Anti-oxidant and anti-bacterial effects of *Diospyros kaki* leaf extract in Vietnam

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ABSTRACT

Diospyros kaki is a famous commercial plant with its edible fruit (persimmon) and is widely used as a traditional remedy in Vietnam. However, the anti-oxidant and anti-bacterial effects against gastrointestinal microbes of the *D. kaki* leaf extract have not been elucidated yet. In this study, the methanol extract from *D. kaki* leaves contained some chemical groups, such as tannins, flavonoids, phenolic compounds, alkaloids, and saponins, mainly it was rich in polyphenols and flavonoids (528.11 ± 12.96 mg GAE/g and 44.49 ± 6.77 mg QE/g, respectively). The extract exhibited a strong anti-oxidant ability (DPPH scavenging activity of about 91.83 ± 1.02% and total anti-oxidant capacity of approximately 110.58 ± 2.65 mg AAE/g). Furthermore, the anti-bacterial activity of the extract against some gastrointestinal bacteria, including *Staphylococcus aureus*, *Enterococcus durans*, *Enterococcus faecium*, and *Yersinia enterocolitica*, has been reported for the first time (the zone of inhibition ranging from 14.17 - 24.67mm). MIC of the extract was 0.6125 - 1.25 mg/mL, and MBC was 2.5 - 5 mg/mL, which implied the extract exerted a bactericidal effect. These findings suggest *D. kaki* leaf extract is a promising source of extracting bioactive compounds, such as anti-oxidant and anti-microbial agents, and a potential medicine for the treatment of gastrointestinal infections.

1. Introduction

The *Diospyros* genus is a large flowering plant of the Ebenaceae family with over 500 members primarily grown throughout the tropical and subtropical zones (Huang et al., 2023). Several members of the genus provide commercial and valued products such as timbers, fruits, and ornamentals. Note that some of them are well-known remedies in traditional medicines for the treatment of microbial infection-related diseases and gastrointestinal disorders such as *Diospyros mollis* (also known as "Mac nua" in Vietnamese), date plum, giant diospyros, jackalberry, hairy star-apple, bladdernut, Oriental persimmon, American persimmon, etc. (Tran et al., 2024; Rauf et al., 2017; Ribeiro et al., 2023). For example, people in Southeast Asia countries such as Vietnam, Thailand, Laos, Cambodia, and Myanmar have extensively applied *D. mollis* as a remedy to cure gastrointestinal disorders and parasite-related ailments, as well as a restorative beverage to cure emaciation (Tran et al., 2024). Furthermore, the anti-inflammatory,

anti-bacterial, and anti-malaria effects of *Diospyros mespiliformis* have been well documented (Rauf et al., 2017). According to Ribeiro et al. (2023), some natural products extracted from *Diospyros* species, including betulinic acid and plumbagin, exert an anti-bacterial effect against a wide range of bacteria, including both Gram-negative and Gram-positive strains.

