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Research Article

Phytochemical Analysis and Antimicrobial Effect of Essential Oil and Extract of *Loranthus europaeus* Jacq. on *Acinetobacter baumannii, Staphylococcus aureus,* and *Pseudomonas aeruginosa*

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Abstract: With the increase in the use of antibiotics and resistance against them, attention has been paid to natural remedies with the possibility of lower resistance and side effects. In this study, the essential oil of *Loranthus europaeus* Jacq. was identified chemically and the antimicrobial effects of the essential oil and extract on bacterial agents such as *Acinetobacter baumannii, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were investigated. *L. europaeus* leaves were collected from the Ilam mountains and after drying, essential oil and hydroalcoholic extract were prepared. The chemical compositions of the essential oil and extract of *L. europaeus* plant were measured by Headspace-solid phase microextraction (HS-SPME), Gas chromatography-mass spectrometry (GC-MS), and High Performance Liquid Chromatography (HPLC) methods. The results showed that the main component of *L. europaeus* extract was Rutin (223 µg/mL). The bacterial strains were isolated from clinical samples, and the microbroth dilution method by Clinical and Laboratory Standards Institute (CLSI) method were used to evaluate the Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC for the *L. europaeus* leaf extract were 6 µg/mL and 196 µg/mL on *S. aureus,* respectively. It had no significant effect on the strains of *A. baumannii* and *P. aeruginosa. L. europaeus* leaf essential oil also had no antimicrobial effect on bacterial sauces. The results showed that the plant extract of *L. europaeus* might be used to treat infections due to its very low MIC against *S. aureus.*

Keywords: *Loranthus europeaus Jacq., Antibactrial, Phytochemistry, GC-SPME, HPLC, Rutin*

Loranthus europaeus Jacq. Uçucu Yağ ve Özütünün Fitokimyasal Analizi ve *Acinetobacter baumannii, Staphylococcus aureus* ve *Pseudomonas aeruginosa* Üzerine Antimikrobiyal Etkisi

Öz: Antibiyotik kullanımının ve bunlara karşı direncin artmasıyla birlikte daha düşük direnç ve yan etki olasılığı olan doğal ilaçlara dikkat çekilmeye başlanmıştır. Bu çalışmada, *Loranthus europaeus* Jacq. uçucu yağı kimyasal olarak tanımlandı ve uçucu yağ ve ekstraktın *Acinetobacter baumannii, Staphylococcus aureus* ve *Pseudomonas aeruginosa* gibi bakteriyel etkenler üzerine antimikrobiyal etkileri araştırıldı. *L. europaeus* yaprakları Ilam dağlarından toplandı ve kurutulduktan sonra uçucu yağ ve hidroalkolik ekstrakt hazırlandı. *L. europaeus* bitkisinin uçucu yağı ve ekstraktının kimyasal bileşimleri, Headspace-katı faz mikroekstraksiyon (HS-SPME), Gaz kromatografisi-kütle spektrometrisi (GC-MS) ve Yüksek Performanslı Sıvı Kromatografisi (HPLC) yöntemleri ile ölçüldü. Sonuçlar, *L. europaeus* özütünün ana bileşeninin Rutin (223 µg/mL) olduğunu gösterdi. Bakteri suşları klinik örneklerden izole edildi ve minimum inhibitör konsantrasyonu (MIC) ve minimum bakterisidal konsantrasyonu (MBC) değerlendirmek için Klinik ve Laboratuvar Standartları Enstitüsü (CLSI) yöntemine göre mikro broth seyreltme yöntemi kullanıldı. *L. europaeus* yaprak özütünün MIC ve MBC değerleri, *S. aureus* için sırasıyla 6 µg/mL ve 196 µg/mL saptandı. *A. baumannii* ve *P. aeruginosa* suşları üzerine önemli bir etkisi olmadı. *L. europaeus* yaprağı esansiyel yağı, bakteri sosları üzerine antimikrobiyal etkiye sahip değildi. Sonuçlar, *L. europaeus* bitki özütünün, *S. aureus*'a karşı çok düşük MIC'i ile enfeksiyonları tedavi etmek için kullanılabileceğini gösterdi.

