ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Study Of *In-Vitro* Biological Activity Of A Multi-Component Therapeutic Product Designed For Treatment Of Inflammatory Conditions Of The Oral Cavity

Lupanova IA[1*], Mizina PG[1,2], Ferubko EV[1], Sidelnikov NI[1].

[1]National Research Institute of Therapeutic and Aromatic Plants, 7 Grina Str., Moscow 117216, Russia
[2]Russian University of People's Friendship, 6 Miklukho-Maklaya Str., Moscow 117198, Russia

ABSTRACT

We evaluated experimental liquid and solid pharmaceutical formulations using specific enzymatic *in vitro* tests to reveal glutathione-reductase, catalase and NADPH-oxidase activity with an aim to choose the most promising formulations for further study and development of products for treatment of inflammatory conditions of the oral cavity. The above mentioned tests allow identification of substances with antimicrobial and immune stimulating activity respectively. Herbal extracts Eucalimin and Estiphan were used as reference products. As a result, differences in antimicrobial and immune stimulating activity of the formulations were found, and the most promising formulations were chosen for further study and development of products with antimicrobial and immune stimulating activity.

Keywords: test systems, therapeutic agent, herbal products, biological activity.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Development of therapeutic agents for treatment of inflammatory conditions of the oral cavity is an important task of pharmaceutical technology science due to wide prevalence of such conditions, which are seen in 90% of all cases of inflectional diseases with a tendency to increase of the incidence within the last 10 years. Antimicrobial drugs used for treatment of infectious diseases cause negative changes of oral microflora. Herbal therapeutic products are known to contain biologically active complexes, which produce a milder effect on the organism, and are better tolerated and cause side effects significantly less frequently as compared to synthetic agents. It is not by chance that prominent WHO experts believe that treatment by herbal products only is expedient for about 75% of patients. Therefore, development of herbal therapeutic products, which lack the above mentioned drawbacks, is necessary and important. Researchers must focus on combination of antimicrobial, anti-inflammatory and immune stimulating activity in one pharmaceutical formulation. It will allow to produce a multi-component therapeutic effect on the damaged oral mucosa, and to correct local immunity and improve sustainability of the mucosa.

Due to development of new pharmaceutical direction, i.e. nanotechnologies, there is a possibility to create new medical forms on the basis of micellae, liposomes, microemulsions and others [1]. It's become possible to build up drug forms that are easy to use, non toxic, high efficient and bioavailable. Earlier we've developed optimized formulations of LF 1 and LF 2 in the form of micellar solutions with plant extracts on the basis of sodium alginate and methyl cellulose; LF 3 and LF 4 are microemulsions with lecitin on the basis of sodium alginate and methyl cellulose accordingly. To offer an opportunity for a patient to choose between various medical forms of the same drug we have formulated two tablet forms, it is SF 1 with dextrate as a filler, and SF 2 with sucrose. These medical forms are portable, easy to intake, accurately dosed, demonstrate taste masking effect of API, possess long shelf life and are less expensive, than LF.

However, a new therapeutic product must undergo multi-phase studies starting from *in vitro* studies before it reaches patients. One of the methods of *in vitro* studies utilizes specific test systems, which allow preliminary evaluation of the biological activity of pharmaceutical formulations without using laboratory animals. This method shows high selectivity, sensitivity and reproducibility, gives much information in short time, and allows optimization of preclinical studies.

The study was specifically aimed at comparative evaluation of biological activity of liquid and solid experimental pharmaceutical formulations using reference products Eucalimin (a herbal therapeutic product with significant antibacterial activity) and Estiphan (a well-known immune stimulator) in order to develop a product for treatment of diseases of the oral cavity.

MATERIALS AND METHODS

Reagents: nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione, and highly purified glutathione reductase and catalase were from Sigma-Aldrich (USA). Hydrochloric acid, sodium phosphate, potassium phosphate, potassium chloride, magnesium chloride and hydrogen peroxide were of local origin. NADPH oxidase originated from resting polymorhonuclear leucocytes of rabbit peripheral blood [2]. Prothymosine-α is a thymus hormone, a natural immune activator [3].

