

STUDIES ON AGRIMONIAE HERBA SELECTIVE EXTRACTS; POLYPHENOLS CONTENT, ANTIOXIDANT AND ANTIMICROBIAL POTENCY, MTS TEST

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Abstract. Romanian folk medicine recommends agrimony, *Agrimonia eupatoria* L. (*Rosaceae* family) as natural treatment for different respiratory and eye infectious and inflammatory diseases, but also for genital and digestive illnesses, mainly liver diseases, as well as for varicose vein and ulcer leg condition. The work presented hereby was aimed at evaluating the general profile (polyphenols content, antioxidant potency, potential antimicrobial effects and the effects on cell culture in vitro) of some polar, water and ethanol, extracts obtained through the processing the aerial part of common agrimony. The results indicated an abundance of luteolin, quercetin, apigenin and kaempferol derivatives as well as good antioxidant potency, a weak effect against *P. aeruginosa* ATCC 9027 bacteria strain and no toxicity on the cell culture (MTS test).

Key words: *Agrimonia eupatoria* L., polar extracts, antioxidant, antimicrobial, MTS test

Introduction

Agrimonia eupatoria L. (fam. *Rosaceae*), commonly agrimony, is generally described as a mild antiseptic and astringent phytomedicine mainly recommended for sore throat and gastrointestinal ailments, also known for antiviral properties. Concerning scientific data, studies on different agrimony derived products and plant parts indicated important antioxidant effects, proved through both chemical and biological tests. For example, studies on healthy volunteers indicated that the consumption of agrimony's tea *has potential in improving markers of lipid metabolism, oxidative status and inflammation in healthy adults* [1].

Chemiluminescence studies [2] as well as DPPH assays [3] indicated augmented radical oxygen scavenger activity of the agrimony's water and ethanolic extracts, similar to those demonstrating superoxide anion, peroxy and hydroxyl radicals, hydrogen peroxide, hypochlorous acid and peroxy nitrite selective scavenging efficacy [4], generally attributed to polyphenol content.

Furthermore, the water extract from agrimony also demonstrated hepatoprotective effects on rats with chronic ethanol-induced liver injury (doses of 10, 30, 100, and 300 mg/kg/day), its beneficial effects being attributed to *the suppression of oxidative stress and TLR-mediated inflammatory signalling* [5].

Agrimoniin, found in Genera *Agrimonia*, but also *Potentilla* and *Fragaria* (e.g., in strawberries), has been proved to have potent anti-tumour activity [6], attributed to a complex mechanism which includes the enhancement of the immune response [7], interleukin (IL)-1 production stimulation [8] and/or protective activity against the environmental mutagens and carcinogens [9]; agrimoniin and oenothien B also were revealed with antimicrobial activity on *Helicobacter pylori* Gram-negative bacteria [10]. Moreover, the aqueous extract prepared from the aerial part has been demonstrated to have antiviral potency against hepatitis B virus, HBV [11].

Concerning the chemical profile, hydrolysable tannins, including agrimoniin and oenothien B found in high quantities (4-10 %) in Genera *Agrimoniae*, and proanthocyanidins, catechins, epicatechins, ellagitannins and gallotannins has been evidenced, as well as flavonoids (quercetin, luteolin, kampferol and apigenin glycosides), phenolic acids and triterpenic acids [12, 2].

The work presented hereby was aimed at evaluating the antimicrobial activity and potential toxic effects (MTS assay) of a standardized ethanolic extract obtained by processing the aqueous extract from the aerial part of *Agrimonia eupatoria* L. in order to extend our former studies [2] reporting good antioxidant potency and valuable chemical composition of *Agrimoniae herba* derived products.

Materials and methods

Plant material description

Agrimonia eupatoria L. (*herba*) has been purchased from a specialised Romanian Plant Product Company which sells packages of 50 grams of medium size plant powder (3-5 mm). Taxonomic aspect is certified by the Trade Company but has also been verified by the botanists' team at the *National Institute for Chemical - Pharmaceutical Research and Development* (ICCF), Bucharest, Romania and voucher specimen (AEcor) is deposited in ICCF *Plant Material Storing Room*.

Extracts' preparation

Technological studies have as the main purpose the obtainment of a selective ethanolic extract enriched in polyphenols compounds isolated from the aqueous extract thus assuring those phytotherapeutic compounds found in usual water and ethanolic extracts (e.g., teas, macerates, tinctures, infusions and decoctions).

