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THE ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS DERIVED FROM LEAVES AND PSEUDOBULBS OF *COELOGYNE FLACCIDA* LINDL. (ORCHIDACEAE) AGAINST DIFFERENT *STAPHYLOCOCCUS AUREUS* STRAINS

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The present study was conducted to investigate in vitro antimicrobial activity of ethanolic extracts obtained from leaves and pseudobulbs of Coelogyne flaccida against Staphylococcus aureus subsp. aureus Rosenbach (ATCC®25923™) (mecA negative), Staphylococcus aureus subsp. aureus Rosenbach (ATCC®29213™) (mecA negative, Oxacillin sensitive, weak β -lactamase-producing strain), Staphylococcus aureus NCTC 12493 (mecA positive, Methicillin-resistant, EUCAST QC strain for cefoxitin). The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). The leaves and pseudobulbs of C. flaccida plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Freshly leaves and pseudobulbs were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1:19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

The cultivation medium was trypticase soy agar (Oxoid™, UK), supplemented with 10 % defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37°C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration.

The examined ethanolic extracts obtained from leaves and pseudobulbs of C. flaccida showed remarkable antibacterial activities against different S. aureus strains. The observed activity may contribute to the reasons why orchids are used for infectious and inflammatory conditions in ethnomedicine. The research showed that ethanolic extracts of C. flaccida possess the highest antibacterial potency against S. aureus NCTC 12493 strain. These findings led the authors to suggest that these extracts may be used as natural antiseptics and antimicrobial agents in medicine and veterinary practice. Nevertheless, despite the promising results, more research should be carried out to further evaluate the roles of particular compounds, isolated from all parts of orchid plants, attributable to antimicrobial activity.

Keywords: antimicrobial activity, extracts, orchids, Coelogyne, Staphylococcus aureus strains, disk diffusion method

Staphylococcus aureus is an opportunistic pathogen causing a wide range of acquired infections with high morbidity [1]. Diseases caused by the Gram-positive bacterium range from skin infections to life-threatening illnesses, such as septicemia, pneu-

monia, endocarditis, meningitis [1, 13, 18]. This is in part due to its ability to adhere to the surface of indwelling medical devices (e.g. catheter) and develop biofilm, a multi-layered structure comprising of bacterial communities embedded within the extracellular hydrated polymeric matrix [20]. A biofilm is a natural form of bacterial growth ubiquitously in environmental niches. In particular, biofilm production by *S. aureus* has been identified as an important factor of pathogenesis, protecting against the immune system and antibiotics, and is considered to be responsible for chronic or persistent infections. It was shown that bacteria living in biofilms show a highly elevated pattern of adaptive resistance to antibiotics and other disinfectants [21]. Bacterial biofilms are known to cause more than 75 % of microbial infections in humans [14].

S. aureus has long been associated with livestock. Livestock can be carriers of *S. aureus*, but can also become infected. The best-known infection is bovine mastitis [15]. An increasing number of reported detections of methicillin-resistant *S. aureus* (MRSA) in food animals has led to the assumption that there is an emerging zoonotic problem with livestock-associated (la)MRSA potentially aggravating the MRSA problem in humans [24]. Pigs are often carriers and are only rarely infected [24]. In chickens, several disease manifestations have been described, such as comb necrosis [25], bacterial chondronecrosis, which is a cause of leg weakness and lameness [22], and septicemia [23]. Bacterial chondronecrosis associated with *S. aureus* has been identified as an important cause of leg weakness in commercial broilers [22]. These diseases may affect a significant proportion of a flock [15].

Over 50 % of all modern drugs are of natural product origin and they play an important role in drug development programs of the pharmaceutical industry [2]. Moreover, the significance of plant-derived medicines has been increasing all over the world. Since PérezGutiérrez (2010) highlighted the importance of orchid species as a source of active compounds, interest in orchid properties has been growing [27]. A wide range of chemical compounds has been identified from medicinal orchids [29].

