Phytochemical characterization and effects on cell proliferation of lentisk (*Pistacia lentiscus*) berry oil: a revalued source of phenolics

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Abstract

The ethno-pharmaceutical use of the edible fixed oil produced from lentisk (*Pistacia lentiscus*) berries covers a long tradition in several Mediterranean regions. Many of the health-promoting properties of lentisk berry oil (LBO) have been associated with the content of polar (poly)phenolic compounds. However, the polar fraction (methanol 80%, v/v) of LBO (LBO-pf) remains poorly and inadequately characterized. We assessed the phytochemical composition (fatty acids, phytosterols and polyphenols) of cold-pressed LBO produced in Cilento (Campania region, Italy) over four years of production (2015-2018). Main phenolic compounds present in LBO-pf were identified and semi-quantified combining ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) and HPLC with diode array detection. Phenolic compounds, also responsible for oil stability and antioxidant properties, are relatively abundant in LBO, compared to other edible oils. LBO-pf induced clear dose-dependent effects on the growth of HT-29 cell line derived from human colorectal adenocarcinoma, as evidenced by the cell cycle arrest. Our data support the health-promoting properties of cold-pressed LBO, which is obtained with good yield from spontaneous plants growing in semiarid regions.

Keywords *Pistacia lentiscus*; lentisk berry oil; phenolic compounds; UHPLC-ESI-MS/MS; HT-29 cell line; cell cycle arrest

Introduction

The mastic tree, also known as the lentisk (*Pistacia lentiscus* L.), is an evergreen spontaneous dioecious shrub or tree of the *Anacardiaceae* family, widely spread in the semiarid Mediterranean areas. The use of berries, galls, resin and leaves of *P. lentiscus* in folk medicine to treat a large series of diseases dates back to the ancient Greeks. State-of-art investigations have provided scientific support to the use of mastic gum and related essential oil to treat several pathologies, including infections and cancer [1-4].

The essential oil (*i.e.* mastic tree oil extract) of *P. lentiscus* is considered a promising source of natural cytotoxic compounds possessing anticancer properties [5-7]. The ability of essential oils from several

species of the *Pistacia* genus (*P. lentiscus, P. lentiscus L. var. chia, P. terebinthus, P. integerrima, and P. vera*) to interfere with cell proliferation in human adenocarcinoma cell lines (MCF-7, 2008 and LoVo from breast, ovarian, and colon cancers, respectively) was most likely associated with the presence of phytocomplexes, which include terpenes, sesquiterpenes, phenolics and fatty acids (FAs) [6]. Mastic tree oil extracts containing a combination of terpenes (mainly α-pinene and myrcene) and polyphenols also inhibit growth and survival of human K562 leukemia cells [5]. The antiproliferative activity of mastic tree oil extract has been confirmed in animal models [8, 9]. Phytoconstituents with pharmacological properties have been reported in almost all the parts of the *P. lentiscus* plant [10]. For example, the essential oil from the aerial parts (leaves, twigs and berries) of *P. lentiscus* exhibited antibacterial and anti-inflammatory effects [3, 4, 7]. Lentisk berries can be considered oleaginous drupes, as they contain averagely high amounts of an edible fixed oil exceeding 40% (w/w) on a dry weight basis [11]. Cold-pressed oil yield from fresh berries approaches 12% (w/w) [12], whereas solvent or Soxhlet extraction increases oil recovery up to 15-18% (w/w). However, oil production can vary depending on the maturation stage of the berries. Lentisk drupes have been defined in previous articles as “berry”, “seeds” or “fruits” interchangeably; thereinafter, we will refer to them exclusively as “berries” to avoid misinterpretation. Lentisk berry oil (LBO) can ameliorate diabetes, gastric ulcers, asthma and possesses antidiarrheal, anti-helminthic, anti-inflammatory properties and can prevent wound infections [11, 13-15]. The antimicrobial properties previously attributed to the mastic gum have been recently described for LBO as well [16-19], proposing LBO as a natural microbial modulator to prevent biofilm-associated infective diseases [19]. Notably, the anti-inflammatory effects of LBO have been demonstrated also *in vivo* [20]. Early studies suggested using lentisk berry compounds for cancer treatment, similar to mastic tree oil extract [21]. However, despite *P. lentiscus* extracts obtained from mastic and leaves show an antiproliferative activity against cancer cell lines, the role of LBO in modulating cancer cell growth remains circumstantial. To this regard, a polyphenol enriched extract obtained from leaves, berries and stems of a Tunisian variety of *P. lentiscus* exerted an antiproliferative activity against two cultured cancer cells [22]. The antioxidant properties and

