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Asian Journal of Plant Science and Research, 2015, 5(5):28-33

ISSN : 2249-7412
CODEN (USA): AJPSKY

Total phenolics content, flavonoids profiling and antioxidant activity of *Lippia multiflora* leaves extracts from Burkina Faso

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ABSTRACT

Polyphenolics, and particularly flavonoids are known and sought for their multiple biological properties. The present study aimed to assess the total phenolics (TP), total flavonoids (TF) contents and the antioxidant activity (AOA) of Lippia multiflora leaves extracts, an aromatic plant of subtropical Africa whose traditional practices reveal enormous potential. The dried leaves of Lippia multiflorawere extracted successively with hexane, dichloromethane, ethyl acetate and ethanol by cold maceration for 24 hours. After a phytochemical screening, the TPCwas determined using the Folin-Ciocalteu reagent, the TFC by aluminum chloride test and the AOA by the DPPH test and the lipid peroxidation inhibitionassay. The results showed that ethanolic crude extract had significant phenolics and total flavonoids contents and also good AOA. Furtherfractionation of this extract by column chromatography, using ethyl acetate - methanol (80: 20), afforded a fraction which AOA (IC_{50} = 23.68 µg/mL) is more important than those of crude extract (IC_{50} = 38.20 µg/mL) and vitamin C (IC_{50} = 80.33 µg/mL). This fraction also inhibited 29.19, 33.57 and 67.15 % lipid peroxidation at 250, 500 and 1000 µg/mL respectively. Lippia multiflora ethanolic extract may provide a source of natural antioxidants and these preliminary results allow further investigation of other biological activities including anti-cancer and antidiabetic properties to enhance the extracts of this plant in phytomedicine

Key words: *Lippia multiflora*, polyphenolics, flavonoids, antioxidant activity, phytomedicine

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INTRODUCTION

Polyphenolics, and especially flavonoids are one of the most important groups of bioactive compounds in plants [1]. They include several classes of compounds with similar structures and have a broad range of biological activities. They have a protective role in carcinogenesis, inflammation, artherosclerosis and have high antioxidant activities [2]. Due to their potent biological activities, polyphenolics in general and particulary flavonoids attracted much interest. Their level in plants depends of the part of plant used and the solvent used for extraction [3, 4]. They are increasingly looking in plants with medicinal applications.

The genus *Lippia* (Verbenaceae) includes approximately 200 species of herbs, shrubs and small trees. The species are mainly distributed throughout the South and Central America countries and Tropical Africa territories [5]. Most of them are traditionally utilized as gastrointestinal and respiratory remedies [6]. Some *Lippia* species have shown antimalarial [7], antiviral [8] and cytostatic activities [9]. Reported results on *L. multiflora* show that the specie from South Africa contains as major non-volatile compounds verbascoside, iso verbascoside, nuomioside A, isonuomioside A, lutueolin and lutueoli-7-O-glucuroninique and isonuomioside is the most effective radicals scavenger [10]. In Burkina Faso, four species of lippia have been reported and *L. multiflora* is widely spread in the

country [11]. Many results have been reported about the essential oil of the specie in Burkina Faso [11-13] but no report about non volatiles compounds. Locally the species is used to cure malaria, high blood pressure, hepatitis, insomnia, yellow fever, etc. Regarding its multiple uses in traditional medicine, our research work has been again focused on this plant.*Lippia multiflora* from Burkina Faso may contain some compounds with higher bioactive activities.

The purpose of this study is to evaluate the total polyphenolics content, total flavonoids content and anti-oxidant activity of *Lippia multiflora* leaves extracts from Burkina Faso obtained using hexane, dicholoromethane, ethyl acetate and ethanol. Because of good correlation between total phenolics, total flavonoids, total antioxidant activity and many biological activities, this preliminary study is prior tothe investigation of other biological activities including anti-cancer and antidiabetic properties, in order to enhance the extracts of this plant in phytomedicine

MATERIALS AND METHODS

I.1. Plant material

The leaves of *Lippia multiflora*were collected between May and June 2013 and dried out of direct sunlight. Plant has been identified and described by the Laboratory of Vegetal Biology and Ecology of University of Ouagadougou, where a specimen was filed. After drying, the plant material was crushed.

