Polygonumins A, a newly isolated compound from the stem of Polygonum minus Huds with potential medicinal activities

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Polygonumins A, a new compound, was isolated from the stem of *Polygonum minus*. Based on NMR results, the compound’s structure is identical to that of vanicoside A, comprising four phenylpropanoid ester units and a sucrose unit. The structure differences were located at C-3″″′. The cytotoxic activity of polygonumins A was evaluated on several cancer cell lines by a cell viability assay using tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The compound showed the highest antiproliferative (p < 0.05) activities against K562 (Human Leukaemia Cell Line), MCF7 (Human breast adenocarcinoma cell line), and HCT116 (Colorectal cancer cells) cells. Cytotoxic studies against V79–4 cells were carried out and showed that polygonumins A was toxic at 50 \(\mu g/ml\), suggesting that this compound may be used as an anticancer drug without affecting normal cells. Polygonumins A also showed promising activity as an HIV-1 protease inhibitor with 56% relative inhibition. Molecular docking results indicated that the compound possesses high binding affinity towards the HIV protease over the low binding free energy range of -10.5 to -11.3 kcal/mol. *P. minus* is used in Malaysian traditional medicine for the treatment of tumour cells. This is the first report on the use of *P. minus* as an HIV-1 protease inhibitor.

The use of traditional herbal medicine is widespread, and plants are sources of many natural antioxidants that might serve as leads for the development of novel drugs. Natural antioxidants and bioactive compounds derived from traditional herbal medicines have received increasing attention for their potential use in treating certain human diseases. For example, traditional herbal medicine has been used widely in cancer patients\(^1\) and to treat neurodegenerative disorders\(^2\).

*Polygonum minus*, also known as *kesum*, is an aromatic plant in the Polygonaceae family, which primarily grows in temperate regions. The plant originates from Southeast Asian countries such as Malaysia, Thailand, Vietnam, and Indonesia, where it is widely used as a spice and flavouring agent in the food industry. *P. minus* is abundant in Malaysia, where it has recently been listed in the National Agro-Food Policy by the Malaysian government to promote its production as a way to boost the agricultural economy. *P. minus* has also been recognized by the Malaysian government in the Herbal Product Blueprint as an essential oil-producing crop\(^3\). *Kesum* oil is a source of natural aliphatic aldehydes and could be commercially produced in North East Victoria and Australia\(^4\). In Japan, China, and Europe, *kesum* has long been used as a hot spice. The sprouts of *kesum* are a traditional vegetable in Japan and are often used in ‘sashimi’\(^5\).

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In addition to its use as a spice in the food industry, *P. minus* has been reported for its use as a herbal medicine. *Kesum* leaf decoctions are traditionally used in indigestion relief, as ingredients in shampoo to remove dandruff, *Kesum* and as postnatal tonics. *P. minus* has been reported to exhibit anti-microbial activity, anti-inflammatory activity and cytotoxic activity against HeLa (human cervical carcinoma) cells and gastric cytoprotective activity. *P. minus* has also been reported to be a source of natural antioxidants for its high total phenolic content and reducing capacity. The pharmacological properties of *P. minus* are based on its chemical constituents. Therefore, numerous studies on the profiling of metabolites have been performed on *P. minus* in the search for its active compounds.

Encouraged by the aforementioned findings, we aimed to isolate novel bioactive compounds from *kesum* in our continued investigations on *kesum* as a medicinal plant. In the present study, we isolated a new polyoxygenated aromatic compound (polygonumins A) from the stem of *kesum*. Polygonumins A demonstrated cytotoxic activities against several cancer cell lines, which indicated that it has potential value in the treatment of cancers such as leukaemia. These data provide baseline information for possible use in controlling cancer diseases, particularly leukaemia. In addition, the present study was undertaken to evaluate the antioxidant and anticholinesterase activities of *P. minus*. We also examined the potential of polygonumins A to serve as an HIV-1 protease inhibitor. Finally, a molecular docking study was performed to support our findings, which could lead to a new drug discovery in the near future.

**Results and Discussion**

**Polygonumins A structure.** Spectroscopy data indicated that polygonumins A has a molecular formula of C_{52}H_{50}O_{21}, as supported by HRESIMS [M + Na]^+ at m/z 1010.2697 (1010.9642 [M + Na]^+ calc.) or [M]^+ at m/z 1010.2697 (1010.9642 [M]^+ calc.). FTIR spectra showed absorption bands at (cm⁻¹) 3334.8 (hydroxyl); 2953.3 (C-H aliphatic); 1704 (C-carbonyl) and 1629.4, 1513.7 (aromatics rings). 1H NMR spectra (acetone-6) δ_H 4.41 (m, 1 H, H-1a); 5.62 (d, 7.8, 1 H, H-3); 4.83 (dd, 13.2, 10.2, 3.6, 1 H, H-4); 4.31 (m, 1 H, H-5); 4.61 (dd, 12.0, 3.0, 1 H, H-6a); 4.47 (d, 3.6, 1 H, H-1); 4.67 (t, 7.8, 1 H, H-2′); 4.47 (m, 1 H, H-3); 4.98 (t, 10.2, 1 H, H-4′); 4.11 (m, 1 H, H-6′a); 4.21 (m, 1 H, H-6′b); 7.55 (d, 8.4, 1 H, H-2′′); 6.94 (d, 8.4, 1 H, H-3′); 6.94 (m, 2 H, H-3′′); 7.78 (d, 15.6, 1 H, H-7′′); 7.72 (d, 15.6, 1 H, H-7′′′); 7.68 (d, 10.2, 1 H, H-7′′′); 7.64 (dd, 13.2, 1.2, 1 H, H-7′′′′); 6.51 (d, 14.4, 1 H, H-8′); 6.48 (d, 13.8, 1 H, H-8′′); 6.43 (d, 12.6, 1 H, H-8′′′); 6.42 (d, 16.2, 1 H, H-8′′′′). 13C NMR spectra (acetone-6, 125 MHz) δ_C (ppm) 65.0 (C-1); 102.7 (C-2); 78.2 (C-3); 72.7 (C-4); 80.4 (C-5); 64.2 (C-6); 89.1 (C-1′); 73.2 (C-2′); 68.6 (C-3′); 71.4 (C-4′); 68.8 (C-5′); 63.1 (C-6′); 126.1 (C-1′′); 130.2 (C-2′′); 115.9 (C-3′′); 159.8 (C-4′′); 115.9 (C-5′′); 130.3 (C-2′′′); 130.2 (C-3′′′); 130.4 (C-2′′′′); 115.8 (C-3′′′′); 115.9 (C-3′′′′′); 116.0 (C-3′′′′′); 159.8 (C-4′′′); 159.9 (C-4′′′′); 160.2 (C-4′′′′′); 169.6 (C-5′′′); 170.1 (C-5′′′′); 20.0 (C-methyl 1) and 20.1 (C-methyl 2).

