Alkenylated phenolics from *Syzygium lineatum* with antiproliferative activity against chronic myeloid leukemia cells

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INTRODUCTION

Among all diseases, cancer poses the highest economic, social, and clinical burden in terms of cause-specific disability-adjusted life years [1]. With a 20.2% lifetime risk of developing cancer and a mortality rate that is second worldwide after ischemic heart disease, the need to address cancer is paramount. In the Philippines, the latest statistics show that cancer is responsible for more than 60,000 deaths in the year 2017. Over the years, patients with cancer are treated with chemotherapy, radiotherapy, and surgery. However, it is common for cancer cells to develop resistance to treatment [2]. Furthermore, patients with late-stage diagnosis are often ineligible for surgery. Hence, there is a clear and urgent need for effective drugs for the treatment of patients with cancer. Recent advances in cancer treatment include the application of targeted therapies which have been successful in reducing mortality and minimizing toxicity [3]. Targeted therapy is an approach using designed drugs that specifically bind to aberrant proteins, such as those implicated in malignant transformation. The design of drugs used in targeted therapy is continuously influenced and inspired by natural products [4]. These natural products are unrivaled sources of anticancer compounds in this modern era of drug discovery due to applicability, accessibility,
and diminished cytotoxicity. They act by modulating the cancer microenvironment and various signaling pathways [5].

Chronic myeloid leukemia (CML) is one of the many types of cancer. It is a clonal malignancy with a global incidence of around 2 per 100,000 persons. In the Philippines, CML along with other leukemias ranked fifth leading cause of cancer-related mortalities [6]. With this, the occurrence of leukemia and its changing trends were highly varied, and more preventive strategies must be adapted to each country [7]. A large majority of this disease is caused by chromosomal translocation between chromosomes 9 and 22, which results in the formation of the hybrid gene BCR::ABL1. The gene product BCR::ABL kinase is responsible for the impaired cellular signaling, thereby dysregulating normal cell proliferation, invasion, migration, and apoptosis which are hallmarks of cancer pathophysiology [8,9]. Thus, anti-CML agents have been continuously discovered and developed to target this hybrid protein [10,11]. The chemotherapeutics imatinib, nilotinib, and ponatinib being BCR-ABL-targeting tyrosine kinase inhibitors (TKIs) mitigate strongly CML progression. In the case of imatinib, which is an inhibitor of tyrosine kinase, it allowed CML patients to experience a life expectancy that is near-normal [12]. Due to concerns related to drug resistance brought by BCR::ABL mutations and overexpression, more chemical-based CML therapeutics are still warranted.

In search for new anticancer agents, natural products have long been tapped due to their purported efficacy and safety. Antiproliferative natural products comprised of alkaloids, flavonoids, glycosides, lignans, polyketides, and oxidized terpenoids inhibit CML proliferation via induction of apoptosis. Apart from differentiating CMLs into monocyte/erythroid cell types, natural products also prevent the occurrence of multidrug resistance in CML cells. The Philippines being a megadiverse archipelagic country hosts native and endemic plants. Among them are medicinal plants reported for their anticancer activities [13–16]. The Philippines’ Department of Health enlisted the “Ten scientifically validated” Philippine medicinal plants in 1992 (R.A. No. 8423—Philippine Institute of Traditional and Alternative Health Care), in which Momordica charantia (ampalaya) and Quisqualis indica (niyu-gniyogan) were identified to have anticancer properties [17]. In the Northern Philippines, locals and natives from provinces such as Apayao and Cagayan utilize Syzygium lineatum (Roxb.) (DC.) Merr & Perry (locally known as “Malubeg” and “Alebadu”), a traditional medicine used to treat cancer and as a souring agent for cooking. Syzygium lineatum is a fruiting tree that grows 4–5 m in height and belongs to the family Myrtaceae [18,19]. Different species of the genus Syzygium have been reported to exhibit anticancer activities [20–22]. Syzygium natural products such as dimethyl cardamonins, phenolics, oleanolic, and betulinic acids have been reported to inhibit cell proliferation and induce apoptosis [23]. In this paper, we report the antiproliferative and cytotoxic activities of Syzygium lineatum extracts, sub-extracts, and its alkenylated phenolic compounds 5-(8Z-pentadecenyl)resorcinol (gingkol, 1) and 5-(8Z-pentadecenyl)resorcinol (bilobol, 2) as well as β-sitosterol (3) and fatty acids 4–6 against HUVEC, K-562, and HeLa cell lines (Fig. 1). To understand the mechanism of action of the antiproliferative compounds against CML, in silico binding characteristics were interrogated against clinically approved target kinases in CML such as BCR::ABL kinase, c-Src kinase, and protein kinase B.

