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Original Article

Phytochemical, Antioxidant, Cytotoxic, Antibacterial Activity, and Enzymatic Inhibition Study of *Piper Betle* Leaves

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Abstract

The *Piper betle* plant, known for its pharmacological properties, has been traditionally used in Asian cultures. The objective of the current study is to assess the antioxidant, cytotoxic, antibacterial, and enzymatic inhibitions of leaf extracts of the *P. betle*. DPPH radicals were used to assess the antioxidant potential. The Lethality Assay for Brine Shrimp was used to assess the cytotoxic potential of plant extracts. The disc diffusion method was used to measure the leaves' antibacterial activity against various Gram stains. The inhibitory potential of four enzymes linked to different diseases was screened spectrophotometrically. Chromatographic procedures were used to isolate active substances, and their structures were determined using spectroscopic approaches. The results indicated that the *P. betle* leaves extracts to possess potent antioxidant activity, highest with the EAPB (ethylacetate, *P. betle*) followed by DCPB (dichloromethane, *P. betle)*, HPB (n-hexane, *P. betle*), BPB (n-butanol, *P. betle)* and AQPB (aqueous *P. betle)*. For cytotoxic activity, the EAPB has the most potent cytotoxic activity among the tested extracts. AQBP and HPB showed activity against all bacteria used, while (EAPB and DCPB displayed good activities against all organisms except *Streptococcus aglaciate*. For anti-diabetic activity, DCPB, and EAPB both showed high percent inhibition and low IC_{50}. The identified compound, allylpyrocatechol, isolated from the EAPB fraction of betel leaf attributed higher inhibitory activity than the standard against α-glucosidase with an IC_{50} of 42.61 ± 1.27. For anti-ulcer activity, the DCPB had the highest urease % inhibition and the lowest IC_{50} value, while (BPB) and AQBP fractions had lower levels of inhibition and higher IC_{50} values. DCPB and EAPB exhibited neurodegenerative therapeutic potential by targeting prolyl endopeptidase with good activity having IC_{50} values of 26.82 ± 0.36 and 52.92 ± 21.30 μg/mL). Furthermore, these extracts were tested for their therapeutic potential for skin diseases by targeting the tyrosinase enzyme. Interestingly, HPB and DCPB displayed good inhibitory capability with IC_{50} values of 36.14 ± 0.72, and 44.72 ± 1.28 μg/mL, respectively, compared to the standard kojic acid (IC_{50} = 7.49 ± 0.21 μg/mL). In conclusion, crude extracts of Piper betle leaves exhibit potent antioxidant, antibacterial, and moderately cytotoxic effects. It also has strong anti-ulcer and anti-diabetic properties. Additionally, it exhibits strong anti-pigentation properties and good neurodegenerative potential.

Keywords: *Piper betle*; Timbac; Anti-diabetic; Anti-ulcer; Cytotoxic; Antioxidant; Phytochemical; Pharmacological.

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1. Introduction

Plants have been used by human societies and civilizations since the beginning of time and have been associated with herbal treatment. *Piper betle* is a member of the Piperaceae family. Betle has more than 700 species that are distributed widely around the world and is considered a widely cultivated and popular plant in the Indian subcontinent, South and East Asia, in addition to Eastern Africa and Southern Arabia. *Piper betle*, known as Betle or Sireh, is a climbing vine with glossy, heart-shaped leaves [1]. Betel leaf is a traditional food plant that is both nutritive and therapeutic. The scientific information on betel leaf (Piper betle L) dietary values and health advantages is critical for increasing betel leaf usage in everyday life. Piper betle L is also known as the Neglected Green Gold of India due to its great nutritional and dietary value, which includes fiber, vitamins, and minerals. Betel leaf has nutritional and medicinal benefits, and it is consumed by around 15-20 million people in India on a regular basis. Furthermore, betel leaf contains a variety of phytochemicals and nutritious components. These compounds have a variety of important pharmacological activities, including antibacterial, antidiabetic, antiulcer, anti-inflammatory, anticancer, antimutagenic, and antioxidant activity [2].

