ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF EXTRACTS OF BETEL LEAVES (*PIPER BETLE*) FROM SOLVENTS WITH DIFFERENT POLARITIES

KY Pin[1,] *, A Luqman Chuah[2], A Abdull Rashih[1], MP Mazura[1], J Fadzureena[1], S Vimala[1] & MA Rasadah[1]

[1]*Forest Research Institute Malaysia, 52109 Kepong, Selangor Darul Ehsan, Malaysia*
[2]*University Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia*

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PIN KY, LUQMAN CHUAH A, ABDULL RASHIH A, MAZURA MP, FADZUREENA J, VIMALA S & RASADAH MA. 2010. Antioxidant and anti-inflammatory activities of extracts of betel leaves (*Piper betle*) from solvents with different polarities. The influence of solvents with different polarities on the antioxidant and anti-inflammatory properties of betel leaf extracts (*Piper betle*) was investigated. The solvents used were water, ethanol, ethyl acetate and hexane. High performance liquid chromatography (HPLC) was used to determine the chemical profiles and concentrations of the active compounds, namely, hydroxychavicol (HC) and eugenol (EU). The antioxidant potential of the extracts was evaluated using two *in vitro* assays—xanthine/xanthine oxidase superoxide scavenging assay (SOD assay) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (DPPH assay). The anti-inflammatory assays used were hyaluronidase (HYA), xanthine oxidase (XOD) and lipoxygenase (LOX) inhibition assays. The HPLC results revealed that HC and EU were detected in all types of extracts and the concentrations were highest in the water extract. The highest extraction yield was obtained using water. All the extracts were highly active in both antioxidant assays with water extract showing the strongest inhibition. The extracts also exhibited significant inhibition in XOD and LOX assays. The results indicated that the bioactivity of the extracts was related to HC and EU.

Keywords: Medicinal plant, bioactivity, high performance liquid chromatography, *in vitro* assay

PIN KY, LUQMAN CHUAH A, ABDULL RASHIH A, MAZURA MP, FADZUREENA J, VIMALA S & RASADAH MA. 2010. Aktiviti antioksida dan antiradang ekstrak daun sirih (*Piper betle*) daripada pelarut pelbagai polariti. Kesan pelarut pelbagai polariti terhadap ciri-ciri antioksida dan antiradang ekstrak daun sirih (*Piper betle*) dikaji. Pelarut yang digunakan ialah air, etanol, etil asetat dan heksana. Kromatografi cecair prestasi tinggi (HPLC) digunakan untuk menentukan profil kimia and kandungan sebatian aktif iaitu hidroksikavikol (HC) dan eugenol (EU). Keupayaan antioksida ekstrak dinilai menggunakan dua asai *in vitro*—asai pencari xantina/xantina oksidase superoksida (asai SOD) dan asai pencari radikal bebas 1,2-difenil-2-pikrilhidrazil (asai DPPH). Asai antiradang yang digunakan ialah asai perencat hialuronidase (HYA), xantina oksidase (XOD) dan lipoksigenase (LOX). Keputusan HPLC menunjukkan yang HC dan EU dikesan dalam semua jenis ekstrak dan kandungan tertinggi terdapat dalam ekstrak air. Hasil pengekstrakan yang tertinggi diperoleh menggunakan air. Semua ekstrak adalah sangat aktif dalam kedua-dua asai antioksida. Ekstrak air memberi perencatan yang paling kuat. Ekstrak-ekstrak juga aktif dalam asai XOD dan LOX. Keputusan menunjukkan yang bioaktiviti ekstrak berkaitan dengan HC dan EU.

INTRODUCTION

Betel (*Piper betle*) belongs to the genus *Piper* of the family Piperaceae. This plant originates from central and eastern Peninsular Malaysia and is locally called sirih (Jaganath & Ng 2000). It is distributed throughout east Africa and the tropical region of Asia. It is a commercial crop that is widely cultivated in many parts of India and Sri Lanka (Guha 2006).

