ARTICLE

Chayote leaves display seasonal variation in phytoestrogenic activities and phenolic content

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ABSTRACT

Chayote (*Sicyos edulis* Jacq.) is a popular vegetable in the Philippines and has been recognized for its nutritional value and bioactive compounds, including phenolic acids found in its edible parts. Hence, this study sought to determine whether extracts from *S. edulis* shoots can elicit a bioactive estrogenic response. Specifically, the study determined whether the location of cultivation or monsoon season of collection would influence the estrogenic activities of *S. edulis* extracts. Here, we found that a specific fraction (F5.6) of *S. edulis* shoots isolated through solid-phase extraction (C-18 and HLB) inhibited the transactivation of an estrogen-induced luciferase reporter gene in an estrogen receptor-positive cell line T47D without affecting cell viability, independently of the farm source and the season of sample collection. This inhibition was also seen in the E2-induced expression of superoxide dismutase 2 *(SOD2)*, an antioxidant enzyme upregulated in advanced breast cancer cells. In contrast, the phenolic content in the F5.6 fraction varied across seasons, with a higher content found during the northeast monsoon season (0.175 ± 0.027 mg GAE/mg sample) than the southwest monsoon seasons (0.043 ± 0.015 and 0.051 ± 0.012 mg GAE/mg sample). The high phenolic content increased the ratio of estrogen receptor alpha to beta transcripts and the

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mRNA expression level of the estrogen-responsive gene, growth regulation by estrogen in breast cancer 1 (*GREB1A),* in T47D cells. Overall, this study showed how seasonal variation may affect the phenolic content of *S. edulis* and influence the plant's bioactivity, providing a potential cultivation strategy in obtaining extracts with the intended bioactivity.

INTRODUCTION

Estrogen is a steroid hormone involved in various physiological processes such as reproduction, cardiovascular health, bone metabolism, and liver function (Darbre 2015; Deroo and Korach 2006). Estrogen has beneficial roles in normal physiology, but this hormone has also been implicated in the progression and development of a wide range of diseases including breast cancer, ovarian cancer, and endometriosis. In most cases, estrogen mediates its effects through the estrogen receptor (ER) isoforms ESR1 and ESR2 (also called ERα and ERβ) present within target cells (Darbre 2015; Deroo and Korach 2006). The binding of estrogen to ESR1, for example, activates downstream signaling pathways which enhance tumor growth and metastasis in hormone-related cancers (Liu et al. 2020). In endometriosis, the activation of ESR2 alters the expression of genes which encourage cell growth and proliferation, and it enhances the invasiveness of endometrial cells to implant at extrauterine sites (Han et al. 2015; Simmen and Kelley 2016). Given the wide range of illnesses associated with estrogen, strategies that can interfere with its negative effects may help in preventing the development of these disorders.

Dietary patterns and food intake affect hormone signaling, both positively and negatively. Plant-derived foods, for example, contain phytoestrogens which activate estrogen receptors due to their structural similarities with the hormone (Cos et al. 2003; Strauss et al. 1998). In plants, phytoestrogens such as phenolic compounds, serve as their defense systems against UV light stress, pathogens, and predators (Mbaveng et al. 2014). These compounds also possess a wide range of biological activities, which prompted researchers to explore their potential benefits in treating many diseases in humans (Mbaveng et al. 2014; Strauss et al. 1998). However, concerns have also been raised regarding the potential risks associated with consuming diets rich in phytoestrogens. Phytoestrogens can disrupt hormone action by directly or indirectly interacting with receptors and intracellular signaling pathways, potentially contributing to the development of estrogen-related disorders (Strauss et al. 1998). Consequently, it may be necessary to monitor the consumption of food containing phytoestrogens more carefully and make appropriate dietary choices.

Chayote (*Sicyos edulis* Jacq.), a member of the Cucurbitaceae family, is an herbaceous plant cultivated in the tropics for human consumption. It is locally known as '*sayote*' in the Philippines, with its leaves and fruits being part of the Filipino diet. Chayote is mainly grown in mountainous regions in the Philippines, especially in Benguet and other areas of the Cordillera Administrative Region. It is known for its ease of cultivation and adaptability to diverse climatic conditions, leading to its expansion into lowland areas to bring the produce closer to urban cities. It is an underutilized crop that eventually gained importance because of its reported nutritional value and biological significance (Lombardo-Earl et al. 2014; Vieira et al. 2019). For instance, aerial parts of chayote have diuretic, antioxidant, antimicrobial, cardiovascular, and anti-inflammatory properties (Ordoñez et al. 2006, 2003). Root and leaf extracts lower blood pressure in rats and dogs (Aung et al. 1990; Lombardo-Earl et al. 2014) and fractions from the leaves ameliorate hyperuricemia in rats (Wahyuningtyas et al. 2022). These properties of chayote have then led to a strong interest to identify the potential components responsible for its bioactivity. Many studies on *S. edulis* focused on identifying phenolic compounds in the plant and discovered that its edible parts are rich in flavonoids, the largest group of naturally occurring phenolic compounds, with the leaves having the highest concentrations of apigenin and luteolin (Siciliano et al. 2004). The fruit also contains high concentrations of flavones, an important subgroup of flavonoids, accounting for about 60.6% of the total compounds in the produce (Díaz-de-Cerio et al. 2019). With the high concentration of phenolic compounds in *S. edulis*, we sought to investigate whether extracts from the plant would elicit estrogenic or anti-estrogenic activities. Specifically, the study focused on determining whether site (highland versus lowland) or monsoon season (northeast versus southwest monsoon seasons) of cultivation would influence estrogenic or anti-estrogenic activities of *S. edulis* extracts.