The leaves of *P. betle L.* Are used extensively as a masticatory throughout Asia because of their powerful, pungent flavor [3]. There are various qualities attributed to the leaves (digestive, stimulant). The leaves can be used as medicine for pulmonary and catarrhal conditions [4]. The phenolic component allyl pyrocatechol from the leaves has shown efficacy against the halitosis-causing obligate oral anaerobes. In laboratory rats, the leaf extract notably enhances both pancreatic lipase activity [5] and superoxide dismutase activity in a manner that correlates with dosage. [6]. In some countries, they are chewed with slaked lime, and in others, they are used as traditional herbal medicine, especially for antibacterial, antidiabetic, antioxidant and neuroprotective effects [7-15]. Also, the essential oil of *Betel Leaf (P. betle L.)* With high scavenging activity against DPPH radicals has been reported [16]. Moreover, bioactive chemicals extracted from varying ages of betel leaf essential oil can be used in a variety of biological applications, as well as the food and pharmaceutical industries [17]. A study found that the methanol extract of betle leaves inhibits α-glucosidase and has a favorable correlation with total phenolic content [18]. α-Glucosidase is a validated therapeutic target for preventing hyperglycemia-related problems [19]. α-glucosidase inhibitors (AGIs) have gained popularity for managing hyperglycemia. More than 411 compounds exhibiting α-glucosidase inhibitory activity were summarized and isolated from different medicinal plants [20]. The last stage of the digestion of

carbohydrates is catalyzed by α-glucosidase. Enzyme inhibitors reduce the breakdown of carbohydrates into monosaccharides, which slows down the bloodstream's absorption of glucose and, as a result, returns blood glucose levels in diabetes patients to normal. [21]. Thus, the identification of new inhibitors from natural and synthetic sources is essential to developing a therapeutic approach for the treatment of diabetes mellitus [22-24].

The metalloenzyme urease (EC 3.5.1.5), which is dependent on nickel, aid in the hydrolysis of urea to yield ammonia and carbamate [25]. As a soil enzyme, it is present in many types of bacteria, fungus, plants, and invertebrates. It gives *helicobacter pylori* a favorable alkaline environment in which to flourish, resulting in severe infection and ulcers of the stomach and small intestine [26]. Since ammonia is a basic molecule and urease activity tends to produce ammonia as a result, the pH of the surroundings tends to rise [27]. Thus, novel plant-based urease inhibitors are important for managing associated problems. The cytosolic enzyme prolyl endopeptidase (PEP; EC 3.4.21.26) from the human uterus was initially identified [28]. PEP is a member of a distinct family of serine proteases that catalyze the cleavage of internal proline residue at the C-terminus to release proline-containing bioactive peptides [29]. Because PEP breaks down several neuropeptides, including substance-P, TRH, and AVP substrates, it is linked to learning and memory [30]. So, PEP inhibitors could return the altered neural network activity and revert neuropeptide intensities to normal [31, 32]. Thus, it can be utilized for the development of new therapies against diseases such as post-traumatic stress disorder, depression, and schizophrenia.

The multi-copper enzyme tyrosinase is found in many different organisms and is crucial to enzymatic browning and melanogenesis [33]. As a result, its inhibitors may appeal to the food and agriculture sectors as anti-browning agents as well as the cosmetics and pharmaceutical businesses as depigentation agents [34]. This prompted scientists and researchers to concentrate on the discovery, synthesis, isolation, and characterization of novel, highly effective tyrosinase inhibitors for use in the food, cosmetics, and pharmaceutical industries [35]. Due to its crucial part in melanogenesis and browning, numerous studies have been conducted to date on the identification of tyrosinase inhibitors of both synthetic and natural (fungi, bacteria, and plants) sources [36]. Because of its critical role in metagenesis, it has become a popular target for the treatment of skin diseases, including skin melanoma, albinism, age spots, ephelides, melasma, and senile lentigines.

The traditional importance of the above-mentioned plant, the present investigation is being accomplished for the first time on the leaves by conducting in vitro tests including antibacterial, cytotoxic, antioxidant, enzyme inhibitory potentials.

2. Materials and Methods

2.1. Reagents/Chemicals/Instrumentation

Sigma-Aldrich Chemical Company (St. Louis, MO, USA) provided the analytical-grade reagents for the investigation. The organic solvents were distilled and evaporated using a Yamato RE801 rotary evaporator. The BIO-

RAD xMark Microplate Absorbance Spectrophotometer is housed in Hercules, California, USA. Fisher Scientific (Loughborough, UK) supplied all organic solvents. Merck KGaA of Darmstadt, Germany provided Whatman Grade 1 qualitative filter paper, TLC silica gel $60F_{254}$ glass plates (20×20 cm), and aluminum sheets (20×20 cm). Nizwa Hospital provided bacterial strains such as gram-negative *Escherichia coli*, and gram-positive *Staphylococcus aureus, Streptococci agalactiae, Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumonia*.

