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ANTIBACTERIAL PROPERTIES OF COMMERCIALLY AVAILABLE CAYEPUT ESSENTIAL OIL AGAINST DIFFERENT GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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Essential oil and leaf extracts of Melaleuca leucadendra L. demonstrated a series of biological activities of interest, including antioxidant, antimicrobial, antitumoral and anti-inflammatory properties. The aim of the current study was an in vitro evaluation of the antimicrobial activity of the cajeput essential oil against Gram-negative strains such as Escherichia coli (Migula) Castellani and Chalmers (ATCC® 25922™), Escherichia coli (Migula) Castellani and Chalmers (ATCC® 35218™), Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) and Gram-positive strains such as Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 29213™), Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 25923™), methicillin-resistant (MRSA), mecA positive Staphylococcus aureus (NCTC® 12493), Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (resistant to vancomycin; sensitive to teicoplanin) and Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™) to assess the possible use of this oil in preventing infections caused by these pathogens. The cajeput essential oil was provided by Polish essential oil manufacturers (Bamer®, Włocławek, Poland). Antimicrobial susceptibility of the tested strains was performed by the Kirby-Bauer disc diffusion method. Our research has shown that cajeput essential oil exhibits antibacterial properties. Gram-positive bacteria were the most susceptible to commercial cajeput oil, which may indicate that the active biological substances in cajeput essential oil (including phenolic acids, tannins, etc.) may be potential agents against bacterial infections. Among Gram-negative bacteria, only Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) strain was resistant to the cajeput essential oil. We did not observe statistically significant changes in the zone of growth inhibition after the application of cajeput essential oil compared to the control samples (96% ethanol). Our study may suggest that the use of cajeput essential oil may be helpful for a wide range of bacterial infections in veterinary, aquaculture, medicine, and beyond.

Keywords: **cajeput essential oil, antibacterial properties, Gram-negative/positive bacterial strains, Kirby-Bauer disc diffusion method.**

Antibiotics have always been considered one of the wonder discoveries of the 20th century. The extraordinary genetic capacities of microbes have benefitted from animal's overuse of antibiotics to exploit every source of resistance genes and every means of horizontal gene transmission to develop multiple mechanisms of resistance for each and every antibiotic introduced into practice clinically, agriculturally, or otherwise [26]. The origin of antibiotic resistance genes now possessed by animal pathogens can be traced back to environmental microorganisms [17]. Consequently, ecological connectivity, founder effect, and fitness costs are important bottlenecks that modulate the transfer of resistance from environmental microorganisms to pathogens [10, 27]. Knowing the general principles involved in the acquisition of antibiotic resistance is therefore of interest to clinicians, biologists, ecologists, etc. The origin of antibiotic resistance genes now possessed by animal pathogens can be traced back to environmental microorganisms [15]. Consequently, a full understanding of antibiotic resistance requires the study of natural environments, as well as clinical environments. Now it is to look for alternatives in veterinary medicine tied to antibiotic resistance, among other things [13].

In recent years there has been an increasing interest in the use of natural substances, and some questions concerning the safety of synthetic compounds have encouraged more detailed studies of plant resources [20]. Plant secondary metabolites are antimicrobial host defense substances expressed in all higher plants. Performing a significant role in plants, they display potent activity against a wide range of pathogens. Plant extracts contain large amounts of bioactive compounds, mainly polyphenols. Polyphenols inhibit the growth of microorganisms, especially bacteria. Their mechanism of action is still not fully understood but may be related to their chemical structure [29]. They can cause morphological changes in microorganisms, damage bacterial cell walls, and influence biofilm formation. Polyphenols also influence protein biosynthesis, change metabolic processes in bacteria cells and inhibit ATP and DNA synthesis (suppressing DNA gyrase) [3, 6, 28].

Essential oils, odorous and volatile products of plant secondary metabolism, have a wide application in folk and traditional medicine, veterinary, food flavoring, and preservation as well as in fragrance industries. The antimicrobial properties of essential oils have been known for many centuries. In recent years, a large number of essential oils and their constituents have been investigated for their antimicrobial properties against some bacteria and fungi in more than 500 reports [16, 23]. The interest in using natural antimicrobials instead of chemical preservatives in food products has been increasing in recent years. In regard to this, essential oils-natural and liquid secondary plant metabolites are gaining importance for their use in the protection of foods, since they are accepted as safe and healthy. Although research studies indicate that the antibacterial and antioxidant activities of essential oils are more common compared to other biological activities, specific concerns have led scientists to investigate the areas that are still in need of research [9, 11]. Also, the use of essential oils in the control of veterinary ectoparasites, such as a range of arthropod ectoparasites, particularly lice, mites, and ticks, is an area that holds considerable potential for the future, and research into their use is still at an early stage [7].