Anahtar sözcükler: *Loranthus europeaus Jacq., Antibakteriyel, Fitokimya, GC-SPME, HPLC, Rutin*

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Introduction

Loranthus europaeus Jacq. is a parasitic plant of the *Loranthaceae* family belonging to the genus *Loranthus* [1]. *Loranthus* is currently a major pest infecting a large part of oak trees [2]. This plant clings to the trunks of the host trees for germination, establishment and absorbing water and materials from it [3]. It is not harmful in its natural state, but it can damage trees and shrubs [4,5]. *Loranthus micranthus* is used in traditional medicine to treat diabetes, schizophrenia, blood clots, and as an immune system stimolator [6]. It is also used to treat infertility, epilepsy, cardiovascular disease, menopausal syndrome, rheumatoid arthritis, and agglutination [7]. It has been shown that there are components such as quercetin, rutin, and epicatechin in this plant [2].

Infectious diseases are widespread diseases in the world that impose great costs on human societies. The diseases death rate is increasing in the world day by day. In recent decades, synthetic antibiotics have played an important role in the treatment of infectious diseases [8]. Today, with the increasing use of antibiotics, the resistance of pathogenic microbial species is increasing and spreading. Resistant pathogenic bacteria have made it difficult to treat infectious diseases. So, researches to discover antibiotic compounds from new sources have been increased [9,10]. Therefore, researchers are searching in nature to discover compounds that have therapeutic potential for use in infectious diseases [11]. The antimicrobial properties of medicinal plants have been considered and used in the treatment of infectious diseases [12].

In this study, the essential oil of *L. europaeus* was identified and the antimicrobial effects of the essential oil and extract on bacterial infectious such as *Acinetobacter baumannii*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* were investigated.

S. aureus stays as our natural flora, and yet sometimes threatens our life as a tenacious pathogen. In addition to its ability to outwit our immune system, its multi-drug resistance phenotype makes it one of the most pathogenic bacteria in the history of antibiotic therapy. Therefore, the presence of natural and herbal compounds that have strong antibacterial properties and are not harmful to human health can be very useful in the treatment of nosocomial infections. The aim of this study was to chemically analyze *S. aureus* plant and determine the active ingredients of this plant and to investigate the antibacterial properties of this plant.

Material and Methods

This study was conducted with the approval of Ilam University of Medical Sciences with the ethics code IR.MEDILAM.REC.1400.074.

Plant Collection

This experimental study was performed in Biotechnology and Medicinal Plants Research Center, Ilam University of Medical Sciences, Ilam, Iran (June 2020 to November 2020). The leaves of the Laurentus plant were collected in June 2020 from the Qalandar region of Ilam city (Ilam province, western Iran) and dried in the shade at room temperature. The identity of this plant was authenticated by the voucher specimens (NO 623) deposited in the Department of Horticulture, Faculty of Agriculture, Ilam University.

Preparation of Essential Oil and Phytochemical Analysis of the Plant

Plant essential oil was extracted by the HS-SPME (Head-space-solid Phase Microextraction) method and the chemical composition of plant leaves was identified by the GC-MS (Gas Chromatography-Mass Spectrometry) method [13]. Extraction of chemical compounds of *L. europaeus* plant was done by HS-SPME method. In this study, the essential oil of *L. europaeus* seed was extracted by HS-SPME technique. In the HS-SPME technique, about 2 grams of dried plant powder was placed in a vial and the vial temperature rised between 60 and 70°C (Extraction time: 20 min). The temperature condition was in the optimal state, causing the extraction of vapors of substances in plant essential oil in the space above the solid surface in a saturated form. The SPME syringe was then placed in the upper space of the container with the lid closed and the material in the plant vapors was absorbed by the silica phase in the needle of the device. After sufficient time and saturation of silica fiber, the volatile compounds of the fiber were placed directly in the input part of the GC-MS device and due to the temperature of the input port, the material in the fiber was desorbed and entered into the GC-MS device and identified.

GC-MS Device Conditions

The device condition was as follows:

The gas chromato-graph (Agilent 6890N) was coupled to the Agilent 5973 bulk detector. Column: HP-5. (30 m length, 0.25 mm (ID), 0.25 µm fixed phase thickness). Type of injection: split/gap and column temperature program: 50°C, holding time 0.00 min; 200°C temperature, holding time, 0.00 min and 5°C/min and 240 min temperature, holding time 0.00 min and 10°C/min. Carrier gas: He (99.999%); Injection type: no gap; Library: Willey 7n; Injector temperature: 250°C and flow rate: 0.9 mL per minute. Extraction mode: (HS-SPME); SMPE fibers: PDMS thickness 100 micrometers (SUPELCO); Sample weight: 0.5 g; Extraction temperature: 60°C; Extraction time: 20 min; Ultrasound time: 10 min (Euronda ultra-sound instrument, Italy) and disposal time in GC-MS injector port: 3 min [14].