**MATERIALS AND METHODS**

**Plant material**

The leaves of *S. lineatum* were collected at Adams, Ilocos Norte, Northern Philippines (18.460268°N; 120.903896°E), in December 2016 and were identified by Mr. Michael A. Calaramo of Northwestern University Ecotourism Park and Botanic Gardens (NUEBG) and Dr. Cecilia Banag-Moran of University of Santo Tomas Herbarium (USTH). Voucher specimens were submitted to USTH (USTH015667) and the Herbarium of NUEBG (HNUL14950).

**Extraction and isolation**

The air-dried and ground leaves of *S. lineatum* (2 kg) were percolated with 1:1 dichloromethane-methanol (16.0 l). The combined extracts were evaporated until dry under reduced pressure to afford a green syrup (313.0 g). A suspension of the crude extract in water was sequentially extracted with petroleum ether (4.7 l), followed by dichloromethane (6.9 l), and finally with n-butanol (2.0 l) yielding after evaporation 94.5 g of the petroleum ether sub-extract (SIP), 58.9 g of dichloromethane sub-extract (SID), and 32.4 g of the n-butanol sub-extract (SIB). The petroleum ether sub-extract was subjected to silica gel vacuum liquid chromatography with increasing amounts of ethyl acetate (EtOAc) in petroleum ether as eluent (10%). Fractions were pooled according to their thin layer chromatography (TLC) profile. Iterative separation using silica gel column chromatography of the SIP fraction 2 (20.8 g) with 20:1 petroleum ether: EtOAc resulted in the isolation and purification of 3-(8Z-pentadecenyl)phenol or gingkol (1) along with the known compounds β-sitosterol (3) and a mixture of fatty acids (4–6). Meanwhile, repetitive separation of SIP fraction 4 (SIPE4) yielded compound 2 (bilobol) [24].

**Antiproliferative and cytotoxicity assay**

To measure the antiproliferative and cytotoxic activities of *S. lineatum* extracts, sub-extracts, and compounds, inhibitory and cytotoxic concentrations against CML (K-562), human umbilical vein endothelial cord (HUVEC), and HeLa cell lines were measured using the CellTiter-Blue® viability assay based on the method developed by Krauth et al. [25]. The GI50 and CC50 values were determined as the value at the intersection of the dose curve with the 50% line, compared to the untreated control. Imatinib and doxorubicin, which are standard anticancer drugs, were used as positive controls [26].

**Molecular docking studies**

**Ligand and protein preparation**

To elucidate potential binding propensities and modalities of the compounds, they were formatted as ligands and were prepared and optimized in Avogadro (1.20). Target proteins were prepared and minimized in UCSF Chimera...
RESULTS

In vitro antiproliferative and cytotoxic activities of S. lineatum leaf extracts and fractions, and compounds 1–6

Antiproliferative and cytotoxicity assessments of the crude extract (SlC), petroleum ether (SlPE), dichloromethane (SID), and butanol sub-extracts (SIB) were carried out using a CellTiter-Blue assay. The antiproliferative activity versus normal HUVEC and human leukemia cells (K-562) is reported as GI_{50}, while cytotoxicity versus cervical cancer cells (HeLa) as CC_{50}. The petroleum ether sub-extract (SIPE) showed the strongest anti-proliferative and cytotoxic activity among the three sub-extracts against the three cell lines (Table 1). In addition, fraction 4 of the petroleum sub-extract (SIPE4) exhibited antiproliferative activity against K-562 at 12.6 (±1.1) μg/ml and cytotoxicity against HeLa at 22.2 (±0.9) μg/ml. Chromatographic purifications of fractions SIPE4 and SIPE2 (the next antiproliferative and cytotoxic fraction) afforded six compounds and were spectroscopically identified as 3-(8Z-pentadecenyl)phenol or gingkol (1), 3-(8Z-pentadecenyl)resorcinol or bilobol (2), β-sitosterol (3), and mixture of fatty acids (4–6). Compounds 1–6 were subjected to CellTiter-Blue assay wherein 1 and 2 exhibited potent cytotoxic activity against K-562 cells. Bilobol (2), an alkenylated resorcinol, showed better selective antiproliferative activity compared to gingkol (1), a phenolic congener (Table 2). For comparison, imatinib was used as a positive drug control.

