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Journal of Medicinal Plants Research

Review

Genus *Etlingera* - A review on chemical composition and antimicrobial activity of essential oils

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Essential oil from plants belonging to several species has been extensively used as natural resources in the prevention and treatment of a large number of diseases. The medicinal and aromatic plants of genus *Etlingera* produces high percentages of essential oil from its every organ and is recommended for a variety of health problems by traditional systems of medicine in south-east Asia. Essential oils from this genus demonstrated promising antimicrobial properties. As antibiotic resistance is emerging at an alarming rate, many infectious diseases have become difficult to treat. Thus, the genus *Etlingera* can be considered as a good source of essential oils, and extensive studies of biological activities of these oils may lead to the identification of new compounds which can be used in modern medicine, cosmetics and pharmaceutical industry, primarily as antimicrobial agents. This review summarizes the characteristics of essential oil of *Etlingera* species with particular attention to the chemical composition and antimicrobial activities from the data in the recent literature.

Key words: *Etlingera*, essential oil, chemical composition, antimicrobial activity.

INTRODUCTION

Etlingera is a genus of medicinal plants native to the Indo-Pacific region. It has been used by indigenous communities for its flavor, culinary and medicinal properties since antiquity. The well-established traditional uses may be explained by the presence of biologically active volatile components in this genus. However, despite the increasing scientific interest in this field, there is a lack of summarized data on herbal medicine composition, therapeutic applications and risks associated to their consumption. Therefore, the purpose of this article is to provide an overview of the published data results regarding chemical composition and antimicrobial activities of essential oil of *Etlingera* species.

Essential oils (EOs), also known as volatile or ethereal oil or aetherolea, are concentrated hydrophobic liquid containing volatile aroma compounds obtained from aromatic plants. An oil is “essential” in the sense that each plant oils contains characteristic “essence” of the plant's fragrance from which it is derived. Essential oils are generally highly odorous, volatile with penetrating taste. Although, their consistency is more like water than oil, it is lighter than water and give transparent to pale yellow color. It is a complex mixtures of various compounds containing about 20-100 components at quite

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different concentrations. Chemically, they are derived from terpenes and their oxygenated compounds and also include non-terpenic compounds such as alcohols, aldehydes, ethers, ketones, phenols, oxides and phenylpropanoids. Many essential oils have limited solubility in aqueous solutions but are soluble in alcohol, non-polar or weakly polar solvents, waxes and oils.

The term 'essential oil' was used for the first time in the sixteenth century by the Swiss reformer of medicine, Paracelsus von Hohenheim, who named the effective component of a drug as Quinta essential (Guenther, 1948). They are not essential for health but have been used to relieve a wide variety of human maladies including bronchitis, pneumonia, pharyngitis, diarrhea, periodontal disease, wounds and numerous other illnesses for thousands of years (Boire et al., 2013). Today, the term 'essential oils' is used to describe complex mixtures of low molecular weight (usually less than 500 daltons) compounds extracted from aromatic plants using conventional techniques. At present, around 3000 essential oils are known, of which 300 are commercially important especially used as flavoring agents in food products, drinks, perfumeries, pharmaceuticals and cosmetics.

Morphology of *Etlingera* species

The genus *Etlingera* belonging to Zingiberaceae family consists of more than 100 species. They are perennial herbs mainly grown in perhumid forest (Poulsen, 2007). The leafy shoots of some of the *Etlingera* species can be up to eight m tall and the bases of these shoots are so stout as to seem almost woody. Many of the *Etlingera* species grow as clumps of leafy shoots, while others have such long creeping rhizomes that each of their leafy shoots can be more than a meter apart. True, *Etlingera* is characterized by its unique and distinctive flowers which have exceptional ornamental value. The inflorescence shoots are found short and do not emerge from the ground. The flowers are characterized with prominent bright red petal-like structures (labella) radiating outward, with the flower tubes and ovaries being below ground level. The brightly colored flowers are thought to be pollinated by insects. Fruits ripen below ground, and the seeds are thought to be dispersed by wild pigs. These plants are very aromatic because of the high content of essential oil in its leaves, stems, flower, fruit and rhizomes.

Distribution

Etlingera is dominant in Indo-pacific terrestrial, native to India, Bangladesh, Burma, China, Laos, Cambodia, Vietnam, Thailand, Malaysia, Singapore, Indonesia, Philippines, Brunei, Papua New Guinea, Queensland and several Pacific Islands, predominantly close to the equator between sea level and 2500 m (Poulsen, 2006; Wojdyło et al., 2007). *Etlingera* are also naturalized in other warm places such as Hawaii, Puerto Rico, Trinidad, Central America, Mauritius and the islands of the Gulf of Guinea (Govaerts et al., 2011). These species are also cultivated in gardens, especially in Mexico and western parts of Indonesia (Java) as an ornamental plant and a source of condiment and spice. A total of 155 names of *Etlingera* species have been accepted in the World Checklist of Selected Plant Families facilitated by the Royal Botanic Gardens at Kew, England (Govaerts et al., 2011). Borneo rainforest is exceptionally rich and presently, at least forty species are known in this forest (Poulsen, 2006). Of these species, eighty-five percent of the *Etlingera* species in Borneo are endemics and thirty-three percent are found in Brunei Darussalam. Three new species of *Etlingera: Etlingera rubromarginata* (from Sabah, Sarawak and Brunei), *Etlingera belalongensis* (from the Temburong District of Brunei), and *Etlingera corrugata* (presently only known from Danum Valley, Sabah) from northern Borneo have recently been described (Poulsen et al., 1999). *Etlingera kenyalang* from Sarawak and *Etlingera palangkensis* from Central Kalimantan have also been reported (Poulsen and Christensen, 2003; Takano and Nagamasu, 2006). Fifteen *Etlingera* species in Malaysia Peninsular, nine species in Java, Indonesia and only three species (one endemic and one introduced) have been recorded in China (Chen and Boufford, 2000).

Traditional application

Plants of *Etlingera* have been used since ancient times as spice and vegetable as well as for medicinal purposes. The common traditional uses of different *Etlingera* species are shown in Table 1.

More specialized uses of few species include perfume (rhizome of *Etlingera baramensis*), shampoo (fruit of *Etlingera elatior* and *Etlingera pyramidosphaera*) and spice (rhizome of *Etlingera punica)* (Chan et al., 2013). Several other species of *Etlingera* also have been used as medicine in the prevention and therapy of diseases (e.g. rheumatism: *Etlingera foetens*; jaundice, fever, urinary ailments: *E. belalongensis;* stomach-ache*: E. pyramidosphaera;* snake bite: *Etlingera sessilanthera,* diarrhea: *E. pyramidosphaera*) (Poulsen, 2006; Sabli et al., 2012; Sirirugsa, 1997).

ESSENTIAL OILS IN COMMERCIAL PREPARATIONS

Several European countries have developed some essential oil-based industry in last few decades. Carvon, the principal constituent of the dill and caraway seeds EO, is currently marketed as Talent® in the Netherlands.

Table 1, Traditional uses of different plant parts of *Etlingera* species.

***Etlingera* species**	**Plant parts and preparation**	**Traditional use**	**Reference**
E. elatior	Young shoot, flower bud	As condiment	Abdelmageed et al. (2011b) and Noweg et al. (2003)
	Fruit decoction	In treating earache	
	Leaf	As wound cleaner, and body odor remover	
	Inflorescence	Food	Chan et al. (2007)
E. brevilabrum	Leaf (green)	To treat dry skin of the legs	Mahdavi (2014)
	Leaf (roasted)	To treat fever by rubbing on the bodies of children	
	Sap of heated young stem	To treat sore eyes	Poulsen (2006)
	Stolon	To cure stomachache	
E. linguiformis	Leaf and shoot	As vegetable	Kithan and Daiho (2014) and Ramana et al. (2012)
	Rhizome	To treat jaundice, sore throat, stomachache, rheumatism and respiratory complications	
	Sliced rhizome (with betel leaf)	To cure sore throat	Hossan et al. (2013)
E. labellosa	Juice of pseudostem	To cure body ache	Poulsen (2006)
E. littoralis	Young shoot, Flower bud, Fruit (raw or cooked)	As condiment	Noweg et al. (2003)
E. rubrolutea	Decoction of rhizome	To treat stomach ache, and as carminative and heart tonic	Chuakul and Boonpleng (2003)
E. coccinea, E. sessilanthera, E. volutina, E. rubromarginata	Inner sheath of leafy shoot	As condiment	Chan et al. (2007) and Poulsen (2006)
E. fimbriobracteata	Fruit	As condiment	
E. maingayi	Flower	As vegetable	

The preparation is used to inhibit the growth of storage pathogens and to suppress sprouting of potatoes in the warehouse (Hartmans et al., 1995). Soil Technologies Corporation (USA) has developed two natural products named Fungastop™ and Armorex™ which are commercially available for the control of various plant diseases in agriculture (Dubey et al., 2012). Eugenol based formulations (eugenol-Tween®;

eugenolethoxylate) showed potent inhibitory effect against four apple pathogens (*Phlyctema vagabunda, Penicillium expansum, Botrytis cinerea* and *Monilinia fructigena)* and thus used in post-harvest disease management of apple fruit (Amiri et al., 2008). Cinnamite™ (cinnamon), Valero™ (rosemary), Promax™ (thymus) and several other essential oil based pesticides are already commercially available (Prakash et al., 2015). EOs or their components (α-bisabolol, geraniol, elemene, *d*-limonene, diallyl trisulfide (DATS) and Eucalyptol) have been shown to exhibit cancer suppressive activity against glioma, colon cancer, gastric cancer, human liver tumor, pulmonary tumors, breast cancer, leukemia and others (De Angelis, 2001). Essential oils rich in terpinolene and/or eugenol have shown antioxidative activity against low density lipoprotein (LDL) oxidation thereby reducing the chance of atherosclerosis (Edris, 2007). Essential oils and their components are exploited for antibacterial properties in diverse commercial products as dental root canal sealers, antiseptics and feed supplements for lactating sows and weaned piglets (Burt, 2004). A few preservatives containing EOs are already available in the market, such as DMC Natural base, which comprises 50% essential oils (Speranza and Corbo, 2010). Beside these, essential oil and their individual constituents exhibited antiviral, antimycotic, antiparasitic and insecticidal properties (Bakkali et al., 2008; Dubey et al., 2010).

EXTRACTION PROCEDURES OF ESSENTIAL OIL

Extraction of essential oils can be achieved by various methods such as distillation, solvent extraction, effleurage, aqueous infusion, cold or hot pressing, supercritical fluid extraction, solvent free microwave extraction (SFME) and phytonic process. The method of extraction is normally dependent on what type of botanical material is being used. It is the key step that determines the quality of the oil as wrongly executed extraction method can damage the oil and alter the chemical signature of the essential oil. Today, hydro-distillation (with a collecting solvent that is then removed under vacuum) and steam distillation are widely used for extracting essential oils from plants. Volatile components in these methods can be distilled at temperatures lower than their individual boiling points and are easily separated from condensed water. Losses of some volatile compounds, low extraction efficiency, degradation of unsaturated or ester compounds of these widely used conventional methods have led to the consideration of the use of new “green” technique in essential oil extraction, which typically use less solvent and energy, such as supercritical fluids, ultrasound and microwave extraction. Berka-Zougali et al. (2012) describes a new innovative method, solvent free microwave extraction (SFME), which yields an essential oil with higher amounts of more valuable oxygenated compounds and allows substantial savings of costs, in terms of time, energy and plant material. However, essential oils obtained by SFME were quantitatively (yield) and qualitatively (aromatic profile) similar to those obtained by conventional method (hydro-distillation), while SFME is highly effective for reducing extraction time (30 min for SFME against 180 min for hydro-distillation) (Périno-Issartier et al., 2013). In *Etlingera* species, hydro-distillation technique is extensively used for extracting essential oils except from the flower of *E. elatior,* where steam distillation is used.

Yield and chemical composition of essential oil of *Etlingera*

Essential oils are derived from almost all parts of plant (leaf, stem, flower, peduncle, bark, rhizome, seed and fruit) in *Etlingera* species. Total oil content in this genus was found very low and rarely exceeds 1% by mass. For example, the essential oil yields in leaf, stem, flower, peduncle, rhizome and whole plant of *Etlingera* species were found in the range 0.031 - 1.94%, 0.001 - 0.02%, 0.014 - 0.9%, 0.005 - 0.1% 0.006 - 1.4% and 0.004 - 0.07% (w/w) respectively as shown in Table 1. Leaves showed the highest yield, while least percentage was obtained from stems. The ranking was in the order: leaf>rhizome>flower>peduncle>whole plant>stem. Essential oil yields of the same plant parts can also vary on the time of collection (Vahirua-Lechat et al., 2010). Leaves of *Etlingera cevuga* showed the highest percentage of yield than other *Etlingera* species. The most extensively studied plant in *Etlingera* species is *E. elatior* and the plant part is rhizome.