Melaleuca leucadendra (L.) L. (Myrtaceae), commonly known as the paperbark tree, is widespread in Australia, Southeast Asia, New Guinea, and the Torres Strait Islands. Historically, various preparations from the bark, leaves, and fruits of this species have been used in folk medicine as tranquilizers, sedatives, painkillers, and in the treatment of various skin disorders [22]. Essential oil and extracts derived from leaves of *M. leucadendra* demonstrated a series of biological activities of interest, including antioxidant, antimicrobial, antitumoral, and anti-inflammatory properties [8, 12, 14, 22]. Phenolic acids, tannins, and flavonoids have been identified as the main ones responsible for the biological effects described for this species [5, 19].

In the current study, *in vitro* antimicrobial profiling of commercial Cajeput essential oil derived from leaves of *M. leucadendra* (Natural essential oil – Cajeput Bamer®) was performed, exhibiting inhibitory activity against Gram-negative strains such as *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™), *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27853™) and Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™), methicillin-resistant (MRSA), mecA positive *Staphylococcus aureus* (NCTC® 12493), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (resistant to vancomycin; sensitive to teicoplanin) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™).

Materials and Methods. *Cajeput essential oil.* The cajeput EO was provided by Polish essential oil manufacturers (Bamer®, Włocławek, Poland). The investigated sample did not contain additives or solvents and was confirmed to be natural by the manufacturers. The samples were stored in resalable vials at 5°C in the dark but were allowed to adjust to room temperature prior to investigation. Geographical origins were excluded as information was mostly not available. Product description: Natural essential oil – Cajeput Bamer®. The highest quality, pure, natural essential oil, obtained from fresh leaves and twigs of the cajeput tree (*Melaleuca leucadendra* Leaf Oil). Laboratory tested.

About the manufacturer: Bamer® has been offering 100% natural, pure essential oils and fragrance compositions since 1993. Application studies on patients conducted at the Medical Academy confirmed the effectiveness of Bamer® oils in aromatherapy and cosmetics. The products are not tested on animals. Safety assessments, numerous certificates and approvals, compliance with the latest pharmacopoeia Ph.Eur. and IFRA, positive opinion of the National Institute of Hygiene guarantee the highest pharmaceutical and cosmetic quality of oils. Bamer® oils have been submitted to the European Commission via CPNP (Cosmetic Products Notification Portal). Bamer® essential oils are certified by the National Institute of Hygiene, IFRA and laboratory analyses.

Determination of the antibacterial activity of plant extracts by the disk diffusion method. The testing of the antibacterial activity of lavender EO was carried out *in vitro* by the Kirby-Bauer disc diffusion technique [4]. In the current study, Gram-negative strains such as *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™), *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27853™) and Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™), methicillin-resistant (MRSA), mecA positive *Staphylococcus aureus* (NCTC® 12493), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (resistant to vancomycin; sensitive to teicoplanin) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™) were used.

The strains were inoculated onto Mueller-Hinton (MH) agar dishes. Sterile filter paper discs impregnated with cajeput EO were applied over each of the culture dishes. Isolates of bacteria with lavender EO were then incubated at 37 °C for 24 h. The Petri dishes were then observed for the zone of inhibition produced by the antibacterial activity of cajeput EO. A control disc impregnated with 96% ethanol was used in each experiment. At the end of the 24-h period, the inhibition zones formed were measured in millimetres using the vernier. For each strain, eight replicates were assayed (n = 8).

The Petri dishes were observed and photographs were taken. The susceptibility of the test organisms to the cajeput EO was indicated by a clear zone of inhibition around the discs containing the cajeput EO and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤ 10 mm [18, 24].

Statistical analysis. Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of the cajeput EO tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v.13.3 software (TIBCO Software Inc., Krakow, Poland) [30].

