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By Arum Setiawan

A Phenolic Compound of Endophytic Fungi Isolated from Stem of *Syzygium aqueum* and its Diuretic Activity

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Abstract: Guava (*Syzygium aqueum*), a typical Indonesian crop plant, has diuretic properties. Plants used as diuretic medicines can also be valuable sources for endophytic fungi containing diuretic drugs. The aims of this study were to isolate and identify endophytic fungi from *S. aqueum* stems, to isolate their secondary metabolites, and to conduct in vivo tests of the diuretic activity of an endophytic fungus extract on white male rats of the Wistar strain. Endophytic fungi were isolated by a direct plating method, and fungal isolates were identified molecularly. A phenolic compound was isolated by chromatography, and the chemical structure was identified spectroscopically. Doses of 100, 200, and 300 mg/kg BW were administered to white male rats divided into six groups (normal control, negative control, positive control, and the three treatment groups). Diuretic tests included urine volume; sodium, chloride, and calcium electrolyte levels; and pH. Phylogenetic analysis identified the endophytic fungal isolate as *Trichoderma ghanense* (isolation code SA1). The secondary metabolite isolated from the *T. ghanense* extract was a phenolic compound. Diuretic tests using the endophytic fungus extract showed the highest urine volume with a dose of 300 mg/kg BW. The diuretic test results showed an increase in urine volume and levels of sodium, potassium, and chloride ions in the urine. The extract of *T. ghanense* isolated from the stem of *S. aqueum* has the potential as initial therapy for hypertension because it contains phenolic secondary metabolites that show diuretic activity.

Keywords: diuretic; endophytic fungi; secondary metabolite; *Syzygium aqueum*.

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1. Introduction

Treating ailments such as hypertension, nephrotic syndrome, cirrhosis, and heart conditions includes diuretics to flush out excessive fluids within the body [1]. Diuretics cause an increase in the production and excretion of urine by the kidneys to promote the removal of toxins, excess water, and accumulated metabolic products, such as urea, from the body [2]. However, their use is sometimes accompanied by a loss of electrolytes, such as sodium, chloride, and potassium. Diuretics function to rid the body of excess fluid (edema) accumulated in tissues due to various disease states [3,4].

One plant with traditionally reported diuretic properties is guava (*Syzygium aqueum*), a typical crop plant from Indonesia. This plant has also been used in various parts of the world to treat various diseases, including hypertension, diabetes, diarrhea, asthma, inflammation, skin diseases, fever, digestive complaints, and high cholesterol [5], [6]. One study stated that most of the 87 secondary metabolites isolated from parts of the *Syzygium aqueum* plant were phenolics or phenolic derivatives [7]. People in South Sumatra, Indonesia, have used a decoction of the leaves and stems of *S. aqueum* as a diuretic medicine and to treat hypertension and diabetes [8].

The implantation of *S. aqueum* and other medicinal plants in Indonesia still faces many obstacles in terms of production, including the unprofessional implementation of medicinal plant implantation activities, the inability of farmers to maintain the quality and quality of medicinal plants, and the lack of attention from the medicinal plant industry regarding the results of scientific research in product development. One potential strategy for working around these obstacles is to use endophytic fungi, which are fast becoming important biotechnological tools [9,10]. Endophytic fungi are microscopic living organisms that live in plant tissues for a certain period by forming colonies without harming their hosts [11]. These endophytic fungi can produce metabolites similar to those produced by their host or different new compounds; therefore, this microbial group has huge biotechnological potential for new drug discovery [12–14]. Compounds produced by endophytic fungi from plants are often used as antioxidants, antidiabetics, cancer, and diseases caused by oxidative damage [15–17]. *Syzygium aqueum* contains compounds such as flavonoids, phenolics, tannins, terpenoids which are often used as antioxidants, anticancer, antiviral, anti-inflammatory [7]. The endophytic fungi are also believed to contain the same compounds and activities. From two of *Syzygium aqueum*, an antioxidant compound identified as 4-hydroxy-3-(4-hydroxyphenyl)-5-oxotetrahydrofuran-2-yl has been isolated [11]. The endophytic fungi associated with the plant *Syzygium aqueum* could represent ideal sources of diuretic drugs.