This way, one hundred grams of plant product (fine powder) were extracted with 2000 mL of distilled water at boiling temperature (100°C) for 1 hour. The aqueous extract was concentrated at low pressure (*Büchi* Rotary Evaporator) at 50 mL final volume after that cooled at room temperature and treated with four volumes of (cold) ethanol 96 % (v/v). The suspension resulted has been filtered (at low pressure) and the filtrate used to prepare the *selective Agrimonia herba* (20 %, v/v) *ethanolic extract* (SAE20) with exactly 5 mg total phenols content expressed as gallic acid derivates (GAE) *per* 1 mL vegetal sample.

Chemicals, reagents and references

Chemicals (sodium carbonate, sodium acetate and aluminium chloride), reagents (commercial Folin-Ciocalteu, Natural Product and PEG4000 - NP/PEG) and solvents (ethanol, ethyl acetate, formic acid and glacial acetic acid) similar to *reference products* rutin (min. 95 %), hyperoside (>97 %), cosmosiin (97 %), vitexin (>96 %), chlorogenic acid (>95 %), caffeic acid (99 %) and cynaroside (>98 %) were purchased of *Fluka* and *Sigma-Aldrich* Co (Bucharest, Romania).

Qualitative analytical determination

Studies were performed according to *Plant Drug Analysis* [13] and *High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plant* [14], polyphenols assessment, as described in our previous paper (*System A*) [15].

Briefly, volumes measuring from 0.5 to 3 µL test vegetal product (SAE20) as well as reference samples (mixtures of 3-5 *Fluka* and *Sigma-Aldrich* phenolics) were loaded as 8 mm band length using Linomat 5 CAMAG instrument (Muttentz, Switzerland). Spots' assignment has been done by using reference compounds data and plant product literature data as well.

Estimation of Total Flavones content

Total flavones content was estimated by *Romanian Pharmacopoeias* method [16]. Briefly, (three) aliquots of 50 to 100 µL *test sample, Agrimoniae herba* derived products respectively, were treated with 600 µL of 2.5 % aluminium chloride and 1000 µL of 10 % sodium acetate (w/w) then finished at 5000 µL with (50 %, v/v) ethanol. Mixtures were incubated for 30 minutes at room temperature then the absorbance at maximum absorption wavelength (410 nm) measured. Rutin flavonol has been used as standard compound and calibration curve subject matter ($r^2=0.988$) and the results expressed as total flavones, mg (R) / 1 mL *test sample*.

Estimation of Total Phenols Content

Total phenols content was estimated by *Folin-Ciocalteu* reagent and *Romanian Pharmacopoeias* method too [16].

Briefly, (three) aliquots of 25 to 50 μL *test sample* were treated with 200 μL of *Folin-Ciocalteu* reagent then finished at 5000 μL with 5 % (w/v) sodium carbonate. Flasks were mixed and left at room temp. for 5 min then the absorbance at 750 nm measured. Total phenols content was estimated by using gallic acid (ref.) standard calibration curve ($r^2=0.9997$) and the results expressed as total phenols, mg (GAE) / 1 mL *test sample*.

Antioxidant activity assay

Studies have been done on the *test samples* prepared in 70% (v/v) ethanol; dilution series x1, x2, x5, etc...x50. Luminol solution at $p\text{H}=8.6$ was used as chemiluminescence reaction and radical oxygen species production indicator [17].

Briefly, aliquots of 50 μL *test sample* (dilution series respectively) were mixed with 200 μL 10^{-3}M luminol (prepared in DMSO solvent), 700 μL 0.2M – TRIS-HCl ($p\text{H}$ 8.6, prepared in bidistilled water) and 50 μL 10^{-3}M H_2O_2 (prepared in bidistilled water). Similarly, a reference sample consisting in 50 μL 70% (v/v) ethanol solvent mixed with identical quantities of luminol, TRIS-HCl and H_2O_2 has been done. CL reaction intensity (activity units/a.u. at each 5 seconds, 60 seconds' total times) of the reference sample and *test sample* (dilution series respectively) have been measured and the obtained values, a.u. at 5 seconds, were further used for antioxidant activity (AA%) estimation (see formula). IC_{50} value (described as concentration, $\mu\text{g}/\text{mL}$, of the sample inhibiting fifty percent of the reactive oxygen species/ROS production) was calculated and compared with Rutin (R) and Gallic acid (GAE) reference compounds values.

$$\frac{\text{CL reaction intensity of the reference sample (a.u.)} - \text{CL reaction intensity of the tested sample (a.u.)}{\text{CL reaction intensity of the reference sample (a.u.)}} \times 100$$

Microbiological Test

Studies, cylinder method in plates respectively, were performed according to *Romanian Pharmacopoeias* [16] and detailed in our previous work [15].

