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Original article

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ABSTRACT

In the present study, the effect of *Ecbalium elaterium* seed oil on adhesion, migration and proliferation of human brain cancer cell line (U87) was determined. Treatment of U87 cell line with the seed oil resulted in strong inhibition of their adhesion to fibrinogen (Fg), fibronectin (Fn). It also reduced their migration and proliferation in a dose-dependent manner without being cytotoxic. Concomitantly, by using Matrigel™ assays, the oil significantly inhibited angiogenesis. The anti- tumor effect of the oil is specifically mediated by αvβ3 and α5β1 integrins. The presence of integrin antagonists in seed oil from *E. elaterium* could be used for the development of anticancer drugs with targeted "multi-modal" therapies combining anti-adhesif, antiproliferative, antimetastasic and anti-angiogenic, approaches.

1. Introduction

Glioblastoma is one of the most frequent and aggressive intracranial neoplasms in humans, and its prognosis remains poor despite the advancement of basic and clinical research studies [1]. Because of its strong vascular proliferation, invasiveness, diminished apoptosis, radio-and chemoresistance [2], treatment of gliobastoma remains one of the most challenging task in clinical oncology, and up to now there is no licensed drug for this malignant tumor [3]. Considering that the onset and progression of cancer is mediated by a bidirectional signalling of several integrins, search on integrin-inhibitors as novel therapeutic targets is of promising alternative to improve and develop treatment of glioblastoma.

Integrins are a family of adhesion molecules constituted of 24 heterodimeric transmembrane receptors for extracellular matrix (ECM) proteins, playing crucial roles in tumor angiogenesis, progression, invasion and metastasis [4,5]. They also have functional relationships with other membrane receptors such as ion channels, growth factor receptors, immunoglobulins and matrix degrading proteases [6]. Among integrin ligands, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ are the most important ones and their relevance during tumor angiogenesis and development is well-documented [7]. In fact, the expression of the former integrin $\alpha_v\beta_3$ in tumor cells (particularly glioblastomas, melanomas, breast and prostate) and vascular endothelial cells leads to tumor progression and invasiveness, while the up-regulation of $\alpha_5\beta_1$ integrin fibronectin receptor participates to tumor cell dissemination and metastasis *via* enhancement of cell adhesion to fibronectin [8].

Considering their role in angiogenesis, proliferation and invasiveness of tumor cells, $\alpha_v\beta_3$ and $\alpha_5\beta_1$integrins have been considered as prime targets for the development of anticancer therapeutic agents. In this direction, attempts aimed at developing potent inhibitors have successfully lead to the identification and development of a plethora of integrin inhibitors including functionally blocking monoclonal antibodies such as vitaxin and CNTO95 [9,10], peptides with RGD motifs such as cilengitide [4,11], desintegrins namely salmosin, jararhagin, eristotstatin, contortrostatin, obtustatin and endostatin [7] paclitaxel, doxorubicin, campthotecin, fumgillin [3] and Nutlin-3a [8] among others.

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However, most of the developed anticancer-drugs are exorbitant cost and cytotoxic and can cause detrimental side effects including hypersensitivity, hypertension, thrombocytopenia, neutopenia, anaemia, liver injury, lung disease, renal failaure, cardiac disorders cutaneous reactions and cancer recurrence [12]. To minimize such effects, the use of plant phytochemicals as anti-cancer agents or as adjuvant of chemotherapeutic drugs is regarded as a valid strategy in cancer therapy.

In connection with this topic, earlier investigations have pinpointed the efficiency of some phytochemicals to treat various cancers types. They include allyl sulphur compounds from *Allium* vegetables [13], tea phenolic compounds (catechin derivatives) [14], flavonoids (i.e. rutin, quercetin, apigenin, chrysin, kaempferol, genistein, silibinin, curcumin etc.) [15,16] alkaloids (acridone, vinblastine, vincristine, solamargine etc.) [17], essential oils (carvacrol, limonene, linalyl acetate, citral, camphene etc.) [18], and fatty acids (*n*-3 fatty acids, oleic acid; conjugated linoleic acids) [19,20], among others. The anticancer efficacy of the latter components against various cancers has been reported [21]. In this context, earlier *in vitro* studies have showed that docosahexanoic acid was able to reduce the invasive profile of renal cell carcinoma [21,22]. Later, conjugated linoleic acids have been found to possess anti-tumor effect in brain-tumor bearing rats [23] and human glioblastoma cells [24]. In another report, α-linolenic acid was found to suppress lipophosphatidic acid (LPA)-induced migration, adhesion, focal adhesion formation, and calcium mobilization in cancer cells [25]. The underlying mechanisms of anticancer effects of fatty acids, specifically n-3 series were also elucidated and they include anti-angiogenic effects mediated through inhibition of angiogenic factors (VEGF, MMP, FGF, etc.), anti-inflammatory by inhibition of TNF, ILs and other pro-inflammatory mediators, inhibition of integrins, modulation of cancer cell proliferation and invasion, as well as induction of apoptosis [25,26]. However, most of these studies have directed attention towards n-3 fish oils, and little is known about the anticancer activity of plant fatty acids. Consequently, it will be of interest to assess the anticancer activity of plant derived fatty acids. With regard to this topic, the species *Ecbalium elaterium* could be a potential candidate for such an assay.