2.2. Plant collection, identification, and fractionation.

The plant *P. betle* sample was collected from Salalah al Hasila and transferred to the lab for identification **Figure 1**.

Figure 1. *Piper betle* from Al Haseelah, Salalah, Oman.

After proper identification by the plant taxonomist, the leaves were cleaned and dried by putting them between two newspapers in the shade for several days. The dried leaf part of the *P. betle* (195.5 g) was ground and then immersed in methanol for one week. The extract was filtered through the Buchner apparatus and concentrated at 45 ºC using a rotary evaporator. Then the residue was suspended in ethanol: water mixed at a ratio of 1:1 and finally extracted with n-hexane (HPB), dichloromethane (DCPB), ethyl acetate (EAPB), n-butanol (BPB), and aqueous (AQPB) fractions successively (**Table 1**). The solvents were removed in a vacuum. Residues were weighed and transferred into vials for further processing. The % yields of each extract compared to the amount of dried leaf part was calculated using the below formula. The pure compound (allylpyrocathecol) was isolated from the EAPB fraction (0.15 g) of the plant using 10 % ethyl acetate/n-n-hexane solvent system.

% Yield = (weight of the extract/weight of the dry plant) × 100

Table 1. The extraction efficiency of total powdered *P. betle* leaves (195.5 g) using different polarity solvents in % yield.

Name of extract	Mass obtained in g	% Yield
HPB	2.16	1.1
DCPB	7.66	3.9
EAPB	0.36	0.1
BPB	0.52	0.2
AQPB	6.0	3.0

2.3. Antioxidant activity

The antioxidant activity of the extracts was determined using the Brand Williams method with some modifications [37]. Different concentrations for different extracts were prepared in methanol (40, 60, 80, 100, and 200 ug/ml). From each sample, 3 mL was

withdrawn and mixed with 1 mL of a methanol solution containing 0.3 mM DPPH (2,2-diphenyl-1-1-picrylhydrazyl). The reaction mixture was strongly shaken and kept in the dark for 30 minutes at room temperature. Then absorbance was measured at 516 nm against a blank consisting of 1 mL of extract and 3 mL of methanol. The control was prepared in the same way but without adding the extract. The radial scavenging activity of the tested concentrations was estimated as the inhibition percentage by using the following equation.

% Radical Scavenging Activity = (A° - As) × 100 / A° (equation 1)

- A° represents the absorbance of the control sample.
- As represented the absorbance of the test sample.

2.4. Antibacterial activity test

The disc diffusion method described by Weli et al., [37], and Al Senidi et al. [38] was used to test the antibacterial activities of *P. betle* leaf extracts. Serial dilutions (1000, 500, 250, and 125 µg/mL) of dimethyl sulphoxide (DMSO) solutions were prepared from the stock solution. Different concentrations of leaf extract were tested using 6 mm of sterile filter paper, which was incubated with different types of gram-negative and gram-positive bacteria. After one day of incubation, at 37 °C, the inhibition zone was measured in millimeters.

2.5. Cytotoxicity test (Brine Shrimp Lethality Assay)

From each extract residues of *P. betle*, a stock solution containing 20 mg of the plant extract in 10 mL of dimethyl sulfoxide (DMSO) was prepared. Solutions corresponding to 1000, 500, 250, 125, and 62.5 µg/mL were prepared in five separate vials by serial dilution of the stock samples. A total of 10 larvae was transferred into each vial containing 1 mL of each concentration diluted to 5 mL using artificial seawater. The vials were illuminated and maintained at room temperature for 24 h. The number of survivors was counted, and the main % mortalities of the larvae after 24 h were calculated and recorded. The stock solution contains 38 g of sea salt and 1000 mL of DMSO. Experiments were performed in triplicate for each concentration. Lethal Concentration 50% (LC_{50} value) was calculated using Probit Analysis [39]

2.6. Ureas enzyme inhibition assay

The urease assay was carried out according to the previously reported protocol [40]. Using 200 µL of the reaction mixture, 25 µL of urease enzyme from jack bean (*Canavalia ensiformis*), and 5 µL of each extract residue. After a 15-minute incubation at 30 °C, 55 µL of urea (100 mM) was added. In a 96-well plate, phenolic and alkali reagents were used at 45 µL/well and 70 µL/well, respectively. The phenolic reagent has 1% w/v phenol and 0.005% w/v sodium nitroprusside components, and the alkali reagent is a combination of 0.5% w/v NaOH and 0.1% w/v NaOCl.