Traditionally, this edible plant is used for medicinal purposes. Among the documented traditional medicinal applications, betel leaf is well-known for its use as masticator or better known as betel quid, which consists of fresh betel leaf, betel nut, slaked lime paste with or without tobacco. Betel quid chewing acts as natural tonic and breath refresher to prevent oral malodour. It

* *E-mail: pin@frim.gov.my*

is estimated that there are 2–2.8 million chewers in Taiwan and 200–600 million chewers in the world (Jeng *et al.* 2001, IARC 2004). It is also used for other purposes including to improve appetite, tonic for brain, antiseptic for wounds and treatment for diarrhoea.

Its promising traditional applications have led to many chemical and biological studies. The extract of betel leaves possesses antimutagenic, anticarcinogenic, antidiabetic, anti-inflammatory and antibacterial bioactivities (Amonkar *et al.* 1986, Padma *et al.* 1989, Arambewela *et al.* 2005, Mazura *et al.* 2007, Nalina & Rahim 2007). Hydroxychavicol (HC) and eugenol (EU) are important phytochemicals found in betel leaves. They are reported to contribute to many bioactivities in betel leaves (Rathee *et al.* 2006, Bhattacharya *et al.* 2007, Mazura *et al.* 2007, Nalina & Rahim 2007). HC and EU are phenolic compounds which consist of a monocyclic aromatic ring with an alcoholic, aldehydic or carboxylic group (de Padua *et al.* 1999). The International Union of Pure and Applied Chemistry (IUPAC) name for HC is 3,4-dihydroxyallylbenzene while that for EU is 3-methoxy-4-hydroxyallylbenzene. Chemical structures of HC and EU are illustrated in Figure 1.

This study was aimed at investigating the antioxidant and anti-inflammatory properties of extracts of betel leaves from solvents with different polarities. The results provided information for selection of solvent for solid–liquid extraction of bioactive compounds from betel leaves.

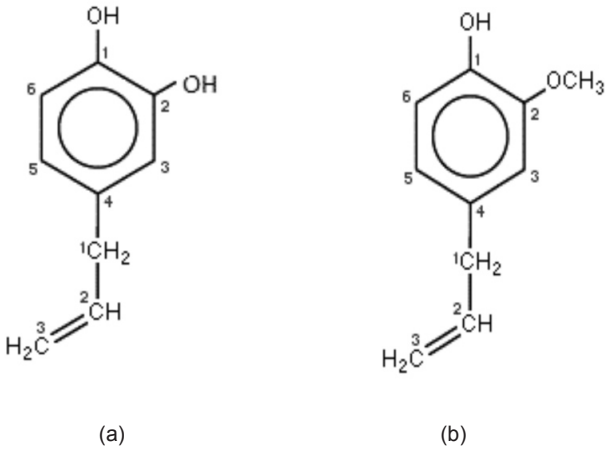

Figure 1 Chemical structures of (a) hydroxychavicol and (b) eugenol

MATERIALS AND METHODS

Materials

Betel leaves were collected and the quality examined using high performance liquid chromatography (HPLC). The voucher specimen of *P. betle* was registered as FRI 45491 and kept at the Forest Research Institute Malaysia.

Extraction process

Four types of solvents were used—water (H_2O), ethanol (EtOH), ethyl acetate (EA) and hexane (Hex). The solvents were selected based on their polarity. H_2O and EtOH are polar solvents while EA and Hex, non-polar. The polarity in increasing order is Hex < EA < EtOH < H_2O. The solvents used were of analytical grade. The extraction was carried out using heating mantle. Round-bottom flask (500 ml) was used in the extraction. The temperature of the extraction was monitored with a thermometer. A low temperature of 50 °C was selected to avoid degradation of phytochemicals. The ratio of solvent to solid solvent and process duration used in the extraction were 30 ml:1 g and 1 hour respectively.

After extraction, the water extract was filtered using vacuum filtration. The filtrates were freeze dried to remove excess solvent. The extracts from EtOH, EA and Hex were recovered by removing the solvents using a rotary evaporator under vacuum. This recovery method is used because the boiling points of the solvents are low. Furthermore, these solvents are not able to freeze and thus freeze drying is not applicable. The evaporation process was conducted at a temperature of 40 °C to minimise possible degradation of the samples. The extraction yield was defined as the percentage of ratio of dried extract recovered to the raw material used, which is given as:

$$\text{Yield} = \frac{W_d}{W_s} \times 100\% \qquad (1)$$

Where W_d and W_s are weights of dried extract and raw material (g) respectively. All experiments were conducted in triplicates.

