

Research Article

Myrtus communis L. Ellagitannins and Flavonoids Protect Cardiomyoblast Cells against CoCl_2 -Induced Hypoxia and H_2O_2 Stress by Improving Oxidative Balance

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Abstract

This study evaluates the potential protective effect of *Myrtus communis* leaves buthanolic fraction (MBF) on CoCl_2 and H_2O_2 -challenged H9c2 cardiomyoblast cells. The identification of the main compounds by LC-ESI-TOF-MS was assessed. Among the 23 identified molecules (12 ellagitannins, 8 flavonoids, 1 coumarins, 1 anthocyanin and 1 myrtucommunone), 6 have been described for the first time in *M. communis*. The MBF displayed high amount of total phenolic (151.09mg of GAE/g of DR), flavonoid and tannin contents (21.81 and 49.54 mg of CE/g of DR, resp.) related to its important antioxidant activity such as DPPH ($\text{IC}_{50} = 3.3\mu\text{g}/\text{mL}$) and ABTS ($\text{IC}_{50} = 14\mu\text{g}/\text{mL}$) tests. Otherwise, exposure of cultured H9C2 cells to CoCl_2 -simulated hypoxia or H_2O_2 -oxidative stress induced over-activity of superoxide dismutase (over 181 %) and Catalase (171 and 500 %, resp.) and decrease in glutathione peroxidase (≈ 48 %) and excess in MDA (≈ 300 %) level. Pretreatment with MBF, attenuated significantly CoCl_2 and H_2O_2 challenged cardiomyoblasts injury by restoring cell viability (≈ 100 %), oxidative balance and MDA level. These results indicate that WBF can be used as a functional food to reduce the side effects of the oxidative stress and can be therapeutically effective candidate in cardiac ischemic injuries.

Keywords: Cytoprotection; CoCl_2 -induced hypoxia; H_2O_2 -induced oxidative stress; H9C2 cells; *Myrtus communis*; LC-ESI-TOF-MS

Introduction

Cardiac cells are sensitive to lack in oxygen (O_2) level and demand a constant supply of O_2 to maintain adequate energy production to ensure their normal function and viability [1]. Oxygen or oxygen-associated processes is determinant to satisfy cardiac metabolic requirements and a simple decrease or lack of myocardial O_2 levels, either during isolated hypoxia or ischemia-associated hypoxia, lead to various physiological changes in the cell [2] and a significantly alteration of gene expression patterns in the heart [3]. Although the mechanism of injury induced by hypoxia is complicated, oxidative stress may be one of the principal causative factors. In fact, deprivation of adequate oxygen supply in cardiac cells lead the production of free radicals which are critical mediators of cardiovascular diseases like cardiac ischemic injury, reversible post-ischemic contractile dysfunction and dysrhythmias [3]. Considerable evidence implicates reactive oxygen and nitrogen (ROS and RNS) species as causes of injury in clinical settings associated with myocardial oxidative stress and are known to inhibit active substances and modulate the signaling of intracellular pathways [4]. It was demonstrated that hypoxia elicits an imbalance in cellular enzymatic and non-enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD) and glutathione reduced (GSH) and induced lipid peroxidation [2,5].

The hypoxia-mimetic agent cobalt chloride (CoCl_2) is well-known to serve as a simple and convenient alternative *in vitro* model to establish a chemical hypoxia-induced cardiomyocyte injury [6]. Formation of ROS, leading to oxidative stress and lipid peroxidation

has been reported to be one of the major pathogenetic mechanisms for CoCl_2 -induced cell injuries [1,4]. Hence, the pharmacologic strategies targeted at scavenging ROS may be an effective way of eliminating the oxidative damage induced by hypoxia in cardiac cells.

Natural antioxidants such as phenolic acids, flavonoids and tannins; already been isolated from different kinds of plants; are specific compounds that protect cells against the damaging effects of free radicals [7]. This activity depends on the structure, the nature of substitutions on the aromatic rings and especially the number and positions of the -OH groups [7]. Additionally, phenolics were highlighted as efficient protectors against cardiac diseases, oxidative stress related disorders [7,8] and some hypoxia related diseases [9]. For example, quercetin supplementation in hippocampal cells significantly inhibited the KCN-hypoxia induced free radicals and lipid peroxidation and restored glutathione peroxidase (GPx) level toward the control one [9]. It was reported that myricitrin, a flavonoid glycoside, protect endothelia cells against ROS-induced apoptosis and prevent diabetic cardiomyopathy by the inhibition of high glucose-induced cardiomyocyte apoptosis [10,11]. It was also reported that myricitrin provides protection to H9c2 cells against hypoxia-reoxygenation-induced oxidative stress and apoptosis, most likely via increased expression of heat shock protein. Additionally, Clinical studies demonstrated that phenols possess antioxidant capacity by modulating genes expression and by inducing the endogenous antioxidant enzymatic defense system [8].

