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LIGUSTRUM VULGARE L.: *IN VITRO* FREE RADICAL SCAVENGING ACTIVITY AND PRO-OXIDANT PROPERTIES IN HUMAN COLON CANCER CELL LINES

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Ligustrum vulgare L. is a semi-evergreen woody branched bush from the family Oleaceae, widespread and frequently used in traditional medicine and pharmacy for treating different diseases. The aim of this study was to determine phenolic and flavonoid contents in *L. vulgare* leaf and fruit methanolic extracts, its free radical scavenging potential and *in vitro* effects on pro-oxidant/antioxidant status of human colon cancer cells (HCT-116 and SW480). The total phenolic content and DPPH free radical scavenging activity, concentration of superoxide anion radical ($O_2^{\cdot-}$), nitrites and reduced glutathione (GSH) in HCT - 116 and SW480 cells were determined spectrophotometricaly. Inducible nitric oxide synthase (iNOS) protein expression was determined by imunoflorescence. Higher concentration of phenolic compounds and flavonoids was observed in leaf in comparison to fruit extracts. Both leaf and fruit extracts had considerably high ability for free radical scavenging by DPPH. The obtained results indicated that *L. vulgare* extracts increased $O_2^{\cdot-}$ and GSH concentrations, inhibited iNOS protein expression and nitrites production in HCT-116 and SW480 cells. All the investigated parameters indicate the presence of oxidative stress in treated cells and pro-oxidative effect of *L. vulgare* extracts on colon cancer cells, which is associated with cytotoxic and apoptotic ability of *L. vulgare*.

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1. Introduction

Wild privet, *Ligustrum vulgare* L. (Oleaceae) is a semi - evergreen woody branched bush, and is often cultivated ornamental plant [1]. Medicinal plant species of the genus *Ligustrum* are very rich in bioactive components and frequently used in traditional medicine and pharmacy [2]. *L. vulgare* is used as a diuretic and antirheumatic, in skin tretments, digestive and respiratory diseases since it has anti-mutagenig, antiinflammatory, antioxidative, antimicrobial effect and liver - protecting effects [3, 4, 5, 6]. Different *in vitro* assays performed with leaves from *Ligustrum* indicated broad pharmacological potential of this plant [7, 8]. To our knowledge there are no data concerning pro-/antioxidant effects of *L. vulgare* extracts on cancer cell lines.

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, nitric oxide and peroxynitrite radicals play an important role in oxidative stress related to the pathogenesis of various important diseases like cancer, cardic reperfusion abnormalities, kidney and liver disease. Oxidative stress occurs when there is an imbalance between generation of ROS and insufficient antioxidant defense systems leading to cell damage [9]. Natural antioxidants, including phenolics, flavonoids, tannins, lignans and others, are widespread especially in food of plant origin and in different medicinal plants. Plant phenolics may function as potent free radical scavengers, reducing agents, quenching ROS, and protecting from lipid peroxidation [10, 11].

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Recently, we showed cytotoxic and proapoptotic effects of leaf and fruit extracts of *L. vulgare* on HCT-116 cell line [6]. Our hypothesis is that these activities of *L. vulgare* leaf and fruit extracts may be due to the disbalance of redox homeostasis in the treated HCT-116 and SW480 cells. Therefore, the basic aim of this research was to determine the contents of phenolics and flavonoids, as potential bioactive substances, their DPPH radical scavenging activity, as well as effects on pro-oxidant/antioxidant status (superoxide anion radical ($O_2^{\cdot -}$) concentrations, nitrite and reduced glutathione (GSH) levels, and inducible nitric oxide synthase (iNOS) protein expression) in human colon cancer cells (HCT-116 and SW480) treated by *L. vulgare* leaf and fruit extracts.

