PHYTOCOMPLEX WITH *ZINGIBER OFFICINALE* EXTRACT, *PIPER NIGRUM* AND *PIPER CUBEBA* OIL - *IN VITRO* ANTIMICROBIAL EFFECT

Cristina Mihaela RÎMBU[1], Cristina Elena HORHOGEA[1], Sabina Andra NECULAI-VĂLEANU[2], Constantin LUNGOCI[3], Oana BARBOI[4], Teodor ROBU[3], Andreea COZMA[2], Mariana GRECU[1]

e-mail:crimbu@yahoo.com

Abstract

Vegetal compounds are known for their therapeutic actions in correlation with their antioxidant activity so that in recent times the interest in their properties has greatly increased.
The phytocomplex obtained by combining the *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil is distributed and recommended in European space as a multi-benefit nutritional supplement for swine, poultry, cattle, horses and others. As the individual properties of the three compounds are known, we aimed to test the antimicrobial activity of the phytocomplex on various Gram negative pathogens.
In the time-kill assay, *in vitro* inhibitory effects were visible after 15 minutes of contact and total inhibition of the species *Samonella enteritidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* was obtained after 24 hours.
The results obtained *in vitro* showed a very good antimicrobial activity which clearly contributes to the overall beneficial effects of the *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil phytocomplex.

Key words: *bacteria, phytocomplex, antimicrobial activity*

Introduction. Herbal extracts and their derivatives have received considerable attention as therapeutic agents for the prevention and treatment of health problems. Plants have always been used for human and animal health due to bioactive plant compounds (Viegi L. et al., 2003). Plants produce secondary metabolites with a role in their own metabolism but also for protection against a multitude of external aggressors. These metabolites are classified into four broad groups: terpenoids, phenolics, nitrogen-containing compounds, and sulfur-containing compounds (Susan G et al., 2007; Mazid M. et al., 2011).

In veterinary medicine, extracts and essential oils obtained from plants are known for their ability to fight certain diseases, regardless of the geographical area of the world (McCorkle, CM, 1992, McCorkle CM et al., 1998; Lev E.,2003). In the tradition medicine, herbal remedies have been strongly anchored in maintaining health being the only option of cheap and easy to administer therapy (Dilshad S.M.R. et al., 2010; McCorkle C.M., 1992). The phenomenon is so widespread that this type of medicine has been called ethnoveterinary medicine (Aziz M.A.et al., 2018) and is an alternative to allopathic medicine. (McCorkle C.M, 1986. et al., 2019). Also, the development of antimicrobial resistance to currently available semi-synthetic antibiotics is another reason why new antimicrobial formulas are sought in nature, given that approximately 25% of current drugs are also extracted from plants (Haghiroalsadat F. et al., 2011).

Medicinal plants and how they are used vary from one geographical region to another, so the reports are sometimes made from different perspectives. Studies conducted in countries in Asia, India, Pakistan, South America predominate and such scientific studies are insufficiently reported in European or North American countries (Aziz MA. et al., 2018).

The benefits of administering plant extracts or oils to animals administered as food supplements or for therapeutic purposes have been described in various studies conducted on various animal species (Dewick P.M, 2001). In pigs, there was an increase in the weight of the carcass muscle mass and improved the compositional quality of

[1] Iasi University of Life Sciences Ion Ionescu de la Brad, Faculty of Veterinary Medicine
[2] Research Station for Cattle Breeding Dancu, Iasi, Iași
[3] Faculty of Agriculture, University of Life Sciences (IULS), Iași
[4] Grigore T. Popa University of Medicine And Pharmacy Iasi

the meat (Szewczyk A. et al.,2006; Hanczakowska E. et al., 2007; Oyebode O. et al.,2004; Hanczakowska E. et al., 2012). Also, in cattle, phytotherapy is successfully applied in intestinal motility disorders, to inhibit pathogenic microorganisms in the rumen that can influence the rate of nutrient assimilation and thus increase production or to prevent and treat mastitis (Wynn S.G. et al., 2007; Neculai - Valeanu A.S. et al., 2021).

In birds, plant extracts have been used prophylactically as an alternative to growth promoters (antibiotics) and immunostimulants. (Hughes P. et al., 2002; Hashemi S.R et al., 2013). A statistic conducted by Tamminen LM et al., In 2018, regarding the interest of researchers in phytotherapy in veterinary medicine at European level, shows that most studies (89%) focused on the preventive effect of phytotherapeutic products administered to poultry, pigs and sheep and only 11% investigated the curative effect of phytocompounds in the treatment of specific diseases of these species, most of them infectious diseases.

