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IN-VITRO SCREENING OF SOME PLANT EXTRACTS FOR THEIR POTENTIAL ANTICANCER ACTIVITY

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Abstract

Background: Natural products have been shown to be reliable sources of anticancer medicines although there is still a consistent demand for new therapeutic natural products for cancer treatment with minimal side-effects.
Materials and Methods: In this study, six plant extracts (*Grevillea robusta; Euphorbia millii; Euphorbia royleana; Aloe grandidentata; Bauhinia corniculate;* and *Cassia fistula*) were screened for the presence of phytochemical metabolites as saponins, tannins, cardiac glycosides, alkaloids, flavonoids, anthraquinones and sterols, using qualitative tests. Antiproliferative screening assay was performed on a panel of three cancer cell-lines (HepG-2, HCT-116 and MCF-7) using MTT assay, and cytotoxicity was determined using WI-38 human fibroblast cell- line.
Results: Some plant extracts reduced cellular growth for the selected cancerous cell-lines. For example, E. royleana and A. grandidentata extracts reduced HepG-2 cellular growth with IC50 of 0.42 and 0.53µg/mL, respectively. Moreover, A. grandidentata and C. fistula reduced cellular growth of MCF-7 with IC50 of 0.37 and 0.67 µg/mL, respectively.
Conclusion: *E. royleana*, *A. grandidentata* and *C. fistula* showed significant anti-proliferative activity against HepG-2 and MCF-7 cell-lines with non-cytotoxic nature. This suggests their potential role as anticancer agents against these types of cancer. The presence of flavonoids, sterols and anthraquinones may suggest their enhanced anti-proliferative activities. Therefore, this study has shed light on the possible use of these extracts as potential sources of natural products-based therapy for cancer.

Keywords: Anti-proliferative, Cancer, Cytotoxic, Natural products, Plant extracts.

List of abbreviations: MDR: Multidrug resistance; OD: Optical density, SI: Selectivity index, DMSO: Dimethyl sulfoxide, IC_{50}: Inhibitory concentration, NCI: National cancer Institute and HSBR: Helwan Structural Biology center.

Introduction

Cancer is the second leading cause of deaths globally, and it is responsible for approximately 70% of deaths (~9.5 million deaths) in low- and middle-income countries in 2018 (Bray *et al*., 2018). In Egypt, the global cancer burden has doubled in the last thirty years of the twentieth century, and it is estimated that this will double again between 2000 and 2020, and nearly triple by 2030 (Ibrahim *et al*., 2014). In Egyptian males, liver cancer is the highest cancer type with crude rate of 39.5%, while in females, breast cancer is the most dominant with crude rate of 35.8% (Ibrahim *et al*., 2014). In addition, colon cancer is considered the fourth prevalent type of cancer in Egypt, which is diagnosed in elderly people one decade younger than the corresponding age in the USA (Bajpai *et al*., 2018; Metwally *et al*., 2018). National population-based cancer registry program showed that 3.47% of male cancers and 3% of female cancers are colon cancers, excluding rectal one. However, from 2015 till now, newly-diagnosed patients are increasing (Ibrahim *et al*., 2014). Chemotherapeutic treatments for all these types of cancers are necessary for both surgery inappropriate patients or after surgical resection to improve prospects for survival and surgery outcomes (Lage, 2008). However, multidrug resistance (MDR) as well as associated severe side-effects such as neurological, cardiac, renal and pulmonary toxicities are seriously affecting the health of the patients and future prognosis (Wen *et al*., 2016).

