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ABSTRACT

EXPERIMENTAL PHARMACOTOXICOLOGICAL EVALUATIONS OF *GALIUM VERUM* L. AND *GALIUM MOLLUGO* L. SPECIES IN SKIN CANCER

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SUMMARY

1. INTRODUCTION

Melanoma is a form of cancer that arises from the uncontrolled proliferation of melanocytes. Cutaneous melanoma is considered the major cause of mortality among skin cancers, which shows a continuous increase in its incidence. The World Health Organization (WHO) reports that melanoma is the fifth most common type of cancer in men and the sixth in women. Melanoma management has many implications, thus several approaches can be taken, from excision, adjuvant therapies (radiation, chemotherapy, and/or surgery), tumor staging and, even therapy through counseling and lifestyle modification.

Nowadays, due to the appearance of resistance to the existing chemotherapeutics and the serious adverse effects that occur following their administration, it is desired to replace them with less toxic therapies. Therefore, interest in natural treatment has gained momentum. Different extracts and phytocompounds obtained from plant barks, roots, leaves, stems, and flowers have demonstrated a promising effect in cancer treatment, an important step in the synthesis of new anti-melanoma drugs. Phytochemical compounds exert a variety of anti-inflammatory and antioxidant actions and have a strong potential to induce chemopreventive effects in melanoma. Phytocompounds, such as polyphenols and terpenoids, show promising antitumor activity, thus being able to be used in the treatment of melanoma.

Since ancient times, *Galium* species have been used in traditional medicine, being a rich source of active compounds such as flavonoids, phenolic acids, terpenes, glycosides, and essential oils. Thus, due to the vast composition, *Galium* species have revealed diuretic, laxative, cicatrizing, antirheumatic, sedative, and

anti-inflammatory properties; and in recent years, emphasis has been placed on the investigation of the plant in cancer therapy.

2. AIM AND OUTLINE

The aim of this thesis was the phytochemical evaluation of *Galium verum* L. and *Galium mollugo* L. extracts and the *in vitro* investigation of the anticancer capacity on human and murine melanoma lines; being an important step in the management of melanoma, a pathology with an increased incidence in recent years.

The first part of this thesis provides information from the recent specialized literature about the management of melanoma and the role of plants and natural compounds in melanoma therapy. Later, the anticancer action of *Galium* plant species and the compounds in the composition were addressed.

The personal contribution consisted, first of all, of the phytochemical investigation of extracts of *Galium verum* L. and *Galium mollugo* L. (procured from the drug store but also collected from the spontaneous flora of Romania) made with different solvents. I used different methods to investigate the most promising extracts such as liquid chromatography coupled with mass spectrometry (LC-MS) to determine the content of compounds, but also Fourier transform infrared spectroscopy (FT-IR) to outline their spectrum, together with the DPPH test to determine the antioxidant potential of the extracts. Subsequently, we investigated the antimicrobial activity using the disk diffusion method, to establish their potential on the most known bacterial strains.

The next step was to determine the *in vitro* action of the extracts. Initially, the safety level was evaluated on the healthy human keratinocyte cell line (HaCaT) and then the anticancer effect was investigated on human melanoma (A375) and murine melanoma (B164A5) cell lines. Thus, the cells were evaluated in terms of morphological changes and the impact on confluence, viability (MTT test),

cytotoxicity (LDH method), potential apoptotic amplitude (Hoechst test), and migratory capacity of cells (using wound healing assay).

The last step was the *in ovo* investigation using the chorioallantoic membrane assay and hen's egg chorioallantoic membrane test of the extracts collected from the spontaneous flora, to determine the tolerance profile in the vascular plexus and irritant action.

