

NATURAL DRUGS

HERBAL PREPARATION EXTRACT FOR SKIN AFTER
RADIOTHERAPY TREATMENT. PART ONE – PRECLINICAL TESTSAGNIESZKA SKALSKA-KAMIŃSKA^{1*}, ANNA WOŹNIAK², ROMAN PADUCH³,
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Abstract: Naran R is a herbal composition made of *Plantago lanceolata folium*, *Malvae arboreae flos*, *Calendulae flos*, *Chamomillae inflorescentia*, *Lamii albi flos* to prepare compresses or to wash skin with inflammations. The extract of this preparation is mixed to be applied as an ointment on patients' skin after radiotherapy. Experiments performed *in vitro* are part of pre-clinical tests with Naran R ointment. This study examined the impact of the plant composition for ethanol-water extract on human skin fibroblasts (HSF) culture. Samples of extract, prepared from patented amounts of herbs, were in the range of 25–225 µg/mL. Six methods were applied: standard spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, neutral red (NR) uptake assay, DPPH free radical scavenging test, labeling of cytoskeleton F-actin, staining of argyrophilic nucleolar organizer regions (AgNORs) and trypan blue coloration. The extract concentration 75 µg/mL was established as safe for application on human skin. In labeling of F-actin with rhodamine-phalloidin dye at this concentration the cytoskeleton was stable. The extract did not influence the membrane stability and had positive influence on the proliferation activity. It was confirmed in AgNOR test during incubation with extract, which led to formation of larger amount of smaller nucleolins. In DPPH scavenging activity test, the extract revealed over 8% higher free-radical scavenging activity in comparison to control. After trypan blue staining, the extract in concentration 125 µg/mL significantly lowered the cell viability. When the cytotoxic and anti-proliferative activity of the extracts were analyzed, MTT and Neutral Red (NR) methods were used. The cells' viability was maintained on a constant level (80–110%) after 24, 48 and 72 h of incubation. During all time of NR test (72 h) and even when 225 µg/mL of extract was applied, the viability of cells was in range 80–110% of control. Positive influence of the extract on investigated cells structure and proliferation, lack of toxicity and increasing anti-oxidant activity enable to consider this preparation as a natural remedy with potential application in skin therapy after radiation.

Keywords: ethanol-water Naran R extract, biological investigations, skin treatment, HSF cells, radiodermatitis

Cells culture is an important element of pre-clinical investigation of new medical formulations. It reflects the influence of biologically active compounds on the whole organism. Knowing how the investigated factor affects the human skin fibroblast cells one may predict its action on patient's skin (1).

Naran R is one of herbal preparation from Naran series. Previous publications were related to investigations of biologically active components of medicinal plants from Naran N formulation (2–4).

The presented publication contains a series of pre-clinical investigations with ethanol-water extract of Naran R. It is a preparation composed of

Plantago lanceolata L. (Plantaginaceae), *Althaea rosea* var. *nigra* Cav. (Malvaceae), *Calendula officinalis* L. (Astraceae), *Chamomilla recutita* L. Rauschert (Astraceae) and *Lamium album* L. (Lamiaceae). Medical raw materials of these plants with anti-inflammatory, scavenging free radicals and skin irritations healing activities are still used as the supporting factors in medicine. This natural herbal composition is intended to be applied, in form of ointment with ethanol-water extract, on the skin of patients after radiotherapy.

Radiation, which is used in cancer therapy, damages healthy tissues before it reaches the tumor.

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The ray beam destructs proliferating tissues, for example, skin with the layer of fibroblasts. Disorders of structure and functions of healthy cells, described as radiodermatitis, are skin damages after radiation (5).

Postradiation skin inflammation is difficult to cure. It has a local character but often is transformed to delayed radiation effect, which may be one of cancer causes (6, 7). The main factors that damage the skin are free radicals originating from irradiated water molecules and from granulocytes in the inflammation area (8).