Therefore, alternative natural products have been the focus of much recent research and became an urge for effective therapeutic strategy to obtain desirable results. The term "natural product" is generally referred to as "organic secondary metabolite" that originated from plants, animals or microorganisms (Kinghorn *et al*., 2009b). These natural compounds have considerable structural diversity that tend to be in the correct chiral form to exhibit biological activity either as single metabolites or in combined mixture of metabolites (Kinghorn *et al*., 2009a). In the last 40 years, natural products have played a very important role in different biological activities either in their natural isolated form or synthetically- modified forms such as doxorubicin, actinomycin and mitomycin C. In the field of drug discovery in oncology, several classes of plant-derived compounds have been documented as natural, bioactive products with potent anticancer activities such as alkaloids, camptothecins and taxane (Lichota and Gwozdzinski, 2018). Others, such as flavonoids, terpenoids, polysaccharides, saponins are also of much interest as anticancer agents which act either individually or in combination with other agent/s to regulate immune function, inducing apoptosis or autophagy, or inhibiting cell proliferation (Rayan *et al*., 2017). Indeed, nature is the best source of natural anticancer agents. Our interest is mainly in the identification of new potent anticancer natural products that overcome the limitations of cell toxicity and adverse reactions.

Grevillea robusta A. Cunn. ex R.Br. (commonly known as "Silky oak") is a flowering evergreen tree belonging to family Proteaceae. It is native to subtropical rainforests of eastern Australia. However, the plant has been cultivated in many regions worldwide, including the African continent, for ornamental purpose. A wide array of bioactive secondary metabolites have been reported from different parts of the tree, with 5-alkylresorcinol derivatives being major phytoconstituents with characteristic antileishmanial and anticancer activities (Ahmed *et al*., 2000; Takahashi *et al*., 2004).

The genus *Euphorbia* is probably one of the largest genera of the Euphorbiaceae family that is commonly distributed in tropical regions. Traditionally, many species of genus *Euphorbia* have been routinely used for the treatment of various human aliments such as pathological skin conditions, headache and intestinal infections. The later has encouraged many researchers for uncovering the biochemical potential of genus Euphorbia. Multiple species of *Euphorbia* have been documented to exhibit diverse biological activities, including anti-inflammatory, antioxidant, antidiabetic and anticancer (Mwine and Van Damme, 2011). In particular, *Euphorbia milii* Des Moul (commonly known as "Christ plant") is a small shrub or subshrub native to Madagascar and also cultivated in many other regions for outdoor ornamental purpose due to its characteristic red flowers. Diverse phytoconstituents have been isolated and characterized from *E. milii* species, with diterpene ingenol derivatives being the characteristic class of secondary metabolites. *Euphorbia royleana* Boiss is another spiny shrub that has been traditionally used in the treatment of various human disorders, including asthma, jaundice, loose motion and pain (Sabeen and Ahmad, 2009). Diverse secondary metabolites have been reported from its latex, with cycloartane triterpene derivatives being the major bioactive constituents with anti-inflammatory and antarthritic attributes (Bani *et al*., 2000).

Aloe grandidentata Salm-Dyck is a prominent member of the family Asphodelaceae that is reputable of being native to Arabian and African regions. The genus *Aloe* is notable for its divergent medicinal attributes such as laxative, wound healing, hypoglycemic and anti- ulcerative activities. Such therapeutic characteristics are inevitably attributed to the wide array of secondary metabolites that have been isolated and chemically characterized form different species of Aloe, such as anthraquinones, sterols, terpenoids and flavonoids. However, limited chemical and biological studies have been published, reporting the chemical and pharmacological profiles of *Aloe grandidentata* (Ibrahim *et al*., 2013).

The genus *Bauhinia* is one of the largest genera of the Fabaceae family, as it comprises approximately more than 500 species of diverse shrubs and small trees located abundantly in tropical regions. A considerable number of species belonging to this genus have been historically implemented in folk medicine of many communities to control various ailments such as inflammation, pain and infection (Filho, 2009). *B. corniculata Benth* is an example of a species that attracted herbal practitioners in folk medicine (Murad *et al*., 2011), yet the chemical and pharmacological profiles of this plant have not been elucidated.