Pseudobulbs are the most commonly used parts of orchid plants to cure various disorders, followed by leaves, roots, tubers/rhizomes, and flowers. Major local uses include aphrodisiacs, energizers, and treatments of skin burns, fractured or dislocated bones (both of humans and cattle), headaches, fever, and wounds. Other uses include insect repellent, blood purifier, skin fungi, antidote against snake bites and scorpion stings, inducement of abortions, and recovery from childbirth. Moreover, many species of the Orchidaceae family also possess antimicrobial activity [19]. Orchids are mainly used as paste, powder, or juice, solely or mixed with milk, honey, or wheat flour. Orchid extracts are either consumed orally or applied externally. Fresh orchid flowers are used to induce vomiting by exposure to a dominant foul smell. Local communities in India and Nepal also commonly eat freshly cut species of *Coelogyne* in the forest when they feel thirsty [29].

Therefore, this study aimed to evaluate the antimicrobial activity of the crude extracts obtained from leaves and pseudobulbs of *Coelogyneflaccida* (Blume) Lindl. against different *Staphylococcus aureus* strains, clinically important microorganisms responsible for many infections, to support the use of these extracts as novel natural products for compounded bacterial-treatment modalities. *C.flaccida* belongs to section *Flaccidae*, distributed from mainland Southeast Asia (Thailand), all over Malesia to the islands in the Pacific Ocean [17].

Materials and methods. Collection of Plant Material. The leaves and pseudobulbs of *C. flaccida* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine (Fig. 1). Since 1999, the whole collection of tropical and subtropical plants (in-

cluding orchids) has the status of a National Heritage Collection of Ukraine. Besides that, the NBG collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environmental Protection, registration No. 6939/19/1-10, June 23, 2004).

Fig. 1. The general views of flowers of *Coelogyne* species screened for anti-bacterial activity. All plant specimens are cultivated under glasshouse conditions at NBG. (Photo by Lyudmyla I. Buyun)

Preparation of Plant Extracts. Freshly leaves and pseudobulbs were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1:19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial test strains and growth conditions. For the current study, a panel of organisms including *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) (*mecA* negative), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™) (*mecA* negative, Oxacillin sensitive, weak β -lactamase-producing strain), *S. aureus* NCTC 12493 (*mecA* positive, Methicillin-resistant, EUCAST QC strain for cefoxitin) were used. The cultivation medium was trypticase soy agar (Oxoid™, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37°C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration.

The Disk Diffusion Method for Evaluation of Antibacterial Activity of Plant Extracts. Strain tested was plated on TSA medium (Tryptone Soy Agar) and incubated for 24 h at 37°C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) [3]. Muller-Hinton agar plates

were inoculated with 200 μ l of standardized inoculum (10^8 CFU/mL) of the bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated by extracts were applied over each of the culture plates, 15 min after bacteria suspension was placed. A negative control disc impregnated by sterile 96% ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 h at 37°C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks. The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well.

Statistical analysis. Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean. All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each extract and each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica software, version 8.0 (StatSoft, Poland) [32]. 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[26].

Results and discussion. Ethanolic extracts obtained from leaves and pseudobulbs of *Coelogyneflaccida* resulted in considerable suppression of the growth of different *S. aureus* strains. Moreover, differential efficacy on the test organisms was noted between extracts obtained from leaves and pseudobulbs tested. Consequently, the extracts from various parts of plants displayed varied antibacterial potency against different *S. aureus* strains (Figs 2 and 3).

Fig. 2. Antimicrobial activity of ethanolic extracts obtained from leaves and pseudobulbs of *C. flaccida* against different *Staphylococcus aureus* strains measured as inhibition zone diameters formed around the disks impregnated by extracts.

Detailed data regarding the zones of inhibition by extracts obtained from leaves and pseudobulbs of *C. flaccida* against different *S. aureus* strains were recorded and presented in Fig. 3.

Fig. 2. Antibacterial spectrum of ethanolic extracts obtained from *C. flaccida* against *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923™) (A – leaves, B – pseudobulbs), *S. aureus* NCTC 12493 strain (C – leaves, D – pseudobulbs) measured as inhibition zone diameter

In the present study, the effect of extracts obtained from leaves and pseudobulbs of *C. flaccida* on the growth of *S. aureus* strains was investigated *in vitro*. The results revealed the antimicrobial potential of these ethanolic extracts. The strains tested were susceptible to ethanolic extracts with inhibition zone diameter ranged from 12.5 to 20 mm (Figs 2 and 3).

