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Phytochemical Analysis of Red Betel (*Piper crocatum Ruiz & Pav*) Stem Extracts and its Antioxidant and Alpha-Glucosidase Inhibitory Potentials

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ABSTRACT

Red betel (*Piper crocatum Ruiz & Pav*) is commonly used in traditional medicine for therapeutic purposes. Although the active ingredients in red betel leaves have been analyzed, there is still limited information on the phytochemical constituents of the stem. Therefore, this research aimed to determine the compounds in red betel stem and evaluate its antioxidant and alpha-glucosidase inhibitory activity. Red betel stem was extracted by successive maceration with n-hexane, ethyl acetate, and methanol. Preliminary phytochemical screening of the extracts was done following standard procedures. The volatile compounds in the extracts were identified by gas chromatography – mass spectrometry (GC-MS). The total phenolic contents of the extracts was determined by the Folin Ciocalteu's method. The antioxidant activity was evaluated using the cupric ion reducing antioxidant capacity (CUPRAC) assay, while the alpha-glucosidase inhibitory activity was detrmined *in vitro* by spectrophotometric method. Phytochemical screening reveal the presence of alkaloids, phenolics and flavonoids in the ethyl acetate and methanol extracts, while steroidal glycosides were found in the n-hexane and methanol extracts. Caryophyllene, butyraldehyde hydroxytoluene, phenol, 2,4-bis (1,1-dimethyl ethyl), octadecadienal, octadecanoic acid, tricosane, 5-cholestene-3-ol, stigmasterol, and sitosterol were among the compounds identified in red betel stem extracts. The methanol extract had the highest total phenolic contents of 939.01 ± 0.02 mg GAE/g. The extracts exhibited strong antioxidant activity with IC_{50} < 50 mg/L. All the extracts exhibited better alpha-glucosidase inhibitory activity compared to acarbose. These findings have shown red betel stem as a potential source of natural antioxidant and alpha-glucosidase inhibitor.

***Keywords*:** Red betel stem, Antioxidant, Alpha-glucosidase, Gas Chromatography-Mass Spectrometry

Introduction

Diabetes is a non-communicable disease and the leading cause of death, along with heart disease, stroke, respiratory problems, and Alzheimer's disease.[1] In Indonesia, there is a total of 10.7 million diabetic cases, ranking 7th among the top 10 countries with the highest prevalence of the disease.[2] This medical condition occurs due to an increase in blood sugar levels arising from hydrolysis of carbohydrates during the metabolic process. Several factors contribute to the development of diabetes, including reduced sensitivity of insulin receptors due to genetic defects,[3] obesity and lifestyle, nutritional intake, bariatric surgery, drugs, particularly sulphonylurea containing medications, and prandial glucose.[4] Alpha-glucosidase enzyme in the small intestine plays a significant role in the breakdown of carbohydrates into glucose.

Efforts to maintain glucose levels in the blood can be achieved through the administration of oral hypoglycemic drugs or injecting insulin to control the activity of alpha-glucosidase enzyme.

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Although acarbose, voglibose, miglitol,[5] thiazolidinediones,[6] and derivatives of pyrrolidine-2,5-dione[7] have been used as synthetic hypoglycemic medicines, their application negatively affects human health. Recognizing this limitation, herbal medicines are increasingly used as an alternative cure for diseases due to their superior bioavailability, solubility, and reduced toxicity.[8] In herbal medicines, *Piper crocatum Ruiz & Pav*, commonly known as red betel, is a widely used plant. The leaf part is used to treat various diseases such as liver disorders, tumors,[9] cancer,[10] and digestive disorders.[11] Red betel leaf extract contains phenolic compounds like hydroxychavicol and eugenol, which have been shown to have anti-fungal, antioxidant, anti-inflammatory, and anti-cancer activities.[9] Other compounds that have been reported include, essential oils, polyphenolic compounds, flavonoids, tannins,[12] saponins, and carvacrol,[9] which are efficacious for curing various diseases.