I.2. Extracts preparation

300 g of *Lippia multiflora* dried leaves were extracted successively (three times) with 1000 mL of hexane, dichloromethane, ethyl acetate and ethanol, by cold maceration under magnetic stirring during 24 hours. The solutions are filtered, concentred under reduced pressure to yield hexane extract (HE), dichloromethane extract (DCME), ethyl acetate extract (EAE) and ethanol extract (EE). Thus, four crude extracts were prepared.

I.3. Phytochemical screening

Each crude extract have been screened for tannins, flavonoids, alkaloids, saponins, carbohydrates, and triterpenes. Chemical tests were carried out on the various extracts using standards procedures as described by Sofowara, Trease and Evans and Harborne [14-16].

I.4. Total phenolicsand flavonoids contents

I.4.1. Total phenolic content

The total phenolics contentwas determined by the Folin-Ciocalteu method as described by Singleton et *al*. [17] and adapted for microplates. Gallic acid is used as standard. A calibration curve is first established by using gallic acid solution at concentrationsranging from0, 25,…,to 100 µg/mL. Then 60 µL of the Folin-ciocalteu reagent (FC-R 1 :10 dilution),were added to the solutions of gallic acid. The mixtures were left to stand at room temperature for 8 min, and then, 120 µL of sodium carbonate solution (Na_2CO_3), 7.5% were added to neutralize the residual reagent. The absorbanceswere measured at 700 nm using a Microplate Autoreader (MP 96 ; SAFAS Instrument) after incubation for 30 min at 37 ° C.

After that, 60 µl of each extract dissolved in DMSO, suitably diluted, were added to 60 µL of FC-R,insteadof Gallic acid. The results, determined from the equation of the calibration curve (y = 0, 0114x + 0, 0888; R^2 = 0.9994), have been expressed in mg of Gallic acid Equivalent (GAE) per gram of dry plant material. Measures were carried out in tripliquat.

I.4.2. Total flavonoids content

Total falvonoids content was measured using aluminiun chloride colorimetric assay as described by Zhishen J. et al. [18] with slight modifications (adapted for a mictoplates reader). Briefly, 25 µL of plant extract or standard solution of quercetin at different concentration were addto 150 µL of ditilled water. Then 10 µL of 5% $NaNO_2$ was added. After 5 min, 10 µL of 10% $AlCl_3$was added. After 6 min, 50 µL of NaOH 1M were added. After incubation for 30 min at 37°C, the absorbances of the mixture were measured using a Microplate Autoreader (MP 96 ; SAFAS Instrument).Measures were carried out in tripliquat.

I.5. Antioxidant activity

Antioxidant activity were evaluated using two methods : 2,2-diphenyl-1-picrylhydrazine (DPPH) scavenging assay and lipid peroxidation (LPO) inhibition method.

I.5.1. DPPH radical scavenging assay

The free radical scavenging activities of the extracts were determined as described by Gulcin et al.[19] with slight modifications. Briefly 10 µL of each extract or standard (ascorbic acid) at different concentration were added to 200

µL of DPPH solution 10^{-4}M. After incubation for 30 min at 37°C, the absorbances were measured at 510 nm usinga Microplate Autoreader (MP 96 ; SAFAS Instrument).Results were expressed as IC_{50} values. IC_{50}represents the concentration of extract that reduces 50% of DPPH free radical. Measures have been carried out in tripliquat.

I.5.2. Lipid peroxidation inhibition assay

Lipid peroxidation inhibition has been evaluated using the method described byHuong et al.[20] and modified by Deepa et al.[21].

In this method, egg lecithin was used as oxidable substratum. The lecithin was extracted from fresh egg yolk.

Extraction of lecithin from egg yolk.

In a 200 mL beaker containing 100 ml of acetone, two egg yolk (isolated from hen eggs) were completely dispersed by stirring with a glass stirrer. The mixture was left for 15-20 min at room temperature and the supernatant was removed. The residue was extracted three to four times by the same quantity of acetone till the yellow colour of the supernatant became clear. The residue was dried à 25°Cto yield awhite powder : lecithin.

Lipid peroxidation inhibition assay

In test tubes, 1 mL of egg lecithin solution [3 mg/mL, prepared by dissolving egg lecithin powder in phosphate buffer (0.1 M, pH = 7.4)] ; 20 µL of $FeCl_3$, 400 mM ;20 µL of L- ascorbic acid 200 mM ;100 µL of extract dissolved in DMSO (or standard) at different concentrations were mixed.