Ecballium elaterium (L.) A. Rich. (Cucurbitaceae) commonly known as squirting cucumber is a wild perennial plant native to the Mediterranean area [27]. It is used since ancient times as valuable medicinal plants for the treatment of sinusitis, hepathitis, fever, cancer, rheumatism, hemorrhoids, liver disorders, earache, and uroclepsia [28]. The antimalarial, antimicrobial, anti-inflammatory, analgesic, antijaundice and anti-proliferative properties of *E. elaterium* extracts have also been reported [29–31]. In a recent study from our laboratory, we have consistently demonstrated that *E. elaterium* seed oil which consists mainly on free fatty acids namely linoleic (C18:2 n-6), punicic (C18:3 n-5) and oleic (C18:1 n-9) acids with hight amounts 48.64%, 22.38% and 15.58% respectively; tocopherols with the isoforms γ- tocopherols (44.23 mg/100 g) and δ-tocopherols (12.44 mg/100 g) being the main ones as well as sterols with β-sitosterol (396.25 mg/100 g) and campesterol (139.63 mg/100 g) as the most abundant ones, had potent antiproliferative effects against Human colonic adenocarcinoma (HT29) and fibrosarcoma (HT1080) cell lines [31]. Prompted by these results, the present study was intended to evaluate the inhibitory effects on αvβ3 and α5β1 integrins, anti-adhesive, anti- migratory, anti-proliferative and anti-angiogenic properties of the seed oil from *E. elaterium* on human brain cancer cell line (U87). It is expected that results from this study will provides new insights on the mechanism of anticancer activity of *E. elaterium* seed oil on one hand, and will contributes to the development of bio-based anticancer drugs on the other hand.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM) and RPMI 1640 medium were purchased from GIBCO (Cergy-Pontoise, France) and fetal calf serum (FCS) from BioWhittaker (Fontenay-sous-Bois, France). Penicillin, streptomycin, human fibrinogen, human laminin and poly-L-lysine were from Sigma (St. Quentin Fallavier, France). Rat type I collagen was from Upstate (Lake Placid, NY, USA) and human fibronectin from Chemicon (Temecula, CA, USA). Human vitronectin was purified according to Yatogho et al. (1988) [32]. Rat monoclonal antibody (mAb) 69.6.5 against αv integrin was produced as previously described [33]. Mouse mAbs LM609 (anti-αvβ3) and P1F6 (αvβ5) were purchased from Chemicon. Mouse mAbs Gi9 (anti-α2β1), SAM1 (anti-α5β1) and C3VLA3 (anti-α3) were from Immunotech (Marseille, France). Rabbit anti-rat was purchased from Sigma. Matrigel™ was from BD Biosciences, Pont de Claix, France. Hexane was purchased from Fluka Chemical Co. (Buchs, Swizerland).

2.2. Cell culture

The human glioma glioblastoma U87 cell line was routinely cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). Human cell lines derived from fibrosarcoma (HT1080) and colonic adenocarcinoma (HT29) were routinely cultured in DMEM containing 10% FCS. Human leukemia (K562) cells were cultured in RPMI 1640 medium containing 10% FCS. HMEC-1 cells were routinely maintained in MCDB-131 medium (Lonza, Levallois-Perret, France) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin and streptomycin (all from Life Technologies, Paisley, UK), 1 mg/mL hydrocortisone (Pharmacia & Upjohn, St-Quentin Yvelines, France) and 10 ng/mLepithelial growth factor (R&D Systems, Minneapolis, MN). HMEC-1 cells were grown on 0.1% gelatin-coated flasks and were used between passages 3 and 12. Cell lines were maintained under humidified 5% CO_2 and 95% air at 37 °C.

2.3. Lactate dehydrogenase (LDH) release assay

Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) activity into the culture medium upon damage of plasma membrane. Total release of LDH (100% toxicity) was obtained by adding 0.1% Triton-X100 in the incubation medium. The supernatants werecollected, clarified by centrifugation for 5 min at 600×g and, then 80 μL of supernatant were submitted to LDH-based cytotoxicity kit (Sigma) in accordance with the manufacturer's instructions.

2.4. Cell adhesion assay

Adhesion assay was performed as previously described [34]. Briefly, 96-well plates were coated with purified ECM protein solutions for 2 h at 37 °C and blocked with 0.5% PBS/BSA. Cells in single cell suspension, pretreated with seed oil or blocking anti-integrin antibodies, were added to wells and allowed to adhere to the substrata for 1 h (HT1080, HMEC1, U87) or 2 h (HT29-D4, K562) at 37 °C. Adhesion of K562 cells was performed in the presence of 1 mM $MgCl_2$ and 100 nM phorbol 12-myristate 13-acetate in order to activate $\alpha5\beta_1$ integrin. After washing, adherent cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 600 nm.

For adhesion assay on antibodies, 96-well plates were coated with 50 μL of rabbit anti-rat IgG (50 μg/mL), overnight at 4 °C. Wells were washed once with PBS and 50 μL of anti-integrin blocking antibodies (10 μg/mL) were added for 5 h at 37 °C. Then, wells were blocked with 0.5% PBS/BSA and adhesion assay was continued as above.

2.5. Cell migration assay

The *in vitro* cell migration assays were performed in modified Boyden chambers (NeuroProbe Inc., Bethesda, MD) with porous membranes pre-coated with 10 μg/mL of fibronectin or 50 μg/mL fibrinogen for 5 h at 37 °C as previously described [35].

Cell velocity was calculated from time-lapse video-microscopy as previously described [36]. Briefly, human glioblastoma cells were trypsinized and then seeded on fibrinogen pre-coated 24-well plates at low confluence (10^4 cells/cm^2) and allowed to adhere at 37 °C for 2 h. The plates were placed on an inverted Nikon microscope equipped with a heated stage and 5% CO2 supply. Three fields per well were imaged and followed at 5 min intervals over 3 h with a Coolsnap HQ camera (Photometrics, Tucson, AZ) operated by NIS-elements AR 2.30 software (Nikon). Manual single-cell tracking was performed by using Metamorph® image analysis software (Roper Scientific, Evry, France). Migration tracks were used to calculate total migration distance, distance to origin, velocity and directional persistence of cell migration. The distance to origin was determined as the net translocation between the initial and the final position. Velocity was calculated as the total migration distance divided by 3 h. Directional persistence was calculated as the ratio of the distance to origin to the total distance migration.

2.6. Cell proliferation assay

Cells were obtained in single cell suspension by treatment of subconfluent cell monolayers with trypsin–EDTA. After centrifugation, cells were washed twice and resuspended with culture medium (5.000 cells/100 μL). After 2 h, seed oil was added at different concentrations (5, 10, 15, 20 and 25 μg/mL) and cells allowed proliferating for 72 h at 37 °C in a cell culture incubator. Cells were washed and then fixed by 1% glutaraldehyde. After staining by 0.1% crystal violet, cells were lysed with 1% SDS and the optical density was measured at 600 nm by a microplate reader [37].

2.7. Tube formation assay

The effect of *E. elaterium* seed oil on *in vitro* angiogenesis was examined by using the HMEC-1 cells capillary-like tube formation assay. Briefly, a 96-well plate pre-coated with 100 μL Matrigel per well was prepared and solidified for 1 h at 37 °C. HMEC-1 cells were plated on Matrigel-coated wells at a density of (5.000 cells/100 μL) and incubated for 6 h at 37 °C and 5% CO_2. The formation of capillary-like tubular networks was observed with a DM-IRBE microscope (Leica, Rueil-Malmaison, France) coupled with a digital camera (CCD camera coolsnap FX, Princeton Instruments, Trenton, NJ). The percentage of tubule area was quantified by image analysis using Metamorph® image analysis software (Roper Scientific, Evry, France) as previously described by Pasquier et al. (2004) [38].

