Antioxidant, anti-inflammatory and cytotoxic activities of the unsaponifiable fraction of extra virgin olive oil

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SUMMARY: The health benefits of olive oil are well-known. In this study, the unsaponifiable fraction of extra virgin olive oil (Unsap) was investigated for reducing power capacity, ferric reducing antioxidant power, ferrous chelating activity and nitric oxide inhibition. The present study was also designed to evaluate the *in vitro* cytotoxic effect of the Unsap against human carcinoma cells. The anti-inflammatory potential of Unsap has been determined via the inhibition of Human Cyclooxygenases. The results showed that Unsap is efficient for ferric reducing antioxidant power and nitric oxide inhibition. Unsap has a selective effect as anti-inflammatory agent. The results showed moderate to good *in vitro* antitumor activities of Unsap against human liver, lung and pancreas cancer cells with IC_{50} ranging from 19.6 to 30.4 μg/mL and good selectivity index (≥ 2). In conclusion, Unsap represents a promising and safe antitumor and antioxidant material that supports the need for further investigation.

KEYWORDS: *Anti-inflammatory; Antioxidant; Cytotoxicity; Olive oil; Unsaponifiable fraction*

RESUMEN: *Actividades antioxidantes, antiinflamatorias y citotóxicas de la fracción insaponificable de aceite de oliva virgen extra.* Los beneficios para la salud del aceite de oliva son bien conocidos. En este estudio, se investigó la fracción insaponificable del aceite de oliva virgen extra (Insap) para reducir el poder antioxidante férrico, la actividad quelante ferrosa y la inhibición del óxido nítrico. El presente estudio también fue diseñado para evaluar el efecto citotóxico *in vitro* del Insap contra las células de carcinoma humano. La potencialidad antiinflamatoria del Insap se ha determinado mediante la inhibición de las ciclooxigenasas humanas. Los resultados mostraron que el Insap es eficiente para reducir el poder antioxidante férrico y la inhibición del óxido nítrico. El Insap tiene un efecto selectivo como agente antiinflamatorio. Los resultados mostraron actividades antitumorales *in vitro* del Insap de moderadas a buenas contra células de cáncer de hígado, pulmón y páncreas humano con una CI50 que varía de 19,6 a 30,4 μg/ml y por su buen índice de selectividad (≥2). En conclusión, el Insap contiene material antitumoral y antioxidante prometedor y seguro que será respaldado por investigaciones adicionales.

PALABRAS CLAVE: *Aceite de oliva; Antiinflamatorio; Antioxidante; Citotoxicidad; Fracción insaponificable*

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1. INTRODUCTION

Extra virgin olive oil (EVOO) is the highest quality olive oil obtained from the olive fruit by mechanical processes. According to the Codex (2015) classification, EVOO category has free acidity, fatty acid ethyl esters, peroxide value and absorbance at 270 nm, and not higher than 0.8%, 35 mg/kg oil, 20 milliequivalents of active oxygen/kg oil and 0.22, respectively. In addition, it should be free of odor and taste defects. It consists of a major fraction of unsaturated fatty acids and the Unsap (1-2%) composed of aliphatic and triterpenic alcohols, sterols, hydrocarbons and α-tocopherol (Servili *et al.*, 2014). Squalene represents the major hydrocarbon in the unsaponifiable fraction of olive oil (Cardeno *et al.*, 2014).