High performance liquid chromatography

The dried extracts from H_2O (1.0 mg) were dissolved in 1.0 ml of water. The dried extracts

(1.0 mg) from EtOH, EA and Hex were dissolved in 1.0 ml of acetonitrile (CH_3CN). The solutions were then filtered using syringe filter (diameter: 17 mm, porosity: 0.45 mm, polyvinylidene fluoride membrane) before HPLC analysis. The filtrate was injected into HPLC for analysis to determine its chemical profiles and concentrations of HC and EU. The HPLC analysis was carried out using Waters 600E System Controller coupled with a Waters 996 Photodiode Array Detector. A Phenomenex Luna C18 100A column (250 × 4.6 mm, 5 µm particle size) was used as stationary phase. The mobile phase was in gradient mode and consisted of 0.1% orthophosphoric acid (H_3PO_4) and 100% CH_3CN. The analysis was carried out following the procedure in Pin *et al.* (2009).

In vitro antioxidant assays

Xanthine/ xanthine oxidase superoxide scavenging assay (SOD assay)

The assay was performed following the method in Chang *et al.* (1996) with slight modifications. Stock solutions of test samples at a concentration of 50 mg ml^{-1} were prepared by dissolving the extracts in ethanol. The reaction mixture was prepared by dissolving 0.53 g sodium carbonate (Na_2CO_3) (pH 10.2), 4.0 mg ethylene diamine tetraacetic acid (EDTA) and 2.0 mg xanthine in 0.025 mM 4-nitro blue tetrazolium chloride (NBT). NBT solution (100 ml of 4.1 mM^{-1}) was prepared by adding 3.15 g trizma hydrochloride (Tris HCl), 0.1 g magnesium chloride ($MgCl_2$), 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4-nitro blue tetrazolium chloride to 100 ml of distilled water. The mixture was kept refrigerated at 4 °C.

The stock solution in 5 µl was mixed with 995 µl of reaction mixture in a microcuvette. The microcuvette was placed in the cell holder of a spectrophotometer and the reading was set zero. The reaction was then initiated by the addition of 0.1 µl of xanthine oxide (XOD) (1×10^{-3} U ml^{-1}). The absorbance of the resulting mixture was measured at 560 nm for 120 s.

The absorbance of the negative control was obtained by replacing the stock solution with 5 µl of reaction mixture. Superoxide dimutase was used as a positive control in this assay. All experiments were performed in triplicates. The percentage of inhibition is calculated as:

$$\% \text{ Inhibition} = \frac{Ab_C - Ab_S}{Ab_C} \times 100\% \qquad (2)$$

where Ab_C and Ab_S are absorbances of control and test samples respectively.

1,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (DPPH assay)

The assay system evaluates the extracts based on the scavenging activity of the stable DPPH radical according to the method described by Vimala *et al.* (2003). The stock solution of test samples was prepared in methanol (MeOH) at a concentration of 0.5 mg ml^{-1}. The reaction mixture, consisting of 4.0 ml of test solution and 1.0 ml of DPPH (1 mM in methanolic solution), was kept in a 5 ml screw-cap bottle. The mixture was shaken and left to stand at room temperature for 3 min. The absorbance of the resulting mixture was measured at 520 nm using a spectrophotometer. The absorbance of negative and positive controls were obtained by replacing the test solution with MeOH and ascorbic acid (Vitamin C) respectively. All tests were performed in triplicates. The percentage of inhibition is calculated as:

$$\% \text{ Inhibition} = \frac{Ab_{C,-ve} - Ab_S}{Ab_{C,-ve} - Ab_{C,+ve}} \times 100\% \qquad (3)$$

where Ab_S is absorbance of test sample, $Ab_{C,-ve}$ and $Ab_{C,+ve}$ are absorbances of negative and positive controls respectively.

