Effect of Myrtus Communis L. Extracts on Attenuation of Liver Normothermic Ischemia-Reperfusion Injury

Henda Ferchichi1, Issam Salouage1,2*, Sarra Bacha1, Dorra Ben Said1, Emna Gaies1,2, Mohamed Boussaid1, Mohamed Lakhal1 and Anis Klouz1,2

1Centre National de Pharmacovigilance – Tunis, Tunisia
2Unité d’Expérimentation Animale, Faculté de Médicine de Tunis, Tunisia
3Institut National des Sciences Appliquées et de Technologie INSAT, Tunisia

Abstract
The Common Myrtle (Myrtus communis L.) is rich in antioxidants, particularly in anthocyanin. It was recognized for its anti-inflammatory and anti-thrombotic effects.

The aim of our work is to evaluate the effect of Common Myrtle on a model of hepatic ischemia-reperfusion in Rat. Two morphs were chosen: white fruit Myrtle and black fruit Myrtle. Within each morph, fruit and leaf were separated and obtained extract were used to determine their effects on the hepatic model of ischemia-reperfusion.

Our work was conducted in three steps (1) Induction of hepatic ischemia (90 minutes) in Wistar Rat (2) injection of the Myrtle extract during 15 minutes before reperfusion (3) and reperfusion (2 hours).

To evaluate the effect of Myrtle on ischemia-reperfusion, we have monitored transaminases levels, Monoethylglycinexylidide (MEGX) concentrations (to assess the liver metabolic capacity) and Malondialdehyde (MDA) concentration.

The determination of total phenol extracts of Myrtle showed a significant difference between black fruit Myrtle (11.3 µg/ml), white fruit (27 µg/ml) and black fruit Myrtle leaves (94.3 µg/ml). The latter presented the highest antioxidant activity (86.54%).

With the extract from the white fruit of Myrtle, we noted a decrease of AST and ALT, respectively, 1321 U/I and 773 U/I compared with I/R group was 5757 U/I and 5404 U/I and an increase in the MEGX concentrations and decrease in MDA.

The testing of extracts of Myrtle in a model of hepatic ischemia showed a difference in the protective power against damage of ischemia-reperfusion, by origin and type of fruit (black or white).

Keywords: Myrtus communis L.; Hepatic ischemia-reperfusion injury; Oxidative stress; Monoethylglycinexylidide; Lipid peroxidation; Antioxidant effect

Introduction
Myrtle (Myrtus communis, Myrtaceae) is a Mediterranean shrub used as a culinary spice and as a folk medicine, for example, in the treatment of diabetes mellitus and as an antiseptic agent. In fact, myrtle extracts have been reported to possess antihyperglycemic [1-3], and antibacterial [4,5] properties. Recent reports have described antioxidant activities of different extracts of myrtle and certain ingredients thereof [6-8], implying potential as medicine for the treatment of diseases related to oxidative stress, such as warm ischemia-reperfusion (I/R).

Ischemia is a state of tissue oxygen deprivation accompanied by a reduced washout of the resulting metabolites [9]. Reperfusion is the restoration of blood flow to the ischemic tissue. Despite the unequivocal benefit of reperfusion of blood to an ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue [10].

Deprivation of oxygen to the liver during ischemia induces severe damage; however, much more damaging reactive oxygen species (ROS) are generated during the reperfusion period (Parks and Granger, 1988). The organ dysfunction that accompanies this condition is generally associated with increased microvascular permeability, interstitial edema, impaired vasoregulation, inflammatory cell infiltration and parenchymal cell dysfunction and necrosis [11,12]. Thus, surgery is well designed to prevent and manage the tissue injury associated with reperfusion.

Free radical ablation for the treatment of reperfusion injury found its first clinical application in the prevention of postsischemic tissue injury after organ transplantation [13,14].

Thus, free radical scavengers and antioxidant agents are thought to be useful in the clinical setting of hepatic I/R damage (Kupiec-Weglinski et al. 2005).

The aim of this work is to evaluate the effect of Myrtle extracts on hepatic ischemia-reperfusion in a rat model. In fact, two morphs were chosen: white and black fruit Myrtle. For each one, fruit and leaf were separated to determine their effects.

