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Molecular mechanisms of immunomodulating effects of Aloe polysaccharide extract and Aloe Liquid Extract on lymphocyte activation markers CD5+ and CD54+ (ICAM-1) in vitro in patients with non-infectious uveitis and viral keratitis

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Background: *Because Aloe polysaccharides, to some degree, determine the high biological activity of the plant, the development of an ophthalmic dosage form based on these compounds is an important and promising field of pharmacology.*

Material and Methods*: We determined relative and absolute expression of the intercellular adhesion marker CD54+ and autoagression marker CD5+ in the peripheral blood lymphocytes of patients with acute non-infectious uveitis and those with acute viral keratitis before and after incubation with Aloe polysaccharide extract and Aloe Liquid Extract. Patients were divided into two groups: group 1 (10 patients with non-infectious uveitis) and group 2 (17 patients with viral keratitis). Mean patient age plus or minus standard deviation was 49.87 ± 3.89 years. Control group was composed of 20 healthy donors of a similar age.*

Purpose*: to investigate in vitro the levels of expression of CD 5+ (a molecular marker of autoimmune activation) and CD 54+ (a molecular marker of intercellular adhesion), in peripheral blood lymphocytes of patients with non-infectious uveitis and viral keratitis before and after incubation with Aloe polysaccharide extract and Aloe Liquid Extract.*

Results: *In both groups of patients, relative and absolute expressions of CD 5+ and CD 54+ in the peripheral blood lymphocytes were significantly increased compared to healthy controls. After incubation with Aloe polysaccharide extract and Aloe Liquid Extract, the relative expression of CD5 in the peripheral blood lymphocytes in group 1 decreased 1.6 times and 1.9 times (to 15.4 ± 1.7% and 13.4 ± 2.1%), respectively. After incubation with Aloe polysaccharide extract, the expression of CD54+ in the peripheral blood lymphocytes in both groups of patients decreased 1.4 times, and this decrease was statistically significant. After incubation with Aloe Liquid Extract, the expression of CD54+ in both groups decreased 1.5 times, and this decrease was statistically significant, too.*

Conclusion: *Aloe polysaccharide extract and Aloe Liquid Extract have significant immunomodulating effect, which was indicated by the normalization of relative expression of intercellular adhesion and autoaggression markers in peripheral blood lymphocytes of patients with non-infectious uveitis and viral keratitis.*

Keywords:

Aloe polysaccharides, markers of lymphocyte activation CD54+ (ICAM-1), CD 5+, immunomodulating effect in vitro, non-infectious uveitis, viral keratitis

Introduction. Biogenic stimulators prepared from Aloe species by the Acad. V.P. Filatov's method are well known tissue preparations. In medical practice, Aloe species are utilized in various dosage forms. Succus Aloes is composed mostly (80%) of the juice from fresh, pulped Aloe leaves; it is applied as a dressing for wounds, burns, and skin inflammation, and in chronic gastritis (in the presence of low gastric acidity), and enterocolitis. Extractum Aloes fluidum (Aloe Liquid Extract) and Extractum Aloes fluidum pro injectionibus (Aloe Liquid Extract for injections) are used in eye disorders and active gastroduodenal ulcer, and Linimentum Aloes have been applied externally in burns and to treat skin lesions during radiation therapy. Sirupus Aloes cum ferro (aloe syrup with iron) stimulates

blood production and has been used in hypochromic anemia. Tablettae Aloes obductae contain crushed aloe leaves and are used as a biogenic stimulator in eye disorders [1-5]. Filatov's Aloe Liquid Extract contains biologically active components of aloe leaves but no calcium chloride: oxyanthraquinones, flavonoids, polysaccharides, vitamins (C, B1, B2, B6, B12, PP), calcium pectate, galacturonic acid, 16 free amino acids (including indispensable amino acids), 18 trace elements (silicon, magnesium calcium, iron, manganese, copper, cobalt, etc.) [6–8].