antiproliferative effects of extracts from varying parts of lentisk have been attributed to tannic acid, gallic acid, digalloyl quinic acid derivative, quercetin and *p*-coumaric acid occurring as the dominant phenolics [22]. Nevertheless, it remains to establish if the observed antiproliferative potential of LBO should be attributed to the simultaneous presence of free FAs or to (poly)phenolic components alone [18]. Formerly produced with temperature-aided extraction processes and used for alimentary purposes by indigent people as a surrogate of the extra-virgin olive oil (EVOO), nowadays LBO is successfully produced by cold-pressing, which improves its peculiar organoleptic traits and preserves most of the nutraceutical components. Thus, in the perspective of obtaining edible oils endowed with health-promoting properties from alternative, neglected and sustainable sources, LBO is gaining renewed economic and nutritional appraisal. The FA composition of LBO obtained by both cold-pressing or solvent extraction has been determined in previous studies [11, 17, 23]. In contrast, the phenolic components of LBO, though evaluated as a class of compounds with generic assays, have not been exhaustively characterized at a molecular level yet. Recently, Mezni et al. [12] attempted elucidating the polyphenolic composition of *P. lentiscus* berry oils obtained from plants growing in several localities of Tunisia. Among the other compounds of LBO, surprisingly they reported at high amount secoiridoids and polyphenols typical of EVOO (from *Olea europea*). In the present work, we aimed at assessing the phytochemical composition (FAs, phytosterols and polyphenols) of cold-pressed LBO produced in Cilento (Campania region, Italy) over four years between 2015 and 2018. The (poly)phenolic fraction of LBO was characterized and semi-quantified by high performance liquid chromatography (HPLC) – diode array detector (DAD) and high-resolution mass spectrometry (MS), providing a different molecular profile in comparison with previous reports [12]. Finally, the effects of LBO polar fraction (LBO-pf) on cell proliferation was assayed against the human colorectal adenocarcinoma cell line HT-29.

Materials and Methods

The material and methods section is reported as Supplementary online resource.

Results and Discussion

Chemical Parameters of LBO

Acidity, peroxide value, total polyphenol content and DPPH % inhibition of LBO samples produced yearly over the 2015-2018 time interval are reported in **Table 1**. Chemical parameters have been determined year-by-year soon after oil production. The level of peroxides was relatively low in all samples, despite the high content of unsaturated FAs. Peroxide values were affected by a relatively high variability, perhaps reflecting a certain oxygen exposure during extraction or early storage phases. It is worth noting that no significant increase of peroxide value was recorded by time, even after two years since oil production (**Table 1**), indicating LBO stability over time, likely because of the co-occurrence of natural antioxidant/radical scavengers. In fact, levels of total polyphenols, determined with the Folin-Ciocalteu spectrophotometric method and expressed as mg_{GAE}/kg oil, were comparable to a high quality EVOO [24]. On the other hand, oil acidity was significantly high in all the samples if compared to other edible oils. Besides possible incomplete triacylglycerol biosynthesis, the high acidity of LBO results at least in part from the technology of berry extraction and oil processing, since oil is separated from water phase by slow decantation that may promote hydrolytic reactions. DPPH inhibition was 9-13%, which is in the same range of values determined under the same conditions for freshly produced EVOO samples relatively high in polyphenols (total amount >250 mg/kg oil of hydroxytyrosol and its derivatives), as determined with the IOC method (data not shown).