Essential oils and their components have gained wide acceptance by consumers because of promising biological activities, safety, and exploitation for potential multi-purpose uses. The chemical composition of the *Etlingera* (around 21 different species including 2 varieties) essential oil has been described by many authors that has been summarized in Table 2.

Table 2 shows that different species *Etlingera* are dominated by different chemical components. Vahirua-Lechat et al. (2010) analyzed the essential oil of *E. cevuga* by capillary gas chromatography and combined GC/MS. Thirty-one components were identified where methyl eugenol (40.9-45.7%) and (E)-methyl isoeugenol were the major constituents. The author also reported that the percentage of major chemical constituents of *E. cevuga* varied with the location of plant. Chemical compositions of essential oil for the *Etlingera* species were found different in different parts of the same plant (Jaafar et al., 2007; Khaleghi et al., 2012a, b). Many reports demonstrated that the fragrance and chemical composition of essential oils can vary based on geography (soil type, climate, altitude, amount of water available, harvesting season) and method of preparation

Table 2. Yield and percentage of major chemical component(s) identified in *Etlingera* species.

Etlingera Species	Plant Source	Plant Part[a]	Yield (%)	Major Constituent	% Composition	Reference
E. brevilabrum	Borneo	R	0.28	Elemicin -	35.6	Vairappan et al. (2012)
				Methyl isoeugenol	19.2	
				β-Farnesene	10.7	
	Malaysia	L	0.24	β-pinene	52.6	Mahdavi et al. (2012)
				α-thujene	28.6	
				o-cymene	7.8	
		S	0.07	limonene	28.6	
				β-pinene	21.6	
				α-thujene	13.9	
		R	0.03	Eucalypto	27.6	
				β-pinene	13.4	
				Caryophyllene oxide	10.5	
				α-thujene	10.1	
E. cevuga	Malaysia	L	0.03	α-thujene	38.1	Mahdavi et al. (2016)
				p-cymen-7-ol	8.0	
		Stolon	0.016	β-pinene	30.6	
				p-cymen-7-ol	25.0	
		S	0.011	δ-3-carene		
				α-thujene	17.7	
		R	0.018	Perilla aldehyde	19.6	
				Bornyl acetate	17.6	
	Tahiti Island	R	NM	Methyl eugenol	47.4	Vahirua-Lechat et al. (2010)
				(Z)- and (E)-methyl isoeugenol	18.8	
		L	0.48-1.94	Methyl eugenol	40.9–45.7	
				(E)-methyl isoeugenol	8.6–16.5	
				α-pinene	6.9-11.6	
				β-pinene	5.6-10.3	
E. coccinea	Borneo	R	0.3	Borneol	28.3	Vairappan et al. (2012)
				L-calamenene	18	
E. elatior	Borneo	R	0.38	1-Dodecanol	46.2	Bhuiyan et al. (2010)
				Cyclodecanol	34.1	

Table 2. Contd.

Malaysia	L	86 mg/100 g	(E)-farnesene	13.6	Chiang et al. (2010)
			(E)-caryophyllene	8.56	
Malaysia	L	0.0735	(E)-ß-farnesene	27.9	Jaafar et al. (2007)
			ß–pinene	19.7	
			caryophyllene	15.36	
	S	0.0029	1,1-dodecanediol diacetate	34.26	
			(E)-5-dodecene	26.99	
			1,1- dodecanediol diacetate	24.38	
	F	0.0334	cyclododecane	40.32	
			α-pinene	6.3	
	R	0.0021	1,1- dodecanediol diacetate	47.28	
			cyclododecane	34.45	
Malaysia	WP	NM	β-pinene	24.92	Abdelwahab et al. (2010)
			1-dodecene	24.31	
			Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl	11.59	
Malaysia	L	0.7	myrcene	13.5	Wong et al. (2010)
			a-humulene	11.8	
			b-caryophyllene	10.7	
	R	0.4	Camphene	18	Wong et al. (2010)
			b-pinene	16.9	
			bornyl acetate	9.2	
Malaysia	F	NM	dodecanal	16.87	Susanti et al. (2013)
			n-dodecyl acetate	16.4	
			1-hexadecanol	16.34	
			cis-9-tetradecen-1-ol	16.29	
			1-decanol	16.27	
Malaysia	L-6[b]	0.1	2-cyclohexen-1-one	93.42	Abdelmageed et al. (2011a)
	L-24	0.1	β-pinene	39.1	
			1-Dodecanol	16.03	
	L-48	0.16	β-pinene	37.3	
			1,6,10-Dodecatriene	-	
			1-Dodecanol	10.9	
	L-72	0.14	1-Dodecanol	42.3	
			Dodecanal	19.21	

Table 2. Contd.

				β-pinene	8.16
		PS-6	0	-	-
		PS-24	0.013	2-Tridecanone	51.5
				1-Tridecyne	12.18
		PS-48	0	-	-
		PS-72	0.007	Dodecanal	22.43
		F-6	0	-	-
		F-24	0.1	-	-
		F-48	0.08	1-Dodecanol	54.48
				Dodecanal	16.53
				Undecanoic acid	10.1
		F-72	0.1	1-Dodecanol	53.12
				Dodecanal	17.27
				n-Decanoic acid	12.33
		R-24	0.047	1-Dodecene	41.6
				Dodecanal	32.9
				1-Dodecanol	48.15
				Dodecanal	25.99
		R-48	0.013	1-Dodecanol	63.64
				Dodecanal	17.01
		R-72	0.02	1-Dodecanol	54.3
				Dodecanal	20.17
				1-Tetradecanol	7.81
	Brazil	F	0.9	Dodecanol	42.5
				α-pinene	22.2
				Dodecanal	14.5
		P	0.1	Dodecanol	34.6
				caryophyllene oxide	22.5
				Dodecanal	21.5
				α-pinene	6.3
	Indonesia	F		1-dodecanol	13.82
				dodecanal	12.10
				17-pentatriacontene	10.52
E. elatior vai Thai queen	Malaysia	L	0.9	a-pinene	24.4
				dodecanol	18.9

Zoghbi and Andrade (2005)

Maimulyanti and Prihadi (2015)

Wong et al. (2010)

Table 2. Contd.

				dodecanal	15.9	
		R	0.6	camphene	15.1	
				dodecanol	12.9	
				dodecanal	10.6	
E. fimbriobractata	Drunei Darussalam	L	0.126	β-pinene	67.8	Ud-Daula et al. (2016)
				α-pinene	17.1	
		AS	0.031	1,8-cineole	37.2	
				β-pinene	18.2	
		BS	0.031	decanal	27.5	
				1,8-cineole	18.7	
				β-pinene	10.6	
		R	0.018	decanal	34.4	
				β-pinene	10	
E. fulgens	Malaysia	L	133 mg/100 g	dodecyl acetate	21.6	Chiang et al. (2010)
				pentadecanol	14.1	
	Malaysia	L	Very Little Amount	(Z)-9-Hexadecen-1-ol	15.04	Khaleghi et al. (2012b)
				cyclotetradecane	8.93	
				oleyl alcohol	8.72	
				(Z)-11-hexadecen-1-ol acetate	7.74	
		S	0.0173	n-dodecyl acetate	16.62	
				cyclodecane	12.22	
				(Z)-9-hexadecen-1-ol	7.21%	
		R	0.0069	n-dodecyl acetate	16.28	
				cyclotetradecane	13.48	
				cyclododecane	11.3	
				cadinene	9.71	
		F	0.0493	n-dodecyl acetate	25.71	
				cyclododecane	22.91	
		F		cyclodecane	9.43	
		P	0.0213	(E)-2-tetradecene	26.24	
				cyclododecane	16.21	
				n-dodecyl acetate	15.18%	
		WP	0.0143	n-dodecyl acetate	18.59	

Table 2. Contd.

				cyclododecane	8.61	
				cyclotetradecane	6.16	
		L	0.2	α-phellandrene	12.1	
				diprene	11	
		S	0.03	1,8-cineole	16.9	
				α-phellandrene	11.5	
				β-trans-ocimene	7.9	
E. hornstedia	Malaysia	R	0.04	1,8-cineole	17.4	Yahya et al. (2010)
				α-phellandrene	9.5	
				1S-α-pinene	9.4	
		WP	0.07	β-pinene	13	
				p-menth-1-en-8-ol	8.5	
				α-pinene	8.1	
				α-phellandrene	8.1	
		L	1.15	eucalyptol	20.02	
				β-pinene	15.7	
				α-pinene	11.76	
	India			linalool	9.48	Kithan and Daiho (2014)
				methyl chevicol	49.93	
E. linguiformis		R	1.4	methyl eugenol	32.3	
				ß-pinene	13.46	
				Asarone	10.5	
		L	1.15	eucalyptol	39.7	
	Bangladesh			β-pinene	13.3	Bhuiyan et al. (2010)
		R	2.4	methyl chevicol	49.9	
				methyl eugenol	32.3	
				(E)-methyl isoeugenol	37.7	
E. littoralis	Malaysia	L	0.2	b-pinene	30.4	Wong et al. (2010)
				b-phellandrene	8.6	
		R	0.2	(E)-methyl isoeugenol	58.1	
E. maingayi	Malaysia	L	1317 mg/100 g	dodecanoic acid	44.6	Chiang et al. (2010)
				decanoic acid	42.6	

Table 2. Contd.

E. megalocheilos	Borneo	R	0.25	Aromadendrene oxide Terpineol oxide Aromadendrene	24.8 13 8.9	Edris (2007) and Vairappan et al. (2012)
E. pavieana	Thailand	R	0.07	trans-anethole p-anisaldehyde d-cadinene (7.2%).	48.6 13.8 7.2	Tachai et al. (2014)
E. punicea	Thailand	R	NM	Methyl chavicol	95.73	Tadtong et al. (2009)
E. pyramidosphaera	Borneo	R	0.45	Lauryl acetate 1-Tetradecano 1-Dodecanol Lauricaldehyde	29.6 20.2 28 11.2	Vairappan et al. (2012)
E. rubrostriata	Malaysia	L	39 mg/100 g	dodecyl acetate dodecanoic acid	6.01 4.41	Chiang et al. (2010)
E. sphaerocephala	Malaysia	L	0.17	δ-cadinene nerolidol	12.49 9.21	Jani et al. (2011)
		S	0.02	nerolidol limonene α-phellandrene	7.64 7.14 6.2	
		R	0.03	isoeugenol α-pinene β-pinene	9.9 9.23 8.4	
E. Sphaerocephala var. grandiflora	Malaysia	L	0.17	α-phellandrene diprene	12.3 10.3	Yahya et al. (2010)
		S	0.02	1,8-cineole α-phellandrene 1S-α-pinene	17.4 9.7 9.5	
		R	0.3	1,8-cineole α-phellandrene β-trans-ocimene	16.8 12.7 8.9	

Table 2. Contd.

		WP	0.05	β-pinene α-pinene p-menth-1-en-8-ol α-phellandrene	12.2 8.6 8.5 8.5	
E. sayapensis	Malaysia	L S R		Carvone cis-carveol a-terpineol linalool formate Linalool formate Eugenol	21.35 13.49 39.86 30.55 25.47 11.84	Mahdavi et al. (2017)
E. venusta	Malaysia	L	0.031	α-Terpineol linalool 1-methyl-3-(1-methylethenyl) cyclohexene (9.7 %),	13.4 10.9 9.7	Khaleghi et al. (2012a)
		AS	0.001	1-methyl-3-(1-methylethenyl) cyclohexene α-terpineol α-pinene	8.3 8.1 7.9	
		R	0.006	dodecanoic acid (37.8 %), cyclododecane (Z)-13-octadecen-1-ol acetate	37.8 15.8 10.9	
		F	0.014	Cyclododecane dodecanoic acid cyclododecanol	17.1 15.6 11.5	
		P	0.005	(E)-2-tetradecene cyclododecane cyclotetradecane	28.5 17.6 15.7	
		WP	0.004	Carene dodecanoic acid n-hexadecanoic acid	8 6.6 6.1	
E. yunnanensis	Vietnam	L	0.25	germacrene D β-pinene α-amorphene	19.2 11.6 11.2	Guo et al. (2015)

Table 2. Contd.