Material and Methods
Chemicals
All solvents used in this investigation were analytical grade: Folin

Keywords: Myrtus communis L.; Hepatic ischemia-reperfusion injury; Oxidative stress; Monoethylglycinexylidide; Lipid peroxidation; Antioxidant effect
and Ciocalteu’s phenol agent: 2N, d = 1.27 (SIGMA – F9252). Methanol, acetic acid, ethyl ether and hydrogen chloride (CHEMLAB).

Reagents used for antioxidant activity assessment were also of analytical grade: 2-thiobarbituric acid (TBA; Merck), trichloroacetic acid (TCA; Prolabo), FeCl₃ (Riedel-De Haen), FeCl₂·4H₂O (Merck) and 2,2-diphenyl-2-picrylhydrazyl free radical (DPPH) 60 μM (SIGMA).

Standard solutions of Quercetin, Catechin, Kampferol and Rutin were prepared as 0.05% solutions in methanol (SIGMA).

**Plant material and extraction procedure**

Two morphs were chosen: white fruit Myrtle and black fruit Myrtle.

Common Myrtle has been collected in August 2007 at the locality Djebel Bargou (North West of Tunisia). The fresh plants were sorted out and dried in the drying room with active ventilation at ambient temperature. Identification was confirmed by Prof. Mohamed Boussaid (Botanic Biotechnology Department of National Institute of Applied and Technology of Tunis: INSAT)

Plant material was reduced to a fine powder and was mixed with 40 ml of saline solution (NaCl 9%). The mixture undergoes maceration for 5 days in the refrigerator (4°C). Then it was centrifuged at 4500 rpm for 10 min.

The extracts were sterilized through micro filters. The samples were stored in sterile tubes at 4°C.

We obtained four extracts from black fruit of Myrtle (E I), leaves of Myrtle Black fruit (E II), white fruit of Myrtle (E III) and leaves of Myrtle White fruit (E IV).

**Determination of total phenolic content**

Total phenolic content was estimated by the Folin–Ciocalteu (FC) colorimetric method.

Briefly, 50 il of the plant extract was diluted 10-fold with NaCl 9% and mixed with 750 il of FC reagent, previously diluted 10-fold with distilled water. The mixture was incubated at obscurity for 5 min at 37°C. 750 il of Na₂CO₃ (60 g/l) solution was added to the mixture. After 90 min, the absorbance was measured at 725 nm. A range standard was prepared in the same conditions as the samples using different concentrations of alpha tocopherol (10 to 100 mg/ml).

**Thin-layer chromatography (TLC)**

Five grams of plant was refluxed with 100 ml methanol for 24h then filtered and 2 ml of HCl (2N) was added to the filtrate, which was then heated at 100°C for 1h until complete hydrolysis.

Acid hydrolysis of methanol extract was conducted by adding an equal volume of ethyl ether. The supernatant was concentrated under heated at 100°C for 1h until complete hydrolysis.

The supernatant was taken up in methanol. Equal volume of ethyllic ether. The supernatant was concentrated under reduced pressure and then the residue was taken up in methanol.

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We used Thin-layer chromatography (TLC) for flavonoid and phenolic separation.

Chromatography was carried out on silica gel using acetic acid, HCl, water (30: 3: 10/v:v:v) as the developing solvent during 5h.

**2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay**

Due to its unpaired electron, DPPH radical gives a strong absorption band at 517 nm (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting discoloration is stoichiometric with respect to the number of electrons taken up. This test consists in mixing 25 µl of the extract (diluted 10-fold with NaCl 9%) with 1 ml of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) to a final concentration 0.04 mM. Mixtures were vigorously shaken and let for 30 min at obscurity. The control corresponds to 1 ml of DPPH (0.5 mM) that was diluted in 4 ml of NaCl 9%. The antioxidant activity was expressed in percentage of the inactivated DPPH radical according to the following formula [15]:

\[
\text{DPPH radical inactivation} = 100 \times \left( \frac{A_0 - A_p}{A_0} \right)
\]

Where:
- \(A_0\): absorbance of the sample at 517 nm (containing all reagents except the test compound).
- \(A_p\): absorbance of the control at 517 nm.

