Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(11):56-61
(http://www.derpharmachemica.com/archive.html)

Phytochemical Screening, Total Phenols and Flavonoids Contents, Antioxidant and Antibacterial Activities of *Tambourissa comorensis* Fruit Extracts

Hamidou Hamada Soule[1,2], Driss Mrani[3], Hanane El Hajaji[2], Aouicha Elkhamlichi[2], Said Hassane Soidrou[1], Abdellah Farah[4], Mohammed Lachkar[2*]

[1]Department of Faculty of Sciences and Technology, University of Comoros, Moroni, Comoros
[2]Engineering Laboratory of Organometallic and Molecular Materials, Faculty of Sciences, Sidi Mohammed Ben Abdellah University, 30000 Fez, Morocco
[3]Department of Faculty of Sciences and Technology, Moulay Ismail University, Errachidia, Morocco
[4]Department of Laboratory of Applied Organic Chemistry, Faculty of Sciences and Technology, Sidi Mohammed Ben Abdellah University, 30000 Fez, Morocco

ABSTRACT

Largely used in Comoros as folkloric medicine, the Tambourissa genoa is represented by six species in Comoros. T. comorensis is one of them. The present study has the objective to evaluate the phytochemical screening, total phenols and flavonoids content, antioxidant and antibacterial activities of T. comorensis extracts (ethyl acetate and methanol). Phytochemical screening concerns the presence of flavonoids in the ethyl acetate and methanol extracts and tannins only on the methanol extract. Total phenolic and flavonoids contents results are showed in a large dominance in methanol extract. The antioxidant activities were examined by 3 different methods namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, reducing power scavenging activity and total antioxidant capacity. All tests showed significant dose dependent antioxidant activities. The ethyl acetate extract shows the high activity in DPPH radical scavenging activity but in reducing power assay, it's the methanol extract which manifested the high activity. Our results on the antimicrobial activity showed the inefficiency of the extracts against most of the tested strains.

Keywords: *Tambourissa comorensis,* Antioxidant activity, Total phenolic, Total flavonoid, Antibacterial activity

INTRODUCTION

Energy used by all living organisms is produced by oxidation processes and diverse molecules named reactive oxygen including free radicals and their precursors. These processes are controlled by several cellular mechanisms [1]. These molecules are used for their beneficial reactivity in a lot of processes, as weapon in defense mechanisms or as signal inside or inter cells. Their useful level is maintained by equilibrium between the generating system producing free radicals and antioxidant systems [2]. However, the excessive production of free radicals and unbalanced mechanisms of antioxidant protection result in oxidative stress [3]. Oxidative stress is partly responsible for a great number of age-related diseases as cancer, cardiovascular disorders, and neurodegenerative diseases [2,3]. Oxidation, mediated by free radical reactions is also responsible of the rancidity of unpreserved rich food in unsaturated fatty acids [4]. Therefore, there is a growing interest in substances exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as specific preventative pharmaceuticals [3]. Plant kingdom is a good source of natural bioactive compounds which can be used for different applications. During last decades they have become a major area of research of natural antioxidants [5-7]. Nowadays, several studies including epidemiological and experimental studies on medicinal plants and vegetables strongly supported the idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems such as cardiovascular diseases, arthritis, chronic inflammation and cancers [5,8-12].

Known for their richness biodiversity, Comoros archipelago has panoply of medicinal and aromatic plants but few studies are held on their chemical characterization and their biological properties [13]. Largely used in Comorian folkloric medicine, the *Tambourissa* genoa is represented by six species in Comoros with fifty percentage of endemicity. These plants are, aromatics, and sometimes heterophyllum. The fruit carries inside many drupes included in its accrescent wall. About Comorian *Tambourrissa* species, only one study exists. It is concerned *Tambourissa leptophylla* essential oil [14]. In this study we are interested on *T. comorensis*, an endemic species for chemical constituents of the fruit, their antioxidant and antibacterial activities. This study is the first phytochemical study about nonvolatile compounds from *Comorian, Tambourissa* species.

MATERIAL AND METHODS

Plant material

Included in laurale order, *T. comorensis* is classified in Monimiaceae family. Their fruit are harvested in Dimadjou Hamahamet forest (N/E of Ngazidja Island at 629 m of altitude) in January 2013. Vouchers specimens (P00196479) were identified by Andiliyat Mohamed Abderehmane and have been deposited in the Herbarium Department of Botany (Faculty of Sciences and Technology University of Comoros).