The aromatic and medicinal shrub *Myrtus communis* L.,

(common Myrtle) belonging to the Myrtaceae family is widely used for spice purposes and as flavor ingredients in alcoholic beverages widespread in the Mediterranean regions [12]. Leaves of this species have been used extensively in folk medicine as antiseptic, astringent, disinfectant, hypoglycemic, antibacterial and anti-inflammatory agent and as remedy against asthma and respiratory diseases [12]. Myrtle has been reported as very rich in essential oils, flavonoids, phenolic acids, ellagitannins and anthocyanins, responsible to several biological activities [12]. Furthermore, the richness of flavonoids and ellagitannins in myrtle leaves encourages better identification, characterization and quantification of the *M. communis* phenolics. Thus, leaves buthanolic fraction, obtained after the fractionation of the crude methanolic extract, was analyzed by LC-ESI-TOF-MS. Further, the *in vitro* antioxidant capacities and the potential protective effect of leaves myrtle buthanolic fraction (MBF) and its related phenolics compounds on CoCl_2 and H_2O_2 -challenged H9C2 cardiomyoblast cells were assessed. We attempted to determinate the possible mechanisms of the therapeutic efficacies of these bioactive molecules by studying the biochemical markers of enzymatic antioxidant defense system by assessment of SOD and CAT activities and evaluation of GPx and MDA levels.

Materials and Methods

Preparation of *M. communis* buthanolic fraction

The leaves of *M. communis* were collected from Zaghouan region (eastern north of Tunisia) in December 2018, air dried and grinded to a fine powder. Leaves powder (1Kg) was firstly extracted 3 times 1 L of methanol-water (1:3, v/v). After centrifugation, the crude extract was collected and freeze-dried. The *Myrtus* buthanolic fraction (MBF) was collected after a successive fractionations by diethyl ether and ethyl acetate. The final obtained buthanolic fraction (MBF) was collected; freeze dried and stored at -4°C until analysis.

Determination of phenolic levels and analysis of compounds by HPLC-ESI-TOF-MS

Evaluation of total phenolic (TPC) and total flavonoids contents (TF) in MBF were assessed according Dewanto et al. [13]. TPC and TF were expressed respectively as mg GAE/g DR, and as mg catechin equivalent /g DR. Total tannin contents (TTC) were measured according Bourgo et al. [14] and was expressed as

Analysis of individual phenolic compounds was assessed by HPLC-ESI-TOF-MS. by Freeze-dried MBF (2mg) was dissolved in methanol. Molecules separation and identification was carried out using an HPLC system (Agilent 1200) recorded to a time-of-flight mass spectrometer, Agilent MSD TOF (Agilent technologies, Germany). The equipments and characteristics descriptions of the apparatus and the used parameters were assessed according Bourgo et al., [14]. The data recorded was processed with MassHunter software (Germany). LC-TOF-MS analyses were studied in the negative ion mode and these compounds were identified mainly by their UV-spectra and ESI-MS spectra and by comparing with published data.

Determination of antioxidant activities of fraction

The total antioxidant capacity (TAC) of MBF, based on the reduction of Mo(VI) to Mo(V) and the formation of a green phosphate/ Mo(V) complex was assessed according Bourgo et al. [14] and the TAC was expressed as mg GAE/g DR.

ABTS and DPPH scavenging activity of MBF were evaluated according to Bettaib et al. [8]. The antiradical capacity was expressed as IC_{50} ($\mu\text{g}\cdot\text{mL}^{-1}$), which is the inhibiting concentration of 50% of the synthetic radical.

The ferric reducing antioxidant power (FRAP), as proposed by Oyaizu [15], is a colorimetric method based on the ability of extract to reduce the ferric to ferrous ion. The ability of MBF to prevent the bleaching of β -carotene was performed according to Koleva, et al. [16].

Cell culture

H9c2 cells (ATCC, Manassas, VA, USA, ATCC CRL-1446) grown in DMEM, supplemented with 10% fetal bovine serum (FBS) and penicillin ($100 \text{ UI}\cdot\text{mL}^{-1}$) - streptomycin ($100\mu\text{g}\cdot\text{mL}^{-1}$) solution. Cells were incubated at 37°C in a humidified incubator under 5% CO_2 . At 70% of confluence, cells were harvested with trypsin/EDTA and were seeded at a concentration of 4×10^3 cells/well in 96-well plates for MTT tests, or in 60mm cell culture dishes at a concentration of 2.5×10^5 cells/petri dish for oxidative stress parameters measurement.

Cell treatment protocol

CoCl_2 , H_2O_2 , and MBF were prepared into sodium pyruvate-free DMEM supplemented with DMSO (1%) without FBS. Pyruvate is depleted because pyruvate can scavenge H_2O_2 in the medium [17]. In order to explore cytotoxicity of MBF, H9c2 cells were incubated with three concentrations of buthanolic fraction (1, 10 and $100 \mu\text{g}/\text{mL}$) for 24h. To examine the protective effect of MBF on CoCl_2 -induced cell injury or H_2O_2 -induced oxidative stress, cells were pre-incubated with the no cytotoxic concentration of myrtle fraction for 3 hours prior to CoCl_2 ($900\mu\text{M}$) and H_2O_2 ($80\mu\text{M}$) exposure for 24h.

Cytoprotective effect of MBF against CoCl_2 -induced hypoxia and H_2O_2 -induced oxidative stress

The evaluation of cell viability was based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan which is measured spectrophotometrically at 540nm after DMSO solubilization. Cells were exposed to CoCl_2 ($900\mu\text{M}$) or H_2O_2 ($80 \mu\text{M}$) as well as MBF (1, 10 and $100 \mu\text{g}/\text{mL}$) for 24h. The medium was removed and cells were incubated with MTT solution ($5\text{mg}/\text{mL}$) for 3h at 37°C .