The Unsap of EVOO as a natural product has anti-inflammatory and anticancer effects on human colon cancer in murine models (Cardeno *et al.*, 2013). Escrich *et al.*, (2011) attributed the beneficial effect of EVOO on the progression of breast cancer to its oleic acid content and its minor compounds. Dietary EVOO's Unsap prevented the damage in acute colitis and reduced pro-inflammatory protein expression to its basal levels in mice (Sánchez-Fidalgo *et al.*, 2013). The Unsap of virgin olive oil prevented the development and progression of neuroinflammation-related diseases in obese mice (Toscano *et al.*, 2019). A diet rich in EVOO exerts its effect on breast cancer by reducing the level of DNA damage (Solanas *et al.*, 2010). Several studies have confirmed that the consumption of olive oil is effective in the prevention and treatment of cardiovascular diseases, and obesity and reduces the risk of cancer, especially breast, lung and stomach cancer (Covas *et al.*, 2009). Squalene has chemopreventive activity against colon cancer (Rao *et al.*, 1998). Smith *et al.*, (1998) reported that a diet that contains olive oil or squalene could effectively inhibit lung tumorigenesis and decrease lung tumor multiplicity. Warleta *et al.*, (2010) suggested that the squalene in virgin olive oils could be partially responsible for a lower incidence of breast cancer due to its protective activity against oxidative DNA damage in normal mammary cells. Liver cancer is one of the most widespread malignant diseases (Bray *et al.*, 2018). A polyphenolic extract of EVOO reduced cell proliferation and increased cell death of liver cancer cell lines (HepG-2, Huh7 and Hep3B) (De Stefanis *et al.*, 2019). Olive oil phenols such as hydroxytyrosol and oleuropein are not Unsap materials (Boskou, 2015). Although, squalene and γ-Tocopherol proved to have antioxidant and anticancer activities when applied as pure substances individually (Newmark, 1997; Abraham *et al.*, 2019), Cardeno *et al.*, (2014) reported that there is a great lack of knowledge on the biological activity of the Unsap of EVOO. The combination of these active compounds may be useful for enhancing the effectiveness, eliminating the free radicals' destructive effects or minimizing the doses needed for inhibiting the growth of cancer cells. Therefore, the novelty of this study is the evaluation of the combined effect of the constituents present in the Unsap of EVOO on scavenging radicals, reduction in COX-2 activity and selectivity against cancer cells compared to normal cells.

2. MATERIALS AND METHODS

2.1. Materials

EVOO was obtained from the Agriculture Research Center, Giza, Egypt. Ascorbic acid, butylated hydroxytoluene (BHT), MTT and trypan blue dye were purchased from Sigma (St. Louis, MO, USA). Fetal Bovine serum, DMEM, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza (Walkersville, USA). Cell lines were obtained from the VACSERA Tissue Culture Unit, the Holding Company for Biological Products and Vaccines, Egypt.

2.2. Extraction of the unsaponifiable matter

The saponification of EVOO (5g) and extraction of the unsaponifiable matter were carried out according to the IUPAC (1992) method.

2.3. Antioxidant assays

2.3.1. Ferric reducing antioxidant power (FRAP)

The reducing power of Unsap was evaluated using the method of Oyaizu (1986). This method is based on the reduction in ferricyanide in relation to different concentrations of Unsap (from 3.9 to 2000 μg/mL, in a geometric sequence). The absorbance was measured at 700 nm against a blank using a spectrophotometer (Milton Roy, Spectronic 1201). BHT was used as reference standard. The reducing capacity percentage (%) was calculated according to Canabady-Rochelle *et al.*, (2015) as follows:

$$\text{Reducing capability } (\%) = 100 - \left[\frac{A_0 - As}{A_0} \times 100 \right] \quad (1)$$

Where, A_0: absorbance of the control solution. As: sample absorbance.

2.3.2. Ferrous metal chelating activity

Different concentrations of the Unsap and reference standard (from 3.9 to 2000 μg/mL, in a geometric sequence) were added to a solution of $FeCl_2$ (2 mM). The reaction was initiated by the addition

of ferrozine (5 mM) and the mixture was shaken vigorously and left to stand at room temperature for 10 min. Then, the absorbance of the solution was measured at 562 nm using a Milton Roy (Spectronic 1201) spectrophotometer according to Gülçin *et al.*, (2003). Decreasing absorbance in the metal chelating activity showed that metal ions were chelated before ferrozine bonding. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the formula (2):

$$\text{Ferrous ion chelating activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (2)$$

Where, A_0 is the absorbance value of the control (in the presence of ferrozine and Fe^{2+} ions), and A_1 is the absorbance value in the presence of the tested sample or standard. BHT was used as a reference standard.