Although, red betel leaf has shown strong antioxidant activity with IC_{50} value of 16.15 mg/L in a previous study by Irawan *et al*. (2017),[13] its chemical and pharmacological studies are still very limited. Therefore the need for the chemical and pharmacological investigations of red betel becomes very imperative. Hence, the present study is aimed at analyzing the chemical constituents of red betel stem extracts as well as the evaluation of its antioxidant and alpha-glucosidase inhibitory potentials.

Materials and Methods

Plant Collection and Identification

Red betel plant was obtained from a private cultivation located in the Bogor area, West Java Province, Indonesia on December 2022 with voucher number: 638/IPH.1.02/II.8/V.

Preparation of Extracts

The stem of red betel was removed, washed, and dried at room temperature. The dried stem was grinded into a coarse powder. The powdered stem (50 g) was macerated with 5 L of n-hexane (Merck, Germany) at room temperature for three days. The extract was filtered and the residue was macerated with 5 L of ethyl acetate (Merck, Germany) at room temperature for three days. After filtering the extract, the residue was again macerated with 5 L of methanol (Merck, Germany) at room temperature for three days. Thereafter, it was filtered, and all three filtrates were evaporated using a rotary evaporator (RE 100-Pro, China) at reduced pressure to obtain n-hexane extract, ethyl acetate extract, and methanol extract.

Phytochemical Screening of Red Betel Stem Extracts

Phytochemical analysis was performed on each red betel stem extract to detect the presence of alkaloids, flavonoids, phenols, terpenoids, tannins, saponins, unsaturated steroids, and steroid glycosides following the procedure previously described by Rajkumar *et al*. (2022).[14]

Identification of Volatile Compounds in Red Betel Stem Extracts

The identification of volatile compounds *in* red betel stem extracts was done by GC-MS analysis. The components of the extracts were separated with the Agilent 7890A GC mass spectrometer. The carrier gas was helium, and was maintained at a flow rate of 20 mL/min throughout the column. The column temperature was initiated in an oven (Daihan LabTech, Indonesia) at 60°C for 5 min and slowly raised to 300°C, which was maintained for 41 min. During this process, the ion source operated at 250°C, with an interface temperature of 305°C, and the cut time (MS measurement time) began at 3.75 min. The peaks in chromatograms obtained were identified by comparison with the database of the Wiley Library. Compounds with composition greater than 4% were categorized as dominant, while a conformity level of 90% was classified as certain.

Determination of the Total Phenolic Content of Red Betel Stem Extracts

The total phenolic content of red betel stem extracts was estimated using the Folin-Ciocalteu (FC) (Merck, Germany) method. First, 400 µL of the extract was diluted with distilled water to make 6 mL, and transferred into a 10 mL measuring flask. Subsequently, 1 mL of FC reagent was added and allowed to stand for 3 min, followed by the addition of 2.5 mL of 10% sodium carbonate (Merck, Germany). The solution was mixed thoroughly and incubated at room temperature for one hour in a dark room. The absorbance of the resulting mixture was measured at 650 nm using a UV-Vis spectrophotometer (Specord 200 Plus by Analytik Jena Germany). As a comparison, gallic acid (Merck, Germany) at concentration range of 0-10 mg/L was used as standard.[15]

Evaluation of Antioxidant Activity of Red Betel Stem Extracts

The antioxidant activity of red betel stem extracts was evaluated by the cupric ion reducing antioxidant capacity (cuprac) method. A total of 1 mL of CUPRAC solution was added to each 20 µL, 40 µL, 60 µL, 80 µL, and 100 µL red betel stem extract solution (1000 mg/L in methanol). Subsequently, the mixture was calibrated with methanol and mixed thoroughly. The mixture was and incubated at 37°C for 30 min. The absorbance was measured using a UV-Vis spectrophotometer (Specord 200 Plus by Analytik Jena Germany) at a wavelength of 459 nm. Butylated hydroxytoluene (BHT) (Sigma Aldrich, USA) at concentrations ranging from 0.25 to 0.75 mg/L was used as standard.[15]