Among them, *Diospyros kaki* is a good candidate for medical utilization because of its abundant bioactive compounds and effectiveness in treating various ailments. *D. kaki* is a valued commercial tree with the well-known product persimmon and is recognized as a remedy in traditional Asian medicine with a long history. According to Do (2022), *D. kaki* is mainly distributed in the Northern region of Vietnam, China, and Japan and can relieve cough, nocturia, and bloating. Many parts of *D. kaki* can be used as remedies, such as persimmon calyx, fruit, and leaves and the presence of some bioactive compounds including catechins, proanthocyanidins, tannins, phenolic compounds, flavonoids, terpenoids (ursolic acid, oleanolic acid, and betulinic acid), as well as carotenoids have been reported in the literature (Butt et al., 2015; Direito et al., 2021; Xie et al., 2015). Moreover, the bioactivity of *D. kaki* also attracts the interest of researchers worldwide. For example, the anti-oxidant and anti-diabetic effects of peel and pulp of *D. kaki* fruit have been proven (Allouache et al., 2024). In the previous report, *D. kaki* extract also has been suggested as an anti-bacterial, anti-diarrheal, and anti-oxidant agent (Dhawefi et al., 2021). However, the bioactivity of *D. kaki* leaves, one of the abundant agriculture by-products, from Vietnam has not been studied yet. In this study, we investigated phytochemical screening, anti-oxidant, and anti-bacterial effects of the *D. kaki* leaf extract, especially against some gastrointestinal-related microbes, such as *Staphylococcus aureus*, *Enterococcus durans*, *Enterococcus faecium*, *Escherichia coli*, *Yersinia enterocolitica*, and *Shigella boydii*. Among six bacterial strains, *S. aureus* is commonly located in the patient's nose, pharynx, and skin. It causes mild to severe skin infections, abscesses, urinary tract infections, food poisoning, and gastrointestinal tract infections. Additionally, *E. faecium* is mainly located in human and animal gastrointestinal tracts and causes some severe infections in other organs, such as urinary tract infections, intraabdominal infections, endocarditis, bacteremia, and even meningitis (Knoll et al., 2012; Venkataraman et al., 2023). Furthermore, *E. durans* is found in infected patient mouths and animal intestines and is usually classified as a commensal bacterium (Tarek et al., 2022). However, *E. durans* also caused a small portion of bacteremia in hospital patients (Ryu et al., 2019). *E. coli* usually lives in human bowels, and some pathogenic *E. coli* can lead to diarrhea, throwing up, and abdominal cramping. *Y. enterocolitica* commonly infects and colonizes in patient guts, which in turn causes yersiniosis, diarrhea, and enteritis. *S. boydii* is mainly found in patient bowels and feces, one of the leading causes of shigellosis. Therefore, investigating the anti-bacterial effect of *D. kaki* leaf extract provides a promising and impactful measure for managing these gastrointestinal-related microbes.

2. Materials & methods

2.1. Plant material and extract preparation

The fresh *D. kaki* samples were grown and collected from Don Duong District, Lam Dong Province, one of the most popular persimmon cultivated regions in the High Centrals of Vietnam. An herbalist from the Faculty of Pharmacy, Ton Duc Thang University, Ho Chi Minh City, Vietnam, identified the voucher specimens and kept them in the herbarium for further reference. To prepare the methanol extract of *D. kaki* leaves, the Tran et al. (2023) procedure was conducted. The fresh leaves of *D. kaki* were dried at 55^{o}C until the moisture was below 10%; the sample was subsequently crushed into small powder. The powder was submerged in 95%

methanol for 04 days with shaking at 150rpm. The sample was treated in an ultrasound bath for 10 minutes, and the extract was collected via filtration with Whatman filter paper. The procedure was repeated once, and the resulting extract was obtained by combining several extraction times. The residue solvent was eliminated via rotary evaporation and air-dried in the refrigerator for 01 weeks. The leaf extract (DKE) was stored at 4^{o}C for further experiments (Figure 1).

Figure 1

Preparation of the DKE Extract

Note. A. The fresh leaves of *Diospyros kaki*; B. the leaf samples after drying; C. The crushed powder; D. The DKE extract
Source. The researcher's data analysis

2.2. Phytochemical screening

The presence of some phytochemical classes was determined via a standard procedure (Harborne, 1998; Tran et al., 2023). Briefly, saponin was detected using foam test, alkaloid was detected via the Bouchardat/Mayer/Dragendorff tests, flavonoid was determined via lead subacetate test, and the phenolic compound was tested by ferric chloride reagent, tannin was detected by gelatin test. Protein was confirmed via biuret reaction, while lipid was detected using the Sudan III test.

2.3. Determination of total polyphenol content and total flavonoid content

The total polyphenol content was examined using the Folin-Ciocalteu reagent method (Nguyen & Tran, 2018; Singleton et al., 1999). The total polyphenol content of the sample was plotted from a standard curve of gallic acid recording at the wavelength of 760nm. The result from the triplicate experiment was expressed as mg of gallic acid equivalents per g extract (mg GAE/g). The total flavonoid content was determined using an aluminum chloride colorimetric assay (Do et al., 2014). The total flavonoid content of the sample was calculated from a standard curve of quercetin recording at the wavelength of 415nm. The result from the triplicate experiment was exhibited as mg of quercetin equivalents per g extract (mg QE/g).

