Characterization of antimicrobial, antioxidant, anticancer property and chemical composition o*f Piper betle* L. leaf extract

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ABSTRACT

This study was carried out to characterize antimicrobial, antioxidant and anticancer activities of Piper betle leaf extract as well as its chemical composition. The main objective of the present study is to reveal the potential of P. betle leaf to be used as a medicinal drug. Antimicrobial property of *P. betle* leaf extract revealed by using two fold microdilution method whereas antioxidant activity of the extract was determined with α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging method. The anticancer property of the plant extract was revealed through Colorimetric MTT (tetrazolium) assay against human breast adenocarcinoma (MCF-7). Chemical compounds of the plant extract were screening and identified by using gas chromatography-mass spectrometry (GC-MS). The result of the present study showed that the minimum inhibition concentration (MIC) values of the plant extract against the tested bacterial isolates ranged from 7.81 to 31.25 mg/l in which the plant extract was found inhibited the growth of all the tested bacterial isolates namely A. hydrophila, E. tarda, E. coli, Flavobacterium sp., *Klebsiella sp*., P. aeruginosa, Salmonella sp., V. alginolyticus, V. cholerae and V. parahaemolyticus. The value IC50 of the plant extract against DPPH and MCF-7 cells was 4.41 ± 0.03 ppt and 19.4 ± 0.3 µg/ml, respectively. A total of 25 chemical compounds was successfully identified where Benzoic acid 40.44 % and Phytol 14.52 % were the major compounds. The findings of the present study indicated that the plant extract medicinal values are promising.

تحديدالتركيب الكيميائي و الخاصيّات المضادات للميكروبات و الأكسدة و السرطان لمستخلص أوراق *Piper betle L*

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الكلمات الدالة

مضادات الأكسدة، مضادة السرطان، مضادات الميكروبات، مركب كيميائي، ورقة Piper betle L.

المُستلخص

أجريت هذه الدراسة لتوصيف الأنشطة المضادة للميكروبات ، ومضادات الأكسدة ومضادة السرطان من مستخلصات أوراق بيتل بايبر وكذلك التركيب الكيميائي لها. الهدف الرئيسي من هذه الدراسة هو الكشف عن إمكانية استخدام P. betle leaf كدواء طبي. خاصية مضادات الميكروبات من مستخلص أوراق بيتل P. كشفت عن طريق استخدام طريقتي التخفيف الجزئي أضعاف بينما تم تحديد نشاط مضادات الأكسدة في المستخلص باستخدام طريقة الكسح الجذري α ، α-diphenyl-β-picrylhydrazyl (DPPH) تم الكشف عن الخاصية المضادة للسرطان لمستخلص النبات من خلال فحص اللونية MTB) التترازوليوم) ضد سرطان الثدي البشري (MCF-7). تم فحص وتحديد المركبات الكيميائية للمستخلص النباتي باستخدام القياس الطيفي الكتلي للغاز (GC-MS). أظهرت نتائج هذه الدراسة أن قيم تركيز تثبيط الحد الأدنى لمستخلص النبات مقابل العزلات البكتيرية المختبرة تراوحت ما بين 7.81 إلى 31.25 ملغم / لتر ، حيث وجد أن المستخلصات النباتية تمنع نمو جميع العزلات البكتيرية المختبرة. وهي A. hydrophila و E. tarda و E. coli و Flavobacterium sp. و Klebsiella sp. و P. aeruginosa و Salmonella sp. و V. alginolyticus و V. cholerae و V. parahaemolyticus. بلغت القيمة IC50 للمستخلص النباتي ضد خلايا DPPH و MCF-7 4.41 ± 0.03 جزء لكل تريليون و 19.4 ± 0.3 ميكروغرام / مل ، على التوالي. تم تحديد ما مجموعه 25 مركبة كيميائية ناجحة حيث كان حمض البنزويك 40.44 ٪ و Phytol 14.52 ٪ المركبات الرئيسية. أشارت نتائج هذه الدراسة إلى أن نبات استخراج القيم الطبية واعدة.