Results and discussion. The results of the current study revealed that cajeput essential oil (EO) resulted in a statistically significant increase in the diameters of the zone inhibition against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain (by 130.5%, $p < 0.05$) compared to the control samples (18.49 ± 0.79 mm *vs*. 8.02 ± 0.61 mm). We obtained similar results after *in vitro* application of cajeput EO against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™), where we also observed a statistically significant increase in the diameters of zone inhibition (by 131.9%, $p < 0.05$) compared to the control samples (16.23 ± 0.88 mm *vs*. 7 ± 0.64 mm). We recorded a statistically significant increase in the diameters of zone inhibition against *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™) strains after exposure with cajeput EO compared to 96% ethanol (by 111, 3%, $p < 0.05$ and 84.4%, $p < 0.05$), i.e. (15.91 ± 0.91 mm *vs*. 7.53 ± 0.6 mm; 15.08 ± 0.62 mm *vs*. 8.18 ± 0.55 mm) (Fig. 1).

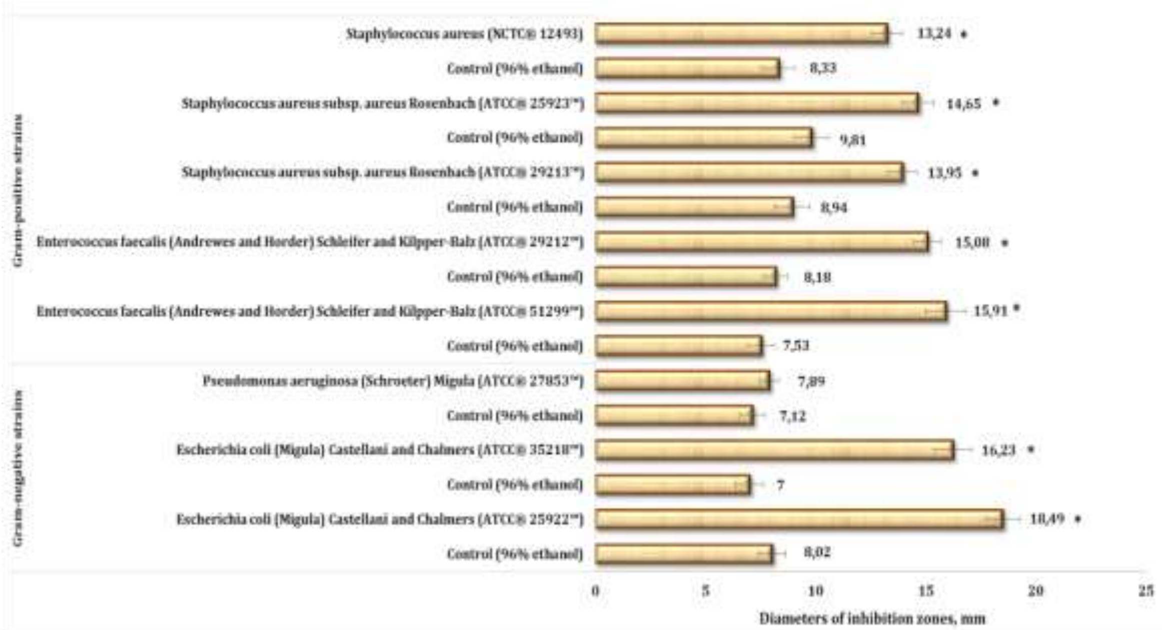

Fig. 1. The antibacterial activity induced by cajeput essential oil estimated as diameters of growth inhibition zones of examined Gram-positive and Gram-negative strains.

The data were presented as the mean ± the standard error of the mean (S.E.M.).
* denote significant differences between the control (96% ethanol) and cajeput EO ($p < 0.05$).

We observed a statistically significant increase in the diameters of zone inhibition of the *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™) strain (by 56%, $p < 0.05$) compared to the control samples (13.95 ± 0.65 mm *vs*. 8.94 ± 0.79 mm). Similar results were obtained after the application of cajeput EO against *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) and *Staphylococcus aureus* (NCTC® 12493) strains, where we recorded a statistically significant increase in the diameters of zone inhibition of pathogenic bacteria (by 49.3%, $p < 0.05$ and 58.9%, $p < 0.05$, respectively) compared to 96% ethanol (14.65 ± 0.71 mm *vs*. 9.81 ± 0.77 mm; 13.24 ± 0.69 mm *vs*. 8.33 ± 0.74 mm). Also, a similar, but not statistically significant, increase in the diameters of zone inhibition was observed after *in vitro* application of cajeput EO against *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27853™) strain (by 10.8%, $p > 0.05$) against 96% alcohol (7.89 ± 0.44 mm *vs*. 7.12 ± 0.56 mm) (Figures 1 and 2).