2. Materials and Methods

2.1. Chemical and drug.

Alcohol 70%, distilled water, sodium hypochlorite (NaOCl), chloramphenicol (Chloromex[®] Actavis, Indonesia), Potato Dextrose Agar (PDA) Merck, Potato Dextrose Broth (PDB) Merck, Silica gel 60 (70-230 mesh) Merck, thin layer chromatography (TLC), Silica gel 60 F254 Merck (Art.5554), methanol, aseton, ethyl acetate, chloroform, n-hexane (Merck), furosemide (Lasix[®], Aventis Indonesia Pharma, Indonesia), tween 80 1%.

2.2. Plant material.

The fresh stem of *Syzygium aqueum* was collected from Martapura, South Sumatra, Indonesia (4.17248 ° S, 104.20472° E). Plant identification was carried out at the Indonesian Institute of Sciences, Plant Conservation Center, Purwodadi Botanical Gardens, Certificate of Identification No.: B-301/III /KS.01.03/1/2021.

2.3. Isolation and identification of endophytic fungi.

Syzygium aqueum stems were washed under running water to remove dirt. The stem surface was sterilized by immersion in 70% ethanol for 2 min, followed by 4% NaOCl for 1

min and two rinses in sterile distilled water. The plant stems were dried on sterile filter paper and then cut aseptically into 1 × 1 cm pieces, which were then planted directly in a Petri dish containing PDA plus chloramphenicol and incubated at 25 °C for 2–5 days. The tip of the hyphae growing out of each segment was transferred to a Petri dish containing new PDA and then re-incubated for approximately 14 days. Pure cultures were obtained after several subcultures and grown on PDA plates without antibiotics [8].

Identification of endophytic fungi was carried out based on partial genetic analysis on the Internal Transcribed Spacer (ITS) of fungal ribosomal DNA. PCR amplification using ITS Primer 4:5'- TCC TCC GCT TAT TGA TAT GC – 3' and ITS Primer 5:5' -GGA AGT AAA AGT CGT AAC AAG G –3'. The purified polymerase chain reaction (PCR) product was then precipitated, followed by cyclic sequencing. The result was purified again by the ethanol purification method. Sequencing was performed using an automated DNA sequencer. Sequence data were trimmed and assembled, followed by alignment BLAST using genomic data at the NCBI (National Center for Biotechnology Information) for analysis of homology/similarity and creation of a phylogenetic tree [19].

2.4. Cultivation and extraction.

Endophytic fungal suspensions were inoculated in 300 mL potato dextrose broth (PDB), placed in 1 L bottles (9 L in 30 bottles), and incubated for 4 weeks at room temperature under static conditions. The endophytic fungal mycelium in each bottle was removed using tweezers, and the medium was filtered through filter paper. The filtrate was extracted with ethyl acetate at a 1:1 ratio. The filtrate and solvent were separated in a separating funnel to obtain an ethyl acetate extract, which was concentrated using a rotary evaporator [11].

2.5. Isolation and identification of secondary metabolites.

The endophytic fungal extracts from *Syzygium aqueum* were separated by column chromatography (stationary phase: silica gel) and eluted with a graded eluent (n-hexane: ethyl acetate: methanol). The eluate droplets were collected in a 10 mL bottle and analyzed by TLC to group the bottles into subfractions. Subfractions identified as having secondary metabolites were further purified by column chromatography until pure compounds were obtained. The structures were identified by 1D and 2D NMR [20].