Treatment of radiodermatitis is complicated and clinically relatively weak results are obtained. In our study, we propose that the plant extract of the composition can be used in form of an ointment for external use (9). We suggest that it can enrich the panel of materials used in patients treatment. In biological investigations the safe concentration of Naran R ethanol-water extract was determined and it can be used for water/oil (W/O) emulsion ointment preparation.

MATERIALS AND METHODS

Ethanol-water extract was made of Naran R. It is a medical composition made of dried and crumbled plant materials: *Plantago lanceolata folium*, *Malvae arboreae flos*, *Calendulae flos*, *Chamomillae inflorescentia*, *Lamii albi flos*. These raw materials were derived from natural state and plant crops near Lublin (Dys), collected in July 2011. The amounts of all components are patented (10). Distilled water and ethanol 95% were added (1 : 1 v/v). Extraction at temperature of 50°C, under reflux was conducted for 5 h. Then, the extract was filtered and evaporated to dryness in a rotary evaporator HB 4 Basic RV 05-ST (IKA, Łódź, Poland). For investigations with cultures of HSF cells the dry residue was dissolved in dimethyl sulfoxide (DMSO) to obtain appropriate concentrations.

In biological experiments, to prepare the HSF cell culture freshly excised small fragments of human skin were used, which were washed two times using RPMI (1640) medium (Gibco™, Paisley, UK) supplemented with antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) (Gibco) and then placed into wells of 24-well plate. The tissue fragment was cut out from the forearm of one of the study's co-author (Roman Paduch). He agreed to use the tissue for presented experiments and whole procedure of explants preparation and cell culture establishment was performed by himself. The explants were then

overlaid with a warm 1 : 1 (v/v) mixture of 1% agarose and RPMI 1640 medium. The culture was performed by adding the culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco) on top of agarose gel and incubated at 37°C in a humidified 5% CO₂/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured. For experiments, HSF cells obtained from two donors were used.

In investigations of extract toxicity, HSF cell cultures in a density of 1×10^5 cell/mL were used. Incubation was conducted for 24 h. When investigations of extract activity were performed, HSF cell cultures with a density of 2×10^4 cell/mL were used. Incubation was conducted for 72 h.

The HSF cells were cultured as monolayers in 25 cm² culture flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% FBS (v/v) and antibiotics at 37°C in a humidified atmosphere with 5% CO₂. For experiments, the total number of cells was estimated by counting in hemocytometer. A 100 µL sample of cell suspension (2×10^4 cells/mL for cells proliferation activity tests or 1×10^5 cells/mL for toxicity tests) was added to appropriate wells of 96-well flat-bottomed microtitre plates (MTT and NR methods). After 24 h of incubation, the medium was discarded and new medium containing 2% FBS and appropriate plant extract concentrations in 25–225 µg/mL range was added. As a control, HSF cells suspended in 100 µL of culture medium with 2% FBS without plant extracts was used. The total cell number was equivalent to that in the sample wells. Additional controls without cells but containing appropriate plant extract concentrations in 2% FBS culture medium were prepared to exclude non-specific dye reduction (MTT method) (11). As blank control culture medium with 2% FBS was used. Sensitivity of cells to Naran R extract activity was determined by a standard spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann (11). MTT test is based on conversion of yellow tetrazolium salt by viable cells to purple crystals of formazan. The reaction is catalyzed by mitochondrial succinyl dehydrogenase. The cells grown in 96-well multiplates in 100 µL of culture medium supplemented with 2% FBS were incubated for 3 h with MTT solution (5 mg/mL, 25 µL/well) (Sigma, St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 10% sodium dodecyl sulfate (SDS) (Sigma) in 0.01 M HCl. The product was quantified spectrophotometri-

cally by absorbance measurement at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

NR cytotoxicity assay is based on the uptake and lysosomal accumulation of the supravital dye, neutral red. Dead or damaged cells do not take up the dye (12). In neutral red (NR) uptake assay the cells were grown in 96-well multiplates in 100 μ L of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of Naran R extract (25–225 μ g/mL). Subsequently, the medium was discarded and 0.4% NR (Sigma) solution in 2% FBS medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO₂/95% air incubator. After incubation, the dye-containing medium was removed, the cells fixed with 1% CaCl₂ in 4% paraformaldehyde and thereafter the incorporated dye was solubilized using 1% acetic acid in 50% ethanol solution (100 μ L). The plates were gently shaken for 20 min at room temperature and the extracted dye absorbance was spectrophotometrically measured at 540 nm.