The mixture was then incubated at 37°C (in an owen) for 1 hour. After incubation, 2 mL of trichloroacetic acid 15% (m/v) followed by 2 mL of Tiobarbituric acid (0.37% (m/v) were added.

The resulted mixture was boiled for 15-20 min. After cooling, the mixture was centrifugated (2000 trs/min) and the absorbance of the supernatant was measured at 532 nm.

The results were expressed as % of inhibition by the following relation :

$$\%\ inhibition\ = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

The blank tube contained all the reagent without sample (or standard).

I.6. Column chromatography

Column chromatography concerned only ethanol crude extract since it was the most potent when screening antioxidant activity.

5 g of ethanol crude extract, have been loaded on a silica gel column chromatography and eluted with the following solvent systems : hexane-DCM ; DCM-ethyl acetate ; ethyl acetate-methanol ; methanol-water. 42 fractions have been collected and gathered to 10 fractions based on TLC analysis ; no fractions were collected using hexane – DCM as mobile phase. The 10 fractions were concentred to rotavapor and dried (table 1).

Tableau 1 : Fractionation of ethanol crude extract with different mobile phases

Fractions N°	Mobile phase	Composition of mobile phase	Volume of solvent (mL)	Masse of fraction (mg)
1	DCM-EtOAc	85:15; 80:20	100x2	45
2	DCM-EtOAc	75:25 to 40:60	100x5	87
3	DCM-EtOAc	30:70 to 10:90	100x3	42
4	DCM-EtOAc EtOAc-MeOH	0 :100 95:5; 90:10	100x1 100x2	38
5	EtOAc-MeOH	85 15	100x1	15
6	EtOAc-MeOH	80:20; 70:30	100x2	193,5
7	EtOAc-MeOH	60:40 to 10:90	100x6	1018
8	EtOAc-MeOH	0 :100	100x1	21
9	MeOH-water	90 :10	100x1	15,3
10	MeOH-water	80 :20	100x1	23,8

RESULTS AND DISCUSSION

II.1. Phytochemical screening

The results are gathered in table 1. We note that, except alkaloids which are absent in ethyl acetate extract, all the chemical groups are found in ethanol and ethyl accetate extracts. Furthermore, only hexane and DCM extracts did

not contain certain chemical groups such as saponins and flavonoids. Tannins and carbohydrates are almost present in most of the extracts prepared. *Lippia multiflora* leaves, whatever the extract type, contain various chemical groups and that might justify the use of this plant in traditional medicine to cure many diseases.

Table 2 : Chemical groups in *Lippia multiflora* leaves extracts

Extracts	Chemical groups					
	tannins	carbohydrates	saponins	alkaloids	flavonoids	triterpenes
Hexane	-	+	-	-	-	+
DCM	+	+	-	+	-	-
Ethyl acetate	+	+	+	-	+	+
Ethanol	+	+	+	+	+	+

+ : presence ; - : absence.

II.2. Total polyphenolics content, total flavonoids content and DPPH radical scavenging activity of crude extracts and fractions of ethanol crude extract.

Table 3 : Values of total polyphenolics content, total flavonoids content and DPPH radical scavenging activity of crude extracts and fractions of ethanol crude extract

Crude extracts	Fractions	Antioxidant activity IC_{50} (µg/mL)	TPC (µg of GAE/mg of extract)	Flavonoids content (µg of QE/mg of extract)
hexane	-	nd	nd	nd
DCM	-	175.60 ± 0.1	6.71 ± 0.00	6.16 ± 0.1
Ethyl acetate	-	370.50 ± 0.3	6.62 ± 0.4	13.72 ± 0.2
	crude	**38.20**± 0.1	**71.17**± 0.2	**40.79**±0.2
	Fraction 2	>1000	1.30 ± 0.1	0.02 ± 0.00
	Fraction 6	**23.68**± 0.2	**37.18**±0.1	**17.07**± 0.04
Ethanol	Fraction 7	192.30 ± 0.0	25.99 ± 0.1	**7.55**± 0.1
	Fraction 8	276.20 ± 1.04	8.08 ± 0.00	**7.47**± 0.1
	Fraction 9	948 ± 0.5	nd	nd
	Fraction 10	261.30 ± 0.3	nd	nd
ascorbic acid		**80.33± 0.1**		

