PROCEEDINGS
The 4th International Seminar of Indonesia Society for Microbiology and IUMS-ISM Outreach Program on Food Safety

INDONESIAN MICROBIAL RESOURCES: DIVERSITY AND GLOBAL IMPACT

22nd-24th June 2011, Udayana University Denpasar-Bali, Indonesia

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On behalf of the organizing committee, I would like to extend my warmest greeting to all of you. I would also like extend my appreciation to all of you, especially to those who have traveled far away to Bali from their origin to attend this conference. This conference has been designed to gather scientists, engineers, practitioners, and industries in microbiological related disciplines, so that they can discuss and share their expertise in the fields of microbiology related issues. From this intense discussion, it is expected that some brilliant ideas to be used to improve the quality of human life can be formulated, so that it is in line with the theme of the conference: *"Indonesian Microbial Resources: Diversity and Global Impact".*

In this opportunity I would like to report briefly about this conference to President of the Indonesian Society for Microbiology. The conference will consist of 12 plenary presentations delivered by International invited speakers from Italy, Netherlands, Japan, Thailand, Singapore, and Indonesia, covering general aspects of Microbiology. Besides this plenary session, we will also have three satellite symposia, covering areas of **Health and Pharmacy**, **food and agriculture**, and **Industry and environment**. Totally, 175 contribution papers (oral and poster presentation) will be presented in this conference and they are distributed according to the areas previously mentioned. The efforts of the presenters to prepare their contribution papers for this conference are highly appreciated.

This Conference is financially supported by PT Merck Indonesia, Olympus, Biomeureux, PT Dipa Puspa Lab Sain, PT Yakult Indonesia, PT New Moduls International, PT Agarindo Biological Co, and participants. Therefore, in this occasion, on behalf of the committee, I would like to acknowledge their financial supports.

My thanks should also go to all people who have been involved in the committee of the conference. Without their hard working and efforts, I am afraid we will not be able to make this event to happen.

Last but not least, I hope you all can enjoy your time in Bali, not only at the venue of the conference, but also enjoy the beauty of Bali and the friendliness of the people, so that you all bring home some unforgettable memories about the island of Bali.

Thank you!

Chairman of the Organizing Committee
Dr. dr. Dewa Made Sukrama, M.Si., Sp.MK.
Dear Distinguished guests, Invited speakers, and all other participants. The main aim of this 4th International Seminar of Indonesian Society for Microbiology and IUMS-ISM outreach Program in Food Safety is to gather scientists from all over the world in a venue to share their expertise in microbiology-related discipline and build scientific network, so that they can develop microbiological-based methods for improving the quality of human life in the future.

In this opportunity, on behalf of the University, I welcome you all to Bali. Bali is well known as a favorite tourist destination in the world. Recently, it is also a favorite site for holding International events, such as International Conference. When people hear Bali as a site of an International conference, a lot of them will be interested to attend the event. By attending such an event in Bali, they can do two things at once. They can present scientific papers and share their expertise with other scientists known to have International reputation, and at the same time they can also enjoy the beauty of the Bali Island and the culture of Bali which is considered to be unique by foreign tourists. Here, I would also like to acknowledge the National and International invited speakers for their willingness to come miles away to Bali and present their high standard papers. I understand that you all spend much time for this conference, and therefore I must give high appreciation on all of those effort and dedication. I hope this International Conference becomes an ideal forum for communication and sharing ideas as well as experience in microbiological-related disciplines in the future. I also hope that this forum can serve as a forum for promoting advanced microbiology with regard to economic growth and social welfare.

Finally, I wish you most successful conference and hope that it may provide new ideas and strategies for the application of microbiology in the industries.

Rector of the Udayana University,
Prof. Dr. dr. I Made Bakta, Sp.PD (KHOM).
OPENING REMARK OF THE PRESIDENT OF THE INDONESIAN SOCIETY FOR MICROBIOLOGY

The Indonesian Society for Microbiology is proud to present the 4th International Seminar of Indonesian Society for Microbiology, in June 22-23, 2011 with the theme “Indonesian Microbial Resources: Diversity and Global Impact”. I am also very proud to inform you that the International Union of Microbiological Societies (IUMS) in collaboration with the Indonesian Society of Microbiology (ISM), the International Commission on Food Mycology (ICFM) and the International Committee on Food Microbiology and Hygiene (ICFMH) is also organizing an IUMS-Outreach Program in Food Safety on 22-24 June 2011. I have the great pleasure in welcoming all of you to these events.

Over the past decades, there has been impressive progress in the area of microbiology. Microbiology and biotechnology have been playing important roles in the area of medical and veterinary, food and industry, agriculture and environment. This seminar will be an excellent event for exchange and sharing information, progress and experiences among the participants and more importantly to encourage collaboration and business interaction among participants. The workshop on food safety is also very important especially for countries like Asia because transfer of knowledge I believe will be very useful.