Preparation of Hydroalcoholic Extract

The dried Laurentus plant was ground with a mill and poured 30 g of the resulting powder into the cartridge and in Soxhlet using 400 mL of 30% ethanol solution, 40% methanol, and 30% water at 75°C. It was placed for 3 h to complete the extraction. The resulting green hydroalcoholic solution (400 mL) was placed in a rotary apparatus. The solvents in the extract were removed by the rotary device (IKA HB 10, Germany) at 30 rpm and 50°C for one hour to evaporate ethanol, water, and methanol. The resulting *L. europaeus* extract was dried for 3 days. Then the extract was lyophilized and kept stored at -20°C. The amount of extract obtained was 1. 788 g (5.96%).

Preparation and Identification of Active Ingredients

Rutin standards were purchased from Sigma Aldrich (USA) and ethanol and methanol solvents with HPLC (High-performance liquid chromatography) analysis grade were purchased from Merck Company. To identify and determine the exact amount of Rutin in the extracted extract, high-performance liquid chromatography (HPLC) was performed based on a previously reported method. Under similar conditions, a standard solution of methanol-soluble Rutin was also analyzed. The standard Rutin solution was prepared in pure methanol at a concentration of one-tenth of a milligram per milliliter and then diluted in the same solvent to concentrations of 10, 20, 40, 80, 160, and 320 µg/mL. Two samples prepared from rutin extract, diluted 1:50 and 1:100 with methanol, were centrifuged and then filtered to precipitate impurities. All prepared concentrations were injected separately by HPLC. The samples were identified and measured after passing through the C18 column at a wavelength of 254 nm and the chromatogram curve of each was plotted. The curves of the compounds of *Laurentus hydroalcoholic* extract were compared and calculated with the standard Rutin [15].

HPLC Conditions

Analyzes were performed with the HPLC device of Knauer company (Germany) (PLATIN BLUE model). Finally, the best conditions were: Column type: HPLC Column, 250 x 4.6 mm, Eurospher II, 100-5 C18. Mobile phase: formic acid solution with a concentration of 0.2% and acetonitrile 70% and 30%, respectively. Moving phase flow rate: 1 mL per min; Column chamber temperature: 25°C; Injection loop volume: 50 microliters.

Preparation of Bacterial Strain and Bacterial Stock

S. aureus strain (ATCC 12600) and *P. aeruginosa* (ATCC 27852) were purchased from the Iranian Research Organization for Science and Technology. *A. baumannii* isolates associated with infection in patients admitted to the burn ward of a teaching hospital in Ilam were identified as *A. baumannii* by API 20NE and gyrB multiplex PCR [16].

Antimicrobial Method (Microbroth Dilution)

Antibacterial activities of *Loranthus* essential oil and leaf extract (MIC and MBC) were determined using the micro-broth dilution method. In microbiology, the minimum inhibitory concentration (MIC) was considered the lowest concentration of a chemical, which prevented the visible growth of a bacterium or bacteria. The Minimum Bactericidal Concentration (MBC) was considered the lowest concentration of the antibacterial agent required to kill the bacterium over the fixed. Initially, a stock of the plant extract was prepared. In a 96-well microtiter plate, 50 µL of Muller-Hinton broth culture was added to each well. Then, 50 µL of prepared stocks (essential oil/extract) was added to rows 1 and 2, and the dilution was performed from the second to the tenth row. Finally, 50 µL of *A. pneumoniae*, *S. aureus*, and *P. aeruginosa* (24-h culture), equivalent to half-MacFarland 5×10^5 CFU/mL, was added to wells two to ten. gentamicin, cholestin and methicillin, and dimethylsulfoxide (DMSO) was used as a positive and negative control, respectively. Plates were incubated at 37°C for 24 h. After incubation, 30 µL of 2, 3, and 5-trinyl tetrazolium chloride was used as bacterial growth visual index [17]. The antimicrobial test was repeated three times for each group. The proposed CLSI protocol (2014) was also used to determine the MBC [17]. Standard antibiotics methicillin, cholestin, and gentamicin were used as controls [18].

