Article

Antiproliferative activity of neem leaf extracts obtained by a sequential pressurized liquid extraction

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Abstract: *Azadirachta indica* A. Juss (neem) extracts have been used in pharmaceutical applications as antitumor agents, due to their terpenes and phenolic compounds. To obtain extracts from neem leaves with potential antiproliferative effect, a sequential process of pressurized liquid extraction was carried out in a fixed bed extractor at 25 ºC and 100 bar, using hexane (SH), ethyl acetate (SEA) and then ethanol (SE) as solvents. An extraction using only ethanol (EE) was also conducted to compare the characteristics of the fractionated extracts. The results obtained by liquid chromatography-electrospray ionization mass spectrometry suggested the highest concentration of terpenes for SEA extract in comparison to SH, SE and EE extracts. Therefore, antiproliferative activity showed SEA extracts were the most efficient inhibitors to human tumor cells MCF-7, NCI-H460, HeLa, and HepG2 between all other extracts studied. However, hepatocellular normal cells were more resistant to SH, SEA, SE, and EE compared to malignant cells of breast, lung, hepatocellular, and cervical. Neem fractioned extracts obtained in the present study seem to be more selective for malignant cells compared to the normal cells.

Keywords: Neem leaves; Sequential pressurized liquid extraction; Antiproliferative activity

1. Introduction

Neem (*Azadirachta indica* A. Juss) is a tree of the Meliaceae family found worldwide in semi-tropical and tropical climates [1]. Neem leaves extracts are related to medicinal properties, due to the presence of salannin, nimbin, gedunin and nimbolide [2], among others terpenes and phenolic compounds. This neem chemical composition has been important to the management of several diseases [3, 4].

Neem-compounds has exhibited a chemopreventive and anticancer efficacy, due to their cellular and molecular mechanisms of action, such as immunomodulatory, carcinogen-detoxification, cell-

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cycle arrest, programmed cell death, and anti-metastatic [5]. The anticancer activity of neem constituents can be able to inhibit the growth of a variety of human cancers, such as lung, breast, oral, prostate, skin, liver [6, 7] and cervical [8]. Pharmacological bioactive compounds can be obtained by different extraction methods such as maceration, soxhlet, and pressurized liquid extraction (PLE) [9]. Furthermore, it is important to select an accurate method for natural compounds extraction [9, 10].

PLE shows a potential for the maximum extraction of metabolites from vegetable matrices [13], due to the possibility of using a variety of polar and non-polar solvents under high pressure, which improves the efficiency of the extraction process [14, 15]. PLE reduces the time of extraction and amount of solvent used, contributing for a better extractive process optimization [16, 17]. Moreover, this method reported above has been used to the exhaustive extraction of analytes in one or more clean-up steps [18]. According to Garmus et al. and Monroy et al., the sequential PLE is a good process to obtain natural compounds [11-13].

Neem extracts exhibit different chemical composition depending on the solvent used (methanol, hexane, ethyl acetate, ethanol, and water) and, therefore, the potential medicinal activity of the extracts are related to the solvent chosen [4, 10]. Hexane, ethyl acetate, and ethanol are efficient solvents to extract terpenes and flavonoids, compounds important to human health [19]. The aim of this study was developed a method to obtain extracts with antiproliferative effects from neem leaves, by a sequential process of pressurized liquid extraction using hexane (SH), ethyl acetate (SEA), and ethanol (EE) as solvents, and evaluate the cytotoxicity of the extracts obtained against human tumor cell lines and non-tumor liver cells.

2. Results and Discussion

2.1. Pressurized liquid extraction process

In the present study, three extracts were obtained from single neem leaf mass (20 g), using three different solvents hexane (SH), ethyl acetate (SEA), and ethanol (SE) by a sequential process of pressurized liquid extraction and an ethanolic extract (EE) using one-step pressurized liquid extraction. The results in Table 1 showed that the increase of the solvent polarity from hexane to ethanol 80% leads to a significant increase in the obtaining of the extract dry mass from neem leaves. Furthermore, SE and EE are not significantly different $P > 0.05$. Thus, the previous extractions with hexane and ethyl acetate did not reduce the ethanol extractive capacity. However, both hexane and ethyl acetate show lower capacity to obtain extract dry mass from neem leaves compared with ethanol 80% (Table. 1).