2.6. Design of the test animal experiments animals.

The test animals were male Wistar rats (weight 150–250 g) obtained from Abduh Tikus Palembang, South Sumatra, Indonesia. All animals were housed at 25 ± 2°C and a 12 h light/dark cycle in polypropylene cages with clean rice husks as bedding. The test animals were acclimatized in the laboratory for 7 days. All animals were provided standard rodent food and drinking water ad libitum. The animal experiment protocol was approved by the Research Ethics Committee of Ahmad Dahlan University, Yogyakarta, Indonesia, with approval number: 022107033 dated August 27, 2021.

Table 1. Test animal treatment group.

No	Category	Treatment
1	Normal control	Aquadest
2	Negative control	Aquadest + NaCl 4,5% + tween 80 suspension 1%
3	Positive control	Aquadest + NaCl 4,5% + furosemide suspension

No	Category	Treatment
4	Treatment 1	Aquadest + NaCl 4,5% + mushroom extract suspension dose 100 mg/kgBB
5	Treatment 2	Aquadest + NaCl 4,5% + mushroom extract suspension dose 200 mg/kgBB
6	Treatment 3	Aquadest + NaCl 4,5% + mushroom extract suspension dose 300 mg/kgBB

2.7. Preparation for assays.

Suspensions of endophytic fungal extract at doses of 100 mg/kg BW, 200 mg/kg BW, and 300 mg/kg BW were prepared by grinding the endophytic fungal extract from *Syzygium aqueum* in a mortar according to the dose calculation (1.5 g in 50 mL for the highest dose of 300 mg/kg BW), then dissolving in phosphate-buffered saline containing tween 80 1%. The next dose was prepared by diluting the mother liquor (the highest dose).

2.8. Test of diuretic effect.

The test animal was divided into 6 treatment groups, consisting of 4 rats (Table 1). Before testing, the animals fasted for 18 h. Samples were given using a gastric probe. After treatment, the test animals were put into metabolic cages to collect their urine. Urine volume was measured after administration of the extract at 1, 2, 3, 4, 5, and 6 h, and cumulative urine volume was determined after 24 h. Drinking water at 1 to 6 h was controlled by giving 2.5 mL of 4.5 ml NaCl every 3 h orally to each test animal, except for the normal group, which was given 2.5 mL distilled water. During the treatment, the test animals were not fed. The percentage potency of the diuretics was determined using the following equation [21]:

$$\% \text{ Diuretic Potency} = \frac{\text{extract treatment volume} - \text{negative treatment volume}}{\text{Positive treatment volume} - \text{negative treatment volume}} \times 100\%$$

Urinary Na⁺, K⁺, and Cl⁻ levels were measured by atomic absorption spectroscopy (AAS) at wavelengths of 766.5 nm and 589.0 nm [22]. The pH was determined with a pH meter. The urine was fresh or preserved so that ammonia was not produced by urea breakdown.

Data Analysis

The results of the study were expressed as mean ± standard error of the mean (SEM). The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by a Tukey post hoc multiple comparison test. P-values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Isolation and identification of endophytic fungi.

The endophytic fungi isolated from the stems of *Syzygium aqueum* were given the isolation code SA1 (Figure 1). The results of the molecular identification of the 649 bp sequence of the SA1 endophytic fungus were as follows: GATCCTTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACCTCCCAA ACCCAATGTGAACGTTACCAATCTGTTGCCTCGGCGGGATTCTCTGCCCGGGC GCGTCGCAGCCCCGGATCCCATGGCGCCCGCGGAGGACCAACCAAACCTTTTTT TCTCTCCGTCGCGGCTTCCGTCGCGGCTCTGTTTTAACCTTTGCTCTGAGCCTTTCT CGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAACTTTCAACAACGGATCT CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT

10 g) and eluted with hexane: ethyl acetate (7:3–4:6) to yield four subfractions (SA1.7.1–SA1.7.4). The SA1.7.3 fraction (55 mg) was further purified by column chromatography until compound 1 (43 mg) was obtained.

