

Chemistry and Biological Activity of Feed Additive for Poultry Farming

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Abstract—Essential oils are one of the most important groups of biologically active substances present in plants. Due to the chemical diversity of components, essential oils and their preparations have a wide spectrum of pharmacological action. They have bactericidal, antiviral, fungicidal, antiprotozoal, anti-inflammatory, spasmolytic, sedative and other activities. They are expectorant, spasmolytic, sedative, hypotensive, secretion enhancing, antioxidant remedies. Based on preliminary pharmacological studies, we have developed a formulation called “Phytobiotic” containing essential oils, a feed additive for poultry as an alternative to antibiotics. Phytobiotic is a water-soluble powder containing a composition of essential oils of thyme, clary, monarda and auxiliary substances: dry extract of liquorice and inhalation lactose. On this stage of research, the goal was to study the chemical composition of provided phytobiotic, identify the main substances and determine their quantity, investigate the biological activity of phytobiotic through *in vitro* and *in vivo* studies. Using gas chromatography-mass spectrometry, 38 components were identified in phytobiotic, representing acyclic-, monocyclic-, bicyclic-, and sesquiterpenes. Together with identification of main active substances, their quantitative content was determined, including acyclic terpene alcohol β -linalool, acyclic terpene ketone linalyl acetate, monocyclic terpenes: D-limonene and γ -terpinene, monocyclic aromatic terpene thymol. Provided phytobiotic has pronounced and at the same time broad spectrum of antibacterial activity. In the cell model, phytobiotic showed weak antioxidant activity, and it was stronger in the ORAC (chemical model) tests. Meanwhile anti-inflammatory activity was also observed. When fowls were supplied feed enriched with phytobiotic, it was observed that gained weight of the chickens in the experimental group exceeded the same data for the control group during the entire period of the experiment. The survival rate of broilers in the experimental group during the growth period was 98% compared to 94% in the control group. As a result of conducted researches probable four different mechanisms which are important for the action of phytobiotics were identified: sensory, metabolic, antioxidant and antibacterial action. General toxic, possible local irritant and allergenic effects of phytobiotic were also investigated. Performed assays proved that formulation is safe.

Keywords—Clary, essential oils, monarda, phytobiotics, poultry, thyme.

I. INTRODUCTION

THE abundance of infections caused by multi-resistant microbes and the complexity of combating them has delivered sizeable reputation to biologically active substances of plant origin. Essential oils have unique place among plant origin biologically active antibacterial substances as they have strong antibacterial, antioxidant and immune modulating

modulating activity [1], [8], [10], [15], [18], [21], that is why they can be extremely useful in stock rising and poultry farming. The use of essential oils prevents the development of diverse intestinal infections, which notably impacts the productiveness and maintenance of stock and fowls. In addition, essential oils provide a pleasant aroma and make food attractive, additionally they have anti-stress activity, increase production of digestive enzymes, even improve chicken's mood [22]. Essential oils are products of secondary metabolism that contain numerous easily evaporated substances: terpenes, terpenoids, phenolic products, aliphatic and aromatic components [2]. The mechanism of their action on microorganisms is following: different organic compounds within it change speed of biochemical reactions, resulting in their destructive effects on microorganism's mesosomes and cytoplasmic membranes, thus reducing oxidative phosphorylation activity, also inhibit cellular respiration [2], [16].

Lately in the scientific community interest in medicinal plants and herbal extracts has increased significantly in terms of antimicrobial activity. Researchers [5] found that essential oils of bergamot, carnation, cypress, big fennel, eucalyptus, lavender, rosemary, peppermint, clary, thyme show pronounced antibacterial activity against various pathogens. That is why for today there is no doubt concerning use of essential oils as new antibacterial chemical modifiers, on the basis of which it is possible to create different compositions with therapeutic, prophylactic effects [19]. Studies have also established the antioxidant and antibacterial activity of *Salvia sclarea* L. Among well-known essential oils one with high antibacterial activity is the essential oil of Thyme (*Thymus vulgaris*) containing 50% thymol [14]. Chemistry, antioxidant, antibacterial and antiviral activity of essential oils of *Thymus transcaucasicus* Ronniger, widespread in Georgia, was studied in details [10]. In this case, it is possible to inhibit different processes of metabolism in the microbial cell at the same time, leading to its rapid death and significantly inhibits the development of resistance in microorganisms.