3. RESULTS

3.1. PHYTOCHEMICAL SCREENING OF *GALIUM VERUM* L. AND *GALIUM MOLLUGO* L.

Using LC-MS, it was obtained that extracts of *Galium* sp. present significant amounts of polyphenolic compounds. In the GvEtOH extract (from the plant material purchased from a local specialty store) 7 compounds were identified, in the highest percentage were found isoquercitrin and rutin (17,765 µg/mL and 14,811 µg/mL, respectively); similar to GvEtOH, in the GvEtOAc extract isoquercitrin was identified in the largest amount (20,384 µg/mL), together with other phenolic compounds. In the case of GvDEE, 7 compounds were also detected, but the presence of new phytocompounds was identified (p-coumaric acid and ferulic acid), the most abundant this time being quercetol; while in the GvBuOH phase, the presence of isoquercitrin, rutin and chlorogenic acid was found in small quantities. Moreover, in the GvPEE phase, 4 polyphenolic compounds were present but were not quantified because they were below the detection limit (0.1 µg/mL), while in the GvH_2O phase, 2 compounds were quantified. On the other hand, in the GVEtOH extract from *Galium verum* L. collected from the spontaneous flora, 6 compounds were identified (rutin - 25.945 µg/mL), including hyperoside, a compound not identified in the other extracts; and in GmEtOH from *Galium mollugo* L., 4 compounds were identified, 3 of which were quantified (chlorogenic acid, rutin and isoquercitrin). In addition, the

presence of epicatechin was identified in the GvEtOH and GvDEE extracts, while in the GvEtOAc phase, the presence of gallic acid was quantified in a small amount (0.34 µg/mL).

Considering the match between the absorption bands recorded at a certain wave number and then comparing them with the frequency of the absorption band in the library, we were able to identify the main polyphenols contained in the dry extracts, through the FT-IR qualitative investigation method, data that have was in agreement with the results obtained by the LC-MS method.

Analyzing antioxidant capacity, it was found that all tested extracts of *Galium* sp. possess antioxidant potential compared to the standard (ascorbic acid). Regarding the highest concentration tested (1 mg/mL), the antioxidant activity of each extract respects the linearity GvEtOAc > GvDEE > GvEtOH > GvBuOH > GvH_2O > GvPEE (Figure 1).

Therefore, the phytochemical results indicate the presence of bioactive compounds; rutin is the main compound identified, the highest concentration is found in the ethanolic extracts of *G. verum* L. (GVEtOH > GvEtOH), followed by GvBuOH. Moreover, significant amounts of isoquercitrin and chlorogenic acid were identified in all extracts. All tested extracts exhibited significant antioxidant potency, in a concentration-dependent manner, the most significant being for the GvEtOAc extract.

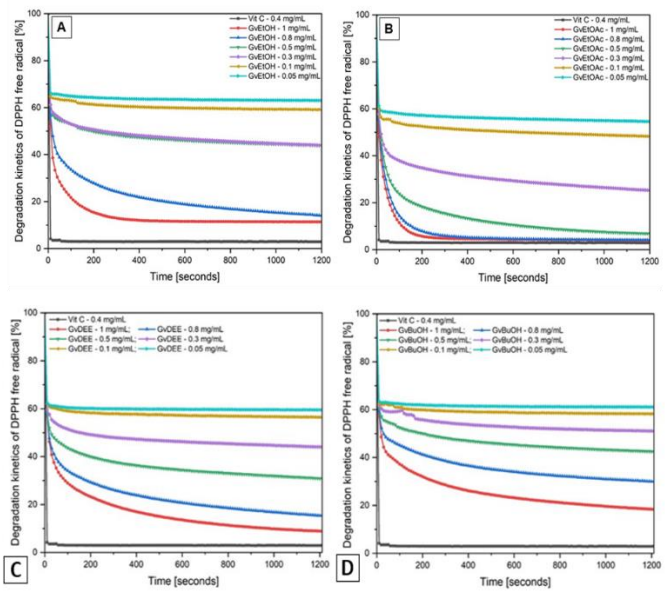

Figure 1. The time-dependent degradation kinetics of DPPH free radicals are provided by the ethanol (A), ethyl acetate (B), diethyl ether (C) and butanol (D) *Galium verum* L. extracts as well as by the ethanolic solution of vitamin C (black line).