Introduction

Piper betle is a tropical plant from the family of Piperaceae. This plant was widely distributed in Southeast Asia countries such as Sri Langka, India, Malaysia, Thailand and Taiwan. It is widely used as masticatory in Asia including Malaysia (Dasgupta and Bratati, 2004) in which *P. betle* leaves and betel quid are added together for chewing (Row & Ho, 2009). *P. betle* is called as sirih in Malaysia, Tanbol in Arabic, Vettilai in Tamil and Burg-e-Tanbol in Persian (Thomas et al., 2013). Punuri et al. (2012) highlighted that this plant pose an important role in world economic and medicinal practices. Traditionally, this plant was used as wet paste for external uses, treatment for headaches, arthritis and joint pain. Recently, a report showed that *P. betle* leaf extract showed antileishmanial activity (Sarkar et al., 2008). Sandeep et al. (2009) claimed that compound hydroxychavicol isolated from *P. betle* leaves by using chloroform possess antimicrobial, antioxidant and anti-inflammatory property. In order to extent the information on the biological activities and chemical compound in the *P. betle* plant extract, the works of the present study are focus on antimicrobial, antioxidant and anticancer activity of *P. betle* as well as its chemical compounds.

Material and methods

Plant material

The plant sample was purchased from herbal nursery located at Pasir Puteh, Kelantan, Malaysia. The fresh plant sample was oven dried at 37 °C for 4 days. Next, the plant sample was freeze dried prior to extraction using 70% methanol and concentrated at 1 g/ml. Finally, the plant extraction was kept in -20 °C until further use.

Bacterial isolates

All bacterial isolates were provided by University of Malaysia Kelantan namely *Aeromonas hydrophila, Escherichia coli, Edwardsiella tarda, Flavobacterium spp., Klebsiella pneumonia, Salmonella typhi, Vibrio alginolyticus, V. parahaemolyticus, V. cholerae and Pseudomonas aeruginosa.* These bacteria were isolated from various aquatic animals and kept in tryptic soy agar (TSA) for further uses.

Minimum inhibitory concentration (MIC) determination

The values of minimum inhibitory concentration (MIC) of *P. betle leaf* extract against bacterial isolates were determined through a two-fold broth micro dilution method (Lee et al., 2011a; Lee and Wendy, 2013). The bacterial isolates were cultured in tryptic soy broth for 24 h at room temperature and the concentration of these cultures were adjusted to 109 CFU mL-1 by using physiological saline. The concentration was cross check with a Biophotometer (Eppendorf, Germany). The bacterial suspensions were then inoculated into a microtiter plate that contained a serial dilution of *P. betle leaf* extract and positive control. The microplate was then incubated at room temperature for 24 h. The MIC values were defined as the lowest concentration of the P. betle leaf extract and positive control (without plant extract) in the wells of the microtiter plate that showed no visible through visually turbidity after 24 h incubation. The experiment was done in triplicate.

Determination of antioxidant activity with α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging method

DPPH radical scavenging method was conducted as described by previous works with some modifications (Blois, 1958; Brand-Williams et al., 1995; Yen and Duh. 1994, Gadow et al., 1997; Lee et al., 2011a). The assay was carried in a 96 wells elisa plate with three replicates. 5 µl of the sample (0.5 mg/ml) solution was added into the well followed by 200 µl DPPH. The absorbance of the sample was recorded by using ELISA reader for every interval 6 s. The percentage inhibition of DPPH radical was calculated based on the absorbance.

Cancer cell lines

The human breast adenocarcinoma (MCF-7) cell line was derived from Institute of Marine Biotechnology, Universiti Malaysia Terengganu. All the cells were grown in standard cell medium (RPMI 1640) supplemented with 5 % fetal bovine serum in a 5 % CO2 atmosphere. The cells was then transferred into microplate at the concentration of 1 X 102 cells per well for cytotoxicity test of the plant extract. At 48 h, proliferation was measured by the MTT colorimetric assay. The experiment was done triplicate. The IC 50 value was calculated from the following formula as described previous works (Lee et al., 2011b)

$$\log_{10}(IC_{50}) = \frac{\log_{10} C_L (I_H - 50) + \log_{10} C_H (50 - I_L)}{I_H - I_L}$$