Cell lysis and protein quantification

The cells were rinsed with ice cold PBS, scraped and sonicated at 4°C with $200\mu\text{L}$ of lysis buffer (Tris/HCl 0.1M, pH 7.4). After homogenization and centrifugation at 1000 for 10min at 4°C , the supernatant was used for measurement of oxidative stress parameters. Protein content was determined according Bradford [18]. Briefly, sample was mixed with Bradford reagent and incubated at ambient temperature for 5min. The absorbance of the wavelength was read at 540nm and protein concentrations were calculated from an internal standard of BSA.

Evaluation of antioxidant enzymes activities and lipid peroxidation

Superoxide dismutase (SOD) activity was assessed according to Kuthan et al. [19] by measuring the ability of MBF to inhibit the photoreduction of nitroblue tetrazolium (NBT) to in a blue formazan. Catalase activity (CAT) was determined as Aebi, [20] by

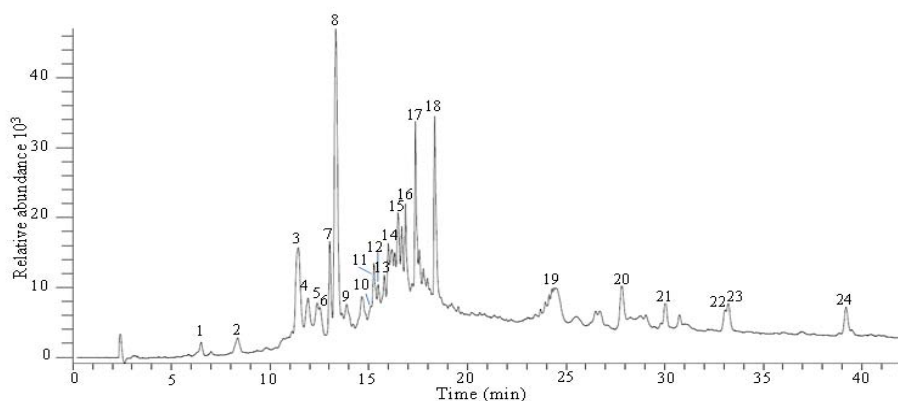


Figure 1: Chromatographic profile of phenolic compounds from *Myrtus communis* buthanolic fraction obtained by HPLC-DAD at 254nm.

evaluating for 3 min, the decrease in absorbance of H₂O₂ at 240 nm. GPx activity was determined as previously described by Rotruck et al. [21] by quantifying the residual glutathione using 5,5'-dithio-bis(2-nitrobenzoic acid). Malondialdehyde (MDA) content was assessed according to Draper and Hadley [22]. Enzyme activities were normalized by protein content (U mg⁻¹) and results were expressed as a % of enzyme activity of different treatment upon control.

Data analysis and statistics

The statistical analysis was performed using one-way ANOVA followed by Bonferroni's test using the software GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) for comparison between groups. Differences between means were considered significant at p values of less than 0.05.

Results and Discussion

HPLC-DAD-ESI-MS screening

In order to characterize the main bioactive compounds of MBF, these were analyzed by HPLC-DAD-ESI-MS. The chromatographic profile shown in Figure 1 exhibits the separation of 24 molecules at 250nm. Identification and assignment of individual phenolic compounds were determined considering the HPLC-DAD analysis together with electrospray mass spectrometry (MS) data in the ESI negative ion mode and by comparison with several databases and published data. Among the identified compounds in MBF, hydrolysable tannins represent the predominant phytochemical group followed by flavonoids, coumarin derivative, anthocyanin and myrtucommulone.

Hydrolysable tannins: Ellagitannins or hydrolysable tannins dominated the MBF. Twenty of these compounds have been isolated from leaves of *Myrtus* buthanolic fraction (1-3, 5-9, 11, 12, 13 and 18).

Compounds 1 (R.T = 6.37min), 2 (RT = 8.38) and 5 (RT = 12.43) presented the same [M-H]⁻ ions at *m/z* 633.3 and UV spectrum shape corresponding to the molecular formula C₂₇H₂₂O₁₈. Based on these results, these compounds were identified as strictinin isomers and were previously identified in several part of myrtle [23,24].

Compound 3 occurred a retention time of 11.47min and exhibited a [M-H]⁻ ion at *m/z* 1583 is one of main dimeric ellagitannins identified in MBF. The data on the mass spectra indicated that compound 3 is

a eugeniflorin D2 by comparison with those of known compounds published previously. Eugeniflorin D2 was first isolated from *Eugenia uniflora*, (Myrtaceae) shown to have digestive properties [25] and in leaves [26] and myrtle berries [27].

Compound 6 (R.T = 12.63min) and 8 (R.T = 13.10min) with the same precursor ion at *m/z* 783.27 and UV spectrum shape were assigned as Bis-HHDP-glucose isomers and corresponded to casuariin and pedunculagin. These compounds are previously reported in Myrtaceae family and Myrtle [28].