According to HRESIMS data, polygonumins A has a molecular weight of m/z 1010.2697 and a molecular formula of C_{52}H_{50}O_{21}. FTIR spectra indicated that the compound has various functional groups i.e., hydroxyl absorbance at 3334.8 cm⁻¹, aliphatic carbon absorbance at 2953.3 cm⁻¹, carbonyl absorbance at 1704 cm⁻¹ and aromatic absorbance at 1629.4, 1513.7 cm⁻¹. The results are supported by NMR data (1H and 13C NMR), which showed that the structure of polygonumins A comprises four phenylpropanoid units and a sucrose unit, which could be well distinguished from the structure of vanicoside A. NMR data for vanicoside A and polygonumins A are compared in Table 1 and Fig. 1.

Vanicoside A has a molecular formula of C_{32}H_{38}O_{21} and a molecular weight of 998 mass units. The structural differences between vanicoside A and polygonumins A are located at C-3″″′. Carbon C-3″″′ of vanicoside A binds a methoxyl unit with δ_C 62.2 and δ_H 6.1; δ_C 62.2 and δ_H 6.1 for the substitutents at C-4, C-2″″″ and C-4″″″″. In vanicoside A, the substituents at C-4, C-2″″″ and C-4″″″″ are hydroxyl, ethanoyl and hydroxyl units, respectively. However, the substituents at C-4, C-2″″″ and C-4″″″″ in polygonumins A are ethanoyl, hydroxyl, and ethanoyl units, respectively. In the HMBC spectrum of polygonumins A in particular, the first ethanoyl unit shows long-range 1H-13C correlations between the signal δ_H 4.98 ppm (H-4′) and the carbonyl signal δ_C 170.1 ppm (C-2″″″″) and between the signal δ_H 1.99 ppm (H-1″″″′′) and the carbonyl signal δ_C 170.1 ppm (C-2″″″″′). The second ethanoyl unit of polygonumins A shows long-range 1H-13C correlations between the signal δ_H 4.83 ppm (H-4) and the carbonyl signal δ_C 169.6 ppm (C-2″″″″) and between the signal δ_H 1.99 ppm (H-1″″″′′) and the carbonyl signal δ_C 169.6 ppm (C-2″″″″′) (Fig. 2).

**Biological activities.** It has been recommended that in selecting plant medicines for cancer treatment, ethnopharmacological uses in cancer-relevant diseases such as inflammation, infection, and immune and skin disorders should be taken into account. In the present investigation, polygonumins A, a novel compound isolated from the stem of *P. minus*, was first evaluated for its antiproliferative activities against HCT116, C33A, H1299, MCF7, A549, and K562 cancer cell lines using the MTT assay and then for its activities in radical scavenging and acetylcholinesterase inhibition. The activity towards HIV-1 protease inhibitor was also evaluated, as the newly isolated compound has a structure similar to that of vanicoside A.

The IC_{50} values inhibiting cell proliferation were determined, and the results are summarized in Table 2. The results showed that polygonumins A exhibited cytotoxicity against all tested cell lines except for the A549 cell line. Considering the cutoff point of 4 μg/ml for potential anticancer compounds, values below this set point were obtained for this compound in HCT116 (IC_{50} 3.24 μg/ml), K562 (IC_{50} 2.25 μg/ml) and MCF7 (IC_{50} 2.87 μg/ml), indicating that polygonumins A could be defined as an anticancer compound. Polygonumins A was most effective in inhibiting cell proliferation in HCT116 and MCF7 (IC_{50}, 1.54 and 1.23 μg/ml, respectively) and was less effective in inhibiting cell proliferation in A549 (IC_{50}, 1.76 μg/ml).

In conclusion, polygonumins A is a promising anticancer compound and is a good candidate for developing new drugs for the treatment of cancer.
against K562 (human leukaemia cell line), with an IC\textsubscript{50} of 2.25 µg/ml, lower than that of doxorubicin (2.97 µg/ml), highlighting the anti-leukaemia potential of polygonumins A. It has been well observed that leukaemia is more sensitive to chemotherapy than other malignancies are\textsuperscript{21,22}.