Formulations: the following pharmaceutical formulations were chosen for the study:

- Dry purified extract of manna gum (*Eucalyptus viminalis* Labill) (Eucalimin, ref. ФС 42-3606-98);
- Dry extract of purple coneflower (*Echinacea purpurea L.)* grass (Estiphan, ref. ВФС 42 – 2372-94);
- Experimental liquid formulations (LF):

LF 1 - micellar solution with plant extracts based on sodium alginate;
LF 2 - micellar solution with plant extracts based on methyl cellulose;
LF 3 - microemulsion with lecithin based on sodium alginate;
LF 4 - microemulsion with lecithin based on methyl cellulose.
- experimental solid pharmaceutical formulations (SF):
SF 1 - tablets with herbal extracts, basic excipient is dextrate;
SF 2 - tablets with herbal extracts, basic excipient is saccharose.

ISSN: 0975-8585

METHODS

Enzymatic reaction rate was measured spectrophotometrically using biochemical analyzer Clima CM-15 (Spain). The rate of glutathione reductase (GR) reaction was measured by decrease of optical density at 340 nm due to NADPH oxidation, which is equimolar to reduction of oxidized glutathione according to Aebi [4]; the rate of catalase (CAT) reaction was measured at 410 nm by color reaction of hydrogen peroxide complex with ammonium molybdate [5]; the rate of NADPH-oxidase reaction was measured according to Daniels [6]. Solutions of the studied formulations in concentration range 1.5-50 mcg/ml were added to the test system after addition of the enzymes. The shape of "concentration – reaction rate" curve was determined in advance, and optimal substance concentrations corresponding to maximum of the "concentration – reaction rate" curve were established. Substances' effects on the reaction rate were compared at optimal concentrations of the studied substances.

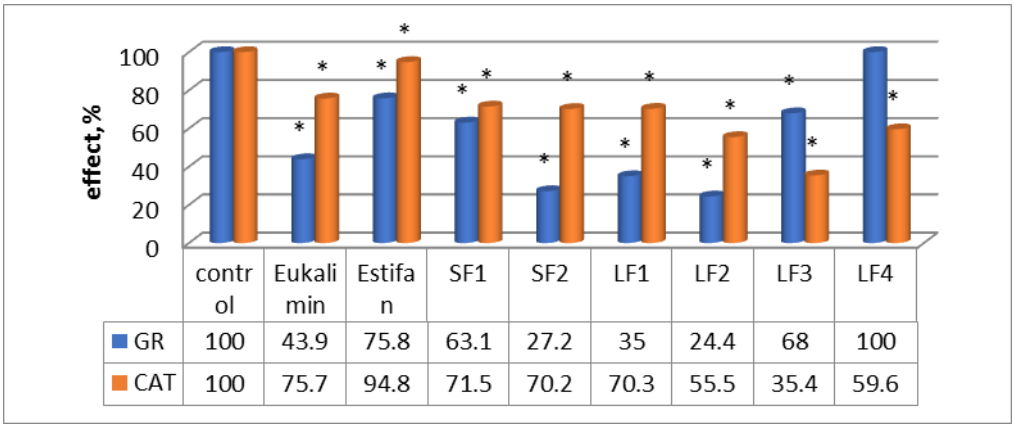
Statistical analysis of the results was done using Statistica 6.0 software (StatSoft, США). Significance of the difference between data sets with close to normal distribution was checked using Student's T-test [7] at threshold significance level for evaluation of statistical hypothesis equal to 0.05. Data are presented in tables as arithmetic mean (M) ± arithmetic mean error (m).

RESULTS AND DISCUSSION

Specific enzymatic biotest systems are included into the Biological Collection of enzymatic biotest systems *in vitro* (BK-SEBTS) of FSBSI VILAR.

Adaptation-improving, antioxidant and antimicrobial activity of the substances was evaluated by their effect on the rate of reactions catalyzed by glutathione reductase and catalase [8, 9, 10]. As shown earlier, adaptation-improving substances selectively accelerate GR reaction and decelerate CAT reaction *in vitro* [8], antioxidant substances accelerate both GR and CAT reactions [9], and antimicrobial substance inhibit GR and CAT reactions [10]. Immune stimulatory activity was evaluated by the substance effect on the rate of reaction catalyzed by NADPH-oxidase [11].