Table 1. Effect of different solvents, hexane (SH), ethyl acetate (SEA), and ethanol 80% (SE and EE) on dry mass extract of neem leaves.

Neem leaves (20g)	One-step extraction (g)	Three-step extraction (g)
Hexane (SH)	____	0.073 ± 0.002^{b}
Ethyl acetate (SEA)	____	0.063 ± 0.004^{b}
Etanol 80% (SE)	____	1.502 ± 0.117^{a}
Etanol 80% (EE)	$1.580 \pm 0.25.89^{a}$	_____

Data are reported as mean ± standard deviation values. Equal letters indicate that there is no difference between the extractions. No performed (___).

2.2. Liquid chromatography analysis

In recent decades, liquid chromatography-mass spectrometry has been used in metabolic studies in the field of analytical chemistry and pharmaceutical analysis, due to their potential to identify compounds [20]. In this study, this methodology was used for chemical characterization of the obtained neem leaves extracts (Fig. 1).

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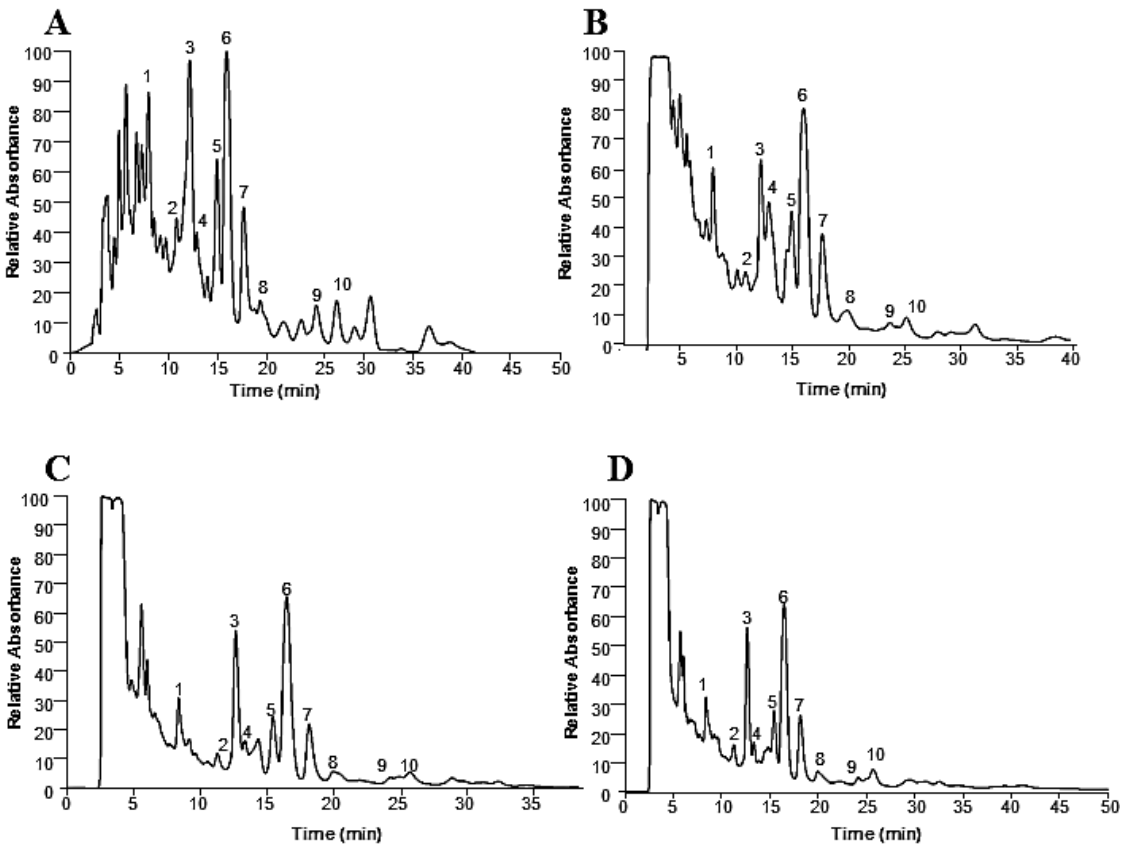

Figure. 1. LC-PDA chromatograms at 210-220 nm of the neem leaf extracts obtained by SH (A), SEA (B), SE (C), and EE (D).

Fig. 1 shows the PDA chromatograms of neem extracts obtained by PLE with different solvents. All extracts presented similar compounds and 10 components were identified, as exhibited in Table 2.