There were used four standard strains, two Gram-negative (*Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739) and two Gram-positive (*Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermitis* ATCC 12228) bacteria; test organisms were purchased from *Mecconti* (*Merck Romania S.R.L.*).

Studies were done on the standardized *selective agrimony ethanolic extract*, SAE20, with exactly 5 mg GAE *per* 1 ml sample/extract. Antimicrobial potency was estimated on basis of the diameter of growth inhibition where diameter less than 10 mm means no antimicrobial activity, diameter from 10 to 15 mm means weak antimicrobial activity, diameter from 16 to 20 mm means moderate activity and diameter more than 20 mm means certain activity.

Cell viability assay – MTS test

The viability test was performed according to Technical Bulletin of *Promega Corporation, CellTiter 96 AQueous One solution Cell Proliferation Assay* [18].

General principle

The MTS tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is bio reduced by cells into a coloured formazan product that is soluble in tissue culture medium (this conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells).

Assay is performed by adding a small amount of the CellTiter 96 AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours then recording the absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells in culture. Each treatment (cell series treated with *test extract* at different doses – dilution series) is carried out in quadruplicate and cell survival is calculated with respect to untreated controls.

Materials

The Cell Proliferation Kit, *CellTiter 96 Aqueous One solution Cell Proliferation Assay* (MTS), was purchased from *Promega Corporation*. Mouse fibroblasts 3T3-L1(ATCC-CL-173) were obtained from ATCC (LGC Standards, Germany) while Dulbecco's Modified Essential Media (DMEM), Fetal Bovine Serum (FBS) and antibiotics, both were purchased from *Sigma-Aldrich* Romania.

Method

The fibroblasts were cultured in DMEM supplemented with 10 % FBS and 1 % antibiotics (10,000 units / mL penicillin and 10,000 µg / mL streptomycin in 0.85 % saline) and incubated at 37°C in 5 % CO₂ humidified atmosphere. After reaching confluence, the cells were detached from the flask with Trypsin-EDTA. The cell suspension was centrifuged at 2000 rpm for 5 min and then suspended in the growth medium. Cells are next seeded in 96-well plates at a density of 4000 cells *per* well in 200 µL of culture medium. Cells were allowed to attach and

achieve approximately 80 % confluence prior to starting test vegetal experiments. This way, different concentrations of the *test vegetal extract* are prepared in complete culture media consisting of DMEM supplemented with 10 % FBS and 1 % antibiotic solution. Subsequently, the effects of the samples on the viability of (3T3 mouse) fibroblasts is evaluated using CellTiter 96 AQueous One Solution Cell Proliferation Assay(a), MTS test respectively. Accordingly, the culture medium from the wells was removed and replaced with 200 μ L new culture media containing the *standardized test vegetal extract* (SAE20) at increasing doses: dilution series 0.5, 5, 50, 100, and 150 μ g / mL respectively. After 12 hours of exposure, the culture medium was removed. After other two hours of incubation with MTS solution, viability of the adherent cells was determined using MTS reduction; the MTS-formazan absorbance was detected by using *Chameleon V Plate Reader, LKB Instruments* and the recorded values, sample absorbance at 490 nm, used for cell viability estimation (formula).

$$\% \text{ cell viability} = \frac{A_{490} \text{ of treated cells}}{A_{490} \text{ of control cells}} \times 100$$

Statistical analysis

Results were calculated as mean \pm SD, n=5.

Results and discussions

Analytical results

Figure 1 shows qualitative (HP)TLC aspects referring to polyphenols content of the test product, selective *Agrimoniae herba* ethanolic extract (SAE20), by comparison with polyphenols compounds, reference products mixtures (ref.).