**Animals**

Adult male Wistar rats, weighing 250 to 350 g were provided by Tunisian Pasteur Institute and then fed in the unit of animal experimentation of the Faculty of Medicine of Tunis. All animal procedures used in this study are in strict accordance with the European Community Council Directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

Animals were subdivided in six groups (n=5 for each one): Sham (not treated group), I/R (normothermic ischemia-reperfusion), BMF (injection of black myrtle fruit extract), BML (injection of black myrtle leaves extract), WMF (injection of white myrtle fruit extract) and WML (injection of white myrtle leaves extract).

**Experimental protocol and hepatic ischemia**

For normothermic liver ischemia, the technique described by Nauta et al. [16].

The surgical procedure was performed under general Anesthesia (Urethane). After section of the ligaments of the liver, hepatic normothermic ischemia was induced for 90 min by hilum clamping of the hepatic pedicles of segments I to V. In order to preclude vascular losses due to portal stasis.

After 120 min reperfusion, animals were killed, and the liver lobes suffering ischemic injury were immediately removed and a blood sample was collected.

**Biochemical assay**

Blood samples were collected for the measurement of liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), in order to evaluate hepatic injury.

Samples were centrifuged, then serum was separated and measurements were made by an automated analyzer (COBAS Integra 800) using the enzymatic technique EMIT (enzyme multiplied immunoassay technique).

Results were expressed in percentage (%) of protection of AST and ALT using the following equation:

\[
\% \text{ Protection AST} = 100 \times \left( \frac{\text{AST}_{\text{Treated}} - \text{AST}_{\text{I/R}}}{\text{AST}_{\text{Treated}} - \text{AST}_{\text{Sham}}} \right)
\]
Liperoxidation test

A portion of liver (5g) was used to quantify malondialdehyde (MDA). Liver homogenate malondialdehyde (MDA) concentration was measured using the thiobarbituric acid (TBA) method. The amount of lipid peroxides (LPO) was measured as the reduction of MDA, which is in combination with TBA.

Briefly, mitochondrial liver (1.25 mg/ml) [18] was incubated during 10 min at 37°C. Lipid peroxidation was initiated by addition of 100 µl of solution of FeCl₂ / FeCl₃ (500/1500µM) and incubated at 37°C for 30 min. Trichloroacetic acid (3% TCA) was added to stop the peroxidation reaction, and centrifuged at 3000 g for 15 min at 20°C.

The supernatant was added to TBA 1%, followed by incubation during 30 min at 95°C. The samples were cooled, and their absorbencies were measured by spectrophotometric method at 530 nm using NaCl 0.9% and TBA (v/v) as an external standard.

\[
\% \text{ liperoxidation inhibition} = \frac{([\text{MDA}]_{\text{sham}} - [\text{MDA}]_{\text{I/R}})}{[\text{MDA}]_{\text{sham}}} \times 100
\]

Evaluation of liver metabolic capacity: Lidocaïne test

Two portions of 2.5 g of liver were used for MEGX test: a test based on the evaluation of liver metabolic capacity by the determination of Lidocaïne and MEGX concentrations. In fact, MEGX is a major metabolite of Lidocaïne which is exclusively metabolized in the liver by the cytochrome P450 [18].

Same equation was used to determine the percentage of protection of ALT and MEGX.

Statistical analysis

All values are given as means ± S.E.M. Statistical comparisons were made by using ANOVA test. A p value < 0.05 was considered statistically significant.

Results

Polyphenol

The total phenols determined by the Folin-Ciocalteu method for the four aqueous extracts of Myrtle (Extracts: EI, EII, EIII and EIV) varied from 103.2 µg/ml for the leaves of Myrtle Black fruit (EII) to 11.3 µg/ml for black fruit of Myrtle (EI).

The total phenolic compound contents in the plant extracts are shown in table 1.