Polysaccharides have received special attention among the compounds contained in plants of the genus Aloe. Acemannan, an aloe vera polysaccharide, has anti-inflammatory, antibacterial, antioxidative, neuroprotective and osteogenic properties. It exhibits antiviral and antitumor activities due to activation of immune response, and has antifungal, hypoglycemic and gastroprotective properties [9–12]. Acemannan has been found to be effective in the treatment of oral aphthous ulceration [13]. Leaf gel extracts of Aloe barbadensis Mill. and Aloe arborescens Mill. have antimicrobial properties. Generally, gram positive were more susceptible than gram negative microorganisms [14]. In Ukraine, preparations made from Aloe arborescens (Folia Aloe arborescens Miller) have been extensively used in surgery, gastroenterology, dermatology, pulmonology and ophthalmology [15].

Extractum Aloes fluidum pro injectionibus has been used as a component of therapy in progressive myopia, myopic chorioretinitis, blepharitis, keratitis, iritis, vitreous opacities, and active gastroduodenal ulcer [16].

Electrophoresis with a mixture of aloe extract, nicotinic acid 0.5% and ascorbic acid 0.5% has been used in macular pigment degeneration and age-related maculopathy to promote vasodilatation and for metabolic stimulation; electrophoresis with a mixture of aloe extract, fibrinolysin and lidasum, to promote resorption of exudation in the anterior chamber and vitreous; electrophoresis with aloe extract, as a component of therapy in glaucoma and ocular hypertension; electrophoresis with a mixture of aloe extract, nicotinic acid 0.5% and drotaverine, to stimulate visual functions, etc. [17].

Aloe polysaccharides, to some degree, determine the high biological activity of the plant, and, given the above, the development of an ophthalmic dosage form based on these compounds, is an important and promising field of pharmacology.

We have investigated the molecular mechanisms of regulation of lymphocyte immune response to uveitis [18], viral keratitis [19], and cancer [20].

In addition, we have demonstrated previously increased expression of both investigated activation markers (CD 54+, CD 5+) in clinically significant active inflammation, which may be an indicator of inflammatory process [21].

CD54 (ICAM-1) is a glycoprotein expressed on the cell surface of numerous cell types, has a molecular weight of 60-114 KDa, contains five Ig-like domains, and is a member of the immunoglobulin (Ig) superfamily. It is expressed on the cell surface of immune, endothelial and epithelial cells, induced by various inflammatory cytokines (interleukin-1-beta (IL-1β), interferon gamma (IFNγ) or tumor necrosis factor alpha (TNFα)), acts as an adhesion molecule and signal receptor in different types of cells to cause inflammatory reactions and enable inflammation and healing. CD 5+ is a member of the superfamily of protein receptors expressed on T and B lymphocytes, a crucial immunomodulator both under homeostatic and inflammatory conditions, and is considered a marker of autoimmune aggression. Previous studies found that aloe preparations have immunomodulating effects on immunocompetent cells, lymphocytes and neutrophils [22].

The purpose of the study was to investigate in vitro the levels of expression of CD 5+ (a molecular marker of autoimmune activation) and CD 54+ (a molecular marker of intercellular adhesion), in peripheral blood lymphocytes of patients with non-infectious uveitis and viral keratitis treated with an Aloe polysaccharide extract and Aloe Liquid Extract.

Material and Methods

The extract from total aloe polysaccharides (calculated as Acemannan) was prepared as follows.

Fresh leaves and side shoots of Aloe arborescens were cut and crashed. A 2,0 g sample of these leaves was collected and placed into a conical flask and 10 ml of distilled water was added into the flask. Over an 8-hour period, the flask was kept at room temperature and agitated every 30 minutes. Thereafter, the sample was filtrated through a paper, then 9 ml of 96 % ethyl alcohol pre-cooled to 4°C was added to 1 ml of the obtained filtrate. Mixture was kept at +4° C for 16 hours then, centrifuged at 8000 rpm for 30 min. The supernatant was carefully decanted, and the precipitate was resuspended in 10 ml of 0,2 M sodium chloride solution, filtered and autoclaved.