Fatty Acids

Composition of LBO FAs was substantially stable over the four-years time range evaluated (**Table 2**). In agreement with previous determinations [23], oleic acid (C18:1 n9*c*) was the dominant FA, followed by linoleic (C18:2 n6*c*) and palmitic acids (C16:0) occurring at similar amounts. Overall, content of unsaturated FAs (MUFA + PUFA) in LBO exceeds 70% of total FAs and, indicatively, the SFA:MUFA:PUFA balance approximates 1:2:1, corresponding to the optimal lipid profile in blood lipoproteins associated with low risk of cardiovascular diseases.

Unsaponifiable LBO Fraction

The composition of the unsaponifiable LBO fraction, including tocopherols and phytosterols, has been determined previously [23]. LBO content of phytosterol and squalene that we measured over four years of production is reported in **Table 3**. The amount of phytosterols appears much higher compared to previous reports [23], while results of the present study are in line with those of oil obtained from seeds of other species belonging to the *Pistacia* genus (e.g. *P. terebinthus*). Similarly, the phytosterol profile of LBO was consistent with those of other edible oils. Under a quantitative standpoint, β-sitosterol was the dominant component of the unsaponifiable fraction with amounts varying in the 705-750 mg/kg range, which are slightly higher than the average amount of other edible oils, as recently quantified [25].

Characterization of the LBO-pf by UHPLC-ESI-MS/MS and HPLC-DAD

To maximize the information about the (poly)phenolic components of LBO-pf, hydroalcoholic extracts were complementarily analyzed combining ultra-performance liquid chromatography coupled with mass spectrometry UHPLC-ESI-MS/MS [26] and HPLC-DAD. Merged collected data on accurate mass, MS/MS fragmentation, UV-absorption profiles and comparison with literature and authentic standards data allowed to profile 21 components (**Table 4 and Figure 1)**. They included phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Assignments were confirmed with comparative analysis of authentic standard compounds, when available. Two compounds initially identified as vanillic and syringic acid, showed different retention time and/or fragmentation with respect to respective authentic standards and therefore were labeled as isomers. Peak no. 6 which was amongst the dominant ones at 280 nm, remained unidentified. This compound, most likely being a simple phenol (unimodal UV-spectrum, Abs max 277 nm), has been quantified based on the calibration curve of 4-hydroxybenzoic acid. However, differently from the other compounds, this peak underwent significant fluctuations when the LBO-pf was evaporated under mild temperature (30 °C), most likely due to a significant volatility. Perhaps, the corresponding compound might be any of the phenol components of the lentisk essential oil, occurring as a major compound in the cold-pressed fixed LBO. Interestingly, several simple phenolics identified in LBO-pf, such as *p*-hydroxybenzoic

acid, have been already reported in resin extracts of *P. lentiscus* [27] or in the polar extracts of *P. vera* oil [28]. Contrarily to a recent characterization of LBO-pf from Tunisia ecotypes [12] no trace of oleuropein aglycone and pinoresinol was detected in our samples. The absence in LBO-pf of typical polyphenols characteristic of olive oil was confirmed by comparative analysis of both standard oleuropein aglycone and hydroalcoholic virgin olive oil extracts. Moreover, since under conditions of acidic hydrolysis oleuropein and ligstroside release hydroxytyrosol and tyrosol, respectively, along with elenolic acid (monitored at 254 nm) [29], we compared LBO-pf and EVOO by HPLC-DAD after incubation with 1% TFA. Differently from EVOO extracts, LBO-pf did not show any hydrolytic products from the above mentioned secoiridoids (not shown). It remains to establish if the conflicting results about the presence of secoiridoids are a consequence of a geographic area-dependent composition of LBO.