Besides, two different galloyl quinic acid derivatives were determined. Compounds 7 (*m/z* 495.32) and 13 (*m/z* 647.35) with respectively the molecular formula C₂₁H₂₀O₁₄ and C₂₈H₂₄O₁₈ were identified as digalloyl quinic acid and trigalloyl quinic acid [24] (Table 1).

Compound 9 (*m/z* 1567.23) with molecular formula C₆₈H₄₇O₄₄ was identified as Oenothien B and was previously identified in myrtle seeds [27]. The molecular ion of 11 (*m/z* 935.17) and 12 (*m/z* 785.39) supporting respectively a molecular formula C₄₁H₂₈O₂₆ and C₃₄H₂₆O₂₂ were previously reported in myrtle berries by D'Urso et al. [24] as casuarietin and tellimagrandin I, respectively. At last, compound 18 (*m/z* 463.43) was characterized as ellagic acid-hexoside as previously reported in *M. communis* leaves [23, 24].

Flavonoids: Regarding flavonoids, 8 compounds were detected. Compounds 14 and 15 with presented the same [M-H]⁻ ions at *m/z* 705.32 corresponding to the molecular formula C₃₂H₃₄O₁₈. Thus in comparison with database and data reported in literature [29,30], it were tentatively identified as acetylated kaempferol glucoside isomers (Kaempferol 3-[2',3',4'-triacyetyl- α -L-arabinopyranosyl-(1-6)-glucoside] and Kaempferol 3-[2''',3''',5'''-Triacyetyl- α -L-Arabinofuranosyl-(1-6)-Glucoside). Compounds 14 and 15 were for the first time identified in the species of *Myrtus communis*.

Compound 16 (RT= 16.93min) and 17 (R.T = 17.3) showed respectively a molecular ion at *m/z* 631.4 and *m/z* 479.4 corresponding to the molecular formula C₂₈H₂₄O₁₇ and C₂₁H₂₀O₁₃ were identified as myricetin galloyl hexoside and myricetin galactoside. These compounds were previously identified in *Myrtus communis* [23,24].

Peak 19 eluting at 24.48min, displayed the [M-H]⁻ ions at *m/z* 609.75. Thus, it was identified as hesperetin-7-O-rutinoside

Table 1: Bioactive compounds identified in *Myrtus communis* leaves buthanolic fraction (MBF) by LC-ESI-TOF-MS analysis in negative ion mode.

Peak N ^o	R T (min)	λ_{\max} (nm)	Measured m/z [M-H] ⁻	Formula	Identified compounds	References
1	6.37	260, 289, 320	633.26	C ₂₇ H ₂₂ O ₁₈	Strictinin isomer 1	[23,24]
2	8.38	265, 290, 320	633.27	C ₂₇ H ₂₂ O ₁₈	Strictinin isomer 2	[23,24]
3	11.47	262	1583.16	C ₆₈ H ₄₇ O ₄₅	Eugeniflorin D2	[27]
4	11.9	257	782.53	C ₃₃ H ₃₅ O ₂₂	Cyanidin 3,5-di-(6-malonylglucoside)	[34]
5	12.43	260, 290, 330	633.34	C ₂₇ H ₂₂ O ₁₈	Strictinin isomer 3	[23,24]
6	12.63	261, 336	783.27	C ₃₄ H ₂₄ O ₂₂	Bis-HHDP-glucose (Casuariin/pedunculagin)	[28]
7	13.04	274	495.32	C ₂₁ H ₂₀ O ₁₄	Digalloyl quinic acid	[24]
8	13.1	262, 336	783.27	C ₃₄ H ₂₄ O ₂₂	Bis-HHDP-glucose isomer(Casuariin/pedunculagin)	[28]
9	13.34	262, 336	1567.23	C ₆₈ H ₄₇ O ₄₄	Oenothetin B	[27]
10	15.28	262, 336	859.38	C ₂₅ H ₃₂ O ₃₃	Unknown	[24]
11	15.33	262, 336	935.17	C ₄₁ H ₂₈ O ₂₆	Casuarictin	[24]
12	15.88	261, 336	785.39	C ₃₄ H ₂₆ O ₂₂	Tellimagrandin I	[24]
13	16.05	257, 336	647.35	C ₂₈ H ₂₄ O ₁₈	Trigalloylquinic acid	[24]
14	16.56	287, 336	705.32	C ₃₂ H ₃₄ O ₁₈	Kaempferol 3-[2'',3'',4''-triacetyl- α -L-arabinopyranosyl-(1-6)-glucoside]	[30,29]
15	16.72	303, 323,	705.32	C ₃₂ H ₃₄ O ₁₈	Kaempferol 3-[2'',3'',5''-Triacetyl- α -L-Arabinofuranosyl-(1->6)-Glucoside	[29]
16	16.93	262, 336	631.4	C ₂₈ H ₂₄ O ₁₇	Myricetin galloyl hexoside	[23,24]
17	17.43	253, 299, 356	479.42	C ₂₁ H ₂₀ O ₁₃	Myricetin galactoside	[23,24]
18	18.42	253, 299, 349	463.43	C ₂₀ H ₁₆ O ₁₃	Ellagic acid-hexoside	[23,24]
19	24.48	243, 277	609.75	C ₂₈ H ₃₄ O ₁₅	Hesperetin-7-O-rutinoside (Hesperidin)	[31]
20	27.95	244	339.68	C ₁₅ H ₁₆ O ₉	Esculetin-O-glucoside	[35]
21	30.17	241, 273	631.6	C ₃₈ H ₄₈ O ₈	Myrtucommulone E	[36]
22	33.15	246	339.68	C ₂₀ H ₁₉ O ₅	C-methylated flavonoid isomer	[32]
23	33.4	246	493.52	C ₂₂ H ₂₂ O ₁₃	Myricetin-3'-methylether 3-O- β -D-galactopyranoside	[33]
24	39.35	247	339.68	C ₂₀ H ₁₉ O ₅	C-methylated flavonoid isomer	[32]