The Indonesian Society for Microbiology, a member of the International Union of Microbiological Societies (IUMS), is a scientific organization in microbiology, with 1067 members spreads out in 20 regions in Indonesia. ISM has also published an English peer-reviewed journal namely Microbiology Indonesia, that has been given the accreditation at level “A” by Department of National Education, since 2001. The journal was published in Bahasa Indonesia from 1999-2006 but later on become English journal. I do expect that the journal will become a real international journal. In this regards, I would like to invite all of you to submit your manuscript and give contribution as editor or reviewer of the Microbiology Indonesia starting from 2012.

The society also conducts Annual Meeting which comprises of scientific meeting both national and international, and take place in the society branch all over Indonesia. Papers presented in the annual meeting are usually selected and published in the journal Microbiology Indonesia. In this regards, the society would like to thank the Department of Higher Education for granting a financial support in collecting appropriate and acceptable papers to be publish in the journal.

We still have to intensify and extend on the networking with international communities. Indonesia being privilege with a large diversity in microbes, we therefore invite our colleague from abroad to explore of the Indonesian microbes based on benefit sharing.

On behalf of the Indonesian Society for Microbiology, I would like to express our sincere appreciation and gratitude to the International Union of Microbiological Societies (IUMS), the International Commission on Food Mycology (ICFM) and the International Committee on Food Microbiology and Hygiene (ICFMH). We would also like to extend our appreciation to Udayana University, research institution, private sector and all supporting parties for the success of the seminar and workshop on food safety.
The 4th International Seminar of Indonesia Society for Microbiology and IUMS-ISM Outreach Program on Food Safety
"Indonesian Microbial Resources: Diversity and Global Impact"

I would like to extend our appreciation to invited speaker and guest from foreign country. I believed your great contribution will be very fruitful and provide significant role in developing microbiology and its application in these region.

Special thanks are addressed to the organizing committee chaired by Prof Dewa Sukrama his excellent effort to conduct this successful seminar.

I do hope that this international seminar and workshop will strengthen our collaboration in exploring the role and application of microbiology.

With warm regards,

Dr. Koesnandar, M.Eng
President of the Indonesian Society for Microbiology
OPENING REMARK OF THE SECRETARY GENERAL
OF THE IUMS

The INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES (IUMS) is one of the 26 Scientific Unions of the International Council of Science (ICSU). It was founded in 1927 as the International Society of Microbiology, and became the International Association of Microbiological Societies affiliated to the International Union of Biological Sciences (IUBS) as a Division in 1967. It acquired independence in 1980 and became a Union Member of ICSU in 1982. IUMS has 109 member societies and 16 associate members, altogether with more than 60,000 microbiologists.

The Divisions are responsible for the organization of their International Congresses (International Congress of Bacteriology and Applied Microbiology, International Congress of Mycology, and International Congress of Virology) and the committees, commissions and federations organize their own meetings. The next IUMS congresses will be in Sapporo Japan from 6 to 16 September 2011 (http://www.congre.co.jp/iums2011sapporo/index.html).

The scientific activities of the Union are conducted by the three Divisions namely Bacteriology & Applied Microbiology (BAM), Mycology and Virology, by six specialist international committees, eight international commissions and two international federations (COMCOFs). Their major activities include the classification and nomenclature of bacteria, fungi and viruses, food microbiology, medical microbiology and diagnostics, culture collections, education, and biological standardization.

In support of its mission to enhance the scientific background and professional effectiveness of basic and applied microbiologists, the IUMS is embarking on a program of educational outreach to developing countries and their microbiologists. The Union envisions an IUMS series of courses that will be offered to groups of microbiologists that may include graduate students, postdoctoral fellows, and practicing professionals from developing countries within a given geographic region. These will be offered periodically in various regions and on different topics of interest and importance.

The first IUMS Regional Course was offered in Singapore during June 15-17, 2010 on Antimicrobial Resistance in Bacteria, Fungi and Viruses and was great success. The IUMS is very happy that the second IUMS outreach programme could be organized together with the Indonesian Society of Microbiology on the topic of Food safety. The International Commission on Food Mycology (Mycology Division) and International Committee on Food Microbiology and Hygiene (Bacteriology and Applied Microbiology Division) kindly sponsored the workshop by providing the experts on food microbiology.

On behalf of the IUMS Executive Board I like to thank the ISM and the local organizers to make this meeting possible and wish you all a very successful and productive workshop.

Robert A Samson - IUMS Secretary General.
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ABSTRACT

