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Philippine ethnobotanicals show anti-proliferative and cytotoxic activities in human breast cancer cells (MCF-7)

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Abstract

The increasing trend and threat of breast cancer had paved the search for novel compounds to discover drugs for its treatment. Since the discovery and development of plant-based anti-tumor drugs, plants has been the focus of several researches to discover species with pharmacological potential. The Philippine *Ilongot-Egongot* ethno botanicals were tested for anti-proliferative and cytotoxic activities to determine their anticancer potential against breast adenocarcinoma (MCF-7) cancer cell line using PrestoBlue® Assay. Nine extracts showed toxic effects on MCF-7 cells: *Cestrum nocturnum, Sarcandra glabra, Oreocnide trinervis, Pittosporum pentandrum, Derris elliptica, Alstonia scholaris, Ageratina adenophora, Ayapana triplinervis* and Lop-lopiit (no known scientific name), Two extracts showed anticancer activity against MCF-7 while being nontoxic to the normal cells (normal primary dermal fibroblast, neonatal (HDFn): *O. trinervis* and *A. triplinervis*. This preferential toxicity makes these enthnobotanicals ideal candidates for anti-cancer drug discovery and development.

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Introduction

Globally, breast cancer is recognized as the most commonly diagnosed and the most frequently occurring cancer in women, comprising almost one – third of all malignancies (Zhao *et al.*, 2009) and the second leading cause of cancer-related death among females in the world. In the Philippines, it occurs in 1 in every 13 Filipino women (Philippine Society of Medical Oncology, 2015) making it the most frequent type of cancer among females in the country and in the world, topping 197 countries in breast cancer cases (Redaniel *et al.*, 2008).

Given the increasing trend and threat, the search for novel compounds to discover drugs for the treatment of cancers, such as breast cancer, has been the focus of several researches to discover species with pharmacological potential. Plants have been one of the immense sources of new compounds with pharmacological activity (Gomes de Melo *et al.*, 2011) and latest research that involved different approaches have pointed out the role of medicinal plants in drug discovery. With the development of plant-based anti-tumor drugs such as Paclitaxel (Taxol®), Vincristine (Oncovin®), Vinorelbine (Navelbine®), Teniposide (Vumon®), and Camptothecin (e.g., Hycamtin®), natural products have proven to be an essential part in cancer chemoprevention that can restrict carcinogenesis. Biologically active compounds from tropical rainforest plants with potential anticancer activity have been discovered (Balunas and Kinghorn, 2005) and more than 60% of the anticancer agents used today are formulated directly or indirectly from natural resources (Gomes de Melo *et al.*, 2011).

In the Philippines, numerous plant species are used by indigenous populations for the purpose of self-care, collectively known as ethno botanicals. These ethno botanicals present a vast potential for the detection of untapped molecules with pharmacological activity. Existing researches on ethno botanicals have revealed that hundreds of local species of flora are used traditionally to remedy numerous conditions (Abe and Ohtani, 2013). However, the use of ethno botanicals is not that well-documented, particularly, not scientifically proven, for they are generally wild, native and can only be found in the areas where the ethnic communities reside. As an archeologically rich country with an abundance of ethnic communities with distinct ways of life, the Philippine ethno botanicals offer a nearly unlimited source of drug leads due to high availability and unparalleled chemical diversity (Sasidharan *et al.*, 2011). The body of existing ethno medical knowledge has led to great developments in health care (Fabricant and Fansworth, 2001).

The ethno botanicals utilized by the Igorot community in Imugan Nueva Vizcaya Philippines have displayed biological activities through recent researches. Despite this, its anticancer potential has never been evaluated. Thus, this research will be of great contribution on the discovery of new sources of compounds with pharmacological activity that would be of great help for the prevention and treatment of cancer. Effective new anticancer drugs should be developed to address rising cancer risk within the population (Torre *et al.*, 2015).