Preparation of extract

150 g of dried and ground fruit of *T. comorensis* were placed in Soxhlet extractor. Briefly, 3 solvents were successively used according their polarity (hexane, ethyl acetate and methanol) and extracts were concentrated by evaporation on rotavapor “Buchi Heating Bath B-490”. The extracts were stored at 4°C for different tests.

Phytochemical screening

The ethyl acetate and methanol extracts were screened in phytochemical constituents (alkaloids, flavonoids and tannins) by using simple qualitative methods from Paris and Nothis using cyanidin reagent for flavonoids, ferrous chloride for tannins and Dragendorf reagent for alkaloids [15].

Total phenolic and total flavonoids contents

Total phenolic content was determined spectrophotometrically by using Folin-Ciocalteu method as described by El Hajaji et al. [5]. This test is a colorimetric method based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectrophotometrically at 765 nm. Extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate solution (25%). The absorbance was measured at 765 nm after 2 h. Concentrations of phenolic compounds were calculated according to the following equation obtained from the gallic acid graph: Absorbance=0.0007 GA (µg)+0.0642 (R^2: 0.9964).

All tests were carried out in triplicate and the results are given as Gallic Acid Equivalents (GAE). Total flavonoids content was also determined spectrophotometrically by using the $AlCl_3$ reagent as described by Soidrou et al. [16]. Briefly, this method consists in making extracts react with an equal volume of an $AlCl_3$ solution (2%). Absorbance was measured after 10 min at 430 nm. Concentrations of phenolic compounds calculated according to the following equation obtained from the quercetin graph: Absorbance=0.0333x+0.0231 (R^2: 0.9961). All tests were carried out in triplicate and the results are given as quercetin equivalents (QE/100 mg of extract).

Antioxidant studies

Free radical scavenging activity by DPPH method

Ethyl acetate and methanolic extracts of fruit from *T. comorensis* were tested in their free radical scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the protocol described by El Hajaji et al. [17]. Briefly, various concentrations of methanol solution or extract were added to a methanol solution of DPPH (1.01×10^{-4} M). The mixture was vigorously shaken and then stored in room in temperature of 30 min in the dark. The absorbance of the mixture was measured at 517 nm by using a double-beam UV-visible Camspec M550 spectrophotometer. Result was expressed in percentage inhibition in this formula:

$$\% \text{ inhibition} = [(A_B - A_S)/A_B] \times 100$$

Where, A_B is the absorbance of control reaction and A_S the absorbance of test compounds. Butylated Hydroxytoluene (BHT) was used in a positive control. The test was carried out in triplicate.

Reducing power assay (Iron reducing activity)

The reducing power of ethyl acetate and methanolic extracts of fruit of *T. comorensis* was determined according to the protocol described by El. Hajaji et al. [17]. Different concentrations of extracts distilled water were mixed with phosphate buffer and potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. A portion of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution was mixed with distilled water and $FeCl_3$ and the absorbance measured at 700 nm. Increasing the absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid, tannic acid and gallic acid were used as standards. For the blank sample, phosphate buffer solution was used. All analyses were run in triplicate and results averaged.

Total antioxidant capacity by phosphomolybdenum method

The antioxidant activity of the extracts was evaluated by using the phosphomolybdenum method as described by El. Hajaji et al. [17]. The test is based on the reduction of Mo(VI) to Mo(V) and the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. In case of blank, methanol was used in place of extracts. The absorbance of the solution was measured at 695 nm using a spectrophotometer. The antioxidant capacity of each sample was served as Ascorbic Acid (AA) equivalent using the following linear equation in using ascorbic acid as standard: [A=0.0037C+0.0343 ; R^2=0.991]. Where, A is the absorbance at 695 nm and C the concentration as ascorbic acid equivalent (µg/ml). The values represent the triplicate analysis.

Determination of antibacterial activity

Bacterial strains

The extracts tested in their antibacterial activity against four bacterial strains. There were two Gram-positive bacteria: *Staphylococcus aureus* ATCC 25922 and *Bacillus subtilis* ILP 14283; as well as two Gram-negative bacteria: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Antibacterial assay

The antibacterial activity from the extracts was examined by disk-diffusion method [18], with some modifications. Briefly, bacterial strains were grouped overnight at 37°C on Luria-Bertani broth, then inoculum consisting of 0.5 McFarland (10^7 CFU/ml) and prepared in physiologic saline. Bacterial inoculum (100 µl) was inoculated in Petri dishes containing a sterile Luria-Bertani Agar medium. Sterile filter paper discs (6 mm diameter) is placed in on medium and impregnated with 10 µl of extract solution (prepared to different concentrations : 1, 2, 10, 20, 30 and 40 mg/ml of DMSO at 2%). The control was performed with discs containing 10 µl of DMSO to 2%. Each experiment was performed in triplicate.