The $^1\text{H-NMR}$ spectrum of compound 1 (Figure 3 (A)) revealed the presence of seven proton signals; four for the sp^3 proton, including a methyl signal at δ_{H} 1.25 ppm (3H, d, $J=1.5$ Hz) and a methine proton signal at δ_{H} 4.17 ppm (1H, m), and three oxygenated methine signals at δ_{H} 4.02 (2H, m), 5.34 (1H, d, $J=2.5$ Hz), and 5.79 ppm (1H, s). The spectrum is in the aromatic chemical shift region, at δ_{H} 7.58 and 8.22 ppm. The spectrum of each signal shows a doublet with the same plot constant $J = 8.0$ Hz, indicating the integration of two protons. Therefore, compound 1 is a para-substituted aromatic compound, so it has two pairs of equivalent protons. Analysis of the $^1\text{H-NMR}$ spectrum revealed compound 1 as a para-substituted aromatic compound with 12 protons attached to a carbon atom. The solvent used in this measurement is CDCl_3 , so the protons bound to the heteroatoms do not appear in the spectrum.

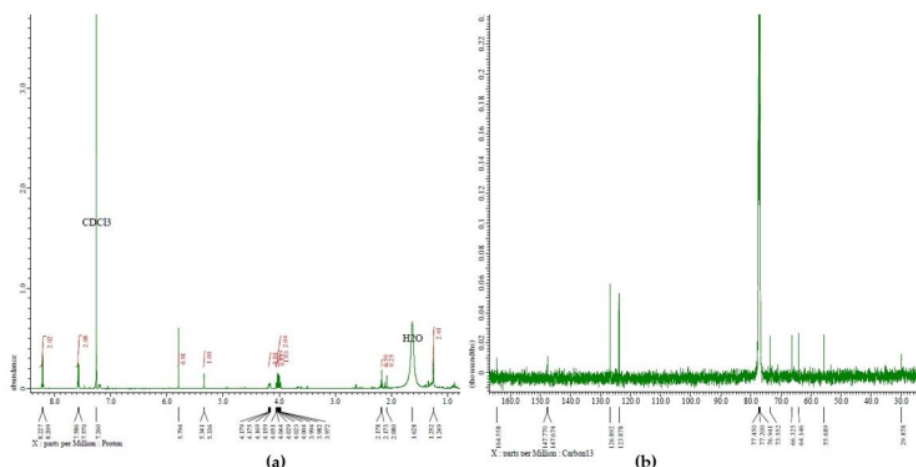


Figure 3. (a) $^1\text{H-NMR}$ spectrum; (b) $^{13}\text{C-NMR}$ of compound 1.

The $^{13}\text{C-NMR}$ spectrum of compound 1 (Figure 3(B)) showed the presence of nine signals: five sp^3 carbon signals, including three signals in the oxygenated carbon area at δ_{C} 64.1, 66.3, and 73.6 ppm. Four other signals appear in the aromatic region as two high-intensity signals, indicating that compound 1 has two pairs of equivalent aromatic carbons (δ_{C} 126.8 and 123.8 ppm), and as two low-intensity signals, indicating the presence of quaternary carbon, one of which is an oxyaryl carbon in the low field (δ_{C} 164.6 ppm). Analyses of the proton and carbon NMR spectra were confirmed by the data on the heteronuclear multiple quantum coherence (HMQC) spectrum shown in Figure 4 and Table 2, namely the $^1\text{H-}^{13}\text{C}$ correlation through one bond. The HMQC spectrum showed seven correlations consisting of two correlations on the aromatic ring, three correlations on oxygenated $^1\text{H-}^{13}\text{C}$, one proton correlation of methine, and one correlation of proton methyl.