2.8. Statistical analysis

All data were expressed as means ± standard deviation of three experiments, and the difference between treated and untreated (control) groups was determined by using one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test (RStudio, Version 0.97, Boston, USA). A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Seed oil of E. elaterium inhibits adhesion and migration of human glioma cell line U87

The effects of *E. elaterium* seed oil on U87 cell adhesion was studied using purified ECM proteins; collagen I (Col I), fibrinogen (Fg), fibronectin (Fn) and vitronectin (Vn). As shown in Fig. 1a and b, the oil significantly ($p < 0.05$) reduced the attachment of the glioma cell line U87 to fibrinogen (IC_{50} = 9.2 μg/mL) and fibronectin (IC_{50} = 34.1 μg/mL) and to a lesser extent on collagen I and vitronectin. When using the integrin-independent substratum poly-L-lysine (PLL), the oil did not affect cell adhesion. The results showed that integrin family of adhesion receptors was involved in the inhibitory activity of the oil.

Due to its inhibitory effect on cell adhesion to fibrinogen and fibronectin, the oil was assayed for its *in vitro* antimetastatic effect using haptotaxis assays in modified Boyden chambers. Fig. 2a and b revealed that the tested oil significantly ($p < 0.05$) inhibited cell migration to fibrinogen (IC_{50} = 9 μg/mL) and fibronectin (IC_{50} = 34 μg/mL) in a concentration-dependant manner.

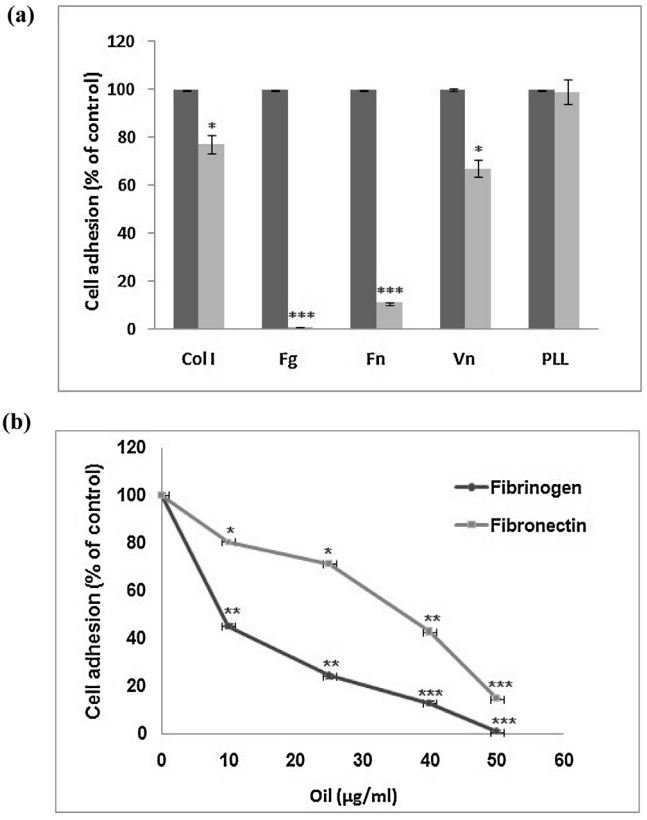

Fig. 1. Seed oil of *Ecballium elaterium* inhibits tumour cell adhesion (a) human glioma cells (U87) were preincubated with oil (50 μg/mL) for 30 min at room temperature. Cells were then added to 96-well microtiter plates coated with different ECM proteins; type I collagen (Col I), fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn), or with poly-L-lysine (PLL) and allowed to adhere for 1 h at 37 °C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Results are expressed as a percentage of adhesion in the absence of seed oil. (b) Dose–effect of oil on U87 cancer cell adhesion to fibrinogen and fibronectin. Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant *: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$.