Aloe Liquid Extract was prepared after aloe leaves were kept in the dark place for 10 days at 4–8°C. The leaves were crushed and extracted thrice with distilled water at 1:1.5, 1:1 and 1:0.5 raw material-to water ratios. After each extraction, the mixture was boiled for 3–5 minutes and added to the previous solution [23].

Necessary dilutions of the concentrated product obtained are used for preparing different dosage forms: liquid and dry extracts of aloe for injections, oral administration, electrophoresis, tablets, capsules, ointments, suppositories, and polymeric films for ocular drug delivery. In addition, they are used for producing biocomponents and nutritional additives in food and beverage industries.

Twenty-seven patients underwent an examination at Immunology laboratory, the Filatov Institute of Eye Disease and Tissue Therapy. They were divided into two groups, group 1 of 10 patients with acute non-infectious uveitis, and group 2 of 17 patients with acute viral keratitis. Patients with a history of diabetes mellitus, pregnancy and acute inflammation of the internal organs were excluded from the study.

Blood samples were collected before treatment. Mean patient age plus or minus standard deviation was 49.87 ± 3.89 years. Control group was composed of 20 healthy donors of a similar age.

The study followed the ethical standards stated in the Declaration of Helsinki, the European Convention on Human Rights and Biomedicine and relevant laws of Ukraine. Written informed consent was obtained from all participants.

Molecular markers of lymphocyte activation were determined using the immunohistochemical peroxidase-anti-peroxidase method with monclonal T-cell antibodies [24]. A 4-5-ml sample of heparinized blood was obtained from the cubital vein with a vacuum system, and twice diluted with 0.9% NaCl.

The method procedure involves separating peripheral blood mononuclear cells from whole blood through density gradient centrifugation using Ficoll separating solution with density of 1.077 g/mL and washing them twice by centrifugation to obtain lymphocyte suspension. The objective lens magnification was set to x80, and the eyepiece magnification, to x15. Positive lymphocytes show a dark brown rim of horseradish peroxidase reaction product.

To assess the specific reactivity of lymphocytes to Aloe polysaccharide extract and Aloe Liquid Extract, we employed our complex methodology (in conjunction with a parallel sampling technique) for assessing the individual's sensitivity to medicaments which has been developed at Immunology laboratory of the Filatov Institute of Eye Diseases and Tissue Therapy [25]. The methodology involves obtaining a lymphocyte suspension, culturing lymphocytes with an examined preparation immunohistochemically (with parallel incubation at 37oC for one hour), and determining the activity of lymphocyte activation markers using the immunohistochemical peroxidase-anti-peroxidase method with monoclonal T-cell antibodies.

STATISTICA 8.0 (StatSoft, Tulsa, OK) software was used for analysis. Data were compared using the Mann–Whitney U test. The level of significance $p \leq 0.05$ was assumed.

Results

Relative and absolute expressions of the autoimmune activation marker CD5+ in the peripheral blood lymphocytes of healthy controls were 10.33 ± 1.96% and 152.11 ± 10.13 cell/µl (Table 1).

In addition, in both groups of patients, relative and absolute expressions of CD5 in the peripheral blood lymphocytes were significantly increased compared to controls. In group 1 (non-infectious uveitis), relative and absolute expressions of CD5 in the peripheral blood lymphocytes were 25.17 ± 0.96% and 463.83 ± 48.54 cell/µl, respectively, at baseline. After incubation with Aloe polysaccharide extract and Aloe Liquid Extract, the relative expression of CD5 in the peripheral blood lymphocytes in group 1 decreased 1.6 times and 1.9 times (to 15.4±1.7% and 13.4 ± 2.1%), respectively.