Therefore, the antimicrobial activity of plant extracts and essential oils has long been known. Although most promising studies are based on *in vitro* testing, their biological potential is demonstrated, and obviously their antimicrobial efficacy has been studied and highlighted, comparable to that of antibiotics. Therefore, they could be used as an alternative to antibiotic treatment, although the scientific community remains reserved when it comes to recommending them in therapy (EMA, EFSA, 2017).

However, in the EU, medicinal plants are increasingly used in veterinary medicine as feed additives and the effects on overall health and productive performance are much more visible to farmers, which will increasingly encourage their use. (Tamminen L.M. et al., 2018).

The phytocompound based on *Zingiber officinale* extract, *Piper nigrum* and *Piper cubeba* oil is used in Europe as a flavoring or food additive administered in farm animal feed.Ginger (*Zingiber officinale*) is one of the most consumed spices in the world and also known for its medicinal properties, having anti-inflammatory, antitumor, antipyretic, antiplatelet, anti-hyperglycemic, antioxidant, antidiabetic, anticoagulant, cardiotonic, cytotoxic, etc. (Wang W.H. et al., 2005; Shahrajabian M. et al., 2019). Scientific studies on ginger extract and its various components demonstrate the medicinal, chemical and pharmacological potential of this plant (Benzie IFF et al., 2011). Ginger obviously has a multitude of other metabolites that have not yet been studied and for which specific molecular targets and mechanisms of action are not known (Wachtel-Galor S. et al., 2011). *Piper nigrum* (black pepper) and *Piper cubeba* (tailed pepper) are two of the more than 700 species of pepper in the *Piperaceae* family, plants native to South Asia but cultivated in many other regions of the world, where there is a warm climate and special lightning (Prasad Ashok K. et al., 2005). In the history of peoples, these plants have been used as traditional medicines and food flavorings. Their complex composition of proteins, fats, carbohydrates, fiber, alkaloids, essential oils, resins, vitamins, etc., has brought many benefits to human health and especially for the treatment of digestive disorders including intestinal parasitosis (Kumar S. et al., 2015,Mansurah A, 2016). Studies have shown their antimicrobial and antioxidant potential, Piper nigrum being recognized for its effectiveness in skin diseases, respiratory diseases, anemia, diabetes, etc. (Sharma MC et al., 2004; Mihăilă B. et al., 2019; Kumar S. et al., 2015) and the species Piper cubeba is recommended in venereal diseases, digestive diseases, asthma (Elfahmi KR et al., 2013). *Piper cubeba* oil is frequently used in phytotherapy, being rich in valuable bioactive compounds such as antioxidants, anti-parasites and insecticides (Yusuf A. et al., 2019).

The aim of these tests was to evaluate the antimicrobial potential of this EU-approved phytocomplex as a flavoring, given the biological properties of the three plant compounds and the therapeutic benefits observed on farms after administration of the plant suspension.

MATERIALS AND METHODS

The tested phytocomplex is marketed in Romania under the name of Respowell, and is a suspension obtained by combining *Zingiber officinale e*xtract, *Piper nigrum* essential oil and *Piper cubeba* oil. The determination of antimicrobial potential was performed by three *in vitro* techniques against four standardized Gram-negative bacterial strains: *Escherichia c*oli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, *Klebsiella pneumoniae* ATCC 13883.

Qualitative determination of antimicrobial efficacy was performed by diffusimetric method (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing - Version 5.0, January, 2019). For this purpose, Muller Hinton solid medium Petri dishes were seeded with bacterial suspensions whose cell density corresponded to the 0.5 McFarland Stardard scale. After dispersing the suspension, filter paper washes (5 mm) were spread on the surface of the

medium, over which 10 ul of Respowell phytocomplex was spotted. A gentamicin microcomprimate (10 ug) was used as an active control. The interpretation of the antimicrobial effect consisted in measuring the diameter of the microbial inhibition and comparing it with the inhibition zone created by the antibiotic.