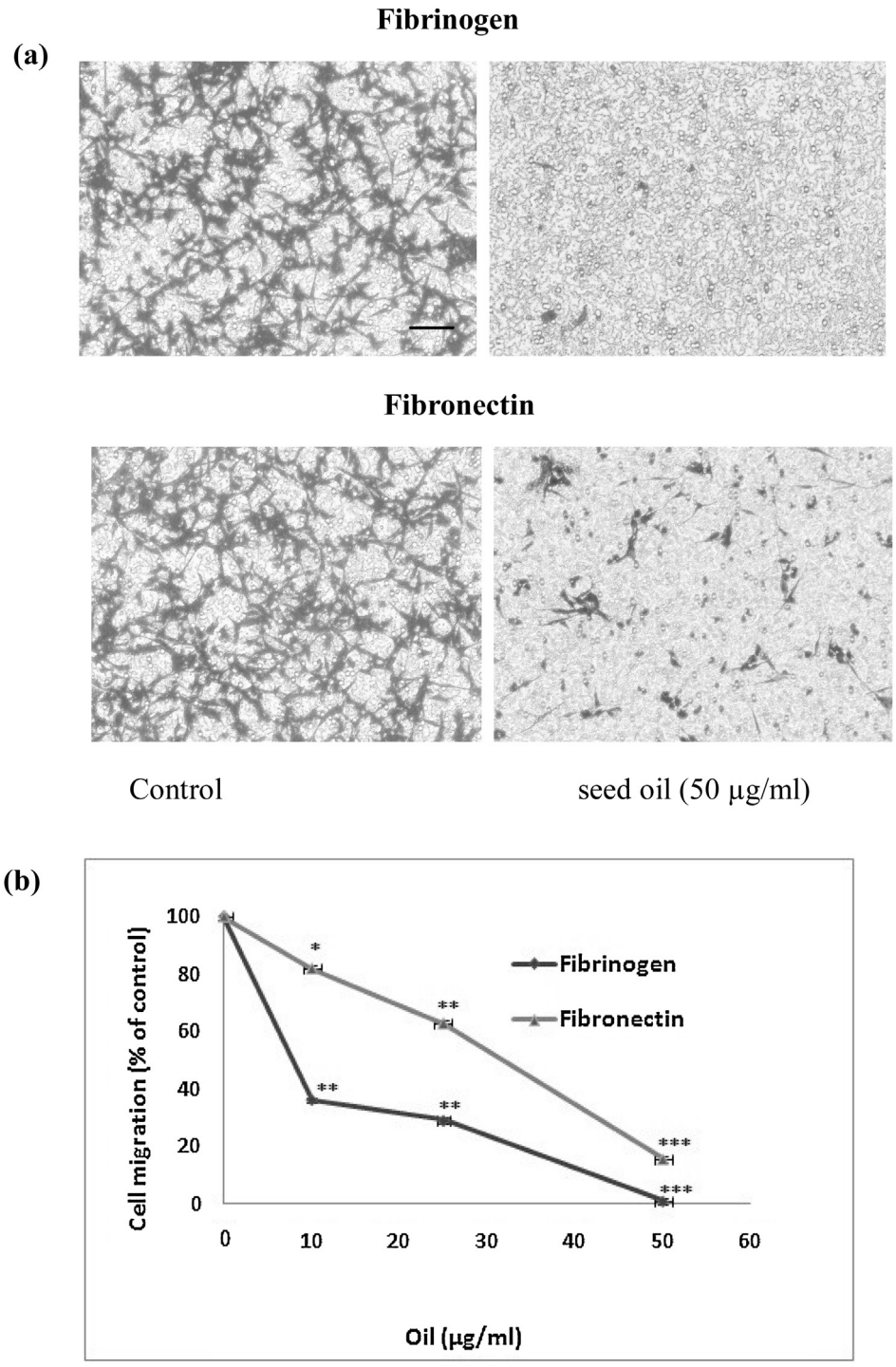

Fig. 2. The oil inhibits cell migration. (a) Cell motility was determined in a modified Boyden chamber using porous membrane precoated with 50 µg/mL fibrinogen or 10 µg/mL fibronectin. After treatment with 50 µg/mL of oil, U87 cells were seeded into the upper reservoir and allowed to migrate through the filter towards the lower reservoir. Cells that migrated to the underside of the filter were stained with 0.1% crystal violet Scale bar: 100 µm. (b) Dose–effect of oil on cell migration to fibrinogen or fibronectin. Results are expressed as a percentage of migration in the absence of the oil. Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$).

Next, a random two-dimensional motility assay by using time-lapse videomicroscopy was performed to screen the inhibitory effects of the tested oil on the motility of U87 cells. Representative migration paths (where the origin of each cell track has been set to the coordinates x = 0, y = 0) for 12 cells are shown in Fig. 3a. They demonstrated that the seed oil significantly ($p < 0.05$) inhibited the motility of U87 cells. The trajectory of oil-treated cells was modified and the distances to origin were decreased by 82.6%. Analysis of the trajectory obtained from time-lapse recordings of each individual cell during 3 h (Fig. 3b), indicated that untreated cells exhibited high migration velocity (0.93 µm/min) (Supplemental data, Video 1), whereas, the migration speed of treated cells decreased remarkably with increasing oil concentration (Supplemental data, Video 2 and 3). The magnitude of cell migration reduction was found to be 0.66 and 0.26 µm/min for 25 and 50 µg/mL, respectively. For the latter concentration (50 µg/mL), the oil

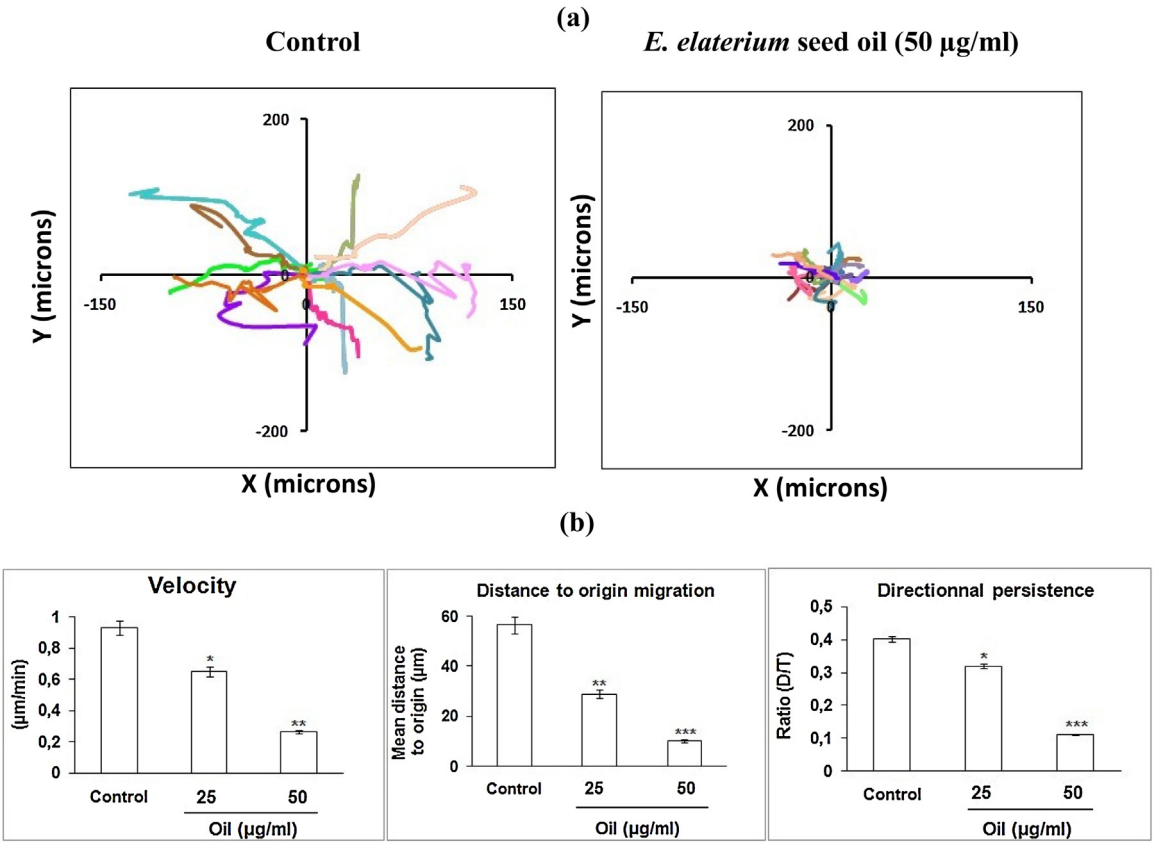

Fig. 3. Evaluation of cell motility by time-lapse video microscopy: Pictures of four fields per well were taken at 5 min intervals during 3 H, the trajectories covered by cells during the 3 h of recording were plotted in with the initial position of each track aligned at x = 0, y = 0. (d) Cell velocity, mean distance to origin and directional persistence were calculated from time-lapse videomicroscopy as described in the Materials section. Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$).

strongly affected cell directionality by 72% (Fig. 3b). The negative effects of the oil on cell velocity and directional persistence may presumably due to the alteration of protrusions formation suggesting a failure in the extension of cell lamellipodia.