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Table. 2. Neem leaves compounds tentatively identified by ESI-MS from their fragmentation (m/z), in positive mode, and respective HPLC areas for different extraction solvents.

Extract	Peak	^{t}R (min)	Area	Compound	Observed ions (m/z)
SH		8.42	45724118		
SEA	1	8.46	38701542	Nimbandiol	371, 401, 421, 425, 441, 444, 457
SE		8.56	20010994		$[M+H]^+$, 474 $[M+H_20]^+$
EE		8.40	25006988		
SH		11.29	29873977		
SEA	2	11.42	22241890	6-Deacetylnimbin	389, 453, 467, 499 $[MH]^+$, 516
SE		11.32	2818278		$[M+H_20]^+$
EE		11.46	14511268		
SH		12.77	81159631		
SEA	3	12.75	37973099	2,3-Dihydronimbolide	178, 315, 426, 433, 441, 450, 469
SE		12.76	28340365		$[MH]^+$, 486 $[M+H_20]^+$
EE		12.84	36750310		
SH		13.91	13066767		
SEA	4	14.02	17659833	Rutin	266, 480, 546, 558, 611 $[M+H]^+$,
SE		13.95	14990955		628 $[M+H_20]^+$
EE		14.02	15674396		
SH		15.56	36960991		
SEA	5	15.59	31070403	Nimonol	274, 293, 353, 421, 439, 453
SE		15.67	15495619		$[M+H]^+$, 470 $[M+H_20]^+$
EE		15.52	20995715		
SH		16.39	70699349		
SEA	6	16.54	86571238	Nimbolide	277, 435, 435,467 $[M+H]^+$,484
SE		16.42	50917437		$[M+H_20]^+$
EE		16.45	57586856		
SH		16.39	70699349		
SEA	6	16.54	86571238	3-Deacetylsalannin	555 $[M+H]^+$, 572 $[M+H_20]^+$
SE		16.42	50917437		
EE		16.45	57586856		
SH		18.22	32928497		
SEA	7	18.18	37398457	6-Deacetylnimbinene	363, 393, 409,441 $[M+H]^+$, 458
SE		18.12	15714996		$[M+H_20]^+$
EE		18.32	21712675		
SH		19.88	15628192		221, 265, 339, 345, 405, 428,
SEA	8	19.93	23156736	Nimbanal	451,453, 455, 471, 482,493, 511
SE		19.87	6245251		$[M+H]^+$, 528 $[M+H_20]^+$
EE		19.74	11010022		
SH		24.96	14175318		
SEA	9	24.86	12952957	Salannin	199, 230, 278, 319, 378, 481, 515,
SE		24.87	5526287		571, 597 $[M+H]^+$, 614 $[M+H_20]^+$
EE		24.93	2517812		

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SH		25.49	17132995		
SEA	10	25.67	13462271	Gedunin	184, 259, 287, 344, 372, 405, 425,
SE		25.75	6429235		451, 483 $[M+H]^+$, 500 $[M+H_20]^+$
EE		25.70	7359673		

In Table 1, it can be observed that compounds extracted by SH and SEA were similar, but with different relative absorbance, as could be observed in Fig. 1. As referred, the extracts from SH and SEA are more concentrated in the compounds in comparison to SE and EE. Peak 6 is the most abundant and its mass spectral analysis suggested it corresponded to the compounds nimbolide and 3-Deacetylsalannin. Fig. 2 presents the mass spectra and respective structures.

