Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(6): 254-258
Received: 05-09-2016
Accepted: 06-10-2016

Etou Ossibi AW
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Morabandza CJ
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Nsonde Ntandou GF
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Tsala D
Animal Physiology Laboratory, Faculty of Sciences, Yaoundé 1 University, P.Box 812, Yaoundé, Cameroun

Elion Itou RDG
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Ouamba JM
Vegetable chemical and life Unity, Sciences and Technical Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Abena AA
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Correspondence
Abena AA
Biochemistry and Pharmacology Laboratory, Health Sciences Faculty, Marien Ngouabi University, P. Box 69, Brazzaville-Congo

Anti-oxidative stress potentiality of aqueous extract of the leaves of *Lippia multiflora* Moldenke (Verbenaceae)

Etou Ossibi AW, Morabandza CJ, Nsonde Ntandou GF, Tsala D, Elion Itou RDG, Ouamba JM and Abena AA

Abstract
Oxidative stress is currently accused in the release and the progression of several diseases. In the present study, the effect of aqueous extract of the leaves of *Lippia multiflora* Moldenke on oxidative stress induced by the DOCA-salt and hydrogen peroxide was studied respectively in rat and mouse. The results obtained show that aqueous extract reduced diminution of catalase activity, GSH and NO contain and augmentation of MDA in rats DOCA-salt treated. The extract also significantly reduce (p<0,001) the concentration of hydrogen peroxide in rat leukocyte cells. These results suggest that aqueous extract of the leaves of *L. multiflora* Mold. have antioxidant potentiality which could contribute to the prevention or the treatment of several diseases.

Keywords: Anti-oxidative stress, *Lippia multiflora* Moldenke, aqueous extract

Introduction
Lippia multiflora Moldenke (*L. multiflora*) is specie belong to the family of Verbenaceae. It is an African plants widely used to treat several pathologies (Ake Assi and Abeye, 1983; Bouquet, 1969; Pascual, 2001) [2, 7, 1] and, abundantly consumed in decoction or aqueous infusion in the form of tea by populations. Former studies revealed that aqueous extract of the leaves is well tolerated by rat and had many pharmacological properties among which those against DOCA-salt induced arterial hypertension (Noamesi, 1985; Pham Huu Chanh, 1988; Abena, 2001; Hondi - Assah, 2003; Etou Ossibi *et al*., 2005; Etou Ossibi, 2012) [20, 23, 2, 18, 9, 10]. Phytochemical studies of aqueous extract of leaves, revealed the presence of the phenolic compounds (Etou Ossibi, 2005) [9]. Many other studies have revealed that phenolic compounds are considered as antioxidant and had capacity to neutralized free radicals responsible of oxidative stress (Akinmoladum and al, 2007; Aruoma, 2003) [3, 4]. Thus, the present study was initiated to evaluate the anti-oxidative stress potentiality of the aqueous extract of the leaves of *L. multiflora* Moldenke.

Materials and Methods
Plant material
The leaves of *L. multiflora* collected in April 2008 in experimental champ of Sciences and Technical Faculty of Marien Ngouabi University (Congo) were used. The specimen was authenticated and compared with the reference specimen n° 2047 of herbarium of National Research in Exacts and Naturals Sciences Institute (IRSEN).

Animals
Female's rats of six weeks, weighing between 100 and 120 g and albinos swiss mice weighing, between 20 and 25 g of Faculty of Sciences of Yaounde I University (Cameroun) were used.
Animals were high under standards conditions (12 H of light/12 H darkness) with free access to salted food (1 g NaCl/100 g of food) and to salted water (NaCl 2%). The control group was subjected to the salt-free diet.

Preparation of aqueous extract
The collected leaves were previously wash and air dried at a temperature of 25° ± 1 °C during 7 days in the laboratory and grounded into powder, using a mortar. 100 g of powder was subjected to decoction in 1000 ml of distilled water during 30 minutes. After cooling, the decoction was filtered and the filtrate was evaporated using a rotavapor type Heidolph and freeze-dried using lyophilisator type Alpha1-CHRIST 2 LD. 12.91 g. The lyophilized form was obtained with a yield of 12.91%.