The determination of the minimum inhibitory concentration (MIC) was performed by the technique of serial microdilutions in 96-well plates (CLSI, 2012). From the stock solution of the phytocomplex *Zingiber officinale* extract, *Piper nigrum* essential oil and *Piper cubeba* oil (1000 mg s.a / lt stock solution) was distributed 100 ul (100 mg s. A.) In the first column (A1-A4). In the other 11 wells (A2-A11; B2-B11, C2-C11, D2-D11) 50 ul of MH broth were distributed. Serial dilutions were performed by transferring from the first well (100 mg.s.a.) 50 ul to the second well, repeating the procedure to well 11. For each dilution, the approximate concentration (mg/ul) was: dil.1/2 (0.0005 mg/µl), dil.1/4 (0.00025 mg/µl), dil.1/8 (0.000125mg/µl), dil.1/16 (6.25E-05 mg/µl), dil. 1/32 (3.13E-05 mg/µl), dil. 1/64 (1.56E-05mg / µl), dil.1/128 (7.81E-06 mg/µl), dil.1/256 (3.91E-06 mg/µl), dil.1/512 (1.95E-06 mg/µl), dil.1/1024 (9.77E-07 mg/µl). The growth control (positive control) was distributed in the last column of wells. From the 0.5 McFarland microbial suspensions (1.8 x 10^8 cfu / ml), 100 ul of inoculum was taken and transferred to each well. The final volume was 200 ul. After distribution, the plates were stirred (State Fax 2200 Awareness). After incubation at 37°C/24h the plates were read by spectrophotometry at (450nm) using Microplate Reader Stat Fax. The MIC of the plant substance was defined as the lowest concentration of the plant substance that inhibited the growth of 80% of the bacterial culture compared to the bacterial growth of the positive control.

The time-kill method is used to test the bactericidal activity of one or more antimicrobial agents against a particular bacterial strain. This is done by counting the viability of bacterial strains at different time intervals. To obtain the time-kill curve the bacterial strains growth rate must be counted at different time intervals starting from 0 hours to 24 hours (CLSI, 1998; ASTM E2315-16, 2016). The bactericidal potential is highlighted when there is a decrease of 3log10 of cfu/ml which is equivalent to the destruction of 99.9% of the bacteria in the inoculum. The antimicrobial activity of the phytocomplex was performed at 15 min, 30 min, 60 min and 24 hours. Then, the percentage of dead cells was calculated by determining the number of living cells (cfu/ml) in each tube and reporting to the positive control (2 x 10 cfu/ml), using the counting method in solid culture plates. For this, the tested bacterial cultures were incubated at 37° C for 24 hours. This testing follows the guidelines set by the Clinical & Laboratory Standards Institute (CLSI, 1999).

RESULTS AND DISCUSSIONS

*Results obtained by diffusimetric method***:**Following the qualitative testing of the antimicrobial potential, modest inhibitory activities were observed compared to the antimicrobial activity of the antibiotic (gentamicin,10µg) (*figure 1*).

Figure 1. Testing the phytocomplex by diffusimetric method against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, *Klebsiella pneumoniae* ATCC ATCC 13883. Muller Hinton agar medium, Gentamicin (Gn / 10 µg)

The best area of microbial inhibition (13 mm) was identified in *Escherchia coli* ATCC 25922, diameter smaller than that obtained in the positive control (20 mm). The areas of inhibition created by the phytocomplex against *Klebsiella pneumoniae* ATCC 13883 (11mm) and *Salmonella enterica* ATCC 13076 (10 mm) were much more modest compared to the inhibitory effects of *Escherichia coli* ATCC 25922 and the positive control. By diffusion method, *Pseudomonas aeruginosa* ATCC 27853, did not show sensitivity to phytocomplex.

The determination of the minimum inhibitory concentration is a quantitative method

and was performed in order to identify the best concentration of plant suspension that can inhibit the selected bacterial strains. The MIC of the plant substance was defined as the lowest concentration of the plant substance that inhibited bacterial growth (*figure. 2*). The results obtained by testing the minimum inhibitory concentration (MIC) are presented in *figure* 3

.

Figure 2 Microdilution broth plate 96 well for phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba* ,

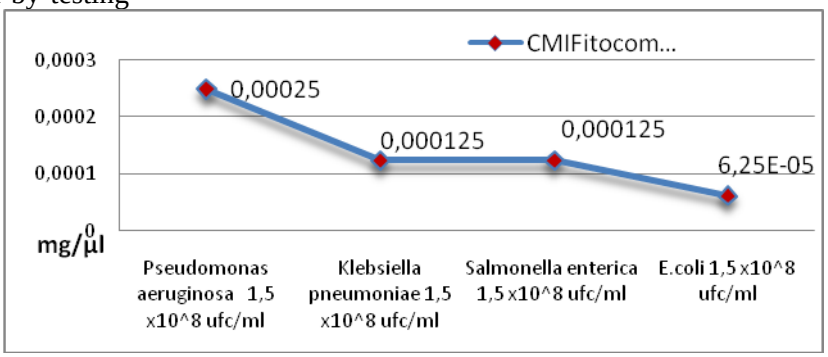

Figure 3 Determination of the minimum inhibitory concentration (MIC) for phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba* (mg/ul) against the bacterial strains tested.