Determination of Minimal Inhibitory Concentration (MIC)

The MICs is determined by using both microdilution assay as previously described by Bouhdid et al. [19], with slight modifications. Agar at 0.15% (w/v) is used as emulsifier and resazurin in bacterial growth indicator. Firstly, 50 µl of Luria-Bertani was distributed from the second to the 12th well of a 96-well polypropylene Microtiter plate. 50 µl of scalar dilution and transferred from the second to the 1th well. The 12th well was considered as growth control. Then, 50 µl of a bacterial suspension was added to each well at a final concentration of approximate 10^7 CFU/ml. Plates were incubated at 37°C for 24 h. After incubation, 10 µl of resazurin were added to each well to assess bacterial growth as indicated by Bouhdid et al. [19]. After further incubation at 37°C for 2 h, the MIC was determined as the lowest extracts concentration that prevented a change in resazurin color. Bacterial growth is detected by reduction of blue dye resazurin to pink resorufin. Experiments were conducted in triplicate. The Minimum Bactericidal Concentration (MBC) corresponded to the lowest concentration of the extracts yielding negative subcultures after incubation at 37°C for 24 h. It is determined by spotting 2 µl from negative wells on LB plates. Experiments were also conducted in triplicate.

RESULTS AND DISCUSSION

Phytochemical screening

As showed in Table 1, phytochemical screening of fruit of *T. comorensis* deals with the presence of flavonoids in ethyl acetate and methanol extract. But only the methanol extract shows the presence of tannins. However alkaloids were absent in both extracts. The major contents in flavonoids and tannins are observed in methanol extract.

Table 1: Phytochemical contents of fruit of *Tambourissa comorensis*

Extracts	Alkaloids	Flavonoids	Tannins
EtOAc	-	+	-
MeOH	-	+	+

+ for present; - for absent

Monimiaceae family which is part of the *Tambourissa* genoa is largely discussed in literature for its phytochemical constituents like alkaloids [20-22], flavonoids [21,22], polyphenols [23] and essential oils [21,22]. But few studies are held about *Tambourissa* genoa. Presence of flavonoids in *Tambourrissa* genoa is showed on *T. trichophylla* from Madagascar [24]. This species contains also tannins compounds [25]. Tannins are also known in *T. purpurea* [25]. Some alkaloids are also identified in this species [25]. About Comorian *Tambourrissa* species, only one study exists. She is concerned the essential oil from *T. leptophylla* [14]. Our investigation is the first phytochemical screening study about nonvolatile compounds from Comorian *Tambourissa* species.

Total phenolic and total flavonoids contents

Total phenolic content was expressed as GAE using the following linear equation as standard: $y=0.0007x+0.0642$, $R^2=0.9964$. The results are given at 0.63 g/l GAE for methanol extract and 0.14 g/l GAE for ethyl acetate extract. For total flavonoids contents, they are made as quercetin equivalent using also the following linear equation with quercetin as standard: $y=0.0333x+0.0231$; $R^2=0.9961$. The results are concerned at 0.14 µg/ml QE for ethyl acetate extract and 0.47 µg/ml QE for methanol extract. These results are showed in a large dominance for methanol extract in total phenolic and flavonoids contents. This observation can be explained by the polarity of solvents and molecules extracted. It's known that polyphenols and flavonoids are molecules with high polarity and also that methanol is polar than ethyl acetate. So it is evident that flavonoids and polyphenols are largely contained methanol extract than ethyl acetate extract.

Antioxidant studies

Antioxidant activity is generally due to different mechanisms such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts [26] and different methods can be used to evaluate the antioxidant activity [6]. In this study, three antioxidant evaluation methods such as DPPH radical scavenging activity, reducing power assay and phosphomolybdenum method were used.