Evaluation of Alpha-Glucosidase Inhibitory Activity of Red Betel Stem Extracts

Alpha-glucosidase (AG) inhibitory activity of red betel stem extracts was evaluated according to the method previuosly described by Irawan *et al*. (2021).[15] Briefly, the test sample (red betel stem extract) was prepared in different dilutions (0 – 10 mg/L). To 17 µL of the sample was added para-Nitrophenyl-α-D-glucopyranoside (PNPG) substrate, and incubated at 37°C for 5 min. Thereafter, 17 µL of AG solution was added to the mixture. The absorbance of the mixture was measured at 405 nm using a UV-Vis spectrophotometer (Specord 200 Plus by Analytik Jena, Germany). Acarbose (PT. Dexa Medica, Indonesia) at concentrations of 30, 60, 90, 120, and 150 ppm was used as standard.

Statistical Analysis

Experiments were done in triplicates and results were expressed as Mean ± Standard Deviation (SD). One way analysis of variance (ANOVA) was used to determine differences between means.

Results and Discussion

Phytochemical Constituents of Red Betel Stem Extracts

Phytochemical screening was done to detect the presence of secondary metabolites in red betel stem extracts. The bioactive compounds analyzed included alkaloids, flavonoids, phenols, terpenoids, tannins, saponins, unsaturated steroids, and steroidal glycosides. The results as presented in Table 1 showed that the extraction solvent has an influence on the levels of secondary metabolites. The extraction of secondary metabolites was found to be correlated with the polarity of the solvent. The positive test for alkaloids was predominant with Wagner reagent, showing a yellow to light brown precipitate. Moreover, this precipitate represented the formation of a potassium-alkaloid complex through coordinate covalent bonds between K^+ ions and nitrogen in alkaloids.[16] The results of this test also revealed the polar nature of the alkaloids present in red betel stems.

The formation of a yellow solution in the flavonoid test indicates a reduction in the flavonoid benzopyrone group to produce the orange-colored salt, which served as a marker for the presence of flavonoid in red betel stem extract. On the other hand, the positive test for phenolic compounds is based on the interaction between the phenolic group and $FeCl_3$ to produce a black-green colour. A similar trend was observed in the test results for flavonoids and phenolic compounds for all the extracts. It is important to note that more phenolic molecules were detected as the solvent's polarity increased as there were more phenolic compounds present in the more polar methanol and ethyl acetate extracts than in the less polar n-hane extract of red betel stems.

Compounds Identified from the GC-MS Analysis of Red Betel Stem Extracts

To get more information on the phytochemical constituents of red betel stem, GC-MS analysis was carried out. Agilent 7890A mass spectrometer was used to analyze the volatile compounds in red betel stem extracts. Figures 1, 2, and 3 show the chromatogram of the n-hexane, ethyl acetate, and methanol extracts of red betel stem, respectively. Meanwhile, the constituents in the n-hexane, and ethyl acetate extracts are presented in Tables 2 and 3.

Sesquiterpene, phenolic, aldehyde, fatty acid, alkane, and steroid groups are among the secondary metabolites found in red betel stem extracts (Tables 2 and 3). Chemical group of compounds such as isoprene, alcohol, aldehyde, alkane, and alkanoic acid, found in the n-hexane extract have been shown to have anti-inflammatory, antibacterial, analgesic, antioxidant, anticancer, antifungal, anti-inflammatory, and antidiabetic activities. While, compounds such as sesquiterpenes and phenolic compounds contained in the methanol extract possess antioxidant, antibacterial, anti-inflammatory, and analgesic abilities.