Cassia fistula Linn (known as "Golden shower") is a deciduous medium-sized tree belonging to the legume family, Fabaceae. The plant is native to tropical and subtropical regions of South Asia and is cultivated as ornamental plant in many other countries worldwide, including Egypt. *C. fistula* has been widely used in traditional medical practice, including Traditional Chinese Medicine (TCM) and Ayurvedic, for treatment and prevention of many diseases such as constipation, skin and liver diseases (Mondal *et al*., 2014). As a consequence of its interesting health benefits, many research groups have focused on elucidating the scientific basis for the traditional uses of *C. fistula* via chemical and pharmacological characterization of its bioactive secondary metabolites. Diverse phytoconstituents, including anthraquinone derivatives, flavonoids, terpenoids and sterols have reported from different extracts of *C. fistula*. An array of pharmacological studies have reported the activity *C. fistula* as antimicrobial, radical scavenging and wound healing effects of the plant in diverse in vitro and in vivo models (Rahmani, 2015).

Herein we report a preliminary antiproliferative effect of the methanolic extract of six plants growing in Egypt that have been used by herbal practitioners in folk medicine of different communities. The MTT-based cell viability assay was used to assess the effect of different doses of the plants extracts on the growth pattern of three cancerous cell-lines (HepG-2, HCT-116 and MCF-7). The in vitro cell safety index was assessed by comparing the antiproliferative IC_{50} values against cancerous cells to the cytotoxic IC_{50} values against the non-cancerous WI-38 human fibroblast cells.

Materials and methods

Collection and identification of plant materials

Aerial parts of *Grevillea robusta* A. Cunn. ex R.Br. (Proteaceae) *Euphorbia milii* Des Moul (Euphorbiaceae), *Euphorbia royleana* Boiss (Euphorbiaceae), *Aloe grandidentata* Salm-Dyck (Asphodelaceae), *Bauhinia corniculate* Benth (Fabaceae) and *Cassia fistula* Linn. (Fabaceae) were collected from Al Waleed Garden, Helwan Agricultural Road, Helwan, Egypt, between June and August 2018. All the plants were at the flowering stage during the time of collection, except *B. corniculate*. The plants were identified morphologically by Dr. Trease Labib, consultant of plant taxonomy at Al Zohria Botanical Garden, Giza, Egypt and voucher specimens' numbers 1Gro/2018, 1Emi/2018, 1Ero/2018, 1Agr/2018, s1Bcor/2018, 1Cfi/2018 were assigned to the collected plants, respectively. Samples were set in shade for a week till dryness and then powdered.

Preparation of extracts for cytotoxic screening

About ten grams of each plant powder were macerated in 100 mL methanol for one week with occasional shaking at room temperature. Methanolic extracts were filtered through Whatman® grade 1 filter paper, dried at 50 °C under vacuum, using Buchi® R-200 rotary evaporator, New Castle, DE, USA.

Chemical reagents

Mayer's (**US Pharmacopeia, USP29**), Dragendroff's **(Khatun *et al.*, 2014)** and Baljet's (**Kokate, 2001**) reagents were freshly prepared and used.

Qualitative detection of main classes of secondary metabolites

Preliminary chemical profiles of the major secondary metabolites of each plant powder was accomplished through the following qualitative chemical tests. We used different solvents to categorize the phytoconstituents according to their degree of polarity.

Aqueous extract

Saponins: Froth test (**Kokate, 2001**)
Tannins: Ferric chloride test **(Houghton and Raman, 2012)**

HCl (1%) extract

Alkaloids: Mayer's and Dragendorff's tests **(Khatun *et al.*, 2014)**
Flavonoids: NaOH and Shinoda tests **(Houghton and Raman, 2012)**

Aqueous methanol (50%) extract

Anthraquinones: Borntrager's test **(Houghton and Raman, 2012)**
Cardiac glycosides: Baljet's test (**Kokate, 2001**)

Dichloromethane extract

Sterols and/or triterpenes: Liebermann-Burchard test **(Houghton and Raman, 2012)**