MATERIALS AND METHODS

Plant materials

Fresh shoot (young and mature) samples of *S. edulis* were sourced from (a) La Trinidad Trading Market, La Trinidad, Benguet, Philippines (Site 1 [S1] – highland), one of the major sources of fresh vegetables in the Philippines, (b) a farm in Pulilan, Bulacan, Philippines (Site 2 [S2] – lowland), and (c) a commercial company (with unknown source) which obtains produce from local growers in the Philippines (Site 3 [S3] – commercial). For Site 1, three sample collection seasons were completed in September 2020 (S1B1), May 2021 (S1B2), and February 2022 (S1B3), two southwest and one northeast monsoon seasons respectively, to assess the seasonal variations in terms of plant bioactivity and concentration of phytochemical components. Shoot samples sourced from S2 and S3 were collected within the same season as S1B3.

Plant DNA extraction, PCR amplification, and purification

The species collected from three different sites were morphologically identified by a plant taxonomist and authenticated using DNA barcoding. DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with some modifications as described previously (Marquez et al. 2019). Proteinase K (0.4 mg/ml) (Sigma-Aldrich, MO, USA) was added to samples and incubated at 60°C for 1 hour followed by centrifugation at 10,000 x g for 3 minutes. Supernatants were then filtered into collection tubes and the DNA was subsequently eluted from filter columns. The isolated DNA from each plant was transferred into a 1.5 ml tube and stored at -20°C. Quality of extracted DNA was analyzed on a 1% agarose gel by gel electrophoresis (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The nuclear ribosomal internal transcribed spacer 2 (ITS2) barcode region was amplified using the following forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA) as described previously (Gu et al. 2013): ITS-S2-F (5'-ATGCGATACTTGGTGTGAAT-3') and ITS-S3-R (5'-GACGCTTCTCCAGACTACAAT-3'). Polymerase Chain Reaction (PCR) was carried out using the thermal cycler (Bio-Rad Laboratories, CA, USA) with the following cycling conditions: an initial denaturation at 94°C for 5 minutes, and 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 10 minutes. PCR products were run on a 1% agarose gel at 120 V for 20 minutes and were then purified using PCR purification kit before submitting for sequencing at Macrogen Laboratory, Korea.

Sequence Alignment and Phylogenetic Analysis
The obtained DNA sequences were trimmed and cleaned prior to sequence alignment using the MEGA10 software (https://www.megasoftware.net). Sequences were then manually refined through visual inspection before performing structural analysis. Phylogenetic affiliation of the genes was determined in reference to the NCBI GenBank database (www.ncbi.nlm.nih.gov) using the BLASTn algorithm. Phylogenetic tree construction was done in the MEGA software, applying Maximum likelihood and Tamura 3-parameter as best fit DNA model. Bootstrap support was analyzed with 10,000 replications.

Crude extraction of S. edulis (Chayote)
Samples were washed under running water, air-dried, and pulverized with a mechanical blender. Approximately 50 g of powdered samples were defatted using 100 ml of HPLC-grade n-hexane (JT Baker, Phillipsburg, NJ, USA) for 48 hours, filtered using vacuum filtration, and dried under the fume hood until the n-hexane (Hex) had fully evaporated. The dried defatted powder was then extracted using 150 ml HPLC-grade ethyl acetate (EA) (RCI Labscan, Bangkok, Thailand) on a mechanical shaker for 48 hours. Resulting crude EA extracts were filtered and concentrated using a rotary evaporator (Heidolph, Schwabach, Germany) at 40°C. Then, extracts were stored at -20°C, protected from light, until further use for SPE clean-up.

Solid Phase Extraction (SPE)
Approximately 50 mg of the EA crude extract from each site and season were subjected to SPE Biotage® Pressure+ 48 (Biotage Charlotte, NC, USA) for sample clean-up and preliminary fractionation using Sep-Pak C-18 6cc Vac 500 mg cartridge (Waters, Milford, MA, USA). The protocol consists of a pre-washing/conditioning and activation step using 18 ml of HPLC-grade methanol (MeOH) (RCI Labscan) and 6 ml of HPLC-grade water (RCI Labscan), respectively. This was followed by an equilibration step using 6 ml of 30% MeOH; a loading step of 12 ml suspension of the crude extract dissolved in 30% MeOH, in which the flowthrough was collected (F1); a washing step of 30% MeOH (F2); and consecutive elution steps using 12 ml each of 50% MeOH (F3), 70% MeOH (F4), 100% MeOH (F5), 50:50 MeOH-Acetonitrile (F6), and 2 rounds of 100% acetonitrile (ACN) (JT Baker) (F7-F8). Eluents were dried at 40°C with direct nitrogen gas flow. Since F1 took too long to evaporate, it was excluded from the testing. Preliminary testing was then conducted on the remaining SPE fractions F2-F8.