The results of the determinations performed showed that the phytocomplex tested had antimicrobial activity against all strains tested. As the concentration of active substances in the stock solution was very low, the calculation of the minimum inhibitory concentrations was performed keeping the ratio, so that the phytocomplex MIC for *Escherichia coli* ATCC 25922 was 6.25E-05 mg/µl, the phytocomplete MIC for *Klebsiella pneumoniae* ATCC 13883 was of 0.000125 mg/µl, the phytocomplete MIC for *Salmonella enterica* ATCC 13076 was 0.000125mg/µl, the phytocomplete MIC for *Pseudomonas aeruginosa* ATCC 27853 was 0.00025 mg/µl.

Time-killing test results. The results obtained at the time-killing test were in close correlation with the tested bacterial strain and the contact time (*figure 4, figure 5, figure 6, figure 7, figure 8, table 1*).

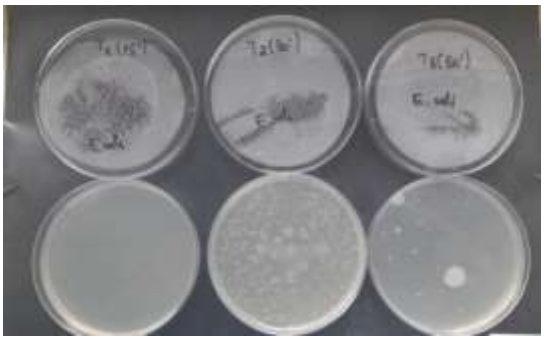

Figure 4. Time-kill assay to *Escherichia coli:* 15 min.(T1), 30min (T2), 60 min (T3)

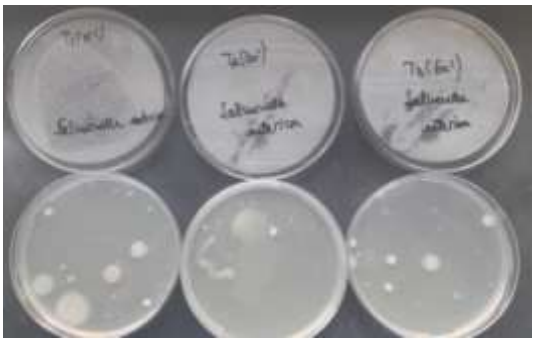

Figure 5. Time-kill assay to *Salmonella enterica*: 15 min.(T1), 30min (T2), 60 min (T3)

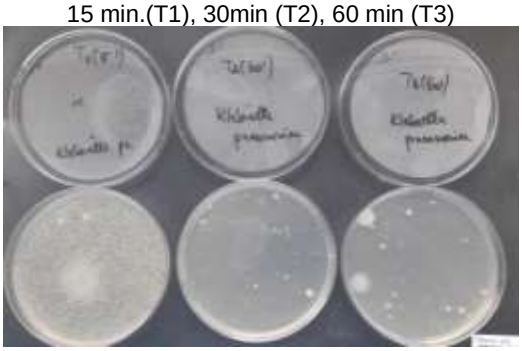

Figure 6. Time-kill assay to *Klebsiella pneumoniae*: 15 min.(T1), 30min (T2), 60 min (T3)

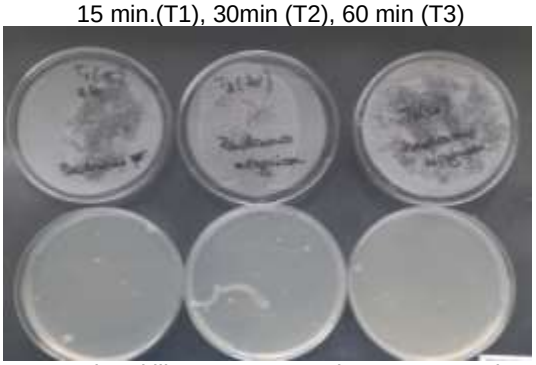

Figure 7. Time-kill assay to *Pseudomonas aeruginosa*: 15 min.(T1), 30min (T2), 60 min (T3)