Effects of the aqueous extract of leaves of *L. multiflora* on some parameters of oxidative stress induced by DOCA-salt

A first group of 5 rats had received simple water *ad libitum* and distilled water (0.5 ml/100 g/day's p.o) during nine weeks. 20 other rats were subjected to salted water *ad libitum* (NaCl 2%) and received DOCA (10 mg/kg, s.c) at the dose of 0.1 ml/100 g of body weight of rat, twice per week during six weeks (Terris, 1976; Badyal, 2003) [25, 5]. At the end, the rats were separated into 4 groups of 5 rats each one and, treated during three additional weeks as follows: DOCA (10 mg/kg, s.c) and distilled water (0,5 ml/100 g/day, p.o), DOCA (10 mg/kg, s.c) and furosemide (5 mg/kg/day, p.o) and DOCA (10 mg/kg, s.c) and *L. multiflora* Moldenke at the dose of 100 and 300 mg/kg/day, p.o. The DOCA (10 mg/kg, s.c) was managed twice per week.

Preparation of homogenates

At the end of treatment, the rats anaesthetized with urethan 15% at the dose of 1 ml/100 g were sacrificed and their heart, aorta, liver and kidneys collected. The taken bodies were weighed then crushed in mortars. The buffer solutions of Tris-HCl 50 mM for liver and kidney, Mc Ewen for aorta and heart were added in the mortars in order to have homogenates at 20%. These homogenates were centrifuged with 6000 turns during 40 min cold (5 °C). The supernatant collected was aliquoted and preserved at the freezer for the determination of some parameters of the oxidative stress.

Study of anti-oxidative stress activity

Concentration of catalase (Sinha, 1972) [24]

50 µl of homogenate and 750 µl of plug phosphates (0.1 M, pH 7.5) were introduced into tests tubes. The chronometer is then started after adds of 200 µl (50 mm) of peroxide hydrogen. After one minute, the reaction is stopped by addition of 2 ml of acetic dichromate/acid solution. The control tube contains 50 µl homogenate and 800 µl of plug phosphates (0.1 M, pH 7.5). Each tube is heated at 100 °C during 10 min. After cooling, the optical density is read using spectrophotometer (standard Genesys 20) at 570 nm. For each tube the quantity of hydrogen peroxide remaining in the solution after addition of the perchromic acid was evaluated by using the calibration curve. The catalase specific activity is expressed in µM H_2O_2/ protein min/mg.

Concentration of reduced glutathione (GSH) (Ellman, 1959) [13]

1 ml of homogenate is precipitated with 1 ml of sulfosalycilic acid. Thus the samples prepared are preserved at – 4 °C during one hour and centrifuged at 1200 turns during 15 min at 4 °C.

2.7 ml of buffer phosphate (0.1 M, pH 7.4) is added with 0.1 ml of supernatant. Developed yellow coulours is immediately read using spectrophotometer (standard Genesys 20) at 412 nm. The concentration of reduced glutathione is calculated using the molar coefficient of extinction ε = 13600/mole.Cm.

Concentration of nitric oxide (NO)

A seriated dilution of the $NaNO_2$ solution is made in 13 test tubes; the bigger concentration being 1 mM. Dilutions are carried out with the half. For the first tube, to 100 $NaNO_2$ µl is added 100 µl of reagent of GRIESS. The unit is homogenized by using the vortex and 100 µl taken for tube 2. The optical density is read at 570 nm after 10 min. Into each of the twelve other tubes are introduced initially 100 µl of distilled water. The dilution of $NaNO_2$ is then made in the following way; to the contents of tube 2 is added 100 µl of taken solution in tube 1, the unit is homogenized with the vortex and 100 µl of this mixture is taken and added in tube 3 and so to the tube 13. For last tube 13, 100 µl of the mixture is taken and thrown. To the contents of tubes 2 to 13 is then added 100 µl of reagent of GRIESS and the absorbance of each tube is read after 10 min at 570 nm. This first series of tubes makes it possible to establish the calibration curve. The evaluation of the quantity of NO in various samples was made in the following way: into each test tube is introduced 100 µl of homogenate of bodies and 100 µl of reagent of GRIESS. After homogenization of the mixture, the optical density of each tube is given by using spectrophotometer (standard Genesys 20) after 10 min at 570 nm.