S	0.20	β-pinene	23.7
		1,8-cineole	11.0
		α-pinene	9.6
Ro	0.31	β-pinene	31.9
		α-pinene	13.7
		1,8-cineole	9.4
R	0.14	estragole	65.20
		β-caryophyllene	6.4
		1,8-cineole	6.5

[a] L - Leaf, S - stem, PS - pseudostem, R - rhizome, Ro-root, F - flower, P - peduncle, WP - whole plant. [b] drying time in hours, NM: not mentioned.

(Luna, 2002). Essential oil concentration is also fluctuated during its different stages of ripening (Wannes et al., 2009). These variations in chemical composition lead to the notion of chemotypes, which are generally defined as a chemically distinct entity in a plant with different compositions of the secondary metabolites(Djilani and Dicko, 2012). An essential oil chemotype can distinguish *Etlingera* of different origins, as well as seasonal variations throughout the vegetative cycle of plants.

Because chemotypes are defined only by the most abundant secondary metabolite, *Etlingera brevilabrum* may be assigned to two different chemotypes, depending on dominant component of the essential oil; elemicin and eucalyptol. Such chemotypes may be indicated as *E. brevilabrum* ct. elemicin, or *E. brevilabrum* ct. eucalyptol, although such indication has no taxonomic standing. Individuals of these chemotypes have vastly different chemical profiles, varying in the abundance of the kind of the next most abundant chemical. This can be a very qualitative assessment of an individual's chemical profile, under which may be hiding significant chemical diversity. Plant genotype is another important factor that can also affect the changes of the chemical composition of essential oils (Djilani and Dicko, 2012). Therefore, genetic and epigenetic factors influence the biochemical synthesis of essential oils in a given plant resulting in different chemical compositions and therapeutic activities.

Essential oil compositions of *Etlingera* were also varied on drying condition of the plants (Abdelmageed et al., 2011a; Mahdavi et al., 2012). The major constituents found in fresh rhizomes of *E. brevilabrum* were elemicin (35.6%) and methyl isoeugenol (19.2%) whereas eucalyptol (27.6%) and β-pinene (13.4%) were abundant in air-dried rhizomes of same species collected from Sabah, Malaysia (Mahdavi et al., 2012). Abdelmageed et al. (2011a) reported that fresh and dried of same plants parts (leaves, pseudostems, flower and rhizomes) of *E. elatior* produced different oil composition with increasing drying time. The author also found that different drying periods had an effect on the percentage of the main compounds and resulted in slight losses in volatile compounds compared with the fresh herb. Some authors also reported that the concentrations of various volatile substances increased after air drying (Díaz-Maroto et al., 2002; Faridah et al., 2010). This might be due to chemical transformation (breakdown of glycosylated forms, dehydration reactions, or oxidation reactions) or loss of compounds during drying process or due to the rupture of the plant cells in which the volatiles are stored.

The major constituents of *Etlingera* leaf essential oil are 1,8-cineole, 1-dodecanol, dodecyl acetate, elemicin, eucalyptol, methyl eugenol, (E)-methyl isoeugenol, α-pinene, β-pinene, and thujene. Stem of the plants contains mainly (E)-5-dodecene, 1,1-dodecanediol diacetate, 1,8-cineole, limonene, β-pinene and α-phellandrene, whereas cyclododecane, 1,1- dodecanediol diacetate, dodecanal, n-dodecyl acetate, 1-decanol and 1-hexadecanol are dominant in flowers. The main essential oil components in the rhizome are aromadendrene oxide, trans-anethole, 1,8-cineole, 1-dodecanol, n-dodecyl acetate, decanoic acid methyl chevicol, lauryl acetate, (E)-methyl isoeugenol as well as other compounds including cyclododecane, dodecanoic acid, linalool, α-pinene, β-pinene and α-

phellandrene. Cyclododecane, dodecanol, dodecanal, (E)-2-tetradecene and caryophyllene oxide are the dominant component in peduncle essential oil and it is followed by α-pinene n-dodecyl acetate and cyclotetradecane. Analysis of whole plant essential oil composition showed that caryophyllene oxide n-dodecyl acetate, α-pinene, β-pinene, nerolidol, phellandrene, cyclododecane and 1-dodecene are abundant in *Etlingera* species.

Classification of *Etlingera* essential oil

Essential oil compounds can be classified into three main categories: terpenes (monoterpene hydrocarbons and sesquiterpene hydrocarbons), terpenoids (oxygenated monoterpenes and oxygenated sesquiterpenes) and non-terpenic compounds. Terpenes are hydrocarbons derived from five carbon atoms attached to eight hydrogen atoms regarded as isoprene units (C_5H_8). They form structurally and functionally diverse classes of organic compounds. They are synthesized in the cytoplasm of plant cells, where two molecules of acetic acid are combined to form mevalonic acid ($C_6H_{12}O_4$) (Dhifi et al., 2016). Terpenes are usually grouped according to the number of isoprene units in the molecule, which can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures. Monoterpenes ($C_{10}H_{16}$) contain two isoprene units; three sesquiterpenes ($C_{15}H_{24}$); four diterpenes ($C_{20}H_{32}$); six triterpenes ($C_{30}H_{48}$); and eight tetraterpenes ($C_{40}H_{64}$). *Etlingera* essential oils are abundant with monoterpenes and sesquiterpenes but longer chains also exist. Themonoterpene hydrocarbons found in *Etlingera* essential oils include α-pinene, β-pinene, α-phellandrene and limonene as seen in Table 3. On the other hand, cadinene, caryophyllene, (E)-farnesene and tetradecadiene are the major sesquiterpene hydrocarbons observed in *Etlingera* oil.

Terpenoids can be thought of as modified terpenes which undergo biochemical modifications *via* enzymes that add oxygen molecules and move or remove methyl groups (Williams et al., 1989). It can be sub-divided according to the number of isoprene units: monoterpenoids (2 isoprene units), and sesquiterpenoids (3 isoprene units) etc. The principle monoterpenoids found in *Etlingera* essential oil are 1,8-Cineole, 1,1-dodecanediol diacetate, eucalyptol, linalool and α-terpineol while caryophyllene oxide and nerolidol are major sesquiterpenoids.

Terpenic compounds are dominant in all parts of *Etlingera* species except *E. fulgens* and *Etlingera venusta.* Monoterpene hydrocarbon was found in greater percentage in essential oil of *Etlingera* species followed by oxygenated monoterpene, sesquiterpene and oxygenated sesquiterpene. Among different plant parts (leaves, stems, flowers, peduncle and rhizomes), leaves are found to contain highest proportion of monoterpene hydrocarbon than any other parts.

Phenylpropanoids (non-terpenic compounds) are a wide-spread class of plant-derived natural products synthesized from the amino acid precursor, phenylalanine. They serve as essential components of a number of structural polymers, provide protection from ultraviolet light, defend against herbivores and microbial attack and acts as signaling molecules (Korkina, 2007).

In *Etlingera* essential oils, most thoroughly studied phenylpropanoids are elemicin, eugenol, methyl eugenol, (*E*)-methyl isoeugenol and methyl chavicol. Out of 16 different *Etlingera* species, they were identified only in 8 species such as *Etlingera bravlabrum* (R), *E. cevuga* (L,R*), Etlingera linguiformis* (R), *E. linguiformis* (R) *Etlingera littoralis* (L,R), *E. pavieana* (R*), E. punica* (R), *Etlingera sphaerocephala* (R)*, E. venusta* (L) (Bhuiyan et al., 2010; Khaleghi et al., 2012a; Tachai et al., 2014; Vahirua-Lechat et al., 2010; Vairappan et al., 2012; Wong et al., 2010; Yahya et al., 2010).

Various other groups of non-terpenic compounds such as alcohol, aldehyde, ketone, esters, carboxylic acid and hydrocarbon were also observed in *Etlingera* species. Examples of this group of compounds are dodecanoic acid, decanoic acid, dodecyl acetate, dodecanol, cyclododecane and (E)-2-tetradecene. *E. fulgen, Etlingera fimbriobracteata* and *E.venusta* are characterized by high proportion of non-terpene hydrocarbon, alcohol and esters (Chiang et al., 2010; Khaleghi et al., 2012a; Ud-Daula et al., 2016).

However, *E. bravilabrum, E. cevuga, E. coccinea, E. elatior* (S, R)*, E. linguiformis, E. littoralis, E. pyramidosphaera* and *E. Sphaerocephala var. grandiflora* are devoid of these compounds (Jaafar et al., 2007; Kithan and Daiho, 2014; Mahdavi et al., 2016; Vahirua-Lechat et al., 2010; Vairappan et al., 2012).

ANTIMICROBIAL ACTIVITY

Test of antimicrobial assays of essential oils in *Etlingera*

An overview of the literature reporting antimicrobial assays of *Etlingera* EOs is presented in Table 4.

Determination of antimicrobial activity of *Etlingera* EOs was generally done by agar disk diffusion, agar dilution and broth micro dilution method. Screening of antimicrobial properties of EOs is generally by the agar disk diffusion method, where a sterile paper disk impregnated with EO is laid on top of an inoculated agar plate.

This method is not considered an ideal method for essential oils, normally used as a preliminary check for antimicrobial activity prior to more detailed studies. Agar dilution or broth microdilution methods are most widely used methods to determine minimum inhibitory

Table 3. Classes of major *Etlingera* essential oils compounds and their bioactivities in different plant parts.

Chemical class	Chemical subclass	Plant parts	Subclass content			Major EO compound	Bioactivity	References for bioactivity
			Highest (%)	Lowest (%)	Absent			
Terpene	Monoterpene hydrocarbons[a] $C_{10}H_{16}$	L	[a]e.b(89.8)	es(7.1)	ef, em, er	β-pinene, α-thujene, myrcene	Stimulant, antiviral, antitumor, decongestant, antibacterial, hepatoprotective	Bakkali et al. (2008) Kalemba and Kunicka (2003)
		S	eb(66.8)	e sp(26.5)	ef	α-pinene, β-pinene, α-phellandrene, limonene,		
		F	ee(47.5)	ev(8.3)	ef	β-pinene,		
		P	ee(6.3)	-	ef,ev	α-pinene, β-pinene		
		R	esv(56)	epv(2.1)	eb(1A), ec, eco, elt, ef, eme, epy, ev	α-pinene, β-pinene, α-phellandrene		
		WP	esv(53.3)	ev(21.9)	ef	α-pinene, β-pinene, phellandrene,		
	Sesquiterpene hydrocarbons[b] $C_{15}H_{24}$	L	ee(45)	eh(0.94)	eb(1B), ec, ef, elt, em, er, egr, ev	(E)-farnesene, dodecyl acetate	Aphicide, Antiviral, Local anaesthetic	Edris (2007) and Nerio et al. (2010)
		S	e sp(13.7)	egr(3.5)	ef	caryophyllene		
		F	ee(9.9)	-	ef,ev	Tetradecadiene		
		P	-	-	ee, ef, ev	-		
		R	eb-1B(47)	eh(3.45)	ec,el, elt,epu, epy, ev,	cadinene, caryophyllene		
		WP	ee(4.5)	egr(3.3)	ev	n-dodecyl acetate		
Terpenoid	Oxygenated monoterpenes	L	ev(58)	esp(6.5)	ec,ef,elt,em,	1,8-Cineole, α-Terpineol, linalool, eucalyptol	Antimicrobial, Antioxidant, insecticidal	Edris (2007) Kalemba and Kunicka (2003) and Nerio et al. (2010)
		S	ee(54.3)	esp(11.5)	-	1,8-cineole, 1,1-dodecanediol diacetate		
		F	ee(30.8)	ev(22.8)	ef	α-Terpineol		
		P	-	-	ee,ef,ev	-		
		R	epy(80.1)	epv(0.6)	ec,ef,elt,epu	1,8-cineole, Borneol		
		WP	ev(31.2)	egr(22.6)	ee,ef	α-Terpineol		
	Oxygenated sesquiterpene[c]	L	esp(17.7)	eh(0.13)	eb(1B),ec,ef,el,elt,em, esv	caryophyllene oxide	Antimicrobial, analgesic, anti-inflammatory	Kalemba and Kunicka (2003)
		S	esp(10.1)	eh(0.32)	ef, egr, ev	nerolidol		
		F	-	-	ee, ef, ev	caryophyllene oxide		
		P	-	-	ee, ef, ev	caryophyllene oxide		
		R	eme(28.6)	eh(0.7)	eb(1A), ec, ee, ef, el, elt, epu, esv, ev	nerolidol		
		WP	ev(7.7)	-	ee, ef, esv	nerolidol, caryophyllene oxide		
Non-terpene	alcohol, aldehyde, ketone, esters, carboxylic acid, hydrocarbon	L	eh(0.29)	ef(85.78)	eb(1B), el, egr	dodecanoic acid ,decanoic acid, dodecyl acetate, (E)-methyl isoeugenol, methyl eugenol	Antimicrobial, Antioxidant, chelating agent	Edris (2007) Kalemba and Kunicka (2003)
		S	ef(65.84)	eh(0.13)	eb(1B), ee, esv	(E)-5-dodecene, n-dodecyl acetate		
		F	ef(84.06)	ev(58.5)	-	Cyclododecane, dodecanol		

Table 2. Contd.

and phenylpropanoids					
	P	ev(96.4)	ee(59.3)	-	(E)-2-tetradecene, dodecanol, dodecanal, cyclododecane
	R	epu(95.8)	eh(0.34)	eb(1B), eco, ee, eme, epy, esv	methyl chavicol ,1-Dodecanol, methyl isoeugenol, eugenol, Dodecanal, trans-anethole
	WP		eh(0.09)	esv	cyclododecane, 1-dodecene

'-' indicates not mentioned. [a]eb: *E. brevilabrum*, ec: *E. cevuga*, eco: *E. coccinea*, ee: *E. elatior*, eet: *E. elatior* vai Thai queen, ef: *E. fulgens*, eh: *E. hornstedia*, elt: *E. littoralis*, el: *E. linguiformis*, eme: *E. megalocheilos*, em: *E. maingayi*, ep: *E. pavieana*, epu: *E. punicea*, epy: *E. pyramidosphaera*, er: *E. rubrostriat*, es: *E. Sphaerocephala*, esv: *E. Sphaerocephala* var. grandiflora, ev: *E. venusta*

concentration (MIC) (Rios et al., 1988).

