Austin Journal of Nutrition & Metabolism

Open Access

Austin Publishing Group

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

Mansour RB[1]*, Megdiche-Ksouri W[1], Nefzi N[1], Bourgou S[1], Ksouri R[1], Giroux-Metgès M-A[2] and Talarmin H[2]

[1]Laboratory of Aromatic and Medicinal Plants, Centre of Biotechnology of Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisia

[2]Université de Bretagne Occidentale, Faculté de Médecine et des Sciences de la Santé, EA 4324 Laboratoire de Physiologie ORPHY, 29200 Brest, France

***Corresponding author:** Rim Ben Mansour, Laboratory of Aromatic and Medicinal Plants, Center of Biotechnology of Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisia

Received: December 20, 2021; **Accepted:** January 31, 2022; **Published:** February 07, 2022

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 myrtucommulone), 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 (IC_{50} = 3.3µg/mL) and ABTS (IC_{50} = 14µg/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 CoCl2 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.,

Citation: Mansour RB, Megdiche-Ksouri W, Nefzi N, Bourgou S, Ksouri R, Giroux-Metgès M-A, et al. *Myrtus communis* L. Ellagitannins and Flavonoids Protect Cardiomyoblast Cells against $CoCl_2$-Induced Hypoxia and H_2O_2 Stress by Improving Oxidative Balance. Austin J Nutr Metab. 2022; 9(1): 1121.

(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 Bourgou 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 Bourgou 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 Bourgou 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} ($\mu g.ml^{-1}$), 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 $UI.mL^{-1}$) - streptomycin (100$\mu g.mL^{-1}$) 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 $4x10^3$ cells/well in 96-well plates for MTT tests, or in 60mm cell culture dishes at a concentration of $2.5x10^5$ 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 µg/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 $CoCl_2$ (900µM) and H_2O_2 (80µM) 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 $CoCl_2$ (900µM) or H_2O_2 (80 µM) as well as MBF (1, 10 and 100 µg/mL) for 24h. The medium was removed and cells were incubated with MTT solution (5mg/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 200µL 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

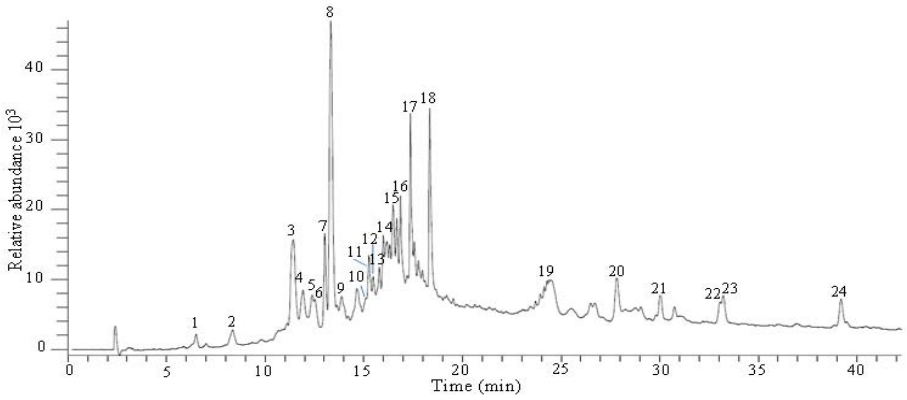

Figure 1: Chromatographic profile of phenolic compounds from *Myrtus communis* buthanolic fraction obtained by HPLC-DAD at 254nm.

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 $C_{27}H_{22}O_{18}$. 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 $C_{21}H_{20}O_{14}$ and $C_{28}H_{24}O_{18}$ were identified as digalloyl quinic acid and trigalloyl quinic acid [24] (Table 1).