Quantitative Analysis of (Poly)phenols by HPLC-DAD

LBO-pf samples were quantified by RP-HPLC-DAD using external calibration curves built for each of the components with a parallel analysis of a 21-components multi-standard solution at different concentrations (**Table 4**). The LBO-pf was dominated by simple phenolics, while flavonoids occurred at relatively low amount as aglycones, likely because the corresponding glycosydes distribute preferentially in the water phase during extraction. A few HPLC-DAD peaks, marked as “unknown”, remained unidentified, being authentic standards and mass information not available. The unambiguous assignment of these components would require the contribution of additional techniques (e. g. NMR). Since many minor peaks remained unassigned as well, an accurate quantification of individual and total (poly)phenols was not possible. Thus, total (poly)phenols were estimated by HPLC at 280 nm, according to the International Olive Oil method for the determination of total biophenols in EVOO [30]. Notably, total (poly)phenols were in the range 199-339 mg/kg oil (**Table 4**), which approached or overcame the threshold level of 250 mg/kg used for EVOO to be considered as “high in polyphenols” [24]. Values of total polyphenols determined with HPLC and Folin-Ciocalteu appeared divergent, especially due to the sensitivity of this latter method to interfering components. In

contrast, the HPLC-based quantification of phenolics, although approximated because based on the peak integration of all the compounds at 280 nm, is in general more reliable [30]. The level of single compounds significantly differed among the four years (**Table 4**), indicating that (poly)phenols are affect by an inherent variability due to a combination of biotic, abiotic and technological factors. Interestingly, these factors might be tuned to maximize the amount of (poly)phenols in LBO.

Antiproliferative Effect of LBO-pf on Colorectal Cell Line

The ability of LBO-pf to interfere with cell proliferation was assayed on HT-29 cell line, derived from human colorectal adenocarcinoma. A crystal violet viability assay with increasing concentrations (50-150 μg/mL, w/v) of LBO-pf 2017 and 2018 for 24 and 48 h showed that both of them reduced cell viability in a dose-dependent manner with a maximum effect in the range of 100-150 μg/mL (w/v) (**Figure 2A, B**). More in detail, 100 μg/mL LBO-pf from 2017 and 2018 preparations was able to reduce HT-29 cell number of about 33% and 48%, respectively, in line with higher polyphenolic content and antioxidant capacity of the 2018 LBO preparation (**Table 1**). The representative microphotographs in **Figure 2C** show the clear reduction of cell number after treatment with LBO-pf at the indicated concentrations. Remarkably, no sign of cytotoxic cell death, e.g. apoptosis or necrosis, was detected following treatment of HT-29 cell lines with LBO-pf (data not shown). Therefore, we investigated the possibility that the reduction in cell number could be related to the capacity of LBO-pf to retard or block cell proliferation. To verify this possibility, we measured cell growth at different time points upon treatment with LBO-pf. As reported in **Figure 3**, 100 μg/mL of LBO-pf (2017, 2018) induced a clear arrest in cell proliferation starting from 24 h of incubation, compared to DMSO treated cells. Previous assays indicated that the antiproliferative activity of *P. lentiscus* berry oil on BHK21 cells was higher compared to its enriched phenolic fraction [18]. Our data are suggestive of a much greater specific activity, as the highest concentration of LBO-pf assayed was about 600-466 fold lower than used by Mezni et al. [18], although assayed with a different cell line. Furthermore, unexpectedly these authors did not evidence any significant difference in cell viability between 24 and 72 h of treatment. We are prone to interpret the cytotoxic effects described by these authors as a possible