2.3.3. Nitric oxide (NO) radical scavenging activity

The NO radical scavenging activity of Unsap was determined according to the method of Marcocci *et al.*, (1994) by using a sodium nitroprusside (SNP). NO radical generated from SNP reacted with oxygen to produce nitrite ions, which were measured by the Greiss reagent. The concentrations of the tested Unsap ranged from 3.9 to 2000 µg/mL in a geometric sequence. The absorbance was measured at 546 nm against the corresponding blank solution (without SNP). Ascorbic acid was used as a reference standard. All the tests were performed in triplicate. The percent inhibition activity was calculated using the formula (3):

$$\text{Inhibition \%} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100 \quad (3)$$

where, $A_{control}$ is the absorbance of the control reaction at 546 nm and A_{test} represents the absorbance of a test reaction at the same wavelength. Tested material concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition percentage against concentration.

2.4. In-vitro anti-inflammatory activity (Cyclooxygenase inhibition assay)

The cyclooxygenase inhibition assay was carried out according to Larsen *et al.*, (1996). Different concentrations of the tested sample (from 7.8 to 1000 µg/mL, in a geometric sequence) were pre-incubated with the cyclooxygenase enzymes (COX-1 and COX-2) at room temperature (25 °C) for 5 min in the presence of hematin. Phenol (500 µM),

1-leuco-dichlorofluorescein (20 µM), arachidonic acid (50 µM) and hematin (1 µM) in 1 mL of 0.1 M Tris-buffer (pH 8) were pre-mixed and added to the enzyme mixture. The absorbance was recorded within 15 seconds of the addition using a Milton Roy (Spectronic 1201) spectrophotometer at 502 nm. A blank was made without the addition of enzymes. Celecoxib was used as a standard. The IC_{50} value (µg/mL), which is the concentration of the Unsap or standard that inhibited 50% of each cyclooxygenase, was calculated from the curve.

2.5. In vitro anti-cancer assay

2.5.1. Cell line propagation

The cytotoxic effects of Unsap were estimated *in vitro* against human Breast (MCF-7), Lung (A-549), Pancreas (Panc-1), Hepatocellular (HepG-2) carcinoma cells and a normal human lung cell line (WI-38). The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 50 µg/mL gentamycin. All cells were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.5.2. Cytotoxicity evaluation

The cytotoxicity assay of Mosmann (1983) was performed as follows: the cells were seeded in 96-well plates at a cell concentration of 1×10^4 cells per well in 100 µL of growth medium. After 24 h of seeding, fresh medium containing different concentrations (from 3.9 to 500 µg/mL, in a geometric sequence) of the tested sample was added. The Microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a period of 24 h. Control cells were incubated without the tested sample. Then, the yield of viable cells was determined by a MTT colorimetric method. The absorbances of the plates were measured at a wavelength of 570 nm using a Microplate reader (SunRise, TECAN, Inc., USA). Treated samples were compared with the cell control in the absence of the tested compounds. Vinblastine sulfate was used as a reference anticancer drug. All experiments were carried out in triplicate. The percentage of viability was calculated as illustrated in the following formula (4)

$$\text{The percentage of viability} = \left[1 - \left(\frac{0D_t}{0D_c}\right)\right] \times 100 \quad (4)$$

Where: $0D_t$ is the mean optical density of wells treated with the tested sample and $0D_c$ is the mean optical density of the untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell

line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}) or the cell cytotoxic concentration (CC_{50}) is the concentration required to cause toxic effects in 50% of intact cells. IC_{50} was estimated from the graphic plots of the dose response curve for each concentration using Graphpad Prism software (San Diego, CA. USA).

2.6. Statistical analysis

The results are expressed as the mean values ± standard deviation (SD) of three experiments. The statistical analysis was carried out using Statistica software (StatSoft Inc., Tulsa, OK, USA). The Tuckey's test was used at a significance level of 5%.

3. RESULTS AND DISCUSSION

Squalene represented the main component of the Unsap while β-sitosterol, and γ-tocopherol were found in minor quantities as reported previously (Cardeno *et al.*, 2014; Galal *et al.*, 2020).

3.1. Antioxidant activity

The antioxidant activity of the Unsap was analyzed by different methods (FRAP, iron chelating activity and nitric oxide radical scavenging activity) to cover various ways of antioxidant protection and the results are illustrated in Figure1.