Compound 9 (*m/z* 1567.23) with molecular formula $C_{68}H_{47}O_{44}$ was identified as Oenothein 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 $C_{41}H_{28}O_{26}$ and $C_{34}H_{26}O_{22}$ were previously reported in myrtle berries by D'Urso et al. [24] as casuarictin 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 $C_{32}H_{34}O_{18}$. 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′-triacetyl-α-L-arabinopyranosyl-(1-6)-glucoside] and Kaempferol 3-[2‴,3‴,5‴-Triacetyl- 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 $C_{28}H_{24}O_{17}$ and $C_{21}H_{20}O_{13}$ 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°	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	$C_{68}H_{47}O_{44}$	Oenothein 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-Alpha-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]

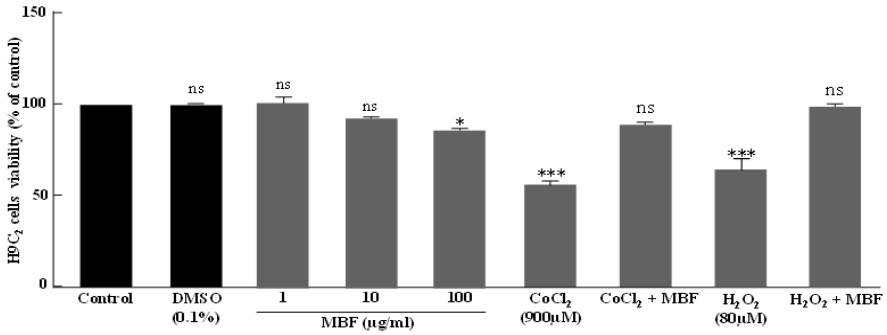

Figure 2: H9c2cell viability in the presence of different concentrations of Myrtus buthanolic fraction (MBF) and effect of MBF (1µg/ml) pretreatment on cell viability in $CoCl_2$ or H_2O_2-challenged H9c2 Cells. Control cells were incubated for 24h with medium alone; DMSO: cells were incubated for 24h with medium supplemented with 0.1% DMSO. H9c2 Cells at 2 x10^4 cells/mL were pretreated with three ranges of MBF concentrations (1, 10 and 100 mg mL^{-1}) for 3h, and then exposed to 900µM of $CoCl_2$ or 80µM of H_2O_2 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 ($C_{20}H_{19}O_5$: [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 *Baeckea 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 $C_{22}H_{22}O_{13}$. 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

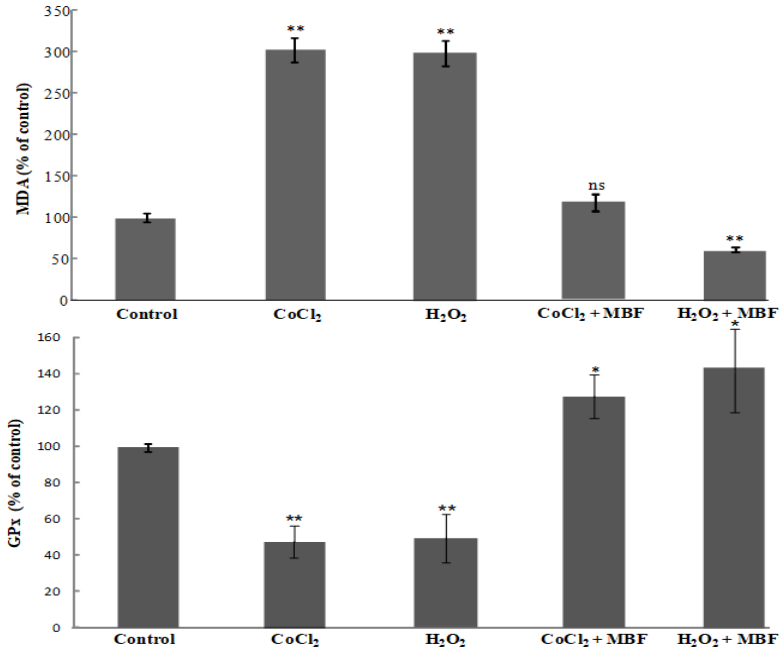

Figure 3: MDA and GPx levels in H9c2 cells stressed with $CoCl_2$ and H_2O_2. Control: cardiac cells were incubated for 24h with medium alone; $CoCl_2$ or H_2O_2 stress: cells were incubated for 24h with 900µM of $CoCl_2$ or 80µM of H_2O_2; $CoCl_2$/H_2O_2 + MBF: cells were pre-treated 3h with 1µg/mL of MBF before stressed with $CoCl_2$ (900µM) or H_2O_2 (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 ($C_{33}H_{35}O_{22}$: $[M-H]^-$ 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 $C_{38}H_{48}O_8$ 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 (EC_{50} = 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 donators and reducing agents [14]. For examples, ellagitannins like oenothein 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- (IC_{50} = 73.5 and IC_{50}=73.6 µM, respectively) and ABTS+ (IC_{50}=54.5 and 65.8 µM, respectively) [39].