2. Experimental

2.1. Chemicals

Methanol, sodium hydrogen carbonate ($NaHCO_3$) and sodium nitrate ($NaNO_3$) were purchased from "Zorka pharma" Šabac, Serbia. Standards of phenolic acids (gallic acid) and flavonoids (rutin hydrate), chlorogenic acid and 2,2 - dyphenyl - 1 - picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu's phenol reagent, 3-tert-butyl-4-hydroxyanisole (BHA) and aluminium chloride ($AlCl_3$) were from Fluka Chemie AG, Buchs, Switzerland. Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, Invitrogen, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The cell culture company), Austria. Dimethyl sulfoxide (DMSO), NADPH and nitro blue tetrazolium (NBT) were obtained from SERVA, Germany. Sulfanilamide and sulfanilic acid were from MP Hemija, Serbia. N - 1 - napthylethylenediamine dihydrochloride was from Fluka chemie GMBH Switzerland. 5,5′ - dithio - bis(2 - nitrobenzoic acid), Sulfosalicylic acid (SSA) and glutathione reductase were purchased from Sigma Chemicals Co., St Louis, MO, USA.All other solvents and chemicals were of analytical grade.

2.2. Plant material

Voucher specimen for *L. vulgare* was confirmed and deposited at the Herbarium of the Faculty of Biology, University of Belgrade, with the number 16696. Leaves of *L. vulgare* were collected in July and fruits in September 2010 in the region of Šumarice, Kragujevac, central Serbia. The collected leaves were air-dried in darkness at room temperature (20 ^{o}C). Harvested fresh fruits were immediately used to prepare extracts.

2.3. Preparation of plant extracts

The air-dried plant material (10 gr) was coarsely crushed in small pieces of 2-6 mm by using a cylindrical crusher and transferred to dark-coloured flasks [12]. The prepared plant samples were mixed with 200 ml of methanol and stored at room temperature. After 24 h, the infusions were filtered through Whatman No. 1 filter paper and the residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 ºC using Rotary evaporator. After evaporation, the extract was completely dried. Immediately after sampling, extracts from fresh fruits of *L. vulgare* were prepared using the same extraction process. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 ºC. All the disolved extracts were sterilized by filtropur S 0.2 for sterile filtration.

2.4. Determination of total phenol content and flavonoid concentrations of the extracts

The phenolic content was determined spectrophotometrically, using Folin–Ciocalteu reagent [13]. Total phenolic content was expressed as gallic acid equivalents (GaE)/g of extract. The total flavonoid concentration was evaluated using aluminum chloride [14].

2.5. Evaluation of DPPH scavenging activity

The ability of the plant extract to scavenge DPPH free radicals was assessed using the method previously described and adopted with suitable modifications [15].

2.6. Cell preparation and culturing

HCT-116 and SW480 cell lines were obtained from American Type Culture Colection. Cells were maintained in DMEM supplemented with 10% FBS, with 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were grown in 75 cm^2 culture bottles supplied with 15 ml DMEM, and after a few passages, the cells were seeded in 96 - well plate for determenation of $O_2^{\bullet-}$, nitrites and GSH levels. After 24 h of cell incubation, the medium was replaced with 100 μl of medium containing various concentrations of *L. vulgare* extracts (1, 50, 100, 250 and 500 μg/ml) for 24 h and 72 h incubation period. For determination of iNOS protein expression, the cells were treated with 50 μg/ml of *L. vulgare* extracts for 24 h.

2.7. Determination of superoxide anion radical (NBT assay)

The concentration of superoxide anion radical ($O_2^{\cdot-}$) in the sample was determined by spectrophotometric method [16], and previously described in detail [17].

2.8. Determination of nitrites (Griess Assay)

The spectrophotometric determination of nitrites - NO_2^- (indicator of the nitric oxide - NO level) was performed by using the Griess method [18], previously described in detail [17].

2.9. Detection of iNOS protein expression - immunocytochemistry

Inducible nitric oxide synthase (iNOS) protein expression on HCT-116 and SW480 cells was detected by immunofluorescence [19]. Cells were cultured in 6-well plates on glass coverslips (Thermo Scientific), 5×10^4 cells/well. When cells were at 70 to 80% confluence, the media was aspirated and the cells were treated with 50 μg/ml *L. vulgare* extracts. After 24 h, the medium was aspirated and the cells were washed with PBS (pH 7.2). Then, the cells were fixed with 4% *p* - formaldehyde in PBS for 20 min at 37 °C. After the fixation, the cells were washed three times with PBS, permeabilized with methanol (−20°C) for 2 min, washed with PBS 3 times and non-specific binding sites were blocked using 1% Bovine Serum Albumin - BSA for 20 min. These fixed cells were stained with 20 μg/ml anti - iNOS specific primary antibody (RD Systems) at 37 °C for 1 h. Sample coverslips were then washed twice and incubated with antimouse secondary antibody conjugated with Alexa448 (Thermo Scientific) at a 1 : 200 dilution in PBS. DAPI was used to stain the cell nuclei (blue) at 1 : 1000 dilutions. Sample coverslips were then washed twice and mounted on glass slide by polyvinyl alcohol as mounting medium. The cells were visualized using Nikon inverted fluorescent microscope (Ti - Eclipse) at 600 x magnification.