epiphenomenon related to the high concentrations of fixed oil applied. Herein, for the first time to our knowledge, we reported the ability of LBO-pf to arrest significantly cell proliferation in HT29 cell line without inducing cytotoxicity, but probably acting on the regulation of the cell cycle checkpoints. This possibility is supported by a significant amount of scientific evidence suggesting the ability of polyphenols to modulate the cell cycle progression at multiple levels [31, 32]. However, it is worth mentioning that also phytosterols and polyunsaturated FAs can show effects on cell cycle regulation. For the former, recent reviews described their capacity to reduce cell cycle progression and/or induce cell cycle arrest [33, 34]. Similarly, circumstantial evidences associate ω-3 FAs, also detectable in LBO at minor amounts (**Table 2**), with the regulation of cell cycle progression in cancer cells [35]. To date, we cannot exclude that the mixtures of FAs and phytosterols present in LBO and listed in **Tables 2** and **3** can possess biological properties independent from those associated with the phenolic compounds. It remains to be investigated if the effect on cell cycle regulation is a unique property of the phenolics present in LBO or can be shared by the other fractions, e.g. FAs and/or phytosterols. The present article was primarily aimed at characterizing polar extracts of LBO and shed light on some controversial aspects emerging from the literature, as discussed above. As an added value, we presented initial data on the biological activity associated with LBO-pf. Therefore, we are aware of the limitations of our work, due to the use of a single cell line and to the absence of molecular markers indicating where the LBO-pf arrests the cell cycle (e.g. expression of cell cycle specific cyclins, cytofluorimetric analysis, etc.). The possible identification of specific biomarkers (e.g. massive increase/decrease of specific cyclins) will facilitate the future identification of the most bioactive compounds among those listed in **Table 4,** responsible for the effects on cell cycle regulation evidenced in **Figure 3**.

Conclusions

All the parts of lentisk plant are receiving increasing consideration as natural and sustainable sources of potentially bioactive molecules. LBO is an edible oil with characteristic sensory properties, obtained from spontaneous crops, growing in semiarid regions, with relatively high yield. Currently, the very

high price of cold-pressed LBO compared to other edible oils, due to the meagre production, limits its diffusion. The relative abundance of polyphenols encourages the use of LBO for nutraceutical and cosmeceutical applications and motivates future investigations in order to complete the LBO-pf profiling, with special focus on the less abundant and volatile compounds. This study represents a preliminary chemical characterization of the (poly)phenol fraction of cold-pressed LBO, which exhibits antioxidant and antiproliferative activity against the HT-29 human cancer cell line. The possible identification of specific biomarkers (e.g. massive increase/decrease of specific cyclins) will facilitate the future identification of the individual bioactive compound(s)**,** responsible for the effects on cell cycle regulation. Work is in progress to fill these gaps in order to elucidate the molecular mechanism(s) of action triggered by phenolics present in LBO-pf. Possible beneficial actions of LBO on human health should be evaluated *in vivo* with dedicate research. Assessing health-promoting effects of LBO is expected to increase its demand and, hence, its production, also inducing significant price reduction.

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Author's Contributions

FS and AC have performed chemical analysis. SM has performed cell culture experiments. MGV, GLR and GP have designed experiments. All the co-authors contributed to draft and revise the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Abbreviations

DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
HPLC	High performance liquid chromatography
LBO	Lentisk berry oil
LBO-pf	Lentisk berry oil - polar fraction
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
TFA	Trifluoroacetic acid
UHPLC-ESI-MS/MS	Ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry
SD	Standard Deviation

Table 1 Chemical parameters of LBO determined of four years. Values are average of triplicate determinations ± SD. [a]Peroxide values determined after 2 years-storage. n.d.: not determined (*significance $p < 0.05$*).

	2015	**2016**	**2017**	**2018**	***p***
Acidity (% oleic acid)	6.28±0.12	3.48±0.15	4.17±0.11	6.17±0.16	*< 0.0001*
Peroxide value ($meqO_2/kg$)	4±1	12±3	8±2	5±1	*0.004*
Peroxide value ($meqO_2/kg$) - 2yrs[a]	6±1	11±2	9±2	n.d.	*0.003*
Total polyphenols (mg_{GAE}/kg)	439±15	435±20	517±18	616±12	*< 0.0001*
Antioxidant activity (DPPH, %I)	9±1	10±2	11±2	13±2	*0.116*