Previous studies indicated that the antioxidant activity of some compounds is correlated with their reducing power. Figure 1a presents the reducing capacity of $Fe^{3+} \rightarrow Fe^{2+}$ transformation in the presence of Unsap compared to the standard. The reducing power of the investigated Unsap and the positive standard BHT was concentration dependent and reached a plateau at concentrations higher than 1000 μg/mL. The reducing power of 1000 μg/mL of the Unsap was not significantly different from that of BHT (p > 0.05) at 250 μg/mL. The IC_{50} value for Unsap (192.05 μg/mL) was higher than that of BHT (54.34 μg/mL), indicating lower activity of the Unsap. Abdallah *et al.*, (2018) found that the polyphenol methanolic extract of olive oil samples, obtained from three Tunisian cultivars, had IC_{50} values of FRAP ranging from 250 to 480 μg/mL. They ascribed this activity to the hydroxytyrosol and decarboxymethyl ligstroside aglycone in the polyphenol extract.

The generation of reactive oxygen species can be avoided by the chelating of metal ions. Extracts with iron chelating activity are effective for the reduction of lipid peroxidation (Atere *et al.*, 2018). Chelating agents disrupt the formation of the Ferrozine-Fe^{2+} complex and decrease the red color of the complex. The chelating effect of the Unsap on ferrous ions compared to BHT is illustrated in Figure1b. The chelating efficiency of 2000 μg/mL of
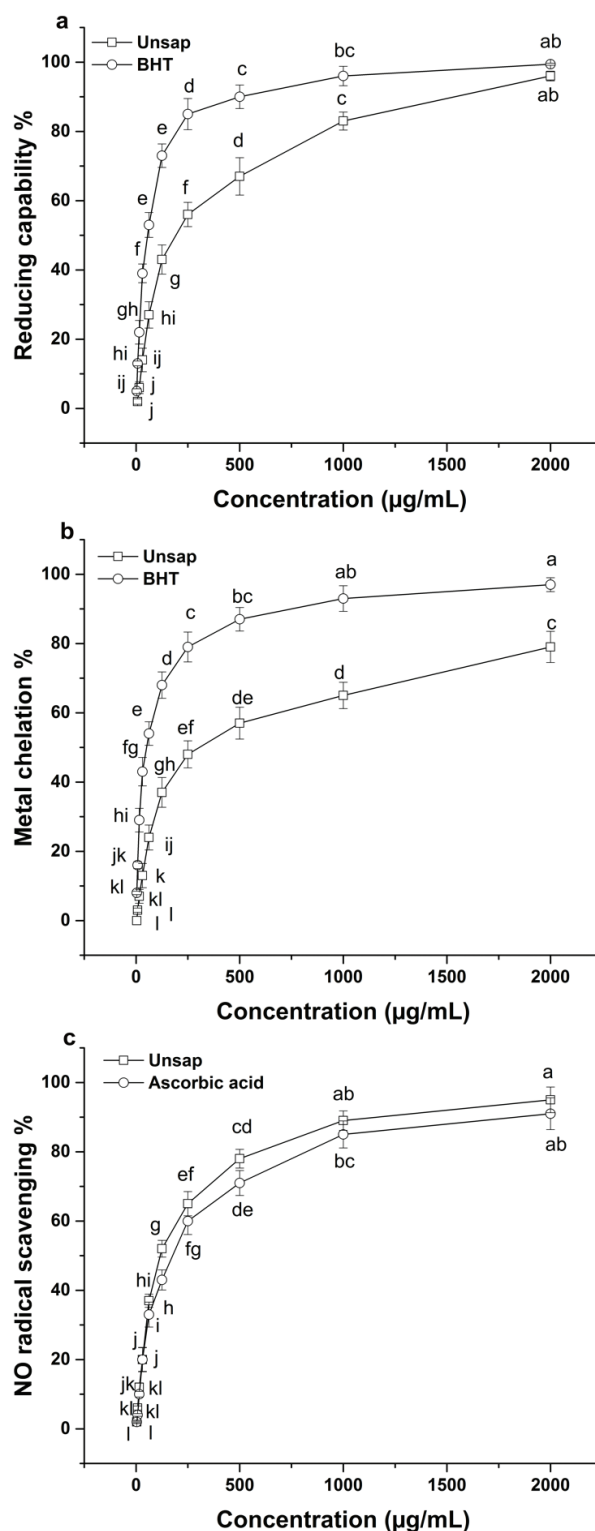