Another round of SPE was conducted on the most promising fraction (F5) using Oasis hydrophilic-lipophilic-balanced (HLB) 6cc Vac 500 mg cartridge (Waters) for further fractionation. Extracts were dissolved in 20 ml of 50% MeOH. The cartridge was conditioned with MeOH, activated with water, and equilibrated using 50% MeOH. The sample was loaded as previously described in which the flowthrough was collected (F5.1). This was then followed by consecutive elution steps using 12 ml each of 60% MeOH (F5.2), 70% MeOH (F5.3), 80% MeOH (F5.4), 90% MeOH (F5.5), 3 rounds of 100% MeOH (F5.6-5.8), and 100% ACN (F5.9). Fractions F5.1 to F5.9 were screened for their estrogenic activity.

Cells and culture medium
Human estrogen-responsive (T47D-kbluc) breast cell lines were used as *in vitro* model to explore the estrogenic activity of *S. edulis* EA leaf extracts. The T47D-kbluc cells were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) with 1% v/v Antibiotic-Antimycotic (AA) (Gibco). Cultures were grown in 25cm^2 or 75cm^2 culture flasks and kept at 37°C in a humidified, 5% CO_2 atmosphere. In all estrogen-response experiments, a steroid-depleted medium consisting of phenol red-free RPMI 1640 (Gibco), 5% hormone-free charcoal-dextran stripped serum (CSS), and 1% v/v AA was used.

ERE Luciferase Reporter Assay
Single luciferase reporter assay was employed to assess the ability of fractions F5.1 to 5.9 from *S. edulis* EA leaf to increase the transcriptional activity of ERE (Estrogen Response Elements) in T47D-kbluc cell line. Cells (10,000 cell/well) were seeded in white wall, clear flat bottom 96-well plates (Corning, NY, USA) in 100 µl steroid-depleted medium and allowed to adhere for 48 hours. Spent medium was aspirated and refreshed with 100 µl steroid-depleted medium containing 3 µg/ml of HLB fractions with 250 pM E2 (Sigma-Aldrich) or vehicle (Dimethyl sulfoxide, DMSO) (RCI Labscan) incubated for 48 hours. After incubation, contents from each well were aspirated and replaced with 50 µl of 1X Passive Lysis Buffer (Promega, Madison, WI, USA), and plates were shaken for 15 minutes at 700 rpm with a microplate shaker (Corning). Cell lysate (30 µl) was mixed with 60 µl of firefly luciferase substrate (Promega) and was read for four times using a Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA, USA) to obtain the relative luminescence units (RLU). Bicinchoninic acid (BCA) protein assay was also performed by mixing 20 µl of cell lysate with 200 µl of BCA reagent (Thermo Scientific, Waltham, MA, USA) in a transparent flat bottom 96-well plate (Corning) and incubated at 37°C for 30 minutes. Plates were read twice at 562 nm in an absorbance UV/Vis spectrophotometer (BMG Labtech, Ortenberg, Germany). The ratio of RLU and absorbance values from BCA was calculated and resulting values were normalized to the vehicle control to obtain the relative luciferase activity per treatment. Experiments were performed in quadruplicate wells per treatment.

Cell Proliferation Assay
Effects of HLB F5.6 on cell proliferation were investigated using the CellTiter Glo® luminescent assay. T47D-kbluc cells (10,000 cells/well) were plated in a white wall, clear flat bottom 96-well plate (Corning) using 100 µl steroid-depleted medium, allowed to adhere for 48 hours, and then treated with 100 µl of the same medium with vehicle (DMSO) or 3 µg/ml of extracts. After 48 hours incubation, plates were processed according to manufacturer's protocol with minor modifications. Specifically, contents of each well were reduced to 25 µl followed by addition of 25 µl of CellTiter Glo® reagent (Promega). Plates were shaken for 2 minutes in a microplate shaker (Corning) to mix the contents and induce cell lysis. Plates were incubated in the dark for 10 minutes and then read in a luminometer (Thermo Scientific) to get the RLU. Relative viability was computed by normalizing RLUs obtained per treatment with respect to the vehicle control. All treatments were performed in quadruplicates.

Thin-Layer Chromatography (TLC) Profiling
The most active fraction, F5.6, was subjected to TLC for comparative profiling. Each fraction from sites and seasons was dissolved in minimal amounts of EA and blotted into pre-coated TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) 10 mm above the bottom of the plates using a glass-blown capillary tube. After sample loading, TLC plates were placed in an equilibrated TLC chamber containing a mobile phase of 1:1 Hex-EA. TLC profiles were observed under visible and ultraviolet light. Rf values were computed as the distance travelled by the solute (in mm) divided by the distance of the solvent front.

Determination of Phenolic Content
All fractions were analyzed for their phenolic content using Folin-Ciocalteu assay as described with minor modifications

(Baluyot et al. 2022). The reaction mixture was prepared by loading 20 µl of 1 mg/ml fractions in a 96-well plate followed by the addition of 20 µl of Folin-Ciocalteu reagent (Loba Chemie Pvt. Ltd., Mumbai, India) diluted in 100 µl of distilled water. After 5 minutes of incubation in the dark at room temperature, 40 µl of 20% (w/v) sodium carbonate (Sigma-Aldrich) was added onto each well and the plate was allowed to incubate for another hour then read at 760 nm using a UV/Vis spectrophotometer (BMG Labtech). A calibration curve was plotted using the average absorbance values obtained at different concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 µg/ml) of gallic acid (Loba Chemie Pvt. Ltd.) as positive control. The concentration of phenolic compounds in the fraction was expressed as milligram of gallic acid equivalent (GAE) per milligram of extract (mg GAE/mg extract). Experiments were done in triplicates per treatment.