Free radical scavenging activity of ethanol-water extract of Naran R was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH \cdot) assay. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH \cdot (Sigma) to the yellow colored diphenyl-picrylhydrazine. Briefly, 100 μ L of DPPH \cdot solution (0.2 mg/mL in ethanol) was added to 100 μ L of different plant extract solution concentrations (25–225 μ g/mL) and standards. Trolox (Sigma) at increasing concentrations (1–50 μ g/mL) was used as a reference for the free radical scavenging activity. After 2, 5, 10, 15 and 20 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower was the absorbance, the higher was free radical scavenging activity of the plant extracts. The activity of each extract concentration was determined by comparing its absorbance with that of a blank solution (reagents without plant extracts) and standard. The capability to scavenge DPPH \cdot radical was calculated by the following formula:

$$\text{DPPH}\cdot \text{ scavenging effect (\%)} = \left[\frac{X_{\text{control}} - X_{\text{extract}}}{X_{\text{control}}} \times 100 \right]$$

where X_{control} is the absorbance of the control and X_{extract} is the absorbance in the presence of plant extract (13).

Argyrophilic nucleolar organizer regions (AgNORs) staining was performed after incubation in 4-well Lab-Tek II Chamber slides; cells were rinsed with PBS and fixed with absolute ethanol/acetic acid solution (1 : 1 v/v) for 10 min.

The silver colloid solution was prepared by 2% gelatin in 1% formic acid mixed in 1 : 2 volumes with 30% aqueous silver nitrate. Cells were immersed in this solution for 5 min at 37°C, rinsed with deionized water and mounted with glycerol. AgNOR proteins were determined using a computer-assisted image analysis system (14).

Phalloidin staining is the useful tool for investigating the distribution of F-actin in cells (15). In labeling of cytoskeleton F-actin, the cells were incubated in 4-well Lab-Tek chamber slides in 1 mL of culture medium supplemented with 2% FBS and plant extracts. After incubation, the cells were rinsed with RPMI 1640 medium and exposed to paraformaldehyde (10%, v/v) solution for 20 min, rinsed three times with PBS, exposed to Triton X-100 (0.2%, v/v) solution for 5 min and rinsed again three times with PBS. A half milliliter of PBS containing tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin, 1 μ g/mL) (Sigma) was added to each well and incubation in the dark at 37°C/5% CO₂ for 30 min was accomplished. Cells observation was conducted under a fluorescence microscope (Olympus, BX51). Quantitative analysis of fluorescent images was performed by AnalySIS imaging software system.

In trypan blue staining, cells were grown in 96-well multiplates in 100 μ L of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of Naran R extract (25–225 μ g/mL). Subsequently, the medium was discarded and 0.4% trypan blue solution in 0.9% sodium chloride was added to each well. The cell walls of dead cells were colored in blue (16).

RESULTS

After trypan blue staining (Table 1) the viability of cells was not lowered when the cells were incubated with the extract at concentrations up to 75 mg/mL. Analysis with MTT method (Fig. 1) revealed that in cells treated with the extract gradual, slight increase of mitochondrial dehydrogenase activity was observed. Cells disintegration appeared for extract concentrations above 125 μ g/mL. NR uptake test showed that HSF cells membranes were stable after fibroblasts incubation with Naran R extract even at the high 225 μ g/mL concentration (Fig. 2).

The analysis with trypan blue coloration is presented in Table 2. Three days' incubation of cells with the extract in high concentrations exceeded 125 μ g/mL significantly lowered the cell viability. When MTT method was used, the cells' viability main-

tained on a constant level (80–110%) after 24, 48 and 72 h of incubation (Fig. 3). During all time of NR test (72 h) and even when the highest concentration of extract was applied, the viability of cells was in range 80–110% of control (Fig. 4).