2.10. Determination of reduced glutathione (GSH) concentration

The measuring of reduced glutathione content was performed by spectrophotometric method [20]. After the incubation of the control and treated cells, the well-plate was centrifuged. The medium was replaced with 100 μl 2.5% SSA and well-plate was kept on ice for 15 minutes. Well-plate was again centrifuged for 15 minutes on 1000 g. 50 μl of each experimental sample was added to wells in duplicate or triplicate. Using a multichannel pipette, 100 μl of reaction mixture was dispensed. This assay was based on oxidation of reduced form of glutathione (GSH)

with active thiol group reagent, i.e. 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) when yellow product of 5′-thio-2-nitrobenzoic acid (TNB) was formed. Colour reaction was measured spectrophotometrically at 405 nm on Microplate Reader following 5-min- incubation. The results were expressed in nmol/ml according to a standard curve established in each test and constituted of known molar GSH concentrations.

2.11. Statistical analysis

The data were expressed as mean ± standard error (SE). Biological activity was examined in three individual experiments, performed in triplicate for each dose. Statistical significance was determined using the Student's t - test. A P value < 0.05 was considered significant. The magnitude of correlation between variables was done using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008).

3. Results

3.1. Total phenolic content and flavonoids concentration

The results of the total phenolic content and the summary of quantities of flavonoids in metanolic extracts from *L. vulgare* are presented in Table 1. Total phenolic content and concentrations of flavonoids were higher in leaf extracts than in fruit extracts.

Table 1. Total phenolic content, flavonoid concentrations and free radical scavenging activity of L. vulgare leaf and fruit extracts.

analysis	leaves	fruits
Total phenolic content (mg GaE/g)	239.47 ± 1.12	180.83 ± 1.01
Flavonoid concentrations (mg RuE/g)	43.78 ± 0.89	23.68 ± 0.96
Free radical scavenging activity, IC_{50} (μg/ml)		
L. vulgare extracts	18.21 ± 0.61	19.41 ± 0.48
Chlorogenic acid	11.65 ± 0.52	

3.2. DPPH scavenging activity

For examination of the antioxidant activity, the values of chlorogenic acid as reference substance were obtained and compared to the values of the antioxidant activity of *L. vulgare* extracts. IC_{50} values of antioxidant activity are given in Table 1. The activity of antioxidant examined by DPPH radical scavenging was approximatly the same for both extracts and similar to the value of chlorogenic acid.

3.3. Superoxide anion production

Nitroblue tetrazolium assay was performed to test whether *L. vulgare* extracts scavenged or stimulated $O_2^{\cdot-}$ production in biological systems. The data presented in Table 2 express the $O_2^{\cdot-}$ concentrations as nmol/ml in control and treated cells, after 24 and 72 h, respectively. In general, the levels of production of $O_2^{\cdot-}$ were higher in the treated cells compared to the controls after both investigated periods. Fruit extracts caused higher $O_2^{\cdot-}$ production than leaf extracts in HCT-116 cells. In SW480 cells, both extracts caused similar changes (higher production) in the treated cells after 24 h; while leaf extracts caused higher production of $O_2^{\cdot-}$ after 72 h. The comparison of incubation periods showed lower differences between control and treated HCT-116 cells after 72 h in comparison to the values after 24 h. Also, their $O_2^{\cdot-}$ concentrations were lower. In SW480 cells, the concentrations of $O_2^{\cdot-}$ were statistically significantly lower in both control and treated cells after 72 h compared to 24 h.