As shown, the selective *Agrimoniae herba* ethanolic extract contains numerous polyphenol compounds such as quercetin derivates (yellow-orange fluorescent/fl. spots s2, s6 and s10), including rutin, hyperoside and quercitrin compounds, luteolin derivates (yellow fl. spots s3, s5 and s8), likely isoorientin, orientin (the major compound) and cynaroside compounds, as well as apigenin (green fl. spot s9) and kaempferol (blue-green fl. spot s11) derivates, likely cosmosiin and tiliroside along with smaller quantities of caffeoyl quinic acid derivates, (neo) chlorogenic (blue fl. zone s4) and caffeic (blue-marine fl. spot s12) acids.

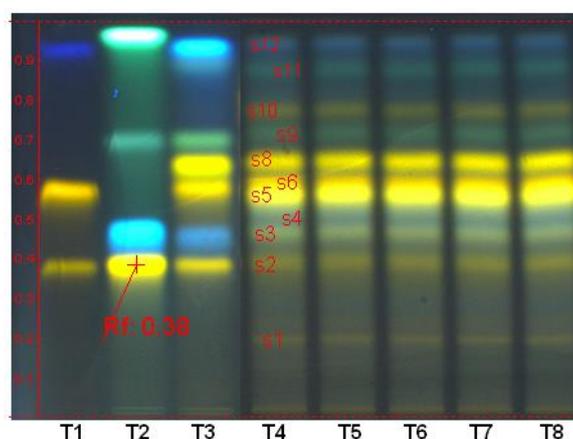


Figure 1. Polyphenols profile of the selective *Agrimoniae herba* ethanolic extract. T1 track – quercetin-3-O-rutinoside/ rutin, quercetin-3-O-galactoside/ hyperoside and protocatechuic acid (ref.); T3 track – rutin, chlorogenic acid, apigenin-7-O-glucoside/ cosmosiin and kaempferol (ref); T2 track – rutin, chlorogenic acid, hyperoside, luteolin-7-O-glucoside/ cynaroside, apigenin-8-C-glucoside/ vitexine and caffeic acid (ref.); T4-T8 tracks: selective *Agrimoniae herba* ethanolic extract.

Therefore, HPTLC analysis suggests that the polar extracts, water and ethanolic type of extracts such as teas, macerates, tinctures and infusions or decoctions, obtained through processing *Agrimoniae herba* plant material contains valuable polyphenol compounds with multiple therapeutic valences, luteolin derivatives being estimated the dominant polyphenol species; this visual appraisal has also been confirmed by an UV-Vis spectrum with maximum absorption wavelength at 410 nm, so that identical with that shown by luteolin reference compound.

Antioxidant activity results

Figure 2 shows antioxidant activity (CL assay, activity units/a.u. at 5 seconds) face to sample concentration ($\mu\text{g} / \text{mL}$) of the two reference compounds, rutin and gallic acid phenolics in order to evaluate their IC_{50} values; it must be noted that rutin and gallic acid references were prepared as 10^{-3}M ethanol solutions (70 %, v/v), eight dilution series (x1 - x2 - x5 - x6 - x7 - x8 - x9 - x10) respectively.

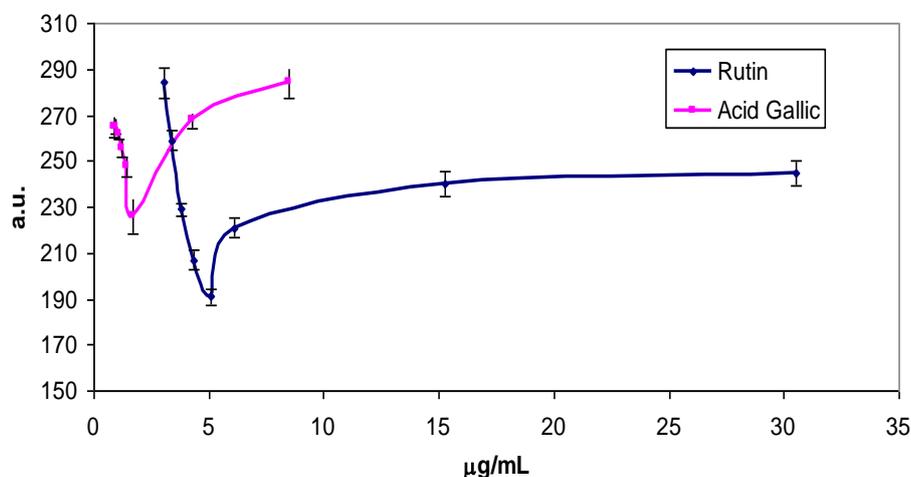


Figure 2. IC₅₀ assay on the two reference samples, rutin and gallic acid phenolics. Similarly, **Figure 3** shows IC₅₀ assay on the test sample, selective *Agrimonia eupatoria* ethanolic extract (SAE20), seven dilution series (x1 - x2 - x5 - x10 - x12 - x15 - x50) respectively.