3.2. E. elaterium *seed oil effects are mediated through αvβ3 and α5β1 integrins*

In keeping with the precedent observations, and in order to identify which integrins are involved in the attachment of U87 cells to fibrinogen and fibronectin, adhesion assays using functional blocking specific antibodies: mAbs anti-αv (69.6.5), anti-αvβ3 (LM609), anti-αvβ5 (P1F6) and anti-α5β1 (SAM1) was performed. Results depicted in Fig. 4a, showed 99% decrease of U87 cells adhesion to fibrinogen in the presence of anti-αvβ3 antibody. Blocking the active site of α5β1 integrin caused a 92% reduction of the U87 cell adhesion to fibronectin. Thus, it appears that the attachment of U87 cells to fibrinogen and fibronectin is mediated through αvβ3 and α5β1 integrins, respectively.

Since integrins are key actors during cell adhesion and migration process, the hypothesis that oil could affect the function of these adhesion receptors make sense. To verify such assumption, we first performed adhesion assays to a panel of immobilized antibodies raised against several integrin subunits. As illustrated in Fig. 4b, when exposed to seed oil, attachment of U87 cells to anti-α5β1 antibodies was dramatically reduced (over 85%) and completely suppressed with the integrin αvβ3-directed antibody (over 98%), suggesting that the oil exerts its effect on human glioma cell adhesion, and migration by inhibiting αvβ3 and α5β1 integrins. However, the oil slightly only decreased U87 cells adhesion on anti-α2β1 (Gi9) and αvβ5 (P1F6) antibodies, while adhesion was not modified on anti-α3β1 (C3VLa3) antibody.

The targeted integrins involved in the inhibitory effects of seed oil were further examined on various cell/ECM protein pairs involving unique integrins: α1β1 (PC12/type IV collagen), α5β1 (K562/fibronectin), αvβ3 (HT1080/fibrinogen), αvβ5 (HT29-D4/vitronectin) and αvβ6 (HT29-D4/fibronectin). As shown in Fig. 4c, the oil blocked the adhesive function of αvβ3 and affected cell adhesion through α5β1 and α1β1 integrins. Interestingly, the tested oil was able to discriminate between αv-containing integrins, since αvβ3 and αvβ6 were inhibited but not αvβ5. These results show that *E. elaterium* exhibits hight affinity towards α5β1and αvβ3 integrins. Supporting data were provided by Fig. 4d, that clearly shows the high efficiency of the oil in inhibiting both integrins with IC50 values of 8.82 and 22.86 µg/mL for αvβ3 and α5β1, respectively.

3.3. *Seed oil of* E. elaterium *inhibit the proliferation of glioma cancer cell lines*

In the proliferation assay, it has been found that the presence of oil in the culture medium dose-dependently reduced the number of U87 cells with an average IC50 value of 5.84 µg/mL (Fig. 5a and b), indicating its strong anti-proliferative effect.

3.4. *Seed oil display anti-angiogenic activity*

As we demonstrated that the oil specifically targeted αvβ3 and α5β1 integrins, and consequently inhibted adhesion, migration and proliferation of Human glioma cells, we next addressed