FIGURE 1. Antioxidant activity of Unsap of olive oil compared to the standard as assessed by reducing capacity compared to butylated hydroxytoluene (BHT) (a), metal chelating activity compared to BHT (b), nitric oxide (NO) radical scavenging activity compared to ascorbic acid (c). The results are presented as average values of three replicates ± SD. Values with different letters indicate significant differences (p < 0.05) according to Tukey's test.

the Unsap was not significantly different from that of BHT (p > 0.05) at 500 µg/mL. The IC_{50} value of the Unsap for chelating activity was 306.73 µg/mL, which was higher than the positive standard BHT (IC_{50} = 50.20 µg/mL).

Based on Figure 1c, the evaluation of nitric oxide radical (generated by sodium nitroprusside at physiological pH) scavenging activity of Unsap and ascorbic acid showed a similar trend. The nitric oxide radical scavenging activity of the Unsap was not significantly different from that of the ascorbic acid (p > 0.05) at all the investigated concentrations. The IC_{50} value for Unsap (116.7 µg/mL) was lower than that of the ascorbic acid (176.5 µg/mL) indicating higher antioxidant activity. Franco et al., (2014) found that NO antiradical activity of the oils of seven Spanish olive varieties ranged from 29.8 to 40.7% at 5.6 mg/mL of olive oil methanol solution.

Based on the data obtained from this study, the tested olive oil Unsap exhibits free radical inhibitor or scavenger activity for NO radical thus acting as primary antioxidant which reacts with free radicals, and may limit free radical damage in the human body.

3.2. Anti-inflammatory activity

The Unsap was investigated for its efficiency to inhibit human cyclooxygenases (COX-1 and COX-2) compared with celecoxib as a standard drug. The results are shown in Figure 2. The COX enzymes were inhibited by the tested Unsap in a dose dependent manner. The investigated Unsap demonstrated a weak potency towards COX-1 (inhibition did not exceed 37.25% at 1000 µg/mL), and moderate potency towards COX-2 (IC_{50} = 412.20 µg/mL). The inhibitory effect of the Unsap at 1000 µg/mL on the activity of COX-2 was not significantly different from that of celecoxib (p > 0.05) at 250 µg/mL. On the other hand, the IC_{50} value of the standard celecoxib on COX-1 and COX-2 was 400 µg/mL and 84.05 µg/mL, respectively.

The inhibition of nitric oxide (NO) and cyclooxygenase-2 (COX-2) production is considered a promising approach for the treatment of various diseases, including inflammation and cancer as reported by Park et al., (2003). Therefore, the tested olive oil Unsap may be a useful anti-inflammatory and anticancer agent.

3.3. Cytotoxic activity of Unsap against cancer cell lines

The cytotoxicity of the Unsap was investigated on different cancer cell lines including MCF-7, A-549, Panc-1 and HepG-2 carcinoma cells, as shown in Figure 3. The inhibitory activity of Unsap against human lung fibroblast normal cell lines was also assayed.
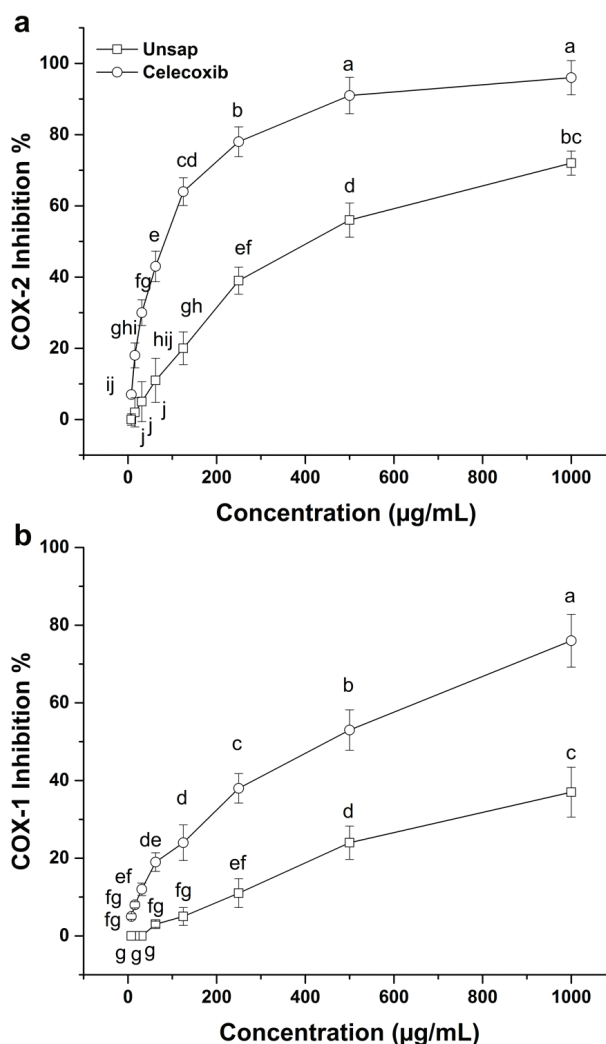

