Antibacterial Activity of Ethanol Extracts from "Basa Genep" Spices Against *Escherichia coli*: A Comparison of Disk and Well Diffusion Methods

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Abstract. "Basa Genep" is a fundamental spice blend in Balinese cuisine, comprising ingredients such as garlic, shallots, cayenne pepper, red chili, ginger, galangal, turmeric, and aromatic ginger. These spices contain bioactive compounds that act as antioxidants and antimicrobials. This research aims to evaluate the antimicrobial activity of ethanol extracts from the "Basa Genep" spice blend against *Escherichia coli*, thereby providing insights into the potential health benefits and applications of these traditional spices in food safety and preservation. This study used disk and well diffusion methods to investigate the antimicrobial activity of ethanol extracts from these spices against *E. coli*. The spices were extracted by maceration with 96% ethanol. The antibacterial tests revealed varying levels of inhibitory activity, with the results of the disk diffusion method showing zones of inhibition ranging from 7.45 mm to 15.84 mm, and the well diffusion method showing zones of inhibition from 10.89 mm to 27.72 mm. Turmeric exhibited the highest antibacterial activity, while garlic had the lowest Effect against *E. coli*.

1 Introduction

"Basa Genep" seasoning is a traditional Balinese seasoning with a distinctive flavor profile characteristic of conventional Balinese cuisine [1]. The composition of "Basa Genep" varies according to individual and regional tastes, but in principle, it has 5 (five) taste elements: bitter, sour, sweet, spicy, and salty. "Basa Genep" is typically used to prepare traditional dishes such as *jukut ares, lawar, betutu chicken*, and *tum*. The seasoning "Basa Genep" consists of 14 components of herbs and spices such as galangal (*Alpina galanga*), ginger (*Zingiber officinale*), aromatic ginger (*Kaempferia galangal*), shallots (*Allium cepa*), garlic (*Allium sativum*), turmeric (*Curcuma longa*), red chilli (*Capsicum annuum*), cayenne pepper (*Capsicum frutescens*), candlenut (*Aleurites moluccana*), black pepper and white

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pepper (*Piper nigrum*), coriander (*Coriandrum sativum*), nutmeg (*Myristica fragrans*), cloves (*Syzygium aromaticum*) and Javanese chilli *(Piper retrofractum* Vahl).

Herbs and spices are food ingredients derived from plants, such as seeds, rhizomes, bark, leaves, and fruits, that have distinctive tastes and aromas. Spices are natural and artificial ingredients that act as food flavourings and are used in fresh or wet form. Meanwhile, spices are parts of plants with a strong aroma or taste and are used in small amounts in food as preservatives or flavour enhancers. The "Basa Genep" components are rich in bioactive compounds that function as antioxidants, antimicrobials, and other bioactive substances [1]. Overall, incorporating herbs and spices into cooking is crucial for creating a distinctive taste and aroma, as well as providing numerous health benefits for the body. The use of herbs and spices can help maintain food quality and extend its shelf life. Spices are essential in cooking because they give food a distinctive taste, aroma, and colour. Using spices in cooking provides both flavor and aroma, improves health, enhances nutrition, and maintains the quality of food.

At average concentrations, the spice components commonly used in daily food processing are insufficient to preserve food directly. However, at these concentrations, herbs and spices can synergistically enhance the inhibitory effects of other ingredients on microbial growth in foods [2]. Each antimicrobial compound has a different inhibitory ability against specific microbes. The plants containing flavonoid compounds, phenols, and essential oils may exhibit antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*. *E. coli* is a normal flora found in the digestive tract, but it can potentially cause disease. *E. coli* will become pathogenic if the number in the digestive tract increases, such as consuming contaminated water or food or entering a body with a low immune system, such as infants, children, older people, and people with low immunity.