Based on experimental studies carried out by the authors of the presented article [3], [4], [7], [20] obtained and studied antibacterial activity of Clary sage, Eucalyptus, Perilla essential oils [6], [11]-[13], [17].

The antibacterial activity of thyme, clary, monarda and perilla essential oils and their compositions have been determined on the basis of biological studies. Based on

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biopharmaceutical studies, the formulation of water soluble phytobiotic containing these essential oils is established [9].

II. RESEARCH GOAL

The goal of the study was to determine the antioxidant, anti-inflammatory activity, antibacterial spectrum and safety of already formulated and designed phytobiotic by given research group and investigate its growth stimulating effect in farm chickens.

To achieve the goal the following tasks have been set:

- Investigation of components of phytobiotic by gas chromatography-mass spectrometry (GC-MS/MS), determination of quality features and standardization;
- Determination of biological activity of phytobiotic - antioxidant, anti-inflammatory and antibacterial range;
- Study of growth stimulation in farm chickens by phytobiotic.
- Determination of phytobiotic's safety.

III. OBJECTIVES AND METHODS

The research object was phytobiotic and following methods were used:

GC-MS analysis is performed in following conditions:

- Agilent Technologies 7000 GC/MS/MS Triple Quad mass spectrometer
- Column - Elite 5-MS; 30M X 250 μm X 0.25 μm ;
- Oven temperature: 60-310 $^{\circ}\text{C}$
- Injector temperature: 250 $^{\circ}\text{C}$
- Transfer line temperature: 310 $^{\circ}\text{C}$
- Carrier gass: Helium 1 ml/min
- Ion source: EI-70 ev
- Scan regime: TIC. 35-500 Amu

In vitro biological activity of Essential Oil and Phyto-biotic Cell Culture: Healthy human cell lines WS1 (ATCC CRL-1502) and the murine macrophage RAW 264.7 (ATCC TIB-71) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in a humidified atmosphere at 37 $^{\circ}\text{C}$ in 5% CO_2 , in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 1 \times solution of sodium pyruvate, 1 \times vitamins, 1 \times non-essential amino acids, 100 IU of penicillin.

Bacterial Strains: The *in vitro* antimicrobial activity of essential oils composite was tested against gram-negative *Escherichia coli* (ATCC 25922) and gram-positive *Staphylococcus aureus* (ATCC 25923). Bacterial strains were provided by the Chicoutimi Hospital, Saguenay, Canada.

Culture Methods: Bacteria were stored at -80 $^{\circ}\text{C}$ until use. For culturing, all bacteria were placed in a nutrient broth base (Difco) for 16–18 h at 37 $^{\circ}\text{C}$; *C. perfringens* were grown in an anaerobic vial. We measured the cellular density of the inoculum via optical density measured at 600 nm for *E. coli* [24], 660 nm for *S. aureus* [25] using a Multiskan™ GO Spectrophotometer (Thermo Fisher Scientific). Based on the results the inoculum was re-diluted in the nutrient broth to obtain the required bacterial concentration.

Measurement of Anti-Inflammatory Activity: The inhibition of nitric oxide (NO) production by phytobiotic was evaluated. Control L-NAME was used as a positive control. The murine macrophage RAW 264.7 cells were incubated with phytobiotic solution in DMSO, then the cells were stimulated using 100 $\text{ng}\cdot\text{mL}^{-1}$ Lipopolysaccharide (LPS) and incubated at 37 $^{\circ}\text{C}$. After 24 h, the cell-free supernatant was collected and the NO concentration was immediately determined using the Griess reaction. The absorbance was read at 540 nm and we quantified the presence of nitrite by comparing with a NaNO_2 standard curve.