*Table 2. Effect of methanolic leaf and fruit extracts from L. vulgare, on $O_2^{\cdot-}$ level (nmol/ml) in HCT-116 and SW480 cell lines after 24 and 72 h of exposure. * statistically significant differences (P < 0.05) compared to values in control cells; # statistically significant differences (P < 0.05) compared to values for 24 h.*

Cell line	Type of extract	0 μg/ml	10 μg/ml	50 μg/ml	100 μg/ml	250 μg/ml
24 h						
HCT-116	leaves	28.00±0.22	28.76±0.04	29.04±1.76	29.24±2.52	32.00±1.48
	fruits		33.20±1.36*	32.12±0.20*	36.04±0.12*	32.04±1.32*
SW480	leaves	35.12±0.13	36.16±0.56	36.70±0.92	40.28±1.00*	39.24±1.56*
	fruits		38.16±0.16	40.80±2.40*	41.12±0.96*	40.48±2.41*
72 h						
HCT-116	leaves	26.48±0.42	26.55±2.28	26.04±1.24#	26.60±0.60#	28.48±0.80#
	fruits		27.80±0.56#	28.80±0.8#	36.44±0.52*	30.76±0.04*
SW480	leaves	25.12±0.34#	28.08±1.76#	30.04±1.16*#	30.20±1.56*#	35.64±1.96*#
	fruits		28.76±1.92#	28.56±0.92#	29.44±0.96#	29.96±2.64*#

3.4. Production of nitrites

The determination of nitrite concentration demonstrated that *L. vulgare* extracts were able to reduce concentration of nitrites, as indicators of NO content (Table 3). In HCT-116 cells, fruit extracts caused higher inhibition of NO production compared to leaf extracts. In SW480 cells, both leaf and fruit extracts caused similar changes in reduction of NO concentration after 24 h, but after 72 h, fruit extracts caused higher inhibition. When periods of incubation were compared, there were statistically significant lower concentrations of nitrites in the cells after 72 h in comparison to 24 h.

*Table 3. Effect of methanolic leaf and fruit extracts from L. vulgare, on nitrite level (nmol/ml) in HCT-116 and SW480 cell lines after 24 and 72 h of exposure. * statistically significant differences (P < 0.05) compared to values in control cells; # statistically significant differences (P < 0.05) compared to values for 24 h.*

Cell line	Type of extract	0 μg/ml	10 μg/ml	50 μg/ml	100 μg/ml	250 μg/ml
24 h						
HCT-116	leaves	18.43±0.23	14.75±1.47*	15.26±0.46*	15.98±1.86	15.23±0.20
	fruits		15.06±1.72*	15.73±2.32	14.57±0.67*	10.48±0.25*
SW480	leaves	20.26±0.52	18.17±0.63	14.91±1.58*	13.88±0.69*	11.97±2.55*
	fruits		16.99±0.36*	16.74±1.63*	14.08±0.44*	13.25±0.20*
72 h						
HCT-116	leaves	16.18±0.36	11.33±0.02*#	11.01±1.63*#	10.13±0.38*#	10.52±0.87*#
	fruits		11.35±0.53*#	11.07±0.60*#	12.01±0.13*	11.75±0.58*
SW480	leaves	11.42±0.40#	10.49±0.10#	10.16±0.20#	10.27±0.18#	10.30±0.11
	fruits		10.11±0.48*#	11.04±0.11#	11.33±0.20#	10.48±0.29*#

3.5. Protein expression of iNOS

In order to determine whether the decrease in nitrite level is consequence of lower endogenous NO production, we followed iNOS protein expression. There was a decreased iNOS

protein expression (Figure) in the treated cells compared to control cells. Higher inhibition in iNOS protein expression was observed in SW480 cells compared to HCT-116.