The extraction method used is the maceration method. The maceration extraction method is a simple extraction process that utilizes a solvent with several stirrings at room temperature for 24 hours. The primary advantage of the maceration extraction method is that the procedures and equipment used are straightforward, and the process does not involve heating, thereby ensuring that natural materials are not decomposed and bioactive compounds are not damaged. Solvent selection is a crucial factor in the extraction process. The polarity of the solvent greatly influences the extraction of target compounds from the raw material. This research uses ethanol because it is an ideal solvent that can dissolve flavonoid and phenolic compounds from plants [3]. The type of extraction solvent also influences the amount of active compounds contained in the extract, according to the "like dissolves like" concept, which states that polar compounds dissolve in polar solvents and non-polar compounds dissolve in non-polar solvents.

The antibacterial test is a method used to determine the sensitivity limit of an antibacterial compound to a particular bacteria. One of the antibacterial test methods is the diffusion method. The diffusion method is often used to analyse antibacterial activity. There are three ways in which the diffusion method can be used, namely the well method, the disc method, and the cylinder method. The working principle of the diffusion method is the diffusion of antibacterial compounds into a solid medium where the test microbes have been inoculated. The observation results indicated whether a transparent area formed around the paper disc, indicating an inhibitory zone for bacterial growth [4]. Based on the background information above, this research was conducted to determine the antimicrobial activity of compounds in the "Basa Genep" seasoning against *E. coli* bacteria using the disk and well diffusion methods.

2. Materials and methods

This research was conducted from April to September 2023 at the Food Analysis Laboratory, Faculty of Agriculture, Warmadewa University, Denpasar. This research employs an exploratory, experimental method, presenting data in tables and graphs to assess the antimicrobial activity of the spices that comprise the "Basa Genep" seasoning against the Gram-negative bacterium *Escherichia coli* FNCC 0091. The tools used are test tubes, tube racks, dropper pipettes, micropipettes, spatulas, Petri dishes, tube needles, autoclaves, discs, measuring cups, Erlenmeyer, stirring rods, spatula, aluminium foil, analytical balance (Electronic Scale), oven, desiccator, evaporator, blender, 60 mesh sieve, glass bottle, litmus paper, digital Calliper, Hirayama laminar airflow (LAF), Maspion (Electric Stove), Wina vortex type 70 mixer, tweezers and Cork borer. This research is an experimental research and the data is analyzed descriptively.

The ingredients used in this research were cayenne pepper (*Capsicum frutescens* L.), red chilli (*Capsicum annuum* L.), garlic (*Allium sativum*), shallot (*Allium ascalonicum* L.), ginger (*Zingiber officinale*), turmeric (*Curcuma domestica* Val), aromatic ginger (*Kaempferia galanga* L.), galangal (*Alpinia galanga* L.), ethanol 96%, potato, agar, distilled water, potato, yeast extract, peptone, glucose, NaCl, KH_2PO_4, Na_2HPO_4, alcohol, spirits, and bacteria *Escherichia coli* FNCC 0091.

2.1 Research Procedures

Sampling was conducted at Badung Market, Dauh Puri Kangin Village, Denpasar City, Bali Province. Sampling was performed by selecting ingredients that make up "Basa Genep," provided they are fresh and have the ideal shape. The ingredients that make up the "Basa Genep" seasoning were collected and wet sorted, separating the main part of the plant from other plant components, dirt, or other foreign materials. The collected materials were then washed to remove any adhering dirt. Washing was performed using running tap water, followed by draining and drying in an oven at 40 °C to achieve a water content of approximately 10% [5].

The dried herbal material is sorted dry, separating foreign objects during the drying process. The dried herbal material is then powdered using a blender. The dried herbal material powder is sieved with a 60-mesh sieve and stored in a glass jar to prevent contamination and other impurities before extraction [6].

Dried "Basa Genep" spices in 100 g were used, then macerated using ethanol solvent, respectively, with a ratio of 1:3 (w/v). Maceration was carried out for 2 × 24 h in a closed container, and stirring was performed for six h with a stirring time of 5 min. A second maceration was carried out using the same solvent and treatment for 24 hours. Second maceration was carried out to remove the remaining compounds left behind during the first maceration. After maceration, the filtrate is separated from the residue using a vacuum filter, which is then evaporated using a rotary evaporator at 40 °C and 100 rpm until the solvent stops dripping, resulting in a thick extract [7].