3.2. MICROBIOLOGICAL ASSESSMENT OF *GALIUM VERUM* L. AND *GALIUM MOLLUGO* L. EXTRACTS

The Disk diffusion method provides evidence that the extracts show important antimicrobial action, especially on the Gram-positive bacterial strains and less on the negative ones. Furthermore, it was highlighted that the GVEtOH extract possessed the strongest activity on *Streptococcus pyogenes* and *Staphylococcus aureus*, along with GvDEE. By comparison, we can see that the GvEtOH extract (procured from the pharmacy) has a lower antimicrobial effect than GVEtOH (from the spontaneous flora), in addition, GmEtOH exerts a stronger activity than GvEtOH.

3.3. EXPERIMENTAL BIOLOGICAL EVALUATION OF *GALIUM VERUM* L. AND *GALIUM MOLLUGO* L. EXTRACTS

Following the *in vitro* studies carried out on keratinocytes, it can be concluded that the studied extracts do not significantly decrease the viability of healthy cells, nor do they interfere with cell morphology up to a concentration of ≈55 µg/mL, after this concentration, a decrease in viability is evident for the GVEtOH and GmEtOH extracts (Figure 2).

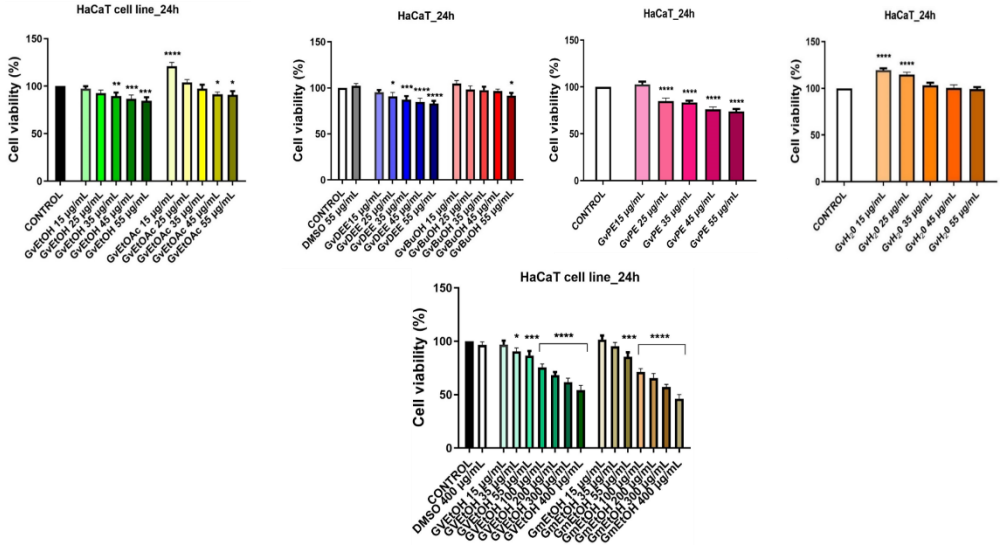

Figure 2. Viability percentage of HaCaT cells after stimulation with the *G. verum* L. and G. *mollugo* L. extracts at 24 h post-stimulation.

At the level of tumor cells, it was observed that at concentration of 55 µg/mL, GvPE decreases the viability of A375 cells in the highest proportion (55.4%), but this may be due to the solvent because, from a phytochemical point of view, no polyphenolic compounds were identified to attest to its effect. In accordance with the composition, GvEtOAc decreased the percentage of viable cells, followed by GVEtOH. Between the two ethanolic extracts of *G. verum* L, no significant

differences were observed at the highest tested concentration – 55 µg/mL (GVEtOH – 70.2% vs GvEtOH - 77.8%) (Figure 3).