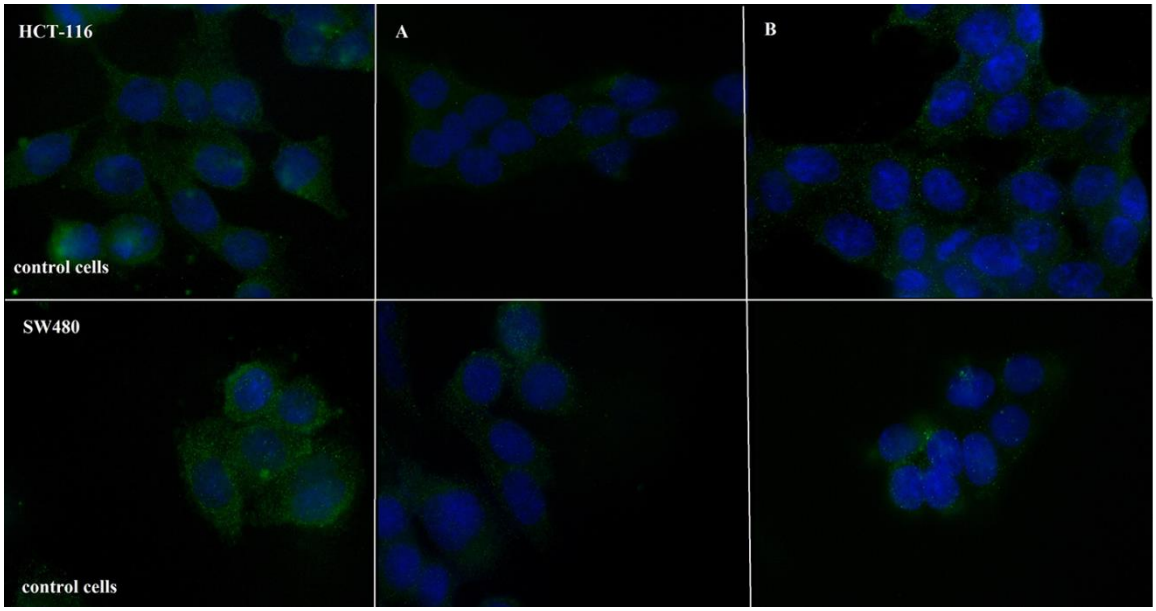

Fig.. Protein expression of iNOS in HCT-116 and SW480 control cells and cells treated by 50 µg/ml of L. vulgare methanol leaf (A) and fruit (B) extracts. Cells were incubated with extracts for 24 h. The images were taken using fluorescence microscopy at 600 ×. Nuclei were stained blue, iNOS was stained green.

3.6. Reduced gluthatione level

The data presented in Table 4 express the GSH level as nmol/ml. The treatments showed increased level of GSH content (negative correlation with applied doses of *L. vulgare* extracts), or caused no statistically significant changes when compared to the control cells.

*Table 4. Effect of methanolic leaf and fruit extracts from L. vulgare, on the reduced gluthatione level (nmol/ml) in HCT-116 and SW480 cell lines after 24 and 72 h of exposure. * statistically significant differences (P < 0.05) compared to values in control cells; # statistically significant differences (P < 0.05) compared to values for 24 h.*

Cell line	Type of extract	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	250 µg/ml
24 h						
HCT-116	leaves	15.09±0.30	22.42±1.33*	20.19±0.38*	20.17±1.17*	20.31±1.78*
	fruits		21.49±3.40*	20.57±2.22*	17.32±0.06	16.56±0.22
SW480	leaves	18.21±0.12	21.52±0.82	20.98±0.41	16.15±1.08	16.65±0.54
	fruits		17.96±0.63	17.16±0.92	17.40±1.56	15.22±0.19
72 h						
HCT-116	leaves	15.85±0.12	20.47±0.98*	20.03±0.85*	18.91±0.06	17.80±1.36#
	fruits		19.58±0.98	17.70±0.06#	19.80±1.71*	17.03±1.81
SW480	leaves	17.13±0.02	25.25±0.98*#	21.84±1.88*	19.74±0.63#	18.34±1.01
	fruits		19.87±0.57	18.45±1.19	18.47±1.27	15.54±1.01

4. Discussion

Many plants rich in naturally phytochemicals, including phenolics and flavonoids, are very important source of antioxidant agents. Phenolic hydroxyl groups are good hydrogen donors: hydrogen - donating antioxidants can react with reactive oxygen and nitrogen species [21] in a termination of reaction, which breaks the cycle of generation of new radicals. Considering high level of phenols and flavonoids in *L. vulgare* extracts, powerful biological activity was expected.

DPPH method has also been used to quantify antioxidants in complex biological systems in recent years and it is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. *L. vulgare* extracts have a powerful scavenger activity, approximately equal to chlorogenic acid, which is used as reference substance for antioxidant activity. Leaf extracts with higher concentrations of phenolics and flavonoids in comparison to fruit extracts, have better antioxidant activity *in vitro* (non-biological system), with lower IC_{50} values.