The heteronuclear multiple bond correlation (HMBC) spectrum (Figure 5) shows the $^1\text{H-}^{13}\text{C}$ correlation through two or three bonds. The aromatic proton signal at δ_{H} 8.22 ppm indicates the existence of two correlations, one to carbon δ_{C} 123.9, which is the carbon equivalent, and the other to δ_{C} 147.7, which is the aromatic quaternary carbon. Another aromatic proton at δ_{H} 7.58 ppm has three correlations, each with carbon at δ_{C} 126.9 ppm, which is the carbon

equivalent, δ_C 147.7 and δ_C 73.6 ppm, which is oxygenated carbon directly bonded to the aromatic ring. Oxygenated methine protons at δ_H 5.34 ppm also have three correlations to carbons at δ_C 66.3, 147.7, and 126.9 ppm.

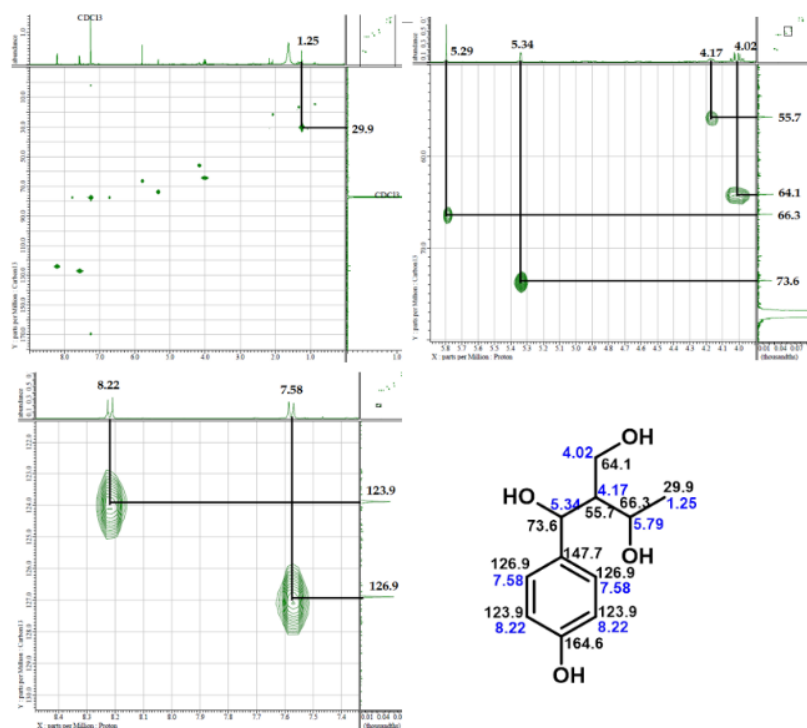


Figure 4. The HMQC spectrum and placement of the ^1H and ^{13}C shift in compound 1.

Table 2. The NMR data of Compound 1.

No. C	δ_C ppm	δ_H ppm (Σ H, multiplicity, J (Hz))	HMBC	COSY
1	73.6	5.34 (1H, d, J=2.5 Hz)	66.3; 147.7; 126.9	4.17
2	55.7	4.17 (1H, m)		5.34
3	66.3	5.79 (1H, s)		
4	29.9	1.25 (3H, d, J=1.5 Hz)	55.7	
5	64.1	4.02 (2H, m)	73.6	
1'	147.7			
2'	126.9	7.58 (1H, d, J=8.0 Hz)	126.9; 147.7; 73.6	8.22
3'	123.9	8.22 (1H, d, J=8.0 Hz)	123.9; 147.7	7.58
4'	164.6			
5'	123.9	8.22 (1H, d, J=8.0 Hz)	123.9; 147.7	7.58
6'	126.9	7.58 (1H, d, J=8.0 Hz)	126.9; 147.7; 73.6	8.22

The correlation indicates that the oxygenated methine group at δ_H 5.34 ppm is directly bound to the aromatic ring and is para-substituted with a hydroxyl group. The proton hydroxyl signal does not appear on the spectrum because the pure compound is measured with CDCl_3 as the solvent. The 1D and 2D NMR spectral data for compound 1 are shown in Table 2.