In our study, the marked antibacterial efficacy of ethanolic extracts obtained from leaves and pseudobulbs of *C. flaccida* was observed against *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923™) strain (mean diameter of inhibition zones was 16.5 ± 1.2 mm and 18.0 ± 1.1 mm, respectively), *Staphylococcus aureus* subsp. *aureus* (ATCC® 29213™) (mean diameter of inhibition zones was 13.5 ± 1.2 mm and 13.8 ± 1.0 mm, respectively), and *S. aureus* NCTC 12493 (mean diameter of inhibition zones was 16.1 ± 0.9 mm and 18.5 ± 1.3 mm, respectively). A statistically significant increase ($p < 0.05$) in the inhibition zone diameters for *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923™) growth was 85.4% (for leaves-derived extract) and 106.9% (for pseudobulb-derived extract), for *Staphylococcus aureus* subsp. *aureus* (ATCC® 29213™) growth was 66.7% (for leaves-derived extract) and 68.3% (for pseudobulb-derived extract), for *S. aureus* NCTC 12493 strain growth was 114.7% (for leaves-derived extract) and 134.2% (for pseudobulb-derived extract).

The results obtained from the present study are consistent with earlier reports, which found that other species of *Coelogyne* have noticeable phytochemical, antimicrobial, antioxidant, and anticancer activity [28]. In our previous study [4-12, 16, 30, 31], we have reinforced the assumption that *Coelogyne* species could be a potential antibacterial or antifungal resource. The orchid extracts from various species of the *Coelogyne* genus displayed varied antimicrobial and antifungal potency. We have determined the antifungal potential of eleven species of orchids namely *C. viscosa* Lindl., *C. cristata* Lindl., *C. lawrenceana* Rolfe, *C. pandurata* Lindl., *C. assamica* Linden & Rchb.f., *C. fimbriata* Lindl., *C. ovalis* Lindl., *C. asperata* Lindl., *C. flaccida* (Blume) Lindl., *C. tomentosa* Lindl., and *C. brachyptera* Rchb. f. against *Candida albicans* [31]. Moreover, ethanolic orchid leaf extracts resulted in considerable suppression of the growth of *S. aureus* [30]. Among orchids selected, marked antimicrobial efficacy was observed for *C. cristata* (mean diameter of growth of inhibition zones was 27.5 mm), *C. tomentosa* (26 mm), *C. lawrenceana* (26 mm), *C. brachyptera* (26 mm), *C. viscosa* (25.5 mm), *C. pandurata* (24.5 mm), and *C. fimbriata* (24 mm). Thus, our results showed that different extracts of epiphytic orchids from the *Coelogyne* genus have potent antimicrobial properties against *S. aureus* [30].

Previously, we have also investigated the antibacterial effects of ethanolic extract of *C. brachyptera* leaves against specific Gram-positive (*S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* locally isolated) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) as well as fungal strain (*Candida albicans*) [12]. Our results showed that the ethanolic extract of *C. brachyptera* leaves showed strong activity against the Gram-positive bacterial strains (20 mm diameter of inhibition zone for *S. aureus* and 26 mm for methicillin-resistant *S. aureus*), and mild activity against Gram-negative bacteria (14 mm for *E. coli* and 11 mm for *P. aeruginosa*). Extract of *C. brachyptera* has displayed strong inhibitory activity against test fungus *C. albicans* (mean diameter of inhibition zone was 17.2 mm) [12]. The ethanolic extract of *C. ovalis* leaves also showed strong activity against *S. aureus* (27 mm of inhibition zone diameter), while the ethanolic extract from pseudobulbs revealed less activity (22 mm). Methanolic and ethyl acetate extracts obtained from *C. ovalis* leaves also showed appreciable antimicrobial activity (32 mm and 35 mm, respectively), whereas those extracts from pseudobulbs revealed no antibacterial activity against *S. aureus* [4].