Results

In this study, the essential oils and volatiles of Lorenthus leaves were extracted by solid-phase microextraction in the upper space (HS-SPME) and the chemical compounds were phytochemically analyzed by GC-MS technique. The results of the phytochemical analysis showed that the lorenthus leaf had 35 chemical compounds. According to the results, the most essential compounds of Laurentus leaf essential oil included phytol (18.91%), dodecane (10.30%), trans-Caryophyllene (5.86%), 3-Hexen-1-ol, benzoate, (Z) (5.21%), Neryl acetone (4.78%), and beta lonone (4.53%), respectively. The results of the percentage of other chemical compounds are detailed in *Table 1* and *Table 2*. Also, the chromatogram of *Laurentus* essential oil is shown in *Fig. 1*.

The HPLC results are shown in *Fig. 2*. Accordingly, the main composition of *Laurentus* hydroalcoholic extract was Rutin (223 µg/mL) based on HPLC. HPLC-PDA chromatograms and UV spectra of Rutin are shown in *Fig. 3*.

The microbroth dilution method was used to determine the MIC and MBC of lorenthus extract and essential oil. According to the results, the MIC and MBC for the hydro-alcoholic extract of lorenthus leaf were 6 µg/mL and 196 µg/mL *S. aureus*, but this extract did not affect

Table 1. *Chemical compounds of Laurentus leaf essential oil*

Compound	Retention Time	Percent	Molecular Formula	Compound	Retention Time	Percent	Molecular Formula
Dodecanal	12.018	2.02	$C_{12}H_{24}O$	Neryl acetone	23.42	4.78	$C_{13}H_{22}O$
2-Octanone	12.252	0.30	$C_8H_{16}O$	Heneicosane	23.557	1.46	$C_{21}H_{44}$
Menthone	13.761	1.47	$C_{10}H_{18}O$	Beta.-Ionone	24.403	4.53	$C_{13}H_{20}O$
Menthol	14.539	1.86	$C_{10}H_{20}O$	Pentadecane	24.62	2.23	$C_{15}H_{32}$
Dodecane	15.19	10.30	$C_{12}H_{26}$	Hexadecane	25.369	1.66	$C_{16}H_{34}$
Decanal	15.487	1.46	$C_{10}H_{20}O$	Hexatriacontane	25.838	3.52	$C_{36}H_{74}$
Fenchyl acetate	15.956	0.73	$C_{12}H_{20}O_2$	Decane, 5,6-dimethyl-	25.986	3.23	$C_{12}H_{26}$
Isobornyl acetate	18.191	0.58	$C_{12}H_{20}O_2$	Tetratetracontane	26.278	0.67	$C_{44}H_{90}$
Menthyl acetate	18.465	2.61	$C_{12}H_{22}O_2$	3-Hexen-1-ol, benzoate, (Z)-	26.666	5.21	$C_{13}H_{16}O_2$
Tridecane	18.562	1.91	$C_{13}H_{28}$	Farnesol	27.004	1.09	$C_{15}H_{26}O$
Tridecane, 5-methyl-	20.311	2.97	$C_{14}H_{30}$	Phytol	27.198	18.91	$C_{20}H_{40}O$
2-Methyltetradecane	20.848	2.06	$C_{15}H_{32}$	Dotriacontane	28.364	1.71	$C_{32}H_{66}$
Copaene	21.111	1.16	$C_{15}H_{24}$	Heptadecane	29.564	1.11	$C_{17}H_{36}$
2-Tetradecene, (E)-	21.665	3.79	$C_{14}H_{28}$	1-Eicosanol	29.656	3.71	$C_{20}H_{42}O$
Tetradecane	21.768	0.37	$C_{14}H_{30}$	Heneicosane	31.37	1.29	$C_{21}H_{44}$
Trans-Caryophyllene	22.466	5.86	$C_{15}H_{24}$	Cyclopentadecane	31.97	1.09	$C_{15}H_{30}$
Camphene	22.746	0.46	$C_{10}H_{16}$	Hexadecanoic acid	33.182	3.41	$C_{16}H_{30}D_2O_2$
Alloaromadendrene	23.037	0.48	$C_{15}H_{24}$				