2.2 Antimicrobial Activity Testing Procedure

The tools are washed until clean and then dried, after which they are covered tightly with cotton and parchment paper. Then, please place it in the autoclave and close the lid tightly. Then, it was sterilized for 15 min at 121 °C. Potato Peptone Glucose Agar (PPGA) media is divided into agar PPGA media and liquid PPGA media. PPGA agar media is made with a

composition of 200 g of potatoes and 800 mL of distilled water, heated on a stove for 20 min, then mix 20 g of agar, 5 g of peptone, 5 g of glucose, 2.5 g of KH_2PO_4 and 1 gram of Na_2HPO_4 into an Erlenmeyer flask then distilled water is added until it reaches 1000 mL. Liquid PPGA media was prepared by mixing 12 g of potatoes with 50 mL of distilled water, heating on a stove for 20 minutes, and then adding 1.5 g of peptone and 1 g of glucose to an Erlenmeyer flask. This mixture is transferred into 5 test tubes, each containing 10 mL. PPGA agar and liquid PPGA media were sterilized in an autoclave for 15 min. After cooling, the PPGA agar medium is poured into a petri dish. Additionally, leaving the culture medium in the autoclave for too long can cause chemical changes in the medium, resulting in poor plant growth. A total of 1 dose of test bacteria was inoculated into liquid PPGA media and incubated for 24 h at 37 ℃ until bacterial colonies formed and the fluid media became cloudy. Classification of clear zone diameter and inhibitory response to bacterial growth is shown in Table 1.

Table 1. Classification of Clear Zone Diameter and Inhibitory Response to Bacterial Growth

Clear Zone Diameter	Growth Barrier Response
≥20 mmm	Very strong
10-20 mm	Strong
5-10mm	Weak
≤5 mmm	Very weak

Source: Karayiğit *et al.*, 2024 [8]

a) Disk Diffusion Method

The diffusion method using discs involves disc paper as a medium for absorbing antimicrobial materials, which is then saturated with the test material. After that, the paper disc is placed on the surface of the agar medium, which has been inoculated with the test microbial culture and then incubated for 18-24 hours at 37 ℃. For each thick extract of the spice ingredients that make up "Basa Genep", the positive control in levofloxacin and the negative control in the form of ethanol solvent will be prepared. Four sterile paper discs with a diameter of 6 mm will be placed in the thick extract and left for 15 minutes until they are evenly absorbed. This will result in a disc containing the extract with a concentration of 100% using an ethanol solvent. The paper disc containing the thick extract was transferred to agar media in a petri dish containing the test bacterial suspension. After incubation, inhibitory power is characterized by the formation of a clear zone (halo) around the test bacterial colony on the paper disc. The diameter of the halo formed is obtained by averaging the measurements on each paper disc using a caliper.

b). Well Diffusion Method

The well method makes perpendicular holes in solid agar inoculated with the test bacteria. The number and location of the holes are adjusted to the research objectives, and then the holes are filled with the sample to be tested. Wells are made using the tip of a sterile pipette. There are two wells in the petri dish. In each well, a thick extract of the spice ingredients that

make up "Basa Genep" is poured; the positive control is levofloxacin, and the negative control is 20 µL of ethanol solvent. Incubate at 37 °C for 24 h. After the incubation period, inhibitory power is characterized by the formation of a clear zone (halo) around the test bacterial colony in the well. The diameter of the halo formed is obtained from the average of the measurement results at each wellbore using a calliper.

3. Results and Discussion

This research uses the maceration method with the organic solvent ethanol. Ethanol is often used as a solvent in the maceration process because it can dissolve various compounds, including polar compounds (such as flavonoids, alkaloids, and organic acids) and non-polar compounds (such as terpenoids and lipids). This makes ethanol effective for extracting a wide variety of bioactive components from plant materials, which supports its effectiveness in extracting active compounds from plant materials. Ethanol is an organic solvent with polar properties, due to its hydroxyl group (OH), which allows it to bind with polar or ionic molecules. Polar solvents are widely used to extract the polar components of natural materials and are known as universal solvents. The polar components of a natural substance in an ethanol extract can be extracted using extraction techniques through a separation process. The maceration method was chosen because it can effectively extract active compounds through soaking without heating, thereby avoiding damage to unstable and heat-sensitive compound components. Stirring aims to increase the contact between the sample and the solvent, thereby maximizing the extraction process.