IC50 = 10log10(IC50)
Where:
I H : I% above 50%
I L : I% below 50%
C H : High drug concentration
C L : Low drug concentration

Colorimetric MTT (tetrazolium) assay

Colorimetric MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, USA) assay was carried out as described by Mosmann (1983). 10 µl of MTT solution (5 mg/ml) was added to all wells of 96 wells micro plate followed by 4 h incubation at 37 oC. Acid isopropanol was added to all wells for dissolving the dark blue crystals. The microplate plate was then read on an ELISA reader at wavelength 570 nm within 1 h after adding isopropanol (Lee et al., 2011c). The experiment was done in triplicate.

Bioactive compound characterization

The chromatographic procedure was carried out using a Shimadzu QP2010-GC-MS with autosampler. The sample was diluted 25 times with acetone and 1 µl of sample was injected into a column. A fused silica capillary column HP5-MS (30 m x 0.32 mm, film thickness 0.25 µm) was used. Helium was the carrier gas, and a split ratio of 1/100 was used. The oven temperature used was maintained at 60 oC for 8 min. The temperature was then gradually raised at a rate of 3 oC per min to 180 oC and maintained at 180 oC for 5 min. The temperature at the injection port was 250 oC. The components of the test solution were identified by comparing the spectra with those of known compounds stored in internal library (Lee et al., 2011e). The experiment was done in triplicate.

Results and discussion

The MIC values of the plant extract against the tested bacterial isolates ranged from 7.81 to 31.25 mg/l in which the plant extract at the concentration of 7.81 mg/l was able to inhibit the growth of *Edwardsiella tard*a, Escherichia coli, Flavobacterium sp., Pseudomonas aeruginosa and Vibrio cholerae whereas at the concentration of 15.63 mg/l of the plant extract was found can control the growth of *Aeromonas* hydrophila, Klebsiella sp., Salmonella sp. and Vibrio alginolyticus. Vibrio parahaemolyticus was failed to grow at the concentration of 31.25 mg/l of the plant extract. The value IC50 of the plant extract against DPPH and MCF-7 cells was 4.41 ± 0.03 ppt and 19.4 ± 0.3 µg/ml, respectively.

A total of 25 chemical compounds was successfully identified namely Benzoic acid 40.44 %, Phytol 14.52 %, 4-Nitrobenzoyl chloride 4.94 %, 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- 4.10 %, n-hexadecanoic acid 2.70 %, Acetic acid 2.31 %, γ - sitosterol 1.76 %, Phenol 1.67 %, 9,12-Octadecadienoic acid, methyl ester, (E,E)- 1.56 %, 4-Chromanol 1.21 %, 8-hexadecyne 1.17 %, Phenol, 2-methoxy-4-(1-propenyl)- 1.11 %, N-Benzyl-2-phenethylamine 1.11 %, 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)- 1.07 %, Hexadecanoic acid, methyl ester 0.96 %, 2-propanone, 1-hydroxy-0.71 %, 2-cyclopenten-1-one, 2-hydroxy- 0.51 %, Eugenol 0.49 %, Borneol 0.24 %, Thiourea, methyl- 0.23 %, Bicyclo [3.1.1] heptanes, 2,6,6-trimethyl, [1R-(1 α, 2 β, 5 α)]- 0.17 %, 2-Ethoxyamphetamine 0.14 %, Pyrrole 0.13Cyclohexanecarboxylic acid, 1-(1,1-dimethylethyl)- 0.12 %, Carbamic acid, methylnitroso-, ethyl ester 0.11 % and another 12 unidentified compounds 16.52 %.