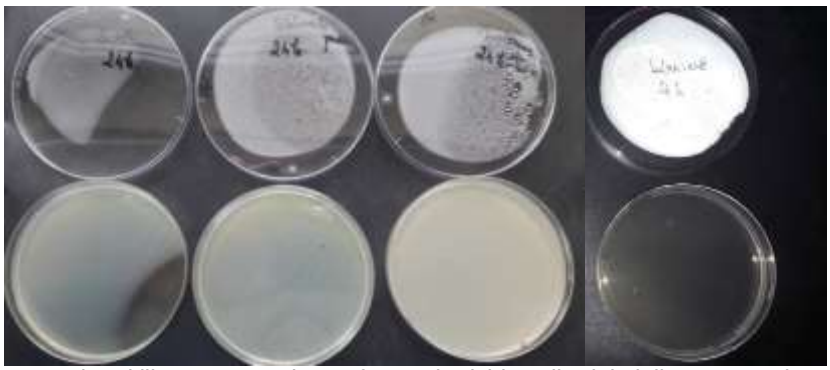

Figure 8. Time-kill assay to 24 hours for *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica*.

In the time-kill assay, the results presented in terms of the changes in the log10 cfu/mL of viable colonies indicated that the extract exhibited significant bactericidal activity.

Table 1

Time-kill test result

Bacteria species	Martor ufc/ml	Times contact with phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba*			
		15 min ufc/ml	30 min ufc/ml	60 min ufc/ml	24 ore ufc/ml
Escherichia coli	1,8 x 10^8	3 x10^7	28 x10^4	3,48 x10^2	0
Log_{10} (ufc/ml)	8,26	7,48	4,45	2,58	0
LR (Log10 reduction)	8	0,7782	2,808	5.844	0
%Reduction		**83,333**	**99,844**	**100**	**0**
Klebsiella pneumoniae	1,8 x 10^8	42 x10^4	34x10^2	12 x10^1	0
Log_{10} (ufc/ml)	8,26	4,45	3,53	2,08	0
LR (Log10 reduction)	8	2,632	4,724	6,176	0
%Reduction		**99,767**	**99,998**	**100**	**0**
Salmonella enterica	1,8 x 10^8	8,2 x10^1	4,9 x10^1	4,7 x10^1	0
Log_{10} (ufc/ml)	8,26	1,91	1,69	1,67	0
LR (Log10 reduction)	8	6,974	6,564	6,583	0
%Reduction		**100**	**100**	**100**	**0**
Pseudomonas aeruginosa	1,8 x 10^8	9,2 x10^1	5 x10^1	2,9x10^1	0
Log_{10} (ufc/ml)	8,26	1,96	1,70	1,46	0
LR (Log10 reduction)	8	6,91	6,55	7,091	0
%Reduction		**100**	**100**	**100**	**0**

Logarithmic reduction (LR) is the method by which microbial reduction can be quantified and the microbicidal potential of a suspension can be determined.

The antimicrobial efficacy test by the time-kill method of the phytocomplex was performed on microbial suspensions whose microbial density was equivalent to 8.26Log10 (cfu/ml).

.