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

In the absence of exogenous $CoCl_2$ or H_2O_2, 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

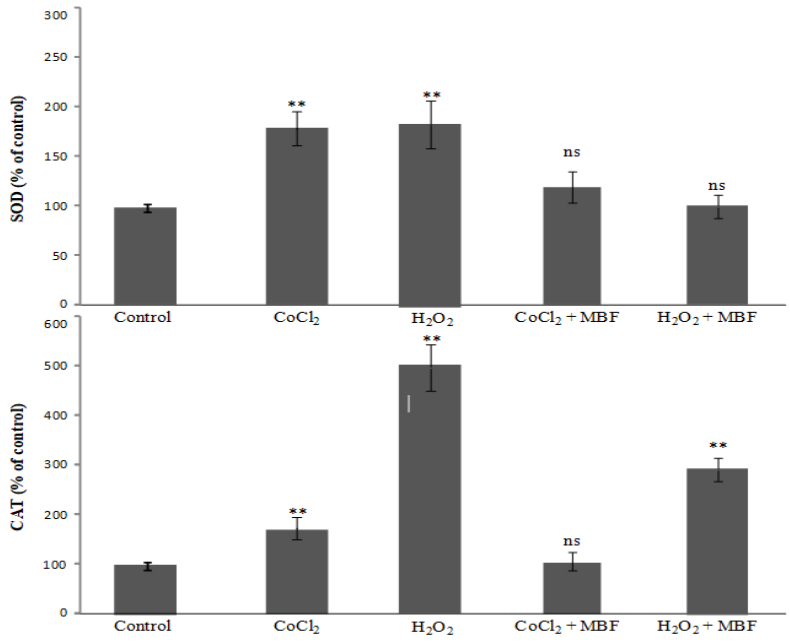

Figure 4: Effect of MBF on $CoCl_2$ or H_2O_2 induced SOD and CAT activities alteration. Cells were incubated for 24h with medium alone; $CoCl_2$: cells were incubated for 24h with 900µM of $CoCl_2$; H_2O_2: cells were incubated for 24h with 80µM of H_2O_2; $CoCl_2$ + MBF: cells were pre-incubated with 1µg/mL MBF then treated by $CoCl_2$ 900µM; H_2O_2 + MBF: cells were pre-incubated with 1µg/mL MBF then treated by H_2O_2 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 (IC_{50} values), β-carotene bleaching test (BCBT) and ferric reducing activity power (EC_{50} 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 H9c9 cells to hypoxia and oxidative stress

The present study demonstrated that 24h of $CoCl_2$ or H_2O_2 treatment, significantly decreased cell viability in H9c2 cells (58.65% and 65%, respectively). $CoCl_2$ and H_2O_2 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 $CoCl_2$ and H_2O_2-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, $CoCl_2$ 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, $CoCl_2$ 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 $CoCl_2$ and H_2O_2 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 $CoCl_2$ or to 80µM H_2O_2 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 $CoCl_2$ and H_2O_2; hence, ellagitanins 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 50μM 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, $CoCl_2$ and H_2O_2 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 H_2O_2 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 H_2O_2 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 $CoCl_2$-induced injury in H9c2 cells.

After 24h exposure of 900μM $CoCl_2$ or 80μM H_2O_2, 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 (1μg/mL). $CoCl_2$ or H_2O_2 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 $CoCl_2$ and or H_2O_2 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 $O_2^{-}\bullet$ increases in hypoxic condition [5], which leads to SOD activation. Thus, this enzyme transforms superoxide anion to H_2O_2, which can explain also the increase of catalase after 24h of $CoCl_2$ treatment. Indeed, H_2O_2 accumulates in response to hypoxia [48]. Accordingly, high H_2O_2 level is pro-oxidant, which leads to CAT antioxidant defense activation, CAT can transform H_2O_2 into oxygen and water [49]. As MBF was added during three hours before $CoCl_2$ its protection really reflects a preventive effect against the multifaceted oxidative stress induced by chemical hypoxia. The protective effect of MBF against $CoCl_2/H_2O_2$ -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.