FIGURE 2. Inhibition % of COX-1 and COX-2 by Unsap of olive oil compared with Celecoxib standard. The results are presented as average values of three replicates ± SD. Values with different letters indicate significant differences (p < 0.05) according to Tukey's test.

The Unsap exhibited a potential anticancer effect on the four tested cancer cell lines in a dose dependent manner. The inhibitory activities (IC_{50} value) of the Unsap against MCF-7 (Figure 3a), A-549 (Figure 3b), Panc-1 (Figure 3c) and HepG-2 (Figure 3d) carcinoma cells were 45.7 ± 2.1, 30.4 ± 0.7, 19.6 ± 0.8 and 22.4 ± 0.4 µg/mL instead of 5.9 ± 0.9, 24.6 ± 0.7, 2.89 ± 0.12 and 3.48 ± 0.22 µg/mL for the drug vinblastine sulfate, respectively.

With regard to MCF-7, the Unsap at 125 µg/mL showed significantly (p < 0.05) the same inhibition activity of 62.5 µg/mL of the vinblastine sulfate drug. Treating Panc-1 cancer cells with Unsap at 62.5 µg/mL significantly induced (p < 0.05) the same inhibition effect as the standard drug at 15.6 µg/mL. The inhibition effect of the Unsap at 62.5 µg/mL on HepG-2 cancer cells was significantly (p < 0.05)
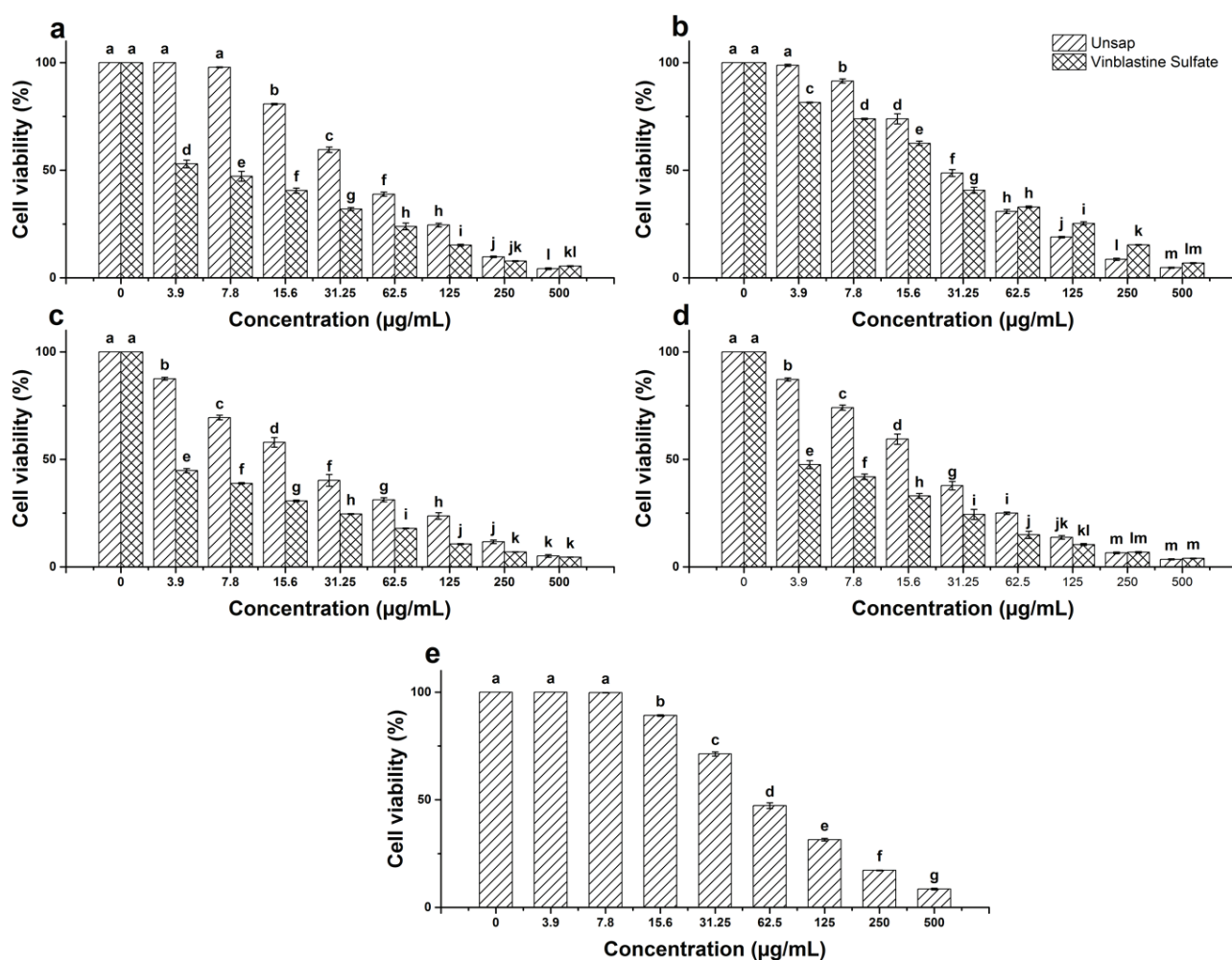
FIGURE 3.   Cytotoxicity effect of Unsap and Vinblastine sulfate concentrations (µg/mL) against MCF-7 human breast cancer cell line (a), A-549 human lung cancer cell line (b), Panc-1 human pancreas cancer cell line (c), HepG-2 human liver cancer cell line (d), WI-38 human lung fibroblast normal cells (e). The results are represented as average values of three replicates ± SD. Values with different letters indicate significant differences (p < 0.05) according to Tukey's test.