RNA isolation, cDNA synthesis, and gene expression by quantitative real-time PCR (qPCR)

T47D-kbluc cells (250,000 cells/well) were cultured in 6-well plates for 48 hours in steroid-depleted medium prior to treatment with 3 µg/ml of F5.6 S1 or vehicle, alone or in combination with 250 pM E2 for another 48 hours. RNA isolation by TRIzol™ Reagent (Thermo Scientific) was performed, and total RNA was measured using NanoDrop 2000 (Thermo Scientific). cDNA was synthesized from 500 ng of RNA samples from each treatment group using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with the following conditions: 25°C for 10 minutes, at 37°C for 120 minutes, then 85°C for 85 minutes. Twenty microliters (20 µl) of the resulting cDNA were diluted to 100 µl with UltraPure DNase/RNase-free water (Thermo Scientific). qPCR was conducted using SensiFAST Master Mix (Bioline, London, UK) according to the manufacturer's protocol with the following forward and reverse primers (Integrated DNA Technologies) (Table 1) and PCR conditions: denaturation for 25 seconds at 95˚C, followed by 45 cycles of annealing for 3 seconds at 95°C, and elongation for 30 seconds at 60°C. Relative mRNA expression was expressed as $\log_2$ fold change (-ΔΔCt). All treatments were performed in triplicates.

Table 1: Primer sequences for qPCR

Gene name	Forward (5'-3')	Reverse (5'-3')
ESR1	CGACTATATGTGTCCAGCCAC	CCTCTTCGGTCTTTTCGTATCC
ESR2	CAGGTCTGGGGTTGGAGAAT	AGTAACTCAAGGGGCCAGTC
GREB1A (Isoform a)	CTGAAGCTAGACACGGAGGC	AGAGGTTATGAACAGTGCTACTCAC
SOD2	AGTGTGCGGCACCAGC	TTGATGTGAGGTTCCAGGGC
ACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Statistical Analysis

All data were presented as mean ± SEM. Data analyses were performed in GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Comparisons between treatment groups were anQalyzed using Student's t-test and differences among multiple groups using ANOVA with Dunnett's post-hoc analysis. Comparison of phenolic contents of *S. edulis* fractions obtained from the different sites and seasons was performed using one-way ANOVA with Tukey post-hoc analysis. *P* values at $p<0.05$ were considered statistically significantly different.

RESULTS

Anti-estrogenic activity of S. edulis fractions in T47D cells

In this study, pro- and anti-estrogenic activities of nine HLB fractions (F5.1-F5.9) from S1B1 were assessed using the T47D-kbluc cell line that contains the estrogen receptors alpha and beta and a stably transfected estrogen-responsive luciferase reporter gene (Wilson 2004). While none of the fractions when used in singly showed pro-estrogenic activities, three fractions (F5.3, F5.6, and F5.7) significantly inhibited E2 activation of the ERE luciferase gene **(Figure 1A)**. Among the three active fractions, F5.6 was the only fraction that did not significantly reduce cell viability when treated alone or in the presence of E2 **(Figure 1B)**, suggesting a low to no observable toxicity of this fraction. Since F5.6 also had the highest anti-estrogenic effect and had the most yield compared with the two active fractions that were tested, succeeding experiments were performed using this fraction.

To compare whether the observed anti-estrogenic activity in *S. edulis* is consistently present in plants grown in lowlands, we repeated the extraction process and SPE fractionation on samples collected from a Bulacan farm (S2). F5.6 from this site also inhibited E2-induced ERE luciferase activity **(Figure 2A)**, without affecting cell viability **(Figure 2B)**. A similar anti-E2 activity was also observed in F5.6 from a commercial source (S3). Hence, these data suggest that the Philippine *S. edulis* F5.6 fraction independent of the source has comparable anti-estrogenic activities.

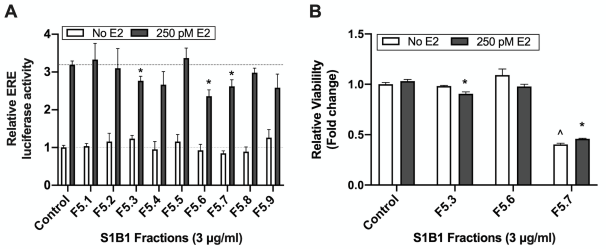

Figure 1: S1B1 fractions inhibit estrogenic activity in T47D-kbluc cell line. Cells (10,000/well) were seeded in steroid-free media for 48 hours, and then treated with (A) 3 µg/ml of F5.1 to F5.9 from S1B1 with or without 250 pM E2 for 48 hours. (B) Active fractions from A were subjected to CellTiter Glo™ assay. Experiments were conducted in quadruplicates per treatment. All data were expressed as mean ± SEM. Caret (^) indicates significant difference of individual fractions relative to no E2 control, and asterisk (*) indicates significant difference of co-treatments relative to E2 control at $p<0.05$ by one-way ANOVA with Dunnett's post-hoc analysis.