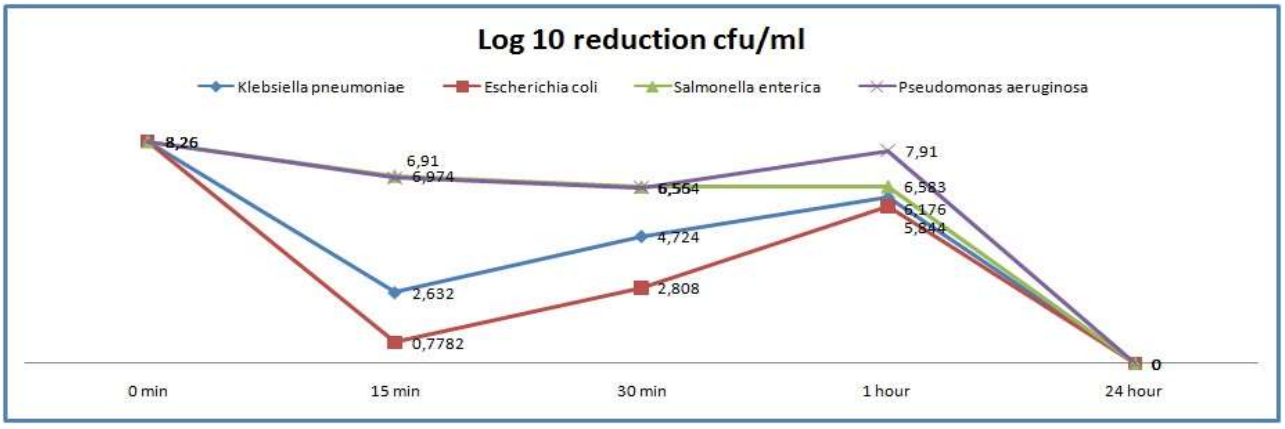

Figure 9. Microbial reduction as log reduction values (LR) when testing microbial suspensions with phytocomplex *Zingiber officinale extract/ Piper nigrum /Piper cubeba*

After 15 minutes of incubation, the best logarithmic reductions were 6.91Log10 cfu/ml in *Pseudomonas aeruginosa* and 6.97Log10 cfu/ml in *Salmonella enterica* for which a 100% logarithmic reduction was calculated. Compared to *Klebsiella pneumoniae*, a reduction of 2.632 Log10 cfu/ ml (99.767% reduction) was obtained and for *Escherichia coli* it was 0.778Log10 cfu/ml (83.33%) (*figure 9*.).

After 30 minutes of incubation, logarithmic reductions of 6.55Log10 cfu/ml (100%) were detected for *Pseudomonas aeruginosa*, 6.564Log10 cfu/ml (100%) for *Salmonella enterica*, 4.724Log10ufc/ml (99.99%) for *Klebsiella pneumoniae* and 2.808Log10 cfu/ml (99.84%) for *Escherichia coli*.

After 60 minutes of incubation, a logarithmic reduction of 5.844 Log10 cfu/ml (100%) for *Escherichia coli* and 6.176 Log10 cfu / ml for *Klebsiella pneumoniae* was identified. The other two bacterial species, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, logarithmically reduced the number of viable cells continued to increase, reaching 7.091 Log10 cfu/ml (100%) for *Pseudomonas aeruginosa* and 6.583 Log10 cfu/ml (100%) for *Klebsiella pneumoniae*.

After 24 hours of incubation, all bacterial species tested were inhibited by the phytocomplex *Zingiber officinale* extract/*Piper nigrum* essential oil/*Piper cubeba* oil. This may be an advantage, given the way this product is administered.

Various antimicrobial susceptibility tests are used in research on the antimicrobial activity of plant complexes to determine their effectiveness against various potentially pathogenic microorganisms. Plant extracts, essential oils or certain constituents extracted from plant complexes are evaluated and verified by different techniques so as to determine their therapeutic concentrations and their usefulness (Ncube NS et al., 2008)

These *in vitro* tests can also determine the sensitivity of pathogenic microorganisms that have developed resistance to semisynthetic antimicrobial agents (Wiegand I et al., 2008). Of all the techniques, dilution methods for determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (CMB) are the most widely used and can be easily determined with or without a spectrophotometer. To further facilitate the interpretation of results, colorimetric indicators such as resazurin, which is oxidized blue and turns pink when reduced by viable bacterial cells, can be added to dilution methods (Mann, C. et al., 1998).

Although the results of *in vitro* tests clearly show the antimicrobial effect of a plant product, additional studies are needed to validate their effectiveness in in vivo administration.

The use of medicinal plants in human and animal therapy has very old attestations in the history of medicine and their use is still current. Studies conducted until 2014 show that about 80% of the world's population uses plants and their extracts therapeutically. Subsequently, the incidence of studies conducted in six countries was reconsidered, also showing that their use is declining, including in the regions among the top users of natural therapies (Oyebode O et al., 2016). However, plants are an integral part of traditional medicine used as adjunctive therapy in modern medicine in countries such as India and China and are still the main source of therapy in certain diseases in humans and animals, especially when modern treatments are either unavailable or inefficient (Oyebode O et al., 2016).

Preliminary tests performed on the phytocomplex obtained by combining *Zingiber officinale* extract/*Piper nigrum* essential oil/*Piper cubeba* oil demonstrate the antimicrobial efficacy of this plant suspension, so we consider it appropriate to mention this inhibitory capacity against opportunistic Gram-negative bacteria that have the potential to be pathogens for animals. A limitation of our study is that the test was performed only on Gram-negative bacterial strains and no additional testing was possible to demonstrate the mechanism of action of the main compounds in the plant suspension.