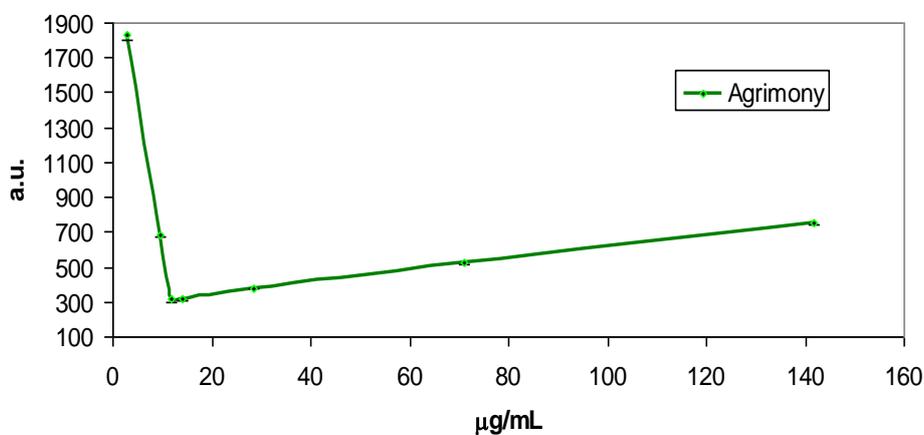


Figure 3. IC₅₀ assay on the selective *Agrimonia eupatoria* ethanolic extract (SAE20)

Accordingly, chemiluminescence (CL) studies and IC₅₀ assays indicated high antioxidant potency of the two reference compounds: rutin has been estimated with IC₅₀ = 2.54 µg / mL while gallic acid resulted in IC₅₀ = 0.85 µg / mL. Comparatively, the selective *Agrimonia eupatoria* ethanolic extract (SAE20) has been estimated with IC₅₀ = 5.91 µg / mL thus suggesting good antioxidant potency of *Agrimoniae herba* derived products, polar extracts respectively.

Microbiological results

Antimicrobial activity screening was purchased on four standard microbial strains, two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739) and two Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermitis* ATCC 12228). Test products were placed on the plate as follows: in each cylinder was added 0.2 mL of sample, one cylinder containing the solvent sample and the other three the selective agrimony ethanolic extract (SAE20) this meaning 100 µg total phenols (gallic acid equivalents) *per* each cylinder. Results are presented in Table 1.

Table 1. Antimicrobial assay on selective *Agrimoniae herba* ethanolic extract (SAE20)

Plant extract	Microbial strain	Diameter (mm) of inhibition zone
Selective <i>Agrimoniae herba</i> ethanolic extract (SAE20)	<i>Staphylococcus aureus</i> ATCC 6538	< 8
	<i>Escherichia coli</i> ATCC 8739	< 8
	<i>Staphylococcus epidermitis</i> ATCC 12228	< 8
	<i>Pseudomonas aeruginosa</i> ATCC 9027	15

Values are mean inhibition zone (mm) ± S.D. of three replicates. Where diam. < 10 mm means *no activity*, diam. 10-15 mm means *weak activity*, diam. 16-20 mm means *moderate activity* and diam. > 20 mm means *certain antimicrobial activity*.

The obtained results (**Table 1**) suggest the lack of antimicrobial activity of the *test extract* SAE20 on *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermitis* ATCC 12228 strains, and only weak inhibitory activity on *Pseudomonas aeruginosa* ATCC 9027. It must be noted that the extract solvent, 20 % ethanol, does not show any antimicrobial activity either.

In vitro pharmacological studies results – SAE20 cytotoxicity

The main purpose of the studies was the evaluation of SAE20 potential toxicity on 3T3 cells. As it has been presented (Section 2.9.), the effects of SAE20 on 3T3 viability were evaluated using a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTS into purple coloured formazan crystals. The quantity of formazan product, as measured by absorbance at 490nm, is directly proportional to the number of living cells in culture, so that the effect of *the sample* on the proliferation of 3T3 cells can be calculated as cell viability percentage (%).