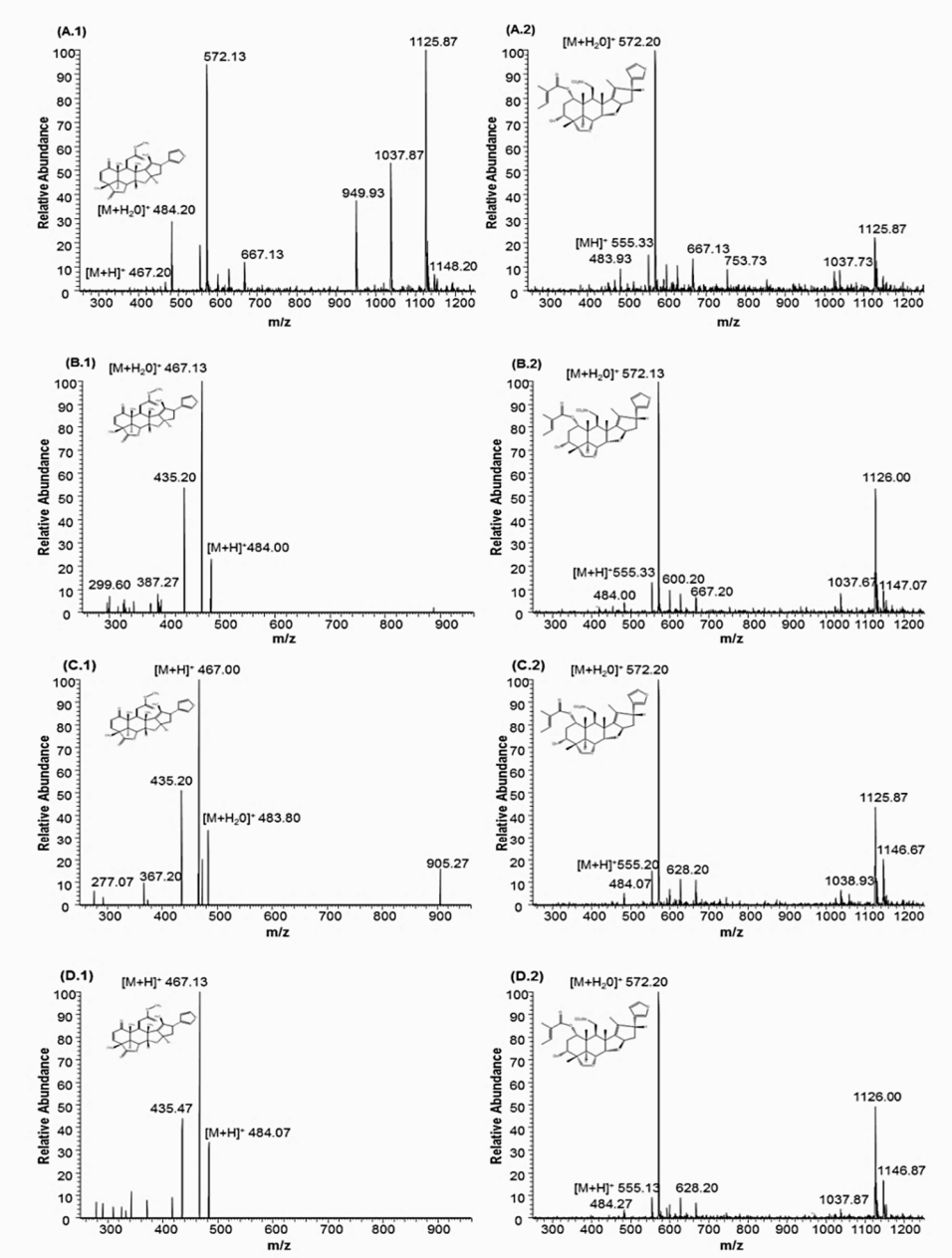

Figure. 2. Mass spectra of nimbolide (A.1, B.1, C.1, and D.1) and 3-Deacetylsalannin (A.2, B2, C2, and D2) terpenoids extracted by pressurized liquid extraction. Capital letters A, B, C, and D correspond to SH, SEA, SE, and EE, respectively.

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These compounds have already been described in neem leaves [5, 21-24]. Fig. 1A and 1B show a relatively higher absorbance (about 20%) for the peaks 1, 3 and 6. Accordingly, these solvents (hexane and ethyl acetate) have a low contribution to overall extraction yield, but a high contribution for several compounds (this is the case of 1, 3 and 6).

Comparing the solvents, it seems that the chemical profile of the polar solvents presents more similarity among them when compared with the non-polar one (Fig. 1A). Table 2 summarizes the compounds tentatively identified by mass spectrometry and retention time of bioactive compounds, according to previously published data [25-30].

Nimbolide mass is 466.199 and its molecular formula is $C_{27}H_{30}O_7$, and the standard shows a mass spectrum $[M+H]^+$ at 467.211 m/z [21]. In Fig. 2, the spectral analysis shows a peak $[M+H]^+$ corresponding to nimbolide in all extracts obtained. The molecular formula $C_{32}H_{42}O_8$, corresponding to 3-Deacetylsalannin $[M+H]^+$ at 555.211 m/z, has been identified in neem leaves [22]. The neem compounds identified by LC-MS show the ability to make adducts with H_2O, forming an additional fragment $[M+18]^+$. Other fragments can result from the rupture of ester bonds from $[M+H]^+$ [31], thereby corroborating the identification of some compounds from Table 2.