Introduction: Betel (Piper betle, L) leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, ototrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries etc as folk medicine. Objectives: This research has the objective of comparing the antibacterial activity as well as the mechanism of inhibition between essential oil betel leaf (Piper betle, linn) and eugenol as the basis for choosing a substance to be developed as antimicrobial drug. Materials and Method: The betel leaf was obtained from Balito Bagor, distilled by steam distillation process. The essential oil of betel leaf that obtained was analyzed by Gas Chromatography-Mass Spectroscopy. The activity test of anti-bacterial of essential oil of betel leaf (Piper betle, linn) and eugenol was carried out by using the method microdilution against the bacterium Proteus mirabilis, Proteus vulgaris, Salmonella thypinurium, Shigella flexneri, and Streptococcus mutans. For detect the mechanism of cell damage process of bacteria was observed leakage of proteins and nucleic acids by using Ultra Violet-Visible spectrophotometer and the leakage of cations K+ and Ca2+ observed using Atomic Absorption Spectrum. While bacterial cell morphology change was observed by using Scanning Electron Microscope (SEM). Results and Conclusion: From the research was obtained, a minimum inhibitory concentration (MIC) of essential oil of betel leaf for Proteus mirabilis 9% (v/v), Proteus vulgaris 6% (v/v), Salmonella thypinurium 5% (v/v), Shigella flexneri 11% (v/v), and Streptococcus mutans more than 17% (v/v), while for eugenol MIC values for Proteus mirabilis 0,4% (v/v), Proteus vulgaris 0,5% (v/v), Salmonella thypinurium 0,2% (v/v), Shigella flexneri 0,5% (v/v), and Streptococcus mutans 0,4% (v/v). Test results nucleic acid and protein leakage and leakage of cations K+ and Ca 2+ greatly increased from 1 MIC to 2 MIC concentration of the tested bacteria compared with the normal controls. While the observations that was detected by using Scanning Electron Microscope showed worse cell damage by giving the higher doses to bacteria. Plasma cell membrane damage and coagulation of the nucleoid by eugenol worse than essential oil of betel leaf (Piper betle, linn).

Keywords: Essential oil of betel leaf, eugenol, antibacterial, Proteus mirabilis, Proteus vulgaris, Salmonella thypinurium, Shigella flexneri, Streptococcus mutans.

INTRODUCTION

The development of resistance is a natural biological process that will occur, sooner or later, with every drug. The use of any antimicrobial for any infection, in any dose, and over any time period, forces microbes to either adapt or die in a phenomenon known as "selective pressure". The microbes which adapt and survive carry genes for resistance, which can be passed on from one person to another and rapidly spread around the world. It is well known that intensive use of an antibiotic is often followed by the appearance of resistant strains. Therefore, the search for new antimicrobial agents continues unabated.

Medicinal plants are promising resources. The use of medicinal plants as screening pool for novel antibiotics has several advantages related to safety, availability, and minimizing the risk of side effects and addiction. The World Health Organization adopted major policy change in accepting that most developing nations would have to make use of more traditional medical practices for primary health care. (Chan, 2011).

The leaves of Piper betle L have been used in traditional medicine as carminative, stimulant, antiseptic, antifungal, and antibacterial agent. The volatile oil known as Betel oil is the chief constituent of the leaves. Piper betle L. can be of great benefit in treating diseases caused by bacteria and fungi. Previous studies on the betel leaves, roots and whole extract (mixture of volatile and non-volatile) of the green variety showed a very strong antimicrobial activity (Jenie, 2001). Eugenol is the main content of the essential oil of betel leaf. Nevertheless, eugenol that produced from natural product isolated from essential oil of clove, because the content of eugenol in clove essential oil is higher than the content of essential oil of betel leaf. Even though so far, betel leaf essential oil and eugenol are known have properties as
antibacterial, however, how the comparison antimicrobial activity between essential oil of betel leaf with eugenol. This publication has not been found in the literature. Therefore in this study was attempted to test the antimicrobial activity of essential oil of betel leaf and eugenol against bacteria; Proteus mirabilis, Proteus vulgaris, Salmonella thypimurium and Shigella flexneri by measuring the diameter of inhibition and minimum inhibitory concentration. On the other hand also was conducted observation of leakage of nucleic acids, proteins, ion K⁺, ion Ca²⁺ from cells membrane of bacteria and morphological changes due to giving of essential oil of betel leaf and eugenol.

**MATERIALS AND METHOD**

Betle (Piper betle. L) leaves was obtained from Research Institution of Spices and Medicinal Plant (Balitro), Bogor. Eugenol was obtained from E-merck.

**Isolation of Essential Oil of Betel Leaf**

The collected leaves were washed, dried on papers and air-dried. The leaves (10 kg) were cut into small pieces for extraction using steam distillation and isolated by rotary evaporation for 4-6 hours, then added NaSO₄ anhydrous to remove water content, and then filtered. Calculate the results was obtained.

**Chemical Analysis of Essential Oil of Betel Leaf.** (Rawat et al, 1989)

Identification components of essential oil of betel leaf was done by using Gas Chromatography-Mass Spectroscopy (GC-MS) instrumentaion Varian Saturn 2000. Sample preparation of essential oils was done by addition diethyl ether. Type of column is VF-17 MS, length 30mm and ID 0.25 mm. Carrier gas was helium with a flow rate of 1.3 ml/ min and a pressure of 10.7 Psi. Column temperature was programmed from 50 °C to 250 °C with two-stage increase. In the early stages of the column temperature was maintained constant at 50 °C for 3 minutes, then raised up to 150 °C with a speed increase in temperature of 5 °C/min, and subsequently raised to 250 °C at 3 °C temperature rise/min. This condition was maintained for 3.67 minutes. Injector temperature as long as analysis was programmed at a constant temperature (230 °C). While the interface temperature was 250 °C and autosampling as much as 2 mL. Solvent cut time as long as 3 minutes and Scan MS 50-450 (M/Z).