In vitro anti-inflammatory assays

Hyaluronidase inhibition assay (HYA assay)

The assay was performed following the method of Ling *et al.* (2003) with slight modifications. Stock solutions of test samples and apigenin at a concentration of 5 mg ml^{-1} were prepared by dissolving the extracts in dimethylsulphoxide (DMSO). The assay medium, which consisted of 100 µl of hyaluronidase (1.00–1.67 U), 100 µl of sodium phosphate buffer (0.02 M, pH 7.0) with 77 mM sodium chloride and 0.01% bovine serum albumin (BSA), was mixed with 25 µl of test solution and incubated for 10 min at 37 °C. The reaction was then initiated by the addition of 100 µl of the substrate in the form of hyaluronic

acid (0.03% in 300 mM sodium phosphate, pH 5.35) solution and incubated for 45 min at 37 °C. The undigested hyaluronic acid was precipitated with 1 ml acid albumin solution made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, pH 3.75. After standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm using a spectrophotometer. The absorbance in the absence of enzyme was used as the control value for maximum inhibition. Apigenin was used as the positive control in this assay. All experiments were performed in triplicates in 96-well UV microplate. The percentage of inhibition is calculated as:

$$\% \text{ Inhibition} = \frac{Ab_S}{Ab_C} \times 100\% \qquad (4)$$

where Ab_C and Ab_S are absorbances of control and test samples respectively.

Xanthine oxidase inhibition assay (XOD assay)

Xanthine oxidase inhibition activity was determined using spectophotometric method with modifications from the method by Noro *et al.* (1983). Stock solutions of test samples and allopurinol at a concentration of 20 mg ml^{-1} were prepared by dissolving the extracts in DMSO. Potassium phosphate buffer 130 µl (0.05 M, pH 7.5), 10 µl of test solution and 10 µl of xanthine oxidase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by adding 100 µl of the substrate in the form of xanthine solution. The enzymatic conversion of xanthine to uric acid and hydrogen peroxides was measured at absorbance 295 nm using a spectrophotometer. Another reaction mixture (control) was prepared by replacing 10 ml of test solution with 10 ml of DMSO in order to obtain maximum uric acid formation. The performance of the assay was verified using allopurinol as positive control. All tests were performed in triplicates in 96-well UV microplate.

The percentage of inhibition is calculated as:

$$\% \text{ Inhibition} = \frac{Ab_C - Ab_S}{Ab_C} \times 100\% \qquad (5)$$

where Ab_C and Ab_S are absorbances of control and test samples respectively.

Lipoxygenase inhibition assay (LOX assay)

Lipoxygenase inhibition activity was determined using spectophotometric method with modifications from the method reported by Riaz *et al.* (2004). Stock solutions of test samples and nordihydroguaiaretic acid (NDGA) at a concentration of 20 mg ml^{-1} were prepared by dissolving the extracts in DMSO. Sodium phosphate buffer of 160 µl (0.05 M, pH 7.5), 10 µl of test solution and 20 µl of soybean lipoxygenase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 µl of the substrate in the form of sodium linoleic acid solution. The enzymatic conversion of sodium linoleic acid to (9Z, 11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate was measured by monitoring the change of absorbance at 295 nm over a period of 6 min using a spectrophotometer. Another reaction mixture (control) was prepared by replacing 10 ml of test solution with 10 ml of DMSO in order to obtain maximum uric acid formation. NDGA was used as the positive control in this assay. All tests were performed in triplicates in 96-well UV microplate. The percentage of inhibition is calculated as:

$$\% \text{ Inhibition} = \frac{Ab_C - Ab_S}{Ab_C} \times 100\% \qquad (6)$$

where Ab_C and Ab_S are absorbances of control and test samples respectively.

Statistical analysis

The results reported were the averages of at least two measurements. Statistical comparisons were made using one way analysis of variance (ANOVA) with SPSS statistical program (version 14.0). Only variables with a confidence level superior to 95% ($p < 0.05$) were considered as significant.

RESULTS AND DISCUSSION

Extraction yield and phytochemicals content of extract

Figure 2 shows the comparison of yield of extraction for four types of solvents. The order of increasing yield in different solvent extraction systems was Hex < EtOH < EA < H_2O. From the

ANOVA analysis, the extraction yield of H_2O was significant compared with the others. This indicates that the major phytochemicals in betel leaves are mostly high in polarity and soluble in water. Markom *et al.* (2007) reported a similar result in the extraction of *Phyllanthis niruri,* in which the highest yield was obtained from H_2O. This observation implies that polar compounds are easier to be extracted compared with non-polar compounds.