The next stage of investigations consisted in determination of cell ability to division under influence of ethanol-water extract of Naran R.

Argyrophilic nucleolar organizer regions (AgNORs) staining was analyzed. The cell cultures were observed under visual microscope. In extract concentrations up to 75 µg/mL r-RNA was detected, which suggested cells proliferation (Fig. 5).

Cell cytoskeleton organization was determined by labeling of F-actin with rhodamine-phalloidin dye. Incubation with plant extract showed positive

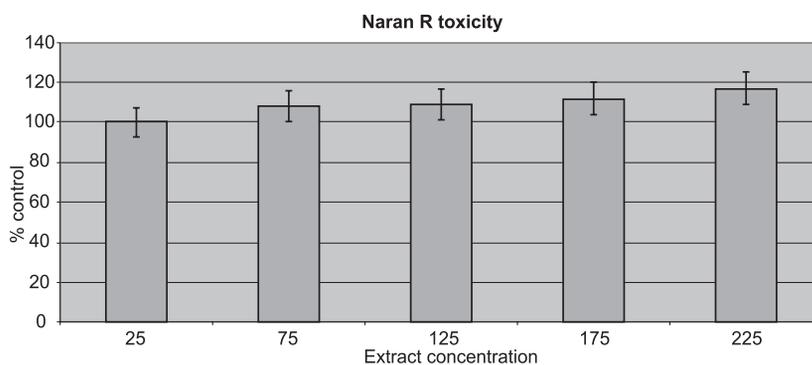


Figure 1. Toxicity analysis of Naran R ethanol-water extract. HSF cells (1×10^5 cells/mL) after 24 h incubation. MTT method. Extract concentration (µg/mL) (n = 4)

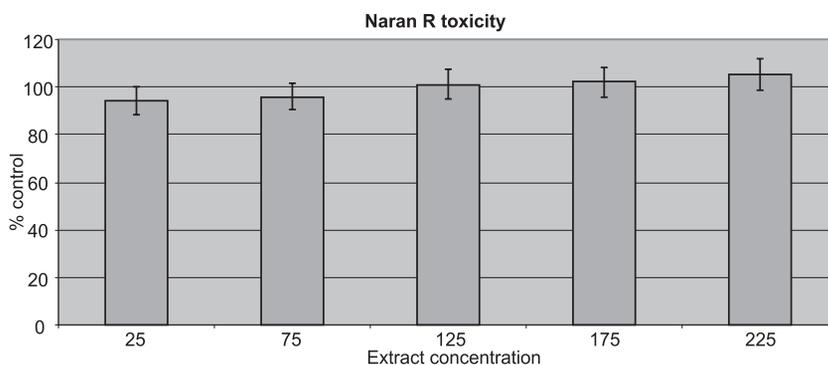


Figure 2. Toxicity analysis of Naran R ethanol-water extract after 24 h of incubation. HSF cells (1×10^5 cells/mL) incubation. Neutral red test. Extract concentration (µg/mL) (n = 4)

Table 1. Toxicity analysis of Naran R ethanol-water extract. HSF cells (1×10^5 cells/mL) after 24 h incubation. Trypan blue coloration.

	Extract concentration (µg/mL)	% viability
	Ethanol-water extract of Naran R	25
	75	95.7 ± 1.2
	125	94.3 ± 1.5
	175	91.7 ± 0.6
	225	85.7 ± 1.5
Control		98.3 ± 0.6