**Preparation of Bacterial Suspension**

The bacterial used were Proteus mirabilis, Proteus vulgaris, Salmonella thypimurium, Shigella flexneri. Culture stocks were obtained from microbiology laboratory, Indonesian Institute of Sciences, Cibinong, Bogor. The stocks of these bacteria were kept in nutrient agar, then inoculated into the Mueller-Hinton Broth and for Streptococcus mutans was added 5% blood, incubation at shaker incubator with speed 120 rpm, temperature 37 °C, for 24 hours until reach the active phase. The amount of bacteria was adjust approximately 1 X 10⁵ CFU/ mL by using methode of McFarland. Bacteria Streptococcus mutans was inoculated into agar slope medium, added 5% blood by using a Dose that was sterilized by heat in the Bunsen flame, and then incubated at 37 °C for 24 hours.

**Preparation of Test Solutions for the Essential Oil and Eugenol**

Preparation of test solutions (betel leaf essential oil) emulsion was made by mixing the essential oil of betel leaf with the solvent 0.5% tween 80, 2% absolute ethanol and aquadest. The concentration was made as follow: 4%, 5%, 6%, 7%, 8%, 9%, 10% and 11%. Especially for testing Streptococcus mutans was made concentration as follow: 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13% 14% 15% 16% and 17%.

Preparation for the solution of eugenol emulsion was made by mixing 0.5% tween 80, 2% absolute ethanol and distilled water. Concentrations was made as follows: 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, and 0.8%.

**Determination of Diameter of Inhibition Area.** (Suppakul et al, 2006, Caburian, et al 2010)

Determination of diameter of inhibition was done by using paper disc diffusion method. As much as 0.1 ml of the solution was placed on the plate and incubated at 37 °C for 24 hours. Then the plate was observed for halo or clear zone around the paper disc as zone of inhibition. The diameter of the inhibition zone was measured in millimeters using a caliper. This determination was repeated for each concentration of the solution in triplicate. The average diameter of the inhibition zone was calculated as follows:

\[
\text{Diameter of Inhibition} = \frac{\text{Total Diameter}}{\text{Number of Observations}}
\]

The results were compared with the diameter of inhibition of the control (sterile water). The inhibition was considered significant if the diameter of inhibition was greater than the diameter of inhibition of the control.

**Results and Discussion**

The results of the determination of diameter of inhibition area showed that the essential oil of betel leaf and eugenol had antibacterial activity against the tested bacteria. The essential oil of betel leaf with a concentration of 0.5% had the largest diameter of inhibition against Proteus vulgaris, Salmonella thypimurium, and Shigella flexneri. While the eugenol solution with a concentration of 0.5% had the largest diameter of inhibition against Proteus mirabilis, Proteus vulgaris, and Shigella flexneri.

**Conclusion**

The essential oil of betel leaf and eugenol has antibacterial activity against the tested bacteria. The essential oil of betel leaf with a concentration of 0.5% had the largest diameter of inhibition against Proteus vulgaris, Salmonella thypimurium, and Shigella flexneri. While the eugenol solution with a concentration of 0.5% had the largest diameter of inhibition against Proteus mirabilis, Proteus vulgaris, and Shigella flexneri.
as 20 ml of sterile Mueller-Hinton Agar was poured into each petri dish (except for Streptococcus mutans blood added as much as 5%). Let freeze at room temperature. Bacteria was suspended 0.1 ml into petri dishes. Spread bacterial suspension inoculum over entire surface with glass rod spreader and allowed to stand for approximately 15 minutes. Paper discs was placed on solid agar medium that has been frozen. Drop sample test preparation with a certain concentration as much as 10 µl on paper discs. Then incubated for 24 hours at 37°C. Measure diameter of inhibition was formed around the discs.

**Determination of Minimum Inhibition Concentration (MIC)** *(Suppakul et al, 2006)*

Determination of minimum inhibition concentration was used micro-dilution method by using sterile microplate. At 1-10 wells was filled with 100µl Mueller-Hinton Broth and into each wells was inoculated 100 mL bacterial suspension. Then add 50 mL of essential oil with a concentration of 4%, 5%, 6%, 7%, 8%, 9%, 10% and 11% (except for Streptococcus mutans concentration was 4% -17%). While For eugenol was made concentration 0.1%; 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7% and 0.8%. As a control dosage were used 4 types of control, namely; wells A containing medium and bacteria, wells B contain only a control medium, wells C containing medium and solvent that used to dissolve the essential oil and eugenol. Wells D containing medium, solvent and the suspension of bacteria. Then microplate was incubated on shaker incubator with 150 rpm for 24 hours at 37°C. Determination of minimum inhibition concentration was carried out by culturing the test sample on Nutrienn Agar medium after incubation for 24 hours. The smallest concentration that causes the bacteria did not grow on bacterial culture was expressed as the MIC for the test solution of essential oil and eugenol. Each experiment was conducted with two times.