Acknowledgment

This work was supported by the Tunisian Ministry of Higher Education and Scientific Research (LR19CBBC06).

References

1. Seta KA, Spicer Z, Yuan Y, Lu G, Millhorn DE. Responding to hypoxia: lessons from a model cell line. Sciences STKE. 2002; re11.

2. Cervellati F, Cervellati C, Romani A, Cremonini E, Sticozzi C, Belmonte G, et al. Hypoxia induces cell damage *via* oxidative stress in retinal epithelial cells. Free Radical Research. 2014; 48: 303-312.

3. Lakshmi SV, Padmaja G, Kuppusamy P, Kutala VK. Oxidative stress in Cardiovascular Disease. Indian Journal of Biochemistry and Biophysique. 2009; 46: 421-440.

4. Pialoux V, Mounier R. Hypoxia-induced oxidative stress in health disorders, Oxidative and Medicinal Cellular Longevity. 2012.

5. Turrens JF. Mitochondrial formation of reactive oxygen species. Journal of Physiology. 2003; 552: 335-344.

6. Baskaran R, Kalaiselvi P, Huang CY, Padma VV. Neferine, a

bisbenzylisoquinoline alkaloid, offers protection against cobalt chloride-mediated hypoxia-induced oxidative stress in muscle cells. Integrative and Medicinal Research. 2015; 4: 231-241.

7. Mattera R, Benvenuto M, Giganti MG, Tresoldi I, Pluchinotta FR, Bergante S, et al. Effects of polyphenols on oxidative stress-mediated injury in cardiomyocytes. Nutrients. 2017; 9: 523.

8. Bettaib J, Talarmin H, Zar Kalai F, Giroux-Metges M-A, Ksouri K. *Limoniastrum guyonianum* prevents H_2O_2-induced oxidative damage in IEC-6 cells by enhancing enzyamtic defense, reducing glutathione depletion and JNK phosphorylation. Biomedicine and Pharmacotherapy. 2017; 95: 1404-1411.

9. Pandley AK, Patnaik R, Muresanu DF, Sharma A, Sharma HS. Quercetin in hypoxia-induced oxidative stress: Novel target for neuroprotection. International Review of Neurobiology, chapter II: New Perspectives of Central Nervous System Injury and Neuroprotection edited by Hari Shanker Sharma, USA. 2012.

10. Sun GB, Qin M, Ye JX, Pan RL, Meng XB, Wang M. Inhibitory effects of myricitrin on oxidative stress-induced endothelial damage and early atherosclerosis in $ApoE^{-/-}$ mice. Toxicology and Applied Pharmacology. 2013; 271: 114-126.

11. Lin X, Lin C-H, Liu R, Li C, Jiao S, Yi X, et al. Myricetin against myocardial injury in rat heat stroke model. Biomed Pharmacother. 2020; 127: 110194.

12. Sumbul S, Aftab Ahmad M, Asif M, Akhta M. *Myrtus communis* Linn. A review. Indian Journal of Natural Product and Resources. 2011; 2: 395-402.

13. Dewanto V, Xianzhong W, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. Journal of Agricultural Food Chemistry. 2002; 50: 3010-3014.

14. Bourgou S, Bettaieb Rebey I, Mkadmini K, Isoda H, Ksouri R, Megdiche Ksouri W. LC-ESI-TOF-MS and GC-MS profiling of Artemisia herba-alba and evaluation of its bioactive properties. Food Research International. 2017; 99: 702-712.

15. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition and Dietetics. 1986; 44: 307-315.

16. Koleva II, van Beek TA, Linssen JPH, de Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis. 2002; 13: 8-17.

17. Long LH, Halliwell B. Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. Biochemichal and Biophysical Research Communication. 2009; 388: 700-704.