TLC separation

TLC (methanolic extracts of Myrtle) indicated the presence of 7 compounds for black fruits of Myrtle, 8 compounds for leaves of Myrtle white fruit and Myrtle black fruit and for white fruit of myrtle.

We identified four compounds: Quercitrin (RF = 0.47), Catechin (RF = 0.6), Kampferol (RF = 0.68) and Rutin (RF = 0.78).

Only samples of leaves from both morph (EII and EIV) contained Kampferol and Rutin exist in all extracts (EI, EII, EIII and EIV). (Figure 1)

DPPH

In order to evaluate the antioxidant capacity of the samples, a method based on the reduction of DPPH was performed. DPPH radical is one of the few stable organic nitrogen free radicals, which has been widely used to determine the free radical-scavenging ability of various samples. Results of DPPH reduction by the 4 extracts are shown in table 1.

Quantitative analysis revealed that the leaves of Myrtle (black and white fruit) (EI and EIV) extracts showed the strongest DPPH radical scavenging property (86.5% and 90.7% respectively). The other extracts (EII and EIII) present a low antioxidant activity (35.1% and 21.2% respectively).

Liperoxidation test

The MDA content as an index of lipid peroxidation was significantly higher after reperfusion in I/R group (6.4 ± 1.1 µM) than in sham group (1.4 ± 0.4 µM).

For BML group, MDA decreased significantly 2.6 ± 0.6 µM (p < 0.05) compared with I/R group.

Serum transaminases levels

The ALT and AST serum levels of the sham group were 153 ± 27 U/l and 74 ± 18 U/l respectively. Serum ALT and AST levels increased significantly after reperfusion in I/R group compared with sham group (5757 ± 383 U/l and 5404 ± 1453 U/l respectively).

Pretreatment with Myrtle significantly decreased AST and ALT levels compared with I/R group.

With BML group, we noted a significant decrease of AST and ALT, respectively, 1537 ± 197 U/l and 1007 ± 182 U/l compared with the I/R group (p=0.03<0.05).

For WML group, Rats died few minutes after injection of the extract (Table 2).

Monitoring of MEGX formed

This test evaluates the metabolic capacity of liver. The concentration of the formed MEGX reached 660 ± 44 µM in the sham group and decreased significantly to 81 ± 13 µM for the I/R group.

This concentration increased, after injection of myrtle extracts, to reach values of 222 ± 48 µM, 177 ± 32 µM and 275 ± 39 µM for BMF, BML and WMF groups respectively.

The differences between treated groups and I/R group are shown in Table 2.

| Sample | ALT (U/l) | AST (U/l) |
|--------|-----------|-----------|
| Sham   | 153 ± 27  | 74 ± 18   |
| I/R    | 5757 ± 383| 5404 ± 1453|
| BML    | 1537 ± 197| 1007 ± 182|
| WML    | 1007 ± 182| 1007 ± 182|

Table 1: Quantitative analysis of liver peroxidation. (MDA).

Key to the spots: Ca – catechin, Q – quercetin, Kf – kampferol, Ru – rutin

Figure 1: Schematic TLC separation on silica gel, with acetic acid, HCl, water (30: 3: 10v: v: v) as the mobile phase, of flavonoids and phenolic acids (1-9) in leaves and fruits of Commun Myrtle.
significant with probabilities lower than 0.05. (Table 2).

For WML group, Rats died few minutes after injection of the extract.

Discussion

Many vegetal extracts show an important antioxidant activity that is linked to the presence of phenols compounds. The antioxidant activity of Myrtle (Myrtus communis L.) extract from different materials has been studied in animal models, so this activity depends on phenolic compounds concentrations and their natures in plant extracts [8].

Antioxidant activity of Myrtle

In the first part of this study, we focused our attention on the identification (Thin-layer chromatography) and the quantification (total phenols concentration) of the Myrtle phenols and testing their antioxidant activity (DPPH).

The extracts of leaves of Myrtle (Black and White) showed a higher polyphenol concentration and may be rich sources of antioxidants compounds. Meeker et al. found that the activity of polyphenolic compounds is indeed conditioned by their chemical composition [19].