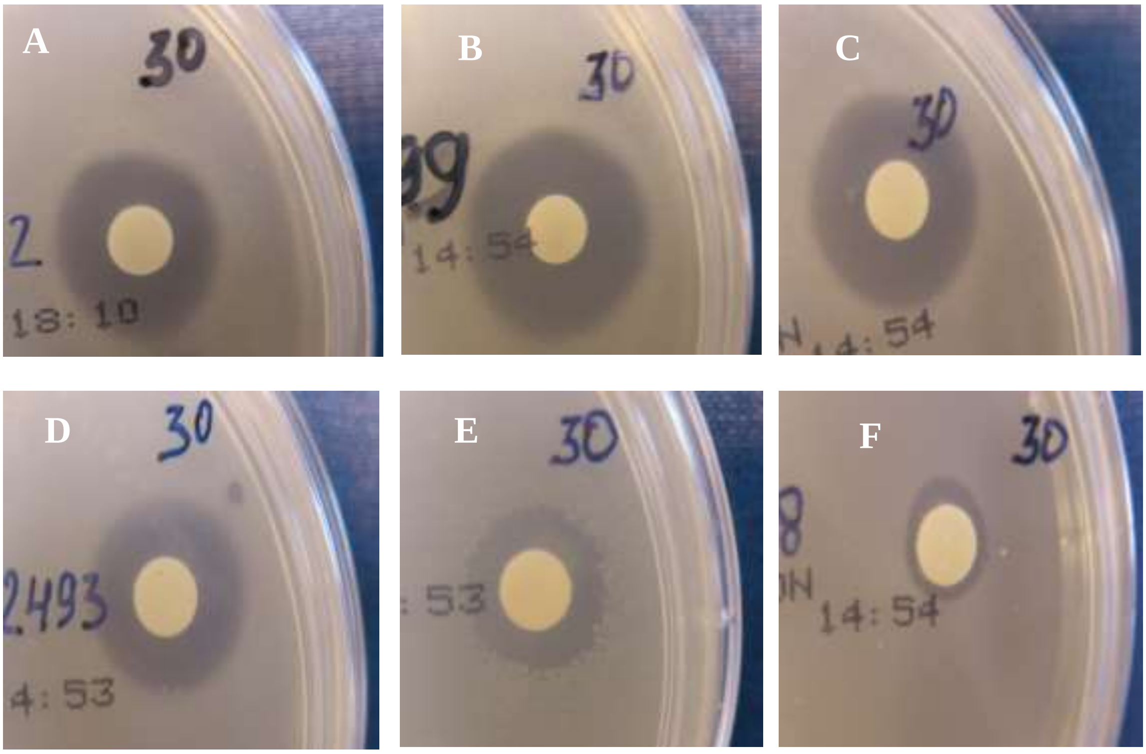

Fig. 2. Inhibition growth zones induced by cajeput essential oil against *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™) (A), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (B), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™) (C), *Staphylococcus aureus* (NCTC® 12493) (D), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) (E), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™) (F).

Thus, the results of our study revealed that cajeput essential oil was effective against *E. coli* strains, resulting in the highest increase in diameters of zone inhibition. More resistant to the cajeput essential oil was *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27853™) strain, exhibiting the lowest growth inhibition (Figs 1 and 2).

Similar results according to the antibacterial activity of cajeput essential oil and extracts derived from *M. leucadendra* were obtained by other researchers. For example, in the study of Valdés and co-workers [25], extensive *in vitro* antimicrobial profiling was performed for three medicinal plants grown in Cuba, namely *Simarouba glauca* DC., *Melaleuca leucadendron* and *Artemisia absinthium* L. Ethanolic extracts were tested for their antiprotozoal potential against *Trypanosoma b. brucei*, *Trypanosoma cruzi*, *Leishmania infantum*, and *Plasmodium falciparum*. Antifungal activities were evaluated against *Microsporum canis* and *Candida albicans* whereas *Escherichia coli* and *Staphylococcus aureus* were used as test organisms for antibacterial activity. Cytotoxicity was assessed against MRC-5 cells. According to these authors, only *M. leucadendron* extract exhibited selective activity against microorganisms tested. Although *S. glauca* exhibited strong activity against all protozoa, it must be considered nonspecific [25].