In group 2 (viral keratitis), relative and absolute expressions of CD5 in the peripheral blood lymphocytes were 27.82 ± 0.57% and 556.18 ± 43.11 cell/µl, respectively, at baseline, and were increased compared to group 1. After incubation with Aloe polysaccharide extract and Aloe Liquid Extract, the relative expression of CD5 in the peripheral blood lymphocytes in group 2 decreased 1.7 times and 1.9 times (to 16.5 ± 1.7% and 14.8 ± 0.9%), respectively.

Therefore, it was demonstrated that both examined samples had a desensabilization effect.

Relative and absolute expressions of the intercellular adhesion marker CD54+ in the peripheral blood lymphocytes of healthy controls were 14.2 ± 3.1% and 165.11 ± 32.1 cell/µl (Table 2).

In group 1 (non-infectious uveitis), relative and absolute expression of CD54+ in the peripheral blood lymphocytes at baseline were 28.67 ± 0.67% and 596.29 ± 48.95 cell/µl, respectively, and were significantly higher compared to controls. In group 2 (viral keratitis), relative

Table 1. Expression of the autoagression marker $CD5^+$ in peripheral blood lymphocytes in healthy individuals (controls) and patients with non-infectious uveitis and viral keratitis, before and after incubation with Aloe polysaccharide extract and Aloe Liquid Extract (mean plus or minus standard error of mean)

Markers of lymphocyte subsets in the peripheral blood	Controls, n = 20	Group 1 (uveitis), n = 10	Group 2 (viral keratitis), n = 17
$CD5^+$ %, relative numbers	10.33 ± 1.96	25.17 ± 0.96*	27.82 ± 0.57*
CD 5^+, counts, cell/µl	152.11 ± 10.13	463.83 ± 48.54*	556.18 ± 43.11*
CD 5^+ %, relative numbers after incubation with Aloe polysaccharide extract	9.3 ± 1.2	15.4 ± 1.7 #	16.5 ± 1.2**
CD 5^+ %, relative numbers after incubation with Aloe Liquid Extract	8.9 ± 1.6	13.4 ± 2.1 #	14.8 ± 0.9**

Note: *, significant difference ($p < 0.05$) between controls and group 1 or group 2; #, significant difference ($p < 0.05$) between group 1 baseline values and post-incubation with examined samples; **, significant difference ($p < 0.05$) between group 2 baseline values and post-incubation with examined samples; n, number of patients

Table 2. Expression of the intercellular adhesion marker CD54+ in peripheral blood lymphocytes in healthy individuals (controls) and patients with non-infectious uveitis and viral keratitis, before and after incubation with Aloe polysaccharide extract and Aloe Liquid Extract (mean plus or minus standard error of mean)

Markers of lymphocyte subsets in the peripheral blood	Controls, n = 20	Group 1 (uveitis), n = 10	Group 2 (viral keratitis), n = 17
CD54+ %, relative numbers	14.2±3.1	28.67±0.67*	27.94±0.7*
CD 54+, counts, cell/μl	165.11±32.1	596.29±48.95	520.67±35.47
CD 54+ %, relative numbers after incubation with Aloe polysaccharide extract	13.8±1.1	20.4±2.7#	19.5±1.6**
CD 54+ %, relative numbers after incubation with Aloe Liquid Extract	13.2±1.3	19.2±1.1#	18.8±1.9**

Note: *, significant difference ($p < 0.05$) between controls and group 1 or group 2; #, significant difference ($p < 0.05$) between group 1 baseline values and post-incubation with examined samples; **, significant difference ($p < 0.05$) between group 2 baseline values and post-incubation with examined samples; n, number of patients

and absolute expression of CD54+ in the peripheral blood lymphocytes at baseline were significantly increased compared to group (27.94 ± 0.7% and 520.67 ± 35.47 cell/μl, respectively). After incubation with Aloe polysaccharide extract, the expression of the molecular marker of autoagression CD54+ in the peripheral blood lymphocytes in both groups decreased 1.4 times, and this decrease was statistically significant. In addition, after incubation with Aloe Liquid Extract, the expression of CD54+ in the peripheral blood lymphocytes in both groups decreased 1.5 times, and this decrease was statistically significant, too.