The difference in the composition in polyphenols may explain the difference in the antioxidant action (DPPH inhibition), obtained by each Myrtle extract (fruit and leaves of the white and black morph).

Previous studies have shown that phenols have antioxidant activities, which contribute to the explanation of the protective effect of plants against some diseases [20].

The capacity of extracts of Myrtle to scavenger free radicals can be attributed to flavonoids and phenolic acids [8]. The variation of the antioxidant activity of Myrtle extracts according to the morphs (black or white) and according to organs (fruits or leaves), can be explained by a difference in the flavonoids composition revealed by TLC in one hand, and by a difference in total phenols content on the other hand. Thus, the most important quantity of total phenols was detected in extract of leaves of Myrtle Black fruit.

Liver ischemia-reperfusion

The second part of this study, we aimed to find solutions to protect liver against lesions induced by I/R, specially the oxidant generation using vegetal extracts.

I/R injury of the liver is a major complication of liver resection, hepatic trauma, and liver transplantation. During the last two decades, a significant progress has been made in understanding liver ischemia-reperfusion injury and in the development of strategies to avoid or reduce this injury in clinical practice. [21].

Reactive oxygen species have been implicated in the pathogenesis of ischemia-reperfusion injury.

The oxidative stress during reperfusion after hepatic ischemia has been reported by assessing MDA [22].

Liver Malondialdehyde levels, a marker of oxidative stress, permitted us to obtain a quantitative estimation of the membrane lipid oxidative damage (Esterbauer et al. 1990).

In the present study, I/R caused significant increases in the hepatic malondialdehyde levels, end products of lipid peroxidation. This observation is in agreement with the previous studies, where elevated levels of lipid peroxidation products were increased from 40% to 80 above basal values [23-25]. Furthermore, our results demonstrated that leaves of Myrtle Black fruit treatment can inhibit the production of oxygen radicals and prevent them from injury to liver tissue.

A major target site of lipid peroxidation damage is the cellular membrane because it contains polyunsaturated fatty acids. Toxicity of partially reduced oxygen species arises from the peroxidation of polyunsaturated fatty acid of membrane phospholipids, which may cause membrane disintegration. In liver ischemia-reperfusion injury, this mechanism is exacerbated. The consequent damage to liver cells by reperfusion results in mitochondrial dysfunction and it can also induce enzymes release.

Devinder et al. [26] showed, in their study, that oxidative stress induced by I/R was associated with enzymatic liver increase in plasma (transaminase).

Transaminase levels (AST and ALT) are considered as excellent markers of hepatocellular injury and a good indicator of structural membrane damage [27]. Thus, this assay allows us to test the ability of these plant extracts to protect the liver against the deleterious effects of ischemia [28].

| Table 1: | Dosage of total phenols and % of DPPH inhibition of each Myrtle extracts. |
|----------|--------------------------------------------------------------------------------|
| Biochemical parameter | Total phenols (µg/ml) | % of DPPH inhibition |
| Sham | 215 ± 27 | 274 ± 18 | 660 ± 44 | 1.4 ± 0.4 |
| I/R | 5757 ± 383 | 5404 ± 1453 | 81 ± 13 | 6.4 ± 1.1 |
| BMF | 4610 ± 237 | 4584 ± 318 | 222 ± 48 | 5.5 ± 1.4 |
| BML | 1537 ± 197 | 1007 ± 182 | 177 ± 32 | 2.6 ± 0.6 |
| WMF | 1321 ± 610 | 773 ± 558 | 275 ± 39 | 5.2 ± 1 |
| WML | - | - | - | - |

Values are expressed as mean ± standard deviation of five rats at p < 0.05. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Monooctylglycine xylidide (MEGX) and malondialdehyde (MDA). *Significantly different from sham versus I/R group at p < 0.05. #Significantly different from I/R versus treated groups at p < 0.05. Sham (non treated group), I/R (normothemic ischemia-reperfusion), BMF group (injection of black myrtle fruit extract), BML group (injection of black myrtle leaves extract), WMF group (injection of white myrtle fruit extract) and WML group (injection of white myrtle leaves extract).