The SAE20 results are summarized in Figure 4.

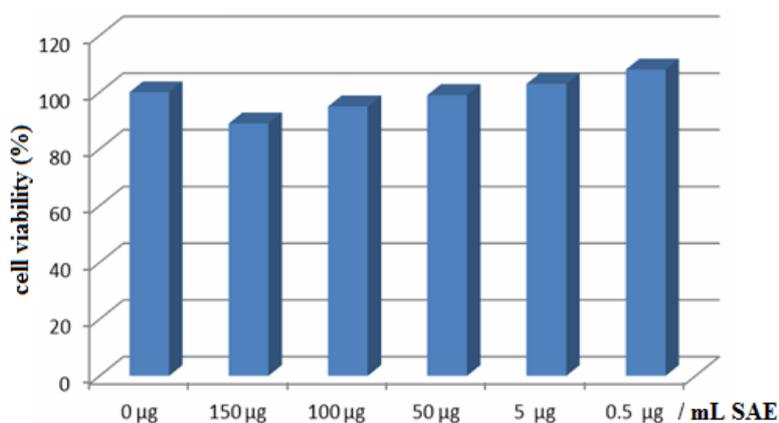


Figure 4. MTS assay, percentage of cell viability on 3T3 fibroblasts exposed to different concentrations of selective *Agrimonia eupatoria* ethanolic extract (SAE20)

As shown in Figure 4, clearly the cells are viable in all studied concentrations of SAE20. Moreover, it can be seen that the smallest SAE20 concentration (0.5 and 5 µg GAE / mL sample) increased the cell's viability; with increasing extract concentration (over 100 µg GAE / mL), the cell viability percentage slightly decreased.

Therefore, the test indicates the safety of usual doses of *Agrimoniae herba* polar, water and ethanolic extracts, the highest doses of the test extract, respectively SAE20 over 50 µg GAE / mL, not being of practical or pharmacological use.

Conclusions

The official document elaborated by European Medicines Agency - EMA [19] presents common agrimony (*Agrimonia eupatoria* L.) as a medicinal plant, in powder form (the dried plant parts) or prepared as liquid extract with water and alcohol, recommended for external use in different skin ailments and as bath additive, but also for internal treatment in mild diarrhoea and mouth and throat inflammation.

Our studies on a selective ethanol extract (SAE20) isolated through processing the origin aqueous extract (thus assuring those phytotherapeutic compounds found in usual teas, macerates, tinctures, infusions and decoctions) obtained by processing *Agrimoniae herba* plant material indicated a valuable chemical qualitative content (HPTLC analysis) consisting in numerous polyphenol compounds such as luteolin derivates – the major polyphenols (e.g., isorientin, orientin and cynaroside), along with quercetin (e.g., rutin, hyperoside

and quercitrin), apigenin (cosmosiin) and kaempferol (tiliroside) derivatives as well as some smaller quantities of caffeoyl quinic acid derivatives (e.g., neo/chlorogenic and caffeic acids).

Antioxidant activity assay, respectively chemiluminescence studies, indicated good antioxidant potency ($IC_{50} = 5.91 \mu\text{g} / \text{mL}$) of the selective ethanol extract (SAE20). For contrast, rutin flavonol glycoside indicated $IC_{50} = 2.54 \mu\text{g} / \text{mL}$ while gallic acid phenolic $IC_{50} = 0.85 \mu\text{g} / \text{mL}$.

Antimicrobial activity screening on four clinically applicable standard bacteria indicated the lack of activity of the selective ethanol extract (SAE20) on *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermitis* ATCC 12228 strains, and only weak activity on *Pseudomonas aeruginosa* ATCC 9027 strain; it must be reminded that the sample tested (SAE20) provided $100 \mu\text{g}$ total phenols (GAE) *per* cylinder.

In vitro pharmacological studies, MTS assay, in order to estimate potential toxicity of the selective *Agrimoniae herba* ethanolic extract (SAE20) on 3T3 cells indicated that the cells were viable in all studied concentrations (0.5, 5, 50, 100, 150 μg GAE / mL). Summing all, MTS assay indicated that agrimony selective extracts with total phenols content measuring from 0.5 to 50 μg GAE *per* 1 mL sample give viability nearest value of the control sample thus showing the lack of toxicity in *Agrimoniae herba* polar, water and ethanol, extracts.

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