The test bacteria used were the Gram-negative bacteria *E. coli*. These bacteria were chosen as test bacteria because they are pathogenic to humans and represent bacteria with different cell wall characteristics. The choice between the disk diffusion and well diffusion methods was made to differentiate the inhibition of herbs against pathogenic microbes. Both methods proved effective in testing antibacterial activity, each offering different advantages. In practice, researchers often employ both methods to enhance the validity of the data and obtain a more comprehensive understanding of the antibacterial activity of the tested compounds. The results of measuring the inhibitory power of the thick ethanol extract, which comprises the seasoning "Basa Genep," at a concentration of 100% are presented in Table 2 and Figure 1. The data in Table 1 show that all the ethanol extracts that comprise the seasoning "Basa Genep" exhibit antibacterial activity against the Gram-negative bacterium *E. coli*. The moderate to powerful inhibitory power category includes the resulting inhibitory power. The higher the concentration of the extract, the greater the diameter of the inhibitory force formed [9].

The component of the "Basa Genep" spice that exhibits the highest inhibitory power against the growth of *E. coli* bacteria is turmeric, with values of 27.72 ± 7.01 mm using the Well Method and 15.84 ± 2.00 mm using the Disk Diffusion Method. Meanwhile, garlic has the lowest inhibitory power, with values of 10.89 ± 4.01 mm using the Well Method and 7.45 ± 0.30 mm using the Disk Diffusion Method (Data in Table 2). In the positive control, the average diameter was 30.38 ± 0.63 mm and 29.94 ± 0.17 mm. Levofloxacin exhibits extreme antibacterial activity due to its fluoroquinolone antibiotic properties, which provide a broad spectrum of activity against both Gram-negative and Gram-positive bacteria. Its mechanism of action involves inhibiting bacterial growth. The negative control did not exhibit inhibitory power, as research indicated that 96% ethanol did not form an inhibition zone, since ethanol with concentrations above 90% or below 50% is less effective in inhibiting the growth of microorganisms. The antibacterial activity contained in the "Basa Genep" seasoning is minimal compared to several constituents of the "Basa Genep" seasoning, such as turmeric, ginger, aromatic ginger, galangal, red chili, and cayenne pepper, in inhibiting the growth of

E. coli bacteria. It is caused by the presence of other constituents, such as spices and other food additives, which reduce the inhibitory power of the "Basa Genep" seasoning to its optimal level.

Table 2. Antibacterial Activity Results Using Disk and Well Diffusion Methods

Sample	Inhibition zone diameter (mm)	
	Disk Diffusion Method	**Well Method**
Cayenne pepper	13.17±1.24	13.39±1.24
Red chili pepper	13.62±2,57	12.44±0.04
Garlic	7.45±0.30	10.89±4.01
Shallots	8.81±0.23	17.50±0.03
Ginger	8.43±0.27	18.71±0.88
Turmeric	15.84±2.00	27.72±7.01
Aromatic ginger	9.66±0.02	18.65±0.96
Galangal	7.96±1.16	22.43±0.36
Genep Basa	9.70±0.20	15.83±0.34
Control+	29.94±0.17	30.38±0,63
Controls -	0.00±0.00	0.00±0.00

Note: +: Standard deviation

Turmeric ethanol extract exhibits extreme inhibitory power, while garlic has moderate inhibitory power against the growth of *E. coli*, fungi, bacteria, and viruses. This indicates that both in vivo and in vitro turmeric exhibits antimicrobial activity capable of killing and inhibiting the growth of fungi, bacteria, and viruses. Turmeric rhizomes are more effective in inhibiting putrefactive bacteria than fungi because turmeric contains curcuminoids and essential oils that can function as antimicrobials. Curcuminoids in turmeric rhizomes are a group of phenolic compounds that inhibit bacterial metabolism by damaging the cytoplasmic membrane and denaturing cell proteins, so bacterial cells die [10]. Apart from that, the antibacterial compounds contained in turmeric are flavonoids and alkaloids. Flavonoid compounds can disrupt cell walls, leading to cell death, whereas alkaloid compounds can denature proteins, impairing enzyme activity and also resulting in cell death; thus, as an antibacterial agent, flavonoids inhibit the metabolic activity of bacterial cells through protein denaturation. [11].