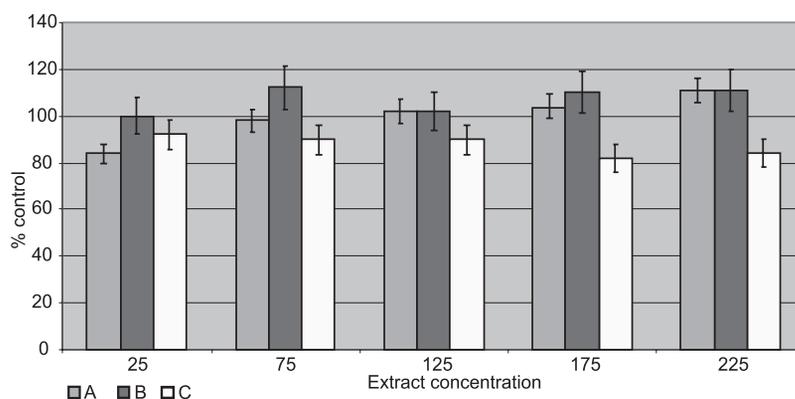


Figure 3. Activity analysis of Naran R ethanol-water extract after 24 (A), 48 (B) and 72 (C) h of incubation with HSF cells (density 2×10^4 cell/mL). MTT test. Extract concentration (mg/mL) (n = 4)

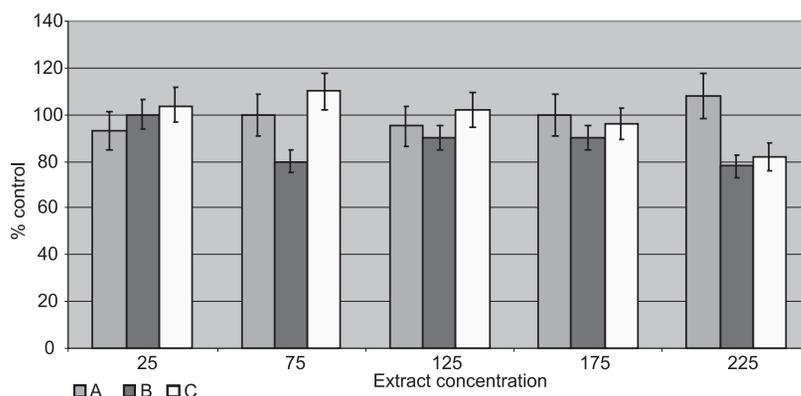


Figure 4. Activity analysis of Naran R ethanol-water extract. HSF cells (2×10^4 cell/mL) after 24 (A), 48 (B) and 72 (C) h of incubation. Neutral red test. Extract concentration ($\mu\text{g/mL}$) (n = 4)

influence on cytoskeleton structure (Fig. 6). At concentration $75 \mu\text{g/mL}$ the cytoskeleton was stable. It changed when extract at concentration $125 \mu\text{g/mL}$ was used.

Moreover, free radical scavenging ability of Naran R was analyzed. Tests were conducted using DPPH[•] reduction method. In this method, the measurement of antioxidant activity is determined by decrease of the oxidized form of DPPH[•] in the sample. In comparison to the control, 8% reduction of free radical DPPH concentration in cultures with Naran R ethanol-water extract was found.

DISCUSSION

Investigations of plant composition for radio-dermitis therapy was motivated by lack of preparations with confirmed efficacy in this type of ailment. If after irradiation erythema and dry epidermis peeling begins, powders (e.g., Alantan), aerosols

(Panthenol) or creams (1% hydrocortisone) are administered. In damp epidermis peeling Silol, Panthenol or Linomag can be used. Chronic postradiation skin inflammation is difficult to cure. Hydrocolloid bandages, interferon α , pentoxiphylline are applied. In complicated cases even surgical skin removal and its grafting are performed (13, 14).

New formulation is planned to be applied on the skin, therefore, the extract was tested with use of cell cultures of HSF. In experiments with use of cell cultures, six methods of assay of biological activity of ethanol-water extract analysis were performed. Succinyl dehydrogenase activity (MTT method), membranes integrity (trypan blue staining), membranes integrity (neutral red test), cells proliferation (r-RNA coloration), cytoskeleton structure (rhodamine-phalloidin fluorescent staining) and antioxidant activity (DPPH method) were tested.

Incubation with the extract resulted in a gradual slight increase of mitochondrial dehydrogenase

activity (MTT) Cells disintegration took place only at higher concentrations. During 24 h investigations of the extract toxicity, no significant modification in cells viability was observed in trypan blue coloration test. In neutral red uptake test cells' membranes were stable and the extract did not disturb the functions of cells even when its high concentrations were used.