The Weatherburn method, which considers the release of ammonia during hydrolysis, was used to investigate urease inhibition of new compounds. After 50 minutes, absorbance measurements were taken with a microplate reader (xMarkTM Microplate Spectrophotometer, BIO-RAD) [39]. Each reaction had a final volume of 200 µL and was

conducted in triplicate. Thiourea, a typical urease inhibitor, was used [40].

2.7. Alpha-glucosidase inhibition assay

This study was carried out by using DMSO as a solvent system to dissolve all tested extract residues of *P. betle*. In this assay, tested samples (20 µL/well) of different concentrations, enzyme solution 2 U/2mL, (20 µL/well), and phosphate buffer 50mM, (pH 6.8) (135 µL/well), were employed to 96-well plate followed by incubation at 37 ºC for 15 min. After incubation 25 µL/well substrate 4-nitrophenyl α-D-glucopyranoside was employed and experiments were run for 30 minutes and changes in absorbance were recorded at 400 nm [16].

2.8. Prolyl Endopeptidase Inhibitory Activity

The inhibitory potential of prolyl endopeptidase was tested using a spectrophotometric assay, with some modifications based on Waumans et al. [28]. In a 96-well plate, add 140 µL of sodium phosphate buffer (50 mM, pH 7.0), 20 µL of each residue of *P. betle* extract (0.5 mM in methanol), and 20 µL of prolyl endopeptidase solution (0.02 Unit/well), for a total reaction volume of 200 µL. In the blank, the extract was replaced with 20 µL of methanol. A positive control was provided with 0.5 mM bacitracin. After adding 20 µL of Z-Gly-Pro-4-nitroanilide (0.4 mM in 1,4-dioxane), the absorbance was continuously measured at 410 nm for 30 minutes using a 96-well plate reader (SpectraMax-384, Molecular Devices, CA, USA). Methanol and 1,4-dioxane were combined at a final concentration of 10% v/v in the reaction mixture. All reactions were carried out in triplicate in 96-well Microplates [28].

2.9. Tyrosinase Inhibitory Assay

The tyrosinase inhibition assay was carried out in a 96-well plate using a spectrophotometer microplate reader, following a method adapted from Masamoto et al [43]. Initially, 10 µL/well of the test samples were added into a 96-well microplate, alongside the positive control, "kojic acid." Followed by the addition of 60 µL/well of phosphate buffer and 10 µL/well of mushroom tyrosinase (30 U/mL in phosphate buffer). After an incubation 15-minute period, 20 µL/well of substrate L-DOPA in phosphate buffer was employed, and the changes in the absorbance were monitored at 480 nm (OD480) for 30 minutes. All reactions were performed in triplicate, and the results were reported as the mean.

2.10. Statistical Analysis

The programs were employed to analyze the attained results for biological activity, the SoftMax Pro package and Excel were utilized. The formula equation was used to calculate % inhibition.

$$(\%\ Inhibition) = 100 - \left(\frac{O.D_{test\ compound}}{O.D_{control}}\right) \times 100 \quad (equation\ 2)$$

EZ-FIT (Perrella Scientific, Inc., USA) was used for IC_{50} calculations of all tested samples. Standard Error of Mean values (SEM) are used to report variances in the results. All experiments were conducted in triplicate to overcome the predicted errors.

$$SE = \sigma/\sqrt{n} \qquad (equation\ 3)$$

2.11. NMR data of the compound (allylpyrocathecol)

^{1}H NMR ($CDCl_3$, 600 MHz): δ 3.25 (d, *J* = 6.6 Hz), 2H, ArCH2), 5.04 (m, 2H, olefn), 5.93 (m, 1H, olefn), 6.59 (broad, 1H, Ar-H), 6.68 (broad, 1H, Ar-H), and 6.77 (broad, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 39.5, 115.3, 115.4, 115.7, 120.8, 133.0, 137.7, 141.9, 143.7; HR-ESI-MS (neg.) 149.0273 $[M-H]^+$ (cald. For $C_9H_9O_2$ ¸ 149.0603).

3. Results and Discussion

3.1. Antioxidant activity

The body's free radical reactions are linked to a wide range of illnesses and conditions, including cancer, neurological disorders, cardiovascular disorders, and pulmonary disorders. Antioxidants may be able to help when these anomalies arise [42]. The outcomes of the antioxidant activity of different extracts of *Piper betle* leaf against DPPH radicals are shown in **Figure 2**.