Caryophyllene is a sesquiterpene compound with anti-inflammatory and antibacterial properties[17], it is found in various food seasonings as the natural active ingredient. The anti-inflammatory activity of caryophyllene is related to its inhibitory effect on major inflammatory mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), nitric oxide synthase (iNOS), interleukin-6 (IL-6), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and activated B cell light chain enhancer nuclear factor (NF-κB).[18] Kumawat and Kaur (2020) reported that the combination of caryophyllene with L-arginine increased insulin production and decreased glucose absorption.[19] Caryophyllene has also been used to treat neuropathic pain

in female rats, and has shown promising potential in the management of depressive-like disorders.[20]

The presence of phenolic compounds such as 2,4-bis (1,1-dimethyl ethyl), phenol, and BHT, which are antioxidants with antifungal, anti-inflammatory, antibacterial, and anticancer activities, play a role in the physiological funtion of red betel stems.[21] The effectiveness of 2,4-bis (1,1-dimethyl ethyl) and phenol, as an antifungal is related to its ability to attach to the active site of the enzyme in the mitochondria.[22]

The abundance of sitosterol and stigmasterol in red betel stem extract significantly contributes to its pharmacological action. This compound has antidiabetic, antioxidant, antifungal, anti-inflammatory, anti-osteoarthritis, antiparasitic, and immunomodulatory activities, it also has neuroprotective activity and play a role in the treatment of various types of cancers.[23] The content of octadecadienal compounds in red betel stems contributes to its antioxidants, antimicrobials, and anti-inflammatory activities, as well as its ability to increase sex pheromones. This unique property contributes to their application in pests control and management.[24,25] The work of Nakaziba *et al*. (2022) has shown the antimicrobial potential of a mixture of octadecanoic acid, hexadecenoic acid, and methyl stearate.[26]

Previous studies have shown that the n-hexane extract of red betel contain the compound 5-cholestene-3-ol. Although the specific pharmacological effects of this compound have not been reported, research on derivatives such as 5-cholestene-3β, and 7α-diol has been conducted, and has shown to be a potential drug candidate for Alzheimer's disease.[27] In the present study, a decrease in the number of secondary metabolites with increasing solvent polarity was observed in the GC-MS analysis of red betel stem extracts. This variation could be attributed to the comparatively low solubility of phenolic chemicals in water, although there are hydroxyl groups attached to aromatic groups.[28] Since all of the bioactives were extracted using n-hexane and ethyl acetate solvents due to the sucessive maceration of red betel stem with increasing solvent polarity, GC-MS analysis of the methanol extract of red betel stem did not detect any volatile metabolites. Furthermore, the presence of abundant amounts of active ingredients, specifically phenolic compounds contributes to the action of red betel stem as a potential source of anti-inflammatory, antibacterial, antidiabetic, antioxidant, anti-osteoarthritis, antiparasitic, antifungal, and anticancer agents.

Total Phenolic Content of Red Betel Stem Extracts

The total phenolic content of red betel stem extracts was determined using the Folin-Ciocalteu (FC) reduction assay. In this assay, the formation of a phosphotungstic-phosphomolybdenum complex – a chromophore with deep blue or purple colour is indicative of a positive result for phenolic compounds.[29] The intensity of the colour is dependent on the number of polyphenols in the extract, and this intensity is determined by measuring the absorbance of the coloured complex at 650 nm using a UV-Vis spectrophotometer.