MIC is generally regarded as the most important parameter to confirm resistance of micro-organisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents (Andrews, 2001). In addition to MIC, some author also stated minimum bactericidal concentrations (MBCs) and bacteriostatic concentration (Susanti et al., 2013).

ANTIMICROBIAL EFFECTS OF *ETLINGERA* EOS

Researchers expedite their relentless venture to discover and synthesize new antimicrobial agents due to emergence of antibiotic resistance, increased population with lower immunity, undesirable effects of current drugs, etc. Plant molecules are well known for their antimicrobial properties; especially many essential oils and their components are known to possess significant activity against wide range of microorganisms (Bakkali et al., 2008; Bassolé and Juliani, 2012). Therefore, detail study of the plant EOs could be helpful to identify novel drugs and targets for controlling the infectious diseases worldwide. The antibacterial activity of *Etlingera* essential oils against gram-positive and gram-negative bacteria were reported in few studies and obtained results is presented in Table 4.

Tadtong et al. (2009) indicated antimicrobial effect of *E. punicea* rhizome essential oil on some pathogenic bacteria, particularly *Staphylococcus aureus*, *Escherichia coli* and *Salmonella albany,* and also possesses fungicidal activity against *Candida albicans*. Essential oils from rhizomes of *E. pyramidosphaera, E. megalocheilos, E. coccinea*, *E. elatior* and *E. brevilabrum* were tested against four strains of clinical bacteria (*S. aureus*, *Staphylococcus* sp., *Streptococcus pyrogenes* and *Salmonella enteritidis*), where *E. megalocheilos, E. coccinea* and *E. elatior* inhibited all the four tested bacteria with MIC values of less than 10 µg/mL, and the other two *Etlingera* species, *E. pyramidosphaera* and *E. brevilabrum* also showed interesting selective inhibition against *S. aureus* and *S. pyrogenes*, with MIC values ranging from 40.0 to 120.0 µg/mL (Vairappan et al., 2012). The author also claimed that the dominant presence of borneol (28.3%) and the availability of camphor (3.8%) could possibly be responsible for potent antibacterial activity.

In the study of Chiang et al. (2010), the oils from leaves of *E. elatior*, *E. fulgens* and *E. maingayi*, and *E. rubrostriata* showed inhibition against gram-positive bacteria of *B. cereus*, *M. luteus* and *S. aureus* with MIC values ranging from 6.3-100 mg/ml and ranking was in the order: *E. maingayi* > *E. rubrostriata* > *E. elatior* > *E. fulgens*. Of the gram-positive bacteria, *M. luteus* was the most susceptible with all four *Etlingera* species having MIC of 6.3 mg/ml, whereas no activity was observed on Gram-negative bacteria of *E. coli*, *P. aeruginosa* and *S. choleraesuis* (Chiang et al., 2010).

Dodecanoic (lauric) acid and decanoic (capric) acid which constitute more than 87% of *E. maingayi* oil content might be responsible for strong antibacterial activity. The oils (leaves, aerial stems, basal and rhizomes) from *E. fimbriobracteata* exhibited moderate to potent broad-spectrum antimicrobial activity against gram positive (*Bacillus subtilis, Bacillus spizizenii*. and *S. aureus*), gram negative (*E. coli*) and fungi (*C. albicans* and *Saccharomyces cerevisiae*) (Ud-Daula et al., 2016). (Reference) *E. sayapensis* essential oils also displayed a broad spectrum of antimicrobial activity against gram positive (*B. subtilis, B. thuringiensis, MRSA, S. aureus*), gram-

Table 3. Antimicrobial activity of essential oils of *Etlingera* species.

Etlingera Species	Plant Part	Microorganism	Test method	Activity	Reference
E. punicea (20 µl/6 mm disk)	R	*Staphylococcus aureus*	Disk Diffusion	0.87 mm	Tadtong et al. (2009)
		Escherichia coli		0.78 mm	
		Pseudomonas aeruginosa		-	
		Salmonella albany		0.82 mm	
		Candida albicans		1.18 mm	
E. brevilabrum	L	*MRSA*	Disk Diffusion	8.1 mm	Mahdavi et al. (2016, 2018)
		Staphylococcus aureus		12.6 mm	
		Bacillus subtilis		-	
		Bacillus thuringiensis		-	
		Escherichia coli		-	
		Proteus vulgaris		10.3 mm	
		Salmonella typhimurium		-	
		Proteus mirabilis		16.4 mm	
		Pseudomonas aeruginosa		-	
	S	*MRSA*		10 mm	
		Staphylococcus aureus		11.2 mm	
		Bacillus subtilis		-	
		Bacillus thuringiensis		-	
		Escherichia coli		13.5 mm	
		Proteus vulgaris		9.5 mm	
		Salmonella typhimurium		-	
		Proteus mirabilis		-	
		Pseudomonas aeruginosa		-	
	R	*MRSA*		12.6 mm	
		Staphylococcus aureus		17.7 mm	
		Bacillus subtilis		-	
		Bacillus thuringiensis		-	
		Escherichia coli		-	
		Proteus vulgaris		-	
		Salmonella typhimurium		-	
		Proteus mirabilis		13.2 mm	
		Pseudomonas aeruginosa		-	

Table 4. Contd.

E. coccinea	R	*Staphylococcus aureus* *Staphylococcus sp.,* *Streptococcus pyrogenes,* *Salmonella enteritidis*		5 µg/ml 10µg/ml 8 µg/ml 8 µg/ml	Vairappan et al. (2012)
E. pyramidosphaera	R	*Staphylococcus aureus* *Staphylococcus sp.,* *Streptococcus pyrogenes,* *Salmonella enteritidis*		90 µg/ml - 120 µg/ml -	
E. megalocheilos	R	*Staphylococcus aureus* *Staphylococcus sp.,* *Streptococcus pyrogenes,* *Salmonella enteritidis*	Agar dilution method	8 µg/ml 8 µg/ml 6 µg/ml 9 µg/ml	
E. brevilabrum	R	*Staphylococcus aureus* *Staphylococcus sp.,* *Streptococcus pyrogenes,* *Salmonella enteritidis*		40 µg/ml - 40 µg/ml -	
E. elatior	R	*Staphylococcus aureus* *Staphylococcus sp.,* *Streptococcus pyrogenes,* *Salmonella enteritidis*		80 µg/ml 90 µg/ml 75 µg/ml 60 µg/ml	
E. elatior	L	*Bacillus cereus* *Micrococcus luteus* *Staphylococcus aureus*	Disk Diffusion	25 mg/ml 6.3 mg/ml 50 mg/ml	
E. fulgens	L	*Bacillus cereus* *Micrococcus luteus* *Staphylococcus aureus*		25 mg/ml 6.3 mg/ml 100 mg/ml	Chiang et al. (2010)
E. maingayi	L	*Bacillus cereus* *Micrococcus luteus* *Staphylococcus aureus*	Disk Diffusion	6.3 mg/ml 6.3 mg/ml 12.5 mg/ml	
E. rubrostriata	L	*Bacillus cereus* *Micrococcus luteus* *Staphylococcus aureus*	Disk Diffusion	12.5 mg/ml 6.3 mg/ml 50 mg/ml	
Etlingera elatior (5 µl/6 mm disk)	WP	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Disk Diffusion	- -	Abdelwahab et al. (2010)

Table 4. Contd.

		Salmonella choleraesuis		-	
		Bacillus subtilis		-	
E. elatior (10 µl/6 mm disk)	F	*Staphylococcus aureus*	Disk Diffusion / Microdilution	12 mm, MIC 33.3 µl/ml, MBC 33.3 µl/ml,	Susanti et al. (2013)
		Bacillus cereus		11.5 mm, MIC 33.3 µl/ml, MBC 33.3 µl/ml,	
		Pseudomonas aeruginosa		-	
		Escherichia coli		-	
		Candida albicans		10 mm, MIC 0.4 µl/ml, MBC 11.1 µl/ml	
		Candida neoformans		20 mm, MIC 0.05 µl/ml, MBC 12 µl/ml	
E. fimbrobractata	L	*Pseudomonas aeruginosa*	Disk Diffusion / Microdilution	-	Ud-Daula et al. (2016)
		Escherichia coli		8 mm, MIC 625 µg/ml	
		Bacillus subtilis		12.33 mm, MIC 78 µg/ml	
		Bacillus spizizenii		9.5 mm, MIC 156 µg/ml	
		Staphylococcus aureus		9.17 mm, MIC 156 µg/ml	
		Candida albicans		17 mm, MIC 78µg/ml	
		Saccharomyces cerevisiae		34.33 mm, MIC 78 µg/ml	
	AS	*Pseudomonas aeruginosa*	Disk Diffusion /Microdilution	-	
		Escherichia coli		10.28 mm, MIC 312.5 µg/ml	
		Bacillus subtilis		15.0 mm, MIC 39 µg/ml	
		Bacillus spizizenii		13.33 mm, MIC 39 µg/ml	
		Staphylococcus aureus		12.0 mm, MIC 156 µg/ml	
		Candida albicans		11.33 mm, MIC 156 µg/ml	
		Saccharomyces cerevisiae		29.83 mm, MIC 39 µg/ml	
	BS	*Pseudomonas aeruginosa*	Disk Diffusion /Microdilution	-	
		Escherichia coli		8mm, MIC 156 µg/ml	
		Bacillus subtilis		12.33 mm, MIC 19.5µg/ml	
		Bacillus spizizenii		9.5 mm, MIC 19.5µg/ml	
		Staphylococcus aureus		9.17 mm, MIC 78µg/ml	
		Candida albicans		17 mm, MIC 78µg/ml	
		Saccharomyces cerevisiae		34.33 mm, MIC 9.7µg/ml	
	R	*Pseudomonas aeruginosa*	Disk Diffusion /Microdilution	-	
		Escherichia coli		12 mm, MIC 156 µg/ml	
		Bacillus subtilis		20.2 mm, MIC 19.5 µg/ml	
		Bacillus spizizenii		16.67mm, MIC 19.5µg/ml	

Table 4. Contd.

		Staphylococcus aureus		13.67 mm, MIC 39 µg/ml	
		Candida albicans		26 mm, MIC 39 µg/ml	
		Saccharomyces cerevisiae		58.67 mm, MIC 2.4 µg/ml	
E. sayapensis	L	*Bacillus subtilis*	Microdilution	0.78 MIC mg/ml	Mahdavi et al. (2017)
		Bacillus thuringiensis		5.29 MIC mg/m	
		Enterococcus faecalis		-	
		MRSA		8.33 MIC mg/m	
		Staphylococcus aureus		0.78 MIC mg/m	
		Staphylococcus epidermidis		4.16 MIC mg/m	
		Aeromonas hydrophila		5.21 MIC mg/m	
		Enterobacter aerogenes		2.6 MIC mg/m	
		Escherichia coli		1.56 MIC mg/m	
		Proteus mirabilis		20.83 MIC mg/m	
		Proteus vulgaris		-	
		Pseudomonas aeruginosa		-	
		Salmonella typhimurium		-	
		Serratia marcescens		5.21 MIC mg/m	
		Shigella sonnei		1.56 MIC mg/m	
		Vibrio parahaemolyticus		6.25 MIC mg/m	
		Candida albicans		4.16 MIC mg/m	
		Candida parapsilosis		3.64 MIC mg/m	
	R	*Bacillus subtilis*		0.78 MIC mg/m	
		Bacillus thuringiensis		4.16 MIC mg/m	
		Enterococcus faecalis		-	
		MRSA		0.52 MIC mg/m	
		Staphylococcus aureus		1.56 MIC mg/m	
		Staphylococcus epidermidis		-	
		Aeromonas hydrophila		3.12 MIC mg/m	
		Enterobacter aerogenes		2.6 MIC mg/m	
		Escherichia coli		3.12 MIC mg/m	
		Proteus mirabilis		1.56 MIC mg/m	
		Proteus vulgaris		-	
		Pseudomonas aeruginosa		-	
		Salmonella typhimurium		-	

Table 4. Contd.