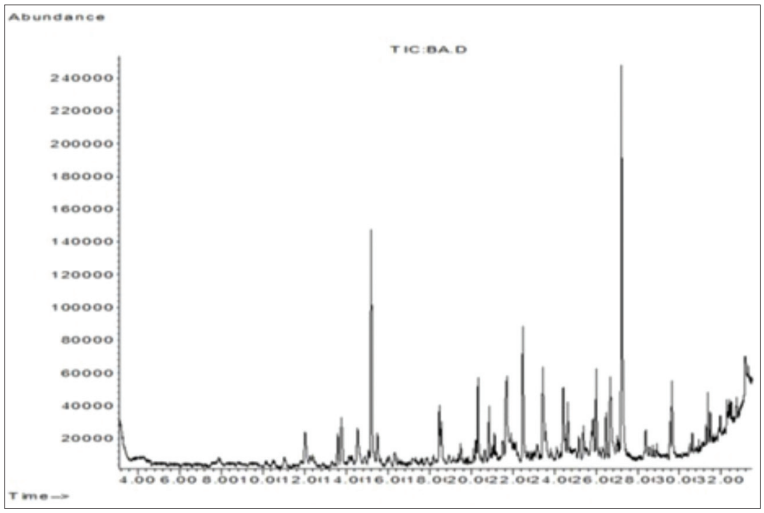

Fig 1. Gas chromatography-mass spectrometry analysis in the essential oil of *Loranthus europaeus*

Table 2. *Active substance, chemical formula and molecular formula of the main components of Laurentus plant*

Active Substance	Chemical Formula
Phytol	$C_{20}H_{40}O$
Dodecane	$C_{12}H_{26}$
Trans-Caryophyllene	$C_{15}H_{24}$
3-Hexen-1-ol, benzoate, (Z)	$C_{13}H_{16}O$
Neryl acetone	$C_{13}H_{22}O$
Beta-lonone	$C_{13}H_{20}O$

the strains of *A. baumannii* and *P. aeruginosa*. Lorenthus leaf essential oil also had no antimicrobial effect on any of the bacterial sauces in this study. The results of the antimicrobial effect of lorenthus essential oil and leaf extract are shown in *Table 3*.

Discussion

Today, the use of medicinal plants in the treatment of various diseases has been considered by researchers. In this regard, some medicinal plants components are used for a variety of disorders and diseases [19]. Phenolic compounds as well as flavonoids, because they are important bioactive compounds for antioxidant effects, have always been considered for the prevention of disease and human health [20].

It has been documented that the fruit extracts of *L. europaeus* (with MIC=0.16±0.04 and 0.28±0.05 $mg.mL^{-1}$ and MBC=0.20±0.01 and 0.38±0.02 $mg.mL^{-1}$) have been effective on *S. aureus* MRSA and *Listeria monocytogenes* species, respectively [21]. The antimicrobial effects of volatile oils and extracts of lorenthus fruit may act as immuomoduialtors during bacterial infection containing substances that act as chemotactic agents for neutrophils and promote macrophage activity [22].

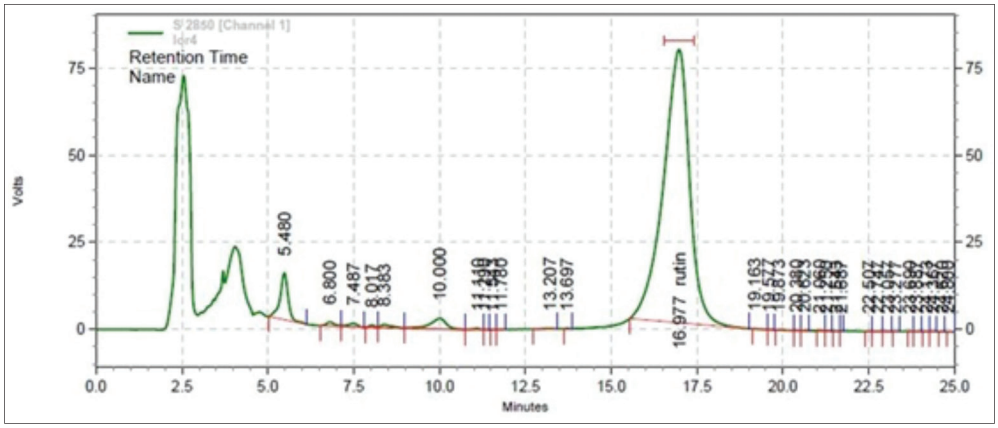

Fig 2. HPLC chromatogram of phenolic compounds present in *Loranthus europaeus* leaves extracts and Rutin