The COSY spectrum in Figure 6 shows the ^1H - ^1H correlation through three bonds. Two types of correlation are evident: the correlation of the aromatic proton at δ_H 8.22 ppm with the aromatic proton at δ_H 7.57 ppm and the proton sp^3 correlation of the substituent group, namely the oxygenated proton of methine at δ_H 5.34 ppm with the proton of methine at 4.17 ppm.

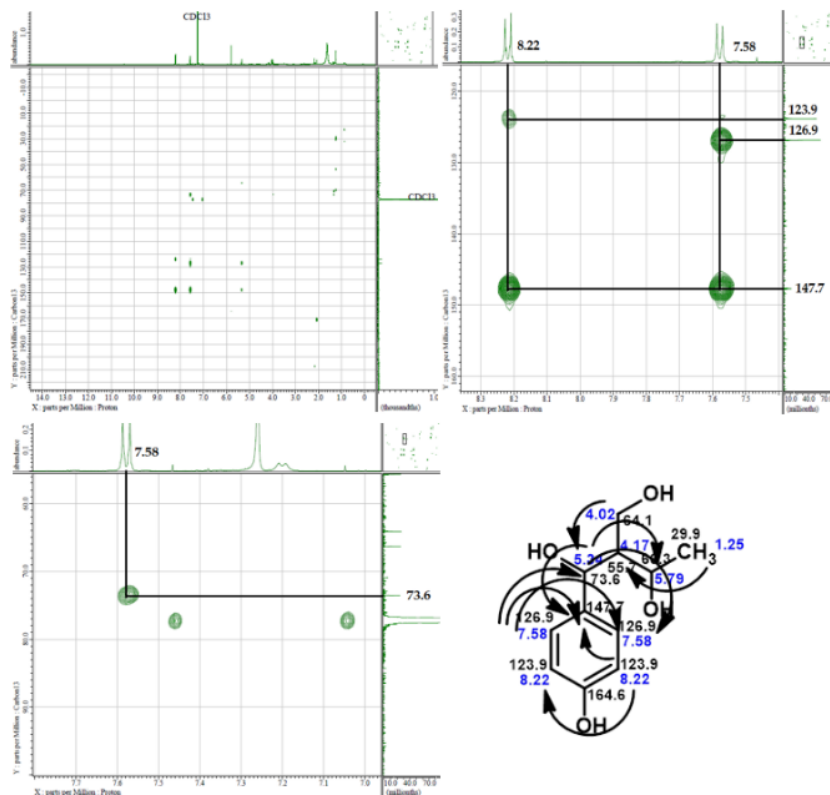


Figure 5. The HMBC spectrum and the correlation ^1H - ^{13}C through two or three bonds in compound 1.

This indicates that the two aromatic equivalent protons are in the ortho position. This strengthens the proposed structure of compound 1 as a benzene ring directly bonded to the oxygenated methine carbon at the para position with a hydroxyl group.

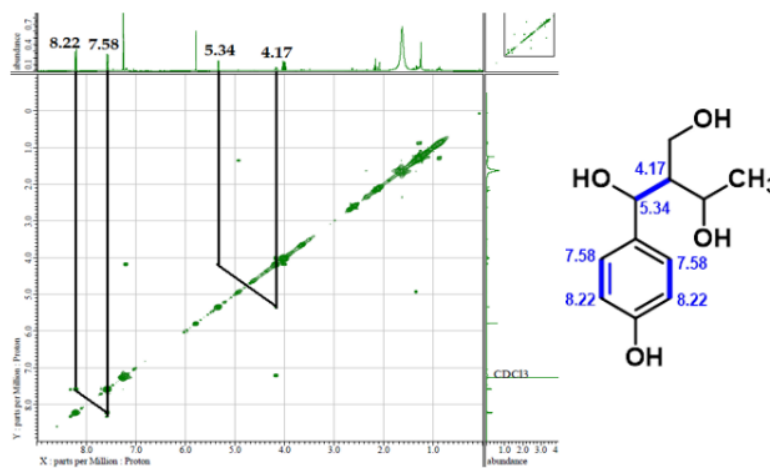


Figure 6. COSY spectrum and the correlation ^1H - ^1H in compound 1.