Evaluation of Antioxidant Activity Using Cell-Based Assays: We incubate human skin fibroblasts WS1 for 1 h with a growing concentration of essential oils composite, phytobiotic or their solution in DMSO. We then add 100 μL of 200 μM tert-butylhydroperoxide and immediately measure fluorescence and again after 90 min. Antioxidant activity is expressed as the concentration of essential oils composite inhibiting 50% (IC50) of DCFH oxidation.

Evaluation of Antioxidant Activity Using ORAC: Group followed the method already described with some modifications [19]. The ORAC assay was carried out in black 384-well microplates (Nunc) on a Fluoroskan Ascent FL™ plate reader (Labsystems) equipped with an automated injector. Gradient of 16 concentrations of the samples (research object dissolved in DMSO) of Trolox (1.56, 3.13, 6.25, and 12.5 μM) in quadruplicate was prepared, without replicating concentrations. The experiment was conducted at 37.5 $^{\circ}\text{C}$ and in a pH 7.4 phosphate buffer with a blank sample run in parallel. The fluorimeter was programmed to record the fluorescence 140 (λ ex.: 485 nm/em.: 530 nm) of fluorescein every minute after the addition of 375 mM of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), for a total of 60 min. The final values were calculated using the net area under the curves of the essential oil composite and phytobiotic concentrations for which a decrease of at least 95% of fluorescence was observed at 60 min. ORAC values were expressed in micromoles of Trolox equivalents (TE) per milligram ($\mu\text{mol TE}\cdot\text{mL}^{-1}$).

Evaluation of antibacterial activity: We tested the antibacterial activity of phytobiotic using the antibacterial hydrophobic assay as described by [23]. Briefly, after microorganisms are passed 16–18 h at 37 $^{\circ}\text{C}$ in a nutrient broth base (Difco), we transferred 20 μL methanol containing growing concentrations of phytobiotic (3.1 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$) onto nutrient agar in 96-well plates. We then add bacterial strains having a concentration of 5×10^3 colony forming units (CFU) per mL of nutrient broth. Bacterial suspension without research objects was used as negative control, and bacterial suspension plus solvent is tested in parallel to demonstrate the absence of solvent toxicity. The blank consisted of a culture medium only and its measurements are extracted from all subsequent results. Research objects are then incubated at 37 $^{\circ}\text{C}$ for 5 h. Then 100 μL of resazurin sodium salt solution having a concentration of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ (Sigma R-2127, St-Louis, MO, USA) was added to each well. Fluorescence was read on Fluoroskan Ascent FL™ plate reader (Labsystems, Milford, MA, USA) after 2 h for *S. aureus* and 3 h for *E. coli*.

The MIC90 is determined as the lowest concentration of phytobiotic resulting in 90% inhibition of bacterial growth.

Spot test: On 2% LB agar plate was made the lawn of 1 ml bacterial suspension and afterwards it was spread all over the plate. After letting plates dry, 10 µL of research object was added to it. Plates were placed in thermostat for incubation for 24 hours. The presence of clear zones around the research sample indicates a positive result.

In vivo Experimental Design in Broilers for Phytobiotic: To use and establish optimal dose using analogue method at Ltd Sabudara 6000 on day old broiler chickens Ross-308 were chosen, they were divided into two groups. In each group live weight was almost same: 40,0-40.02. It indicates high homogeneity of birds among groups. The first group (n = 3000) was the control group and received basic fed, combined foods with antibiotics, without phytobiotic. The second group was the experimental group (n = 3000), which ate food without antibiotics, phytobiotic mixed with chicken drinking water was added to the portion.

During the experiment broiler keeping parameters were identical for each group and they came into accordance with Ross-308 growth requests. Broilers were placed in an insulated isolated room with facilities to control temperature, light, and humidity according to industry standards. They were fed with Nutrinor standard diet for poultry. The feeding program consisted of a starter (1–10 d), growth (11–20 d), and finisher (21–35 d) diet given to broiler ad libitum. The treatment group receive 0.1% phytobiotic in starter and growth diet, and 0.05% phytobiotic in the finisher diet. The weight is daily checked by using an automatic balance installed in the hen house. Mortality was recorded daily and at the end of the experiment the total amount of received food was determined.