Concentration of Malondialdéhyde (MDA) (Wilbur and al, 1949) [27]

2 ml of homogenate is introduced into tests tubes and in control tube, 1 ml of buffer Tris-HCl 50 mM, KCl 150 mM, pH 7.4. In each tube, are added 1 ml of trichloroacetic acid (TCA) 20% and 2 ml of thiobarbituric acid (TBA) 0.67%. The tubes are stopped using the balls of glass and are incubated during 10 min at 90 °C with the Marie bath. They are then cooled with water and centrifuged at 3000 tours/minute during 15 min at ambient temperature. The supernatant is elutriated and the D.O is read at 530 nm in rapport of negative control. After D.O reading, the malondialdehyde concentration is calculated using the molar extinction coefficient of ε = 1.56 × 105 Cm²/mM.

Effect of the aqueous extract of *L. multiflora* on the H_2O_2 concentration in leukocytes of mouse

The method describes by Etou Ossibi, (2010) [11] was used. After anesthesia with halothane and, sacrifice of the mice using the guillotine to recover a great possible quantity of blood in eppendorf tube containing two to three heparin drops. Six groups of five tubes each one containing a recovered blood of the five mice were used:1 group negative control tubes being used to evaluate the basic intracellular hydrogen peroxide concentration; 1 group control positive tubes to which was added hydrogen peroxide ; 3 batches of tubes treated with 5 µl of aqueous extract of *L. multiflora* with the concentrations of 5, 10 and 20 mg/ml and 1 group of tube treated with the chlorogenic acid at the dose of 0.05 mg/ml. 100 µl of heparinized blood were introduced in each tube to which were added 2 ml of a lyses diluted solution to the tenth. H_2O_2 concentration was measured on the total lymphocytes, polynuclear and monocytes by flow Cytometry at 525 nm. The event number is fixed at 10000 cells within a homogeneous leucocytes population.

Statistical analysis

Results were analyzed using the software Graph Pad in Stat version 3.0 and were expressed on mean ± ESM. Analysis of variance (ANOVA) was made for the comparison of groups. The post test of Dunnett was applied to determine the difference between groups; ANOVA test was significant at least with the threshold $p<0.05$.

Results

Effects on some parameters of the oxidative stress

Effects on catalase activity

Figure 1 shows a significant reduction of catalase activity in aorta, heart, liver and kidneys of treated rat with DOCA-salt

and distilled water respectively at 68.81 ($p<0.01$); 65.16 ($p<0.01$); 69.95 ($p<0.01$) and 82.35% ($p<0.01$) compared to the control group (distilled water). However, in the treated rats with DOCA-salt and furosemide or DOCA-salt and aqueous extract of *L. multiflora*, catalase activity increased significantly to the higher values than those of the treated rats with the DOCA-salt and distilled water. Level increased significantly ($p<0{,}001$) at dose of 100 mg/kg is 80.54; 87.93 and of 89.57% respectively at heart, liver and kidneys.

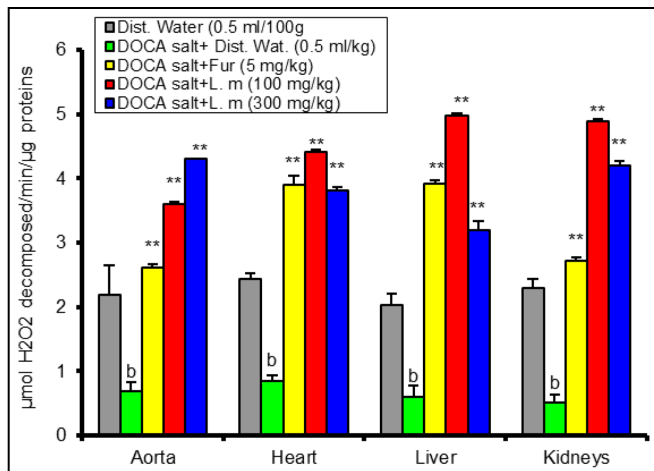

Fig 1: Effect of aqueous extract of *L. multiflora* (L. m) and furosemide (Fur.) on catalase activity from DOCA-salt treated rat. Values are mean ± SEM of n = 5. $^{b}p < 0.01$ vs respective control group and $^{**}p < 0.01$ vs respective DOCA-salt plus water distilled group.