In addition to its sensitivity towards the leukaemia cell line, polygonumins A exhibited cytotoxic activities against human breast cancer and colorectal cancer cells. This finding could well explain why polygonumins A has

| C   | Vanicoside A* | Polygons A | 1H (300 MHz) | 13C (75 MHz) | 1H (600 MHz) | 13C (125 MHz) |
|-----|--------------|------------|--------------|--------------|--------------|--------------|
| 1   | 4.25, 4.6 m  | 63.80      | 4.21 (m, 1 H), 4.41 (m, 1 H) | 65.0         |
| 2   | —            | 102.12     | —            | 102.7        |
| 3   | 5.61 d (8.4) | 77.64      | 5.62 (d, 7.8, 1 H) | 78.2         |
| 4   | 4.65 m       | 72.65      | 4.83 (ddd, 13.2, 10.2, 3.6, 1 H) | 72.7         |
| 5   | 4.3 m        | 79.82      | 4.31 (m, 1 H) | 80.4         |
| 6   | 4.6 m        | 64.55      | 4.61 (dd, 12.0, 3.0, 1 H), 4.47 (m, 1 H) | 64.2         |
| 1'  | 5.66 d (3.6) | 89.18      | 5.77 (d, 3.6, 1 H) | 89.1         |
| 2'  | 4.69 m       | 73.01      | 4.67 (t, 7.8, 1 H) | 73.2         |
| 3'  | 3.9 m        | 70.79      | 4.47 (m, 1 H) | 68.6         |
| 4'  | 3.51 dd (9.4, 9.4) | 70.66 | 4.98 (t, 10.2, 1 H) | 71.4         |
| 5'  | 4.3 m        | 70.79      | 4.11 (br t, 9.6, 1 H) | 68.8         |
| 6'  | 4.3, 4.35 m  | 64.49      | 4.41 (m, 1 H), 4.21 (m, 1 H) | 63.1         |
| 1″, 1‴ | —           | 125.69     | —            | 126.1        |
| 2″  | 7.33 d (1.8) | 110.08     | 7.55 (d, 8.4, 1 H) | 130.21       |
| 3″  | —            | 148.97*    | 6.94 (d, 8.4, 1 H) | 115.9        |
| 4″  | —            | 147.62*    | —            | 159.81       |
| 5″  | 6.81         | 114.94     | 6.94 (d, 8.4, 1 H) | 115.9        |
| 6″  | 7.11 d (1.8, 8.2) | 123.24 | 7.55 (d, 8.4, 1 H) | 130.21       |
| 7″, 7‴ | 7.58, 7.62, 7.64, 7.72 ca d (16) | 144.92 | 7.72 (d, 15.6, 1 H) | 146.1        |
| 8″, 8‴ | 6.33, 6.40, 6.45, 6.53 ca d (16) | 145.91 | 7.68 (d, 10.2, 1 H) | 145.2        |
| 9″, 9‴ | —           | 165.93     | —            | 166.0        |
| 10″−10‴ | —          | 165.96     | —            | 166.04       |
| 11″−11‴ | —          | 166.46     | —            | 166.5        |
| 12″−12‴ | —          | 166.64     | —            | 166.6        |
| 13″−13‴ | —          | 159.53     | —            | 159.84       |
| 14″−14‴ | —          | 159.63     | —            | 159.9        |
| 15″−15‴ | —          | 159.81     | —            | 160.2        |
| 16″−16‴ (C−O) | —        | 170.12 (1) | —            | 170.1 (1)    |
| 17″ | 2.06 s       | 20.03 (1)  | 1.99 (s, 3 H) | 20.0 (1)     |
| 18″ | 3.85 s       | 20.25 (1)  | 1.99 (s, 3 H) | 169.6 (2)    |
| 19″ | 7.33 d (1.8, OH phenolic) | 55.25 | 7.83, 7.64 (4 OH phenolic) | 20.1 (1)     |

Table 1. Comparison of NMR data (1H and 13C) between vanicoside A and polygonumins A.
been widely used to treat digestive disorders. It is believed that the sugar moiety, a sucrose unit, in its structure plays an important role in determining its pharmacological and biological activities. Structure and activity relationship (SAR) analysis of several related compounds containing sugar moieties, such as CCL-34 and its natural analogues, revealed that the sugar moiety was essential to their anticancer activity. In particular, the sugar moiety was recognized to be critical to the topoisomerase inhibition activity of anthracyclines as antitumour drugs. It has also been suggested that the sugar structure in daunorubicin plays a critical role in determining its anticancer activity. To date, there is no report on the cytotoxicity activity of vanicoside A, except in an MCF cell line. However, vanicoside A has been classified as a member of the family of protein kinase C inhibitors, which exhibit antitumour effects.

The equivalence between the therapeutic effect on cancer cell lines and the toxicological effect on target human organs is an important criterion of the applicability of an anticancer compound. Therefore, we conducted toxicological studies on the V79–4 cell line (lung fibroblast cell line derived from Chinese hamsters) to determine the safe concentration range of polygonumins A. This test has been widely used to evaluate general cytotoxicity and target organ toxicity. In some cases, this test may also provide information about lethal dose in vivo. The results indicated a loss of viability of V79–4 fibroblasts at concentrations above 50 µg/ml following approximately 24 hours of exposure. Thus, the compound is approximately 10 times as toxic towards cancer cells and normal cells at these concentrations, as it is at its therapeutic concentration.