The five crude extracts with different polarities were able to gradually discolor DPPH radicals. The AQPB extract had the lowest level of free radical scavenging activity (67.08% inhibition), whereas the DCPB and EAPB extracts had the highest level (90.08%-95.28% inhibition). All extracts showed good radical inhibition up to 95.28% at 500 and 1000 µg /mL except AQPB. Similar results were obtained by Widawati et al 2010 [45]. These findings also showed that *P. betle* leaf extracts could be utilized as a shield against human bodily harm due to their anti-free radical properties.

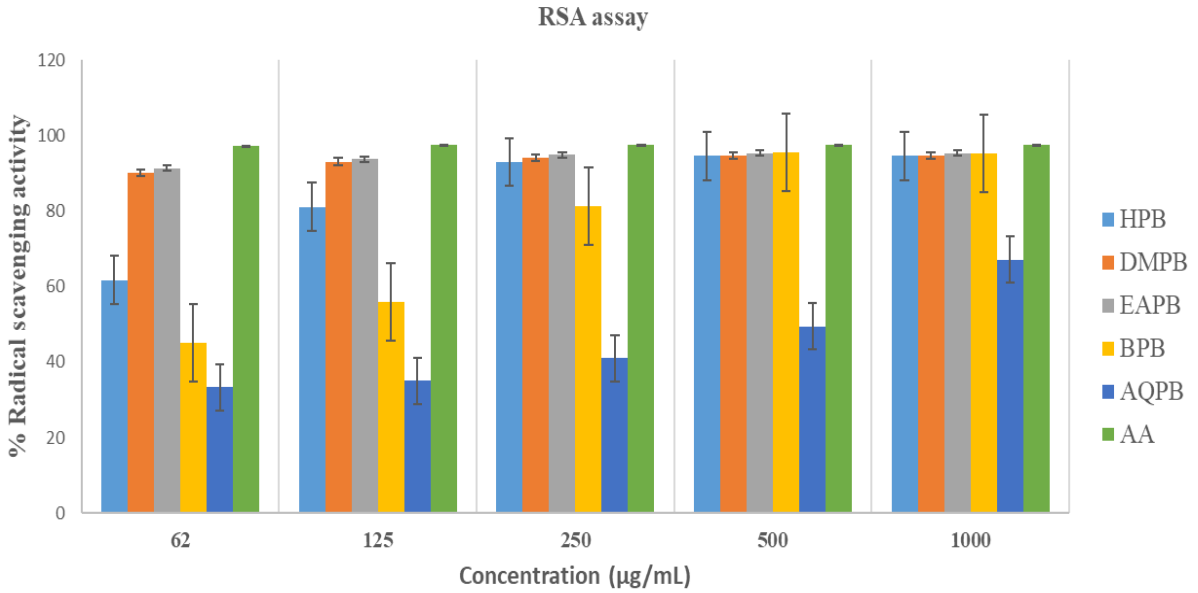

Figure 2. Antioxidant activity of *Piper betel* leaves extracts presented as percent of DPPH radical inhibition.

3.2. Cytotoxic activity

The cytotoxicity (BSLT method) test is a crucial preliminary test for anticancer characteristics. Tests for cytotoxicity on human cancer cells can proceed if the results indicate cytotoxicity. The cytotoxic activity test of the various extracts was carried out using the simple and affordable BSLT technique [41]. The results of cytotoxic activity after 24 and 48 hours are presented in **Table 2**.

Table 2. % mortality of brine shrimp larvae for different polarity leaf extracts of *Piper betle*.

Fractions	Con.	24 h.		48 h.	
		Live	Dead	Live	Dead
HPB	1000	2	8	0	10
	500	3	7	0	10
	250	5	5	2	8
	125	8	2	4	6
	62.5	10	0	8	2
DCPB	1000	2	8	0	10
	500	7	3	6	4
	250	9	1	6	4
	125	10	0	7	3
	62.5	10	0	9	1
EAPB	1000	2	8	0	10
	500	5	5	3	7
	250	9	1	6	4
	125	10	0	8	2
	62.5	10	0	9	1
BPB	1000	9	1	7	3
	500	10	0	10	0
	250	10	0	10	0
	125	10	0	10	0
	62.5	10	0	10	0
AQPB	1000	10	0	8	2
	500	10	0	9	1
	250	10	0	10	0
	125	10	0	10	0
	62.5	10	0	10	0

Con.: Concentration.