Although ethanol and water contain hydroxyl group which can form hydrogen bonding with the solute, water is more effective in extracting the solute because it has higher polarity and shorter chain. These characteristics improve its capability to extract the polar compounds. This explains the significant difference observed between the extraction yield of H_2O and EtOH. The yield of H_2O was about two times greater than EtOH. Markom *et al.* (2007) also observed about two times difference while Xu and He (2007) reported four times difference between the extraction yields of H_2O and EtOH of other medicinal plants. The difference in yields may be due to other factors such as phytochemicals in plants, extraction temperature and ratio of solvent to solid.

Both targeted compounds, namely, HC and EU, were detected in all the extracts but they differed in amounts (Figure 3). The chemical profile of H_2O extract varied significantly from the other extracts because it contained only two major peaks. HC was the major component in the aqueous extract of betel leaves using gas chromatography mass spectrometry but EU was not detected (Nalina & Rahim 2007).

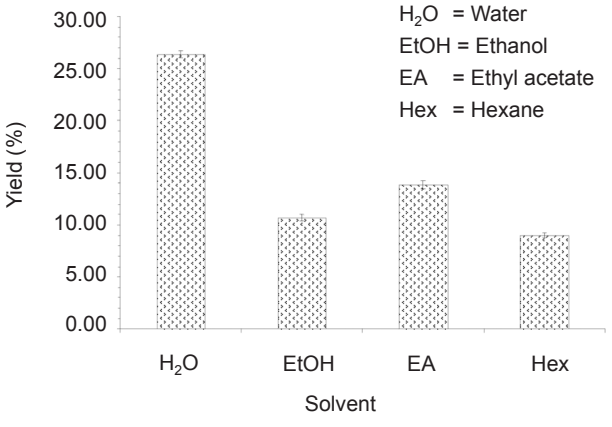

Figure 2 Yield of extraction from different solvents

The absence of EU could be due to the high extraction temperature (100 °C), which resulted in the degradation of EU.

The chromatograms of EtOH, EA and Hex extracts showed six major peaks. The chemical profiles of EtOH and Hex extracts were similar but the peak of HC was smaller in Hex extract. The peaks of HC and EU were smaller in the EA extract. The increase in peaks indicated that EtOH, EA and Hex were capable of extracting more types of phytochemicals from betel leaves compared with H_2O.

It is obvious that HC which has two hydroxyl groups is more soluble in the high polar solvent, i.e. H_2O (Figure 4). The order of increasing concentration of HC in the different solvent extracts was Hex < EA < EtOH < H_2O. The highest concentration of EU was found in Hex extract. EU is a hydrophobic volatile compound and soluble in organic non-polar solvents (Geng *et al.* 2007). In this study, Hex was more effective in extracting EU. The order of increasing concentration of EU in different solvent extracts was H_2O < EA < EtOH < Hex. Comparing the concentration of EU in EA and EtOH, the presence of hydroxyl group in EtOH was advantageous in extracting EU because its hydroxyl group could bind with the hydroxyl group in EU.

Antioxidant activity of betel leaf extracts

All the extracts were active in both SOD and DPPH assays because the percentages of inhibition were greater than 50% (Table 1). Based on the ANOVA analysis, the SOD inhibition activity of H_2O was significantly ($p < 0.05$) higher than the other extracts. The DPPH inhibition activities of H_2O, EtOH and Hex extracts were not significantly different ($p > 0.05$). H_2O extract exhibited the highest inhibition in both assays. The antioxidant property of the phenolic compounds mainly depends on the number and position of the hydroxyl group (Rice-Evans *et al.* 1995). The high percentage of inhibition of H_2O extract could be due to the availability of two hydroxyl groups in HC which is the major component found in H_2O extract. HC was reported to contribute to the antioxidant activity of betel leaf extract (Chang *et al.* 2002, Rathee *et al.* 2006). The presence of EU, that contains single hydroxyl group, in H_2O extract could also give rise to the scavenging activity.