Cell culture

The different cancer cell-lines, including human colon cancer cell-lines (HCT116), human liver cancer cell-line (HepG2) and breast cancer cell-line (MCF-7) in addition to normal human lung fibroblast cell-line (WI-38) were obtained from Egyptian company for production of vaccines (VACSERA) and deposited in HSBR laboratory. All cell-lines were cultured in their suitable media (HCT116: McCoy's 5a; HepG2 and MCF-7: DMEM-high glucose and WI-38: EMEM) supplemented with 10% FCS, 2mM-glutamine and 100 units each, of penicillin and streptomycin at 37 °C with 5% CO2. Culture media were replaced every 3–4 days. Upon 85-90% confluency, cultures were passaged using appropriate techniques for downstream application using 0.25% trypsin/EDTA solution.

Plant extract solutions

All plant extract residues were prepared in stock solutions (as shown in Table 1) by dissolving them in DMSO (Sigma-Aldrich) and the aliquots were stored at −20°C in the dark. Working aliquots were diluted using fresh cell culture media and ensuring complete solubility.

Proliferation assay

Assessment of relative numbers of viable cells will be done using MTT tetrazolium assay (SERVA, Germany) **(Styczynski *et al.*, 2002)**. Briefly, all cells were seeded in 96-well plate at density of 20,000 cells/well for 24-hour incubation at standard conditions. Next day, all plant extracts were evaluated in this assay in triplicates at

five doses concentration for 24 hours as indicated in Table 1. Ten µL of MTT reagent in fresh media (5 µg/mL) was added to each well. The reaction was stopped after 4-hour incubation by adding 100 µL of DMSO for 20 min at 37°C to form violet formazan crystals, and the OD was measured at 550 nm with a microplate reader (800TSUV Biotek ELISA Reader). Cells treated with 5-fluorouracil (5-FU) as standard chemotherapeutic agent are positive control. Negative control cells are those treated with 0.1% DMSO solvent vehicle only. The SI was calculated and compared to WI-38 normal cell-line.

Results

Phytochemical analysis

Flavonoids and sterols were detected in all tested extracts, while alkaloids and cardiac glycosides were also absent in all (Table 1). Moreover, tannins were found in all extracts except *G. robusta*, and saponins were detected only in *Euphorbia* species. Anthraquinones were observed only in *A. grandidentata* and *C. fistula*

Anti-proliferative activity

All tested plant extracts were evaluated using different cell-lines for calculation of IC_{50} after treatment in 2- and 10-fold dilution concentrations according to Table 2, for reducing the rate of growth and proliferation in previously mentioned cancer cell-lines compared to normal cell-line (WI-38). *E. royleana* and *A. grandidentata* showed strong antiproliferative activity against HepG-2 cancer cell-line (IC_{50} = 0.42 and 0.53µg/mL) respectively, with maximum selectivity index compared to mild effect of *G. robusta* and *E. milii* (IC_{50} = 89.5 and 87.1µg/mL) respectively (Figure 1, Table 3). *B. corniculate* extract was the only plant extract that showed anti-proliferative activity against HCT-116 cell-line (IC_{50} = 71.6 µg/mL) with maximum selectivity index. *A. grandidenta*, and *C. fistula* showed strong anti-proliferative activity against MCF-7 (IC_{50} = 0.37 and 0.67µg/mL) respectively, with maximum selectivity index (Figure 1, Table 3).

Table 1: Phytochemical analysis of the main secondary metabolites in the plant extracts studied.

Secondary metabolites	*G. robusta*	*E. milii*	*E. royleana*	*A. grandidentata*	*B. corniculata*	*C. fistula*
Saponins	-	+	+	-	-	-
Tannins	-	+	+	+	+	+
Alkaloids	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+
Anthraquinones	-	-	-	+	-	+
Sterols and/or triterpenes	+	+	+	+	+	+

Qualitative tests were repeated three times to confirm and to ensure the accuracy and consistency of results. +: present, -: absent

Table 2: Stock and calculated working solution concentrations used in proliferation assay for the plant extracts studied.