Previously, we have also investigated the antibacterial effects of ethanolic extracts obtained from leaves and pseudobulbs of plants belonging to the *Coelogyne* genus [7, 8]. For example, the antifungal potential of eight species of orchids, i.e. *Coelogyne cristata* Lindl., *C. fimbriata* Lindl., *C. flaccida* Lindl., *C. huettniana* Rchb.f., *C. ovalis* Lindl., *C. speciosa* (Blume) Lindl., *C. tomentosa* Lindl. and *C. viscosa* Lindl. against fungus strain, *Candida albicans* was determined in the study of Buyun and co-workers (2018). Marked antifungal efficacy was observed in the case of ethanolic extracts derived from leaves of *C. flaccida* (mean diameter of inhibition zones was 19.5 mm), *C. viscosa* (18.6 mm), *C. huettniana* (18.2 mm), and *C. fimbriata* (17.5 mm). Extracts of *C. cristata*, *C. ovalis*, and *C. tomentosa* displayed less inhibitory activity against test fungus (mean diameter of inhibition zones ranging from 16 to 17.5 mm). The ethanolic extracts from the pseudobulbs of eight species from the *Coelogyne* genus exhibited strong activity against *C. albicans* (inhibition zone diameter ranged from 16 to 23.5 mm). Moreover, it has been observed that ethanolic extract obtained from pseudobulbs of *C. speciosa* revealed the highest antibacterial activity (21 mm as the diameter of the inhibition zone) among various species from the *Coelogyne* genus. The results also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results [7, 31].

Also, the results obtained from the present research showed the antimicrobial potential of *C. flaccida* extracts against different *S. aureus* strains. So, ethanolic extracts can be used as antiseptics and antimicrobial agents in medicine and veterinary. The antibacterial activity in *Coelogyne* may be due to the presence of alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes, and terpenoids. Clinical studies confirmed diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, antimicrobial, anticonvulsive, relaxation, neuro-protective, and antiviral activities [27].

The presence of these phytochemicals provides antimicrobial, antitumor, anti-inflammatory, antiviral activities, etc. Several members of the orchid family are used as a potent inhibitor against bacteria and also proved to be a potent antimicrobial agent. The methanolic extract from different parts of orchids has shown antimicrobial activity. A broad-spectrum antibacterial activity (24 bacteria and protozoan) was exhibited by *Galeolafoliata* leaves and stem bark extracted with petrol, dichloromethane, ethyl acetate, butanol, and methanol. A very good level of activity was demonstrated by the dichloromethane and ethyl acetate fractions [19].

Conclusions. The examined ethanolic extracts obtained from leaves and pseudobulbs of *C. flaccida* showed remarkable antibacterial activities against different *S. aureus* strains. The observed activity may contribute to the reasons why orchids are used for infectious and inflammatory conditions in ethnomedicine. The research showed that ethanolic extracts of *C. flaccida* possess the highest antibacterial potency against *S. aureus* NCTC 12493 strain. These findings led the authors to suggest that these extracts may be used as natural antiseptics and antimicrobial agents in medicine and veterinary practice. Nevertheless, despite the promising results, more research should be carried out to further evaluate the roles of particular compounds, isolated from all parts of orchid plants, attributable to antimicrobial activity.

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АНТИМИКРОБНАЯ АКТИВНОСТЬ СПИРТОВЫХ ЭКСТРАКТОВ, ПОЛУЧЕННЫХ ИЗ ЛИСТЬЕВ И ПСЕВДОБУЛЬБ *COELOGYNE FLACCIDA* LINDL. (ORCHIDACEAE) ОТНОСИТЕЛЬНО РАЗЛИЧНЫХ ШТАММОВ *STAPHYLOCOCCUS AUREUS*

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Целью нашего исследования было изучение антимикробной активности *in vitro* этанольных экстрактов, полученных из листьев и псевдобульб *Coelogyne flaccida* Lindl. в отношении *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) (*mecA*-отрицательный), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 29213™) (*mecA*-отрицательный, чувствительный к оксациллину, штамм, продуцирующий слабую β -лактамазу), *Staphylococcus aureus* NCTC 12493 (*mecA*-положительный, метициллин-резистентный, штамм EUCAST QC для цефокситина). Листья и псевдоклубни растений *C. flaccida*, культивировали в тепличных условиях, они были отобраны в Национальном ботаническом саду им. Н.Н. Гришко Национальной академии наук Украины. Свежие листья и псевдоклубни промывали, взвешивали, измельчали и гомогенизировали в 96% этаноле (в пропорции 1:19) при комнатной температуре. Затем экстракты фильтровали и исследовали их антимикробную активность.

Питательной средой служил триптиказо-соевый агар (Oxoid™, Великобритания), дополненный 10 %-ной дефибрированной овечьей кровью при 37 °C. Затем культуры разбавляли стерильным раствором 0,9 % нормального физиологического раствора, чтобы приблизить плотность до 0,5 стандарта Макфарланда. Стандарт McFarland готовили путем инокуляции колоний тест-штамма бактерий в стерильном физиологическом растворе и подгонки плотности клеток до заданной концентрации.