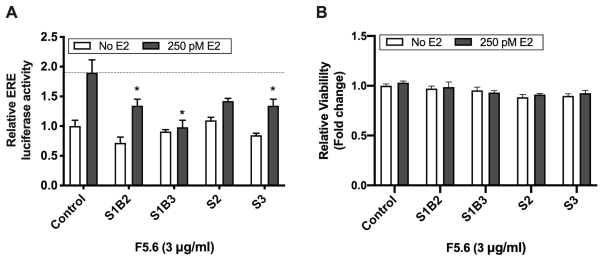

Figure 2: F5.6 from different sites and monsoon seasons displayed similar anti-estrogenic activities towards T47D-kbluc cell line. Cells (10,000/well) were seeded in steroid-free media for 48 hours, and then treated with 3 µg/ml of F5.6 from different sites collected during the southwest (S1B2) and northeast (S1B3, S2, and S3) monsoon seasons, with or without 250 pM E2 for 48 hours. Experiments were performed in quadruplicates per treatment. All data were expressed as mean ± SEM. Asterisk (*) indicates significant difference of co-treatments relative to E2 control at $p<0.05$ by one-way ANOVA with Dunnett's post-hoc analysis.

To verify the taxonomic identity of the samples used in this study, phylogenetic tree was constructed using ITS2 sequences of Philippine samples and previously published *S. edulis* ITS2

sequences in NCBI (www.ncbi.nlm.nih.gov). Here, we found that *S. edulis* samples sourced from different locations within the Philippines clustered together, indicating a similar genetic make-up. Philippine *S. edulis* samples also clustered with cultivars from Mexico and Japan (**Figure 3**), suggesting that these samples are more genetically related to each other, yet

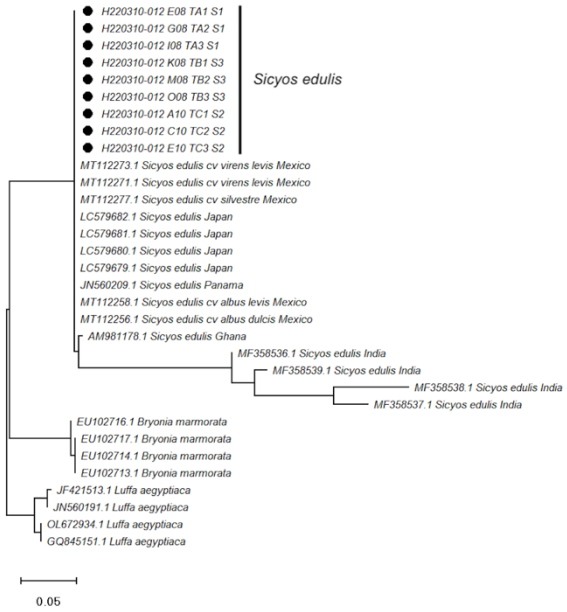

Figure 3: Phylogenetic analysis using maximum likelihood of ITS2 sequences display clustering of *S. edulis* obtained from three different sites. Sequences from *S. edulis* samples are indicated by black circles whereas reference sequences are indicated by accession number. Bootstrap values shown at the nodes were obtained from 10,000 bootstrap replicates and presented as percentages.

genetically distinct from Indian cultivars. Interestingly, historical documents indicate that chayote originated and was first domesticated in Mexico and Guatemala, and its distribution to different regions, including the Philippines and Japan, occurred towards the end of 19th century (Amano et al. 2021; Cadena-Iñiguez et al. 2007; Newstrom 1991).

To explore whether the collection season influences the observed anti-estrogenic activity in *S. edulis,* samples were also recollected in May (S1B2) and February (S1B3), during the early southwest and late northeast monsoon seasons, respectively. However, these samples retained the anti-estrogenic activity, like the samples collected in September (S1B1), during the late southwest monsoon seasons **(Figure 2A)**. Hence, the anti-estrogenic activity of *S. edulis* is observed independent of the season of sample collection.

High phenolic content in S. edulis collected during the northeast monsoon season

F5.6 from the different seasons were further subjected to TLC to compare the constituents present in the fractions. Four distinct bands were developed in the TLC plate for S1B3, but fainter and fewer bands were observed in F5.6 from S1B1 and S1B2 (**Figure 4, Supp. Table 1**). Among the fractions generated from site 1, S1B3, which was collected during the northeast monsoon season, displayed the highest phenolic content (0.175 ± 0.027 mg GAE/mg extract), followed by S1B2 (0.051 ± 0.012 mg GAE/mg extract) and S1B1 (0.043 ± 0.015 mg GAE/mg extract sample), which were both collected during the southwest monsoon season (**Figure 5**). F5.6 from S2 and S3 also displayed a similar banding profile to that of S1B3, with phenolic concentrations (S2 = 0.140 ± 0.045 mg GAE/mg extract; S3 = 0.138 ± 0.017 mg GAE/mg extract) of about thrice as much as those detected for S1B1 and S1B2, albeit not statistically significant. These concentrations fall between 39 and 125 µg GAE/g dry weight of the plant, which is lower than the total phenolic content of the EA extract, usually at around 680 µg GAE/g dry weight (Chang et al. 2021). This is expected given the additional SPE extraction steps performed prior to quantification. Nevertheless, these data suggest that while the anti-estrogenic activities of *S. edulis* fractions remain comparable across seasons, fractions from samples collected during the northeast monsoon season have higher concentrations of phenolics than from those collected during the southwest monsoon season.