	Serratia marcescens	0.52 MIC mg/m
	Shigella sonnei	6.25 MIC mg/m
	Candida albicans	2.6 MIC mg/m
	Candida parapsilosis	0.52 MIC mg/m

'-' indicates no inhibition.

negative bacteria (*A. hydrophila, E. aerogenes, E. coli, P. mirabilis, S. marcescens, S. sonnei, V. parahaemolyticus*) and fungi (*C. albicans* and *C. parapsilosis*) (Mahdavi et al., 2017).

Abdulwahab et al. (2010) reported that essential oils from whole plant of *E. elatior* failed to inhibit gram positive (*B. subtilis)* and gram-negative (*S. choleraesuis* and *P. aeruginosa)* bacteria. However, Susanti et al. (2013) demonstrated the activity of *E. elatior* flowers essential oil against Gram positive bacteria of *S. aureus*, *Bacillus cereus* with zone of inhibition of 13 and 12.3 mm, respectively but not sensitive against *P. aeruginosa* and *E. coli*. The oil also showed potent inhibitory effects against fungi with mean inhibition zones 10 mm for *C. albicans* and 20 mm for *C. neoformans. C. neoformans* was found as the most susceptible among all four microorganisms with MIC value of 0.05 µL/mL. antifungal property of *Etlingera* is suspected to be associated with their high contents of monoterpene hydrocarbon and phenylpropanoids.

It has been generally reported that gram-negative bacteria are more resistant than gram-positive bacteria. Similar observations were also found in *Etlingera* species where Gram-negative bacteria (*E. coli* and *P. aeruginosa*) showed less susceptibility to the *Etlingera* essential oil than Gram positive bacteria (Abdelwahab et al., 2010; Susanti et al., 2013). Of the gram-negative bacteria, *P. aeruginosa* was found resistant to the action of essential oil. This could be due to highly restricted outer membrane of these bacteria that slows down the passage of essential oils, whereas lacking of outer membrane enables Gram positive bacteria to be more susceptible to *Etlingera* essential oils.

Mode of antimicrobial action

Different modes of action are involved in the antimicrobial activity of essential oils, because of the variability of quantity and chemical profiles of essential oil. Antimicrobial action of essential oils and their components may be attributed by any or a combination of six possible mechanisms which include: (1) disintegration of cytoplasmic membrane, (2) damage of membrane proteins (ATPases and others), (3) degradation of cell wall with the release of lipopolysaccharides, (4) leakage of ions and other cell content, (5) coagulation of cytoplasm and (6) inhibition of enzyme synthesis (Bakkali et al., 2008; Bouhdid et al., 2010; Burt, 2004). Until now, the mechanisms of antimicrobial action of *Etlingera* essential oil and their components are not demonstrated in any published paper. Precise mechanisms of antibacterial activity of few individual pure essential oil components from different genera were reported. Carvacrol and thymol are able to disintegrate the outer membrane, increasing membrane fluidity, which in turn increases the permeability of the cytoplasmic membrane to ATP (Ultee et al., 1999). Eugenol - a major component (approximately 85%) of clove oil binds with membrane protein of both gram positive and negative bacteria altering their structure and increases permeability. Farnesol, nerolidol and plaunotol has been regarded as a cause for the loss of potassium and sodium ions in *S. aureus* (Carson and Hammer, 2011). *p*-cymene, terpinen-4-ol, 1,8-cineole, terpinolene and γ-terpinene increase membrane fluidity that breaches membrane integrity and allows small intracellular components such as hydrogen, potassium and sodium to pass through the cell membrane and ultimately causes cell death.

AREAS OF FUTURE RESEARCH

All the literature in the last 15 years mainly focuses on the chemical composition of essential oil of *Etlingera* species. Only three literatures demonstrated the antimicrobial properties of essential oil in which two papers reported antifungal properties. Also, the oil from *E. punicea* and *E. elatior* showed potent antifungal properties than bacteria. However, some limitations have also been identified in the investigation of antimicrobial activity (only 2 species of fungi were used). Fungi are eukaryotic cells and

consequently most agents that are toxic to fungi are also toxic to the host, hence development of antifungal agents has lagged behind that of antibacterial agents. Fungi grow slowly and many of them have multicellular forms which complicates in developing new antifungal agents and in understanding the existing ones. The action of essential oil and their components against bacterial and fungal cell is not fully identified and is a focal area for future research. Thus, elucidation of mechanism of action of essential oil and their components would provide insights that may lead to identification of new antibiotic target and exploitation of novel biochemical pathways.

CONCLUSION

This review summarizes and characterizes the importance of essential oils obtained from different parts of *Etlingera* species which comprise diverse chemical constituents. The essential oil of *Etlingera* showed promising antimicrobial properties that could be an alternative source of synthetic antibiotics in order to combat emerging drug resistance. Since, essential oil and their components possess many important medicinal activities, seeking new drugs from aromatic and medicinal plant like *Etlingera* is crucial. Many studies have demonstrated the chemical composition of this plant but only few researchers have investigated the antimicrobial properties of essential oil. Thus, more studies are needed to understand and elucidate the mechanism of action of essential oil and their constituents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Cytotoxic potential of 14 Passiflora species against cancer cells

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This work aimed to evaluate the cytotoxic potential against cancer cells of Passiflora genus plant species cultivated in Brazil and identify the mechanism of cytotoxicity induced by the most promising extract. Ethanolic extracts from the leaves of 14 Passiflora species were obtained by accelerated solvent extraction and *in vitro* cytotoxicity evaluated against cancer cell lines using MTT assay at a single concentration of 50 µg/ml. Additionally, the IC_{50} of the *Passiflora alata* (ELPA) leaf extracts was determined against both cancer (HCT-116, SF-295, OVACAR-8, and HL-60), and non-cancer cells (PBMC). The ELPA flavonoids were identified by HPLC-DAD and UHPLC-MS/MS. The morphological analyses, using light and fluorescence microscopy, and cell cycle and DNA fragmentation analysis, using flow cytometry, were evaluated to study the mechanism of cell death induced by ELPA in HL-60 cells. Among the Passiflora leaf extracts evaluated; ELPA stood out with high cytotoxic activity, followed by *Passiflora capsularis* and *Passiflora quadrangularis* with varying high and low cytotoxic activity. ELPA presented high cytotoxic potency in HL-60 (IC_{50} 19.37 µg/ml), and without cytotoxicity against PBMC, suggesting selectivity for cancer cells. The cytotoxic activity of ELPA may well be linked to the presence of ten identified flavonoids. Cells treated with ELPA presented the hallmarks typical of apoptosis and necrosis, with cell cycle arrest in the G_2/M phase. From among the studied species, ELPA presented greater cytotoxic activity, possibly a consequence of synergistic flavonoid action, which induces cell death by apoptosis and necrosis.

Key words: Cancer, cytotoxicity, ethanolic extract, Passiflora, *Passiflor*a *alata.*

INTRODUCTION

Cancer is a progressive disease, characterized by a gradual accumulation of genetic mutations and epigenetic change that causes cellular mechanism imbalances, which progressively transform normal cells into malignant cells (Hedvat et al., 2012; You and Jones, 2012). Together with their associated treatment modalities

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for curative or palliative purposes, there are more than 100 types of cancers (Ge et al., 2017; Palumbo et al., 2013). Most therapeutic agents promote programmed cell death (apoptosis) in tumors. However, due to the relative similarity between malignant and normal cells, severe side effects are often observed; this is a limiting factor for therapeutics (Hedvat et al., 2012).

Despite existing treatment modalities, cancer is one of the most common causes of morbidity and mortality worldwide, with estimates that 14.1 million new cancers and 8.2 million cancer deaths occured in 2012, anticipating an increase of at least 70% by 2030, or 20 million new cases of cancer annually by 2025 (Antoni et al., 2016; Ferlay et al., 2015).

Finding more effective and selective compounds that reduce this growing public health problem is a challenge, and nature is the alternative (Dias et al., 2012). Historically, natural plant and animal products have been the origin of most medicinal preparations and natural products continue to provide discovery clues towards pharmacologically active compounds, particularly anti-cancer agents (Dias et al., 2012; Harvey et al., 2015). A continuing study beginning in 1981 through 2014 demonstrated that 83% of the world's registered anticancer drugs were in one form or another either natural products, based therein, or mimetics (Newman and Cragg, 2016). Despite these successes, most plant species have not been systematically investigated (Harvey et al., 2015), and though Brazil has the greatest diversity of plant species in the world, discoverable cancer cell line cytotoxicities have been poorly studied (Ferreira et al., 2011).

The purpose of this study was to carry on the search for new natural product derived anticancer drugs. Within the gigantic diversity of species of the genus Passiflora, some such as *Passiflora edullis* Sims (Kuete et al., 2016), *Passiflora ligularis* Juss (Carraz et al., 2015), *Passiflora incarnata* (Kapadia et al., 2002; Ma et al., 2014; Sujana et al., 2012), *Passiflora molíssima* (Chaparro et al., 2015) and *Passiflora tetandra* (Perry et al., 1991) have been described in the literature as cytotoxic to cancer cell lines, with *in vivo* chemopreventive or antitumor activity. This raises the hope that related species could exhibit similar activities.

Facing treatments with low curative power, a rising cancer mortality rate in the world, a great variety of plant species not yet having been studied in relation to their antitumor activity, and the fact that certain species of the genus Passiflora present anticancer potential, this study aimed to evaluate the cytotoxic potential of leaf extracts from 14 plant species of the genus Passiflora against human cancer cell lines, and to identify the mechanism of cytotoxicity presented by most promising extract.

MATERIALS AND METHODS

Collection of plant

The leaves of 14 species of Passiflora were collected in July 2011 in the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), in the city of Cruz das Almas, Bahia, Brazil (12°04'10"S, 39°06'22"W). The species were registered in the active germoplasm bank (BGP) with access number BGP 08 (*Passiflora giberti* N. E. Brown), BGP 32 (*Passiflora maliformis* L.), BGP 77 (*Passiflora cincinnata* Mast.), BGP 104 (*Passiflora vitifolia* Kunth), BGP 105 (*Passiflora tenuifila* Killip), BGP 107 (*Passiflora morifolia* Mast.), BGP 109 (*Passiflora galbana* Mast.), BGP 114 (*Passiflora mucronata* Sessé & Moc.), BGP 125 (*Passiflora capsularis* L.), BGP 152 (*Passiflora suberosa* L.), BGP 157 (*Passiflora quadrangularis* L.), BGP 163 (*Passiflora alata* Curtis), BGP 170 (*Passiflora malacophylla* Mast.), BGP 172 (*Passiflora racemosa* Brot.) and BGP 237 (*Passiflora setácea* DC.). The leaves of all species were placed in a drying oven at 30°C for seven days and then powdered (only particles between 0.5 and 1.0 mm were utilized for the extractions). The leaves of all species were placed in a drying oven (30°C) for 7 consecutive days and after spraying

Preparation of extracts and samples

Ethanolic extracts of the dried and powdered leaves were prepared by accelerated solvent extraction (ASE 100, Dionex Corporation, Sunnyvale, CA, USA). Each extract was prepared to weigh exactly 6.0 g *P. alata*, *P. capsularis*, *P. cincinnata*, *P. gibertii*, *P. maliformis*, *P. malacophylla*, *P. morifolia*, *P. mucronata*, *P. quadrangularis*, *P. racemosa*, *Passiflora setacea*, *P. suberosa* and 3.0 g of *P. vitifolia* and *P. tenuifila*. The plant material was extracted under optimized conditions in five extraction cycles with 64% (w/w) ethanol at 80°C, 1500 psi, and a static cycle timing of 10 min. The cells were then rinsed with fresh extraction solvent (100% of the extraction cell volume) and purged with N_2 gas for 60 s. The solvent was removed under reduced pressure at 55°C to yield the corresponding crude extracts, which were stored under refrigeration and protected from light (Gomes et al., 2017). For HPLC analysis, hydroethanolic crude extract of *P. alata* was purified by solid-phase extraction (SPE) using the typical method with minor modifications (Pereira et al., 2005). A C_{18} cartridge (Agilent SampliQ, 3 ml/200 mg) was conditioned with 3 ml of methanol, followed by 1 ml of water. Next, 2 ml of a methanolic solution of the sample (5 mg/ml) was added, and the flavonoid fraction was obtained by elution with 3 ml of 60% (w/w) methanol. All samples were prepared and analyzed in triplicate.