Interestingly, vanicoside A also showed significant β-glucosidase inhibitory activity, indicating promising therapeutic potential in the treatment of metastatic cancer and human immunodeficiency virus infection (HIV). As polygonumins A possesses a structure similar to that of vanicoside A, we performed an HIV-1...
protease inhibition test against the newly isolated compound. Based on our results (Table 3), polygonumins A showed potential activity as an HIV-1 protease inhibitor. A relative inhibition level of 56% towards HIV-1 protease was detected compared with pepstatin A as a positive control. This is the first report indicating that Polygonum spp have been reported to possess anti-HIV properties, with compounds such as flavonoid glycoside, quercetin and phenolics playing an essential role in anti-HIV activity. Based on the results of this study, we believe that the phenyl propanoid glycoside moiety in the structure of polygonumins A is associated with the activation of anti-HIV protease activity. Moreover, previous study have shown that the phenylpropanoid glycoside group acts as an inhibitor of HIV-1 integrase activity, thus supporting our findings.

Several techniques have been used to determine antioxidant capacity. One of the methods we used is based on scavenging activity, in which we measured the ability of polygonumins A to donate electrons to a free radical to scavenge potential damage. The antioxidant activity determined by the DPPH free radical-scavenging method
(Table 2) was not consistent with our expectations. The measured IC$_{50}$ value of 812 µg/ml indicated that polygonumins A is far less potent than gallic acid and ascorbic acid. It has been reported that ethanolic, methanolic, and aqueous extracts of P. minus exhibit antioxidant activity as remarkable as that of gallic acid and ascorbic acid$^{13,32}$. 

Figure 3. Hydrogen bond interaction of polygonumins A (a), vanicoside A (b) and pepstatin (c) with HIV-1 protease at respective lower binding energy.
This activity is due to the phenylpropane group of *P. minus*. The four units of the phenylpropanoid group in the structure have been shown to produce lower scavenging activity. However, the high antioxidant activity of *P. minus* extracts has been attributed to high contents of polyphenolic compounds. Therefore, we measured the total phenolic content of polygonumins A by the Folin-Ciocalteu method. Phenols consist of hydroxyl groups that are able to destroy free radicals to form stable phenoxyl radicals. Although the results of the DPPH assay showed a weak scavenging ability, the total phenolic content of this compound is relatively high at 124.0625 ± 0.88 mg GAE/g when compared with that of crude extract. In our previous studies, we found that the total phenolic content in crude *P. minus* ethanolic extract produced the highest scavenging activity, ranging from 100 to 140 mg GAE/g. Perhaps the antioxidant activity of this compound is not due to DPPH scavenging activity alone. Because antioxidant activity can be realized by multiple mechanisms or a single mechanism, we analysed the reducing power capacity of polygonumins A. This assay determines a substance’s ability to reduce Fe$^{3+}$ to Fe$^{2+}$. The presence of antioxidants in the extracts result in the reduction of the ferric cyanide complex (Fe$^{3+}$) to the ferrous cyanide form (Fe$^{2+}$). We found that polygonumins A may act as an electron donor (a hydrogen electron donor), supporting its antioxidant activity, as the compound showed a comparatively high reducing capacity, with an EC$_{50}$ of 89.3 µg/ml, near the value of its reference compound gallic acid (Table 2). The reducing power of this compound increased rapidly with its concentration. Based on this antioxidant activity, the compound’s high total phenolic content was correlated with its reducing power. Hence, it can be inferred that polygonumins A could act as a strong antioxidant agent.

It is well documented that the use of antioxidants may minimize neuronal degeneration and slow the progress of Alzheimer’s disease. Anticholinesterase activity in compounds has also been reported to be related to radical-scavenging activity. Moreover, based on our previous findings on crude extracts of polygonumins A, the compound showed promising anticholinesterase activity. Therefore, we evaluated the anticholinesterase activity of polygonumins A relative to that of tacrine. However, the compound showed only weak anticholinesterase activity at concentrations of up to 2 mg/ml (Table 2), and the compound did not show potential to serve as an anticholinesterase drug. The IC$_{50}$ for this compound could not be determined due to its low activity; but we managed to determine the IC$_{20}$ value for this compound (1980 µg/ml).

| Residue | Position |
|---------|----------|
| Polygonumins A | ASP | 29 |
| | GLY | 27 |
| Vanicoside A | ASP | 30 |
| | LYS | 45 |
| | ILE | 50 |
| | ILE | 150 |
| | THR | 180 |
| Pepstatin | ASP | 29 |
| | ASP | 129 |
| | GLY | 48 |
| | GLY | 127 |
| | GLY | 27 |

Table 5. Residues of HIV-1 protease involved in hydrogen bond interactions.