Studied plants	*Stock solution* (µg/mL)	*Working solutions conc.* (µg/mL)				
		Conc. (1)	Conc. (2)	Conc. (3)	Conc. (4)	Conc. (5)
G. robusta	22,00	220	110	55	5.5	0.55
E. milii	35,00	350	175	87.5	8.75	0.88
E. royleana	79,000	395	197.5	19.75	1.98	0.20
A. grandidentata	8,000	160	80	40	4	0.40
B. corniculate	16,000	160	80	40	4	0.40
C. fistula	35,000	350	175	87.5	8.75	0.88

All plant extracts were prepared and dissolved as stock solutions in DMSO and working solutions were prepared in aqueous cell culture consequently ensuring complete dissolution.

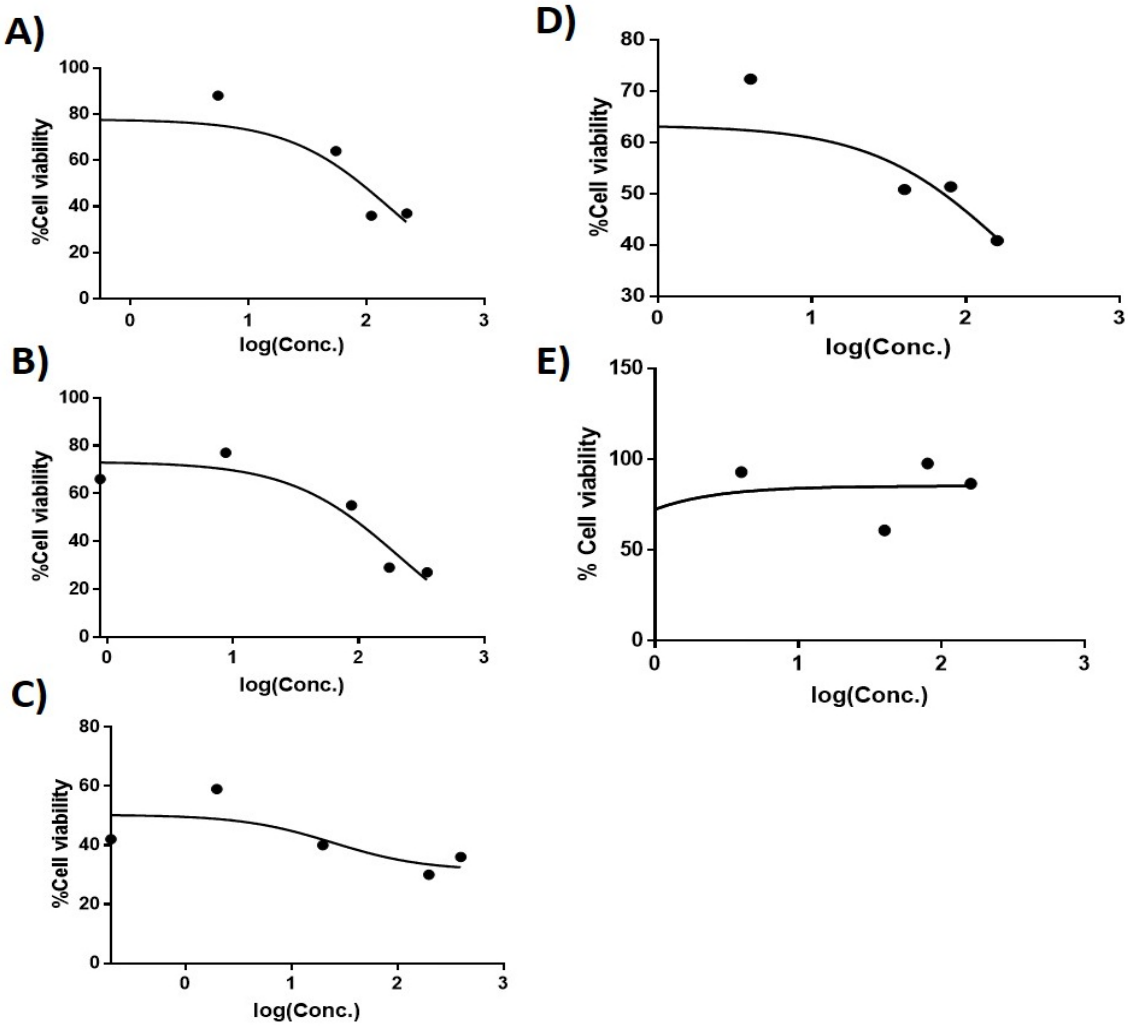

Figure 1: Dose-response curves showing antiproliferative effects of tested plant extracts on sensitive cell-lines; (A) *G. robusta* on HepG-2 (B) *E. milii* on HepG-2 (C) *E. royleana* on HepG-2 (D) *Ba. corniculate* on HCT-116 and (E) *A. grandidentata* on MCF-7. Log concentrations (in µg/mL) were used against percentage cell viability to present the nonlinear sigmoidal graphs (using three parameters).

Table 3: In-vitro anti-proliferative activities of tested extracts and standard chemotherapy 5-FU.