**Analysis of Proteins and Nucleic Acids.** *(Carson et al, 2002)*

Suspension of test bacteria that had been incubated for 24 h in Muller-Hinton Broth (MHB) medium. Centrifuged at 3500 rpm for 20 minutes. Subsequently the filtrate discarded and the pellets in the tubes were washed with phosphate buffer pH 7.0. Washing conducted 2 times. Then add the essential oil and eugenol with treatment 1 MIC and 2 MIC which add phosphate buffer up to 10 ml. Incubated for 24 hours in a shaker 150 rpm. Then the suspension is centrifuged again for 15 minutes at 3500 rpm, and supernatant fluid was taken. Perform measurement of absorbance with spectrophotometer UV/Vis at a wavelength of 260 nm and 280 nm. For this testing was only done to the bacteria Shigella flexneri, because Shigella flexneri is having middle sensitive among the test bacteria. In this case, Shigella flexneri was considered to represent the five others bacteria. So also for the Analysis of Metal Leakage and scanning of changes in cell morphology by SEM

**Analysis of Metal Leakage.** *(Carson et al, 2002)*

Analysis of leakage of metals was measured in the form ions Ca²⁺ and K⁺. Leakage of ions Ca²⁺ and K⁺ from the bacterial cell membrane due to treatment with essential oil of betel leaf and eugenol. Preparation of samples for analysis of leakage of ions is the same as preparation for the leakage of proteins and nucleic acids. Leakage was expressed with the measurability of metal ions contained in the test bacteria after contact with essential oils in treatment 1 MIC and 2 MIC. Leakage of ions Ca²⁺ and K⁺ was detected by using Atomic Absorption Spectrophotometer (AAS).

**Scanning of Changes in Cell Morphology by SEM.** *(Carson et al, 2002)*

Suspension of test bacteria that had been incubated for 24 h in MHB medium. Centrifuged at 3500 rpm for 20 minutes. Filtrate discarded and the pellets in the tubes was washed with phosphate buffer (pH 7.0) twice. Added the essential oil and eugenol with treatment 1 MIC and 2 MIC up to 10 ml. Incubated for 24 hours in a shaker 150 rpm. Bacterial cell suspension results of contact with essential oil and eugenol were centrifuged at 3500 rpm for 15 minutes. Pellet was washed with a buffer solution fospat and the liquid separated by centrifuge, the treatment was repeated twice. Filtrate discarded and the pellet was immersed in glutaraldehyde dehide and buffer for 4 hours. Then the centrifuged and the supernatant was discarded. Pellets immersed again with 2% tannin acid in the buffer chodolate for 12 hours, then centrifuged again, supernatant discarded and pellets was immersed in 1% osmium tetraoksida solution for 2-4 hours. Washed with buffer cdolite, then centrifuged and pellets washed with ethanol 50%, allow 10 minutes and
centrifuged again (done twice). Then washed again with 70% ethanol, 80% and 95% respectively for 10 minutes and then centrifuged. Pellet was washed with absolute ethanol and allowed to stand for 10 minutes and the centrifuged again (this treatment was performed 2 times). Washed again with terbutanol allowed to stand for 10 minutes and centrifuged (done twice). Added a little terbutanol in the sediment cell. Applied to cell smears on a glass slip, then placed on the stub for coating with gold for 1 hour in a vacuum. Observed by using Scanning Electron Microscopy (SEM)

RESULTS AND DISCUSSION

From this study was obtained the yield of the essential oil of betel leaf from BALITRO 0.35% (W/V). There were 66 essential oil fractions that obtained. Eugenol (Cis-iso-Eugenol) was a fraction the highest with a concentration 47.47%. Eugenol was a fraction of the 41 on the GC-MS chromatogram with a retention time of 26.389, as shown in Figure 1. Content essential oil of betel leaf varies widely, from 0.08% to 0.4% (Guha 2006, Tyler et al 1998, Parmar et al, 1997). While, the content of eugenol from essential oil of betel leaf between 13.9% to 64% (Rawat et al, 1989). Content essential oil of betel leaf from Balitro, Bogor was 0.35% and eugenol was 47.47%. It means the quality betel leaf from BALITRO has a good quality.

![GC-MS Chromatogram of Essential oil of Betel Leaf](image)

Figure 1. Profile GC-MS Chromatogram of Essential oil of Betel Leaf

The compounds obtained from the distillation with a retention time (RT) and percentage concentration (%) were as follows:

1. 1-Felandrena type 1 (RT 8.122, C 0.2%); 2. α –Pinena (RT 8.475, C 0.77%); 3.Kamfena (RT 9.415, C 1.11%); 4.Sabinina type1 (RT 10.326, C 2.26%); 5.2- β–Pinena (RT 10.534, C 0.01%); 6. β–Pinena (RT 10.727, C 0.66%); 7.1-Felandrena type 2(RT 11.487, C 0.23%); 8.α –Terpinena (RT 11.848, C 1.22%); 9. dl-Limonena (RT 12.212, C 0.25%) 10. Sabinina type 2 (RT 12.552, C 1.28%); 11.1,3,6-oktatrien, 3, 7-Dimetil-(E)(RT12.889, C 0.82%); 12. 1,8-Sineol (RT 13.038, C 0.25%) 13.
- Terpinena type 1 (RT 13.559, C 1.48%); 14. α-Terpinolen (RT 14.575, C 0.48%); 15. Linalol (RT 14.575, 0.58%); 16. 2-β-Pimena (RT 16.30, C 1.01%); 17. γ -Terpinolen type 2 (RT 17.129, C 0.09%); 18. 3-sikloheksan-1-01-4-metil-1-(1-metiletil) (RT 18.431, C 3.11%) 19. Not detection (RT 18.837, C 0.06%); 20. α -Terpinineol (RT 19.093, C 0.27%); 21. Trans-piperit (RT 19.433, C0,05%); 22. Trans-Anetol (RT 20.316, C 0.47%); 23. β-elemen (RT 20.801, C 0.32%); 24. α -Kubeben (RT 21.1, C 0.06%); 25. 1-(1-etil-2,3-dimetil-siklopen-2-enil) (RT 21.447, C 0.1%); 26. Salvia-4(14)-en-1-one (RT 21.873, C 0.11%); 27. α -Kopaen (RT 22.07, 0.21%); 28. β -Patchoul (RT 22.758, C 5.14%); 29. β -Elemena (RT 22.758, C 0.75%) 30. α -Bergamot (RT 23.350, C 0.22%); 31.1,3-Benzioxidoks, 5-(2-propenil). (RT 23.500, C 0.21%); 32. (Z,E), ε-Farnesen (RT 23.662, C 0.08%); 33. 3(1H)-Azulenol,2,3,4, 5, 5, 8a-heksahidro-6,8a-dimetil-3-(1-metiletil) (RT 23.816, C 1.04%); 34. Kariofen (RT 23.951, C 3.29%); 35. Trans- β-Farnesen (RT 24.986, C 0.15%); 36. Benzaldehida,2-etil (RT 24.986, C 5.63%); 38. 4α,5,6,8a-oktahidro-7-metil-4- metilene-1-(1-metiletil) (RT 25.812 C 1.15%); 39. Fenol,2-methoxi-4-(2-propenil) (RT 25.52, C 1.19%); 40. Naphtalen,1,2,3,4a,5,6,8a-oktahidro-7- metil-4-metilene-1-(1-metiletil) (RT 25.812 C 1.56%); 41. Cis-iso-Eugenol (RT 26.389, C 47.47%); 42. α -Selinena (RT 26.565, C 2.93%); 43. δ -Guaien (RT 26.732 C 0.08%); 44. Naptenal,1,2,3,4 4a,5,6,8a-oktahidro-7-metil-4- metilene-1-(1-metiletil) (RT 26.923 , C 0.26%); 45. α -Patchoul (RT 27.314, C 0.59%); 46. β -Kardinena (RT 27.473, C 0.55%); 47. 7-epi- α -Selinena (RT 27.619, C 0.41%); 48. α -Kubebin (RT 27.912, C 0.07%); 49. Nerolidol (RT 28.511, C 0.06%); 50. Elemol (RT 29.179, C 0.06%); 51. Benzena,1,2-dinmethoxy-4-(1-propenil) (RT 30.339, C 0.09%); 52. (--)Kariophilen oksid (RT 30.664, C 0.18%); 53. Agaruspil (RT 31.222, C 0.09%); 54. Humulan-1,6-dien-3-ol (RT 31.669, C 0.26%); 55. 1R,4S,7S,11R,2,4,8- Tetrametiltrisiklo [5,3,1,0 2,3]undec-8 ene (RT 31.796, C 0.26%); 56 Fenol-2-methoxi-4-(1-propenil)-asetat (RT 32.115, C 4.53%); 57. α -Gurjenen (RT 32.516, C 0.20%); 58. α -Kardinena (RT 32.662, 0.18%); 59. Veridiflorol type 1RT 32.843, C 0.09%); 60. Naptenal,1,2,3,4,4a,5,6,8a-oktahidro-7-metil-4-metilena-1-(1-metiletil) (RT 33.003, C 0.19%); 61. β -Selinena (RT 33.285, C 0.5%); 62. Veridiflorol type 2 (RT 33.724, 0.36%); 63. Patchouli alkohol (RT 34.444, C 2.39%); 64. β -tumorin (RT 35.099, C 0.08%), 65. 4-Alil-1,2-diaceotoks -benzen (RT 36.014, C 1.48%); 66. Benzilbenzoat (RT 41.246, C 0.12%), T O T A L = (100%)

From 66 fractions of betel leaf essential oil compounds were obtained, It can be classified into 6 groups. Phenyl Propanoid group is the highest with a concentration 49.31%, while the others group is the group with the lowest concentration 6.01%. As shown in Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Groups</th>
<th>Betel leaf essential oil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Monoterpenes</td>
<td>10.54</td>
</tr>
<tr>
<td>2.</td>
<td>Monoterpenes Alkohol</td>
<td>9.56</td>
</tr>
<tr>
<td>4.</td>
<td>Sesquiterpenes alkohol</td>
<td>2.59</td>
</tr>
<tr>
<td>5.</td>
<td>Phenyl Propanoid</td>
<td>49.31</td>
</tr>
<tr>
<td>6.</td>
<td>Others</td>
<td>6.01</td>
</tr>
</tbody>
</table>