Data about direct effect of the substances on the rate of GR and CAT reactions in experimental conditions *in vitro* are presented in fig.1.



	control	Eukalimin	Estifan	SF1	SF2	LF1	LF2	LF3	LF4
GR	100	43.9	75.8	63.1	27.2	35	24.4	68	100
CAT	100	75.7	94.8	71.5	70.2	70.3	55.5	35.4	59.6

Fig 1. Direct effect of the substances on the rate of GR and CAT reactions in experimental conditions *in vitro*

Evaluation of the effect of two solid formulations on the rate of GR and CAT reactions (fig.1) showed that solid formulation 2 (SF2) significantly influenced the rate of GR and CAT reactions in experimental *in vitro* conditions. At final concentration 6.6 mcg/ml SF2 decreased the rate of CAT reaction by 29.9% and the rate of GR reaction by 72.8%, thus demonstrating significant antimicrobial activity according to [10]. Solid formulation 1 (SF1) also decelerated both GR and CAT reactions, though to a lesser extent, thus demonstrating antimicrobial activity.

ISSN: 0975-8585

All 4 liquid formulations showed inhibition of GR and CAT reactions, and LF2 demonstrated the highest antimicrobial activity with deceleration of CAT reaction by 44.5% and GR reaction by 75,6%.

Reference product Eucalimin, which is a known antimicrobial drug [12], inhibited GR reaction by approximately 50% and CAT reaction by 25%. Estiphan, which is a known immune stimulator [13], did not affect the CAT reaction in vitro, but significantly decreased the rate of GR reaction, this demonstrating moderate antimicrobial activity.

Data about direct effect of the studied substances on the rate of NADPH-oxidase reaction in experimental conditions *in vitro* are presented in fig.2. NADPH is inactive in resting (non-activated) leucocytes [10], but is activated upon addition of an immune stimulator. Direct stimulating effect of the studied substances on NADPH-oxidase reaction *in vitro* was qualitatively compared after data normalization to the rate of this reaction in leucocytes treated with immune activator prothymosine-α, with the latter value taken as 100%.

The rate of NADPH -oxidase reaction in control sample (without any substance) was equal to 0. Prothymosine-α addition to homogenate of resting leucocytes resulted in induction of NADPH-oxidase, and NADPH was oxidized at a rate of 69.8 nmol/min/10 mcl, which was taken as 100%.

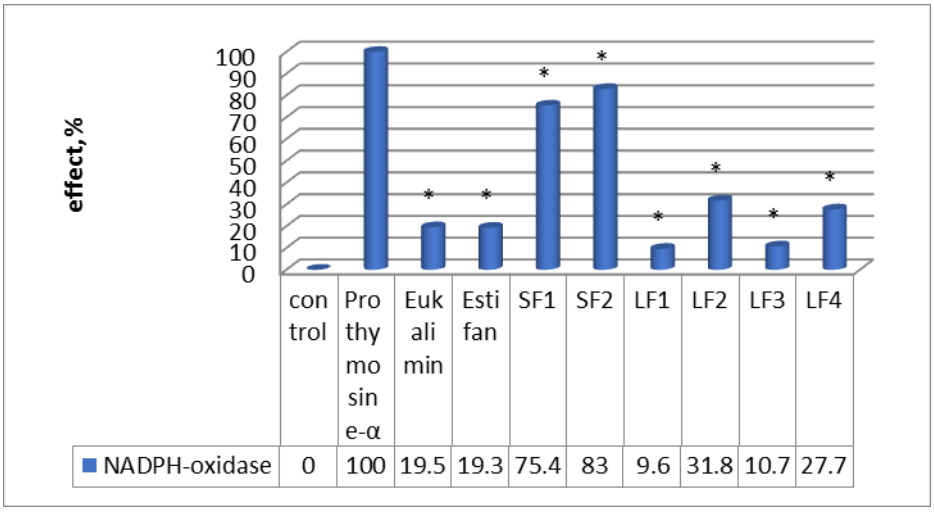


Fig 2. Direct effect of the substances on the rate of NADPH-oxidase *in vitro.*

Remarks: * - statistical significance variation from the control, p<0,05.

Eucalimin addition in a lower concentration (3.3 mcg/ml) resulted in NADPH oxidation at a rate of 20.3 nmol/min/10 mcl, which makes 29% of prothymosine-α effect. This effect matches the previously shown Eucalimin immune stimulating effect *in vivo* with elevation of opsonization-phagocytic index 1.5-2-fold [12].