Safety Research: To study for general toxicity, mice were given a phytobiotic solution at different concentrations as a single and repeated injections. Saline solution was given as control. To determine chronic toxicity phytobiotic was supplied to mice together with drinking water during 30 days. They were put under observation during this period and after passing 24 hours from the last day dissection of animals was performed and internal organs were examined. To test phytobiotic on local irritating effect it was applied on preliminarily depilated murine skin. Olive oil was used as control. In order to evaluate allergic reactions, the phytobiotic was spread on skin daily within 14 days.

Statistical processing of results: The results are processed using the statistical program Sigma STAT.

IV. RESULTS

In order to identify the target substances in the research object, the mass spectra of peaks on the chromatograms were compared with the data of the substances in the NIST database (Fig. 1).

GC-MS studies have identified up to 38 individual substances in essential oils (Table I).

TABLE I
IDENTIFIED INDIVIDUAL SUBSTANCES OF ESSENTIAL OILS IN THE STUDY OBJECT

	Component	Formula	Retention time	%
1	α -Thujene	C10H16	4.06	
2	α -Pinene	C10H16	4.09	
3	β -Pinene	C10H16	4.53	
4	3-Carene	C10H16	4.71	
5	D-Limonene	C10H16	4.88	30.68
6	γ -Terpinene	C10H16	5.11	8.43
7	Terpinolene	C10H16	5.34	
8	β -Linalool	C10H18O	5.41	7.14
9	Neo-allo-ocimene	C10H16	5.63	
10	Trimethyl-3-cyclohexenyl-1-carboxaldehyde	C10H16O	5.94	
11	(-)-4-Terpineol	C10H18O	6.11	
12	L- α -Terpineol	C10H18O	6.21	
13	Isopulegyl acetate	C12H20O2	6.37	
14	Linalyl acetate	C12H20O2	6.55	15.74
15	1,3-Heptadiene, 3-ethyl-2-methyl-	C10H18	6.68	
16	Thymol	C10H14O	6.86	22.28
17	Nerol acetate (cis-Geranyl acetate)	C12H20O2	7.3	
18	Geranyl acetate	C12H20O2	7.42	
19	Copaene	C15H24	7.54	
20	(+)-Valencene	C15H24	7.6	
21	Caryophyllene	C15H24	7.86	
22	α -Humulene	C15H24	8.09	
23	γ -Muuroleone	C15H24	8.17	
24	β -Chamigrene	C15H24	8.35	
25	(+)- δ -Cadinene	C15H24	8.44	
26	trans-calamenene	C15H22	8.48	
27	trans- α -Bergamotene	C15H24	8.54	
28	Caryophyllene oxide	C15H24O	8.7	
29	trans-Z- α -Bisabolene epoxide	C15H24O	8.73	
30	trans- β -Ocimene	C10H16	12.59	
31	β -Ocimene	C10H16	12.85	
32	Neodihydrocarveol	C10H18O	13.46	
33	trans-2-Caren-4-ol	C10H16O	15.57	
34	Isopulegol	C10H18O	16.98	
35	cis-Verbenol	C10H16O	17.18	
36	Carvacrol	C10H14O	18.46	
37	α -Terpinyl acetate	C12H20O2	19.38	
38	Alloaromadendrene	C15H24	20.22	

The results of the *in vitro* study on biological activity are presented in Tables II-IV.

MIC90 indicates minimum concentration of phytobiotic which inhibits the growth of bacterial strains by 90%. The data show that phytobiotic does not have antibacterial activity against the investigated bacterial strains. The study of antibacterial activity was also carried out by the Spot test method. The results are given in Table III. The data (Table III) show that phytobiotic has a broad spectrum of antibacterial activity.

In the final stage of the research, an *in vivo* test for phytobiotics was conducted at the Ltd Sabudara poltry fabric based in Gamarjveba.