The result diameter of zone of inhibition of antibacterial activity test between essential oil of betel leaf compared with eugenol with concentration 15%, shown in Table 1.2. Diameter of zone of inhibition that given by eugenol average larger than that given by essential oil of betel leaf against the five bacteria test with the same dose.. In this case, a sharp difference occurred on Streptococcus mutans, eugenol gives diameter of zone of inhibition 10 mm, while essential oil of betel leaf just gives diameter of zone of inhibition 1 mm. Then for bacteria Proteus mirabilis and Salmonella typhimurium occurred only a thin difference on diameter of zone of inhibition.
Table 1.2. The result diameter of zone of inhibition (mm) of antibacterial activity test between essential oil of betel leaf compared with eugenol with concentration 15%.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Essential oil (mm)</th>
<th>Eugenol (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. mirabilis</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Sal. thypimurium</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Sh. flexneri.</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Str mutans</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

The comparison Minimum Inhibitory Concentration (MIC) values of essential oil of betel leaf compared with eugenol also showed, that eugenol has antibacterial activity more sensitive than the essential oil of betel leaf.

_Salmonella thypimurium_ is the most sensitive, both to the essential oil of betel leaf and eugenol, _Streptococcus mutans_ is much more sensitive to eugenol than the essential oil of betel leaf, to MIC eugenol is 0.4%, while, for essential oil of betel leaf was not obtained the MIC until concentration 17% (v/v), as shown in Table 3.

Something interesting about the antimicrobial activity of essential oil of betel leaf is he has broad-spectrum antimicrobial activity, not only for bacteria but also for fungi. As research was conducted by Row et al (2009). Comparing the antimicrobial activity of essential oil with 4 antibiotics that are widely used for treatment, namely: ampicillin, Ciprofloxacin, Kanaycin, and Cephalotin against _Staphylococcus aureus_, _Escherichia coli_ and the fungi namely _Candida albicans_ and _Malassezia pachydermatis_. The results of research was conducted by Row et al (2009) showed that the anti-microbial essential oils of betel leaf is quite effective against bacteria and fungi, while antibiotics are only effective against bacteria and was ineffective against fungi.

Table 3. The result of Minimum Inhibitory Concentration (MIC) of antibacterial activity test between essential oil of betel leaf compared with eugenol.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Essential oil (% v/v)</th>
<th>Eugenol (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. mirabilis</td>
<td>9</td>
<td>0.4</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>Sal. thypimurium</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>Sh. flexneri.</td>
<td>11</td>
<td>0.5</td>
</tr>
<tr>
<td>Str. mutans</td>
<td>&gt;17</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The test results for essential oil of betel leaf was obtained for 1 MIC at concentration 8% (v/v) and for 2 MIC at concentration 16% (v/v), while for eugenol for 1 MIC at concentration 0.8% (v/v) and for 2 MIC at concentration 1.6% (v/v). Results from these concentrations were used for analysis of nucleic acids and proteins. The results of nucleic acid and protein leakage was obtained, the greater the concentration the greater the leakage that occurred, both for the essential oil of betel leaf as well as for eugenol. The Leakage of nucleic acids that caused by the essential oil of betel leaf at dose 1 MIC was 1.018, 2 MIC was 1,366, for eugenol at dose 1 MIC was 1,446 and for 2 MIC was 1,842. While The Leakage of proteins that caused by the essential oil of betel leaf at dose 1 MIC was 1.351, 2 MIC was 1,639, for eugenol at dose 1 MIC was 1,552 and for 2 MIC was 1,709, as shown in Table 4.
Table 4. The results of absorbance values due to leakage of nucleic acids (260 nm) and protein (280 nm) with UV-Vis of volatile oil and eugenol against *Shigella flexneri*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Essential oil (nm)</th>
<th>Eugenol (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0,283</td>
<td>0,260</td>
</tr>
<tr>
<td>1 MIC</td>
<td>1,018</td>
<td>1,351</td>
</tr>
<tr>
<td>2 MIC</td>
<td>1,366</td>
<td>1,630</td>
</tr>
</tbody>
</table>

Some antimicrobial agents cause gross membrane damage and provoke whole-cell lyses and this has been reported previously for essential oils from oregano, rosewood, and thyme (Carson et al, 2002). On essential oil of betel leaf, there are some agent that can cause cell membrane damage, such as eugenol, Benzilbenzoat, Patchouli alcohol and the compounds of group monoterpene alcohol and sesquiterpene alcohol (Caburian et al, 2010, Cox et al, 2000).

In this case, as shown in Table 4. And Figure 2. that caused by the effects of compounds that work damage the cell membrane. There were occurred some increase in leakage of nucleic acids and proteins from dose 1 MIC to dose 2 MIC compared with the control dose. As well as for leakage of protein from the cell membrane.

