E. arvense* >*E. purpurea* >*A. millefolium* > polyherbal extract. Therefore, the polyherbal extract presented a significant ($p < 0.05$) higher radical scavenging activity than individual plant extracts, but significant ($p < 0.05$) lower than BHT, a well-known synthetic antioxidant.

The antioxidant activities of plant extracts evaluated by ABTS assay varied from 98.13 µM Trolox equivalents/g dry extract for *E. arvense* to 176.19 µM Trolox equivalents/g dry extract for *A. millefolium* (Table 1). The polyherbal extract had the highest antioxidant activity (254.88 µM Trolox equivalents/g dry extract). This result was in accordance with that obtained by DPPH assay and correlated with its phenolic compounds content.

Table 1.
Antioxidant activities of plant extracts and polyherbal formula extract evaluated by ABTS assay

Plant species	ABTS assay (µM Trolox equivalents/g dry weight)
Hyssopus officinalis	171.73 ± 4.54
Echinacea purpurea	168.22 ± 8.48
Achillea millefolium	176.19 ± 4.96
Equisetum arvense	98.13 ± 3.84
Polyherbal extract	254.88 ± 12.67*

*$p < 0.05$, compared to each plant extract

All these data showed that the polyherbal extract exhibited higher phenolics content and antioxidant capacity, compared to its component extracts of *A. millefolium*, *E. purpurea*, *E. arvense* and *H. officinalis*. As a result, the polyherbal extract was tested in subsequent analyzes.

Chemical composition of the polyherbal extract

The established HPLC method was applied as analytic approach to determine the major compounds of the polyherbal extract. The recorded HPLC profile presented nine main peaks, at 1.312, 6.352, 18.616, 21.899, 24.819, 25.169, 29.190, 29.541 and 45.067 min (Fig. 3).

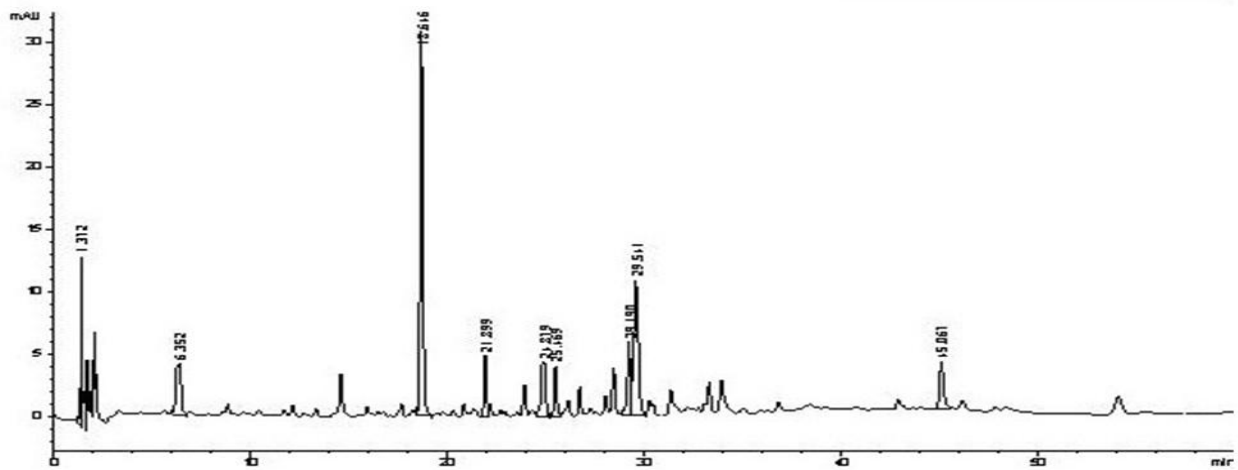

Fig. 3 Chromatographic profile of polyphenolic constituents from the polyherbal extract recorded by HPLC

A mixture of known pure compounds was also chromatographed and used as external standards of phenolic acids (gallic acid, chlorogenic acid, caffeic acid, coumaric acid and ferulic acid) and flavonoids (rutoside, myricetin, luteolin, quercetin and apigenin). The values of retention time for these standards and their calibration curves parameters are presented in Table 2.

Table 2.
Analytical results of calibration curves of ten polyphenolic compounds used as standards in HPLC analysis

Standard	Retention time (min)	Regression equation of the calibration curve[a]	Correlation factor R^2
Gallic acid	4.451	y = 28.963x + 41.860	0.985
Chlorogenic acid	14.485	y = 14.856x + 19.038	0.988
Caffeic acid	17.440	y = 24.325x + 39.374	0.984
Coumaric acid	23.115	y = 19.319x + 33.477	0.982
Ferulic acid	26.018	y = 24.906x + 42.230	0.982
Rutoside	28.305	y = 14.240x + 20.226	0.987
Myricetin	35.800	y = 33.706x + 99.057	0.903
Luteolin	44.904	y = 58.234x + 179.806	0.920
Quercetin	45.302	y = 45.482x + 139.321	0.914
Apigenin	53.747	y = 33.958x + 121.724	0.908

[a]The calibration curves were plotted in linear regression analysis of the integrated peak area (y) versus concentration (x)

In order to determine the content of the identified compounds in polyherbal extract, quantitative calculations were performed by peak area integration. The results of HPLC analysis showed that the polyherbal extract presented high levels of chlorogenic acid (1.226 mg/g dry extract), rutoside (1.605 mg/g dry extract), apigenin (0.982 mg/g dry extract) and luteolin (0.692 mg/g dry extract) (Table 3). Low levels of caffeic acid, coumaric acid and quercetin were quantified in the polyherbal extract (Table 3).