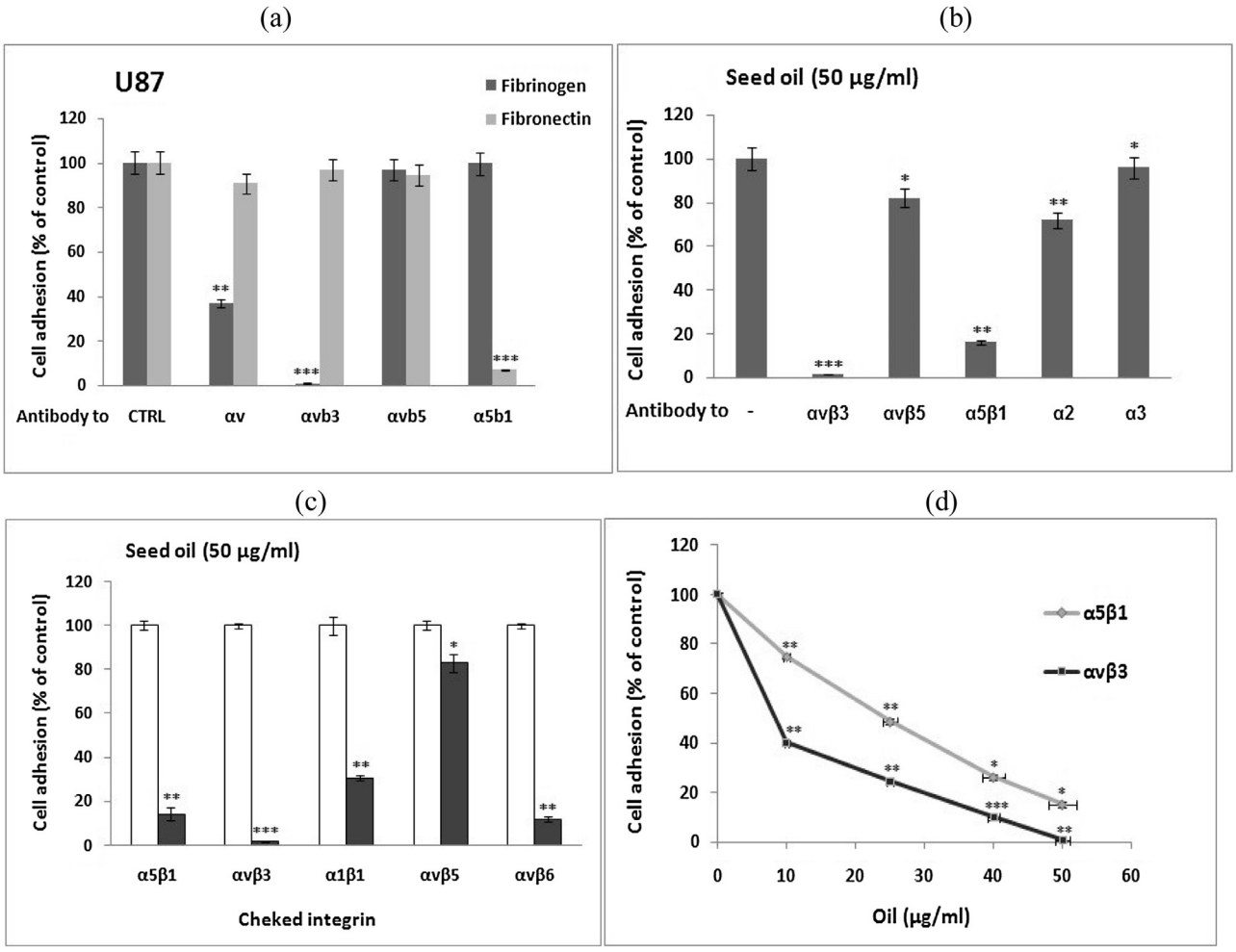

Fig. 4. *Ecballium elaterium* seed oil inhibits αvβ3 and α5β1 integrin-mediated adhesion in glioma tumor cell. (a) The adhesion of U87 cell line to fibrinogen and fibronectin was performed in the presence of function-blocking anti-integrin antibodies. (b) U87 cells were preincubated with 50 µg/mL oil and tested for adhesion on microtiter plates coated with antibodies raised against the indicated integrin subunits αvβ3, αvβ5, α5β1, α2, α3 as described in the experimental section. (c) Adhesion assays were performed with various cell/ECM protein pairs involving unique integrins: α1β1 (PC12/type IV collagen), α5β1 (K562/fibronectin), αvβ3 (HT1080/fibrinogen), αvβ5 (HT29/vitronectin) and αvβ6 (HT29/fibronectin). Before adhesion, cells were preincubated without (open bar) or with 50 µg/mL oil (shade bar) for 30 min at room temperature. (d) Cell adhesion through αvβ3 and α5β1 was measured by using increasing concentrations of seed oil. Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$).

whether that oil modulates angiogenesis by targeting these integrins. In this assay, we assessed capillary-like tube formation by human microvascular endothelial cell (HMEC-1) *in vitro*.

As illustrated in Fig. 6a, exposure of HMEC-1 to seed oil for 6 h hindered the capillary-like tube formation in a dose-dependant manner with a maximal inhibition seen at 50 µg/mL. Similar results were observed when specific α5β1 and αvβ3 blocking antibodies are used, suggesting that the oil had antiangiogenic activity which was mediated by α5β1 and αvβ3 integrins.

4. Discussion

The potentially important roles of integrins, the large family of cell-surface receptors, in mediating cancer cell adhesion, migration, proliferation, apoptosis and angiogenesis is well established [39]. Thus, targeting these key molecules could be a promising strategy to cancer therapy. In the context of glioblastoma, considered as the most common intracranial neoplasm; clinical trials showed that the administration of cilengitide which is a cyclized arginine-glycine-aspartic acid-containing pentapeptide that selectively bind the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins inhibits angiogenesis and tumor growth [40]. However, despite its relative efficiency, this integrins inhibitor was associated with side effects including thrombosis, myalgia, arthralgia, thrombocytopenia, anorexia, hypoglycemia and hyponatriema [41]. To overcome such side effects, the use of natural products as anticancer agent could be a promising alternative. With regard to this topic, our earlier *in vitro* study has showed that *E. elaterium* seed oil was coveted with antiproliferative effect on human colonic adenocarcinoma (HT29) and fibrosarcoma (HT1080) cell lines [31].

In the present study, the *in vitro* treatment of human glioma cell line U87 with *E. elaterium* seed oil remarkably reduced cell adhesion to fibrinogen and fibronectin in the range concentration of 10–60 µg/mL. In an attempt to decipher the mechanism by which the oil exerts such inhibitory activity, additional experiments using integrins inhibitors and integrin-independent substratum have been performed and the results demonstrate that αvβ3 and α5β1 integrins were the specific targets of the tested oil. It was also found that the oil dose-dependently inhibited the migration of U87 to fibrinogen and fibronectin by using haptotaxis assays in modified Boyden chambers. Such effect was tightly associated with a remarkable decrease in cell velocity and directional persistence as assessed by time-lapse videomicroscopy. Most importantly is that effect was not due to cytotoxicity, as seed