Among the 10 compounds identified in Table 1, just the compound 4 (peak 4) was not a terpene: it corresponds to rutin, a flavone $[M+H]^+$ at 611m/z [32]. However, the terpenes obtained in this study are more soluble in less-polar solvents such as n-hexane and ethyl acetate compared with the polar solvent ethanol. The affinity of the targeted compounds with the solvent used in the extraction is very important to obtain bioactive compounds such as anthocyanins, flavones, and terpenes [9, 33]. According to the results, ethyl acetate (SEA) and n-hexane (SH) seem to be good options to obtain terpenes from neem leaves by sequential pressurized liquid extraction. Moreover, in this study, it was also demonstrated that the sequential extraction in fixed bed extractor cell using SEA in the second step improves the extraction of terpenes such as nimbolide and 3-Deacetylsalannin, compared with the other solvents.

2.3. *Cytotoxicity evaluation of neem leaves extracts*

The biological efficacy of the neem extracts was evaluated against four human tumor cells and one normal cell line. The obtained results are summarized in Table 3.

Table. 3. Cytotoxicity of neem leaves extracts obtained by PLE against several human cancer cells (MCF-7, NCI-H460, HeLa, and HepG2) and the normal cell (PLP2).

Lines	Extract (µg/mL)			Control (µg/mL)	
	SH	**SEA**	**SE**	**EE**	**Ellipticine**
MCF-7	188.8±6.4 **a**	82.3±4.3 **b**	307.7±26.0 **c**	312.3±19.2 **c**	0.9±0.1
NCI-H460	224.4±14.4 **a**	60.6±4.3 **b**	316.6±16.1 **c**	>400 **d**	1.0±0.1
HeLa	203.9±13.6 **a**	48.8±4.3 **b**	330.2±15.3 **c**	332.4±7.2 **c**	1.9±0.1
HepG2	115.5±14.4 **a**	52.3±4.8 **b**	333.6±23.3 **c**	313.1±20.1 **c**	1.1±0.2
PLP2	>400 **a**	201.3±17.0 **b**	>400 **a**	>400 **a**	3.2±0.7

Sequential Hexane (SH), Ethyl Acetate (SEA), and Ethanol (SE) extracts. Non-sequential Ethanol Extract (EE). Ellipticine positive control. All data <400 are reported as a mean ±

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standard deviation, from 50% inhibition of cell growth (IG_{50}). Equal letters in the line indicates that there is no significant difference in the cytotoxic effects ($P < 0.05$).

As presented in Table 3, all neem extracts could inhibit the growth of human tumor cell lines. Nevertheless, these extracts exhibit different values regarding IG_{50}. SEA extracts show the highest potential to inhibit the growth of tumor cells, presenting IG_{50} value smaller than values found for SH, SE, and EE, suggesting that the clean-up process performed by the sequential PLE extraction was able to produce fractions with high antitumor effects. NCI-H460, HeLa, and HepG2 cells were more sensitive to SEA among the other studied cells. Some studies have demonstrated that plant-derived fractions obtained by high pressure show an antiproliferative potential against cancer cells [34-38]. The results obtained in this study are in agreement with Hao *et al*., who reported that neem extracts have a potential therapeutic effect on the growth of various types of cancer cells [39].

In the present study (Table 2), we found that neem extracts concentrations were more cytotoxic to the MCF-7 and HeLa cells than 50 and 100 μg/mL of neem ethanolic extract combined with 5 μM cisplatin (antitumor agent). According to Sharma *et al*., these combinations have a synergistic effect on cancer cell growth inhibition in 52.2 (MCF-7) and 65% (HeLa) [8]. Moreover, SEA (Table 2) also exhibits higher cytotoxic effect against human tumor cells compared with the leaves methanolic extracts reported by Pereira *et al*., who obtained IG_{50} values with 83 ± 9 (MCF-7), 262 ± 4 (NCI-H460), 160 ± 13 (HeLa) and 100 ± 10 (HepG2) μg/mL of *Thymus vulgaris* and 154 ± 7 (MCF-7), 229±16 (NCI-H460), 224 ± 12 (HeLa) and 111 ± 12 (HepG2) μg/mL of *Mentha x piperita* [40].