The activity of the extract was checked with use of the same methods but incubation was conducted for 72 h. The cells showed stability of cell membranes and intracellular structures. Naran R extract did not cause disorders of mitochondrial dehydrogenase (MTT test) over the incubation time (72 h). Membranes integrity in trypan blue staining after 72 h of incubation was lowered. In NR test, the influence of extract on cells' membranes stability was analyzed. Naran R extract did not damage them. Cell's ability to division and cytoskeleton structure were examined in extract concentrations determined earlier as safe. The extract had positive influence on rRNA synthesis, which is essential before cell division. After cells incubation with the extract, more (in comparison to control) small nucleoli with genetic material to mitotic cell division were observed. The cytoskeleton was steady with contact

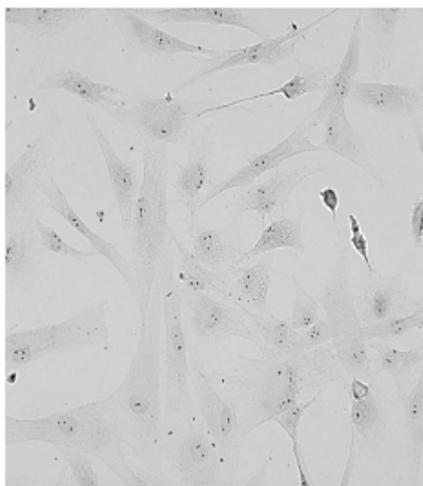
between cells. In the cells, the F-actin fibres were longitudinal, which is the proper structure for HSF cells.

One of the important analysis steps was the determination of antioxidant ability of plants' extract. Free radicals in oxygen breathing organisms arise all the time and take part in many physiologic and also pathologic processes. In the evolution process, a lot of mechanisms occurred to protect organism from the destructive action of free oxygen radicals. Under physiological conditions, without additional exposure on radiation risk, organisms have produced a set of enzymes (superoxide dismutase, catalase, glutathione peroxidase) and natural antioxidants (glutathione, bilirubin, glucose) for tissues protection (8, 15, 16). After X-ray therapy, organism's own mechanisms of prevention are not sufficient. In chain reactions, numerous free radicals are formed and this can lead to long lasting inflammations, and after a long time, to tumors (7).

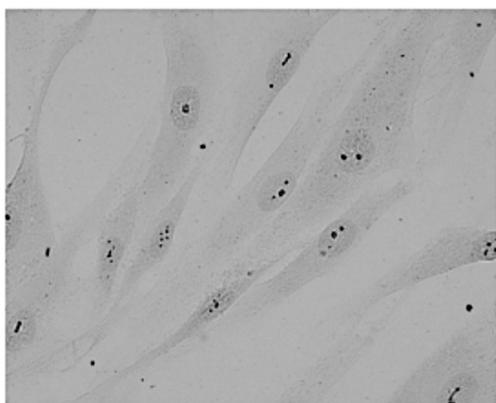
Application of preparation with scavenging free radicals activity should defend tissues against such complications (17, 18). The main source of such compounds are medical plants. In the proposed medicament, ethanol-water extract of five medicinal plants rich in flavonoids, polysaccharides, phenolic

Table 2. Etanol-water extract of Naran R activity after 24, 48 and 72 h of incubation with HSF cells (2×10^4 cells/mL). Trypane blue coloration.