![Figure 2](image1.png)

Figure 2. The level leakage of nucleic acids (260 nm) and proteins (280 nm) by essential oil of betel leaf against *Shigella flexneri* with dose 1 MIC and 2 MIC.

![Figure 3](image2.png)

Figure 3. The level leakage of nucleic acids (260 nm) and proteins (280 nm) by eugenol against *Shigella flexneri* with dose 1 MIC and 2 MIC.
The damage of cells membrane can also be observed with K and Ca metals leakage from the cell membrane as shown in table 5. It also happened an increase leakage of K and Ca metal from dose 1 MIC to dose 2 MIC when compared with the control dose. As shown in Table 5. And Figure 3.

Table 5. The results of measurement concentration (ppm) ions K + and Ca2 + from Shigella flexneri by using Atomic Absorption Spectrophotometer (AAS).

<table>
<thead>
<tr>
<th></th>
<th>Essential oil</th>
<th>Eugenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca2+ K+</td>
<td>Ca2+ K+</td>
</tr>
<tr>
<td>Control</td>
<td>4,21 52,3</td>
<td>4,21 52,3</td>
</tr>
<tr>
<td>1 MIC</td>
<td>27 61</td>
<td>31 78</td>
</tr>
<tr>
<td>2 MIC</td>
<td>32 86</td>
<td>35 90</td>
</tr>
</tbody>
</table>

The level of damage caused by essential oil of betel leaf and eugenol against Shigella flexneri more real, when observed with Scanning Electron Microscope (SEM), as shown in Figure 4. In the Figure 4.a. is Shigella flexneri without treatment by essential oil of betel leaf and eugenol (control normal). The surface of cells looked complete and normal. In Figure 4.b. Shigella flexneri with treatment essential oil of betel leaf 1 MIC, Here seemed the Surface of cells that were not flat. In Figure 4.c Shigella flexneri with treatment essential oil of betel leaf 2 MIC Here seemed the Surface of cells have many holes and rugged. In Figure 4.d. Shigella flexneri with treatment eugenol 1 MIC. Here seemed the Surface of cells that also were not flat. In Figure 4.e Shigella flexneri with treatment eugenol 2 MIC. Here seemed the Surface of cells that also were not flat and have many hole. the greater the dose the greater the damage that occurred, both for the essential oil of betel leaf as well as for eugenol. it is likely that this variability reflects the rate at which its active components diffuse through the cell wall and into the phospholipids regions of cell membrane structures and caused the plasma cell membrane damage and coagulation of the nucleoid.

CONCLUSION

From the results of research that was carried out can be concluded. That essential oil of betel leaf and eugenol have antibacterial properties against bacteria Proteus mirabilis, Proteus vulgaris, Salmonella thyphimurium, Shigella flexneri and Streptococcus mutans.

Salmonella thyphimurium is the most sensitive both to the essential oil of betel leaf and eugenol.

Streptococcus mutans is quite sensitive to eugenol (MIC = 0.4% v/v) and less sensitive to the essential oil of betel leaf (> 17% v/v).

Observations antibacterial activity against Shigella flexneri that carried out by using spectrophotometer UV/Vis, Atomic Absorption Spectrophotometer showed that, the greater the dose the greater the leakage of nucleid acids, proteins, ion K+ and ion Ca2+ from cells membrane, both for the essential oil of betel leaf as well as for eugenol. While the observations that were detected by using Scanning Electron Microscope showed worse cell damage by giving the higher doses to bacteria. Shigella flexneri Plasma cell membrane damage and coagulation of the nucleoid by eugenol worse than essential oil betel leaf.
The surface of complete of cells (Control)

Surface of cells that are not flat

The hole

Figure 2. The result of Scanning of Changes in Cells Morphology by SEM (Magnification 15.000x)

Annotation:
(a). Normal cells of *Shigella flexneri* (control normal)
(b). Cells of *Shigella flexneri* with treatment essential oil of betel leaf 1 MIC
(c). Cells of *Shigella flexneri* with treatment essential oil of betel leaf 2 MIC
(d). Cells of *Shigella flexneri* with treatment eugenol 1 MIC
(e). Cells of *Shigella flexneri* with treatment eugenol 2 MIC
REFERENCES


Chan M., (2011) Statement by WHO Director-General, 6 April 2011.


CERTIFICATE OF APPRECIATION

This certificate is presented to

Muhammad Yani's Mustaja

In recognition of your valuable contribution as Presenter in the 4th International Seminar of Indonesian Society for Microbiology and IUMS-IMS Outreach Program in Food Safety "Indonesian Microbial Resources: Diversity and Global Impact"

22-24 June 2011 | Udayana University, Denpasar-Bali, Indonesia

Organized by: Indonesian Microbiology Society (IMS) and Microbiological Society of Indonesia (SMIK)

Dr. dr. I Dewa Made Sukrama, MSi., SpMK(K)
Head of Organizing Committee

Dr. Ir. Koensnandar, M.Eng.
Head of Indonesian Society for Microbiology