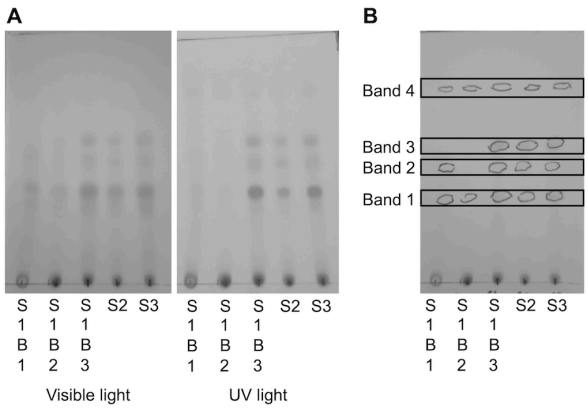

Figure 4: TLC profile of F5.6 S1B1 and S1B2 collected during southwest monsoon season displayed similar banding profiles Extracts were blotted in Silica gel 60 F254 TLC plates (Merck). After sample loading, the TLC plates were placed in an equilibrated TLC chamber containing a mobile phase of 1:1 Hex-EA. (A) The chromatogram was observed under visible light and short-wave ultraviolet light (254 nm). (B) Boxes were used to highlight the number of bands observed in the TLC plates under visible light.

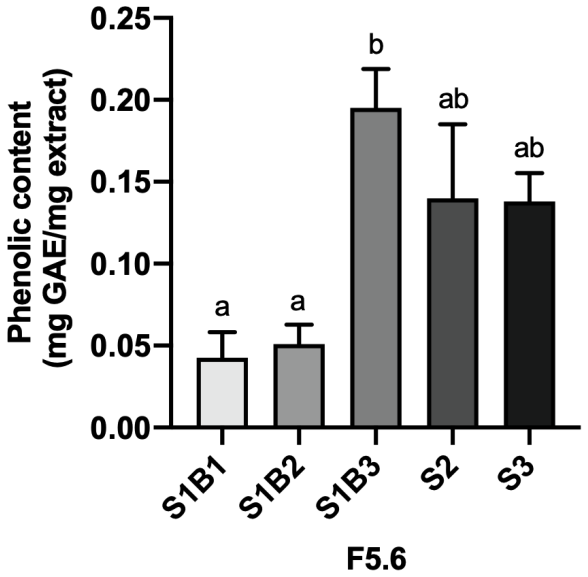

Figure 5: Increased phenolic content of F5.6 were observed from sites collected during the northeast monsoon season. Folin-Ciocalteu assay was used to quantify the phenolic content of F5.6 collected during the southwest (S1B1, S1B2) and northeast (S1B3, S2, S3) monsoon seasons. Concentrations were obtained from the calibration curve using gallic acid as a standard. Data were expressed as mean ± SEM. One-way ANOVA with Tukey post-hoc analysis was performed to compare each fraction. Different letters indicate significant difference at $p<0.05$.

Upregulation of ESR1 and GREB1A by S. edulis collected during the northeast monsoon season

Since plant phenolics may induce the expression of select estrogen-responsive genes, F5.6 collected from the different seasons were also tested for their effects on the mRNA expression of estrogen-responsive genes *GREB1A* and *SOD2*. Consistent with its phenolic content, S1B3 displayed the highest

level of *GREB1A* expression whether cells were exposed to E2 (**Figure 6A)**. Interestingly, while S1B1 and S1B2 have relatively low *GREB1A* gene expression, all fractions inhibited E2-induced SOD2 expression in these cells.

Previous studies have shown that ESR isoforms have opposing effects and that the ratio of ESR1/ESR2 expression is an essential predictor of cellular responses to estrogen (Leygue et al. 1998; Pons et al. 2019; Sotoca Covaleda et al. 2008). We then tested whether F5.6 affected the proportion of *ESR1* and *ESR2* expression in T47D-kbluc cells. Consistent with its phenolic content, S1B3 displayed the highest level of *ESR1* but not *ESR2* expression whether cells were exposed to E2 (**Figure 6A)**. This led to a significant increase in the *ESR1/ESR2* ratio **(Figure 6B)**.

Given the high phenolic content of S1B3, these data suggest that *S. edulis* leaf extracts contain phenolics that may induce the expression of select estrogen-responsive genes, such as *GREB1A*, likely through an ESR1 signaling pathway, and that the concentration of these phenolics depend on the season the plant samples were collected. In contrast, *S. edulis* leaf extracts contain a plant metabolite which can inhibit the estrogen-responsive gene *SOD2*, independent of the season of sample collection.

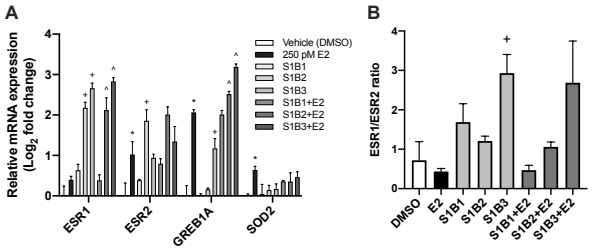

Figure 6: F5.6 from S1B3 increased the mRNA expression of *ESR1* and *GREB1A*. Cells (10,000/well) were seeded in steroid-free media for 48 hours. (A) Gene expression was analyzed after treatment with 3 µg/ml of F5.6 from S1 collected in the southwest (S1B1, S1B2) and northeast (S1B3) monsoon seasons, with or without 250 pM E2 for 48 hours, and (B) *ESR1/ESR2* ratios in samples from each season were calculated. Experiments were performed in triplicates. Data were expressed as mean ± SEM. One-way ANOVA with Dunnett's post-hoc analysis was performed to compare control with each fraction. Symbols indicate significant differences: (*) between controls, (+) relative to no E2 control, (^) relative to E2 control at $p<0.05$.