Тестирование противомикробной чувствительности к препаратам проводилось на агаре Мюллера-Хинтона методом дисковой диффузии (протокол теста на чувствительность Кирби-Бауэра). Исследованные этанольные экстракты, полученные из листьев и псевдобульб *C. flaccida*, показали высокую антибактериальную активность относительно различных штаммов *S. aureus*. Наблюдаемая активность может быть одной из причин, по которым орхидеи используются при инфекционных и воспалительных заболеваниях в этномедицине.

Исследования показали, что этанольные экстракты *C. flaccida* обладают наивысшей антибактериальной активностью в отношении штамма *S. aureus* NCTC 12493, и могут использоваться в качестве естественных антисептиков и антимикробных средств в медицине и животноводстве. Вместе с тем, несмотря на многообещающие результаты, необходимо провести дополнительные исследования для дальнейшей оценки роли конкретных соединений, выделенных из всех частей растений орхидеи, обусловленных антимикробной активностью.

Ключевые слова: антимикробная активность, экстракты, орхидные, *Coelogyne*, штаммы *Staphylococcus aureus*, диско-диффузионный метод

АНТИМІКРОБНА АКТИВНІСТЬ СПИРТОВИХ ЕКСТРАКТІВ,
ОТРИМАНИХ З ЛИСТЯ І ПСЕВДОБУЛЬБ *COELOGYNE FLACCIDA* LINDL.
(ORCHIDACEAE) ЩОДО РІЗНИХ ШТАМІВ *STAPHYLOCOCCUS AUREUS*

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Метою дослідження було вивчення антимікробної активності *in vitro* етанольних екстрактів, отриманих з листя і псевдобульб *Coelogyne flaccida* Lindl. щодо *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC®25923™) (mecA-негативний), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC®29213™) (mecA-негативний, чутливий до оксациліну, штам, який продукує слабку β-лактамазу), *Staphylococcus aureus* NCTC 12493 (mecA-позитивний, метицилін-резистентний, штам EUCAST QC для цефокситину). Листя і псевдобульби рослин *C. flaccida*, культивованих в тепличних умовах, були відібрані в Національному ботанічному саду ім. М. М. Гришко Національної академії наук України. Свіже листя і псевдобульби промивали, зважували, подрібнювали і гомогенізували в 96% етанолі (в пропорції 1:19) при кімнатній температурі. Потім екстракти фільтрували і досліджували їх антимікробну активність.

Живильним середовищем служив триптиказо-соєвий агар (Oxoid™, Великобританія), доповнений 10%-ною дефібрированою овечою кров'ю. Культури вирощували аеробно протягом 24 год. при 37°C. Потім культури розбавляли стерильним розчином 0,9% нормального фізіологічного розчину, щоб наблизити щільність до 0,5 стандарту Макфарланда. Стандарт McFarland готували шляхом інокуляції колоній тест-штаму бактерій в стерильному фізіологічному розчині і підгонки щільності клітин до заданої концентрації.

Тестування протимікробної чутливості до препаратів проводилося на агарі Мюллера-Хінтона методом дискової дифузії (протокол тесту на чутливість Кірбі-Бауера). Досліджені етанольні екстракти, отримані з листя і псевдобульб *C. flaccida*, показали високу антибактеріальну активність щодо різних штамів *S. aureus*. Спостережувана активність може сприяти тому, що орхідеї використовуються при інфекційних і запальних станах в етномедицині.

Дослідження показали, що етанольні екстракти *C. flaccida* мають найбільшу антибактеріальну активність відносно штаму *S. aureus* NCTC 12493. Ці результати привели авторів до припущення, що ці екстракти можуть бути використані в якості природних антисептиків і антимікробних засобів в медицині і тваринництві. Проте, незважаючи на багатообіцяючі результати, необхідно провести додаткові дослідження для подальшої оцінки ролі конкретних сполук, виділених з усіх частин рослин орхідеї, обумовлених антимікробною активністю.

Ключові слова: антимікробна активність, екстракти, орхідні, *Coelogyne*, штами *Staphylococcus aureus*, диско-дифузний метод