Figure 4. Superimposition of the structures revealed polygonumins A (red), vanicoside A (blue), and pepstatin (green) were docked in a similar binding domain.
| Protease Mutant (PDB ID) | Affinity (kcal/mol) | Residues involved in hydrogen bond interaction with ligand : hydrogen bond distance |
|--------------------------|---------------------|----------------------------------------------------------------------------------|
| 3KT5                     | −7.3                | ASP 1029: 3.038, LYS 1045: 2.937, ASP 1036: 3.459                                |
|                          | −8.5                | GLY 1027: 3.315, GLY 48: 3.244                                                  |
| 3NU4                     | −8.3                | ASP 29: 3.346, LYS 45: 2.912, LYS 45: 3.176                                   |
|                          | −8.6                | ASP 29: 2.761, LYS 45: 2.912, ASP 29: 3.463                                    |
| 3NU5                     | −9.1                | LYS 145: 3.386, LYS 145: 2.800, GLY 148: 3.285                              |
|                          | −8.3                | GLU 21: 3.345, VAL 50: 3.225, GLY 150: 3.171                               |
| 3NU6                     | −8.3                | LYS 45: 3.126, ARG 87: 3.111, ARG 87: 3.039                               |
|                          | −8.3                | ARG 8: 2.982, ARG 8: 3.257, ARG 8: 3.335                                |
|                          | −8.3                | ASP 29: 3.004, ILE 50: 3.012, ASP 130: 3.269                           |
| 3NU9                     | −9.3                | ASP 130: 3.158, ASP 130: 3.269, PRO 181: 3.491                           |
| 3NUJ                     | −8.6                | ASP 30: 3.004, ILE 50: 3.180, ILE 50: 3.280                              |
|                          | −8.1                | ARG 8: 3.222, MET 46: 3.620, ARG 8: 3.381                               |
| 3NUO                     | −9.3                | ARG 8: 3.381, ARG 8: 2.944, GLY 150: 3.171                                |
| 3PWM                     | −10.2               | ASP 30: 2.895, ILE 150: 3.098, ASP 30: 3.371                             |
|                          | −9.8                | ASP 130: 3.158, ASP 130: 3.269, PRO 181: 3.491                           |
| 3S43                     | −8.3                | ASP 30: 3.004, LYS 45: 2.965, ARG 8: 3.222                               |
|                          | −8.4                | ILE 50: 3.280, MET 46: 3.620, ARG 8: 2.937                                |
|                          | −8.4                | ASP 130: 3.269, ASP 130: 3.292, ASP 30: 3.335                           |
| 3TH9                     | −7.5                | ASP 30: 3.004, ASP 30: 3.371, LYS 145: 3.094                             |
|                          | −8.4                | ILE 150: 3.160, GLY 181: 3.317, ARG 8: 2.937                               |
|                          | −8.4                | ASP 130: 3.269, ASP 130: 3.292, ARG 8: 3.335                           |
| 3VF5                     | −9.5                | ASP 30: 3.004, ASP 30: 3.371, ASP 30: 3.335                             |
|                          | −9.7                | ASP 130: 3.269, ASP 130: 3.292, ASP 30: 3.335                           |
| 3VFB                     | −9.4                | ASP 29: 2.917, ASP 30: 3.371, LYS 45: 2.939                             |
|                          | −9.7                | ASP 83: 3.022, ASP 30: 3.335, PRO 181: 3.522                         |
|                          | −9.7                | ASP 29: 2.917, ASP 30: 3.371, LYS 45: 2.939                             |
|                          | −9.7                | ASP 30: 3.335, ASP 30: 3.371, ASP 30: 3.371                         |
| 4GB2                     | −7.5                | ASP 30: 3.004, ASP 30: 3.371, ASP 30: 3.371                             |
|                          | −8.3                | ASP 30: 3.335, ASP 30: 3.371, ASP 30: 3.371                         |
| 4HDB                     | −11.4               | ASP 130: 3.527, ASP 130: 3.527, ASP 130: 3.527                        |
|                          | −11.2               | ASP 130: 3.527, ASP 130: 3.527, ASP 130: 3.527                      |

Continued
The positions of pepstatin and polygonumins A are depicted in Fig. 5. This analysis indicated that polygonumins A possesses the potential to inhibit a range of HIV-1 protease mutants that are currently known to resist available antiretroviral drugs. The potential anti-HIV activities against different protease mutants were predicted based on properties through enzymatic assay37,38, and thus, pepstatin was included as a reference in a comparative analysis to engage in potential interactions with mutant HIV-1 proteases with the following PDB IDs: 3PWM, 4HDB, 4HDF, 4HEG, and 4YHQ; the affinities recorded were lower than −10 kcal/mol. The potential interaction was found to be particularly significant for the mutant 4YHQ, for which the affinity was as low as −13.8 kcal/mol.

Table 6. Affinities of polygonumins A and pepstatin docked in HIV-1 protease mutants. Residues of HIV-1 protease mutants involved in hydrogen bond interactions with polygonumins A and pepstatin.

| Protease Mutant (PDB ID) | Affinity (kcal/mol) | Residues involved in hydrogen bond interaction with ligand: hydrogen bond distance |
|-------------------------|---------------------|------------------------------------------------------------------------------|
|                         | Polygonumins A | Pepstatin | Polygonumins A | Pepstatin |
| 4HDF                   | −10.8            | −11.2     | GLY 48: 3.356   | GLY 48: 3.023   |
|                         |                   |           | GLY 48: 3.298   | GLY 48: 3.146   |
|                         |                   |           | ARG 8: 3.335    | GLY 27: 3.341   |
|                         |                   |           | ARG 8: 3.296    | ASP 25: 3.124   |
|                         |                   |           | ASP 25: 3.295   | GLY 48: 2.759   |
|                         |                   |           | GLU 21: 3.436   | ASP 25: 3.462   |
| 4HEG                   | −11.4            | −11.3     | GLY 48: 3.381   | GLY 48: 3.288   |
|                         |                   |           | GLY 48: 3.243   | GLY 48: 3.135   |
|                         |                   |           | ASP 30: 3.125   | GLY 48: 3.037   |
|                         |                   |           | ASP 49: 3.551   | GLY 48: 3.141   |
|                         |                   |           | ASP 30: 3.186   | GLY 48: 3.166   |
|                         |                   |           | ASP 30: 3.186   | GLY 48: 3.175   |
| 4YHQ                   | −13.8            | −12.2     | ARG 8: 3.193    | ASP 29: 2.803   |
|                         |                   |           | ARG 8: 2.931    | GLY 48: 3.098   |
|                         |                   |           | VAL 82: 3.170   | GLY 48: 2.935   |
|                         |                   |           | ASP 29: 3.268   | GLY 48: 2.955   |
|                         |                   |           | ASN 30: 3.325   | GLY 48: 3.178   |
|                         |                   |           | ASN 30: 3.184   | GLY 48: 2.946   |
|                         |                   |           | LYS 45: 3.258   | GLY 48: 2.629   |
|                         |                   |           | GLY 48: 3.261   | GLY 48: 3.292   |
|                         |                   |           | GLY 48: 2.964   | ASN 30: 2.953   |
|                         |                   |           | ASN 30: 3.208   | GLY 48: 2.946   |
|                         |                   |           | ASN 30: 3.478   | GLY 48: 2.629   |
|                         |                   |           | ASN 30: 3.384   | ASN 30: 2.953   |