Effects on reduced glutathione level

DOCA-salt causes a reduction in the reduced glutathione (GSH) level in heart and kidneys respectively at 5.84 ± 0.75 ($p<0.05$) and 2.42 ± 0.88 mol/l ($p<0.05$) against 7.46 ± 0.70 and 4.53 ± 0.47 mol/l in the control group (figure 3). In heart, kidneys and liver; aqueous extract of *L. multiflora* at the doses of 100 and 300 mg/kg is opposed significantly to this reduction. At 300 mg/kg, percentage augmentation of in heart, kidneys and liver is respectively 51.93 ($p<0.01$); 36.87 ($p<0.05$) and of 75.43% ($p<0.001$). In aorta, the variations of GSH level are not significant in all groups of rats (figure 2).

Effects on nitric oxide level

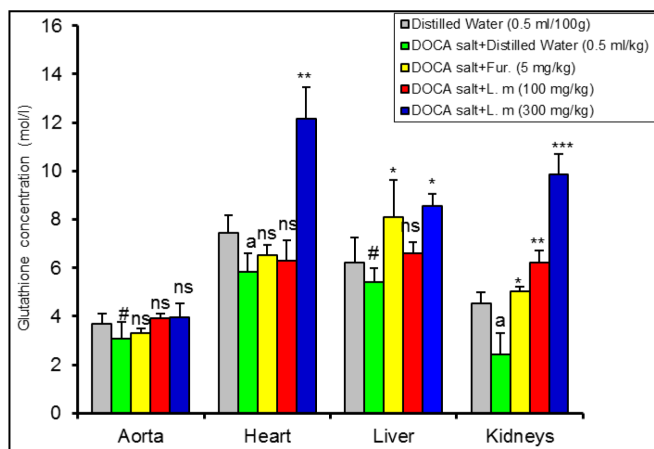

Fig 2: Effect of the aqueous extract of *L. multiflora* (L. m) and furosemide (fur.) on glutathione concentration from DOCA-salt treated rat. Values are mean ± SEM of n = 5. $^{a}p < 0.05$ vs respective control group (water distilled) and # no vs respective control group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs respective DOCA-salt plus water distilled group.

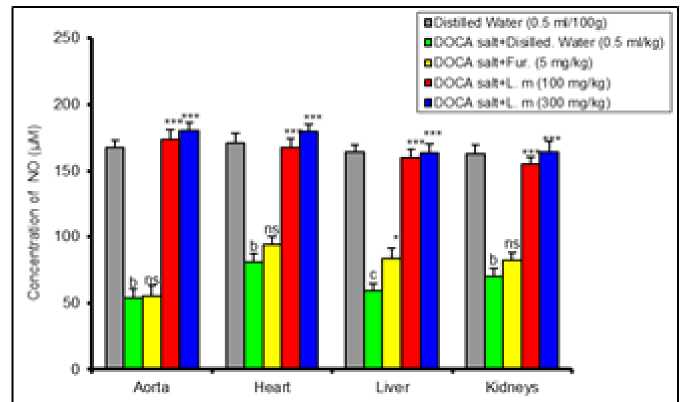

Fig 3: Effect of the aqueous extract of *L. multiflora* (L. m) and furosemide (fur.) on NO concentration from DOCA-salt treated rat. Values are mean ± SEM of n = 5. $^{b}p < 0.01$ and $^{c}p < 0.01$ vs respective control group (water distilled) . $^{*}p < 0.05$ and $^{***}p < 0.001$ vs respective DOCA-salt plus water distilled group.

Figure 3 shows that DOCA-salt causes a significant reduction in the concentration of nitric oxide (NO) in aorta, heart, liver and kidneys with percentages of 67.91 ($p<0.01$); 52.56 ($p<0.01$); 63.66 ($p<0.001$) and 56.55% ($p<0.01$) respectively. On the other hand, in the treated rats concomitantly with DOCA-salt and aqueous extract of *L. multiflora* (100 and 300 mg/kg), it is observed a significant increase ($p<0.001$) of the concentration of NO in these various bodies compared to the treated rats with DOCA-salt and distilled water. This increase is respectively of 70.23 ($p<0.01$); 55.03 ($p<0.05$); 63.48 ($p<0.05$) and 57.05% ($p<0.05$) at 300 mg/kg.