In silico binding propensities and interactions between test compounds 1, 2 and target CML-associated proteins

To further elucidate the potential mechanism of action of compounds 1 and 2 against CML (K-562), these were docked onto the adenosine triphosphate (ATP)-binding site of an active, inactive, and T334I_D382N imatinib-resistant BCR::ABL kinase, c-Src kinase (2SRC), and protein kinase B (4EJN) were selected as potential therapeutic targets due to their established roles in CML pathophysiology [28–30].

Docking simulation

Molecular docking, an in silico tool to assess binding affinities of compounds, especially natural products, to target receptors or proteins, was performed using UCSF Chimera (1.16) [31]. A grid-based “flexible ligand into flexible active site” protocol was followed in the entire simulation [32]. To visualize and enumerate the amino acid residues involved in the binding as well as evaluate the kind of intermolecular interactions, BIOVIA Discovery Studio (4.1) was utilized.

ADME properties and BOILED-Egg prediction

To predict the drug-likeness of the compounds, their absorption, distribution, metabolism, and excretion (ADME) properties were assessed based on the number of violations in Lipinski’s rule of five (LRo5). Meanwhile, pharmacokinetic behaviors [gastrointestinal (GI) absorption and brain penetration] were predicted using the Brain Or IntestinaL EstimatedD permeation (BOILED)-Egg methodological model [33,34]. The SMILES notations of the ligands were inputted in SWISSADME (http://www.swissadme.ch/index.php) and parameters such as molecular weight, lipophilicity, and number of H-acceptors and donors were recorded [35].

RESULTS
Table 3. Binding energies (BEs) and interactions between test compounds 1–2 and target proteins. Imatinib served as positive control.

<table>
<thead>
<tr>
<th>Target proteins</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Binding energy (BE) (kcal/mol)</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib-resistant BCR:ABL kinase</td>
<td>M337 E335 (H-bond), L267, L389, Y272, (pi-sigma), F336 (pi-pi), F401, I334, K290,</td>
<td>V318, A399, M309, V275 (alkyl/alkyl)</td>
<td>−8.1</td>
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Table 4. Growth inhibitory and cytotoxic concentration at 50% (IC50) of gingkol (1) and bilobol (2) against HUVEC, K562, and HeLa cell lines. The IC50 values were determined by MTT assay (n=3).

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<tr>
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<td>Gingkol (1)</td>
<td>18.2 ± 0.3</td>
<td>36.4 ± 1.1</td>
<td>47.4 ± 0.9</td>
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<td>Bilobol (2)</td>
<td>26.2 ± 3.8</td>
<td>42.4 ± 1.2</td>
<td>22.2 ± 0.9</td>
<td>46.8 ± 1.0</td>
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<td>Imatinib</td>
<td>31.8 ± 0.2</td>
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Table 5. Growth inhibitory and cytotoxic concentration at 50% (IC50) of gingkol (1) and bilobol (2) against HUVEC, K562, and HeLa cell lines. The IC50 values were determined by MTT assay (n=3).

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2 was predicted to be passively absorbed by the GI tract based on BOILED-Egg.

**DISCUSSION**

In this study, extracts, sub-extracts, and alkenylated phenolic constituents gingkol (1) and bilobol (2) from the leaves of *S. lineatum* exhibited promising anticancer activities against HUVEC, K-562, and HeLa cell lines *in vitro*. In general, extracts from several members of the genus *Syzygium* have been reported to exhibit anticancer properties based on *in vitro* cell viability biological assay evidence. For example, *S. aromaticum* extracts showed cytotoxic activity versus breast cancer cell lines MCF-7 and MDA-MB-231 in a dose-dependent manner by inducing cell cycle arrest at the S phase and promoting apoptotic activities [36,37]. Interestingly, *S. aromaticum* conferred anticancer properties against colon, cervical, liver,
ovarian, and pancreatic cancer cell lines. Water and ethanolic extracts of *S. aqueum* also conferred anticancer properties against MCF-7 cells. In addition, other *Syzygium* species such as *S. samaranense* and *S. jambo* extracts exhibited cytotoxic activities versus colon cancer and liver cancer cells, respectively [38–40]. In the Philippines, *S. lineatum* is among the traditional medicinal plants used by Ilocano natives and locals against cancer and other diseases [18,19]. This study contributes to the growing interest in *Syzygium* species as a bioresource of novel potential anticancer agents. More significantly, our results confirm the anticancer potentials and use of *S. lineatum*.