Free radical scavenging activity

Two extracts from the fruit of *Tambourissa comorensis* were tested for their radical scavenging properties using DPPH radical (Figure 1). The effect of antioxidants on DPPH radical scavenging activity was thought to be due to their hydrogen-donating ability [27]. All extracts tested showed an antioxidant activity and this activity increased by the increasing concentration as observed in other studies [3,6]. It is known that, a strong correlation was observed between the radical scavenging capacity and polarity of the extracts [3]. But in the current study, the methanol extract, our polar extract, was manifested less activity, and the high activity was established at 31%. The highest scavenging activity showed by ethyl acetate extract. The most scavenging percentage established at 78% and IC_{50} was estimated at 0.5 g/l. However, none of samples evaluated showed a strong activity than BHT, a synthetic antioxidant molecule used as control, with IC_{50} of 0.2 g/l.

Reducing power assay

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [28].

In this study, we are used the direct correlation observed between antioxidant activities and reducing power of certain plant extracts to evaluate the possible antioxidant effect of *T. comorensis* extract. Ethyl acetate and methanol extracts of *T. comorensis* fruit are showed a strong power reducing activity at low doses compared with tannic acid and ascorbic acid (Figure 2).

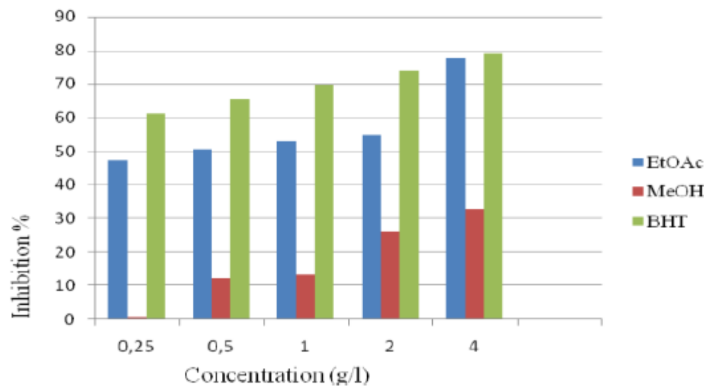

Figure 1: Free radical scavenging activity of *Tambourissa comorensis*

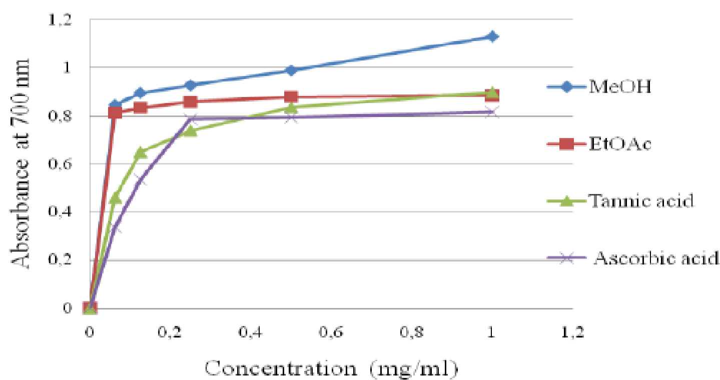

Figure 2: Total reducing power of ethyl acetate and methanol extracts of *Tambourissa comorensis*

The power reducing activity observed in our extracts is high than tannic acid and ascorbic acid. The strong activity was observed on methanol extract with an increasing concentration. Contrarily to the DPPH radical scavenging, the ethyl acetate extract manifested the less antioxidant activity.

Total antioxidant activity

Total antioxidant capacity of the *T. comorensis* fruit extract, described as the number of gram equivalents of ascorbic acid, is shown in Figure 3. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm.

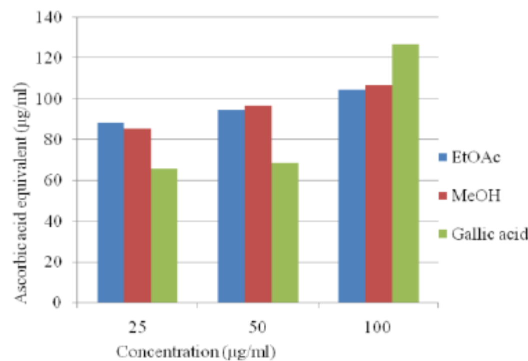

Figure 3: Total antioxidant capacity of *Tambourissa comorensis* extracts

All extracts showed antioxidant capacity. In low concentrations, extracts manifested a highest antioxidant capacity than gallic acid our antioxidant control. But for a strong concentration, gallic acid manifested a strong activity than *T. comorensis* fruit extracts. The concentration of 25 µg/ml, ethyl acetate extract manifested strong activity than methanol extract. However for concentrations ≥ 50 µg/ml, methanol extract gives a strong activity than ethyl acetate extract.