Non-tumor liver PLP2 cells have been used to evaluate toxicity effect for liver normal cells [40-41]. These normal cells were more resistant than human tumor cells (Table 2) to the treatment with SH, SEA, SE and EE. This result can contribute to the alternative therapy development against the growth malignant cells.

4. Materials and Methods

4.1. Neem samples

Neem (*Azadirachta indica* A. Juss) leaves were collected in the Brazilian Agricultural Research Center - Embrapa Coastal Tablelands, in Aracaju, Sergipe, Brazil. All leaves were dried at 45 ºC for 36 h in an oven with hot-air circulation. After that, the leaves were milled and the granulometry classified in the range from 8 to 16 mesh, using a series of Tyler sieves. The obtained product was stored under refrigeration and protected from light until the extractions.

4.2. Pressurized liquid extraction process

Neem leaves (20g) were used for the sequential pressurized liquid extraction with n-hexane (SH) in a first step, ethyl acetate (SEA) in a second step, and water/ethanol (20:80 v/v) mixtures (SE) in a third step. Carbon dioxide from 20 to 0 bar at 25 ºC was used for total removing the n-hexane and ethyl acetate solvent from the sample, before second and third extraction step. Thereby was achievable the use of only one distinct solvent for each extraction step. One-step pressurized liquid extraction using only a water/ethanol (20:80 v/v) mixtures (EE) was carried out for comparison the effects of extraction sequential. All extractions were performed in triplicate, under experimental conditions of 100 bar, 25 ºC, and a flow rate of 1 mL/min during 60 minutes for each extraction solvent.

4.3. HPLC-PDA-ESI-MS analysis

The extracts were analyzed by HPLC-PDA-ESI-MS using a Finnigan Surveyor Plus High-Performance Liquid Chromatography (HPLC) system fitted with a photodiode array (PDA, at 210-220 nm) and a liquid chromatography quaternary pump. The system was coupled to a Finnigan LCQ Deca XP max mass detector equipped with electrospray ionization source (ESI). A LIChroCART® RP-18 column (150 mm x 4.6 mm, 5 μm) (Merck Millipore) was used. The mobile phase was acetonitrile/water (60:40 v/v) at a flow rate of 0.50 mL min^{-1}, and the run time was 40 min with a sample volume injection of 25 μL. The mass spectrometry analysis was performed under positive

electrospray ionization (ESI+). The mass spectra were obtained in the scan range of 250-1200 m/z [21], controlled by Xcalibur software version 2.2.

4.4. Cytotoxicity assays

The cell lines used were: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma) and PLP2 (non-tumor liver primary culture). Each of the cell lines was grown in a 96-well microplate, at a density of 7.5×10^3 cells/well for MCF-7 and NCI-H460, and 1.0×10^4 cells/well for HeLa, HepG2, and PLP2. The cells were allowed to attach for 24 h. After this period, distinct neem extract concentrations (1.56-400 µg/mL) or Ellipticine (positive control) were added to the cells and incubated for 48 h. After that, a prechilled trichloroacetic acid (TCA 10%, 100µL) was added and incubated for 60 min at 4 ºC to improve the adherence of the cells. The plates were washed with deionized water, dried and after the addition of a solution of sulforhodamine B (SRB 0.1% in 1% acetic acid, 100 µL), the mixture was incubated for 30 min at room temperature. Subsequently, the plates were washed with acetic acid (1%) to remove the unbound SRB and dried. The bounded SRB was solubilized with Tris (10 mM, 200 µL) and the absorbance measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) [40, 42].

4.5. *Statistical analysis*

Statistical analysis was determined by one-way ANOVA, followed by a post-hoc Tukey's test using Prism version 5.0 software. Statistical significance was concluded with $p< 0.05$.

5. Conclusions

This study demonstrated that sequential-PLE is an efficient methodology for extraction of bioactive compounds from neem leaves. The use of three different solvents for the extraction process provides extracts with different concentrations of bioactive compounds. The ethyl acetate extract (SEA) was the richest extract in terpene compounds. Moreover, SEA was the most efficient growth inhibitor of tumor cells among all extracts tested. Human tumor cells are more sensitive than normal cells to all neem extracts. The present study provides a process to obtain extracts of neem leaves with potential for application antiproliferative against malignant cells.

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