Table 2 Determination of LBO fatty acids over four years of production. Values are average of triplicate analyses ± SD. (significance $p < 0.05$)

Component (FAMEs), area %	**2015**	**2016**	**2017**	**2018**	***p***
Palmitic, C16:0	23.82±1.01	22.42±0.85	23.01±1.35	22.85±2.05	*0.678*
Palmitoleic, C16:1	1.70±0.15	1.97±0.23	1.87±0.18	1.77±0.12	*0.321*
Stearic, C18:0	1.36±0.09	1.46±0.16	1.42±0.13	1.40±0.11	*0.804*
Oleic, C18:1 n9*c*	44.20±3.60	44.22±2.98	44.88±2.50	44.47±1.98	*0.985*
Linoleic, C18:2 n6*c*	24.83±1.95	25.33±1.50	24.98±2.01	25.12±1.88	*0.984*
Arachidic, C20:0	0.15±0.02	0.17±0.01	0.17±0.01	0.16±0.01	*0.228*
cis-11-Eicosenoic, C20:1	0.15±0.01	0.17±0.01	0.16±0.01	0.17±0.01	*0.601*
Linolenic, C18:3 n3*c*	0.66±0.05	0.64±0.04	0.60±0.07	0.64±0.05	*0.602*
Σ-SFA	25.33±1.01	24.05±0.86	24.60±2.05	24.41±2.05	
Σ-MUFA	46.05±3.60	46.36±2.99	46.91±2.51	46.41±1.98	
Σ-PUFA	25.49±1.95	25.97±1.50	25.58±2.01	25.76±1.88	

Table 3. Determination of LBO phytosterols over four years of production. Values are average of triplicate analyses ± SD. (significance $p < 0.05$)

Unsaponifiable fraction (mg/kg)	**2015**	**2016**	**2017**	**2018**	***p***
Squalene	480±33	520±52	462±25	495±31	*0.327*
Campesterol	38±2	42±3	35±3	40±4	*0.107*
Stigmasterol	14±1	16±1	12±1	15±1	*0.006*
β-sitosterol	714±36	750±28	705±15	745±51	*0.364*

Table 4 Identification and semi-quantification of polyphenols in the LBO polar extracts. Values are average of triplicate analyses ± SD. (*significance* $p < 0.05$).
tr = trace amounts; amount lower than limit of quantification. Compounds without MS data have been assigned by HPLC-DAD comparison with authentic standard. The definitive identification requires further confirmation.