Effects on malondialdehyde level

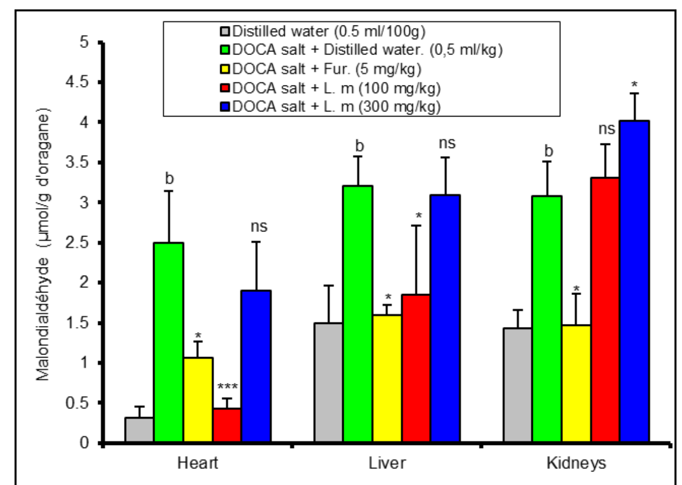

Fig 4: Effect of the aqueous extract of *L. multiflora* (L. m) and furosemide (fur.) on malondialdehyde concentration from DOCA-salt treated rat. Values are mean ± SEM of n = 5. $^{b}p < 0.01$ vs respective control group (water distilled) . $^{*}p < 0.05$ and $^{***}p < 0.001$ vs respective DOCA-salt plus water distilled group.

Figure 4 shows a significant increase of malondialdehyde level from 0.31 ± 0.15 to 2.50 ± 0.64; 1.50 ± 0.46 to 3.20 ± 0.37 ($p<0.01$) and 1.43 ± 0.23 to 3.08 ± 0.42 µmol/g of body ($p<0.01$) respectively in heart, liver and in kidney in the rats which received DOCA-salt and distilled water compared to the control group rats. In the treated rats with additional DOCA-salt and aqueous extract, the malondialdehyde levels are lower in heart and liver. The decrease of percentages are respectively 82.80 ($p<0.001$) and 42.37% ($p<0.05$) at 100

mg/kg. On the other hand, in these rats, it is observed a significant increase of malondialdehyde level in kidneys.

Effects of aqueous extract of the leaves of *L. multiflora* on the hydrogen peroxide level in leucocytes of mouse

Table I shows that addition of hydrogen peroxide (H_2O_2) in treatment increase significantly ($p<0.001$) his level in lymphocytes, polynuclear and monocytes. H_2O_2 level is significantly ($p<0.001$) weaker in the cells to which was added the aqueous extract of *L. multiflora* (5, 10 and 20 mg/ml) or chlorogenic acid (0.05 mg/ml). These two products cause with at the studied concentrations a significant reduction ($p<0.001$) of the H_2O_2 level in these cells.

Table 1: Effect of aqueous extract of *L. multiflora* on hydrogen peroxide (H_2O_2) concentration

Traitements	Conc. (mg/ml)	Peroxyde d'hydrogène (H_2O_2) level (%)		
		Lymphocytes	Polynucléaires	Monocytes
Negative Control (Without H_2O_2)	/	1.78 ± 0.67	1.63 ± 0.23	1.47 ± 0.38
Positive Control (With H_2O_2)	/	95.21 ± 4.49^c	96.65 ± 5.17^c	87.32 ± 1.44^c
L. multiflora	5	56.93 ± 1.75***	60.54 ± 2.91***	57.72 ± 0.94***
	10	59.97 ± 2.25***	60.88 ± 1.16***	60.61 ± 1.82***
	20	57.24 ± 1.05***	60.65 ± 1.75***	57.71 ± 0.87***
Ac. Chloro.	0,05	16.27 ± 1.90***	14.41 ± 1.93***	13.50 ± 1.71***

Values are mean ± SEM, $^c p < 0.001$ vs respective negative control group and ***p < 0.001 vs respective positive control group. Conc. = Concentration, Ac. chloro. = chlorogénic acide.