Estiphan addition in a higher concentration (6.6 mcg/ml) resulted in NADPH oxidation at a rate of 13.5 nmol/min/10 mcl, which makes 19.3% of prothymosine-α effect. This effect matches the previously shown dose-dependent Estiphan immune stimulating effect *in vivo* [13].

Dose-dependent immune stimulating effect was also found for SF1 and SF2. Bothe substances in optimal concentrations increased the rate of NADPH-oxidation by mean 80% of reference substance effect.

As to liquid forms, the strongest immune stimulating effect was found for LF2, which increased NADPH-oxidation rate by 31.8% (added volume 10 mcl) or by 24,4% (added volume 20 mcl) of prothymosine-α effect.

CONCLUSIONS

Evaluation of in vitro biological activity of different liquid and solid multi-component formulations containing extracts of manna gum (*Eucalyptus viminalis* L.) and of purple coneflower (*Echinacea purpurea L.)*

grass demonstrated significant immune modulating and antimicrobial activity of solid formulations SF1 and SF2 and liquid formulations LF2 and LF4. Activity of solid formulations increased with elevation of product concentration, while liquid formulations were more active at lower concentrations (added volume 10 mcl).

The strongest immune modulating and antimicrobial activity was shown for SF2 and LF2, which are, therefore, most promising for further study and development of products for treatment of inflammatory diseases of oral cavity.

REFERENCES

[1] Kwon G.S. Polymeric micelles for delivery of poorly water-soluble compounds. Crit Rev Ther Drug Carr Syst. 2003; 20: 357-403.

[2] Mineeva MF. Tyrosine hydroxylase of blood leukocytes. Bulletin of Experimental Biology and Medicine. 1987; 104: №7: 99-101 (in Russian).

[3] Gabdulhakova AG, Popova NB, Hodyakova AV. Effect of recombinant protimosin-α on the activity of human NADPH-oxidase of human neutrophils. International journal of immunoreabilitation. M. 1999; 12: 15 (in Russian).

[4] Aebi H. Glutathione reductase. Methods in enzymatic analysis. H. V. Bergmeyer (red.). 1974; 2: 673-678.

[5] Korolyuk MA, Bvanova MI, Mayorova IG., Tokarev VE. Method of catalase activity determination. Laboratory science 1988; 1: 1- 8 (in Russian).

[6] Daniels R. H., Elmore M. A., Hill. M. E., Shimizu, Y., Lackie, J. M. Finnen, M. J. Priming of the oxidative burst in human neutrophils by physiological agonists or cytochalasin B results from the recruitment of previously non-responsive cells. Immunology 1994; 4: 465 – 472.

[7] Habriev RU. The study guide of experimental (preclinical) study of new pharmacological substances, 2 Revise and additional, Moscow, Public Corporation "Medicine" 2005; p. 832 (in Russian).

[8] Russian-owned patent №2181890 (Russia). A method of the detection of substances with adaptogenic properties, *in vitro*, Bikov VA, Mineeva MF, Dubinskaya VA, Rebrov LB, Kokhir VK.

[9] Russian-owned patent № 2181892. A method of the detection of substances with antioxidant properties, *in vitro*, Bikov VA, Mineeva MF, Dubinskaya VA, Rebrov LB, Kokhir VK.

[10] Russian-owned patent №2181891. A method of the detection of substances with antimicrobial and anti-viral properties, *in vitro*, Bikov VA, Mineeva MF, Dubinskaya VA, Rebrov LB, Kokhir VK.

[11] Lupanova IA. Investigation of molecular mechanisms of action of biologically active substances on the example of triterpene and flavonoid glycosides. Dissertation boil.sciences, Moscow 2011; P. 131 (in Russian).

[12] Vichkanova SA. New aspects of eucalimin. Questions of biological, medical and pharmaceutical chemistry, 2012; 1: 214-220 (in Russian)

[13] Bortnikova VV, Krepkova LV, Sokolskaya TA, Shkarenkov AA, Kuznetsov UB, Borovkova MV, Botcharova IV, Yartsev DP, Gnutov VB. The study of immunostimulatory properties of drugs from purple coneflower grass. Materials of the VIII International Congress "Actual problems of creating new drugs with natural origin. 21-23 June, 2004; 61-65 (in Russian)