Table 6. Affinities of polygonumins A and pepstatin docked in HIV-1 protease mutants. Residues of HIV-1 protease mutants involved in hydrogen bond interactions with polygonumins A and pepstatin.

Molecular docking of polygonumins A, vanicoside A and pepstatin in HIV-1 protease (pdb ID: 3OXC). Polygonumins A appeared to possess high binding affinity towards HIV protease (pdb ID: 3OXC), as indicated by low binding free energy range of −10.5 to −11.3 kcal/mol (Table 4) recorded in 3 independent runs. The low binding free energies are comparable to those of vanicoside A (−10.5 to −11.7 kcal/mol) docked to the same protein, as illustrated in Fig. 3. Moreover, pepstatin (positive control) exhibited low binding free energies of −8.9 to −9.5 kcal/mol in 3 independent runs. Pepstatin has been used as positive control drug that exhibits anti-HIV properties through enzymatic assay37,38, and thus, pepstatin was included as a reference in a comparative analysis of molecular docking to determine the compound’s activity as an anti-HIV protease based on its potential binding affinity. The structures possessing the lowest binding free energy were visualized, and hydrogen bond analysis was conducted using the surface/binding analysis tool in UCSF Chimera version 1.11. The residues involved in hydrogen bond interactions are summarized in Table 5. Moreover, superimposition of the structures showed that polygonumins A, vanicoside A and pepstatin were docked in a similar binding domain (Fig. 4). Recent studies have reported the anti-HIV activity of several bioactive compounds derived from plant extracts37,38 and mushroom39. The current study examined the anti-HIV activity of polygonumins A, and the compound’s potential activity was further evaluated to identify the potential interactions of the compound with mutant HIV-1 proteases through molecular docking simulation. Mutant HIV-1 proteases available in the protein databank were downloaded and submitted to docking analysis using VinaAutodock. Polygonumins A was found to engage in potential interactions with mutant HIV-1 proteases with the following PDB IDs: 3PWM, 4HDB, 4HDF, 4HEG, and 4YHQ; the affinities recorded were lower than −10 kcal/mol. The potential interaction was found to be particularly significant for the mutant 4YHQ, for which the affinity was as low as −13.8 kcal/mol.

Experimental procedures

Plant material. The stem of *P. minus* was originally collected from Ulu Yam, Malaysia, and a voucher specimen was deposited in the UKMB Herbarium, Universiti Kebangsaan Malaysia. Specimens were identified by a taxonomist and further confirmed by ITS sequencing41. Samples were washed and stored at −80°C prior to use.

Isolation of polygonumins A. A total of 5 kg of *P. minus* stem bark powder (230–250 mesh) was extracted using methanol 3 times for 24 hours each time at room temperature. The extracts were concentrated by a vacuum rotary evaporator at low pressure to yield a dark-green gum (260 g). A total of 150 g of methanol extract was fractionated by vacuum liquid chromatography (VLC) using a column (Φ 10 cm) with silica gel 60PF34 (0.063–0.200 mm) as an adsorbent and a chloroform:methanol mixture with increasing polarity (100% chloroform,
90–10%, 80–20%, and MeOH 100%) to yield 5 fractions i.e., F₁ (2.1 g), F₂ (4.4 g), F₃ (8.4 g), F₄ (10.3 g), and F₅ (88.3 g), respectively. Based on the spot size produced by thin layer chromatography (TLC), F₄ was refractionated by the VLC method using a column (Φ 5 cm) with silica gel 60PF₂₅₄ (0.063–0.200 mm) as an adsorbent and a chloroform:methanol mixture (100% chloroform, 90–10%, 80–20%, and MeOH 100%) as an eluent to produce 4 fractions: F₄₁ (0.8 g), F₄₂ (1.3 g), F₄₃ (1.8 g) and F₄₄ (3.3 g), respectively. The selected fractions, F₄₃ and F₄₂ (1.8 g), were combined and further purified by radial chromatography (RC) with silica gel 60PF₂₅₄ containing gypsum as an adsorbent with a chloroform:methanol mixture as an eluent with increasing polarity (95:5% 100% MeOH) to yield Fraction 4232 (0.4 g). Radial chromatography was then performed iteratively using the same eluent mixture.
to obtain polygonumins A (107 mg) as a white amorphous compound with a melting point of 150.2–152.0 °C. The schematic representation of polygonumins A isolation is summarized in Fig. 6.