Studies demonstrated that Aloe polysaccharide extract and Aloe Liquid Extract had a desensabilization effect on the markers of intercellular adhesion and autoagression.

Discussion

In the current work, we investigated immunomodulating effects of extract of Aloe arborescens polysaccharides and liquid extract of Aloe arborescens in vitro.

Other researchers [11, 26] reviewed the biological activities (the presence of immunoregulative, anticancer, anti-oxidative, wound healing, neuroprotective, antiviral effects, etc.) of Acemannan, an aloe polysaccharide, in vitro and in vivo. Li and colleagues [27] reported that Aloe polymeric Acemannan inhibits the cytokine storm in mouse pneumonia models by modulating macrophage metabolism. A study by Konovalova [28] demonstrated inflammatory effect of Aloe electrophoresis in the treatment of the sequelae of posterior tubercular uveitis.

Ahluwalia and colleagues [29] conducted an experimental study to determine the metabolite composition of various commercial extracts of Aloe barbadensis and assess their effects on human blood T cell activity in vitro. Aloe extracts differing in their standard composition had varying effects on T cell activation, proliferation, apoptosis, and cell-death in vitro, although this was not related to the acemann content. Given the above, it is important to determine, whether the extracts obtained by us have effects on inflammation. Since extract of Aloe arborescens polysaccharides and liquid extract of Aloe arborescens were tested on blood T cells of patients with non-infectious uveitis and viral keratitis, we selected molecular markers of autoimmune and molecular processes (CD 5+ and CD 54+, respectively).

We found the expression of molecular markers of autoimmune and molecular processes (CD 5+ and CD 54+, respectively) at baseline to be significantly increased in patients with non-infectious uveitis and viral keratitis showed compared to healthy controls. After incubation with Aloe polysaccharide extract, the expression of the intercellular adhesion marker CD 54+ in patients with non-infectious uveitis decreased by 29%, and in patients with viral keratitis, by 30%. In addition, after incubation with Aloe Liquid Extract, the expression of the intercellular adhesion marker CD 54+ in both groups of patients decreased by 33%. Moreover, after incubation with Aloe polysaccharide extract, the expression of the molecular marker of autoimmune aggression CD5+ in patients with non-infectious uveitis decreased by 39%, and in patients with viral keratitis, by 41%. After incubation with Aloe Liquid Extract, the expression of the molecular marker of autoimmune aggression CD5+ in both groups of patients decreased by 47%.

The results of our experimental studies indicate the feasibility of further research on the pharmacological efficacy of extract of Aloe arborescens polysaccharides and liquid extract of Aloe arborescens, because bioregulative therapy is an essential component of current treatment. Since Aloe arborescens polysaccharides, to some degree, determine the high biological activity of the plant, the development of an ophthalmic dosage form based on the active pharmaceutical ingredient (API; i.e., the isolated, enriched and purified polysaccharides fraction from the plant) will enable finding a reliable and convenient method of standardization of the amount of API contained in a unit of a given ready-to-use dosage form to meet the requirements of current best practice. The national pharmacopeias of different countries require that not only injection solutions, but also

ophthalmic drugs need to be purified as much as possible from mechanical impurities and microbial strains, have accurate concentrations of substances, and be isotonic, sterile, stable, etc.

Therefore, the current study found that extract of Aloe arborescens polysaccharides and liquid extract of Aloe arborescens have significant immunomodulating effect, which was indicated by the normalization of relative expression of intercellular adhesion and autoaggression markers in peripheral blood lymphocytes of patients with non-infectious uveitis and viral keratitis.

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Disclosures

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Ethics Statement: *This study included human participants, was approved by the local bioethics committee and adhered to the tenets of the Declaration of Helsinki. Appropriate informed consent was obtained. This study did not include animal experiments.*