Abu Bacar and co-workers [1] have evaluated *Melaleuca cajuputi* essential oil in aerosol spray against the dengue vectors *Aedes aegypti* and *Ae. albopictus* at low-cost housing flats in Setapak, Kuala Lumpur, Malaysia. They sprayed 5% and 10% of concentrations for 5 seconds each towards hung mosquitoes in 5 cylindrical net cages. Aerosol weights were recorded before and after spraying to determine discharge rates. High knockdown and mortality were observed in both species of mosquitoes towards MS standard aerosol. There was a significant difference in mortality and knockdown between 5% and 10% of essential oil aerosol and 5% and 10% of essential oil between MS standards. For 5% essential oil, the mean percentage (%) of knockdown and mortality of *Ae. aegypti* displayed slightly higher compared to *Ae. albopictus*. Spraying with 5% *M. cajuputi* essential oil aerosol indicated a knockdown of *Ae. aegypti* 5.60 ± 1.18 and mortality of 22.90 ± 4.22, while *Ae. albopictus* showed 4.60 ± 0.89 knockdown and 20.00 ± 2.85 mortality. The 10% essential oil concentration gave 23.60 ± 1.68 knockdown and 48.05 ± 0.37 mortality for *Ae. aegypti*. *Ae. albopictus* gave 23.00 ± 3.16 knockdown and 44.20 ± 2.10 mortality, respectively [1].

M. cajuputi extracts also possess antioxidant and antibacterial activities. The results of Al-Abd and co-workers [2] revealed that both flower and leaf extracts had significant antioxidant and free radical-scavenging activity. Well diffusion, minimum inhibitory concentration, and minimum bactericidal concentration assays were used to determine antibacterial activity against eight pathogens, namely *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Streptococcus pneumoniae*, and *Pasteurella multocida*. The antibacterial assay of the extracts revealed no inhibition zones with the Gram-negative bacteria tested. However, the extracts demonstrated activity against *B. cereus*, *S. aureus*, and *S. epidermidis* [2].

Schelkle and co-workers [21] have evaluated the efficacy of commercially available products such as Melafix® and Pimafix® against Gyrodactylus turnbulli infections in guppies Poecilia reticulata. Melafix® and Pimafix® are formulated with the essential oils cajuput (*Melaleuca cajuputi*) and West Indian bay (*Pimenta racemosa*) and are marketed against bacterial and fungal infections, respectively. Previous experiments showed high efficacy of emulsified cajuput oil against gyrodactylids; the current study tested Melafix® and Pimafix® and their individual compounds against *Gyrodactylus turnbulli* infecting the guppies *Poecilia reticulata*. In particular, a combination treatment of Melafix® and Pimafix® was highly effective at reducing *in vitro* survival of parasites from 15 to 2 h and eradicating 95% of gyrodactylids *in vivo*. The unexpected high efficacy of this combination treatment is likely explained by the high content of terpenes and phenol propanoids in the cajuput and West Indian bay oils, as well as the anti-helminthic properties of the emulsifier Crovol PK 70. Hence, Melafix® and Pimafix® effectively reduce gyrodactylid burdens on fish, increasing the chances of efficient disease control in ornamental fish [21].

Antioxidant and anti-inflammatory activities of the butanol extract of *M. leucadendron* were evaluated by Surh and Yun [22]. These researchers examined the effects of *M. leucadendron* extracts on oxidative stress and inflammation. *M. leucadendron* was extracted with methanol (MeOH) and then fractionated with chloroform ($CHCl_3$) and butanol (BuOH). The antioxidant activity of the MeOH extract and BuOH fraction was higher than that of both α-tocopherol and butyrated hydroxytoluene (BHT). Total phenol content in the extracts of *M. leucadendron*, especially the BuOH fraction, is well correlated with antioxidant activity. The anti-inflammatory activity of BuOH extracts was investigated by lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production, and cyclooxygenase-2 (COX-2) expression in RAW 264.7 macrophages. The BuOH fraction significantly inhibited LPS-induced NO and PGE2 production. Furthermore, the BuOH extract of *M. leucadendron* inhibited the expression of COX-2 and iNOS protein without an appreciable cytotoxic effect on RAW264.7 cells. The extract of *M. leucadendron* also suppressed the phosphorylation of inhibitor κBα (IκBα) and its degradation associated with nuclear factor-κB (NF-κB) activation. Furthermore, BuOH fraction inhibited LPS-induced NF-κB transcriptional activity in a dose-dependent manner. These results suggested that *M. leucadendron* could be useful as a natural anti-oxidant and anti-inflammatory resource [22].