DISCUSSION

Studies conducted on *S. edulis* have shown that the plant possesses a variety of biological properties, including anti-cancer, anti-inflammatory, and antioxidant activity (Lombardo-Earl et al. 2014; Ordoñez et al. 2006, 2003). However, the ability of *S. edulis* to elicit estrogenicity on estrogen-responsive cells has not yet been explored. In this study, we demonstrated that F5.6 at low doses is sufficient to induce an anti-estrogenic activity on a hormone-responsive breast T47D-kbluc cells.

Several studies have demonstrated that biological activity of the same plant collected from different sites and environmental conditions has varied potencies depending on the concentration of phytochemical ingredients (Ghasemzadeh et al. 2018, 2015; Ghasemzadeh and Jaafar 2013; Muraina et al. 2008). Since chayote is sourced from different areas within the Philippines, this study also investigated whether its bioactivity differed depending on the season and geographical location (highland, lowland, commercial farm). Our study showed that F5.6 across different sites and season of collection displayed similar anti-estrogenic effects. This finding contradicts previous studies where bioactivity was significantly influenced by sampling location. For instance, *Parkia speciosa,* which has been reported to possess antibacterial properties, exhibited significant variations when the extracts from the same plant were collected from different locations. In fact, the potency of the extract was even completely lost against certain bacterial strains and this loss in bioactivity was correlated to the flavonoid and phenolic acid content of the extract (Ghasemzadeh et al. 2018). While our study did not perform identification of putative compounds, we can surmise that the bioactivity of the plant extract is attributed to the presence of potentially similar concentrations of phytochemicals or compounds from the fractions.

This study also identified that *S. edulis* samples obtained from various sites in the Philippines have similar genetic makeup, which could explain the observed comparable anti-estrogenic response. *S. edulis* samples from the Philippines were also genetically related to cultivars from Mexico (Latin America) and Japan, confirming historical records describing that chayote in the Philippines came from Mexico and Guatemala (Amano et al. 2021; Cadena-Iñiguez et al. 2007; Newstrom 1991), likely through the galleon trade from Mexico to the Philippines (Amano et al. 2021; Newstrom 1991).

Many studies were also conducted on the different edible parts of Mexican chayote. For instance, extracts and fractions from chayote roots display antihypertensive and vasorelaxant activity (Lombardo-Earl et al. 2014). Aerial parts of chayote also possess antioxidant, antimicrobial, anti-inflammatory, cardiovascular, and diuretic properties (Ordoñez et al. 2006, 2003). Although these studies did not explore the potential estrogenicity of *S. edulis,* it is tempting to speculate that a similar anti-estrogenic action may also be detected in these cultivars, given their close phylogenetic relationship.

Phytoestrogens, such as phenolics, are estrogen-like substances that can activate estrogen receptors (Cos et al. 2003; Strauss et al. 1998). Because these metabolites occur naturally in plants, this study also explored whether different collection seasons would influence the constituents present in the F5.6. Our study showed differences in TLC profiles of the fraction that were further corroborated by the varying concentration of phenolics per season of collection. The phenolic content of *S. edulis* was more abundant when samples were collected during the northeast monsoon (Feb 2022 [S1B3, S2, S3]) than during the southwest monsoon (Sept 2020 [S1B1] and May 2021[S1B2]) season. The Philippines experiences two distinct seasons: northeast monsoon (amihan), and southwest monsoon (habagat), that bring different weather patterns to different regions of the country. The northeast monsoon brings cool, dry air from the northeast that contributes to colder temperatures over the country. This period is also expected to have less rainfall compared to the southwest monsoon. Interestingly, other studies have shown that a higher phenolic content was observed in response to seasons with lower temperatures and rainfall (Ben Ahmed et al. 2017; Alba et al. 2023). Lower temperatures lead to cold stress which tend to enhance phenolic content of plants as part of their protective response. Conversely, high radiation and elevated temperatures lead to reduced phenolic content, likely associated with the plant's increased metabolic activity and induction of oxidative stress by excessive UV exposure (Ben Ahmed et al. 2017; Yang et al. 2013). Hence, our results agree with these published data.

The phenolic compounds luteolin, gallic, and caffeic acid were previously reported to be present in the leaves (Aguiñiga-Sánchez et al. 2017; Siciliano et al. 2004). Chayote leaves have the highest concentrations of apigenin and luteolin, while trace amounts are found in fruits and roots (Siciliano et al. 2004). *S. edulis* leaves also contain the flavonoids myricetin, morin,

quercetin, and kaempferol, as well as cinnamic and phenolic acids (Chao et al. 2014; Ragasa et al. 2014). Higher amounts of flavonoids were present in leaves of yellow chayote, while quercetin and kaempferol were not detected in leaves of green chayote (Chao et al. 2014). However, it remains unclear whether any of these compounds play a role in the bioactivity of our fraction. Hence, future experiments are needed to confirm this hypothesis.