Results obtained from the BSL assay showed maximum mortalities at 1000 µg/mL. Only 2 cells were alive after 24 h and 0 cells after 48 h after treatment with HPB, DCPB, and EAPB. It is clear that the concentration of *P. betle* extracts directly correlate with the observed percentage of mortality. The HPB, DCPB, and EAPB exhibited cytotoxic activity with LC_{50} 465.36, 684.15, and 463.9 µg/mL, respectively (**Table 3**). The BPB and AQPB extracts did not show any activity.

In conclusion, the results suggest that the EAPB and HPB extracts of *P. betle* leaves have the most potent cytotoxic activity among the tested extracts (**Table 3)**. This finding supports the use of *piper betle* leaves in traditional medicine for their potential anticancer properties. However, further studies are needed to investigate the mechanism of action and safety of the extracts before they can be used as therapeutic agents.

Table 3. LC_{50} of cytotoxic activity for different extracts of *Piper betle* leaf.

Extract	LC_{50} (µg /mL)	1/ LC_{50}
HPB	465.36	0.002148
DCPB	684.15	0.014616
EAPB	463.9	0.002155
BPB	4960	0.000201

3.3. Antibacterial activity

Based on the data provided in **Table 4**, it appears that the antibacterial activity of the different *Piper betle* fractions varies depending on the concentration and the type of bacteria being tested. The HPB extract demonstrated inhibitory effect against all tested bacterial types at greater concentrations, with the maximum activity found against *K. pneumonia* (8.5mm) and *S. aureus* (8) at 1000 µg/ml. At lower concentrations (500, 250, and 125

µg/ml), activity dropped but remained effective against most bacterial species, except for *P. mirabilis*, which showed no activity at 500 and 250 µg/ml. Overall, the HPB extract showed good suppression against all tested bacterial species at high doses.

DCPB extract demonstrated efficacy against various bacterial strains. At a concentration of 1000 µg/ml, the extract inhibited *P. aeruginosa* by 10mm, *E. coil* of 7 mm, *P. mirabilis* by 6mm, *K. pneumonia* by 10mm (second highest value after aqueous extract), *S. aureus* by 7mm, and *S. agalactiae* by no activity.

The inhibition zone decreased with decreasing concentration, with the lowest concentration of 125 µg/mL having activity of 7 mm against *P. aeruginosa*, 6mm against *E. coli*, no activity against *P. mirabilis*, 8mm against *K. pneumonia*, 6 mm against *S. aureus*, and no inhibition against *S. agalactiae*. Overall, the DCPB extract has moderate antibacterial activity against the examined bacterial gram negative and gram positive strains.

Table 4. Antibacterial activity of different crude extracts of the leaves of *Piper betle* against *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus agalactiae* (diameter of inhibition zones in mm).

Extract	**Concentration (µg\mL)**	***E. coli***	***P. aeruginosa***	***P. mirabilis***	***K. pneumonia***	***S. aureus***	***S. aglactiae***
		Inhibition zone in mm					
HPB	1000	7	7	6	8.5	8	7
	500	7	7	NZ	8	8	7
	250	6	6	NZ	8	7	6
	125	6	6	NZ	7	7	6
Levofloxacin	300	18	25	27	15	14	20
DCPB	1000	7	10	6	10	7	NZ
	500	6	8	NZ	9	7	NZ
	250	6	8	NZ	8	6	NZ
	125	6	7	NZ	8	6	NZ
Levofloxacin	300	24	27	28	15	14	18
EAPB	1000	10	10	6	9	8	NZ
	500	7.5	8	6	8	7	NZ
	250	7	8	NZ	6.5	7	NZ
	125	7	7	NZ	6	6	NZ
Levofloxacin	300	30	26	26	16	17	22
BBP	1000	6	6.5	6	9	NZ	NZ
	500	6	6	NZ	7.5	NZ	NZ
	250	NZ	NZ	NZ	7	NZ	NZ
	125	NZ	NZ	NZ	6	NZ	NZ
Levofloxacin	300	30	27	28	15	14	19
AQPB	1000	7	7	8	11	7.5	8
	500	6	7	7	7	7	8
	250	6	6	6	7	7	7
	125	6	6	6	7	6	6
Levofloxacin	300	27	26	27	13	15	25