**Compound identification.** The structure of the purified compound was determined based on spectral data recorded on a Frontier Perkin-Elmer FTIR/NIR (Perkin-Elmer Inc., Norwalk, CT, USA) spectrophotometer and a Bruker NMR 600 MHz Cryo-Probe instrument that could perform 1-D and 2-D NMR measurements (Bruker, Germany). ESIMSs were recorded on a Bruker Daltonics micrOTOF-Q 86 (direct infuse + ve.m). Isolation was carried out by radial chromatography using round glass plates on a Merck Kieselgel 60 PF 254 (art. no. 7749), and the profile was analysed using aluminium sheets measuring 20 × 20 cm on a Merck TLC silica gel 60 F 254 with a thickness of 0.25 mm (art. no. 5554) with UV light detection (254 nm) or CeSO 4 spraying followed by heating.

**Anticholinesterase activity.** Inhibition of acetylcholinesterase (AChE) was assessed using the spectrophotometric method developed by Ellman with slight modifications. Electric eel AchE (electric eel acetyl-cholinesterase, type-VI-S, EC 3.1.1.7, Sigma–Aldrich, St. Louis, USA) and acetylthiocholine iodide (Sigma–Aldrich, Steinheim, Germany) were used as the enzyme and substrate, respectively. Briefly, 125 μl of DTNB (Sigma–Aldrich, Steinheim, Germany) (50 mM Tris-HCl, pH 8, 0.1 M NaCl, 0.02 M MgCl₂·6H₂O), 25 μl of test compound solution in DMSO, and 50 μl of buffer (50 mM Tris–HCl, pH 8, 0.1% BSA) were mixed and incubated at 25 °C for 30 min. DMSO or buffer (25 μl) was added instead of the test compound solution in control experiments. The reaction was then initiated by the addition of 25 μl of acetylthiocholine iodide (0.25 mmol/l), which brought the final volume to 250 μl. The formation of 5-thio-2-nitrobenzoate anion from the enzymatic hydrolysis of acetylthiocholine iodide was monitored based on the absorbance at 412 nm on a 96-well microplate reader (Model 680, Biorad Inc., and Hercules, CA, USA). The reaction rates were calculated from data collected at specific time points over the first 180 s in 20 s increments. Percent inhibition of AChE was determined by the ratio of the reaction rate with the test sample to that with the blank control (DMSO).
in Tris-HCl buffer, pH 8.0) using the formula \((E - S)/E \times 100\), where \(E\) is the activity of enzyme with DMSO and \(S\) is the activity of enzyme with the test sample. The experiments were carried out in triplicate. Tacrine was used as a reference compound.

**Antioxidant activity.** Free radical-scavenging assay. Radical-scavenging activities were determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Various concentrations of the experimental samples were obtained, and the volume was adjusted to 200 µl with ethanol. Approximately 100 µl of 0.5 mM DPPH in methanol was added to the samples or standard (ascorbic acid and gallic acid) in a 96-well plate. After incubation for 30 min in the dark, changes in absorbance at 600 nm were measured on a 96-well plate reader. IC\(_{50}\) was calculated as the sample concentration required for a 50% decrease in the absorbance of a control solution of DPPH.

Reducing power assay. Ferric reducing antioxidant power was determined by the direct reduction of Fe\(^{3+}\) (CN\(^{-}\))\(_{6}\) to Fe\(^{2+}\) (CN\(^{-}\)), and by measuring the absorbance resulting from the formation of the Perl's Prussian Blue complex following the addition of excess ferric ions (Fe\(^{3+}\)). The reducing power method was performed as reported by Oyaizu\(^4\) with slight modifications. Different concentrations of samples (50 µg/ml to 500 µg/ml) were mixed with 2 ml of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 ml of potassium ferricyanide. The mixture was incubated at 50 °C for 20 minutes. After 20 minutes of incubation, 1.25 ml of trichloroacetic acid (10%) was added. Finally, 0.5 ml of FeCl\(_3\) (0.1%) was added to the mixture and incubated for 10 minutes. The intensity of the blue-green colour was measured at 700 nm. Increases in the absorbance of the reaction mixture indicated an increase in the reduction capability. The EC\(_{50}\) value (µg/ml) associated with the reducing power-the extract concentration at which the absorbance was 0.5 was calculated from a graph of absorbance at 700 nm against the extract concentration. Gallic acid was used as a positive control.

**Total phenolic content.** The total phenolic content was determined using the Folin-Ciocalteu method\(^4\). The reaction mixture was prepared by mixing 0.2 ml of sample (1 mg/ml) and 1.5 ml of 10% Folin-Ciocalteu reagent dissolved in water. The mixture was allowed to equilibrate for 5 minutes and then mixed with 1.5 ml of 7.5% Na\(_2\)CO\(_3\) solution. After incubation for 60 minutes at room temperature in the dark, the absorbance of the mixture was read at 725 nm against a blank using a spectrophotometer. The blank was prepared by using DMSO instead of a sample. The sample procedure was repeated for gallic acid at different concentrations, i.e., 0.00, 0.25, 0.50, 0.75 and 1 mg; the results were used to produce a calibration curve. Total phenolic content was calculated as the milligrams of gallic acid equivalent (GAE) per gram of sample (mg GAE/g) by using the gallic acid calibration curve, \(y = 0.0016x + 0.0295\), \(R^2 = 0.9548\).