| Table 2: | Effect of each extracts of Commun Myrtle on the biochemical parameters in rat ischemia-reperfusion liver. |
|----------|--------------------------------------------------------------------------------|
| Biochemical parameter | % of AST protection | % of ALT protection | % of MEGX protection | % of inhibition of liperoxidation |
| BMF | 21 ± 4.2 | 15 ± 6 | 24 ± 8 | 19 ± 0.3 |
| BML | 75 ± 4 | 83 ± 5.4 | 17 ± 6 | 77 ± 1.8 |
| WMF | 79 ± 11 | 87 ± 11 | 34 ± 7 | 24 ± 1.2 |
| WML | - | - | - | - |

Values are expressed as mean ± standard deviation of five rats at p < 0.05. Aminotransferase (ALT), Aminotransferase (AST), Monooctylglycine xylidide (MEGX) and Malondialdehyde (MDA).

| Table 3: | Effect of each myrtle extract on AST and ALT blood levels, on liver metabolic function (MEGX test) and lipoperoxidation in different groups of rats. |
|----------|--------------------------------------------------------------------------------|
| Biochemical parameter | % of AST protection | % of ALT protection | % of MEGX protection | % of inhibition of liperoxidation |
| BMF group (injection of black myrtle fruit extract), BML group (injection of black myrtle leaves extract), WMF group (injection of white myrtle fruit extract) and WML group (injection of white myrtle leaves extract).
The heterogenic phenols composition can explain the difference of anti ischemic effects obtained by every aequous extracts of Myrtle.

The white Myrtle fruit extract presents the most important protection transaminase rate although low ployphenols quantity and low antioxidant effect.

According to the literature, the rate of liver protection against reperfusion period depends of the relationship with his protective antioxidant capacity [29]. This leads us to believe that there is other compound, other that phenols, which can be responsible for anti-ischemic action, present in the Myrtle extract.

The reduction of transaminase rate is the lowest observed in the extract of fruits of the black Myrtle (Table 3).

Myrtle extracts improved metabolic function which was evaluated by liver capacity to transform Lidocaine into MEGX and other metabolites. This test has been used in several studies as a quantitative index of hepatic functions [30]. It has been reported that the hepatic metabolism of Lidocaine to Monoethylglycinexilidide (MEGX) is the basis of dynamic tests of liver function and that it is more discriminatory and recognizes early damage to the liver than conventional individual liver function tests and it shows a good correlation with severity of liver injuries [31-33].

Among all plant extracts used, we obtained a best protection of MEGX rate formation for the white fruit of Myrtle extract (Table 3).

In most cases, the injection of extract of leaves Myrtle white fruit causes cardiac arrest few minutes after injection. In fact, the Myrtle has a proved hypotensive effect [34,35].

Otherwise, flavonoids have attracted great attention in the prevention and treatment of hypertensive disease and it can reduce the risk of artery disease [36,37].

Indeed, catechin has anti-hypertensive proprieties [38]. According to results by TLC, only samples of leaves from both morph (EI and EIV) contained Catechin. We suppose that catechin concentration is very important for the EIV extract.

In summary, in the present study, we found that hepatic I/R caused a significant increase in the serum levels of AST and ALT which reflected the degree of liver injury in association with increased MDA contents and decrease MEGX concentrations in liver tissues. Pretreatment with Myrtle extracts (EI, EII and EIII) could reduce MDA contents and elevate MEGX concentrations in liver tissues, which are associated with the attenuation of liver injury.

But we noted a difference in the protective power against damage of ischemia-reperfusion, by origin and type of fruit (black or white).

The results confirmed that leaves of Myrtle Black fruit has an important protective effects against I/R injury, which may be partly due to its antioxidative effects.

Also, the addition of the white fruit of Myrtle extract permitted us to avoid deleterious effects of ischemia-reperfusion despite this low antioxidant activity.

It possible to use leaves Myrtle white fruit for therapeutic application of the hypotensive effect.

Chemical elucidation of the extracts contents should be the main objective of further investigations. This should lead to the structural identification of the active metabolites and the determination of their antioxidant profile in pure form.

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