Materials and methods

Collection of plant samples

Leaves of *Bidens pilosa*, *Cestrum nocturnum*, *Sarcandra glabra, Oreocnide trinervis*, *Pittosporum pentandrum*, *Derris elliptica*, *Alstonia scholaris*, *Ageratina adenophora*, *Ayapana triplinervis* and Lop-lopiit (no scientific name) were collected along a trail of Mt. Imanduyan, Brgy. Imugan, Sta Fe, Nueva Vizcaya with an elevation of 1,092 meters above sea level. Barangay Imugan is home of the Ikahalahan tribe since the late 19th century (www.santafe.gov.ph) and geographically situated in the Caraballo Mountain Range between the Cordillera Central and Sierra Madre mountain ranges. It displays a unique ecological diversity characterized by a combination and interplay of human communities and immense natural diversity of its flora and fauna. Plants included in the evaluation were pre-determined in an ethno botanical survey (Undan *et al*, 2014) with the permission of the council of elders and local officials.

Ethanol extraction

Leaf samples were rinsed in running tap water to followed by second rinsing using distilled water and then with 70% (v/v) ethanol (Tan *et al.*, 2013). Dry plant materials were ground to fine particles using blender. Fifty (50) grams of each ground dry plant material were soaked in 500 ml of 95% ethanol in a stoppered flask for 72 hours. The mixture was filtered using What man no. 1 filter paper and the solvent was completely removed using a rotary evaporator (Tan *et al.*, 2013). The result extracts were stored in tightly stoppered sterile amber bottles (Srisawat *et al.*, 2007) at temperature between 0 to 5 ºC. The containers at were labeled with the name of the plant, weight of the extract in grams (g), and the date of extraction.

Culture of cell lines

Breast adenocarcinoma (MCF-7)(ATCC, Manassas, Virginia, USA) and normal primary dermal fibroblast, neonatal (HDFn)cell lines regularly maintained at the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Science and Ecological Research at De La Salle University, Manila, were used in this study. Cells were grown following a standard protocol (Freshney, 2000). Cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) and supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1x antibiotic-antimycotic (Gibco, USA) and kept at 37°C in a humidified 10% CO2 incubator.

The cells were grown to 80% confluency and washed with phosphate-buffered saline (PBS, pH 7.4, Gibco®, USA), trypsinized with 0.05% Trypsin-EDTA (Gibco®, USA) and resuspended with complete fresh media.

Cells were seeded in 100 µL aliquots into 96-well microtiter plates (Falcon, USA) in a final inoculation density of 1 x 104 cells/well. Cells were counted following standard trypan blue exclusion protocol using 0.4% Trypan Blue Solution (Gibco®, USA). The plates were further incubated overnight at 37°C with 5% CO2 in a 98% humidified incubator until complete cell attachment (Delos Reyes *et al*, 2015).

Cell viability assay

Presto Blue® (Molecular Probes®, Invitrogen, USA) were used in determining the cytotoxicity of the ethno botanical extracts in the cell viability test. One hundred (100) microliters of filter-sterilized ethanolic extracts were added to corresponding wells at two-fold serial dilutions to make final treatment concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/mL, and incubated for 4 days at 37°C in 5% CO2 and 98% humidity. Twenty microliters of Presto Blue® was added to each well and further incubation was done for 1 hr at 37°C in 5% CO2 and 98% humidity. Negative control without test samples was also prepared. Zeocin TM (Gibco®, USA) served as positive control. Test was done in three replicates (Delos Reyes *et al*, 2015).

Absorbance readings was taken with Bio Tek ELx800 Absorbance Micro plate Reader (Bio Tek Instruments, Inc.) at 570 nm and normalized to 600 nm values as reference wavelength to estimate for the half maximal inhibitory concentration, IC50 (the concentration of the extract which resulted in a 50% cell viability reduction). From absorbance reading, cell viability for each sample concentration was computed.

Statistical analysis

Data were analyzed by non-linear regression using Graph Pad Prism 6.05 (Graph Pad Software, Inc.) to extrapolate the half maximal inhibitory concentration, IC50. The extra sum-of squares F test was used to evaluate the difference in the best- fit parameter (half maximal inhibitory concentration) among data sets (treatment) and to determine the differences among dose-response curve fits according to the software's recommended approach (De Los Reyes *et al.*, 2015).