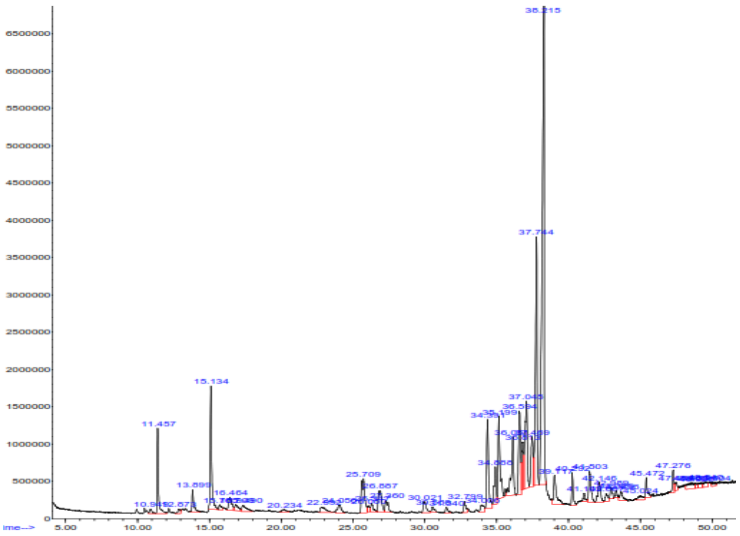

Figure 1: Chromatogram of n-hexane extract of red betel stem

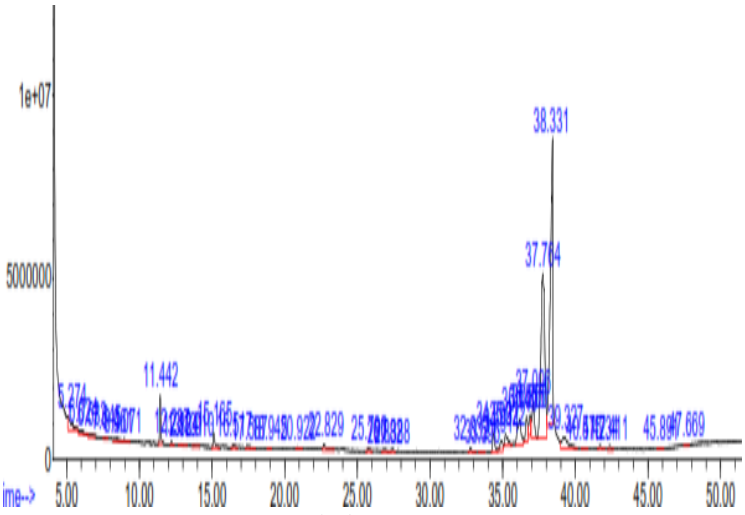

Figure 2: Chromatogram of ethyl acetate extract of red betel stem

Table 1: Phytochemical constituents of red betel stem extracts

No	Phytochemical	Inference		
		n-Hexane Extract	Ethyl Acetate Extract	Methanol Extract
1	Alkaloids	-	-	+
2	Flavonoids	-	+	+
3	Phenolics	-	+	+
4	Terpenoids	-	-	-
5	Tannins	-	-	-
6	Saponins	-	-	-
7	Unsaturated steroids	-	-	-
8	Steroidal glycosides	+	-	+

Gallic acid is a common standard substance used in total phenolic content determination due to its prevalence as an antioxidant compound in plants.[30] The phenolic content is expressed in milligrams of gallic acid equivalent to every gram of sample extract. The linear regression equation for the gallic acid standard curve was y = 0.0996x + 0.0455, with an R^2 value of 0.9829 (Figure 4). The amount of total phenolic compounds determined for the three different extracts were 417.17 ± 0.01 mg GAE/g, 774.35 ± 0.02 mg GAE/g, and 939.01 ± 0.02 mg GAE/g for the n-hexane, ethyl acetate, and methanol extracts of red betel stem. The amount of phenolic compounds in red betel stem varied significantly in different solvent extracts due to variation in the solubility of the phenolic compounds in these solvents as well as the molecular weight of phenolic compounds. Furthermore, the total amount of phenolics obtained was significantly high for each extract. This agrees with the results of the phytochemical screening which showed that the phenolic content improved with increasing solvent polarity. Similarly, from the results of the GC-MS analysis, it is suggested that most of the volatile secondary metabolites have been extracted by n-hexane and ethyl acetate, leaving behind the nonvolatile phenolic compounds which were then extracted with methanol.

Antioxidant Activity of Red Betel Stem Extracts

The antioxidant activity of red betel stem extracts was assessed by the CUPRAC method. This method is based on the ability of an antioxidant compound to reduce Cu^{2+} ions to Cu^{+} ions, as indicated by a colour change from blue to yellow. The IC_{50} value for each solvent extract was obtained from a linear regression equation as illustrated in Figure 5. The IC_{50} values obtained for each solvent extract as well as the standard antioxidant compound are presented in Table 4.