**In vitro cytotoxicity.** Cell lines and cell culture. Cells of the lines K-562 (human leukaemia cell line), C33A (cervical cancer cell line), H1299 (human non-small cell lung cancer cell line) and MCF7 (human breast adenocarcinoma cell line) were purchased from the American Type Culture Collection. H1299 (human non-small cell lung cancer cell line) and A549 (human lung adenocarcinoma epithelial cell line) were provided as a courtesy by Prof. Masa-Ikeda of Tokyo Medical and Dental University. Cells were cultured in DMEM media supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin. All cell lines were cultured at 37 °C with 5% CO\(_2\).

V79 fibroblast cultures. V79–4 cells were grown in tissue culture flasks using DMEM as the growth medium at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cultures were examined daily to ensure they remained healthy. The confluent monolayer was removed by trypsinization, and the number of viable cells was calculated. Cells were seeded into a 96-well plate at a seeding density of 10,000 cells/well and incubated at 37 °C. The test substance was prepared by dilution with an appropriate volume of complete growth medium (DMEM) supplemented with 10% foetal bovine serum (FBS) to obtain the highest working concentration of 100 µg/ml (weight/volume). Procedures were performed aseptically.

The test substance was tested in triplicate at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. Growth medium from each well of a 96-well plate containing healthy culture was replaced with 200 µl of the test substance and controls respectively. The cultures were then incubated for 24 hours at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air.

In vitro cytotoxicity assay. Cells were plated in 96-well microplates and cultured for 24 h. The test compounds were dissolved in DMSO and added at different concentrations (100, 50, 25, 12.5, 6.25, 3.13 µg/ml) to the plate in triplicate. The cytotoxicities against K-562 and H1299 cell lines were measured using a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazolium bromide) assay. Exponentially growing cells were harvested and suspended in DMEM, and a 100 µl cell suspension was added to a 96-well plate. After 24 h incubation at 37 °C with 5% CO\(_2\), the cells were treated with varying concentrations of the test compounds (100 µl). The medium was removed, and cells in each well were incubated with PBS containing 1 mg/ml MTT for 24 h at 37 °C with 5% CO\(_2\). DMSO (100 µl) was added to each well to dissolve the insoluble formazan crystal, and plates were incubated for 4 h at 37 °C for complete solubilization of formazan. The level of coloured formazan derivative was analysed on a microplate reader using the results at wavelengths of 570 nm and 630 nm as references\(^5\). The percent viability of cells was calculated using the following equation:

\[
\text{% viability} = \left( \frac{\text{Absorbance of treated cells}}{\text{Absorbance of cells in solvent}} \right) \times 100
\]
Percent viability was plotted versus compound concentration, and IC₅₀ values at which the compound showed 50% inhibition of tumour cell proliferation were calculated using Microsoft Excel. The compound was tested at each concentration in triplicate.

**HIV-1 protease fluorogenic assay.** The HIV-1 Protease Inhibitor Screening Kit (Fluorometric) from Biovision Incorporated (Milpitas, CA, USA) was used to measure the inhibitory effect of each sample on HIV-1 protease activity. Pepstatin A (1 mM) was used as a known standard for HIV-1 protease inhibition, and 1% DMSO was used as a solvent control. The assay was performed according to the manufacturer’s instructions. Briefly, each sample (1 mg/ml) was incubated with HIV-1 protease enzyme at room temperature for 15 minutes. Then, the HIV-1 protease fluorescent substrate was added. The fluorescence (excitation/emission = 330/450 nm) in kinetic mode over a period of 90 minutes at 37°C was determined using a PerkinElmer EnSpire plate reader.

**Statistical analysis.** One-way ANOVA at 95% confidence level was used for statistical analysis followed by Dunnett’s test in relation to control and standard.

**Molecular docking.** The structure of wild-type HIV-1 protease (PDB ID: 3OXC) was downloaded from the protein databank for molecular docking. The protein model was prepared by removing all non-standard residues that included artificial drug saquinavir, sulfate ion and formic acid in their structure. Water molecules were removed from the structure and hydrogen atoms added using AutoDockTools 1.5.6 prior to conversion of the file format to PDBQT for docking analysis. A grid box with dimensions of 50 × 40 × 40 Å³ was generated with HIV-1 protease as the centroid using AutoDockTools 1.5.6. Vanicoside, pepstatin, and polygonuminss were docked in the HIV-1 protease using AutoDockVina 1.1.2⁴. Docking of the molecules was repeated in three independent runs. Structures were visualized and hydrogen bond interactions were further analysed using UCSF Chimera version 1.11⁵.

**Conclusion**

Polygonuminss A, a new compound isolated from *P. minus*, showed promising anticancer and HIV protease inhibition activities. This compound has been filed for a Malaysian patent (PI 2014700594). In summary, our investigation provided promising results supporting the potential use of *P. minus* in the treatment of cancers such as leukaemia and colorectal and breast cancer and as an anti-HIV agent. Hopefully, these leads can be taken up for the further development of anticancer and anti-HIV drugs.

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**Author Contributions**

S.N.B. and N.M.N. conceived and designed the whole experiments; R.A., I.S., M.T., C.E.L. and C.S. performed the experiments and analysed the data; S.N.B., N.M.N. and S.C. contributed reagents/materials/analysis tools; F.I. and N.F.R. performed the cytotoxicity test; R.A., I.S., C.E.L. C.S, S.C., T.S., T.T. and S.N.B. wrote and edited the paper. All authors reviewed the manuscript.
Additional Information

Competing Interests: The authors declare no competing interests.

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