Table 3.
Content of phenolic acid and flavonoid compounds identified in the polyherbal extract

Compound	Content in the polyherbal extract (mg/g dry weight)
Gallic acid	ND
Chlorogenic acid	1.226 ± 0.025
Caffeic acid	0.252 ± 0.050
Coumaric acid	0.251 ± 0.092
Ferulic acid	0.014 ± 0.003
Rutoside	1.605 ± 0.224
Myricetin	ND
Luteolin	0.692 ± 0.148
Quercetin	0.081 ± 0.008
Apigenin	0.982 ± 0.281

ND - not detected

Previous studies showed that these phenolic compounds presented several pharmacological properties and exerted anti-inflammatory, antioxidant, antiviral, antibacterial and vulnerary activities (Fuchs *et al.*, 1993; Morishita *et al.*, 2001; Song *et al.*, 2008; Lopez-Lazaro, 2009; Kostyuk *et al.*, 2010).

Effect of the polyherbal extract on collagen secretion

Wound healing is a fundamental response to tissue injury. The present knowledge described three phases of this process: inflammatory phase, proliferative phase and remodelling phase. In the proliferative phase, fibroblasts produced a variety of substances, essential for wound repair, including glycosaminoglycans and collagen (Madden *et al.*, 1968). In the remodeling phase, new collagen was formed and tissue tensile strength was increased due to intermolecular cross-linking of collagen, via vitamin C-dependent hydroxylation (Prockop *et al.*, 1979; Stadelmann *et al.*, 1998). In Fig. 4 is presented the influence of polyherbal extract on the synthesis of soluble collagen, after cultivation in different concentrations with L929 fibroblast cells.

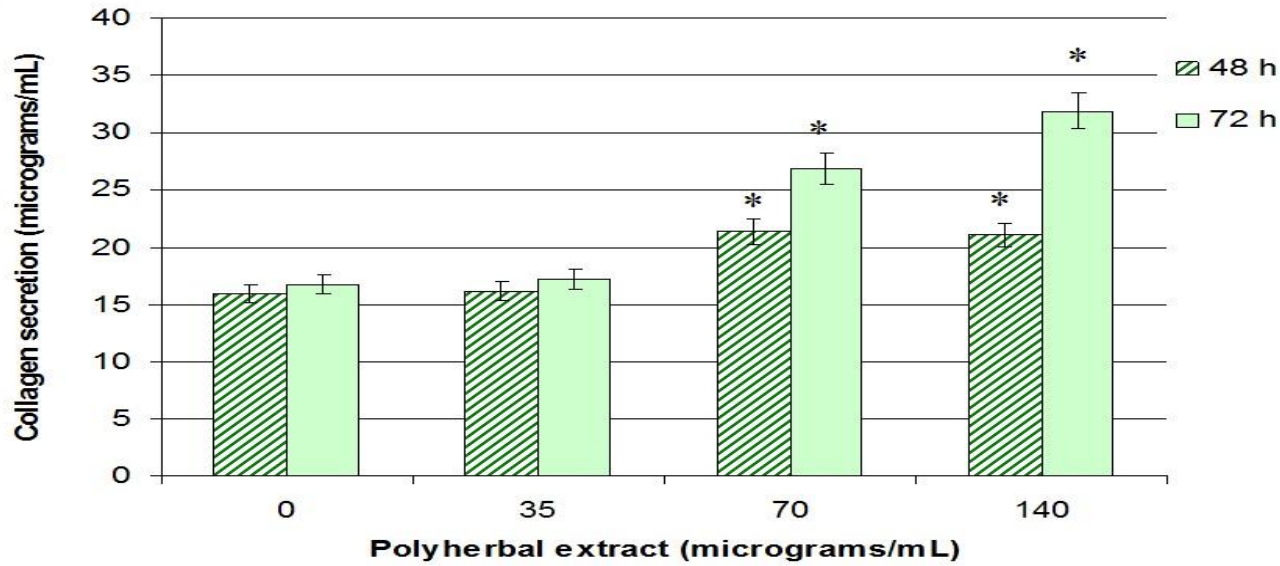

Fig. 4 Determination of collagen secretion by L929 fibroblast cells incubated with different concentrations of polyherbal extract, for 48 h and 72 h, using Sircol assay. Three independent experiments were performed with three replicates for each sample. Values are mean ± SD. *p < 0.05, compared to control (untreated cells).

The results showed a significantly ($p < 0.05$) increase of collagen synthesis in the culture medium of fibroblasts treated with 70 and 140 µg/mL polyherbal extract, after 48 h and 72 h of cultivation. It was observed that the collagen synthesis was almost 2 times higher in cultures treated with 140 µg/mL polyherbal extract, for 72 h, compared to the value obtained in the control group (0 µg/mL polyherbal extract).

Previous studies reported that natural polyphenols presented reducing properties, protection of intracellular lipids from oxidation and influenced collagen synthesis (Mucha *et al.*, 2013). Our experimental data suggested that increased collagen synthesis in L929 fibroblast cell culture was correlated to high phenolics content and, by default, with the antioxidant activity of the polyherbal extract.

CONCLUSIONS:

These results support the traditional use of this four-herb formula for wound care. The polyherbal extract had higher total phenolics content and antioxidant activity, compared to individual plant extracts. It was showed its *in vitro* capacity to stimulate collagen synthesis in a culture of fibroblast cells. Therefore, this combination of plant extracts may be useful as therapeutic agent in wound healing. Future studies could be performed, in order to find out its unexplored efficacy and high potential as a source of natural health care products.

ACKNOWLEDGMENT:

This study was supported by the Executive Unit for Financing Higher Education, Research, Development and Innovation (UEFISCDI), Project No. 62071 and Romanian Project BIODIV No. 102.

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