2.4. Determination of anti-oxidant activity of the extract

The anti-oxidant activity of the extract was determined via DPPH free radical scavenging assay and total anti-oxidant capacity. The DPPH free radical scavenging assay was performed using the Nguyen and Tran (2018) procedure with slight modification (Blois, 1958; Nguyen & Tran, 2018). A volume of sample (final concentration of 5 mg/mL) was combined with 0.2mM DPPH and mixed well via vigorous shaking. The sample was left to stand at room temperature in a dark room for half an hour, and a spectrophotometer was used to record the absorbance at 517nm. Ascorbic acid (final concentration of 120 µg/mL) was used as the

positive reference, and the mixture without a sample was used as a control. The percentage of DPPH radical scavenging was calculated using the following formula: $\%\, DPPH\ radical\ scavenging = (1 - \frac{A_S}{A_0}) \times 100$, in which the A_s denotes the absorbance at 517nm of extract or positive reference, while A_0 denotes the absorbance at 517nm of the control (mixture without the extract or positive reference).

The total anti-oxidant capacity of the extract was evaluated using the phosphomolybdate method with complying with Keffous et al. (2016) procedure (Keffous et al., 2016; Prieto et al., 1999). A volume of the reagent solution, which comprised 0.6M H_2SO_4, 28mM Na_3PO_4, and 4mM $(NH_4)_2MoO_4$, was combined with the diluted extract (final concentration of 5 mg/mL). The same volume of reagent and solvent were used as blank. The mixture was heated at 95^{o}C for 01 hour and a half, then cooled down, and the absorbance at the wavelength of 695nm was recorded. The result was calculated by a standard curve of ascorbic acid ranging from 37.5 to 600 µg/mL and expressed as mg of ascorbic acid equivalents per g of extract (mg AAE/g).

2.5. Microorganisms

Six bacterial strains comprised three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus durans,* and *Enterococcus faecium*) and three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Yersinia enterocolitica* KACC 15320, and *Shigella boydii* KACC 10792) were obtained from the microorganism collection of Laboratory of Pharmacology, Faculty of Pharmacy, Ton Duc Thang University, Ho Chi Minh City, Vietnam. All strains were streaked on the tryptic soya agar plates from glycerol stock and reactivated in tryptic soya broth for 16 hours at 37^{o}C with shaking at 200rpm. The cell suspension was used for further experiments such as Kirby Bauer well diffusion assay, Minimum Inhibitory Concentration (MIC) determination, and Minimum Bactericidal Concentration (MBC) of the extract against tested bacterial strains.

2.6. Anti-bacterial activity of the extract

The anti-bacterial effect of the extract was evaluated via well diffusion assay, MIC, and MBC of bacteria with the extract. The sound diffusion assay was performed using the method that Pham et al. (2024) had described (Magaldi et al., 2004). Briefly, the cell suspension was sub-cultured in a ratio of 1:100 under shaking at 200rpm for 04 hours (except for *E. durians* with 07 - 08 hours). The cell suspension was adjusted to 0.5 McFarland standard. Then, the cell suspension was spread on a tryptic soya agar plate using a sterile swab. A volume (20µL) of the diluted extracts (100 and 50 mg/mL of the extract in DMSO) or positive control (gentamicin, 3 mg/mL) was added into the well. The same volume of DMSO was used as the negative control. The susceptibility of bacteria with the extract was determined by measuring the inhibition zone diameter.

Furthermore, the bacterial strains that showed susceptibility to the extract were selected to determine MIC and MBC (Do et al., 2023; Oliveira et al., 2022). Briefly, a range of the diluted extract was mixed with cell suspension (final concentration ranging from 5.0, 2.5, 1.25, 0.625, 0.3125, 0.15625 mg/mL). The cell suspension was cultured in a shaking incubator at 37^{o}C for 24 hours. A part of the cell suspension was mixed with resazurin solution (0.4 mg/mL) in a 96-well plate for 60 minutes to detect MIC. The MIC (expressed as mg/mL) was defined as the lowest concentration of the extract in which the cell suspension did not turn to turbid, and the resazurin-treated well was blue. A part of the cell suspension was spread on tryptic soya agar and incubated at 37^{o}C for 24 hours, and then colonies were counted. The

MBC was the lowest concentration, eliminated 99.9% of the initial bacterial inoculum, and was expressed as mg/mL (Parvekar et al., 2020).