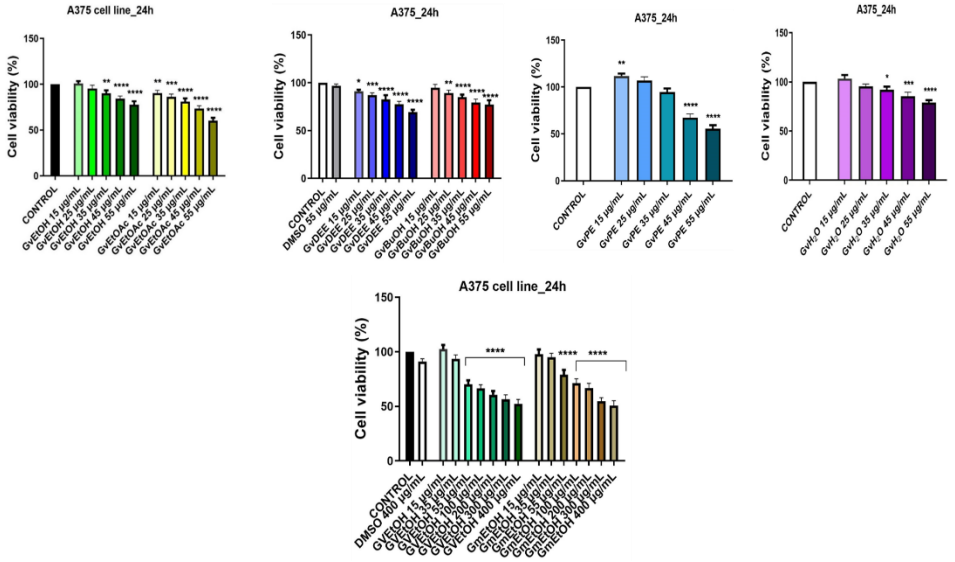

Figure 3. Viability percentage of A375 cells after stimulation with the *G. verum* L. and G. *mollugo* L. extracts at 24 h post-stimulation.

The extracts produced no significant changes in cell morphology of HaCaT, with cells remaining similar to untreated control cells, and had no negative impact on cells' adherence or confluence until the concentration of 55 µg/mL.

In the case of tumor cells, changes in shape and confluence were visible, depending on the extract and the tested concentration. At the highest concentration tested, their shape became round and shrank. More signs of cell death were observed, cells became stressed, detached from the plate and confluence decreased. Moreover, signs of cell apoptosis were evident, with nuclear condensation and the appearance of apoptotic bodies at 55 µg/mL.

Regarding murine melanoma cells, GVEtOH was observed to have a stronger cytotoxic action on the B164A5 cell line than on A375. Also, up to the concentration of 200 µg/mL, GVEtOH demonstrated a more significant inhibitory

effect on B164A5 cells than the GmEtOH extract, but with increasing concentration, GmEtOH becomes more cytotoxic. At the concentration of 15 µg/mL, no cells that have undergone changes are observed, only a slight decrease in the confluency for GmEtOH. From the dose of 55 µg/mL, the confluency drops visibly, especially for the GVEtOH extract, following the same route at the concentrations of 200 and 400 µg/ml. At the highest concentrations, cellular changes are observed, the elongated cells become more rounded and detach from the plate. Moreover, following the Hoechst assay, we can observe at 55 µg/mL that the shape of the nucleus changes and begins to no longer have a normal appearance. At 200 µg/mL, pro-apoptotic signs are seen, such as chromatin condensation. Also, a slight disintegration of the nucleus observed through membranes in waves is exposed. As the dose increases, the same trend continues, but more nuclei are affected, with more prominent apoptotic signs.

3.4. IN OVO EVALUATION OF THE *GALIUM VERUM* L. AND *GALIUM MOLLUGO* L. EXTRACTS

The evaluation of GVEtOH and GmEtOH at concentrations of 400 ug/mL upon the developing chorioallantoic membranes was considered during a period of rapid growth of the vessel branching, to estimate the potential inhibitory effect on the angiogenic process. After 24 hours from the inoculation of the extracts, observation of the developing membranes indicated a normal, intense angiogenic process for this period of administration, with no signs of diminishment of vessel branching pattern (Figure 4).