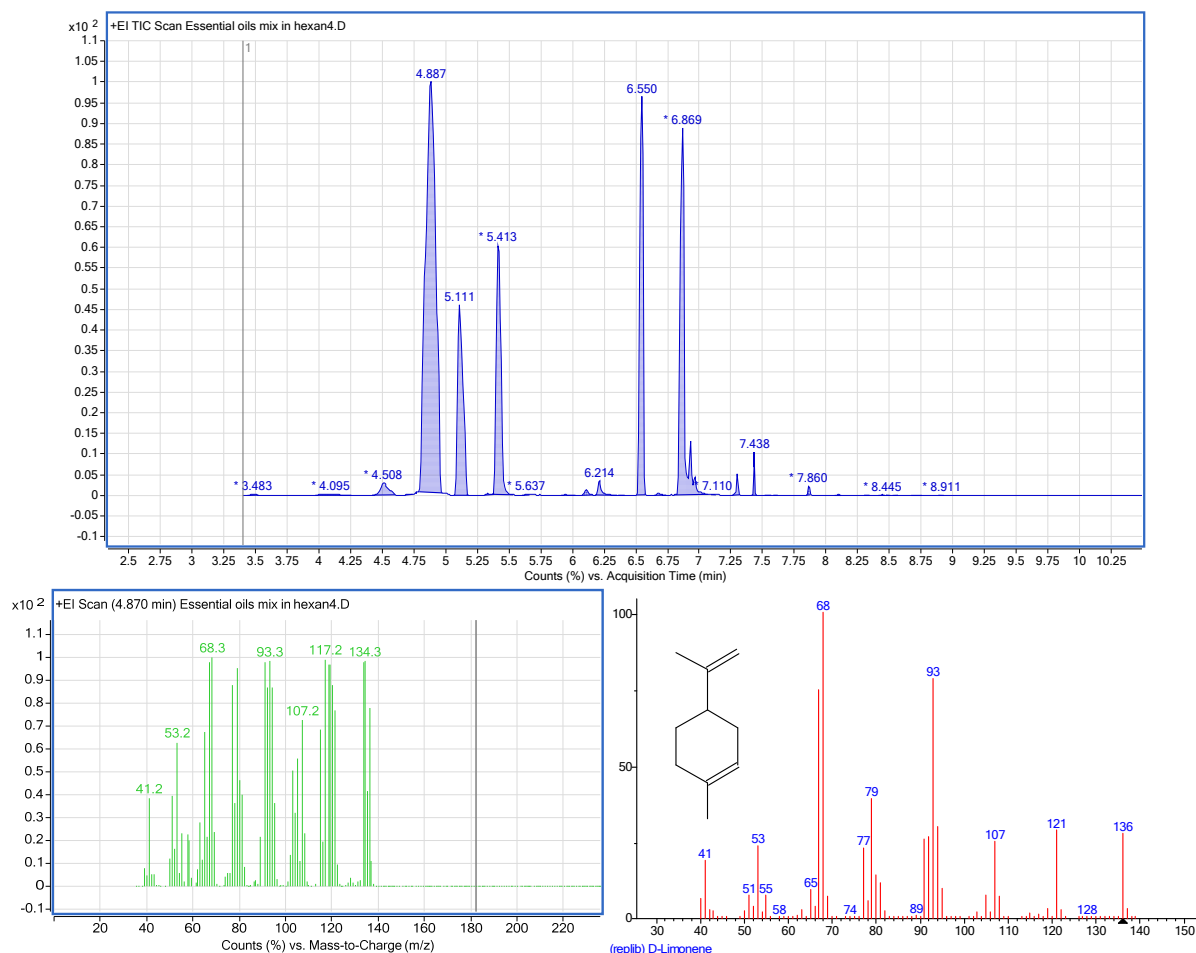


Fig. 1 | D-Limonene (Carvone)

TABLE II
RESULTS OF RESEARCH ON THE ANTIBACTERIAL ACTION OF PHYTOBIOTICS

Research object	Antibacterial activity	
	MIC90 ($\mu\text{g}\cdot\text{mL}^{-1}$)	
	<i>E. coli</i>	<i>S. aureus</i>
Phytobiotic	61 ± 2	30 ± 3