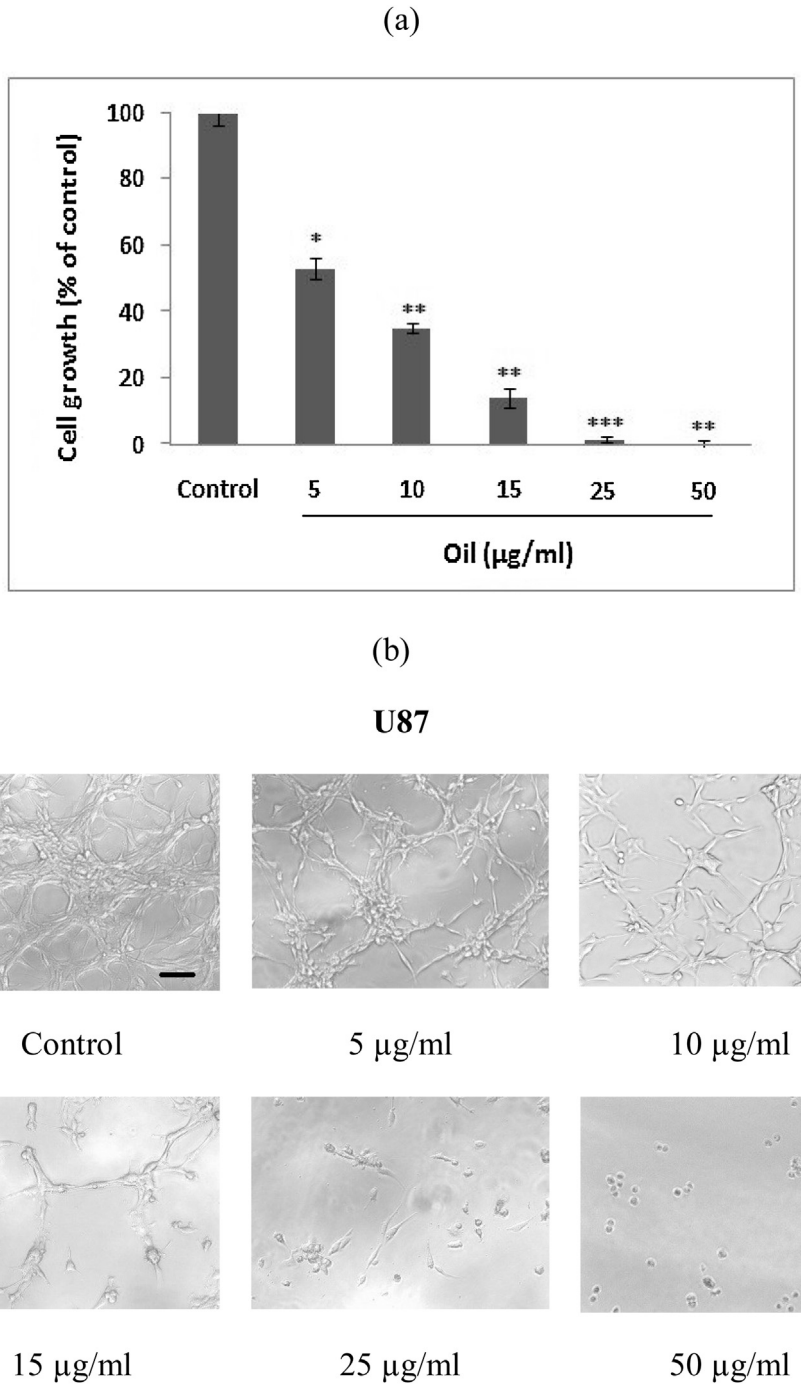

Fig. 5. Seed oil of *Ecballium elaterium* inhibits U87 cells proliferation. (a) Cells (5,000 per well) were seeded in culture medium. After 2 h different concentrations of seed oil was added and cells were allowed to proliferate for 72 h. Cells were quantified by staining with 0.1% crystal violet, solubilization with 1% SDS and measure of absorbance at 600 nm. Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$). (b) Representative visualization of proliferation assay after treatment of cells without (control) or with 5, 10, 15, 20 or 25 µg/mL oil for 72 h. Metamorph imaging software was used to capture images. Scale bar: 70 µm.

oil (up to 100 µg/mL for 5 h) did not significantly induce detectable lactate dehydrogenase (LDH) release by U87 cells (Supplemental data, Fig. S1). Our results demonstrate that *Ecballium elaterium* seed oil showed a potent antiproliferative activity against glioblastoma cell line with a half-maximal inhibition (IC50) of 5.84 µg/mL. In the same way different species of the cucurbitaceae family showed an inhibitory growth effect against various tumor cell lines, *Citrullus colocynthis* extract showed a dose-dependent antiproliferative effect on bresat cancer cell line MCF7 and huamn adenocarcinoma cell line HT29 with an IC 50 of 22.0 and 32.5 µg/mL respectively [42]. *Luffa acutangula* fruit methanolic extract inhibited the growth of the human lung adenocarcinoma epithelial cell line A-549 with an IC-50 of 131 µg/mL [43]. *Momordica charantia* leaf extract inhibit cell proliferation and migration of prostate cancer cell line PLS10 with an IC50 of 150 µg/mL and 25 µg/mL respectively [44].

Because glioblastoma growth, proliferation and metastasis are angiogenesis-dependent processes, we have hypothesized that the

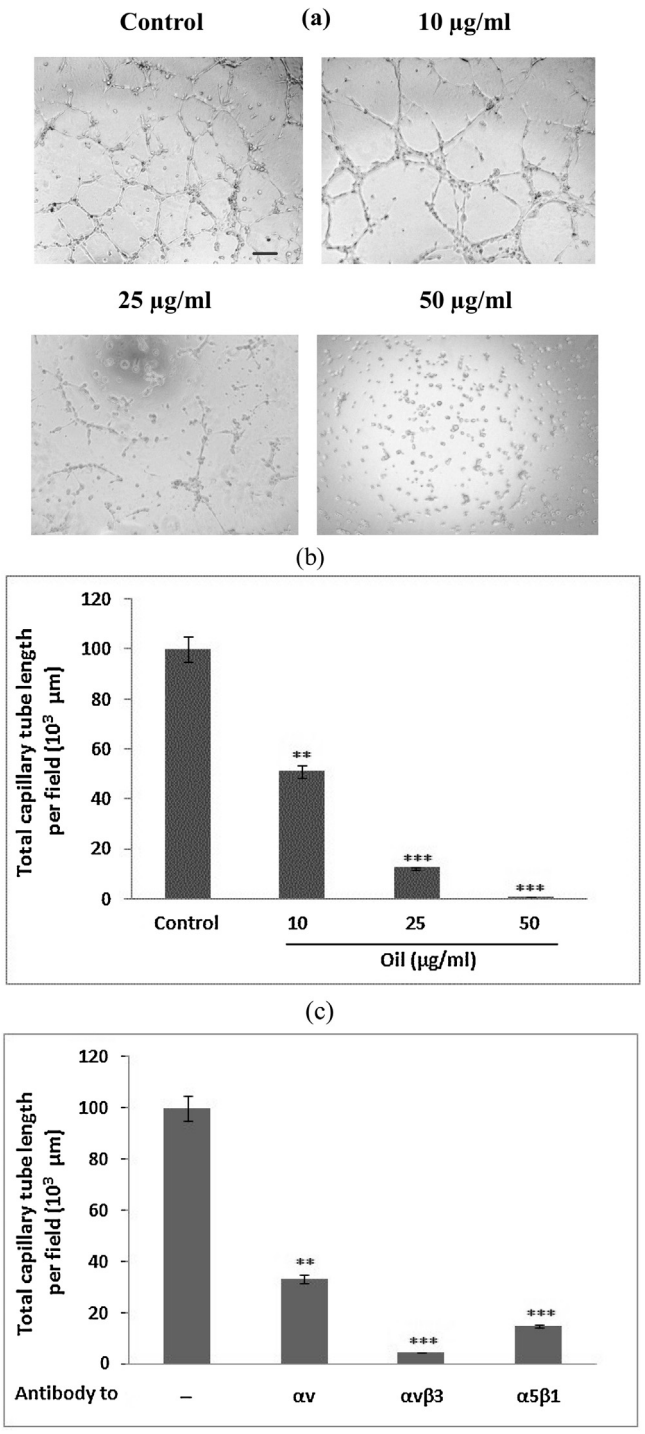

Fig. 6. *Ecballium elaterium* seed oil blocks *in vitro* angiogenesis. (a) Representative visualization of tubulogenesis assay. HMEC-1 cells were incubated without (control) or with 10, 25 or 50 µg/mL seed oil at room temperature. Cells were then added to Matrigel™ and allowed to form capillary-like structures for 5 h at 37 °C. Scale bar: 70 µm. (b) Dose-effect of seed oil at the indicated concentration, on tubulogenesis on Matrigel™. Quantification of tubulogenesis was done as described in experimental section. (c) Tubulogenesis on Matrigel™ was performed as above in the presence of the indicated anti-integrin antibodies (20 µg/mLmL). Data shown are means (±SD) from 3 experiments performed in triplicate. $P < 0.05$ was considered statistically significant (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$).

oil may serve as angiogenesis inhibitor. Our results showed its high ability of inhibiting the formation of capillary-like tube in HMEC-1 cells mediated by αvβ3 and α5β1 integrins inhibition as determined in the *in vitro* Matrigel™ assay. In this context Moon et al. [45] reported that *Benincasa hispida* Cogniaux. (cucurbitaceae) exhbited an anti-angiogenic effect on human umbilical vein endothelial cells (HUVECs).