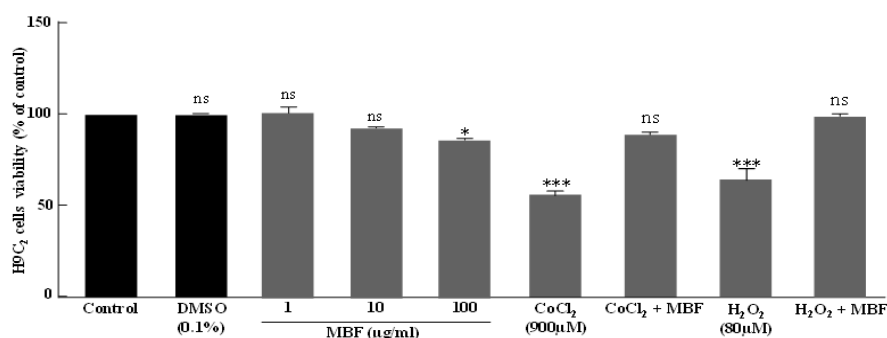


Figure 2: H9c2 cell viability in the presence of different concentrations of *Myrtus* buthanolic fraction (MBF) and effect of MBF (1 µg/ml) pretreatment on cell viability in CoCl₂ or H₂O₂-challenged H9c2 Cells. Control cells were incubated for 24h with medium alone; DMSO: cells were incubated for 24h with medium supplemented with 0.1% DMSO. H9c2 Cells at 2 x 10⁴ cells/mL were pretreated with three ranges of MBF concentrations (1, 10 and 100 mg mL⁻¹) for 3h, and then exposed to 900 µM of CoCl₂ or 80 µM of H₂O₂ during 24h. H9c2 cells survival was quantified by MTT test and the results are expressed as percentages of the control. Data are means ± SEM of three independent experiments. ANOVA followed by Bonferroni's test ***p < 0.001; **p < 0.01; *p < 0.1; ns: not statistically different from control cells.

(hesperidin). This compound was previously identified from the pericarps of *Myrtus communis* by Martin et al. [31].

Compound 22 and 24 eluting respectively at 33.15 and 39.35 min in MBF, matched exactly the same accurate mass (C₂₀H₁₉O₅: [M-H]⁻ 339.68) and UV-vis spectrum shape with a λ_{\max} at 247 nm. Thus, in comparison with database and data reported in literature we suggest

that peak 22 and 24 were C-methylated flavonoid isomers previously identified from aerial part of *Baekkea frutescens* L. a *Myrtaceae* species [32] and in first time in Myrtle. Moreover, compound 23 gave a [M-H]⁻ ions at m/z 493.52. Full scan ESI mass spectra proposed the formula C₂₂H₂₂O₁₃. Thus, it was tentatively identified as myricetin-3'-methylether 3-O- β -D-galactopyranoside. This compound was previously identified from buds of *Cleistocalyx operculatus* belong the

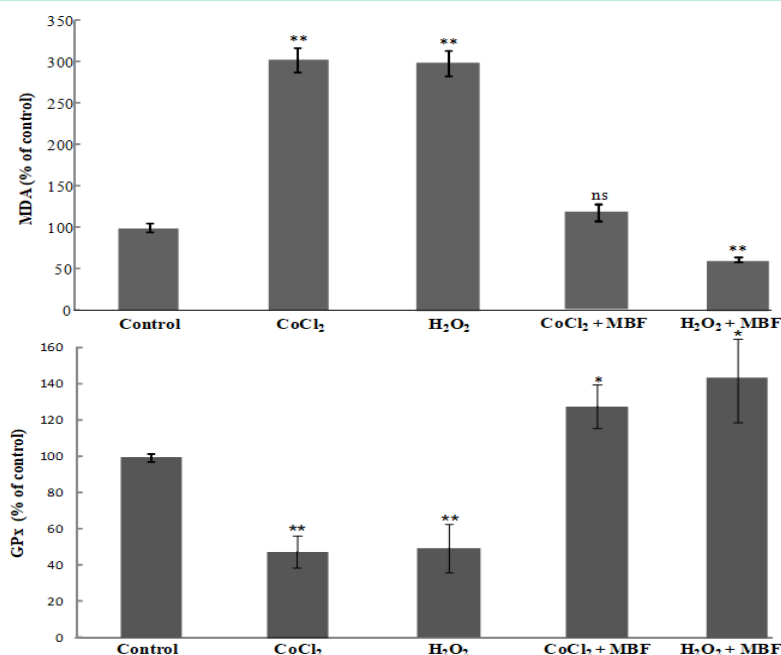


Figure 3: MDA and GPx levels in H9c2 cells stressed with CoCl₂ and H₂O₂. Control: cardiac cells were incubated for 24h with medium alone; CoCl₂ or H₂O₂ stress: cells were incubated for 24h with 900μM of CoCl₂ or 80μM of H₂O₂; CoCl₂/H₂O₂ + MBF: cells were pre-treated 3h with 1μg/mL of MBF before stressed with CoCl₂ (900μM) or H₂O₂ (80μM). The results are expressed as percentages of the control. Data are means ± SEM of three independent experiments. ANOVA followed by Bonferroni's test **p <0.01; ns, not statistically different from control cells.