TABLE III
RESULTS OF DETERMINATION OF ANTIBACTERIAL ACTION OF PHYTOBIOTICS

Strain	Phytobiotic	K
<i>Streptococcus pyogenes</i>	4+	
<i>Escherichia coli</i>	4+	
<i>Enterobacter cloacae</i>	4+	
<i>Salmonella typhimurium</i>	4+	
<i>Klebsiella pneumoniae</i>	4+	+
<i>Proteus vulgaris</i>	4+	
<i>Shigella flexneri</i>	4+	
<i>Enterococcus faecalis</i>	4+	
<i>Staphylococcus aureus</i>	4+	
<i>Pseudomonas aeruginosa</i>	3+	

While providing phytobiotic-enriched poultry feed, it was noted that during the whole experimental period the weight of the experimental group exceeded the weight of the control group, particularly: at 7 days of age, the average live weight of the control group broiler was 170 g, while 179 g was for

research group, which is 5.29% heavier. The mean live weight of the control group broiler at 14 days of age was 415 g and for the experimental group 440 g, or 6.02% more; At 21 days of age, the average live weight of a control group broiler was 898 g and 950 g was for a control group, or 5.79% more; At the age of 28 days, the average live weight of the control group broiler was 1250 g, and for the experimental - 1360 g, or heavier with 8.8%; In the last period of the experiment, at the age of 35 days, the average live weight of the control group broiler was 1783 g, and the experimental group - 1955 g, or 9.65% more. The test group broilers consumed 4.40 kg of food during the whole test period, compared to 4.50 kg of control. Based on the results of the experiments, we observed a significant weight difference between the control and experimental group (fed with phytobiotic feed) broilers. Broilers with a phytobiotic diet were significantly heavier than control broilers with antibiotics, and they showed a noticeable weight gain curve throughout the trial period. The survival rate of the experimental group broiler during the growing period was 98%, compared to -94% of the control group.

Results of tests investigating safety of phytobiotic have shown:

1. Phytobiotic belongs to low-toxic compounds;
2. No systemic side effects were observed in experimental

animals during 30 days of chronic oral administration of phytobiotic;

3. Phytobiotic does not show cumulative toxicity;
4. Phytobiotic does not have local irritant effect;
5. Prolonged use of phytobiotic in experimental animals does not cause allergic reactions, although in some cases, individual hypersensitivity to the components of phytobiotic can take place.

TABLE IV
RESULTS OF DETERMINATION OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTION OF PHYTOBIOTICS

Research object	Antioxidant activity		Anti-inflammatory	
	Using cell culture IC50 ($\mu\text{g}\cdot\text{mL}^{-1}$)	ORAC $\mu\text{mol Trolox}\cdot\text{mg}^{-1}$	IC50 ($\mu\text{g}\cdot\text{mL}^{-1}$)	inhibition $\mu\text{g}/\text{ml}$
<i>Phytobiotic</i>	78 ± 16 $\mu\text{g}/\text{ml}$	6 ± 1	160 ± 8	44% @ 160 $\mu\text{g}/\text{ml}$
<i>Thymus vulgaris</i>	> 100 $\mu\text{g}/\text{ml}$	0,5 ± 0,1	> 160 $\mu\text{g}/\text{ml}$	41% @ 160 $\mu\text{g}/\text{ml}$
<i>Salvia sclarea</i>	> 100 $\mu\text{g}/\text{ml}$	0,4 ± 0,1	> 160 $\mu\text{g}/\text{ml}$	39% @ 160 $\mu\text{g}/\text{ml}$
<i>Monarda didyma</i>	5,4 ± 0,4 $\mu\text{g}/\text{ml}$	9,4 ± 0,3	69 ± 16 $\mu\text{g}/\text{ml}$	81% @ 160 $\mu\text{g}/\text{ml}$

V. CONCLUSIONS

The antibacterial, antioxidant, anti-inflammatory activity of thyme, clary, monarda and essential oils and their composition have been determined on the basis of biological studies. As a result of conducted researches probable four different mechanisms which are important for the action of phytobiotics were identified: sensory, metabolic, antioxidant and antibacterial action.

Conducted *in vivo* study proved effectiveness of using phytobiotic as a feed additive in poultry farming.

Investigation of probable side effects has shown that phytobiotic is a safe formulation.

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