Results and discussion

Figure 1 shows the anti-proliferative and cytotoxicity effects of ethno botanical extracts at different concentrations on MCF-7 cells with the dose – response curves of ethno botanical ethanolic extract showing the relationship between concentration and

cell viability and cytotoxicity. Data exhibited the typical sigmoidal curve. The two-phasal response showed the inhibitory concentration of 0.5 µg/mL of the ethno botanical extracts from 82 to 70% cell viability. In the second phase, cell viability decreased steeply below 27 percent viable cell in log concentration of 0.5 µg/mL to 2 µg/mL. The maximum concentration of ethno botanical extracts (2µg/mL) inhibited 73% of the breast adenocarcinoma cancer cells. Data show higher concentration of ethno botanical extracts led to the decrease in cell viability. The concentration of 1 µg/mL to 1.5 µg/mL is the effective concentration to inhibit 50 percent cell proliferation. The maximal inhibitory response of the extracts is 2 µg/mL.

Table 1. Inhibitory concentration (IC50) of selected ethnobotanical ethanolic extracts against MCF-7.

Scientific name	IC50* value (µg/mL)	Remarks
ZEOCIN (Control)	9.394	Highly toxic
Lop-lopiit	11.69	Highly toxic
Ayapana triplinervis	14.73	Highly toxic
Alstonia scholaris	17.82	Highly toxic
Sarcandra glabra	18.64	Highly toxic
Derris elliptica	20.80	Toxic
Pittosporum pentandrum	22.46	Toxic
Cestrum nocturnum	22.85	Toxic
Oreonicde trinervis	24.85	Toxic
Ageratina adenophora	25.95	Toxic
Bidens pilosa	30.90	Non-toxic

The Inhibitory Concentration(IC50) value of ≤30 µg/mL is considered as having significant cytotoxic effect (Suffness and Pezzuto, 1990) after exposure time of 72 hours (Abdel-Hameed,2012). Moreover, crude extracts with IC50 of less than 20µg/mL is considered highly cytotoxic (Delos Reyes *et al.*, 2015). Table 1 shows the IC50 of the ethno botanicals against MCF-7.

Table 2. Inhibitory concentration (IC50) of selected ethnobotanical ethanolic extracts against normal primary dermal fibroblast, neonatal (HDFn).

Scientific name	IC50* value (µg/mL)	Remarks
ZEOCIN (control)	8.29	Highly toxic
Cestrum nocturnum	4.40	Highly toxic
Derris elliptica	10.94	Highly toxic
Pittosporum pentandrum	14.34	Highly toxic
Sarcandra glabra	17.50	Highly toxic
Lop-lopiit	18.99	Highly toxic
Bidens pilosa	21.05	Toxic
Alstonia scholaris	26.05	Toxic
Ageratina adenophora	29.39	Toxic
Oreonicde trinervis	32.18	Non-toxic
Ayapana triplinervis	36.86	Non-toxic

The ethnobotanicals exhibited high cytotoxicity on MCF-7 with respective IC50 values: Lop-lopiit (no known scientific name) (11.69 µg/mL), *A. triplinervis* (14.73 µg/mL), *A. scholaris* (17.82 µg/mL) and *S. glabra* (18.64 µg/mL). *D. ellipita* (20.80 µg/mL) while *P. pentandrum* (22.46 µg/mL), *C. nocturnum* (22.85 µg/mL), *O. trinervis* (24.85 µg/mL) and *A.*

adenophora (25.95 µg/mL) also showed least cytotoxicity effect to MCF-7.

The ethnobtotanicals and the different concentration applied to the breast adenocarcinoma cancer cells (MCF-7) revealed a decrease in cell viability. Ethnobotanical extracts of *C. nocturnum, S. glabra, O. trinervis, P. pentandrum*, Lop-lopiit (no knwon scientific name), *D. elliptica*, *A. scholaris*, *A. adenophora* and *A. triplinervis* showed anticancer activity against breast adenocarcinoma (MCF-7) cancer cells.