The spectrum analyses by ^1H -NMR, ^{13}C -NMR, HMQC, HMBC, and COSY can explain that compound 1 has a benzene ring that is substituted at the para position with a hydroxyl group and an oxygenated methine group that is part of the substituent group, namely 2- (hydroxymethyl)butane-1,3-diol. The proton at H 5.78 ppm appears as a singlet, probably

because the neighboring proton, which is three bonds apart, is in the farthest geometric position; therefore, cleavage and/or other factors, such as inappropriate resolution, do not occur. Thus, the proposed chemical structure of compound 1 is 2-(hydroxymethyl)-1-(4-hydroxyphenyl)butane-1,3-diol, as shown in Figure 7.

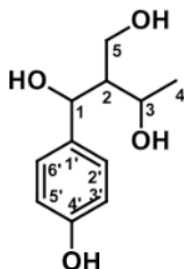


Figure 7. Structure compound of 3-(hydroxymethyl)-1-(4-hydroxyphenyl)butane-1,3-diol.

3.3. Test of diuretic effect.

The total urine volume produced was larger in the treatment group than in the normal and negative groups. This indicates a diuretic effect of the extract on urine excretion. The highest urine volume was produced by the positive control group; the data for the treatment group can be seen in Table 3.

Table 3. Total Urine Volume and Diuretic Potency.

Group	1 hour	2 hour	3 hour	4 hour	5 hour	6 hour	24 hour	% Diuretic Potency
Normal	1.42±0.572	2.42±0.453	2.85±0.433	3.30±0.248	3.42±0.319	3.57±0.278 ^a	6.37±0.327	0
Negative	1.17±0.411	2.02±0.652	2.32±0.850	3.77±1.048	4.37±0.851	4.57±0.797 ^a	7.50±0.659	0
Treatment 1	0.62±0.252	1.52±0.564	2.20±0.696	3.87±1.295	4.75±1.420	5.17±1.546 ^a	8.27±1.981	20
Treatment 2	0.37±0.217	1.20±0.535	1.75±0.590	3.90±0.994	4.77±1.220	5.67±0.900 ^b	8.67±1.697	21.27
Treatment 3	1.45±0.132	2.67±0.406	2.92±0.314	6.70±0.817	7.05±0.790	7.70±0.994 ^c	11.00±1.511	93.95
Positive	1.25±0.221	2.65±0.457	4.35±0.800	7.32±1.145	8.07±1.416	8.42±1.353 ^d	11.22±1.915	100

Each value represents the mean ± S.E.M (Standard Error of Mean); n=4. Positive: furosemide 20 mg/kgBW, Treatment 1 (100 mg/kgBW), Treatment 2 (200mg/kgBW), Treatment 3 (300 mg/kgBW)

Treatment with the extract increased urine volume or diuresis as a ratio between the dose and diuretic potency (R=0.78). The increase in urine volume occurs due to the inhibition of water reabsorption in the kidneys [23]. The increase in urine volume in the treatment group compared to the negative and normal groups occurred at 4–6 hours, due to the working time of furosemide at 4–6 hours, while the treatment groups 1–3 also showed a similar response, indicating that the working time of the extract was the same as the working time of furosemide [24] The treatment dose that had the highest diuretic potential was treatment 3, with a diuretic potency of 93.95% (P<0.003), whereas it was considered 0% (P<0.00) for the control and negative groups because those were the baseline references for the diuretic effect of the extract.