Antibacterial activity

The results of antibacterial activity from methanol extracts were presented in Table 2. The negative control used (DMSO 2%) did not exert any inhibition on the strains tested. In this study, methanol extracts from fruit of *T. comorensis* exhibit is low than antibacterial activity to *Bacillus subtilis*, *Staphylococcus aureus and Escherichia coli.* However, *Pseudomonas aeruginosa* extract has not exhibited to any antibacterial activity.

Table 2: The growth-inhibitory diameters (mm) of methanol extract tested from bacteria

Bacterial strains	**Fruit of *Tambourissa comorensis***
	Methanol extract
Staphylococcus aureus ATCC 25922	9
Bacillus subtilis ILP 14283	8
Escherichia coli ATCC 25922	8
Pseudomonas aeruginosa ATCC 27853	-

MIC and MBC shown in Table 3, thus, as can be seen in this finding, it is interesting to note that the extract showed in a very low without antibacterial activity against all strains tested. Indeed, we have seen the growth of all bacteria strains at all concentrations of the extract used. So any minimal inhibitory concentration could not be determined. In traditional Comorian medicine, *T. comorensis* is largely used against infectious diseases and bacterial diseases [29]. It is also known that biological activities from natural compounds are mainly due to the presence of polyphenolic compounds. As shown in Table 1, methanolic extract from fruit of *T. comorensis* contains flavonoids and tannins, strong polyphenolic compounds. For these two reasons, our methanol extract should show a good antibacterial effect. However, as observed in Tables 2 and 3, very low or any antibacterial activity was manifested. This result could be explained by possible alteration of compounds due to combination of temperature and the time of passage in Soxhlet apparatus.

Table 3: Determination of MBC values of methanol extract against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*

Concentrations (mg/ml)	***Staphylococcus aureus* ATCC 25922**	***Bacillus subtilis* ILP 14283**	***Escherichia coli* ATCC 25922**	***Pseudomonas aeruginosa* ATCC 27853**
	Methanol extract fruit of *Tambourissa comorensis*			
Control	+	+	+	+
50	+	+	+	+
25	+	+	+	+
12.5	+	+	+	+
6.25	+	+	+	+
3.125	+	+	+	+
1.5625	+	+	+	+
0.78125	+	+	+	+
0.390625	+	+	+	+
0.1953125	+	+	+	+
0.09765625	+	+	+	+
0.048828125	+	+	+	+

Key: + for presence of growth; - for absence of growth

CONCLUSION

This study was conducted to determine phytochemical compounds, total phenolic, flavonoids contents, antioxidant capacities and antibacterial activity of *T. comorensis* extracts. Phytochemical screening demonstrates the presence of flavonoids in both extracts and tannin only on the methanol extract. Total phenolic and flavonoids contents results showed a large dominance in methanol extract. For antioxidant activity, all tests showed significant dose dependent antioxidant activities. The ethyl acetate extract leads to a high activity in DPPH radical scavenging activity but in reducing power assay, it's the methanol extract which manifested a high activity. The methanol extracts from fruit of *T. comorensis* exhibit low antibacterial activity. Ethyl acetate extract could not be tested against bacterial strains because it is no soluble in Dimethyl Sulfoxide (DMSO). Indeed, temperature and time in the passage of compounds in Soxhlet apparatus have probably alternate these compounds and affected also the antibacterial effect of methanol extract. So, in future, we must extract these compounds with another protocol without hot temperature in antibacterial tests. These results suggested that *T. comorensis* fruit can be used as possible in natural antioxidant source. It is then necessary to identify and isolate the compounds that are responsible to these antioxidant activities.

ACKNOWLEDGMENTS

We are grateful to Andiliyat Mohamed Abderehmane, responsible of Herbarium of Faculty of Sciences and Technology of University of Comoros, in botanical identification. We would like to acknowledge the support and technical assistance from Interface Regional University Center (University Sidi Mohammed Ben Abdellah, Fez, Morocco), and National Center for Scientific and Technical Research (CNRST-Rabat, Morocco).