The percentages of inhibition of EtOH and Hex extracts were close because both extracts

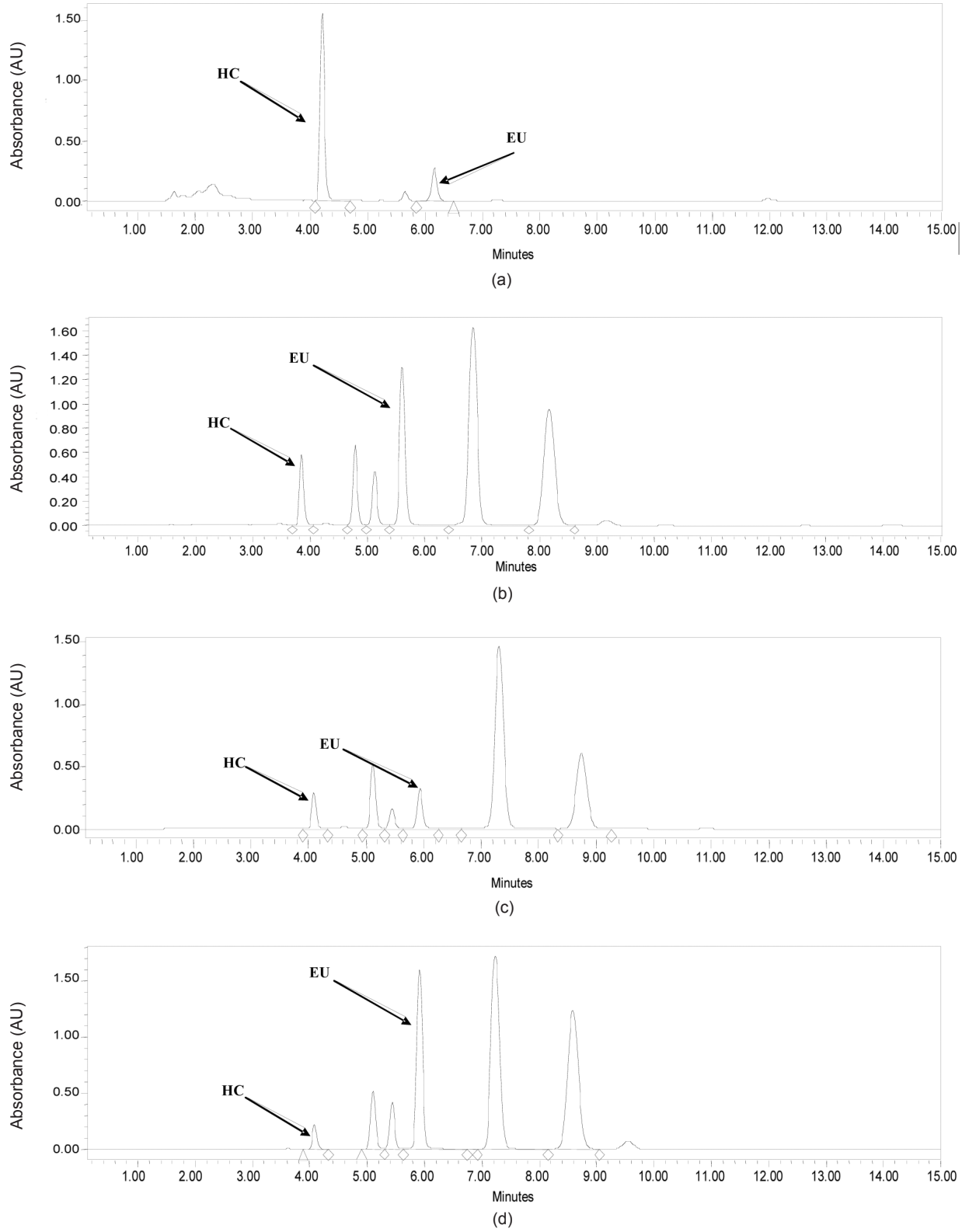

Figure 3 HPLC chromatograms of (a) H_2O, b) EtOH, (c) EA and (d) Hex extracts

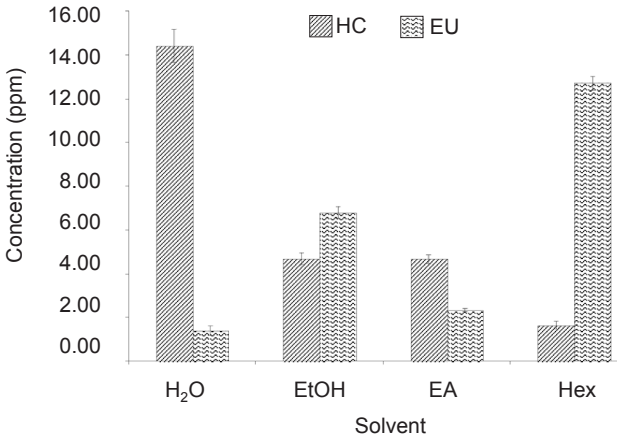

Figure 4 Concentrations of HC and EU in the extracts from different solvents

contained similar amounts of HC and EU. The lowest inhibition was observed in EA extract because the contents of HC and EU which were hydroxyl-group contributors were lowest. This implies that the presence of additional compounds in extracts EtOH, EA and Hex as shown in Figure 3 has no enhancing effect on the antioxidant activity of the extracts.

Anti-inflammatory activity of betel leaf extracts

The extracts were not active in the HYA assay because the percentages of inhibition were below 50% (Table 2). From the XOD assay, all types of extracts exhibited high inhibitory activity as the percentages of inhibition were greater than 70%. The ANOVA analysis revealed that there was no significant difference ($p > 0.05$) between the H_2O, EtOH and Hex extracts. HC and EU were highly active in the inhibition of xanthine oxidase because these two compounds were dominant in H_2O extracts. The EA extracts showed the highest inhibition although it contained lesser amounts of HC and EU. This implies that other phytochemicals present in betel leaves are also active in the assay.

The extracts were highly active in the LOX assay because the percentages of inhibition were greater than 70%. The order of increasing inhibitory activity of the extracts was H_2O < EA < EtOH < Hex. The ANOVA analysis showed that the inhibition of EA, EtOH and Hex extracts