Identification of flavonoids in the extract of *P. alata*

HPLC-DAD

High-performance liquid chromatography (HPLC) *fingerprint* analysis was carried out using an LC system (Thermo Scientific Dionex Ultimate 3000; MA, USA) consisting of a Thermo Scientific Dionex Ultimate 3000 diode array detector (DAD), quaternary pump, on-line degasser and automatic sampler. The chromatographic separation of sample was achieved on a reversed-phase HPLC column (Waters XBridge™, BEH C_{18}, 100 mm × 3.0 mm I.D., 2.5 µm particle size). For HPLC *fingerprint* chromatographic analysis, a binary gradient elution system composed of 0.2% (w/w) formic acid in water (solvent A) and acetonitrile (solvent B) was applied as follows: 0 - 30 min, 5 - 20% B. There re-quilibration duration between individual runs was 30 min. The injection volume was 10 µL per sample, the flow rate was 0.6 ml/min and the column temperature was maintained at 30°C. The flavonoids were detected at 337 nm, and the UV spectra of individual peaks were recorded within a range of 190 to 400 nm. Data were acquired and processed with Chromeleon *software*. The main flavonoids were identified in the extract of *P. alata* based on their retention times (t_R), coinjection of the samples with standards

and comparison of their UV adsorption spectra (Gomes et al., 2017).

UHPLC-MS/MS

Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was utilized to identify and confirm chromatogram peaks, which comprised an ACQUITYTM UHPLC system (Waters Corp., Milford, MA, USA). Separation was performed with an ACQUITY BEH C_{18} column (50 mm × 2 mm, 1.7 µm; Waters, USA). The mobile phase was a mixture of 0.1% formic acid (A) and acetonitrile in 0.1% formic acid (B), with an linear gradient elution as follows: 0-11 min, 5-95% B. The injection volume was 4 µL. The flow rate was set at 0.30 ml/min. The UV spectra were registered from 190 to 450 nm. Eluted compounds were detected from *m/z* 100 to 1000 using a Waters ACQUITY® TQD tandem quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source in the negative mode using the following instrument settings: capillary voltage 3500V; capillary temperature 320°C; source voltage 5 kV; vaporizer temperature 320°C; corona needle current 5 mA; and sheath gas nitrogen 27 psi. Analyses were run in the full scan mode (100-2000 Da). Ion spray voltage: -4 kV; orifice voltage: -60 V. The identification of flavonoids was based on the comparison of the molecular formula with that of the published data, further confirmation was performed by illuminating the quasi-molecular ions and key flavonoids fragmentations, in particular for those isomeric.

Human cancer cell lines and non-cancer cells

The cytotoxicity of these Passiflora species extracts was tested against colon carcinoma (HCT-116), glioblastoma (SF-295), ovarian adenocarcinoma (OVCAR-8) and promyelocytic leukemia (HL-60) human cancer cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin incubated at 37°C in a 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMCs) were isolated from a sample of about 3 ml of human blood plus 5 ml of saline. The steps up to isolation included the addition of 3 ml of Ficoll followed by 15 min of centrifugation at 1500 rpm, and aspiration of the PBMCs present in the intermediate region between the red blood cells and the plasma. The PBMC suspension was transferred to another tube which was added with saline to the 11 ml volume and centrifuged for 5 min at 1000 rpm. The supernatant was discarded, and the PBMC pellet was re-suspended in complete medium (RPMI 1640 plus 20% fetal bovine serum and 10 µg/ml ConA) and counted in a Neubauer chamber for further dilution and plating. The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (# 031019/2013).

Determination of the cytotoxic effect of Passiflora extracts on cancer cell lines

The MTT assay was used to determine the cytotoxic effect of the 14 extracts of Passiflora leaves against cancer cell lines (Mosmann, 1983). For all experiments, HCT-116 cells (0.7×10^5 cells/ml in 100 µL medium), SF-295 and OVCAR-8 cells (0.1×10^6 cells/ml in 100 µL medium) were seeded in 96-well plates and incubated in a humidified chamber with 5% CO_2 at 37°C for 24 h. After this, the 14 extracts of Passiflora were solubilized in dimethyl sulfoxide (DMSO, 0.7%), and added to each well at the concentration of 50 µg/ml. The cells were then incubated for 72 h at 37°C in a 5% CO_2 atmosphere. After 72 h, MTT (0.5 mg/ml) was added, followed by incubation at 37°C in an atmosphere of 5% CO_2 for 3 h. After incubation, the formazan product was dissolved in 150 µL DMSO, and absorbance was measured using a multi-plate reader (DTX 880 Multimode Detector, Beckman Coulter Inc.). The treatment's effects were expressed as the percentage of control absorbance of reduced dye at 595 nm. All absorbance values were converted into a cell growth inhibition percentage (GI %) by the following formula:

$$[GI\% = 100 - [(TE/NC) \times 100\%]$$

In which, NC is the absorbance for the negative control and TE is the absorbance in the presence of the tested extracts. The half maximal inhibitory concentration (IC_{50}) was determined for the extract that caused the greatest GI% against HCT-116, SF-295 and OVCAR-8. The same protocol for the same cells, adding the cell line HL-60 (0.3×10^6 cells/ml in 100 µL medium) was used to determine the IC_{50}, varying only the concentration of the compound from 0.39 to 50 µg/ml, and the potency of the extract. The experiment was performed as three independent experiments, and doxorubicin (0.3 µg/ml) was used as a positive control (Doxorubicin, purity > 98%; Sigma Chemical Co., St. Louis, MO, USA).

Determination of the effect of *P. alata* leaf extract in non-cancer cells

The alamar blue assay was used to determine the effect of extract from the leaves of *P. alata* (ELPA) against proliferation of non-cancer cells (PBMCs) obtained from peripheral blood from healthy human volunteers (Nakayama et al., 1997). Initially, the cells were plated in 96-well plates (100 µL/well of a solution of 3×10^6 cells/ml). After 24 h of incubation, extract from the leaves of *P. alata* (0.39 to 50 µg/ml) was dissolved in 0.3% DMSO, added to each well and incubated for 72 h. Doxorubicin (0.03 - 0.25 µg/ml) was used as a positive control and 0.3% DMSO was used as a negative control. 24 h before the end of the incubation period, 20 µL of alamar blue stock solution (0.312 mg/ml) (resazurin) was added to each well. Absorbances were measured at wavelengths of 570 nm (reduced) and 595 nm (oxidized) using a plate reader (DTX 880 Multimode Detector, Beckman Coulter™).

Determination of the cytotoxicity mechanism induced by *P. alata* leaf extract

Morphological analysis by light microscopy

Differential staining with hematoxylin/eosin was used for morphological analysis. The HL-60 cells, plated at 0.3×10^6 cells/ml were incubated for 72 h with *P. alata* leaf extract (9.69, 19.77, and 38.74 µg/ml) and examined under an inversion microscope. To observe the morphology, 50 µL of the cell suspension was added to the slide centrifuge (Shandon Southern Cytospin™). After cell adhesion on the slide, fixation with methanol for 1 min was completed, hematoxylin followed by eosin was used. Doxorubicin (0.3 µg/ml) was used as a positive control (Veras et al., 2004).

Morphological analysis by fluorescence microscopy

Acridine orange/ethidium bromide (AO/EB) (Sigma Aldrich) staining in HL-60 cells was performed to evaluate the pattern of cell death induced by *P. alata* leaf extract. Cells of the HL-60 strain were plated at 0.3×10^6 cells/ml, and then incubated for 72 h with the extract (9.69, 19.77, and 38.74 µg/ml). The cell suspension was transferred to a microtube and centrifuged for 5 min at low speed (500 rpm). The supernatant was discarded and the cells re-suspended in 20 µL of PBS solution. Then 1 µL of the ethidium

bromide/acridine orange solution was added to each tube and placed under a fluorescence microscope to observe the cellular events. Doxorubicin 0.3 µg/ml was used as a positive control (Geng et al., 2003).

Analysis of cell cycle and DNA fragmentation

Cell cycle analysis was performed to determine cell content, which reflects the cell cycle phases (G_0/G_1, S, and G_2/M). For this, HL-60 cells were plated in 24-well plates at the concentration of 0.3×10^6 cells/ml and incubated for 72 h with *P. alata* leaf extract (9.69, 19.77 and 38.74 µg/ml). Doxorubicin (0.3 µg/ml) was used as a positive control. After the incubation period, cells were harvested in a permeabilization solution containing 0.1% triton X-100 (Sigma Chemical Co. St Louis, MO, USA), 2 µg/mL propidium iodide (Sigma Chemical Co. St Louis, MO, USA), 0.1% sodium citrate and 100 µg/mL RNAse (Sigma Chemical Co. St Louis, MO, USA) and incubated in the dark for 15 min at room temperature (Militão et al., 2006). Finally, cell fluorescence was measured by flow cytometry with a BD LSRFortessa cytometer using BD FACSDiva Software (BD Biosciences) and FlowJo Software 10 (FlowJo Lcc; Ashland, OR, USA). Ten thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

Statistical analysis

Data are presented with mean ± standard error of the mean (SEM), standard deviation (SD), or IC_{50} values; confidence intervals (CI 95%) were obtained by nonlinear regression. Differences among the experimental groups were compared by one-way variance analysis (ANOVA), followed by Newman-Keuls test ($p < 0.05$). All analyses were carried out using the Graphpad program (Intuitive Software for Science, San Diego, CA, USA).

RESULTS AND DISCUSSION

Based on the fact that some species of the genus Passiflora have cytotoxic activity against cancer cell lines and that relatively few species have been evaluated for their possible anticancer activity, we selected 14 species of Passiflora cultivated in Brazil, produced by ASE method to evaluate their potential *in vitro* cytotoxic activity.

The cell growth inhibition percentage (GI%) of the Passiflora extracts was evaluated against three human cancer cell lines (HCT-116, OVACAR-8, and SF-295) in a single concentration of 50 µg/ml and measured by MTT assay after 72 h of incubation (Table 1). The results were analyzed for each cell line tested using a GI% scale as follows: samples with low cytotoxic activity have GI < 50%; samples with moderate cytotoxic activity have GI between 50 and 75%, and samples with high cytotoxic activity have GI > 75% (Mahmoud et al., 2011). The results showed that the extracts of *P. cincinnata* (3)*, P. gibertii* (4)*, P. maliforms* (5), *P. mallacophyla* (6), *P. murchronata* (7), *P. morifolia* (8) *P. racemosa* (10), *P. setacea* (11), *P. suberosa* (12), *P. tenuifila* (13) and *P. vitifolia* (14) have low or nonexistent cytotoxic activity in all human cancer cell lines tested. Extracts from *P. capsularis* (2) and *P. quadrangularis* (9) have high activity against HCT-116, low activity against SF-295, and cytotoxic activity from low (*P. quadrangulares*) to moderate (*P. capsularis*) against OVACAR-8. The extract from the leaves of *P. alata* (1) presented high cytotoxic activity in all human cancer cell lines analyzed. Therefore, among the Passiflora leaf extracts evaluated, *P. alata* (1) stands out with promising cytotoxic activity, followed by *P. capsularis* (2) and *P. quadrangularis* (9).

Although none of the three species highlighted in the present study had been previously evaluated for their cytotoxic activity against cancer cells, other species of the genus Passiflora have also been evaluated. The cytotoxic or antiproliferative activity of species of the genus Passiflora was evidenced for the first time by Perry et al. (1991) through study of *P. tetandra* leaf extract, demonstrating cytotoxic activity against murine leukemic cell line (P388), being possibly mediated by the extract component 4-hydroxy-2-cyclopentane. The ethanolic extract of *P. ligularis* Juss. and the methanolic extract of the *P. edullis* fruit have been respectively described as presenting activity against human hepatocellular carcinoma (Hep3B) and leukemic cell line (CCRF-CEM) (Carraz et al., 2015; Kuete et al., 2016).