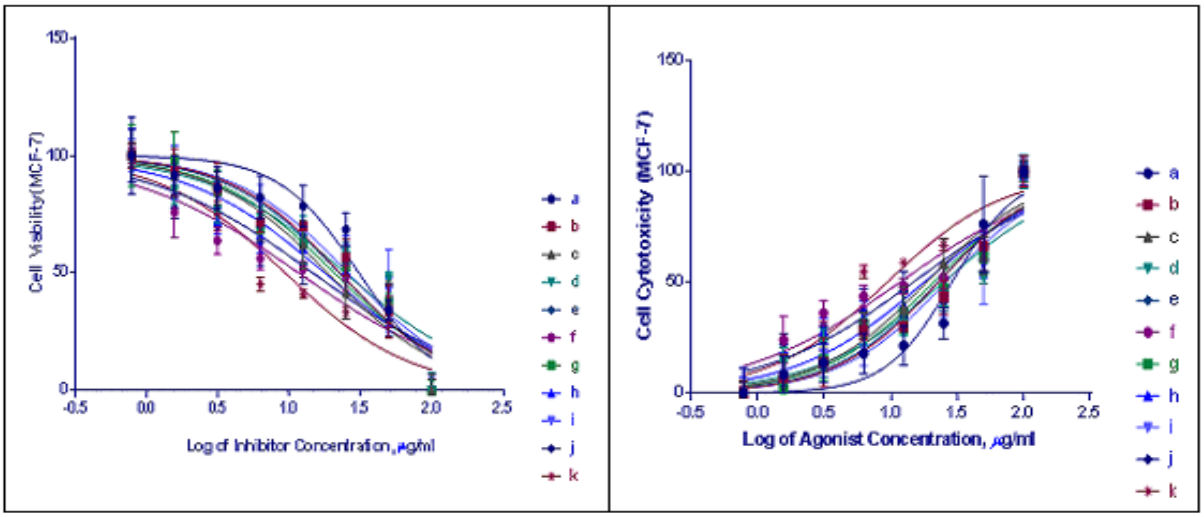

Fig. 1. Dose-response curves showing (A) inhibitory and (B) cytotoxicity activity of (a) *B. pilosa* (b) *C. nocturnum* (c) *S. glabra* (d) *O. trinervis* (e) *P. pentandrum* (f) Lop-lopiit (g) *D. elliptica* (h) *A. scholaris* (i) *A. adenophora* (j) *A. triplinervis* and (k) Zeocin (control) on breast adenocarcinoma cancer cell line (MCF-7). Each line shows the effect of the extracts against the cancer cell line.

Normal Primary Dermal Fibroblast, neonatal (HDFn)

Figure 2 shows the anti-proliferative and cytotoxicity of ethanolic ethno botanical extracts against normal primary dermal fibroblast, neonatal (HDFn). Data show the typical sigmoid curve characteristics of an inhibitory and cytotoxicity dose-response relationship between treatments. Fig. 2 shows the log concentration of the ethno botanical extracts that inhibit cell viability of the normal primary dermal fibroblast and shows the 83 percent to 61 percent decrease at 0.5 µg/mL while 1.5 µg/mL exhibited reduction from 60 to 55 percent of viable cells. Furthermore the cell viability decreased to 40 percent in 2 µg/mL.

The increase in extract concentration lead to gradual decrease in cell viability as higher concentration were found to be cytotoxic to cell. The concentration of 1.5 µg/mL to 2 µg/mL effective concentration exhibit 50 percent cell death to normal primary dermal fibroblast.

Table 2 shows the inhibitory concentration (IC50) values of the selected ethnobotanical extracts that is needed to reduce 50% cell viability on normal primary dermal fibroblast (HDFn). The ethno botanicals that exhibited high inhibition effects on normal primary dermal fibroblast are the following with their respective IC50 values: *C. nocturnum* (4.40 µg/mL); *D. elliptica* (10.94 µg/mL); *P. pentandrum* (14.34 µg/mL), *S. glabra* (17.5 µg/mL), and Lop-lopiit (18.99 µg/mL). *B. pilosa* (21.05 µg/mL), *A. scholaris* (26.05 µg/mL) and *A. adenophora* (29.39 µg/mL) showed least inhibition effect to normal primary dermal fibroblast (HDFn) based on NCI standard value of ≤30µg/mL.