Table 2: Total polyphenol (TPC), flavonoid (TFC) and condensed tannin (CTC) contents from leaves of *Myrtus communis* buthanolic fraction (MBF).

	TPC (mg EAG/g DR)	TFC (mg EC/g DR)	CTC (mg EC/g DR)
MBF	151.09 ± 6.14	21.81 ± 4.12	49.54 ± 5.68

Values are the means of three replicates and standard deviation

Myrtaceae family [33] and as so far we known no reports were found in myrtle.

Other compounds: Compound 4 eluting at 11.9min in MBF, matched the accurate mass (C₃₃H₃₅O₂₂; [M-H]⁻ 782.53) and a λ_{max} at 257nm. Hence, in comparison with several database and data reported in literature, this compound was identified as cyanidin 3,5-di-(6-malonylglucoside) belonging the anthocyanin group. This compound has been previously reported from the flowers of *Asteraceae* species *Dahlia variabilis* [34]. Compound 4 was reported for the first time in *M. communis*.

Compound 20 displayed [M-H]⁻ ions at m/z 339.68, was identified in WBF as Esculetin-O-glucoside according to the literature [35]. Peak 21 occurred a RT of 30.17 min and exhibited a [M-H]⁻ ion at m/z 631.60 in WBF chromatogram. The full MS spectra of this peak allowed the molecular formula C₃₈H₄₈O₈ led to identify this compound as Myrtucommulone E. This compound was previously identified in aerial part of this species [36].

Phenolic content and *in vitro* antioxidant activity

The amounts of the total phenolics (TPC), total flavonoids (TFC) and condensed tannins (CTC) in the MBF are listed in Table 2. Results showed that extraction mode that they adopted yielded higher amounts of polyphenols (151mg GAE/g DR) flavonoids (21.81mg EC/g DR) and condensed tannins (49.54mg EC/g DR).

The high amount of these compounds is in accordance with the results of phenolic compounds characterization obtained earlier in our study. Otherwise, the antioxidant capacity of MBF was assessed by five *in vitro* tests (Table 3). A strong total antioxidant activity (277.87mg GAE/g DR), and scavenging capacity against the radical DPPH. and ABTS.+ (3.3 and 14μg/mL) were observed. However, this fraction exhibited moderate ferric reducing antioxidant power (EC₅₀ = 204μg/mL) and antioxidant activity determined by the β-carotene-linoleic acid system (123.33μg/mL). Our data agree with some recent works in the field, demonstrating that myrtle phenolic extract have great *in vitro* antioxidant potential [37]. This capacity was attributed to phenolic compounds like ellagitannins and flavonoids, to their redox properties, which allow them to act as powerful singlet oxygen quenchers, hydrogen donors and reducing agents [14]. For examples, ellagitannins like oenothien B has been shown to have anticancer, anti-inflammatory, antioxidant, and antifungal properties [27]. Strictinin was also used as an efficient antioxidant in lipid peroxidation [38] and it was reported that both Tellimagrandin I and pedunculagin possessed high scavenging activity against DPPH- (IC₅₀ = 73.5 and IC₅₀=73.6 μM, respectively) and ABTS+ (IC₅₀=54.5 and 65.8 μM, respectively) [39].

Evaluation of cardiomyoblast (H9c2) cells viability in the presence of MBF

In the absence of exogenous CoCl₂ or H₂O₂, buthanolic fraction at 1 and 10 μg/mL had no significant effect on cell death (Figure 2) and the viability was maintained from 100 and 98 %. The high concentration (100μg/ml) exhibited a moderate H9c2-cell toxicity and lowered cell viability rate to by 13.27 %. Hence, 1μg/mL which did not affect H9c2 viability was selected as safe concentration. There has been much attention accorded to study cytotoxicity as a first