NZ – no zone of inhibition

The EAPB fraction showed high antibacterial activity against *P. aeruginosa.* The inhibition was high, with inhibition zone values of 7-10 mm at the tested concentration. The inhibition was moderate only 6mm for *P. mirabilis* at higher concentrations (1000 and 500 µg/mL). The EAPB resulted in moderate inhibition against *S. aureus* 6-8 mm with no effect on *S. agalactiae* at any concentration used (**Table 4**). The BPB gave moderate inhibition against *P. aeruginosa, E. coli and P. mirabilis* with inhibition zone ranging from 6-6.5 mm inhibition, no effect was seen against *S. agalactiae* and *S. aureus*. Potent inhibition was seen against *K. pneumonia,* with about 9 mm of zone inhibition at the concentration of 1000 µg/mL. Finally, the AQPB extract exhibited strong efficacy with an inhibition zone against all tested species. For *P. aeruginosa*, a 7 mm inhibitory zone value at 1000 µg/mL, with a reduction to the fixed value of 6mm for the remaining concentrations. The inhibitory zone value for *E. coli* was 7mm at 1000 µg/mL and 500 µg/mL, but decreased to 6mm at 250 µg/ml and 500 µg/mL concentrations. The greatest inhibition zone was reported for *K.pneumonia* with a value of 11mm at 1000 µg/ml, followed by a 7mm clear zone for the other doses. For *S. agalactiae*, good action and the maximum inhibition activity against these bacteria in comparison with other extracts, with 8mm zone inhibition value at 1000 µg/mL and 500 µg/mL and steady decrease with concentration reduction (7 and 6 mm, respectively). Moderate inhibition zone value of 7.5mm at the concentration of 1000 µg/mL against *S. aureus*, 7 mm clear zone at 500 and 250 µg\mL and 6mm inhibition at 125µg\mL.

3.4. Enzyme inhibition assays

3.4.1. Urease enzyme inhibition

Highly stable and low-toxicity urease inhibitors may be an effective treatment for diseases caused by urease-dependent pathogenic microorganisms. Among the different fractions, the DCPB fraction determined higher inhibition of 94% with IC_{50} values of 17.81 ± 0.74 µg/mL, followed by EAPB (IC_{50} = 48.64 ± 2.16 µg/mL) and HPB (IC_{50} = 83.99 ± 4.29 µg/mL) compared to the standard thiourea (IC_{50}, 8.61 ± 0.31 µg/mL). While three samples (HPB, BPB, and AQPB) exhibited the weak inhibitory capability against urease, with IC_{50} values 83.99 ± 4.29, 190.15 ± 3.16 and 211.36 ± 3.59 μg/mL, respectively. The DCPB fraction could be an excellent source of compounds with good urease inhibitory activity **(Table 5)**.

3.4.2. α-Glucosidase inhibition assay

In the current study, we evaluated five (DCPB, EAPB, HPB, BPB, and AQPB) fractions along with one natural product (allylpyrocatechol) by targeting the α-glucosidase enzyme (**Table 5**). Two fractions (DCPB and EAPB) exhibited potent anti-α-glucosidase activity with IC_{50} values of 9.87 ± 0.56 and 12.20 ± 0.49 μg/mL, as compared to acarbose 377.26 ± 1.17 μg/mL. The remaining three fractions were found inactive. The noteworthy inhibition was observed by the pure compound (allylpyrocatechol, IC_{50} = 42.61 ± 1.27 μg/mL) isolated from ethyl acetate which further strengthened the results.

Table 5. Inhibition concentration of the plant extracts on urease, α-glucosidase, prolyl endo peptidase, and tyrosinase enzymes.

Enzyme	Code	% Inhibition (0.5 mg/mL)	IC_{50} ± µg/mL (SEM)
Urease	HPB	92.54	83.99±4.29
	DCPB	94.39	17.81±0.74
	EAPB	93.87	48.64±2.16
	BPB	87.23	190.15±3.16
	AQPB	81.00	211.36±3.59
	Standard	Thiourea	8.61±0.31
α-Glucosidase	HPB	28	N/A
	DCPB	94.61	9.87±0.56
	EAPB	94.27	12.20±0.49
	BPB	26	N/A
	AQPB	36	N/A
	Allylpyrocatechol	-	42.61±1.27
	Standard	Acarbose	377.26±1.17
Prolyl endo peptidase	HPB	37	N/A
	DCPB	82.90	26.82±0.36
	EAPB	83.5	52.92±21.30
	BPB	42	N/A
	AQPB	41	N/A
	Standard	Z-prolyl-prolinal	0.001±0.0004
Tyrosinase	HPB	78	36.14±0.72
	DCPB	76.90	44.72±1.28
	EAPB	61	93.82±1.30
	BPB	64	86.30±1.62
	AQPB	36	N/A
	Standard	Kojic acid	7.49±0.21