The ability of red betel stem extract to reduce copper (ii) ion increased with increasing polarity of the solvent extract. At 32 mg/L, the methanol extract exhibited the highest copper ion reducing power of 80.69 ± 0.10%, while the n-hexane extract showed the lowest reducing power of 50.10 ± 0.14% at the same concentration. This result correlated with the phenolic contents in the extracts, where the methanol extract had more total phenolic content compared to the ethyl acetate and n-hexane extracts. The IC_{50} values as presented in Table 4 indicated that all three solvent extracts of red betel stem have strong antioxidant activity (IC_{50} < 50 mg/L). Generally, antioxidant properties in plant extract are influenced by the quantity and arrangement of hydroxyl groups in the main tricyclic structure of flavonoid compounds, capable of forming metallic chelates with Fe^{2+} ions and Cu^{2+} ions.[31] Although, the results obtained indicate a strong potential of red betel stem as a natural antioxidant, but the antioxidant capacity is still low compared to the synthetic antioxidant compound butylated hydroxyl toluene (BHT). Various chemical compounds such as alkanes, ketones, esters, aromatics, carboxylic acids, and ketones identified in the n-hexane and ethyl acetate extract of red betel stem may have also contributed to the antioxidant activity of the plant due to their ability to transfer electrons and reduce Cu^{2+} ions.