2.7. Statistical analysis

The experiments were conducted in triplicate mode, and the data were exhibited as mean ± standard deviation. The Statgraphics XV software was employed for the statistical analysis. The ANOVA One-way analysis and multiple range test mode were used to analyze the differences among groups, and the statistical significance threshold was set at 0.05.

3. Result and discussion

3.1. Chemical composition screening of the extract

The chemical composition of the extract is presented in Table 1. Some chemical compound classes, such as alkaloids, flavonoids, phenolic compounds, saponins, and tannins, were confirmed. On the contrary, proteins and lipids were not found in the extract (DKE). The presence of some functional and anti-oxidant groups, such as flavonoid and phenolic compounds, suggested further experiments to elucidate some bioactivities of the extract, such as anti-oxidant activity and anti-bacterial effect. According to Bešlo et al. (2023), dietary phenolic compounds are one of the frontline anti-oxidants to protect human and animal cells from oxidative stress damage. Phenolic compounds can act in both extracellular and intracellular modes, from donating hydrogen atoms and electrons to regulating cells' protein/lipid kinase pathway (Tsao, 2010). Furthermore, flavonoids also act as a prominent factor in managing oxidative stress via scavenging and reducing reactive oxygen species (Speisky et al., 2022). Therefore, we tried to investigate the level of phenolic and flavonoid compounds in DKE to establish a strategy for further bioactivity examinations.

Table 1

Phytochemical Screening of the Extract (DKE)

No.	Chemical compounds	DKE
1.	Alkaloids	+
2.	Flavonoid	+
3.	Lipids	-
4.	Phenolic compounds	+
5.	Proteins	-
6.	Saponins	+
7.	Tannins	+

Notes. (+) detect in the extract, (-): not detect in the extract
Source. Data analysis result of the research

3.2. Total polyphenol content, total flavonoid content, anti-oxidant activity of the extract

As shown in Table 2, the extract (DKE) contained rich polyphenols and flavonoids (528.11 ± 12.96 mg GAE/g and 44.49 ± 6.77 mg QE/g, respectively). Compared to the previous

study, the extract had comparable polyphenol and flavonoids of hexane fraction of flavonoid-rich extract isolated from aerial parts of *Thymus kotschyanus* (Baharfar et al., 2015). In the previous study, the hexane fraction extract showed noticeable anti-oxidant and anti-bacterial effects, which implied the DKE extract was a prominent anti-oxidant remedy. To test the anti-oxidant ability of DKE, we compared the free radical scavenging capacity of the extract with the well-known anti-oxidant, ascorbic acid, at the pre-designed concentration (120 µg/mL). The reference drug (ascorbic acid) reduced 92.71 ± 0.79% of DPPH free radicals in the solution, while the DKE extract at the concentration of 5 mg/mL reduced 91.83 ± 1.02% of DPPH free radicals of the reaction. There was no significant difference between the extract's DPPH free radical scavenging ability and reference, which proved the extract was a potent free radical scavenger ($p > 0.05$). Furthermore, the total anti-oxidant capacity of the extract was 110.58 ± 2.65 mg AAE/g extract. Our results of total anti-oxidant capacity were comparable with the other extracts, which implied the extract as a new source for anti-oxidant compounds. For example, Berber et al. (2014) showed that the methanol extract of mixed materials from different parts of *Adenocarpus complicatus* in the previous study was 105.75 mg AAE/g. The intense anti-oxidant activity of the extract may be due to the high abundance of polyphenols and flavonoids in Table 2.

Table 2

Investigation of Anti-Oxidant Activity and Contents of some Anti-oxidants of the Extract

	DPPH scavenging activity (%)	**Total anti-oxidant capacity (mg AAE/g)**
Ascorbic acid (120 µg/ml)	92.71 ± 0.79[a]	
DKE (5 mg/ml)	91.83 ± 1.02[a]	110.58 ± 2.65
	Total polyphenol content (mg GAE/g)	**Total flavonoid content (mg QE/g)**
DKE	528.11 ± 12.96	44.49 ± 6.77

Note. The superscript lower-case letter "a" implied the same in statistical significance analysis ($p > 0.05$)
Source. Data analysis result of the research