	Culture time (h)	Extract concentration (mg/mL)	% of viability
Ethanol-water extract of Naran R plant composition	24	25	94.7 ± 1.2
		75	94.7 ± 2.1
		125	91.3 ± 1.5
		175	87.3 ± 0.6
		225	86.7 ± 2.1
	48	25	96.7 ± 0.6
		75	95.7 ± 0.6
		125	84.3 ± 2.5
		175	78.3 ± 2.1
		225	73.3 ± 2.1
	72	25	88.0 ± 1.7
		75	67.7 ± 3.2
		125	48.7 ± 4.2
		175	40.7 ± 4.7
		225	30.3 ± 3.5
Control	24		99.7 ± 0.6
	48		99.7 ± 0.6
	72		94.0 ± 1.0



A

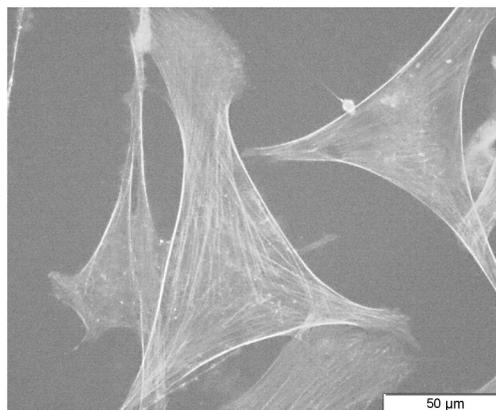


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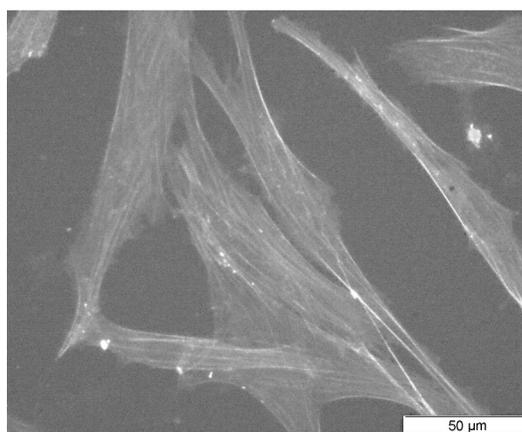
Figure 5. Photograph of AgNORs analysis after cells incubation with ethanol-water extract of Naran R in concentration 75 µg/mL (A) and control image (B). Visual microscope, magnification 200x.

acids, terpenoids and anthocyanins was used. These substances are known antioxidants and due to their presence in the extract the reduction of DPPH[•] concentration is observed (19–21).

To enable patients to apply the extract it is planned to prepare an ointment used in pre-clinical tests, containing the dry residue of ethanol-water extract. The dry residue will be dissolved in DMSO to obtain 75 mg/mL concentration, which was established in biological investigations as safe. This solution will be subjected to emulsifying process with an ointment base to obtain a preparation in the form of a W/O emul-



A



B

Figure 6. Photograph of cells' cytoskeleton staining with the use of rhodamine-phalloidin after their incubation with ethanol-water extract in 75 µg/mL concentration (A). Control image (B). Fluorescent microscope, magnification 400x.

sion. This kind of emulsion is suitable for long lasting inflammations of skin, which occurs after radiation. To prepare the ointment as a base *Unguentum cholesteroli* (22) is recommended. Subsequently, the Naran R ointment is planned to be examined in clinical tests with oncological patients after radiotherapy.

CONCLUSIONS

Investigated herbal composition was prepared for traditional use in form of water infusions and compresses. Naran R was not previously tested in form of extracts with use of different solvents. Presented extract was selected among several others because it showed the best results in biological investigations and it gives the possibility to use it as a plant drug (23).

Six methods describing the structure, functions and ability to proliferation of cells confirmed the profitable influence of the extract on human skin. It is not toxic, its application will improve skin condition of patients and speed up the healing process. By lowering the free radicals concentration, the lowering of delayed radiation effect is expected as well. This should decrease the probability of postradiation skin damages.

The biological investigations of Naran R activity confirmed the safety of its application. The safe concentration of extract established in toxicity and activity investigations is 75 µg/mL. Good results in *in vitro* tests give possibility to carry out tests *in vivo* with oncological patients. Optimal extract concentration (non-toxic, with the best activity) will be employed to get an ointment, which is planned to be used on skin after radiation.

Free radicals reduction, good image of cell cultures, no toxic effect gives possibility of further clinical researches of Naran R ointment. Application of the investigated preparation should reduce the delayed radiation effect on patients' skin.

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