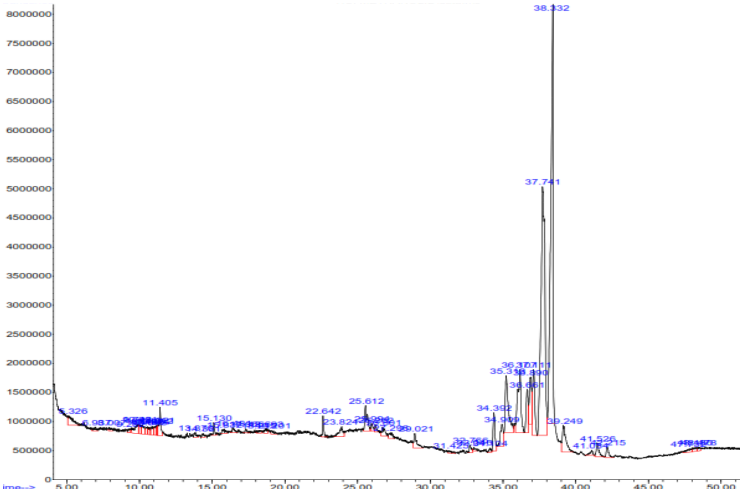

Figure 3: Chromatogram of methanol extract of red betel stem

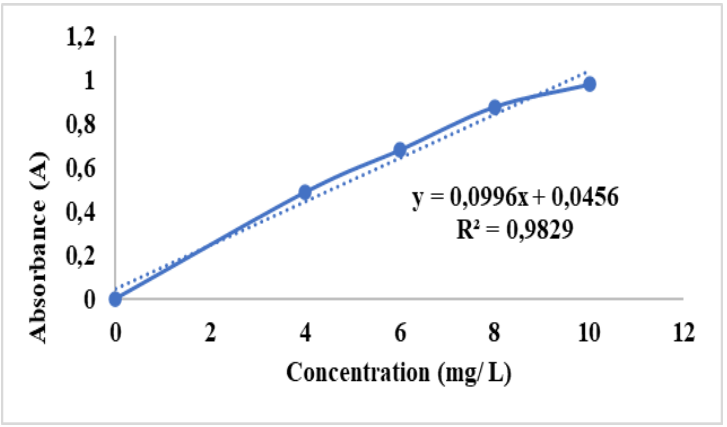

Figure 4: Gallic acid standard curve

Table 2: Compounds identified in the n-hexane extract of red betel stem

S/N	Compound	Retention Time (min)	Area (%)	Similarity (%)	Compound Group	Activity
1	Caryophyllene	11.457	2.88	97	Sesquiterpenes	Anti-inflammatory[17], antibacterial[32], antidiabetic[33]
2	Unknown	13.901	1	96	-	-
3	Phenol, 2,4-bis (1,1-dimethyl ethyl)	15.133	4.52	90	Phenolic	Antioxidant, anticancer, antifungal, antibacterial[21,22]
4	Octadecadienal	25.71	2.74	93	Aldehyde	Pheromone,[34] antioxidant, anti-inflammatory, and antimicrobial[24]
5	Octadecanoic acid	26.886	1.8	97	Fatty acid	Antibacterial, antifungal,[35] antivirus,[26] anti-algae[36]
6	Tricosane	40.294	1.17	96	Alkanes	Antibacterial,[37] antimicrobial[38]
7	5-Cholestene -3-ol	41.112	0.44	94	Alcohol	-
8	Stigmasterol	41.507	1.83	90	Phytosteroks	Anticancer,[39] antioxidants,[40] anti-inflammatory,[41] antidiabetic,[42] antimicrobial[43,23]
9	Sitosterol	42.151	1.37	95	Sterols	Antibacterial,[44] anticancer, anti-inflammatory[45]

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Table 3: Compounds identified in the ethyl acetate extract of red betel stem

No	Compound Name	Retention Time	Area (%)	Similarity (%)	Compound Group	Activity
1	Caryophyllene	11.457	2.88	97	Sesquiterpenes	Anti-inflammatory,[17] antibacterial,[32] antidiabetic[33]
2	Unknown	13.901	1	96	-	-

Table 4: Antioxidant activity of red betel stem extracts and standard (BHT)

Sample	Concentration (mg/L)	Reducing Power (%)	IC_{50} (mg/L)
Methanol extract	8	17.65 ± 1.52	14.80 ± 0.17
	16	62.28 ± 0.16	
	32	80.69 ± 0.10	
Ethyl acetate extract	8	17.65 ± 1.52	17.33 ± 0.15
	16	50.39 ± 0.28	
	32	72.12 ± 0.09	
n-Hexane extract	8	4.91 ± 1.52	31.84 ± 0.11
	16	16.83 ± 1.16	
	32	50.10 ± 0.14	
BHT	0.25	7.01 ± 0.48	0.74 ± 0.01
	0.5	23.87 ± 0.32	
	0.75	52.36 ± 0.64	

BHT = Butylated hydroxy toluene

Alpha-Glucosidase Inhibitory Activity of Red Betel Stem Extracts

Alpha-glucosidase (AG) is an enzyme responsible for hydrolyzing carbohydrates into glucose in the blood. Since a high level of AG can trigger diabetes mellitus, antidiabetic agents are needed to inhibit the function of alpha-glucosidase enzyme. The alpha-glucosidase inhibitory activity of red betel extracts and the standard (acarbose) as a function of concentration is presented in the form of regression analysis (Figure 6). Linear regression equation was used to calculate the IC_{50} value and the results obtained are presented in Table 5. Based on the results, it was discovered that the AG inhibitory activity was proportional to the concentration. The higher the concentration of the extract used, the higher the percentage AG inhibitory activity. All the three extracts of red betel stem (IC_{50} = 5.27 ± 0.01 - 9.76 ± 0.002 mg/L) exhibited a better AG inhibitory activity than acarbose (IC_{50} = 98.67 ± 0.13 mg/L). The presence of anti-diabetic substances such as stigmasterol, L-arginine, caryophyllene, and sitosterol in red betel stem extracts may have significantly contributed to their high alpha-glucosidase inhibitory activity.

Conclusion

The findings from this study have shown that red betel stem contain secondary metabolites in the form of sesquiterpenes, phenolics, aldehydes, fatty acids, and steroids. The total phenolic content varied significantly among the different extracts with the methanol extract having the highest total phenolic content. All three extracts possessed strong antioxidant and alpha-glucosidase inhibitory activity. Furthermore, the extracts exhibited a superior alpha-glucosidase inhibitory activity compared to the standard (acarbose). These observations indicate that red betel stem possess the potential as a source of natural antioxidant and inhibitor of alpha-glucosidase.

Conflict of Interest

The authors declare no conflict of interest.