Previous studies have revealed that phenolics can inhibit the carbonic enzyme anhydrase in the renal tubules. Phenolics increase diuresis because they inhibit the angiotensin-converting enzyme (ACE) [25,26]. Cumulatively, the results for the urine volumes were greater for each treatment group than for the negative and normal control groups. The results of one-way ANOVA for the total urine volume (p = 0.003) indicate a significant difference between the test groups. The homogeneity test resulted in a Levene statistic of 2.257 with p = 0.523, showing that the test data have the same variance or are homogeneous.

The pH value of urine (Table 4) is determined by acid-base regulation in the kidneys. If a certain amount of HCO_3^- is continuously filtered into the renal tubules and excreted in the urine, the urine will be alkaline, as was observed in this study, where the pH value was pH 9–10 [27]. By contrast, if H^+ ions were continuously filtered into the kidney tubules and excreted into the urine, the urine would be acidic [28]. The kidneys have several mechanisms for regulating the concentration of H^+ ions, including secretion of H^+ ions into the tubules, reabsorption of HCO_3^- ions, and production of new HCO_3^- ions. All these mechanisms reduce and neutralize excess H^+ ions in the body [29,30].

Table 4. pH measurement.

Treatment category	Urine pH
Normal	10.00 ± 0.000
Negative	10.00 ± 0.000
Treatment 1	09.50 ± 0.288
Treatment 2	09.00 ± 0.000
Treatment 3	09.50 ± 0.288
Positive	10.00 ± 0.000

Each value represents the mean ± S.E.M (Standard Error of Mean); n=4. Positive: furosemide 20 mg/kgBW, Treatment 1 (100 mg/kgBW), Treatment 2 (200mg/kgBW), Treatment 3 (300 mg/kgBW)

The increase in diuresis caused by the endophytic fungal from *S. aqueum* was also evident in the excretion of electrolytes. The extract significantly increased the excretion of urinary electrolytes, such as Na^+ , K^+ , and Cl^- (Table 5). This increase in electrolyte excretion is thought to be due to the endophytic fungi extract acting through a loop diuretic mechanism, thereby increasing the urine flow rate and in turn causing a decrease in the absorption of Na^+ , K^+ , and Cl^- in the loop of Henle in the kidney [31,32]. This will increase the amount of water and salt excreted in the urine [33]. However, in the present study, the amount of K^+ excreted was not as large as the amounts of Na^+ and Cl^- . This condition results from the antioxidant activity of the endophytic fungal extract, which reduces the side effects of loop diuretics so that the body can still absorb K^+ [34,35]. The saluretic activity of the three test groups was above 100 percent, compared to the positive control using furosemide. These results indicate that the extract has saluretic potential.

Table 5. Measurement of Na^+ , K^+ , and Cl^- levels.

Treatment Category	Na^+ level (ppm)	K^+ level (ppm)	Cl^- level (ppm)	Saturetic level of Na (%)	Saturetic level of Cl (%)	Saturetic level of K (%)
Normal	200±1,154	141,5±3,175	141,5±3,175	0	0	0
Negative	247,5±8,948	330±16,743	386±73,323	0	0	0
Treatment 1	363±16,454	360,5±47,342	360,5±47,342	417.9	161.6	86.9
Treatment 2	272,5±7,794	338±31,754	338±31,754	184.6	145	77.97
Treatment 3	363±15,011	360,5±4,330	360,5±4,330	417.9	161.2	86.9
Positive	239±3,464	393,5±14,145	277±81,406	100	100	100

Each value represents the mean ± S.E.M (Standard Error of Mean); n=4. Positive: furosemide 20 mg/kgBW, Treatment 1 (100 mg/kgBW), Treatment 2 (200mg/kgBW), Treatment 3 (300 mg/kgBW)

4. Conclusions

The endophytic fungi extract from *Syzygium aqueum* contains a phenolic compound 2-(hydroxymethyl)-1-(4-hydroxyphenyl) butane-1,3-diol that has diuretic activity and increases the volume of excreted urine and its ion concentration.

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Conflicts of Interest

The authors declare no conflict of interest.

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