The anticancer activity of the ethno botanicals against breast adenocarcinoma cancer cell (MCF-7) may be accounted to the presence of biologically active

compounds which act directly on the tested cancer cell line. *C. nocturnum* have been proven to have anticancer activity (Podolak *et al.*, 2010) and contains anticancer agents such as flavonoids, saponin and tannins. Flavonoids can inhibit DNA and protein synthesis (Makita *et al.*,1996; Tanaka *et al.*, 1997) and may block several points in the progression of carcinogenesis, including cell transformation, invasion, metastasis, and angiogenesis, through inhibiting kinases (Birt *et al.*, 2001). Saponin induce cycle (G1) arrest on the human breast cancer cell line (Haridas *et al.*, 2009) while tannins has anticancer property (Sadipo *et al.*, 1991; Rashed *et al.*, 2013). Tannin family like maplexins, have anti-cancer property against breast cancer cell line and ellitannin showed inhibit the proliferation of breast cancer (Barrajon-Catalan *et al.*, 2010) by down-regulation of cyclins A and B1 and up regulating of cyclin E, cell-cycle arrest in S phase, induction of apoptosis via intrinsic pathway (FAS- independent, caspase 8-independent) through bcl-XL down-regulation with mitochondrial release of cytochrome c into the cytosol, activation of initiator cascape 9 and effector caspase -3 (Larrosa *et al.*, 2006).

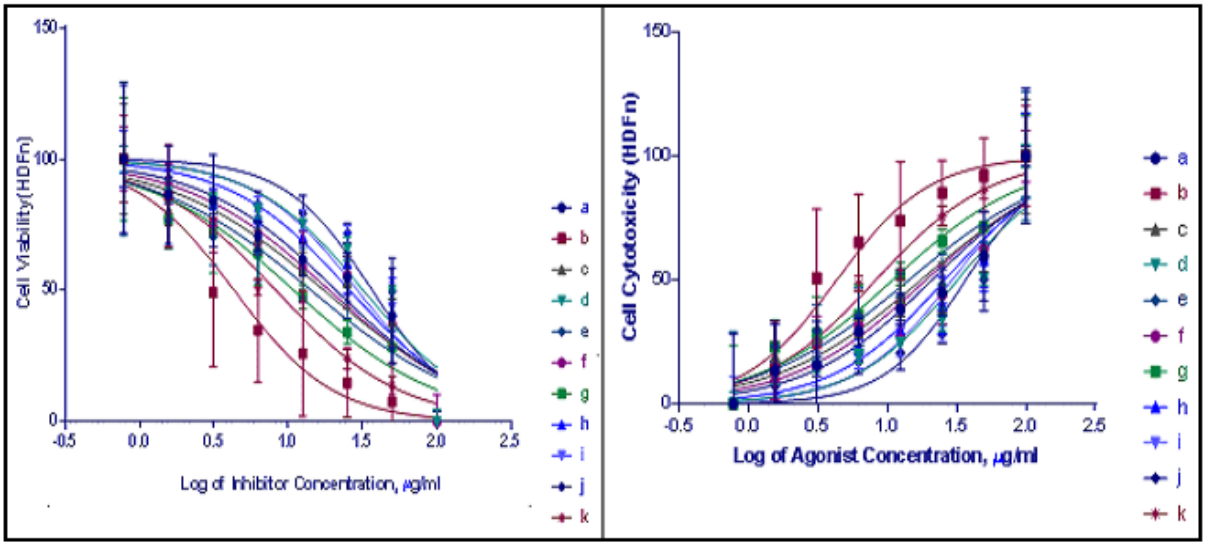

Fig. 2. Dose-response curves showing (A) inhibitory and (B) cytotoxicity activity of (a) *B. pilosa;* (b) *C. nocturnum* (c) *S. glabra* (d) *O. trinervis* (e) *P. pentandrum*; (f) Lop-lopiit (g) *D. elliptica*; (h) *A. scholaris* (i) *A. adenophora* (j) *A. triplinervis* and (k) Zeocin on normal primary dermal fibroblast (HDFn).