The study of antimicrobial property of P. betle

was quite well established in the literature. For instance, Nalina and Rahim (2007) claimed that aqueous extract of P. betle showed the inhibitory activity against Streptococcus mutans whereas Razak and Rahim (2003) reported that this plant extract was able to inhibit the growth of S. mitis, S. saguis and Actinomyces viscosus. Another study of Shitut et al. (1999) claimed that P. betle leaf extract was able to control the growth human pathogenic bacteria (Vibrio cholerae, Staphylococcus aureus, Diplococcus pneumoniae and Klebsiella aerogenes) effectively. P. betle not only possesses antimicrobial property towards bacteria but also able to inhibit the growth of 124 strains of fungi as claimed by Ali et al. (2010). The essential oil of P. betle was also possesses inhibitory activity against S. aureus, E. coli, Candida albicans and Malassezia pachydermatis (Row and Ho, 2009). Similar finding was also observed in the study of Suppakul et al. (2006) in which they claimed that commercial betel oil in Thailand possesses inhibitory activity against various species of gram negative and positive bacteria as well as yeast. Furthermore, Benzoic acid, Octadecatrienoic acid, hexadecanoic acid and Acetic acid were the chemical compounds that found in the present plant extract indicated that the antimicrobial property of this plant is promising.

Till present, the studies of antioxidant property of P. betle were well documented. For instance, Dasgupta and Bratati (2004) showed the inhibitory activity against DPPH. Similar finding was also observed in the present study. The essential oil of P. betle was also possesses antioxidant activity as claimed in the study of Row and Ho (2009). Similar finding was also observed in the study of Suppakul et al. (2006) in which the commercial betel oil in Thailand was able to inhibit the oxidation of β carotene. Furthermore, several compounds such as Benzoic acid, Phytol, Octadecatrienoic acid, hexadecanoic acid, Acetic acid, sitosterol and Phenol were found in the present study plant extract that may responsible to the antioxidant activity indicated the antioxidant property of P. betle is undoubtedly.

The plant extract in the present study exhibited positive response to breast cancer cell in which was able to inhibit the growth of the tested cancer cell. However, contra finding was observed in the study of Fathilah et al. (2010) where they found the P. betle plant extract failed to inhibit the growth of HeLa cell. On the other hand, this plant showed inhibitory activity towards human nasopharyngeal epidermoid carcinoma (KB) cell. Furthermore, Benzoic acid, Phytol, Octadecatrienoic acid, hexadecanoic acid, Acetic acid, sitosterol and Phenol were existed in the plant extract in which these chemical compounds may responsible to the anticancer activity of P. betle.

Table 1. Minimum inhibition concentration (MIC) of Piper betle L. leaf extract against bacterial isolates

Bacterial isolates	MIC (mg/l)
Aeromonas hydrophila	15.63
Edwardsiella tarda	7.81
Escherichia coli	7.81
Flavobacterium sp.	7.81
Klebsiella sp.	15.63
Pseudomonas aeruginosa	7.81
Salmonella sp.	15.63
Vibrio alginolyticus	15.63
Vibrio cholera	7.81
Vibrio parahaemolyticus	31.25

Table 2. Compound composition of Piper betle L. leaf extract

Compound	Compound Composition (%)
Benzoic acid	40.44
Phytol	14.52
4-Nitrobenzoyl chloride	4.94
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	4.10
n-hexadecanoic acid	2.70
Acetic acid	2.31
γ – sitosterol	1.76
Phenol	1.67
9,12-Octadecadienoic acid, methyl ester, (E,E)-	1.56

4-Chromanol	1.21
8-hexadecyne	1.17
Phenol, 2-methoxy-4-(1-propenyl)-	1.11
N-Benzyl-2-phenethylamine	1.11
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	1.07
Hexadecanoic acid, methyl ester	0.96
2-propanone, 1-hydroxy-	0.71
2-cyclopenten-1-one, 2-hydroxy-	0.51
Eugenol	0.49
Borneol	0.24
Thiourea, methyl-	0.23
Bicyclo [3.1.1] heptanes, 2,6,6-trimethyl, [1R-(1 α, 2 β, 5 α)]-	0.17
2-Ethoxyamphetamine	0.14
Pyrrole	0.13
Cyclohexanecarboxylic acid, 1-(1,1-dimethylethyl)-	0.12
Carbamic acid, methylnitroso-, ethyl ester	0.11
12 unidentified compounds	16.52
Total	100.00

Conclusion

Based on the literature survey and the findings of the present study indicating that P. betle possesses the huge potential to be used as antimicrobial, antioxidant and anticancer agents. However, further study should be carried out in the very near future before this plant come to a commercial sense in the medical world.

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