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976; 72: 248-254.

19. Kuthan H, Haussmann HJ, Werringloer J. A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions. Biochemistry Journal. 1986; 237: 175-180.

20. Aebi H. Catalase *in Vitro*, Methods. Enzymology. 1984; 105: 121-126.

21. Rotruck J, Pope A, Ganther H, Swanson A, Hafeman DG, Hoekstra W. Selenium: biochemical role as a component of glutathione peroxidase. Science. 1973; 179: 588-590.

22. Draper HH, Hadley M. Malondialdehyde determination as index of lipid Peroxidation. Methods in Enzymology. 1990; 186: 421-431.

23. Taamalli A, Iswaldi I, Arraez-Roman D, Segura-Carretero A, Fernandez-Gutierrez A, Zarrouk, M. UPLC-QTOF/MS for a rapid characterization of phenolic compounds from leaves of *Myrtus communis* L. Phytochemical Analysis. 2014; 25: 89-96.

24. D'Urso G, Montoro P, Lai C, Piacente S, Sarais G. LC-ESI/LTQOrbitrap/MS based metabolomics in analysis of *Myrtus communis* leaves from Sardinia (Italy). Industrial Crops and Products. 2019; 128: 354- 362.

25. Lee MH, Nishimoto S, Yang LL, Yen KY, Hatano T, Yoshida T, et al. Two macrocyclic hydrolysable tannin dimmers from Eugenia uniflora. Phytochemistry. 1997; 44: 1343-1349.

26. Yoshimura M, Amakura Y, Tokuhara M, Yoshida T. Polyphenolic compounds isolated from the leaves of *Myrtus communis*. Journal of National Medical Association. 2008; 62: 366-368.

27. Franco AM, Tocci N, Guella G, Dell'Agli M, Sangiovanni E, Perenzoni D, et al. Myrtle Seeds (*Myrtus communis* L.) as a Rich Source of the Bioactive Ellagitannins Oenothein B and Eugeniflorin D2. ACS Omega. 2019; 4: 15966-15974.

28. Díaz-de-Cerio E, Arráez-Román D, Segura-Carretero A, Ferranti P, Nicoletti R, Perrotta GM, et al. Establishment of pressurized-liquid extraction by response surface methodology approach coupled to HPLC-DAD-TOF-MS for the determination of phenolic compounds of myrtle leaves. Analytical and Bioanalytical Chemistry. 2018; 410: 3547-3557.

29. Abdel-Salam IM, Ashmawy AM, Hilal AM, Eldahshan OA, Ashour M. Chemical composition of aqueous ethanol extract of *Luffa cylindrica* leaves and its effect on representation of caspase-8, caspase-3, and the proliferation marker Ki67 in intrinsic molecular subtypes of breast cancer *in vitro*. Chemistry and Biodiversity. 2018; 15: e1800045.

30. Simon A, Chulia AJ, Kaouadji M, Allais DP, Delage C. Two flavonol 3-[triacetylarabinosyl(1→6)glucosides] from *Calluna vulgaris*. Phytochemistry. 1993; 33: 1237-1240.

31. Martin T, Rubio B, Villaescusa L, Fernandez L, Diaz AM. Polyphenolic compounds from pericarps of *Myrtus communis*. Pharmaceutical Biology. 1999; 37: 28-31.

32. Ji-Qin H, Heng Z, Jiang-Hong Y, Ling-Jun C, Hao W. New meroterpenoids and C-methylated flavonoid isolated from *Baeckea frutescens*. Chinese Journal of Natural Medicine. 2020; 18: 379-384.

33. Min BS, Cuong TD, Lee J-S, Shin B-S, Woo MH, Hung TM. Cholinesterase Inhibitors from *Cleistocalyx operculatus* Buds. Archives in Pharmaceutical Research. 2010; 33: 1665-1670.

34. Yamaguchi M-A, Oshida N, Nakayama M, Koshioka M, Yamaguchi Y, Ino I. Anthocyanidin 3-glucoside malonyltransferase from *Dahlia variabilis*. Phytochemistry. 1999; 52: 15-18.