Other researches also reported the anticancer activities of *S. glabra* (Jiang *et al.* 2001). The phytochemical composition of *S. glabra* that includes phenolic acid, terpenoids, flavonoids, isofraxidin, triterpenoid, saponins, coumarins and sesquiterpenoids may possibly influence the proliferation of breast cancer. The secondary compounds of sesquiterpenoid such as curcumol and germacrone inhibits cell proliferation of breast cancer (Xu *et al.*, 2005; Zhong *et al.*, 2011). Similar studies shown the anticancer activity of coumarins (Nasr *et al.*, 2014; Bronikowska *et al.*, 2014). This compound exhibited its anticancer activity by reducing the mitochondrial-trans membrane-depolarization potential, regulating the mitochondrial Bcl-2 family pathway, increasing the pro-apoptotic factors Bid, Bad and Box expression and decreasing the expression of Bcl-xl and Mcl-1 (Sashidhara *et al.*, 2013).

O. trinervis has cytoprotective effects (Ansarullah *et al.*, 2011). *O. trinervis* contains flavonoid, tannin and terpenoid reported to have anticancer activity (Khan *et al.*, 1993; Oduro *et al.*, 2009). Terpenoids are known to induce cell death by apoptosis in various tumor cells (Yao *et al.*, 2008).

Shimamura *et al.* (2007) also reported the cytotoxic, antioxidant and antitumor activity of *P. pentandrum*. Formation of cancer cell is affected by the

antioxidants in plants. The antioxidant plays an important role in cancer prevention (Surya *et al.*, 2012) by reducing DNA mutations and adducts caused by cytosolic free radicals and consequently prevent the initiation of cancer through induction of mutations. Fayad *et al.* (2011) mentioned that a plant extract that combines antioxidants and anticancer activities is a promising cancer chemo preventive candidate. Anticancer activity is useful in early eliminating any newly formed neoplastic cells (Fayad *et al.*, 2011).

D. elliptica also showed anticancer activity in similar studies (Sapido *et al.*, 1991). It contains important secondary metabolites such as flavonoids, tannins and terpenoid which is reported to have anticancer activity (Okwu *et al.*, 2004; Chiu *et al.*, 2008) Flavonoids are known to have the capability to treat certain physiological disorders and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity which adds protection against all stages of carcinoma (Okwu *et al.*, 2004).
Ethanolic extract of *A. scholaris* has potent effects against breast cancer cells (Vikas *et al.*, 2010), and the result of this study also proves the anticancer activity of *A. scholaris* as reported by related studies(Kamarajan *et al.*, 1991; Zhisen *et al.*,1999; Ziegler *et al.*, 2002;Kessler *et al.*, 2003;Meena *et al.*, 2011). The plant is a rich source of flavonoids and alkaloids, which are known to have anticancer activity (Zheng *et al.*, 2003; Elisabetsky and Campos, 2006). Secondary compounds of alkaloids such as Etoposide, is a topoisomerase II inhibitor, stabilizing enzyme-DNA cleavable complexes leading to DNA breaks (Liu,1989); taxanes paclitaxel and docetaxel has been shown antitumor activity against breast and other tumor types. It stabilizes microtubules and leading to mitotic arrest (Wani *et al.*, 1971).

A. adenophora (Syn:*Eupatorium adenophorum*)has been reported for itsanti-tumor and anticancer activities mainly due to a sesquiterpene that has inhibited the growth of human cancer cell lines: HCT-8 (colon), Bel 7402 (liver) and A2780 (ovary) (Cao *et al.*, 2009; Ma *et al.*, 2015). Another sesquiterpene, elemene, has been isolated from *A. adenophora* (Hui-Ping *et al.*, 2014), and this phytochemical constituent exhibits broad-spectrum anti-cancer activity against many types of cancer cells including leukemia, brain, breast, prostate, ovarian, cervical, colon, laryngeal and lung carcinoma cell (Yuan *et al.*, 1999; Zou*et al.*, 2001; Li *et al.*, 2005; Wang *et al.*,2005; Yao *et al.*, 2008; Li *et al.*, 2010; Zhu *et al.*, 2011). The inhibition of β- elemene induced cancer cell proliferation mainly due to the apoptotic cell death and cell cycle arrest (Li *et al.*, 2005; Wang *et al.*,2005) through the mitochondrial- mediated caspase activation pathway (Li *et al.*, 2010; Wang *et al.*,2005).