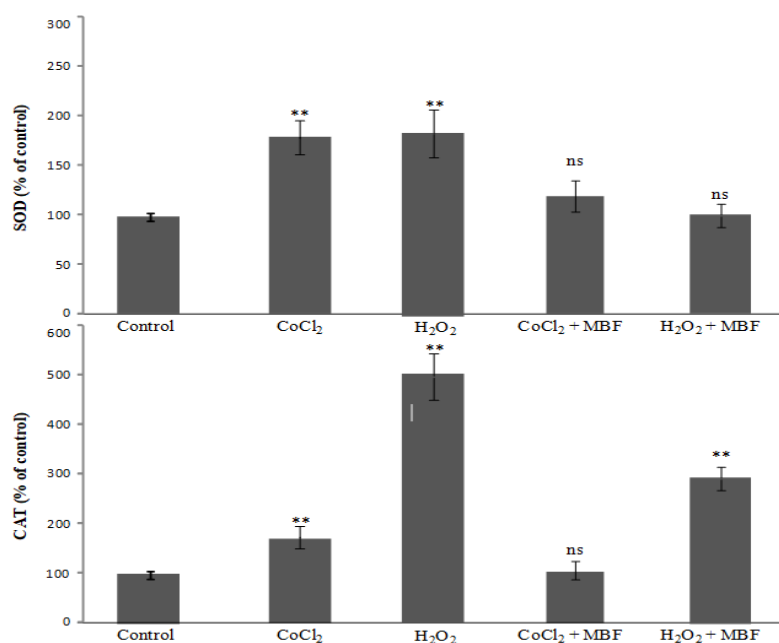


Figure 4: Effect of MBF on CoCl₂ or H₂O₂ induced SOD and CAT activities alteration. Cells were incubated for 24h with medium alone; CoCl₂: cells were incubated for 24h with 900μM of CoCl₂; H₂O₂: cells were incubated for 24h with 80μM of H₂O₂; CoCl₂ + MBF: cells were pre-incubated with 1μg/mL MBF then treated by CoCl₂ 900μM; H₂O₂ + MBF: cells were pre-incubated with 1μg/mL MBF then treated by H₂O₂ 80μM. The results are expressed as percentages of the control. Data are means ± SEM of three independent experiments. ANOVA followed by Bonferroni's test *p <0.05*; **p <0.01; ns: not statistically different from control cells.

Table 3: Total antioxidant activity (TAA), DPPH and ABTS radical scavenging activities (IC₅₀ values), β-carotene bleaching test (BCBT) and ferric reducing activity power (EC₅₀ values) of buthanolic fraction from leaves of *Myrtus communis*.

	TAA (mg EAG/g DR)	DPPH (IC ₅₀ = μg/mL)	ABTS (IC ₅₀ = μg/mL)	BCBT (IC ₅₀ = μg/mL)	FRAP (EC ₅₀ = μg/mL)
MBF	277.87 ± 2.31	3.3 ± 0.17	14 ± 0.01	204.66 ± 7.71	123.33 ± 1.15

step in evaluating the toxicity of plant extracts and its isolated active compounds. However, it was suggested that phenolic compounds have antioxidant properties at low doses, but can exert pro-oxidant effects at high doses [40].

MBF reestablished the viability of exposed H9c2 cells to hypoxia and oxidative stress

The present study demonstrated that 24h of CoCl₂ or H₂O₂ treatment, significantly decreased cell viability in H9c2 cells (58.65% and 65%, respectively). CoCl₂ and H₂O₂ concentrations were selected based on previous results obtained in the laboratory. These data are in line with those of Chen et al. [41]. Importantly, pre-treatment of cells with a low dose of MBF (1μg/mL) prevented CoCl₂ and H₂O₂-induced cell injury by enhancing cell viability to 100% (Figure 2). In this study, under hypoxic and oxidant stress conditions, cells can't maintain normal energy production which is critical for ensuring their normal function [1]. Furthermore, CoCl₂ is known to activate hypoxic signaling by stabilizing the hypoxia inducible transcription factor 1α (HIF1α) and plays a major role in mediating cells toxicity and oxidative stress. Thus, CoCl₂ may help to develop new therapeutic strategies for hypoxia related diseases [41]. The most relevant finding from this study is the protection offered by MBF against the deleterious effects of CoCl₂ and H₂O₂ injuries, by creating a protective environment around H9c2 cells under hypoxic or oxidative conditions. Cardiomyoblasts became then more resistant to support hypoxic stress. In this case, ellagitannins and flavonoids

can have potential to inhibit oxidative stress pathway death related to hypoxia or oxidative stress. Previous studies together with our present experiments show that *M. communis* is rich source of phenolic compounds possessing strong biological properties [12,37].

Effect of *M. communis* buthanolic fraction on MDA level and in improving enzymatic defense

Exposure of cardiomyoblasts to 900μM CoCl₂ or to 80μM H₂O₂ for 24h resulted in a significant increase of MDA (< 300 %) as compared to untreated cells (Figure 3). The accumulation of oxidized lipids and proteins is highly toxic to cells. In this study, it was found that 1μg/mL of MBF was efficiently capable in reduction of lipid peroxidation at hypoxic and oxidative stress conditions and restored amount of MDA as the control levels. Lipid peroxidation is a process under which oxidants attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion, thereby, MDA is an end-product generated by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes [42]. MBF prevents lipid damage caused by CoCl₂ and H₂O₂; hence, ellagitannins and flavonoids play an important antioxidant role with protective functions that can block MDA formation. That is to say, pre-treatment with myrtle can avoid MDA related damages i.e. loss of membrane fluidity, impaired ion transport and membrane integrity, and finally loss of cellular functions [43]. These data corroborate previous studies, indicating that the herbal

extracts can block lipoperoxidation mechanism [44]. Furthermore, phenolic acids, flavonols, flavan-3-ols, flavanones have also reported a decrease of lipid peroxidation in oxidative stress mediated injury on cardiomyoblasts *in vitro* and *in vivo* [7]. Doxorubicin (DOX)/Adriamycin is an anthracycline antibiotic used in chemotherapy and it is associated with cardiotoxicity. The treatment of H9c2 cardiomyocytes with 50 μ M of dihydromyricetin decreased the ROS production, lipid peroxidation and NADPH oxidase activity [45]. The same behavior was also observed in *in vivo* models. Dihydromyricetin (125-500 mg/kg) pretreatment reduced cardiomyocytes necrosis and apoptosis, ROS production and lipid peroxidation and increased the production of antioxidant enzymes in mice and rats exposed to DOX [45].