Conclusions. Our research has shown that cajeput essential oil exhibits antibacterial properties. Gram-positive bacteria were the most susceptible to this oil, which may indicate that the active substances in cajeput essential oil (including phenolic acids, tannins, etc.) may be potential agents against bacterial infections. Among the Gram-negative bacteria, only strain *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27853™) was resistant to the cajeput essential oil. We did not observe statistically significant changes in the diameters of growth inhibition after the application of cajeput essential oil compared to the control samples (96% ethanol). Our study may suggest that the use of cajeput essential oil may be helpful for a wide range of bacterial infections in veterinary, aquaculture, medicine, and beyond.

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АНТИБАКТЕРІАЛЬНІ ВЛАСТИВОСТІ КОМЕРЦІЙНОЇ КАЄПУТОВОЇ ЕФІРНОЇ ОЛІЇ ВІДНОСНО РІЗНИХ ГРАМ-ПОЗИТИВНИХ І ГРАМ-НЕГАТИВНИХ БАКТЕРІЙ

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Ефірна олія та екстракти листя Melaleuca leucadendra L. продемонстрували ряд біологічних активностей, включаючи антиоксидантні, протимікробні, протипухлинні та протизапальні властивості. Метою цього дослідження була оцінка in vitro протимікробної активності каєпутової ефірної олії щодо грам-негативних штамів, таких як Escherichia coli (Migula) Castellani *та Chalmers (ATCC® 25922™), Escherichia coli (Migula) Castellani та Chalmers. (ATCC® 35218™), Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) та грам-позитивних штамів, таких як Staphylococcus aureus subsp. aureus Rosenbach (ATCC 29213), Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 25923™), метицилін-резистентного (MRSA), mecA-позитивного Staphylococcus aureus (NCTC® 12493), Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (стійкий до ванкоміцину; чутливий до тейкопланіну) та Enterococcus faecalis (Andrewes and Horder) Schleifer и Kilpper-Balz (ATCC® 29212™) для оцінки можливого використання цієї олії для профілактики інфекцій, викликаних цими патогенами. Каєпутова ефірна олія була надана польськими виробниками ефірних олій (Bamer®, Влоцлавек, Польща). Антимікробну чутливість штамів визначали методом дискової дифузії. Наші дослідження показали, що каєпутова ефірна олія проявляє антибактеріальні властивості. Грам-позитивні бактерії були найбільш чутливими до дії каєпутової олії, що може вказувати на те, що біологічні активні речовини в цій олії (включаючи фенольні кислоти, дубильні речовини тощо) можуть бути потенційними речовинами щодо лікування та профілактики бактеріальних інфекцій. Серед грам-негативних бактерій, тільки штам Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) був стійкий до каєпутової ефірної олії. У цьому випадку, ми не спостерігали статистично істотних змін у зоні затримки росту після застосування ефірної олії порівняно з контрольними зразками (96% етанол). Наше дослідження може свідчити про те, що використання каєпутової ефірної олії може бути корисним у терапії та профілактиці широкого спектру бактеріальних інфекцій у ветеринарії, аквакультурі, медицині та інших галузях.*

Ключові слова: каєпутова ефірна олія, антибактерійні властивості, грам-негативні та грам-позитивні штами бактерій, диско-дифузійний метод Кірбі-Бауера.

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ORAL VACCINATION AGAINST *YERSINIA RUCKERI:* OXIDATIVE STRESS BIOMARKERS IN THE GILLS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM)

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The aim of this study was to assess the effect of oral vaccination against Yersinia ruckeri based on oxidative stress biomarkers in the gills of rainbow trout (Oncorhynchus mykiss Walbaum). The vaccine consisted of three Y. ruckeri strains (O1 serotype) that originated from rainbow trout cultured on different farms, where fish ex-