Estrogen is known to mediate its effects on target cells through the estrogen receptor isoforms, ESR1 and ESR2, that are known for their opposing functions (Darbre 2015; Deroo and Korach 2006). ESR1 is involved in cell proliferation and anti-apoptosis activities whereas ESR2 is thought to have tumor suppressive properties (Drummond and Fuller 2010; Han et al. 2015; Paruthiyil et al. 2004). Therefore, the ratio between these receptors is a crucial determinant on how substances affect the overall estrogen signaling and other processes, such as cell proliferation, in ER-positive cells (Leygue et al. 1998; Pons et al. 2019; Sotoca Covaleda et al. 2008). Here, we investigated the effects of F5.6 on the mRNA expression levels of *ESR* isoforms and select estrogen-induced proliferation and survival genes on the T47D-kbluc cells. The parental breast cell line T47D, from which T47D-kbluc originated, were reported to have low *ESR1/ESR2* ratios, showing that these cells express more *ESR2* over *ESR1* (Jansson et al. 2006; Pons et al. 2014). Consistent with these studies, we discovered that exposing T47D-kbluc cells to E2 significantly increased the expression levels of *ESR2* but not *ESR1*. A significant increase in the expression of *ESR1/ESR2* ratio and the estrogen-responsive gene *GREB1A* were also observed in cells exposed to S1B3. *GREB1* is an early-response gene involved with ER-responsive breast cell proliferation (Haines et al. 2018). *ESR1* is necessary for *GREB1* activation in breast cancer through the binding of *ESR1* to the estrogen response elements (EREs) upstream of the *GREB1* promoter (Deschênes et al. 2007). While some studies show that increased *GREB1* expression may happen independent of ER activity (Haines et al. 2018), our results may indicate the possible reliance of *GREB1A* with *ESR1,* and that its expression is likely through the *ESR1* signaling pathway. Furthermore, the presence of *GREB1A* expression in conjunction with a high phenolic content may also indicate that chayote contains phenolics with pro-estrogenic action. Although there was no evidence of cell proliferation in our viability assay, we speculate that the pro-estrogenic activity of the extract may be better determined by gene expression analysis. Nevertheless, the pro-estrogenic activity of the extract, which is likely attributable to the presence of phenolics, would be seasonally dependent. Future studies may be needed to determine the association between phenolic content with *GREB1A* and to identify the compound that induces its expression.

The mitochondrial superoxide dismutase 2, encoded by the *SOD2* gene, is an essential antioxidant defense enzyme that protects the cells against oxidative stress and cell death (Huang et al. 2000). In endometriosis, *SOD2* contributes to its pathogenesis by keeping mitochondria functioning to sustain the high energy metabolism of ectopic endometrial stromal cells. *SOD2* was also found to increase cell proliferation and migration in ovarian endometriosis (Chen et al. 2019). Our results showed that E2 induces the expression of *SOD2* in breast cells. Consistent with our findings, other studies also reported that estrogen upregulates *SOD2* in endothelial cells and ovarian cancer. Specifically, increased *SOD2* expression reduces ROS formation in endothelial cells to improve cardiovascular function (Liu et al. 2014). In addition, enhanced SOD2 expression by estrogen protects human ovarian cancer cells from undergoing apoptosis (Huang et al. 2000). We also found that leaf extracts from *S. edulis* inhibited E2-induced *SOD2* expression. In contrast to studies which showed that *SOD2* inhibition reduced proliferation and induced apoptosis in ovarian cells (Chen et al. 2019; Huang et al. 2000), our study showed that the extracts had no apparent reduction in cell proliferation. It may be possible that the reduced *SOD2* expression is related to the anti-estrogenic activity of the extracts which may be attributed to the presence of a metabolite that is unaffected by seasonal variation.

Overall, this study demonstrated that fractions from *S. edulis* shoots have anti-estrogenic activity that is independent of season and geographical location while the pro-estrogenic activity is dependent on phenolic contents that are influenced by seasonal changes. Our findings suggest that chayote can be promoted for consumption and harvested at the optimal time to give anti-estrogenic effects with little estrogenic activity. Since this is the first study to look into the estrogenicity of chayote, future research may focus on the role of metabolites in the extract in the progression and development of ER-positive breast cells.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

CMDM designed and conducted most of the experiments, analyzed the data, and wrote the manuscript with support from RASD and HMC. JCFDC, NSQ, and MGN contributed to the identification of sample sites and the development of the extraction protocol. RASD collected and performed the extraction of plant materials and conducted TLC profiling. HMC performed DNA extraction and constructed the phylogenetic tree. MCV assisted in the study design, revised the manuscript, and supervised the project. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY DATA

Supp. Table 1: Rf[a] values of bands from the TLC profile of F5.6 from different sites and monsoon seasons (Mobile phase: 1:1 Hex-EA).

	S1B1[b]	S1B2[b]	S1B3[c]	S2[c]	S3[c]
Band 1	0.34	0.34	0.34	0.35	0.36
Band 2	0.46	-	0.46	0.47	0.47
Band 3	-	-	0.54	0.54	0.55
Band 4	0.77	0.77	0.77	0.77	0.78

[a]Rf values were computed as the distance travelled by the solute (in mm) divided by the distance of the solvent front. [b]Represents southwest monsoon season. [c]Represents northeast monsoon season.