Based on the promising cytotoxic activity observed in the *P. alata* leaf extract (ELPA), we determined the median inhibitory concentration able to induce 50% of maximal effect (IC_{50}) against four human cancer cell lines (HCT-116, HL-60, OVCAR-8, and SF-295) through the MTT method. The results demonstrated that ELPA has cytotoxic activity in the four cancer cell lines evaluated, respectively being most potent for the cell line HL-60 (IC_{50} of 19.37 µg/ml), followed by HCT-116 (IC_{50} of 20.79 µg/ml), SF-295 (IC_{50} of 21.87 µg/ml), and OVCAR-8 (IC_{50} of 28.26 µg/ml) (Table 2). According to the preclinical cytotoxic drug screening program based on the US National Cancer Institute program, only extracts with IC_{50} values below 30 µg/ml in assays with cancer cell lines are considered promising for the development of anticancer drugs (da Silva et al., 2016; Mahmoud et al., 2011). Thus, ELPA presents promising *in vitro* cytotoxic activity for further study. For a natural compound to progress as a potential anticancer drug candidate, it is necessary to determine the degree of specificity of the drug by evaluating *in vitro* cytotoxic activity against non-cancer cells (Andrade et al., 2015; da Silva et al., 2016). For this, ELPA was subjected to *in vitro* cytotoxic activity evaluation against non-cancer cells (PBMC); the results demonstrated that the compound shows no cytotoxic activity against PBMC (Table 2).

The cytotoxic activity of natural bioactive products against cancer cells is attributed to the chemical composition of the product, and this practice of identification is the most successful source of potential drug discovery and development (Dias et al., 2012).

A total of 4 flavonoids were identified by HPLC-DAD, and 8 flavonoids were identified by UHPLC-MS/MS in the negative mode. They are summarized along with their retention time, molecular formula and MS/MS fragments

Table 1. Cell growth inhibition percentage (GI%) of leaf extracts from 14 species of Passiflora against human cancer cell lines.

Extract	Cells					
	HCT-116	SD	OVACAR-8	SD	SF-295	SD
	GI (%)		GI (%)		GI (%)	
P. alata (1)	97.55	0±1.38	96.03	0±1.38	99.03	0±0.54
P. capsularis (2)	89.99	0±6.54	2.91	0±2.86	52.20	0±16.38
P. cincinnata (3)	-9.93	0±3.92	20.30	0±7.89	21.50	0±2.86
P. gibertii (4)	23.69	0±12.00	-1.76	0±14.19	21.37	0±6.49
P. maliforms (5)	-2.69	0±0.46	-7.20	0±1.97	14.63	0±5.06
P. mallacophyla (6)	33.64	0±10.08	31.70	0±57.17	38.39	0±17.81
P. murchronata (7)	4.54	0±0.05	-7.06	0±1.58	13.75	0±0.71
P. morifolia (8)	4.00	0±1.92	-6.78	0±5.72	18.30	0±0.48
P. quadrangulares (9)	77.75	0±6.08	4.37	0±7.89	40.53	0±15.25
P. racemosa (10)	5.58	0±5.92	-0.44	0±0.49	21.37	0±0.66
P. setacea (11)	-3.07	0±0.46	-3.57	0±7.69	29.00	0±1.31
P. suberosa (12)	2.86	0±34.15	-5.80	0±10.25	18.89	0±5.24
P. tenuifila (13)	12.43	0±5.00	-2.39	0±7.20	19.48	0±3.22
P. vitifolia (14)	-8.73	0±34.00	-11.10	0±5.32	16.74	0±5.18
Doxorubicin	99.12	0±0.51	99.87	0±0.85	99.23	0±0.42

HCT-116 (Colon carcinoma), OVACAR-8 (ovarian adenocarcinoma), and SF-295 (glioblastoma) humans. GI% values are presented as the mean ± SD from three independent experiments measured using the MTT assay after 72 h of incubation. All extracts were tested at a concentration of 50 µg/ml; Doxorubicin was used as the positive control.

Table 2. Evaluation of the cytotoxic activity of *P. alata* leaf extract (ELPA) against cancer and non-cancer cells.

Cell	Histotype	ELPA (µg/ml)	Doxorubicin (µg/ml)
HL-60	Promyelocytic leukemia	19.37 (15.46 - 24.27)	0.21 (0.14 - 0.31)
HCT-116	Colon carcinoma	20.79 (19.06 - 22.68)	0.02 (0.01 - 0.04
SF-295	Glioblastoma	21.87 (18.83 - 25.41)	0.24 (0.17 - 0.36)
OVCAR-8	Ovarian adenocarcinoma	28.26 (22.13 - 29.40)	1.10 (1.00 - 1.50)
PBMC	Peripheral blood mononuclear cells	> 50	0.77 (0.43 - 1.40)

Values were expressed as IC_{50} and obtained by nonlinear regression from three independent experiments, performed in triplicate and measured with the MTT assay after 72 h of incubation with the ELPA; values are means with 95% confidence limits in parentheses. Doxorubicin was used as positive control.

as shown in Table 3. HPLC fingerprint chromatogram of ELPA with 4 identified compounds is shown in Figure 1.

Flavonoid from *Passiflora* spp. are usually *C*-glycosylated with one or more sugar units. Different fragmentation patterns were observed in MS/MS experiments for flavone *C*-glycosides. Based on MS and UV/Vis spectra, all identified flavones were vitexin, isovitexin, orientin, isorientin, 8-*C*-glucosyldiosmetin, 6-*C*-glucosyldiosmetin, 6-*C*-glucoronylvitexin and 8-*C*-glucoronylisovitexin.

Thus, compounds 1 and 2 (t_R = 2.65 and 2.76 min) were identified as vitexin and isovitexin, once the $[M-H]^-$ pseudo molecular ions were registerated at *m/z* 431 and the fragmentation pattern, compared with the literature are also in accordance with these two compounds. In the same way compounds 3 and 4 (t_R = 2.37 and 2.48 min) were identified as orientin and isoorientin, also due the pseudo $[M-H]^-$ ions at *m/z* 446. These compounds (1-4) indicated similarities of fragmentation patterns due to dehydration and cross-ring cleavages of the glucose moiety, that is, 0.2 cross-ring cleavage (-120 amu) and 0.3 cross-ring cleavage (-90 amu) (Figure 2) (Davis and Brodbelt, 2004).

Compounds 5 and 6 (t_R = 2.80 and 2.89 min) were identified as 8-*C*-glucosyldiosmetin and 6-*C*-glucosyldiosmetin, $[M-H]^-$ ions presented at *m/z* 461. In the MS/MS spectra the fragments were at *m/z* 341 $^{0.2}X^-$ $[M-H-120]^-$, which was attributed to the loss of a

Table 3. Identification of the chemical constituents from ELPA by UHPLC-MS/MS.

Peak No.	Retention time (min)	Λmax (nm)	[M-H]-m/z	MS/MS fragments ions	Identification
1	2.65	269; 338	431.43	340.9; 311.0; 295.1; 282.8	Vitexin
2	2.76	270; 339	431.12	341.2; 311.1; 295.0; 282.9	Isovitexin
3	2.37	266; 345	446.72	356.8; 327.2; 297.0; 255.0	Orientin
4	2.48	268; 338	446.72	356.8; 327.2; 298.9; 284.8	Isoorientin
5	2.80	270; 343	461.37	341.0; 313.3; 297.8	8-*C*-Glucosyldiosmetin
6	2.89	270; 343	461.05	341.2; 298.0	6-*C*-Glucosyldiosmetin
7	2.71	269; 339	607.46	487.0; 432.3; 340.7; 322.9; 298.0	6-*C*-Glucoronylvitexin
8	2.78	269; 340	607.12	487.0; 432.3; 341.4; 322.9; 298.0	8-*C*-Glucoronylisovitexin

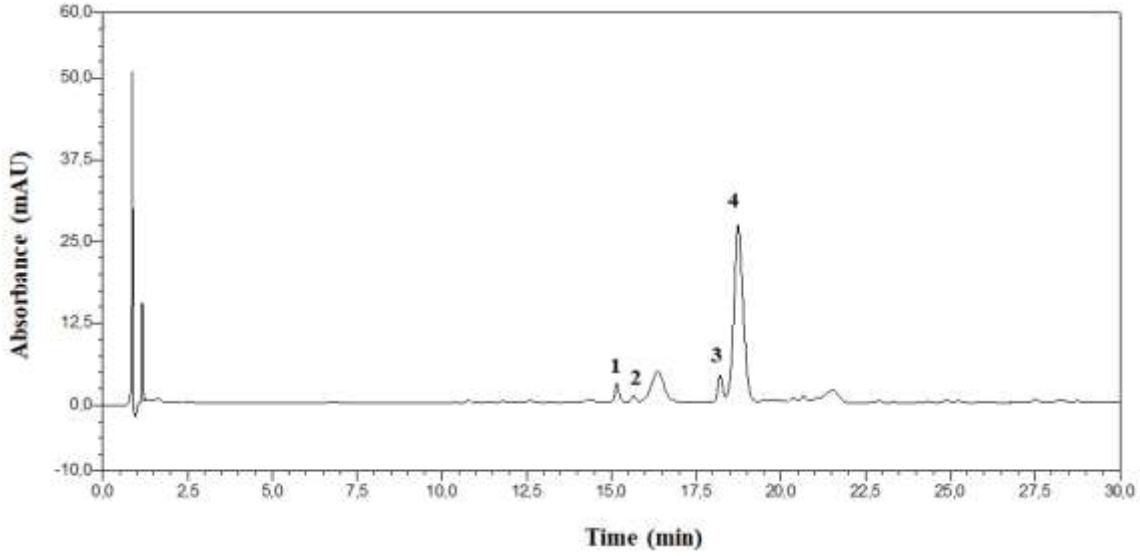

Figure 1. HPLC fingerprint chromatogram (λ=337 nm) of ELPA. Mobile phase: ACN (B): 0.2% (w/w) formic acid (A) (0-30 min, 5-20% of B). Flow rate: 0.6 ml/min. Injection volume: 0.6 µL. Column temperature: 30°C. Peaks: 1 - isoorientin; 2 - orientin; 3 - vitexin; 4 - isovitexin.

glucose moiety.

Compounds 7 and 8 (t_R = 2.71 and 2.78 min) exhibited $[M-H]^-$ ions at *m/z* 607. They were identified as 6-*C*-Glucoronylvitexin and 8-*C*-Glucoronylisovitexin. In the MS/MS spectrum, the same fragment ion at *m/z* 487 $^{0.2}X^-$ $[M-H-120]^-$ indicated the loss of a glucose moiety. The fragment ion at *m/z* 432 suggesting the loss of a glucuronic acid residue $[M-H-175]^-$ (Chen et al., 1998; Schutz et al., 2004; Wolfender et al., 1998).

The extractive process used for the ELPA was ASE, developed to maximize the yield of the extract and simultaneously the content of flavonoids (Gomes et al., 2017). According Pearson et al. (2010) (Pearson et al., 2010) and Saha et al. (2015), the process is promising, because it offers advantages over other extraction processes such as: easy automation, faster sample analysis, better reproducibility, less solvent required, and sample maintenance in an environment without light and free of oxygen. Accelerated extraction systems also allow the operator to control the temperature, pressure, extraction time and number of extractions, which may increase the number of compounds extracted from the plant when optimized. Thus, ASE could have influenced the optimization of extraction and increased the number of flavonoids extracted from ELPA.

The observed cytotoxic activity of ELPA demonstrated in this study might be attributed to the mixture of various flavonoids found in the extract. Flavonoids have the potential to modify many biological cancer events, such as apoptosis, vascularization, cell differentiation and proliferation (Batra and Sharma, 2013). Flavonoids found in ELPA such as vitexin and isovitexin also exert chemotherapeutic potential against breast, hepatic, colorectal, lung, skin, oral, prostate, cervix, ovary,

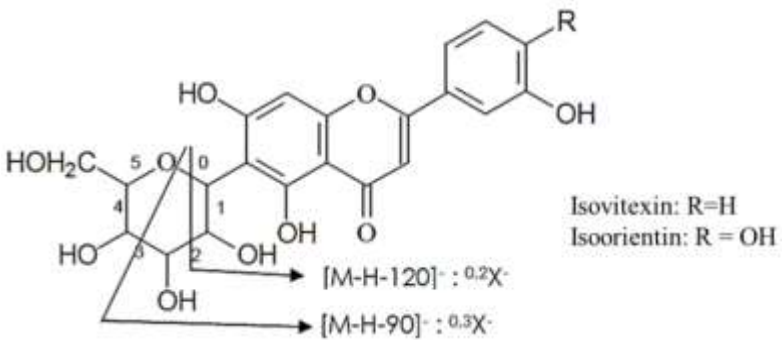

Figure 2. Fragmentation of isovitexin and isoorientin.

esophagus and leukemic cancers (Ganesan and Xu, 2017). Orientin shows an inhibitory effect on the proliferation of esophageal cancer cells (EC-109) and isoorientin demonstrated the ability to inhibit proliferation of liver cancer cells (HepG2) (An et al., 2015; Xiao et al., 2016; Yuan et al., 2012). Diosmetin, found in ELPA conjugated with C-glucosyl groups (8-C-Glucosyldiosmetin and 6-C-Glucosyldiosmetin), is described as having *in vitro* anticancer activity against several cancer cells lines, with potent inhibition against leukemic cells (P-388), and lesser cytotoxic activity against HepG2, Hep3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cancer cells (Patel et al., 2013). These data may justify the *in vitro* cytotoxic effect presented by ELPA.