Similar studies also showed the anticancer activity of *A. triplinervis* (Scio *et al.*, 2003;Riveiro *et al.*, 2004; Kawase *et al.*, 2005; Watanabe *et al.*, 2005;Riveiro *et al.*, 2009). The plant contains thymoquinone and coumarin which are known anticancer agents (Kawase, 2005; Nasr *et al.*, 2014). Thymoquinone suppresses human breast carcinoma both *in vitro* and *in vivo* models (Woo *et al.*, 2013). It demonstrated anti-proliferative and pro-apoptotic effects through its induction effect on p38 (Woo *et al.*, 2013); and reactive oxygen species, ROS signaling (Alhosin *et al.*, 2010; El-Najjar *et al.*, 2010).

The phytochemicals contained in the ethno botanicals tested are also known to possess anti- estrogen property which can affect the cell growth and proliferation of MCF-7. MCF-7 contained variable amounts of estrogen receptor (ER) and progesterone (PgR) (Osborne *et al.*, 2011). The antiestrogenic property induces apoptosis in breast epithelial cells via activation of caspases 9 and 7 to stop the cell proliferation. These biological active compounds are flavonoids, tannin, and coumarins (Musa *et al.*, 2008). Ethnobotanical extract with flavonoids, tannins and coumarins are *C. nocturnum, S. glabra, O. trinervis, P. pentandrum, D. elliptica, A. scholaris, A. triplenervis* and *A. adenophora*.

O. trinervis and *A. triplinervis*while exhibiting toxicity to MCF-7, remained non-toxic to normal cell line, primary dermal fibroblast (HDFn). This finding

makes these ethno botanicals ideal candidates for anti-cancer drug discovery and development.

This preferential toxicity may be due to the presence of phytochemical constituents that has the capacity to recognize cancer cells. Phytochemicals such assaponins can recognize cancer cellswhich are structurally different from normal cells (Rao and Sung, 1995). Cancer cell membrane contain more compounds like cholesterols. Saponin can disrupt cell membrane permeability by binding cholesterol in the membrane of cancer cells (Rao and Sung, 1995; Nainggolan and Kasmirul, 2015).

Saponins also exert antitumor activity through cell cycle arrest (Man *et al.*, 2010) and through the induction of cell death by programmed or nonprogrammed routes, hence used as agents to control cell proliferation (Escobar-Sanchez *et al.*, 2015). Furthermore, these plants contain other phytochemicals such asflavonoids, tannins, triterpenoids, terpenoids, phenolic, coumarins, sesquiterpenoid and polyacetylenes with known activities such as anti-carcinogen, anti-proliferation, cell cycle arrest, induction of apoptosis and inhibition of angiogenesis to different cancer cells (Charhar *et al.*, 2011). Additionally, natural phytochemical constituents have preventive mechanisms on tumor progression that ranges from the inhibition of genotoxic effects, proteases and cell proliferation, and protection of intercellular communications to modulate apoptosis and signal transduction pathways (Nawab *et al.*, 2011).

Aside from anti-cancer activities of the ethnobotanicals shown in this study, these plants also display biological activities such as anti-inflammatory (Judan Cruz *et al.*, 2018b), analgesic (Judan Cruz *et al.*, 2018b), anti-oxidant (Divina, 2015), anti-bacterial (Judan Cruz *et al.*, 2018b; Limos *et al.*, 2018; Padilla *et al.*, 2018) anti-gout (Jose, 2015), anti-quorum sensing (Limos *et al.*, 2018; Judan Cruz *et al* 2018a; Padilla *et al*, 2018). These biological activities can also influence inhibition on proliferation of cancer cells.

Conclusion

This study contributed to the increasing data on this group of ethno botanicals by confirming their anticancer activities which may be helpful in the development and formulation of anti-cancer drugs. Traditionally used by the Igorots of the Philippines, these plants present remarkable potential in drug discovery. Biological activities of these plants confirm this pharmacological significance.

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