Discussion

The present study was initiated to evaluate the anti-stress potentiality of the leaves of *L. multiflora*. The obtained results shows a reduction of catalase activity, GSH and NO level and the increase of MDA level in treated rats with DOCA-salt. According to literature, this reduction of catalase activity and GSH, NO level in organs is the oxidative stress expression (Favier, 2003) [14]. DOCA-salt would thus stimulate the production of the reactive species of oxygen (ERO) and thus the installation of the oxidative stress in rats. The oxidative stress caused by the reactive species of oxygen is the origin of the release and the progression of several diseases such as cancer, diabetes, arterial hypertension, inflammatory, neurodegenerative diseases … (Hadj Salem, 2009). The increase of GSH level and catalase activity in the treated rats with aqueous extract of *L. multiflora* or with furosemide suggests that these two products would stimulate the synthesis of these two enzymes. Aqueous extract would thus have antioxidant potentialities. The antioxidants are implied in prevention and treatment of several pathologies as HTA (Aruoma, 2003; Akinmoladun and al, 2007; Coruh and al, 2007) [4]. Anti-hypertensor effect of aqueous extract of this plant, have been shown in anterior study (Etou Ossibi, 2012) [10]. In addition, the present study showed that the aqueous extract of *L. multiflora* reduced significantly the H_2O_2 level in leucocytes of mouse. The hydrogen peroxide (H_2O_2) is itself oxidizing and can be a precursor of free radicals (Favier, 2003) [14]. These results suggest that aqueous extract of *L. multiflora* would be opposed to the synthesis of H_2O_2 or would stimulate the synthesis of catalase. These corroborate the increase in the activity of the catalase observed in the present study in the rats treated with DOCA-salt and extract. Indeed, catalase is an enzyme which ensures the second stage of detoxification of reactive species oxygenated by eliminating of excess of hydrogen peroxide in water molecule (Favier, 2003) [14]. The antioxidant capacity of the aqueous extract of *L. multiflora* could be explained by the presence of flavonoids (Kunle, 2003; Hondi - Assah, and al, 2003; Etou Ossibi, and al, 2005) [18, 17, 9]. Flavonoids are polyphenols having a powerful antioxidant activity implying the antiradicalaire activity and even the chelation of metals (Urquiaga and Leighton, 2000) [26]. It was shown the probable presence of the cholinomimetic substances in the aqueous extract of *L. multiflora* (Etou Ossibi, 2014) [12]. The increase of NO level in particular in the aorta of treated rats with aqueous extract of *L. multiflora* could be explained by the presence of these substances. As acetylcholine, these substances would be fixed on the vascular receivers muscarinic and would cause the production of NO, responsible of relaxation of the vascular smoothes muscle (Furchgott and Zawadzki, 1980) [15].

In the present study, it was also observed an increase of MDA level in treated rats with DOCA-salt and distilled water compared to the control group rats not having received but distilled water. The MDA is a marker of lipidic peroxidation and, her concentration raised in fabrics would represent an increase in this peroxidation (Nayeemunisa and Kumuda, 2003). As, it is reported at the cardiac level, the MDA is the last product of the peroxidation of lipids of cells membranes due to the free radicals derived from oxygen.

MDA is regarded as a reliable marker of the damage of the myocardic cells (Rao and al, 1983) [23]. The reduction of MDA concentration in heart and liver in treated rats with aqueous extract of *L. multiflora* or with furosemide suggests that these two products would be opposed to the lipidic peroxidation in these organs. The aqueous extract of *L. multiflora* could thus have cardio-protective and hepatoprotective properties. This result joined the one obtain in another work which showed hepatoprotective effect of aqueous extract of *L. multiflora* in rat (Hondi Assah *et al*., 2003; Bouagnon *et al*., 2015) [17, 6].

Conclusion

The present study revealed that aqueous extract of the leaves of *L. multiflora* Moldenke is able to increase concentration of antioxidants (catalase and reduced glutathione) in rat organ and to reduce the oxidative stress markers (malondialdehyde and hydrogen peroxide) concentrations. These results suggest that *L. multiflora* would have the antioxidant potentiality to prevent or to treat several pathologies coming from oxidative stress and, could explain the important traditional use of this species.

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