35. Parejo I, Jauregui O, Sánchez-Rabaned F, Viladomat F, Bastida J, Codina C. Separation and Characterization of Phenolic Compounds in Fennel (*Foeniculum vulgare*) Using Liquid Chromatography-Negative Electrospray Ionization Tandem Mass Spectrometry. Journal of Agricultural and Food Chemistry. 2004; 52: 3679-3687.

36. Shaheen F, Ahmad M, Khan SN, Hussain SS, Anjum S, Tashkhodjaev B, et al. New α-Glucosidase Inhibitors and Antibacterial Compounds from *Myrtus communis* L. European Journal of Organic Chemistry. 2006; 2371-2377.

37. Kanoun K, Belyagoubi-Benhammou N, Ghembaza N, Atik Bekkara F. Comparative studies on antioxidant activities of extracts from the leaf, stem and berry of *Myrtus communis* L. International Food Research Journal. 2014; 21: 1957-1962.

38. Zhou D, Shao L, Spitz DR. Reactive oxygen species in normal and tumor stem cells, Adv. Cancer Research. 2014; 122: 1-67.

39. Chen Y, Wang J, Ou Y, Chen H, Xiao S, Liu G, et al. Cellular antioxidant activities of polyphenols isolated from Eucalyptus leaves (*Eucalyptus grandis* × *Eucalyptus urophylla* GL9). Journal of Functional Foods. 2014; 7: 737-745.

40. Wong WS, McLean EM. Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis in vitro. Toxicology. 1999; 139: 243-253.

41. Chen SL, Yang CT, Yang ZL, Guo RX, Meng JL, Cui Y, et al. Hydrogen sulphide protects H9c2 cells against chemical hypoxia-induced injury. Clin. Exp. Pharmacology and Physiology. 2010; 37: 316-321.

42. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Medicine and Cellular Longevity. 2014.

43. Tandon R, Khanna HD, Dorababu M, Goel RK. Oxidative stress and antioxidants status in peptic ulcer and gastric carcinoma. Indian Journal in Physiology and Pharmacology. 2004; 48: 115-118.

44. Bihamta M, Hosseini A, Ghorbani A, Boroushaki MT. Protective effect of pomegranate seed oil against H_2O_2 induced oxidative stress in cardiomyocytes. Avicenna Journal of Phytomedicine. 2017; 7: 46-53.

45. Zhu H, Luo P, Fu Y, Wang J, Dai J, Shao J, et al. Dihydromyricetin prevents cardiotoxicity and enhances anticancer activity induced by adriamycin. Oncotarget. 2015; 6: 3254-3267.

46. Pan TT, Feng ZN, Lee SW, Moore PK, Bian JS. Endogenous hydrogen sulfide contributes to the cardioprotection by metabolic inhibition preconditioning in the rat ventricular myocytes. Journal of Molecular and Cellular Cardiology. 2006; 40: 119-130.

47. Kaneto H, Katakami N, Matsuhisa M, Matsuoka TA. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. Mediators of Inflammation. 2010; 2010.

48. Vergara R, Parada F, Rubio S, Pérez FJ. Hypoxia induces H_2O_2 production and activates antioxidant defense system in grapevine buds through mediation of H_2O_2 and ethylene. Journal of Experimental Botany. 2012; 63: 4123-4131.

49. Walters CR, Cheng P, Pool E, Somerset V. Effect of temperature on oxidative stress parameters and enzyme activity in tissues of Cape River crab (*Potamanautes perlatus*) following exposure to silver nanoparticles (AgNP). Journal of Toxicology and Environmental Health. - Part A. 2016; 79: 61-70.

50. Rong Z, Pan R, Xu Y, Zhang C, Cao Y, Liu D. Hesperidin pretreatment protects hypoxia-ischemic brain injury in neonatal rat. Neurosciences. 2013; 255: 292-299.

51. Elavarasan J, Velusamy P, Ganesan T, Ramakrishnan SK, Rajasekaran D, Periandavan K. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. Journal of Pharmacognosy and Pharmacology. 2012; 64: 1472-1482.

52. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. Critical Revue of Food Sciences and Nutrition. 2005; 45: 287-306.