HPLC peak	Component	Measured *m/z* $[M-H]^-$	Calculated *m/z* $[M-H]^-$	MS^2	Content (mg/kg)				
					2015	**2016**	**2017**	**2018**	***p***
1	gallic acid	169.0135	169.0137	125.0232	8.2±0.8	8.8±0.6	15.3±1.1	10.1±0.9	*< 0.0001*
2	protocatechuic acid	153.0185	153.0188	135.0970, 109.0131	6.1±0.4	0.5±0.1	0.7±0.2	6.0±0.5	*< 0.0001*
3	*p*-hydroxybenzoic acid	137.0233	137.0239	93.0331	29.6±0.9	9.2±0.8	13.9±0.5	73.8±5.2	*< 0.0001*
4	vanillic acid isomer	167.0343	167.0350	138.9279; 152.0103	6.9±0.5	14.5±1.1	18.0±1.3	11.8±0.8	*< 0.0001*
5	caffeic acid				0.9±0.1	1.0±0.1	0.8±0.1	8.0±0.5	*< 0.0001*
6	unknown								
7	syringic acid isomer	197.0446	197.0455	169.0132	0.8±0.1	0.4±0.1	0.7±0.1	1.6±0.2	*< 0.0001*
8	*p*-coumaric acid	163.0392	163.0395	119.0489	0.9±0.1	5.1±0.4	6.0±0.5	0.8±0.1	*< 0.0001*
9	ferulic acid	193.0502	193.0501	134.0361, 178.0263	2.0±0.3	1.5±0.2	2.5±0.1	0.1±0.0	*< 0.0001*
10	rutin	609.1471	609.1456	300.0276	1.4±0.1	1.3±0.1	1.8±0.2	1.6±0.1	*0.003*
11	quercetin 3-*O*-glucoside	463.0893	463.0882	300.0276	0.9±0.1	26.9±2.1	32.7±2.3	20.1±1.4	*< 0.0001*
12	unknown								
13	myricetin	317.0311	317.0303	151.0124, 178.9980	tr	tr	tr	tr	
14	unknown								
15	eryodictiol	287.0566	287.0561	151.0025, 135.0499	9.7±1.0	11.1±0.9	17.9±1.6	15.7±1.1	*0.0001*
16	quercetin	301.0359	301.0348	151.0024, 178.9979	2.1±0.2	2.4±0.3	3.6±0.4	2.4±0.2	*< 0.0001*
17	luteolin	285.0405	285.0399	133.0282, 151.0023	1.4±0.1	0.8±0.1	1.9±0.2	1.8±0.1	*< 0.0001*
18	naringenin	271.0618	271.0606	135.0439, 153.0181, 151.0024	tr	tr	tr	tr	
19	unknown								
20	kaempferol	285.0406	285.0399	151.0028, 257.0455	0.2±0.0	0.3±0.1	0.4±0.1	0.5±0.1	*0.0001*
21	diosmetin	299.0561	299.0561	284.0329	tr	tr	tr	tr	
Total polyphenols (HPLC method), mg/kg					**199**±18	**261**±23	**257**±21	**339**±20	
Total polyphenols (Folin-Ciocalteu method), mg_{GAE}/kg					**439**±15	**435**±20	**517**±18	**616**±12	

1

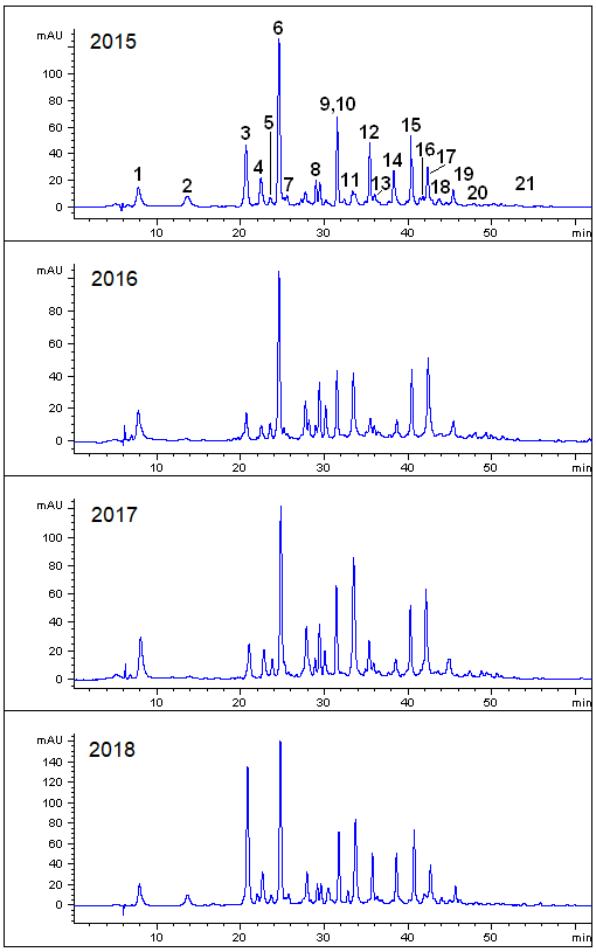

Fig. 1 RP-HPLC-DAD comparison of polar extracts from LBO produced over four years (2015-2018). Peaks are assigned in Table 4.