Integrins play a central role in glioma proliferation and invasion [1], the hypothesis that *E. elaterium* seed oil exerts its anticancer effect by inhibiting the αvβ3 and α5β1 integrins-mediated cell adhesion pathway provides a new understanding of the anticancer activity. Another point to be considered is that the oil targets both tumor cells and vasculature, which make it of particular interest in developing anticancer drugs with targeted "multi-modal" therapies combining anti-adhesif, antiproliferative, antimetastasic and anti-angiogenic, approaches. However, a more in depth *in vitro* and *in vivo* studies employing individual compounds and focusing on the mechanistic aspects could support our assumptions.

Because of the complexity of the oil composition and the possible synergistic, antagonistic and additive interactions between its constituents, it is difficult to ascribe the anticancer activity of *E. elaterium* seed oil to a particular component. However, several lines of evidences indicate that some major components namely fatty acids could be responsible for the anticancer activity. The contribution of some minor components such as γ-and δ-tocopherols as well as β-sitosterol and campesterol is not ruled out.

Among the identified fatty acids, some of them have received particular attention due to their anticancer activity. For example, it has been reported that linoleic acid (C18:2 n-6) promote or suppresses cancer cell growth depending on the cell lines and the concentration of this fatty acid [46]. However, most of the *in vitro*, *in vivo* and clinical studies pinpointed pro-angiogenic properties of linoleic acid [47]. In the case of glioblastoma, previous *in vitro* study indicated that this fatty acid had no effect on the human glioblastoma cell line A-172 [48]. In contrast, linoleic acid was found to activate β1 integrin through selective activation of a specific protein kinase C (PKC), leading to enhanced human breast cancer cell adhesion to collagen IV [49]. Nine years later, an *in vivo* investigation showed that dietary linoleic acid stimulates invasion and peritoneal metastasis of gastric carcinoma cells (OCUM-2MD3) [50]. The study author's also found that the increased mtastasis behaviour was associated with increased activity of cyclooxygenase (COX-1) and extracellular signal-regulated kinase (ERK) [50]. Although data on the anticancer activity of linoleic acid are somewhat controversial, presumably depending on cancer cell line type, *in vitro* and *in vivo* models, its natural occurring isomers commonly known as conjugated linoleic acid (CLA) are unequivocally the best studied anticancer agent [24]. It has been reported that CLA strongly inhibits cell growth, migration, invasiveness and induces apoptosis of human glioma cell line (ADF) through the activation of PPARs (Peroxisome Proliferators Activated Receptors) and a p-ERK (Extracellular signal-regulated kinase) downregulation [24]. Two years later, Kelley et al. (2007) [51] reported that the reduction of invasiveness of tumor cells in response to CLA was mediated through suppression of metalloproteinases (MPP) activity. Moreover, the potential of CLA to inhibit β2 integrins has been reported as one of the putative anti-adhesion mechanism underlying the anticancer their anticancer activity [52].

Punicic acid (C18:3 n-5), is another putative anticancer agent to which numerous biological activities were attributed. It has been found that punicic acid disrupted the mitochondrial membrane potential and induces apoptosis of breast cancer cell lines MDA-MB-231 and MDA-ERα7 [53]. In LnCaP androgen-dependent human prostate cancer cells, exposure to punicic acid (10–100 µM) for 24 h resulted in growth inhibition, DNA fragmentation and apoptosis via a caspase-dependent pathway [54].

The key role of oleic acid (C18:1 n-9) as anticancer agent has also been reported. In this direction, it has been found that exogenous

supply of oleic acid (100 μM) induced apoptosis of C6 glioma cells by altering the *de novo* fatty acid synthesis *via* downregulation of acetyl-Coenzyme A carboxylase (ACC) and 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) activities [55].

The involvement of palmitic acid (C16:0) in cell death through reduction of mitochondrial membrane potential and induction of cell apoptosis has also been described [56].

Collectively, the results obtained herein could be at least, in part, attributed to the presence of these fatty acids. Experiments are in progress to assess the effects of individual fatty acid on human brain cancer cell line (U87).

As indicated above, the contribution of some minor compounds identified in the seed oil of *E. elaterium* is not ruled out. In this context, substantial data pinpointed the potential anti-adhesion, antiproliferative and anti-angiogenic effects of α-, γ-, and δ-tocopherols [57–59]. The latter isoform was found to be the most potent tocopherol in inhibiting the growth and inducing apoptosis of murine glioma C6 cells [57], prostate, mammary and colon cancers [59–61] as well as human lung cancer H1299 cells in a xenograph model [62]. Cell cycle arrest at the S phase or G0/G1 phases, decrease in cyclin D1, cyclin E, p27, p21, and p16 as well as induction of apoptosis, activation of caspase-2 and caspase-9, the disruption of the de novo synthesis of sphingolipids, and anti-angiogenesis, among others, are the mains mechanisms of action of tocopherols [57,59].

In addition to tocopherols, phytosterols namely β-sitosterol and campesterol could be considered as potential contributors to the anticancer activity of *E. elaterium* seed oil. In this direction, earlier studies showed that β-sitosterol was effective against prostate (LNCaP) cancer cell line *via* activation of sphingomyelin cycle and induction of cell apoptosis [63]. Later, Moon et al. (2008) [64] showed that β-sitosterol induces a cell cycle arrest at G2/M phase and apoptosis of cancer cell through the Bcl-2 and PI3 K/Akt signalling pathway. In another *in vitro* study by Vundru et al. (2013) [65], β-sitosterol was found to arrest cell cycle at G1 phase and disturb the mitochondrial membrane potential in breast carcinoma MDA-MB231 cells. More recently, it has been reported that β-sitosterol stabilized microtubule assembly in a manner similar to taxol [66]. Based on these antecedents, it seems logical to suggest that the presence of some putative anticancer agents such as punicic acid, oleic acid, δ-tocopherols and sitosterol could be responsible of the *in vitro* anticancer activity against U87 glioma cell line of *E. elataerium* seed oil.

5. Conclusions

The presence of integrin inhibitors in the seed oil of *E. elataerium* was reported herein for the first time. Most importantly is that activity is mediated through αvβ3 and α5β1 integrins inhibition This original finding provides baseline information towards developing natural drugs and setting chemoprevention strategies for glioma and anti-angiogenic therapies. However, further studies are warranted to identify the main component(s) responsible for such an activity and to decipher its or their exact mode of action in *in vitro* and *in vivo* models.

Conflict of interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2016.10.035.

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