REFERENCES

[1] B. Halliwell, J.M.C. Gutteridge, 3rd Ed Oxford University press, **2007**, 851.
[2] A. Favier, *Actual Chim. Novembre*., **2003**, 108-115.
[3] C. Sarikurkcu, K. Arisoy, B. Tepe, A. Cakir, G. Abali, E. Mete, *Food Chem. Toxicol*., **2009**, 47, 2479-2483.
[4] H.B. Li, C.C. Wong, K.W. Cheng, F. Chen, *LWT-Food Sci. Technol.*, **2008**, 41, 385-390.
[5] H. El Hajaji, N. Lachkar, K. Alaoui, Y. Cherrah, A. Farah, A. Ennabili, B. El Bali, M. Lachkar, *Arabian J. Chem.*, **2011**, 4, 321-324.

[6] C. Sarikurkcu, B. Tepe, D.K. Semiz, M.H. Solak, *Food. Chem. Toxicol.,* **2010,** 48, 1230-1233.
[7] P.R. Venskutonis, *Acta Alimentaria.*, **2004,** 33, 1-5.
[8] C.C. Chen, L.K. Liu, J.D. Hsu, H.P. Huang, M.Y. Yang, C.J. Wang, *Food Chem.,* **2005**, 91, 60.
[9] R.L. Prior, *Am. J. Clin. Nutr.,* **2003**, 78, 570S-578S.
[10] A. Saleem, M. Husheem, P. Harkonen, K. Pihlaja, *J. Ethnopharmaco*., **2002**, 81(3), 327-336.
[11] E. Souri, G. Amin, H. Farsam, H. Jalalizadeh, S. Barezi, *IJPR.,* **2008**, 7, 149-154.
[12] Y. Zhang, S.K. Vareed, M.G. Nair, *Life Sci.,* **2005**, 76, 1465-1472.
[13] S.H**.** Soidrou, N.A. Mohamed, A. Farah, S.O. Said Hassane, D. Bousta, *IJP.,* **2013**, 4, 230-236.
[14] S. Gallori, A.R. Bilia, N. Mulinacci, C. Bicchi, P. Rubiolo, F.F. Vincieri, *Planta Med.,* **2001**, 67, 290-292.
[15] R. Paris, A. Nothis, *Plantes Médicinales et Phytothérapie.*, **1996**, 4, 274-287.
[16] S.H. Soidrou, D. Bousta, M. Lachkar, S.O. Said Hassane, A. El Youbi-Hamsas, J. Benjilali, L. El Mansouri, H. El Hajaji, A. Farah, *Phytothérapie,* **2014**, 1-6.
[17] H. El Hajaji, N. Lachkar, K. Alaoui, Y. Cherrah, A. Farah, A. Ennabili, B. El Bali, M. Lachkar, *Rec. Nat. Prod.,* **2010**, 4, 193-204.
[18] NCCLS., *Clinical and Laboratory Standards Institute*., **2012**.
[19] S. Bouhdid, J. Abrini, A. Zhiri, M.J. Espuny, A. Manresa, *J. Appl. Microbiol.*, **2009**, 106, 1558-68.
[20] J. Bruneton, Paris, **1999**, 421-499.
[21] R. Hegnauer, *Birkhäuser Verlag. Basel.*, **1990**.
[22] G. Leitão, N.K. Simas, S.S.V. Soares, A.P.P. De Brito, B.M.G. Claros, T.B.M. Brito, F. Delle Monache, *J. Ethnopharmacol.*, **1999**, 65, 87-102.
[23] B.M. Claros, B.M, da Silva, A.J, Vasconcellos, M.L, de Brito, A.P, G.G. Leitao, *Phytochem.,* **2000**, 55, 859-862.
[24] A**.** Lhuillier, Thèse de doctorat, Institut National Polytechnique de Toulouse, France, **2007.**
[25] C.M. Hladik, B. Simmen, P. Ramasiarisoa, A. Hladik, Mémoires de la Société de Biogéographie de Paris, **2000**, 105-114.
[26] L.C. Mao, X. Pan, F. Que, X.H. Fang, *Eur. Food Res. Technol.*, **2006**, 222, 236-241.
[27] K. Shimada, K. Fujikawa, K. Yahara, T. Nakamura, *J. Agric. Food Chem.*, **1992**, 40, 945-948.
[28] T. Osawa, *JSSP.*, **1994**, 241-251.
[29] H.H. Soule, S.H. Soidrou, A. Farah, S.O. Said Hassane, A. Chaouch, M. Lachkar, *IJP.,* **2014**, 5, 416-422.