3.3. Anti-bacterial effect of the DKE extract

The anti-bacterial effect of the extract was evaluated via the measurement of the diameters of the zone of inhibition of the extract against tested bacteria (Figure 2). The reference drug had a strong inhibitory effect against all tested strains' bacterial growth, with the inhibition zones ranging from 18.67 to 27.33mm. Gentamicin is a broad aminoglycoside antibiotic that explicitly targets protein synthesis inhibition, particularly in Gram-negative bacterial strains; therefore, it is used as the positive control to validate the experiment (Chaves & Tadi, 2023). Among all strains, the extract exhibited anti-bacterial activity against four strains, including Gram-positive (*S. aureus, E. durans,* and *E. faecium*) and Gram-negative bacteria (*Y. enterocolitis*). On the contrary, *S. boydii* and *E. coli* could not be eliminated by the DKE extract (Figure 2).

Figure 2

Inhibition Zones of the Extract against some Gastrointestinal Disease-Related Bacteria

Note. P: Positive control (gentamicin, 3 mg/mL); N: DMSO; 1: 100 mg/mL of the extract; 2: 50 mg/mL of the extract

Source. Data analysis result of the research

Moreover, among tested strains that showed inhibitory growth, the susceptibility with the diluted extracted (50 mg/mL) also varied with the order at *S. aureus and E. durans* > *Y. enterocolitica* > *E. faecium* (Table 3). The data from the diluted extract at the dose of 100 mg/mL also supported these findings, in which *E. durans* was the more susceptible strain with the extract than *Y. enterocolitica* (24.50 ± 0.87 versus 20.33 ± 1.53mm, $p < 0.05$). The most minor zone of inhibition at the DKE (100 mg/mL) was observed in *E. faecium* (17.33 ± 0.76mm). Note that, in some bacterial strains, the diluted extract (50 mg/mL) showed similar efficacy with the reference drug against some bacteria such as *Y. enterocolitica* and *E. durans*, which implied the DKE extract as a prominent anti-bacterial reagent for treating *Y. enterocolitica* and *E. durans.*

Table 3

Investigation of the Anti-Bacterial Effect of the Extract via Well Diffusion Assay

	Gentamicin (3 mg/mL)	**DMSO**	**DKE (100 mg/mL)**	**DKE (50 mg/mL)**
S. aureus	26.67 ± 1.53^{B}	-	24.67 ± 0.58^{Ac}	23.50 ± 0.50^{Ac}
Y. enterocolitica	18.67 ± 1.15^{A}	-	20.33 ± 1.53^{Ab}	18.83 ± 1.04^{Ab}
E. durans	25.83 ± 1.04^{A}	-	24.50 ± 0.87^{Ac}	24.17 ± 0.76^{Ac}
E. faecium	19.17 ± 0.76^{B}	-	17.33 ± 0.76^{Ba}	14.17 ± 1.26^{Aa}
E. coli	27.33 ± 1.15	-	-	-
S. boydii	25.50 ± 0.87	-	-	-

Notes. In the same column, the superscript low-case letters "a, b, c, d" denoted the significant differences among the bacterial strains. In the same row, the superscript capital letters "A, B, C, D" denoted significant differences between the reference drug and the diluted extracts ($p < 0.05$). The symbol (-) meant the inhibitory zone could not be detected

Source. Data analysis result of the research

Table 4

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentration of the Extract against Testing Bacteria

	MIC (mg/mL)	**MBC (mg/mL)**
S. aureus	0.625	2.5
Y. enterocolitica	0.625	2.5
E. durans	1.250	2.5
E. faecium	1.250	5

Source. Data analysis result of the research

The MIC and MBC data aligned with the above data from the well diffusion assay (Table 4). We observed that the MICs of *Y. enterocolitica* and *S. aureus* were 0.625 mg/mL, while that of the others (*E. faecium* and *E. durans*) was 1.250 mg/mL. Furthermore, all ratios of MBC values/MIC values were below or equivalent to 4, which mentioned the extract's mechanism of action as a bactericidal reagent (Mogana et al., 2020).