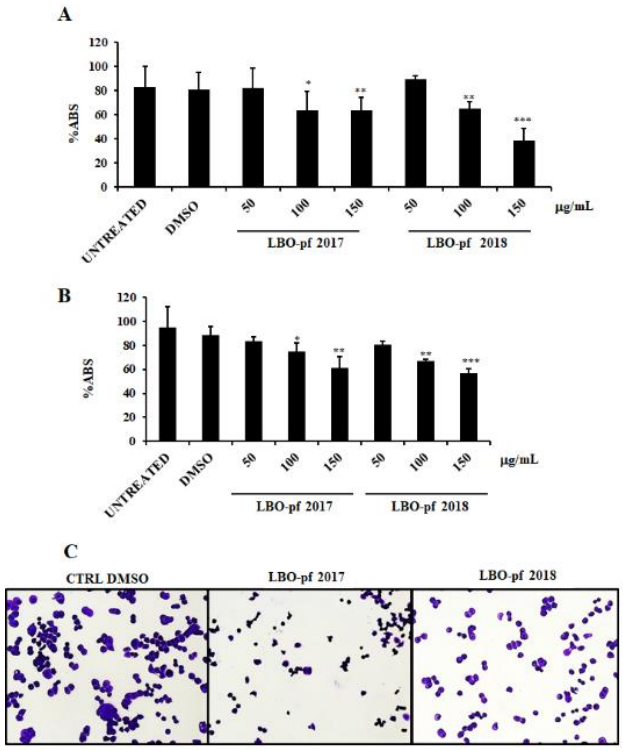

Fig. 2 LBO-pf reduces HT-29 cell number. HT-29 cells were incubated with increasing concentration (50, 100, 150 µg/mL, w/v) of LBO-pf (2017, 2018) for 24 h (A) and 48 h (B). Crystal violet viability assay was performed at the indicated times, as described in Materials and Methods. Bar graphs represent the mean ± SD; symbols indicate significance: ***p < 0.001; **p < 0.01 and *p < 0.05 with respect to untreated cells. Representative images (C) of HT-29 cells treated with LBO-pf (2017, 2018) (100 µg/mL, w/v) for 48 h. Images were taken by microscope Axiovert 200 Zeiss (200x magnification).

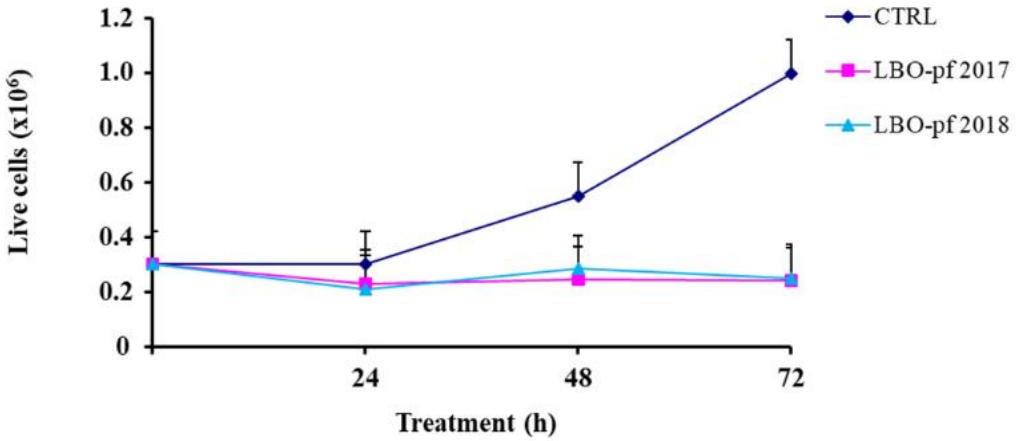

Fig. 3 Effect of LBO-pf on HT-29 cell growth. HT-29 cells were incubated with of LBO-pf (2017, 2018) (100 µg/mL, w/v) and counted at the indicated times using the trypan blue method. Data points epresent the mean ± SD