3.4.3. Prolyl endopeptidase activity

Among five fractions, DCPB and EAPB exhibited neurodegenerative therapeutic potential by targeting prolyl endopeptidase with good activity having IC_{50} values of 26.82 ± 0.36 and 52.92 ± 21.30 μg/mL), as compared to Z-prolyl-prolinal (IC_{50} = 0.001 ± 0.0004 μg/mL), while the remaining fractions did not display any inhibition against the selected enzyme **(Table 5)**.

3.4.4. Tyrosinase bioassay

Furthermore, these extracts were tested for their therapeutic potential for skin diseases by targeting the key metabolic enzyme of melanin. Interestingly, HPB and DCMP displayed good inhibitory capability with IC_{50} values of 36.14 ± 0.72, and 44.72 ± 1.28 μg/mL, respectively, compared to the standard kojic acid (IC_{50} = 7.49 ± 0.21 μg/mL).

On the contrary, samples (EAPB and BPB) resulted in weak inhibitory potential against tyrosinase with IC_{50} values of 93.82 ± 1.30 and 86.30 ± 1.62 μg/mL, respectively **(Table 5)**.

3.5. Allylpyrocatechol

The compound (allylpyrocatechol) was obtained as light-yellow oil and sent to the

Natural and Medical Research Center, University of Nizwa for various spectroscopic methods (including MS and NMR). The molecular formula was deduced as $C_9H_{10}O_2$. ^1H NMR and ^{13}C NMR data are in full agreement with the structure already isolated from the same plant [44] (**Figure 3**).

4. Conclusion

In this investigation, the antibacterial, enzymes, cytotoxic, and antioxidant activities of the *piper betle* leaf extracts were studied. Hexane extract showed excellent cytotoxic activity against brine shrimp larvae, while aqueous extract demonstrated high antibacterial activity. For enzymatic inhibition activity, dichloromethane showed potent alpha-glucosidase and urease enzyme inhibition, followed by ethyl acetate. The isolated allylpyrocatechol compound from ethyl acetate extract showed stronger α-glucosidase inhibition activity than the standard acarbose.

For cytotoxic activity, the ethyl acetate extracts were superior in comparison with the other extracts, while antioxidant activity showed potent results. All these results are of significant interest for the initiation of further studies to detect the active compounds that may be found in hexane extract. Additionally, the investigated plant leaf extracts showed significant anti-urease activity, suggesting their potential for inhibiting *H. pylori*-related infections. Thus, this medicinal plant was found to be a potential source for isolating natural anti-H. pylori and urease inhibitory agents.

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Conflict of interest

The authors declare to have no conflict of interest.

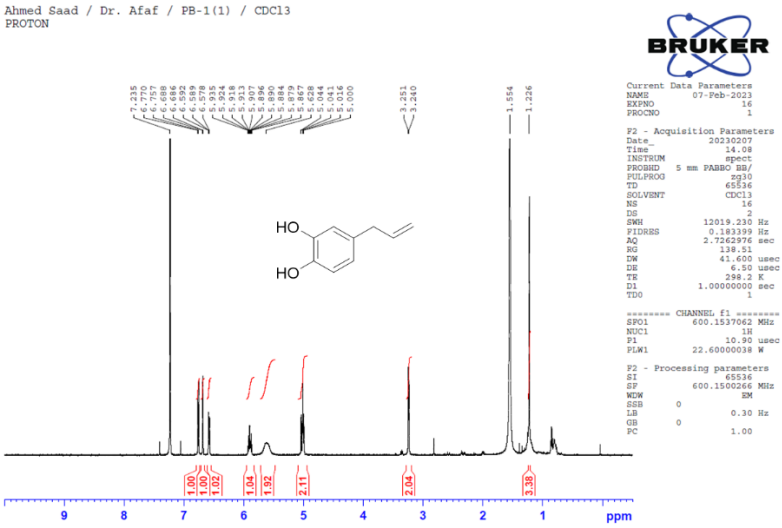

Figure 3. ^1H NMR and structure of allylpyrocathecol.

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