Apart from inhibiting the growth of Gram-negative bacteria, *E. coli*, which makes up the "Basa Genep" seasoning, can also inhibit the growth of Gram-positive bacteria, such as *S. aureus*. This is supported by the results of research conducted by Sapitri *et al*. [12], which show that red chilli ethanol extract exhibits antibacterial activity against both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*). The lowest clear zone was observed at a concentration of 45%, while the highest clear zone was observed at a concentration of

90%. In research by Yunianto *et al*. [13], the activity of ointments containing the active ingredients turmeric and galangal demonstrated inhibitory powers, with average inhibition zones of 14.52 mm and 13.26 mm, respectively, against the growth of *S. aureus* bacteria. In addition to the results of research conducted by Azkiyah *et al*., [14] wrote that at a concentration of 80% ginger rhizome extract, there was an inhibitory power of 14.17 mm against the growth of *E. coli* bacteria and 12.33 mm against the development of *S. aureus* bacteria.

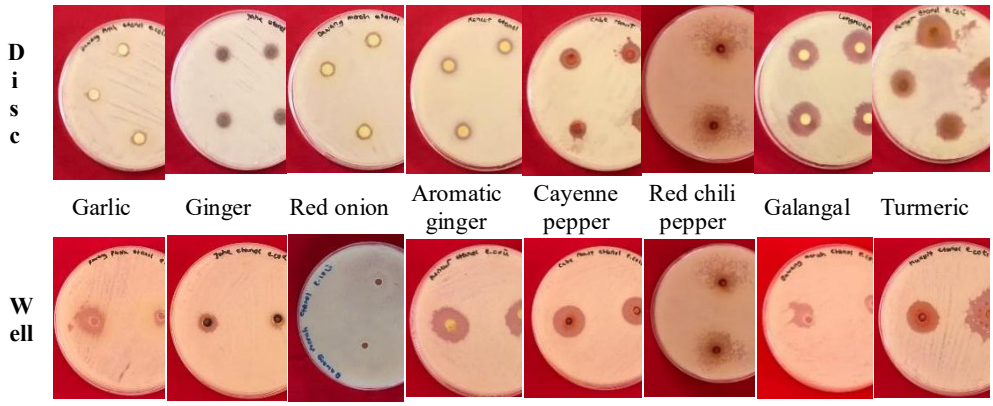

Figure 1. Antibacterial Activity Results Using Disk and Well Diffusion Methods

The antibacterial activity measured using the well method is higher than that measured using the disc method. This is because the sample is inserted into the well-made so that the osmosis process can occur more homogeneously and efficiently, which is more effective in inhibiting bacterial growth [14]. This aligns with research, which states that testing antibacterial activity using the well method can produce a wider area or zone of inhibition. Apart from that, it is supported by the research results of Rasheed *et al*. [15], who studied the well diffusion and disc diffusion techniques to evaluate antibiotic sensitivity against *Escherichia coli* bacteria and found that the well method produced a higher clear zone for antibiotics compared to the disc method.

Conclusion

This study asserts that ethanol extract from the spice "Basa Genep" possesses significant antibacterial potential against Escherichia coli, with turmeric identified as the most effective component in inhibiting the growth of this bacterium. The practical implications of these findings are extensive, ranging from the development of natural alternatives as antibacterial agents in health care and food preservation to applications in pharmaceuticals and cosmetics. Furthermore, these results open avenues for further research that could expand the understanding of optimal spice combinations for more substantial antibacterial effects, as well as the effectiveness of this extract against various clinically and industrially relevant gram-negative and gram-positive bacteria

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