similar to that of the standard drug at 31.25 µg/mL. These results indicated that those carcinoma cells were less susceptible to the cytotoxic effect of the Unsap compared to vinblastine sulfate. It is interesting to note that Unsap and vinblastine sulfate at the concentration of 62.5 µg/mL have significantly (p < 0.05) the same inhibition effect on A-549 lung cancer cells. The results clearly showed that Unsap at concentrations higher than 62.5 µg/mL was significantly (p < 0.05) more efficient in the inhibition of A-549 cancer cells than vinblastine sulfate at the equivalent concentration.

Unsap exerts a low toxicity (CC_{50} = 59 ± 3.9 µg/mL) on WI-38 human lung fibroblast normal cells (Figure 3e). However, the selective index (the ratio between IC_{50} value for normal fibroblast cells and IC_{50} value for cancer cells) towards HepG-2, A-549 and Panc-1 was 2.63, 1.94 and 3.01, respectively, indicating high selective toxicity (≥ 2)

against those cancer cells as reported by Valderrama *et al.*, (2016). Squalene has a chemopreventive effect against colon carcinogenesis (Rao *et al.*, 1998). They attributed this effect to its inhibitory activity of HMG-CoA reductase and to suppressing the activation of oncogene proteins.

4. CONCLUSIONS

Unsap proved to have antioxidant activity and a selective cytotoxic effect against the A-549 cancer cell line. The data reveal that Unsap has anticancer activity on lung cancer cells (A-549) which is comparable to that of the drug vinblastine sulfate. These properties make Unsap a natural antioxidant and a promising anti-tumor agent for new pharmaceutical products to provide protection and treatment against the deleterious effects of free radicals.

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