The anti-microbial activity of species from the *Diospyros* genus has been documented for a long time. The previous study reported the anti-microbial effect of ethanol leaf extract of Diospyros gracilipes against S. aureus, Klebsiella pneumoniae, and Candida albicans. The extracts from *Diospyros malabarica* have MICs ranging from 2 - 5 mg/mL against *Salmonella typhi*, *E. coli*, and *Bacillus subtilis*, which are comparable with our studies (MBCs varying from 2.5 - 5 mg/mL). Recently, Nageswararao and Suneetha (2024) also reported that methanol extract from *Diospyros chloroxylon* leaves could inhibit bacterial growth of 04 human pathogenic microbes, including *Bacillus cereus, S. typhi, E. coli,* and *C. albicans.* The anti-bacterial activity of the compound isolated from *D. kaki leaves,* Kaempferol, against *Streptococcus mutans* has been proven (Yamada et al., 1999). These studies suggest the potential application of leaves of *D. kaki* as an anti-microbial agent. Interestingly, this study found that the *D. kaki* leaf extract inhibited 04 bacterial strains related to gastrointestinal diseases from Gram-positive to Gram-negative bacteria, such as *S. aureus, E. durans, E. faecium, and Y. enterocolitica.* Even *S. aureus* frequently colonizes the skin, throat, and nose, but it can also penetrate the gastrointestinal tract and cause food poisoning and enterocolitis (Kotler & Sordillo, 2010). Moreover, *E. durans* is related to diarrhea and enteritis in animals, and *E. faecium,* which localizes in the gastrointestinal tract and is the cause of urinary tract infection, endocarditis, intraabdominal infections, and neonatal meningitis.

Additionally, *Y. enterocolitica* infection results in various complications in the gastrointestinal tract, from cholangitis to small bowel necrosis (Aziz & Yelamanchili, 2023). This study is the first study investigating the anti-bacterial activity of the *D. kaki* leaf extract against these gastrointestinal bacteria strains. According to Liu et al. (2019), tannin from the young astringent *D. kaki* can control methicillin-resistant *S. aureus*. Moreover, carboxymethylated polysaccharide derived from *D. kaki* benefits human gut microbiota via enhancing *Lactobacillus* sp. - growth and hindering *S. aureus* and *E. coli* growth (Shen et al., 2023). The findings in this study and previous studies shed light on the potential application of *D. kaki* leaf extract in regulating gastrointestinal bacteria.

This study found many bioactive compound classes, such as alkaloids, phenolic compounds, flavonoids, tannins, and saponins. The extract's anti-bacterial activity may be due to its abundant bioactive compounds. For example, tannins of *D. kaki* have been suggested as anti-bacterial reagents, and their anti-microbial effect is related to hydrogen peroxide production (Arakawa et al., 2014). Polyphenol is well known not only for its anti-oxidant activity but also for its anti-bacterial activity (Manso et al., 2022). Flavonoids can also inhibit bacterial growth through the inhibitory effect on nucleic acid synthesis and energy balance, altering the cell membrane function and structure (Shamsudin et al., 2022). The presence of these compounds not only explains the bioactivity of the extract but also suggests the potential extraction of bioactive compounds from this source. These data indicated that DKE extract is a possible remedy for anti-oxidants and anti-bacterial medicines used to treat gastrointestinal diseases.

4. Conclusions

This study reported some bioactive compounds in the D. kaki leaf extract, including saponins, alkaloids, phenolic compounds, flavonoids, and tannins. The extract is rich in polyphenols and flavonoids (528.11 ± 12.96mg GAE/g and 44.49 ± 6.77mg QE/g, respectively) and possesses high free radical scavenging activity (91.83 ± 1.02%) and total oxidant capacity (110.58 ± 2.65mg AAE/g). The anti-bacterial activity of the extract against some gastrointestinal bacteria, such as *S. aureus, E. durans, E. faecium,* and *Y. enterocolitica,* has been reported for the first time with a zone of inhibition ranging from 14.17 - 24.67mm. The extract exerts the bactericidal effect with MIC ranging from 0.625 - 1.250 mg/mL and MBC varying 2.5 - 5.0 mg/mL. This indicates that DKE extract is a promising measure to control gastrointestinal infections and is a potential source for natural product extraction in the pharmaceutical industry.

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