CONCLUSIONS

The antimicrobial potential of the phytocomplex *Zingiber officinale* extract/Piper *nigrum* essential oil/*Piper cubeba* oil was demonstrated by all tests performed, compared to all Gram negative bacterial species tested.

Preliminary results of the tested Phytocomplex demonstrate its antimicrobial potential and a possible therapeutic alternative in veterinary pathology.

REFERENCES

ASTM E2315-16,2016 - Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure, ASTM International, West Conshohocken, PA, www.astm.org

Aziz M.A., Khan A.H., Adnan M., 2018 - *Traditional uses of medicinal plants used by Indigenous communities for veterinary practices at Bajaur Agency, Pakistan*. J Ethnobiology Ethnomedicine 14, 11.

Benzie IFF, Wachtel-Galor S, (editors), 2011- *Herbal Medicine: Biomolecular and Clinical Aspects*. 2nd edition. Chapter 7The Amazing and Mighty Ginger

(authors:Ann M. Bode and Zigang Dong.Boca Raton (FL): CRC Press/Taylor & Francis; 2011.

CLSI, 1999 - *Methods of Determining Bactericidal Activity of Antimicrobial Agents*; Approved Guideline, M26, V. 19, No. 18,

CLSI- M26-A,1998- *Methods for Determining Bactericidal Activity of Antimicrobial Agents.Approved Guideline,* CLSI document M26-A. Clinical and Laboratory Stan-dards Institute, 950 West Valley Roadn Suite 2500,Wayne, Pennsylvania19087, USA, 1998

CLSI- M07-A9,2012- *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically,* Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA,

Dewick, P.M., 2001 - *Medicinal natural products: A biosynthetic approach*. 2nd edition, John Wiley & Sons Ltd. Chichester.

Dilshad S.M.R., Rehman N.U., Ahmad N., Iqbal A., 2010 - *Documentation of ethnoveterinary practices for mastitis in dairy animals in Pakistan.* Pak Vet J.;30:167–7

Elfahmi K.R., Batterman S., Bos R., Kayser O., Woerdenbag H.J., Quax W.J., 2007- *Lignan profile of* Piper cubeba*, an Indonesian medicinal plant*.*Biochem. Syst. Ecol.* 2007;35:397–402.

EMA and EFSA, 2017 - *Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety* (RONAFA).*EFSA J.*, 15:4666 10.2903/j.efsa.2017.4666

Gounder R., 2006 - *Kava consumption and its health effects.* Pac Health Dialog. 2006 Sep;13(2):131-5.

Haghiroalsadat F, Vahidi A, Sabour M, Azimzadeh M, Kalantar M, Sharafadini M., 2011- *The indigenous cuminum cyminum L. of yazd province: chemical assessment and evaluation of its antioxidant effects*. Journal of Shahid Sadoughi University Medical Sciences; 19(4):472–481

Hanczakowska E., Swiatkiewicz M., 2012- *Effect of herbal extracts on piglet performance and small intestinal epithelial villi*, Chzec. J.Animal.Sci., 57,(9), 420-429

Hanczakowska E., Swiatkiewicz M., Szewczyk A., 2007- *Efficiency of herb preparation and gallic acid in pig feeding.* Annals of Animal Science, 7, 131–139

Hashemi S.R, Davoodi H., 2011 - H*erbal plants and their derivatives as growth and health promoters in animal nutrition.Vet Res Commun*.35:169–80.

Hughes P., Heritage J., 2002 - *Antibiotic growth-promoters.* Feed Tech. 6:20–22.

Juneja V.K., Dwived, H.P., Yan X., 2012 - *Novel natural food antimicrobials.* Annual Review of Food Science and Technology, 3, 381–403.

Khalid K., Inayat Ur R., Soares C.E., Ali Niaz, Farhana I.,2019 - *Ethnoveterinary Therapeutic Practices and Conservation Status of the Medicinal Flora of Chamla Valley*, Khyber Pakhtunkhwa, Pakistan , Frontiers in Veterinary Science, 6, 122

Kumar S., Malhotra S., Ashok K. Prasad K.A., Erik V. Van der Eycken V.E., Bracke E.M., Stetler-Stevenson G.W., Parmar S.V., Ghosh B., 2015- *Anti-Inflammatory and Antioxidant Properties of Piper Species: A Perspective from Screening to Molecular Mechanisms*, Curr Top Med Chem.; 15(9): 886–89

Lev E., 2003 - *Traditional healing with animals (zootherapy): medieval to present-day Levantine practice*. Journal of Ethnopharmacology, 85, 107–11

Mann C., Markham J., 1998 - *A new method for determining the minimum inhibitory concentration of essential oils.* Journal of Applied Microbiology, 84: 538-544.