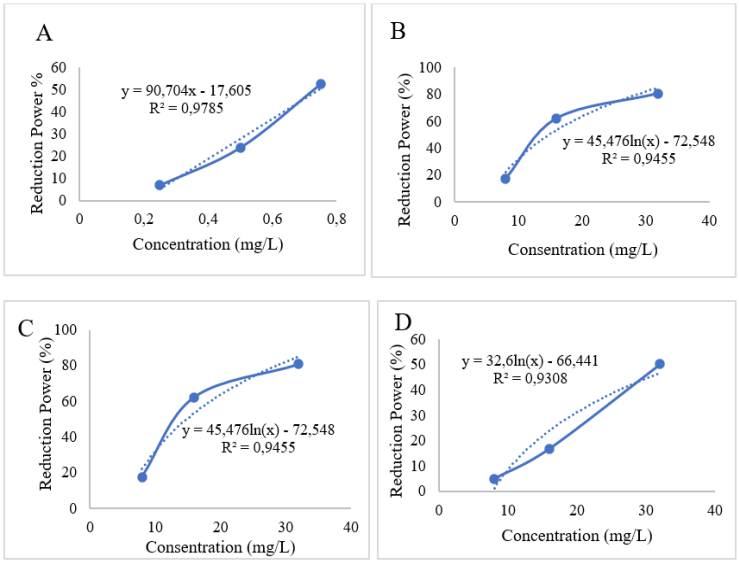

Figure 5: Regression analysis for determination of the IC_{50} value for antioxidant activity. (A): Butylated hydroxy toluene (BHT), (B): Methanol extract, (C): Ethyl acetate extract, and (D): n-Hexane extract of red betel stem

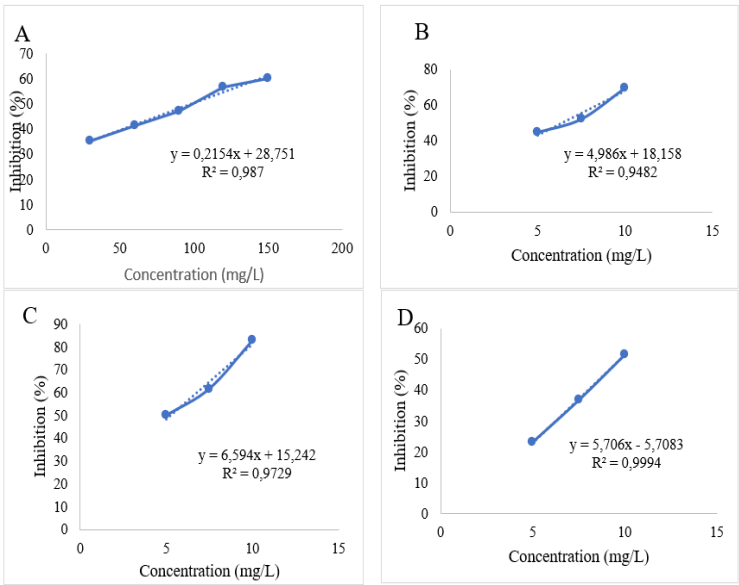

Figure 6: Regression analysis for determination of the IC_{50} value for alpha-glucosidase inhibitory activity. (A): Acarbose, (B): Methanol extract, (C): Ethyl acetate extract, and (D): n-Hexane extract of red betel stem

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 5: Alpha-glucosidase inhibitory activity of red betel stem extracts and standard (Acarbose)

Sample	Concentration (mg/L)	Inhibition (%)	IC_{50} (mg/L)
	5	44.77 ± 0.03	
Methanol Extract	7.5	52.19 ± 0.01	6.39 ± 0.01
	10	69.70 ± 0.03	
	5	49.80 ± 0.04	
Ethyl acatate Extract	7.5	61.52 ± 0.03	5.27 ± 0.01
	10	82.77 ± 0.03	
	5	23.02 ± 0.04	
n-Hexane Extract	7.5	36.69 ± 0.03	9.76 ± 0.002
	10	51.55 ± 0.03	
	30	35.31 ± 0.07	
	60	41.50 ± 0.27	
Acarbose	90	47.23 ± 0.15	98.67 ± 0.13
	120	56.53 ± 0.15	
	150	60.1 1± 0.15	

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