Superoxide anion radical is one of the strongest free radicals *in vivo* and is generated in a variety of biological systems, either by oxidation processes or by enzymes. The concentration of $O_2^{\cdot-}$ increases under conditions of oxidative stress [22]. Moreover, other kinds of cell produce $O_2^{\cdot-}$ [23], damaging free radicals and oxidizing agents. In our experimental biological system (colon cancer cell lines), *L. vulgare* extracts have pro-oxidative effects. Oleuropein, as dominant phenolic compound in *L. vulgare* caused ROS production in some cancer cell lines [24]. Superoxide anion radical production is in correlation with some findings which confirm that phenolics can act as pro-oxidants in cancer cells [25]. It was found that these extracts had a good cytotoxic and proapoptotic effect on HCT-116 cells [6] and some studies suggested that the cytotoxic effects and induction of apoptosis of cancer cells by some polyphenolic compounds were partially due to their pro-oxidant actions [26, 27]. Production of reactive oxygen and nitrogen species can act as initiators of apoptosis by increasing mitochondrial membrane permeability, which resulted in citohrome c relase and induction of apoptosis [28].

Nitric oxide (NO), a diatomic free radical, is synthesized in biological systems by constitutive and inducible nitric oxide synthase (cNOS and iNOS) [29]. *In vitro* data support the ability of NO to protect human carcinoma cells from apoptosis by a variety of mechanisms including enhancing the stability of the anti-apoptotic protein Bcl-2 via S-nitrosylation [30] and inhibiting the pro-apoptotic activity of caspase-3 [31]. *L. vulgare* extracts caused inhibition of iNOS protein expression, directly leading to the decrease of NO. Inhibition of iNOS protein expression and NO depletion enhance sensitivity to treatments, such as cisplatin [32]. Thus, the targeted inhibition of iNOS and iNOS-derived NO may be an effective therapeutic approach for carcinoma and other iNOS - expressing tumors. Our results are in agreement with other studies [33, 34], suggesting that some compounds in extracts (most likely phenolic compounds) may be linked to intracellular target molecules involved in NO production pathway and result in inhibition of NO production by suppressing iNOS expression. Also, NO has a half-life only several seconds in the $O_2^{\cdot-}$ rich environment. Superoxide anion radical had a high affinity for NO forming peroxynitrite anion ($ONOO^-$), probably reducing the concentration of NO in the treated cell samples [35].

Glutathione, the major intracellular non-protein thiol, plays an important role in a number of cellular functions, including enzyme activity, membrane transport, DNA synthesis and inactivation of xenobiotics and reactive intermediates [36]. Oxidative stress in cells generally involves the GSH system, therefore the level of GSH is measured as a very important parameter of oxidative stress in control and treated cells. An increased GSH level and antioxidant capacities were observed in the treated cells, as a response to oxidative stress. The tendency for increased GSH content in the treated cells suggests that phenolics from plant extracts can either enhance the antioxidant status and GSH level [37], or the cells *de novo* synthesize glutathione as a consequence of the production of reactive metabolites, such as $O_2^{\cdot}$-.

5. Conclusions

The presence of high levels of phenolic compounds in the *L. vulgare* leaf and fruit extracts can contribute to the observed intense free radical activities *in vitro* by DPPH method. However, the obtained results in HCT-116 and SW480 cells indicated that *L. vulgare* extracts increased $O_2^{\cdot-}$ and GSH concentration, inhibited iNOS protein expression and NO production. All the investigated parameters indicate the presence of oxidative stress and pro-oxidative effect of *L. vulgare* extracts in colon cancer cells, which is associated with cytotoxic and apoptotic ability of *L. vulgare*. The observed changes in all the redox parameters were not clearly dose-dependent, but generally higher concentrations induced stronger oxidative stress, with the greatest $O_2^{\cdot-}$ concentrations and GSH depletion. SW480 cells were more sensitive on oxidative stress in comparison to HCT-116 cells. In HCT-116 cells, the antioxidant capacity was enhanced, with higher GSH level and lower changes in $O_2^{\cdot-}$ and NO production. This finding is important from a nutritional point of view, because these extracts contain significant amounts of bioactive constituents, which provide health benefits.

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