Table 1 Antioxidant activities of solvent extracts

Solvent	% Inhibition	
	SOD assay	DPPH assay
Water (H_2O)	95.77 ± 0.06 a	88.15 ± 1.80 a
Ethanol (EtOH)	81.50 ± 4.70 b	76.87 ± 5.28 ab
Ethyl acetate (EA)	69.97 ± 3.27 b	65.33 ± 1.34 b
Hexane (Hex)	86.47 ± 3.18 c	76.43 ± 2.04 ab

Values within a column followed by the same letter are not significantly different according to Tukey's test.

Table 2 Anti-inflammatory activities of solvent extracts

Solvent	% Inhibition		
	HYA assay	XOD assay	LOX assay
Water (H_2O)	14.37 ± 1.30 a	86.57 ± 1.53 a	77.00 ± 5.95 a
Ethanol (EtOH)	23.47 ± 1.88 b	89.18 ± 1.36 ab	96.83 ± 0.11 b
Ethyl acetate (EA)	13.14 ± 1.04 a	93.57 ± 1.82 b	90.74 ± 0.25 b
Hexane (Hex)	24.23 ± 1.98 b	88.36 ± 0.77 a	99.17 ± 0.09 b

Values followed by the same letter within a column are not significantly different according to Tukey's test.

was not significantly different ($p < 0.05$). The results revealed that the inhibition of lipoxygenase was related to the concentration of EU. The extract of H_2O which contained the least amount of EU exhibited the lowest inhibition activity. EtOH and Hex extracts with relatively higher content of EU showed inhibitory activities which were close to 100%.

CONCLUSIONS

All the extracts were active in the *in vitro* antioxidant assays. Water extract which contained the highest amount of HC showed the highest antioxidant activity. The extracts also exhibited high inhibition in two of the anti-inflammatory assays. Water gave the highest yield among the selected solvents. Judging from the quantitative and qualitative results, water is the most suitable and effective solvent to obtain betel leaf extracts.

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