The presence of saponins in EFPA may be relevant for its antitumor effect. Saponins exert various pharmacological effects (cardiovascular protective activity, anti-inflammatory, antiviral and immunoregulatory effects) including significant anticancer activity, such as anti-proliferative, anti-metastatic, anti-angiogenic and reverses multiple drug resistance (MDR) effects through mechanisms which include induction of apoptosis and promotion of cell differentiation. In addition to these actions, it is described that saponins may reduce the side effects of radiotherapy and chemotherapy, suggesting that this class of compounds is a promising prospect for anticancer research and development (Man et al., 2010; Xu et al., 2016).

Thus, most of the secondary metabolites identified in the EFPA have proven cytotoxic or anticancer activities, which collaborate with the *in vitro* cytotoxic action presented as a result of the present work, emphasizing the hypothesis that the action of the EFPA is probably mediated by synergistic activity among its constituents.

For drug discovery, cell death related testing is paramount in oncology because resistance to dying is a hallmark of cancer cells (Méry et al., 2017). Knowing the cytotoxic activity demonstrated by ELPA, we decided to identify the mechanism of the cytotoxicity induced by the extract. For this, HL-60 neoplastic cells were selected for subsequent testing based on the fact that the lineage is among the most widely used cellular models of myeloid origin, and simultaneously was the lineage against which ELPA demonstrated it's the highest cytotoxic potency. Three concentrations of ELPA, ½ IC_{50} (9.69 µg/ml), IC_{50} (19.37 µg/ml) and 2 × IC_{50} (38.74 µg/ml) were chosen.

To ascertain the cellular death process in cancer cells, two tests were performed, hematoxylin-eosin coloration analyzed by light microscopy, and ethidium bromide/acridine orange coloration using fluorescence microscopy. After 72 h incubation, the effect of ELPA was evaluated based on cell morphology using hematoxylin-eosin. The HL-60 cells treated with ELPA showed the characteristic morphology of cell death induced by apoptosis (reduction in cell volume, chromatin condensation, and nuclear fragmentation), and necrosis (membrane disruption and cell swelling) as shown in Figure 3.

To confirm the light microscopy findings, we performed a morphological analysis of cells treated with ELPA by staining and examining cells with ethidium bromide/ acridine orange by fluorescence microscopy. The percentages of viable, apoptotic and necrotic cells were calculated. After 72 h, HL-60 cells treated with ELPA showed a reduction in the percentage of viable cells for the concentrations of 9.69 µg/ml (48.82 ± 3.76%), 19.77 µg/ml (41.88 ± 2.58%) and 38.74 µg/ml (34.74 ± 1.25%) when compared with the NC group (91.67 ± 1.72%). The percentage of cells in apoptosis increased for the concentrations of 9.69 µg/ml (30.74 ± 3.22%), 19.77 µg/ml (32.44 ± 3.23%) and 38.74 µg/ml (35.88 ± 3.06%) when compared twith the NC group (4.95 ± 0.98%). Another process of cell death observed was the increase in the percentage of cells in necrosis, for the concentrations of 9.69 µg/ml (20.44 ± 5.86%), 19.77 µg/ml (26.88 ± 5.03%) and 38.74 µg/ml (29.45 ± 3.92%) when compared with the NC group (3.17 ± 1.18%) as shown in Figure 4.

These data reaffirm the results found in the morphological analyses using light microscopy; demonstrating that ELPA can cause cell death by apoptotic and necrotic processes. Apoptosis is a programmed cell death process, and many studies suggest the activation

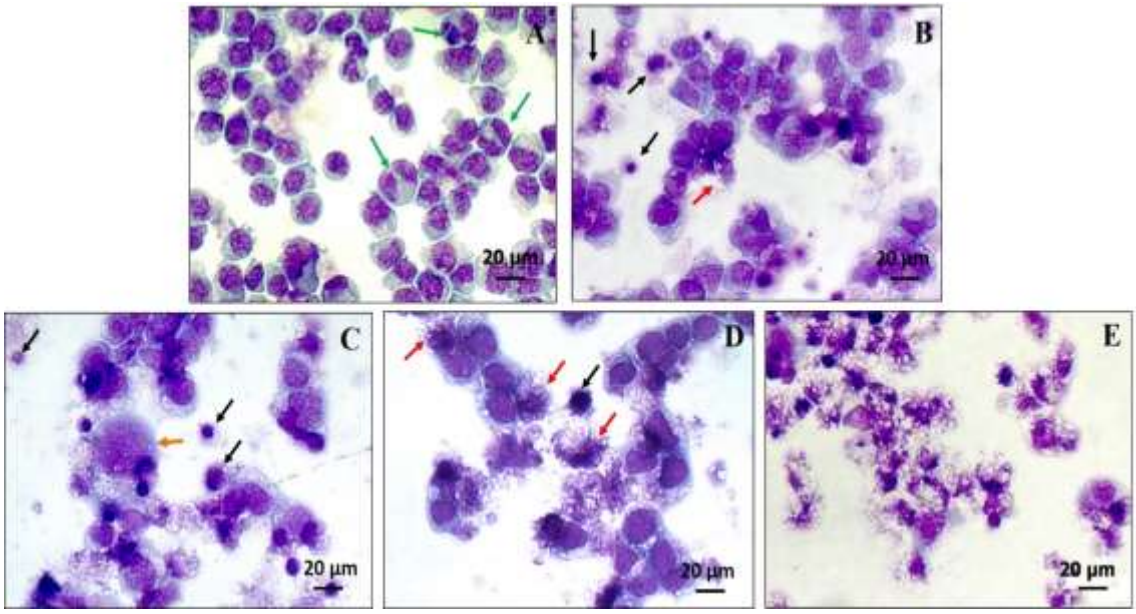

Figure 3. Photomicrography of human cancer cells (HL-60) submitted to hematoxylin/eosin differential staining after 72 h of incubation and analyzed under a light microscope (100X). A - negative control, B - cells treated with ELPA 9.69 µg/ml, C - cells treated with ELPA 19.77 µg/ml, D - cells treated with ELPA 38,74 µg/ml and E - cells treated with doxorubicin. Black arrows indicate cell volume reduction and nuclear fragmentation; red arrows indicate loss of membrane integrity, green arrows indicate cells in mitosis and orange arrow cell swelling.

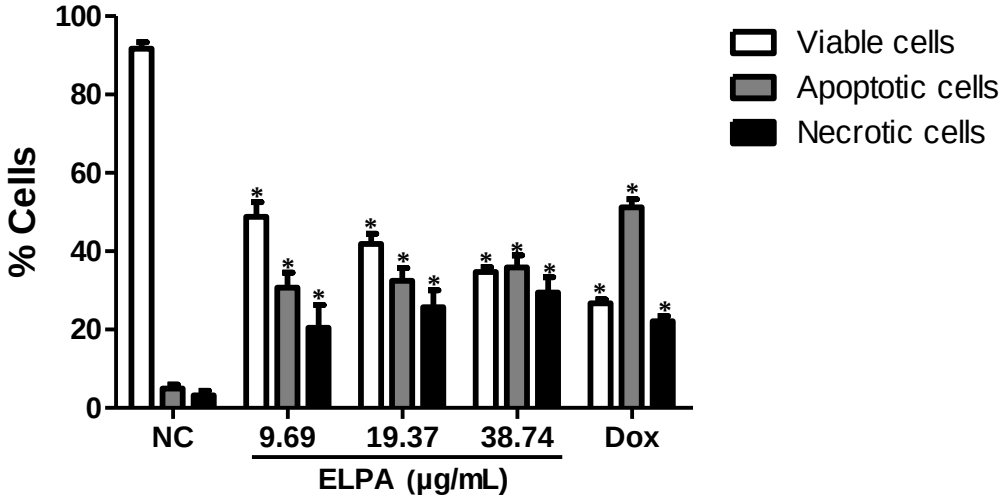

Figure 4. Identification of the type of cell death induced by *P. alata* leaf extract (EFPA) against HL-60. Viable cells (white bar), apoptosis (gray bar) and necrosis (black bar) were determined by fluorescence microscopy using ethidium bromide/acridine orange after 72 h of incubation. The negative control (NC) was treated with DMSO 0.3%, and doxorubicin (Dox), used as a positive control. Data are presented as mean ± SEM of three independent experiments evaluated by one-way variance analysis (ANOVA) with a *Student Newman Keuls* post-test. *$p < 0.05$ compared to the NC group.

of apoptosis as one of the most effective forms of chemotherapy to prevent the development and progression of cancer (Harvey and Cree, 2010; Yuan et al., 2014). Photodynamic treatment and certain antineoplastic agents, such as β-lapachone and DNA alkylating agents induce cell death by apoptosis and

Table 4. Effect of *P. alata* leaf extract (ELPA) on nuclear DNA content in HL-60 cells determined by flow cytometry.

Treatment	Concentration (µg/ml)	DNA Content (%)			
		Sub-G1	G_1	S	G_2/M
NC	-	12.92 ± 1.76	40.13 ± 1.32	36.96 ± 3.46	8.66 ± 3.85
ELPA	9.69	17.79 ± 1.82	34.56 ± 6.15	31.19 ± 3.40	16.87 ± 1.12*
ELPA	19.77	25.08 ± 3.13*	25.03 ± 1.83*	26.51 ± 2.12*	23.77 ± 0.73*
ELPA	38.74	40.05 ± 4.14*	16.69 ± 2.28*	19.08 ± 1.34*	24.21 ± 4.69*
Doxorubicin	0.3	55.42 ± 4.59*	5.99 ± 0.99*	10.59 ± 1.80*	26.38 ± 0.41*

The data presented correspond to the mean ± standard error of the mean of two independent experiments performed in duplicate and evaluated by one-way analysis of variance with *Student Newman Keuls* post-test. *p < 0.05 compared to the negative control. The negative control (NC) was treated with DMSO 0.3%.

simultaneously induce cell death by necrosis in a variety of cancer cells (Li et al., 1999; Zong and Thompson, 2006). Considering the two cell death processes involved, ELPA is a promising candidate for *in vivo* evaluation in animal experimental tumor models.

The morphological analysis in HL-60 indicated that apoptosis is one of the mechanisms of death involved in the cytotoxicity observed in ELPA, we resolved to determine DNA fragmentation and alteration in the cell cycle through flow cytometry. The results concerning cell cycle progression showed that the ELPA at all concentrations tested showed a significant increase in the percentage of cells in the G_2/M phase, being indicative of cell cycle arrest in the specific phase (Table 4).

Assessing DNA fragmentation (Sub-G_1), the data demonstrated a significant ($p < 0.05$) increase in fragmentation in the ELPA-treated groups at concentrations of 19.97 and 38.74 µg/ml (Table 4). Thus, cell cycle arrest may be related to ELPA-promoted DNA damage and subsequent DNA repair attempts. The lesion appears to be intense, as the DNA repair does not occur, and apoptosis is triggered.

Conclusion

The cytotoxic potential of extracts from 14 *Passiflora* spp. obtained by ASE was evaluated. ELPA presented greater cytotoxic potential against cancer cells, without cytotoxicity in non-cancer human cells. The *in vitro* cytotoxic activity may be a consequence of synergistic action between the flavonoids found in ELPA, promoting cell cycle arrest in the G_2/M phase and inducing cell death by apoptosis and necrosis. This work thus demonstrates the potential of Brazilian plants and opens perspectives for ELPA evaluation in experimental *in vivo* tumor models.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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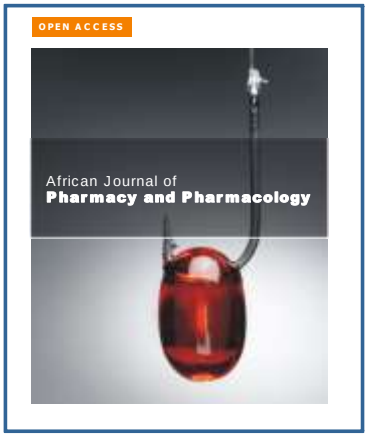

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