Studied plants and reference standard drug	IC_{50} ± SEM and corresponding SI							
	WI-38	HepG-2	SI	HCT-116	SI	MCF-7	SI
G. robusta	249.5±10.7	89.5±6.3	2.8	*N/A*	<1	199.1±25.7	1.25
E. milii	N/A	87.1±9.4	>1	*N/A*	---	*N/A*	---
E. royleana	N/A	0.42±0.7	>1	285.1±19.2	>1	*N/A*	---
A. grandidentata	N/A	0.53±0.5	>1	812.8±30.7	>1	0.37±0.2	>1
B. corniculate	N/A	*N/A*	---	71.6±17.1	>1	*N/A*	---
C. fistula	148.6±12.4	445.6 ±25.8	0.3	*N/A*	<1	0.67±0.6	>1
5-FU	>100	>100	1	>100	1	5.6 ± 0.89	~ 17.8

N/A = not active on the cell line. Data represent mean IC_{50} ± SEM, n=3 and corresponding SI (selectivity index) calculated as IC_{50} plant extract (WI-38)/ IC_{50} plant extract (cancer cell line).

Discussion

Medicinal plants are valuable sources of natural products such as alkaloids, cardiac glycosides, triterpenoids, diterpenes and so forth, while some as flavonoids have been documented to have nutritional values (Chun *et al*., 2007; Middleton, 1996). Many of the isolated natural products have diverse therapeutic applications in both in-vitro and in-