In this study, CoCl₂ and H₂O₂ decreased the GPx production about 48 % and MBF significantly increased and restored GPx levels as the control (Figure 3). GPx is an important antioxidant that uses glutathione to reduce H₂O₂ and lipid hydro-peroxides and was responsible for the removal of hydroperoxides formed in cells, thereby detoxifying and protecting cells against oxidative damage. Indeed, glutathione (GSH) which is required in the reaction for glutathione peroxidase- (GPx-) mediated detoxification of H₂O₂ in cells has a key role in protecting the myocardium against ischemia-reperfusion injury [46]. Therefore, GPx may be involved in the cytoprotective effect of MBF against CoCl₂-induced injury in H9c2 cells.

After 24h exposure of 900 μ M CoCl₂ or 80 μ M H₂O₂, SOD and CAT activities significantly increases (181.62 and 185 %) and 162 and 500%, respectively) comparing to untreated cells (Figure 4). This enzymes surproduction was inhibited with MBF at low dose (1 μ g/mL). CoCl₂ or H₂O₂ treated cells exhibit an increased oxidative stress which is counterbalanced by enhanced antioxidant defense mechanisms, generation of free radicals has a fundamental role in the signaling of CoCl₂ and or H₂O₂ induced cell toxicity [2]. Cardiomyoblast cells express enzymatic antioxidative defense systems, including antioxidant enzymes SOD and CAT to prevent oxidative damage caused by oxidative stress [47]. The mitochondrial production of O₂[•] increases in hypoxic condition [5], which leads to SOD activation. Thus, this enzyme transforms superoxide anion to H₂O₂, which can explain also the increase of catalase after 24h of CoCl₂ treatment. Indeed, H₂O₂ accumulates in response to hypoxia [48]. Accordingly, high H₂O₂ level is pro-oxidant, which leads to CAT antioxidant defense activation, CAT can transform H₂O₂ into oxygen and water [49]. As MBF was added during three hours before CoCl₂ its protection really reflects a preventive effect against the multifaceted oxidative stress induced by chemical hypoxia. The protective effect of MBF against CoCl₂/H₂O₂ -induced oxidative stress and cell death could be mediated through the modulation of the endogenous antioxidant enzyme system, which may contribute to enhance cardiac cells function following hypoxia insult, under this circumstances cardiomyoblast cells can survive.

Besides, the structural diversity of flavonoids offer them to act directly as a free radical scavengers, such as the presence of a catechol group on the B-ring, the presence of OH-groups at the 3 and 5 position and the 2,3- double bond in conjugation with a 4-oxofunction of a carbonyl group in the C-ring [7]. Chemical structure of flavonoids like derived myricetin and kaempferol, hesperidin is probably responsible for the free radical scavenger function of myrtle phenol and can

inhibit the creation of an oxidative environment around and inter the cardiomyoblast cells. In this context, Lin et al. [11] reported that myricetin reduced hyperthermia, hypotension, cardiac inflammatory and normalized the cardiac levels of antioxidant defense enzymes (SOD and GSH/GSSG ratio), as well as accumulation of ROS and MDA in heated rats via enhancing HSP-72 activity. The protective effects of hesperidin against hypoxia-ischemic (HI) brain injury in neonatal rat are demonstrated by Rong et al. [50]. Hesperidin pretreatment significantly reduced HI-induced brain tissue loss and improved neurological outcomes and reduced free radicals and lipid peroxide levels. In addition, administration of hesperidin (100mg/kg/day, p.o. for 90 days) increased the activity of antioxidant enzymes and protected cardiac tissue of aged rats against age-related increase in oxidative stress [51].

Moreover, ellagitannins exhibit a wide range of antioxidant, antimicrobial, antiinflammatory and anticancer properties, as confirmed by numerous studies [47]. Therefore, this group of compounds prevents or reduces oxidative stress, which could otherwise induce carcinogenesis, and which is a major cause of atherosclerosis and cardiovascular diseases [47,52].

Thus, to detoxify ROS and protect cells from their detrimental effects, bioactive compounds such as flavonoids and ellagitannins may act as allosteric effectors for regulatory control and catalytic activation of SOD and CAT enzymes [47] and are considered as attractive candidates for the development of novel pharmaceuticals due to their beneficial properties.

Conclusion

In conclusion, MBF have a potent antioxidant activity and is particularly rich in ellagitannins and flavonoids compounds that, to our knowledge, they have not been extensively investigated until now. Thus, the study indicates that myrtle leaves phenolics can be potentially used as dietary antioxidant in the prevention of different disorders associated with hypoxia and oxidative stress especially cardiovascular disorders and can be used with people which are exposed to risk factors (smokers) or presenting favorable genetic ground. Nevertheless, the influence of *M. communis* phenolics on *in vivo* and clinical system should be continued.

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