Mansurah A. Abdulazeez, Ibrahim Sani, Bolanle D. James, Abdulmalik S. Abdullahi, 2016 - *Essential Oils in Food Preservation, Flavor and Safety*,Chapter 31 - Black Pepper (Piper nigrum L.) Oils,Editor(s): Victor R. Preedy,Academic Press,277-285, ISBN 9780124166417,

Mazid M, Khan TA, Mohammad F., 2011- *Role of secondary metabolites in defense mechanisms of plants*. Biology and Medicine. 2011; 3(2):232–249

McCorkle C.M., Green E.C, 1998 - *Intersectoral health care delivery*. Agric Hum.;15:105–14

McCorkle C.M., Mathias-Mundy, E., 1992 - *Ethnoveterinary medicine in Africa*. Journal of the International African, 62(1), 59–93.

McCorkle CM., 1986 - *An introduction to ethnoveterinary research and development*. J Ethnobiol. 6:129–49.

Mihăilă B., Dinică R.M., Tatu A.L., Buzia O.D., 2019- *New insights in vitiligo treatments using bioactive compounds from Piper nigrum.* Experimental and Therapeutic Medicine, 17, 1039-1044.

Ncube N. S., Afolayan A. J., Okoh A. I., 2008 - *Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends,*" African Journal of Biotechnology, vol. 7, no. 12, pp. 1797–1806,

Oyebode O, Kandala NB, Chilton PJ, Lilford RJ., 2016 - *Use of traditional medicine in middle-income countries: a WHO-SAGE study.* Health Policy Plan. 2016;31(8):984-991.

Prasad Ashok K., Kumar Vineet, Arya Pragya, Kumar, Sarvesh Dabur, Rajesh Singh, Naresh Chhillar, Anil K., Sharma Gainda L., Ghosh Balaram, Wengel Jesper, Olsen Carl E. and Parmar Virinder S., 2005 - *Investigations toward new lead compounds from medicinally important plants* Pure and Applied Chemistry, vol. 77, no. 1, pp. 25-40.

Shahrajabian M. H, Wenli S., Qi C., 2019- *Clinical aspects and health benefits of ginger (Zingiber officinale) in both traditional Chinese medicine and modern industry*, Acta Agriculturae Scandinavica, Section B — Soil & Plant Science, 69:6, 546-556,

Sharma M.C., Chinmay Joshi, 2004- *Plants used in skin diseases of animals*, Natural Product Radiance Vol 3(4).

Szewczyk A., Hanczakowska E., Swiatkiewicz M., 2006- *The effect of nettle (Urtica dioica) extract on fattening performance and fatty acid profile in the meat and serum lipids of pigs.* Journal of Animal and Feed Sci-ences, 15 (Supl. 1), 81–84

Tamminen L.M., Emanuelson U., Blanco-Penedo I., 2018 - *Systematic Review of Phytotherapeutic Treatments for Different Farm Animals Under European Conditions*.Front Vet Sci. 2018;5:140.

Viegi L., Pieroni A., Guarrera P.M., Vangelisti R., 2003- *A review of plants used in folk veterinary medicine in Italy as basis for databank.* Journal of Ethnopharma-cology, 89, 221–224

Wachtel-Galor S, Benzie IFF., 2011- Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs. In: Herbal Medicine: Biomolecular and Clinical Aspects. 2nd ed. CRC Press/Taylor & Francis, Boca Raton (FL);

Wang WH, Wang ZM., 2005- *Studies of commonly used traditional medicine-ginger*. Zhongguo Zhong Yao Za Zhi. 2005 Oct;30(20):1569-73. Chinese.

Wiegand I., Hilpert K.,. Hancock R. E. W. 2008 - *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances*,Nature Protocols, vol. 3, no. 2, pp. 163–175,
Wynn S.G., Fougère B.J, 2007 - Veterinary Herbal Medicine, book, Elsevier Inc., ISBN 978-0-323-02998-8
Yusuf A., Tran D.X., Tran N.Q., Hoang-Dung T., Quang-Tri L., 2019 - *Biological Activities and Chemical Constituents of Essential Oils from Piper cubeba Bojer and Piper nigrum L*.Molecules. 2019 May; 24(10): 1876.
.