vivo studies such as wound healing (Nagori and Solanki, 2011), anti-inflammatory (Zhang *et al*., 2005; Shia, Juang *et al*., 2009; Zengin *et al*., 2016; Wang *et al*., 2019), antibacterial (Sofowora *et al*. 2013; Mujeeb *et al*., 2014) and anticancer activities (Sreelakshmi and Abraham, 2016). First stage of traditional preliminary phytochemical screening is using qualitative tests to determine the presence of any of these key compounds in total or fractions of plant. The next stage is the biological screening, such as anti-proliferation-cytotoxicity screening of natural products represented by IC_{50} and SI, using a panel of different independent cancer cell-lines as well as normal cell-line. This is essential for unbiased model during the interpretation of results to determine the selectivity of plant extracts against different cell types. Based on this hypothesis, the current work introduces a pilot study to evaluate six different phytochemical total extracts from traditional medicinal plants growing in Egypt for their potential anticancer activity against three cancer cell-lines HepG-2, HCT-116, MCF-7 and a normal fibroblast cell-line, WI-38. Both *E. royleana* and *A. granidentata* showed very potent antiproliferative activity against liver carcinoma HepG-2 cells, with IC_{50} values 0.42 and 0.53 µg/mL, respectively. It is worth to mention that NCI defines a cut-off concentration limit as 30 µg/mL in order to consider a plant-derived extract as bioactive by in-vitro screening anticancer assays (Fouché *et al*., 2008). This clearly explains the extraordinary potencies of these extracts, which are is also documented by approximately 70% - folds as potent as the standard cut-off concentration limit set by NCI. In particular, *E. rolyleana* is known to biosynthesize terpenoid class of secondary metabolites, which are prominent to exhibit anticancer properties against diverse cancer cell-lines by targeting multiple cancer-related proteins and oncogenic pathways. Additionally, *A. grandidentata* is also notable for its anthraquinones and flavonoids contents (Table 1). In particular, anthraquinones are reputable for their interference with DNA dynamics, either via inhibition of DNA topoisomerases or direct intercalating with DNA double strands (Qiao *et al*., 2008), which will lead to DNA damage and ultimately hampering the uncontrolled cancer cell proliferation. Besides, the methanolic extract of *C. fistula* exhibited exceptional antiproliferative potency against the breast adenocarcinoma-derived MCF-7 cells with IC_{50} value down to 0.67 µg/mL. Interestingly, the standard anticancer drug 5-FU showed antiproliferative activity with IC_{50} value of 5.6 µg/mL.

This truly indicates the superiority of this plant extract over the standard 5-FU in inhabiting the unrestrained cancer cell division. This potent activity may be, in part, correlated with the phenolic contents of *C. fistula*, tannins and flavonoids. Phenolics have been extensively reported in literature for their promising anticancer effects, mostly via induction of cell cycle arrest and apoptosis (Dai and Mumper, 2010). On the contrary, IC_{50} values of methanolic extracts of *G. robusta*, *E. milii* and *B. corniculata* were all greater than the 30 g/mL against the three tested cancer cell-lines accomplished by the MTT-based cell proliferation assay. These results are quite surprising, despite the fact that these plants share the commonality of flavonoids and tannins based on the obtained results from the preliminary phytochemical screening assays. This can be due, in part, to the empiric fact that the anticancer effects of polyphenols are generally fluctuating and depends on multiple factors, including structural framework, concentration and the type of cancer cells (Yáñez *et al*., 2004). In this regard, it was also reported that the antiproliferative effect of flavonoids is critically affected by the C2-C3 degree of unsaturation, and also the number and nature of substituents on A and B rings. In addition to that, it has been reported that even minor modifications in the chemical structure of flavonoids can result in a strong variation of the biological response (Benavente-Garcia and Castillo, 2008). It was observed also that presence of anthraquinones in both *A. gandidentata* and *C. fistula* can explain the marked enhancement of anti-proliferative activity of these two extracts against HepG-2 and MCF-7, and this was matched with the literature documenting its role for selective inhibition of cancer cells (Bajpai *et al*., 2018; Deitersen *et al*., 2019; Shrestha *et al*., 2015). The SI value of plant extracts represents the safety margin for therapeutic uses of such extracts as a therapy or supplement with minimal adverse effects (Deitersen *et al*., 2019; Molina-Salinas *et al*., 2019), and this was reflected in many of the presented extracts. However, in those extracts with SI>1 and above, further fractionations with single pure compounds will give clear picture on the extent of selectivity of these fractions in therapeutic applications.

Conclusion

In summary, extracts of *E. royleana*; *A. Gandidentata* and *C. fistula* have shown strong reduction in in-vitro tumor cell proliferation assay, suggesting that further studies are needed to identify how these natural extracts could be used as a complementary approach to currently used chemotherapies for different cancers. Also, more studies will be needed to investigate the molecular mechanisms underlying their potential anticancer activities. Altogether, these natural extracts hold a promise as an adjuvant treatment to prevent tumor cell growth.

Conflict of Interest/Competing Interests

All the authors revised the manuscript and agreed on the contents of the article and post no financial, personal or organizational conflicting interest that may affect this research article.

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