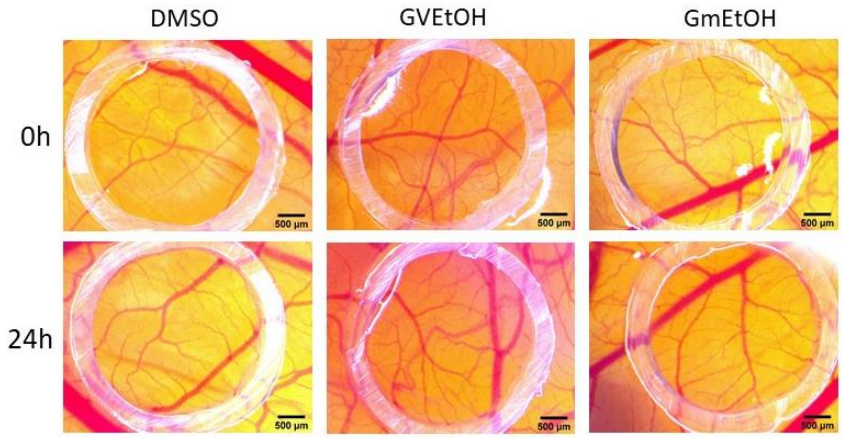

Figure 4. Effects of GVEtOH and GmEtOH extracts on normal angiogenesis on the CAM.

Both *Galium* sp. extracts were evaluated at the concentration of 400 µg/Ml for the potential irritability towards epithelial tissues, using the HET-CAM assay. No sign of toxicity was registered on the chorioallantoic membrane in terms of hemorrage, vascular lysis and coagulability, compared to the positive control, SDS, that induced a strong irritative effect (Figure 5).

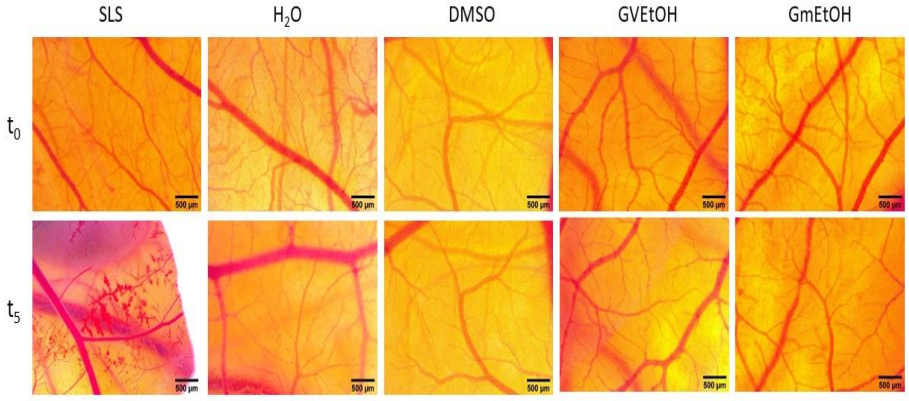

Figure 5. Stereomicroscopic images of the HET-CAM method illustrating effects initially (t_0) and 5 min (t_5) after application of the samples: negative control (H_2O), positive control (SDS), and *Galium* sp. extractS (GVEtOH and GmEtOH)

4. CONCLUSIONS

Following the analyses, we can state that the extracts have antioxidant, antimicrobial, and cytotoxic activity, being a possible candidate for melanoma therapy. Nevertheless, further investigations of the extracts and their phytocompounds are needed to establish in detail the mechanism of action underlying this cytotoxic effect.

The novelty of the study consists in the fact that, although *Galium verum* L.has shown important antitumor potential on different cancer cell lines, its effectiveness in skin cancer has not yet been established, and moreover, *Galium mollugo* L. has not yet been studied regarding its action anticancer. We believe that the results obtained in the present study will complement the lack of information in the specialized literature.