With the high morbidity associated with CML in the present and in the next 20 years which is increased by 60% by then, as well as the continuous occurrence of chemotherapeutic resistance in CML treatment, the discovery and development of new-generation drugs are warranted, thus, ushering the potentials of compounds 1 and 2 as anticancer agents and/or structural drug scaffolds [41,42]. Herein, the study reports the *in vitro* and *in silico* antiproliferative potentials of these two fatty phenolics, especially against K-562 (chronic myelogenous leukemic cell lines), of *S. lineatum* for the first time. We also report for the first time the isolation of alkenylated phenolics gingkol (1) and bilobol (2), and 3–6 from *S. lineatum*. Interestingly, both phenolic compounds also exhibited promising inhibitory activity and selectivity to CML. Comparing the activities of 1 and 2, the alkenylated resorcinol (bilobol, 2) conferred better cytotoxicity against the three cell lines (HUVEC, HeLa, and K-562). This could be attributed to the increase of hydroxyl functionalization in 2. In general, hydroxylation of compounds including alkenylated phenolic natural products has been widely reported to increase chemical stability, bioactivity including inhibitory effects on enzymes in disease pathogenesis, and binding interactions to specific molecular disease targets [43–47].

In addition, three pharmaceutically important kinases (BCR::ABL, c-Src, and protein kinase B) have been targeted by compounds 1 and 2 as shown by the results of the docking simulations. BCR::ABL1 is a well-established target as it is a chimeric constitutively active tyrosine kinase that is associated with CML cell differentiation and survival. It is the protein product of the chromosomal translocation between chromosomes 9 and 22 [48]. Many anticancer drugs like imatinib have been developed as tyrosine kinase inhibitors [49]. In this paper, three BCR::ABL tyrosine kinases were targeted—active, inactive, and imatinib-resistant. Although BCR::ABL possesses both ATP-binding and allosteric sites, the earlier was chosen as a target as this is the usual mechanism of action of clinically approved TKIs. Competitive inhibition in the ATP-binding domain yields impaired protein phosphorylation, thereby decreasing intracellularly mediated cell proliferation, growth, and survival necessary in CML cellular pathophysiology [50,51]. Comparing the results of the docking studies to the three ABL1 kinases, the alkenylated phenolics 1 and 2 from *S. lineatum* conferred the best binding affinity to the imatinib-resistant structure that harbors T315I mutation. Meanwhile, the drug control imatinib showed a weaker propensity to this structure, although this is given due to the associated resistance. Furthermore, several residues such as M337, I332, F336, Y272, A288, V275, L267, and L389 were identified as common targets between imatinib, and the two test compounds and these could serve as initial target hotspots of the compounds. Thus, both compounds 1 and 2 may serve as prodrugs or templates in developing new-generation TKIs against cancer cells harboring chemotherapeutic resistance.

Other than the three ABL1 kinases, c-Src, and protein kinase B also served as CML targets. c-Src is associated with cellular invasion and metastasis brought about by decreased cell-to-cell adhesion when c-Src is overexpressed [26,28,52]. Meanwhile, protein kinase B (or AKT) is a downstream target in the PI3K/AKT signaling pathway which is involved in cell proliferation, apoptosis, and development of cancer chemotherapeutic resistance. Thus, inhibition of AKT negatively affects the PI3K/AKT pathway leading to apoptosis and inhibition of cell growth in many types of cancer [29,53,54]. In our results, both compounds 1 and 2 showed moderate binding to both c-Src and AKT kinases, thereby highlighting their multitargeting potentials as anticancer agents against cell proliferation and drug resistance while promoting apoptosis and cell growth arrest.

**CONCLUSION**

Our study validated the traditional medicinal use of *S. lineatum* against cancer as we report the antiproliferative and cytotoxic activities of its extracts, sub-extracts, and alkenylated phenolics gingkol (1) and bilobol (2) against HUVEC, HeLa, and K-562 cell lines for the first time. *In vitro* results further suggested the sensitivity of K-562 (CML) cells to gingkol (1) and bilobol (2) with the latter showing better selectivity to K-562 cells. Molecular docking experiments supported putative multitargeting binding mechanisms of 1 and 2 in the active pockets of associated target kinases BCR::ABL1, c-Src, and protein kinase. In-depth studies on 1 and 2 are, therefore, warranted to explore further their *in vitro* and *in vivo* mechanisms of action against CML.

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**AUTHOR CONTRIBUTIONS**

FVI, VNODL, JAHM: conceptualization, investigation, data collection and analysis, manuscript preparation, and manuscript editing. ALC, APGM: conceptualization, design, manuscript preparation, editing, and review. All authors have read and approved the present version of the manuscript.

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**CONFLICT OF INTERESTS**

The authors declare that they have no conflict of interest in the publication.
ETHICAL APPROVALS
This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY
All data generated and analyzed are included in this research article.

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