Fig 3. HPLC-PDA chromatograms and UV spectra of Rutin

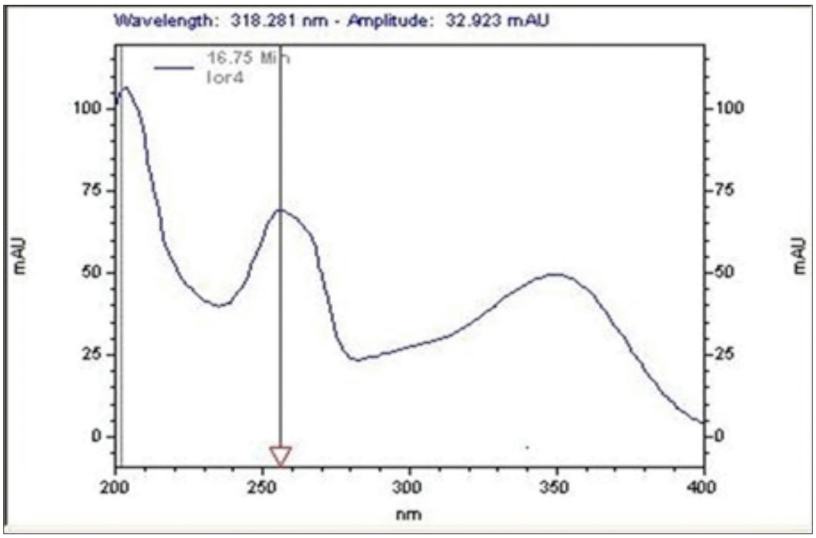

Table 3. *Results of MIC and MBC of Laurentus essential oil and hydroalcoholic extract*

Bacterial Strain	Antibiotics						Essence		Hydroalcoholic Extract	
	Gentamicin		Colestin		Methicillin					
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
Acinetobacter baumannii	IF	IF	75	25	IF	IF	IF	IF	IF	IF
Staphylococcus aureus	IF	IF	IF	IF	384	384	IF	i.f.	196	6
Pseudomonas aeruginosa	12.35	12.35	IF	IF	IF	IF	IF	IF	IF	IF

IF = Ineffective

It has been shown that the alcoholic extract of *L. europaeus* has an inhibitory effect on the methicillin-resistant *S. aureus*, which was 17.28 mm at the concentration of 20 mg/mL, followed by the concentrations of 100, 50, 25 mg/mL which the diameter of inhibition zones were 13.28, 10.57, 8.42 mm, respectively [18]. A previous study showed that lorenthus could affect methicillin-resistant *S. aureus* [23]. In our study, *Loranthus* extract was effective against infection *S. aureus*. Also, our results showed that tannins and flavonoides existed in *L. europaeus*, deposited in the bacterial cell membrane, and inhibited the action of metabolic enzymes leading to bacterial death.

Based on the results obtained from the analysis of plant essential oil, the most compounds in *L. europaeus* had plant essential oil included hexadecanoic acid and 1-eicosanol. It has been shown that the essential oils of *Solanum sisymbriifolium* containing hexadecanoic acid and 1-eicosanol showed antibacterial activity against *S. aureus* in 60 and 80 µg/mL for fruit and flower, respectively [24]. Today, the importance of effective compounds of medicinal plants has become especially important in pharmaceutical science [25,26]. Medicinal plants have different compounds that can be used in various industries such as health, medicine and pharmaceutical industry [27,28]. Many herbal

remedies are derived from compounds found in herbs [29,30]. Rutin ($C_{27}H_{30}O_{16}$) or Phytomelin is a flavonol glycoside found in many plants including buckwheat, lorenthus, and eucalyptus acts as a metabolite and an antioxidant. It is a disaccharide derivative, a quercetin glucoside, a tetra hydroxyl flavone, and a rutinoside [31]. Rutin has many therapeutic effects on blood pressure, diabetes, cancer, sedatives, analgesics, blood cholesterol, cataract [32]. Other Rutin effects include antibacterial, antimalarial, antiviral, and antifungal activities [33]. It has been studied for antimicrobial activity against different strains of bacteria. Also, it has antimicrobial activity against *Escherichia coli*, *Proteus vulgaris*, *Shigella sonei*, *Klebsiella,* and *Bacillus subtilis* [25,34-36]. The main reason for the lorenthus hydroalcoholic extract effectiveness might be the high concentration of Rutin. Extraction of herbal active ingredients and their implications in the treatment of diseases are expanding [35,36]. The results of extract analysis showed that the main ingredient of this plant is Rutin. The very low MIC extract of the *L. europaeus* has the potential to be used as alternative medicine in the treatment of *S. aureus* infections. Its toxicological studies on human cells should be considered.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (N. Abbasi) on reasonable request.

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Competing of Interest

The authors declared no competing interests.

Authors' Contribution

NA, AA and EK conceived and supervised the study. MB, VHK and MP collected and analyzed data. VHK made antimicrobial and MB and AA made phytochemical measurements. The first draft of the manuscript was written by MB and NA and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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