***nd** : non determined ; **QE** : quercetine equivalent ;**GAE** : gallic acide equivalent ; **TPC** : Total Polyphenolics Content.*

As it's shown in table 3 we notethe same total polyphenolics content in DCM and ethyl acetate extract. Among the three extracts types, (DCM, ethyl acetate and ethanol), ethanol crude extract contained high polyphenols and flavonoids content. That's because polyphenols and/or flavonoids are generally well extracted with polar solvent (ethanol, methanol) than non/less polar solvents [4]. Ethanol crude extract contains 71 mg of GAE/g of dried plant, approximately 12 folds polyphenoliccontent than DCM and ethyl acetateextract, as it's clearly showed in figure 1.

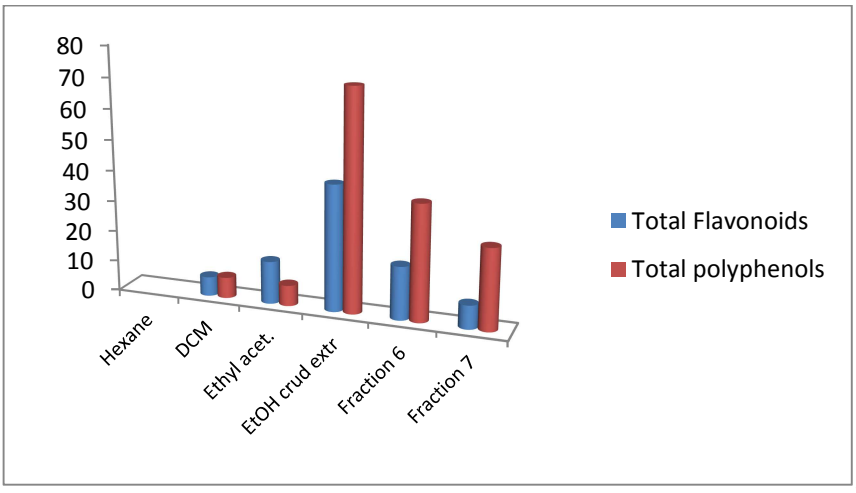

Figure 1 : Total phenolic and total flavonoids content in various extracts of *Lippia multiflora*

Compared to other medicinal plants, Burkina Faso *Lippia multiflora* leaves extracts seem to contain higher level of phenolic compound and flavonoids. Inded, Alain. H.O et al. [22] evaluated phenoliccontents in 13 organs of 10 medicinal plants from Ivory Coast and found some contents ranging from 3.5 to 7.8 mg GAE/g of dried plant. TP and TF contents reported by Eshan K. et al [23] in different part (leaves, stem and roots) of a Malaysian indigenious medicinal herb (*Labisia pumila*) are very low, compared to those of *Lippia multiflora*.

On the other hand, ethanol crude extract also exhibits higher total flavonoids content (40.79 µg of QE/mg of extract) compared to that of DCM (6.16µg of QE/mg of extract) and ethyl acetate extract (13.72µg of QE/mg of extract). Polyphenolics are polar compounds and thus, are easyly extracted with polar solvents such as ethanol, methanol and water. Saikat Sen et al. reported higher total phenolic content (90.08 mg GAE/g of extract) and total flavonoids (58.50 mg QE/g of extract) in methanol extract of the leaves of *Meyna spinosa*, an Indian medicinal plant [24].

The antioxidant activity is expressed as IC_{50} values. An extract is more potent if its IC_{50} is weak. In this consideration, ethanol crude extract (IC_{50} = 38.2 µg/mL) is the most active extract compared to DCM (IC_{50} = 175) and ethyl acetate extracts (IC_{50} = 370 µg/mL), even ascorbic acid (IC_{50} = 80.33 µg/mL) used as positive controle. Subsequently, ehanol crude extract has been submitted to column chromatography to efford 10 fractions of whom fraction 6, obtained by eluting with ethyl acetate-methanol (80 :20 and 70 :30) were found to be more potent (IC_{50} = 23.68 µg/mL) than crude extract itself and ascorbic acid. This fraction 6 contains high total flavonoidscontent, and that may justify its good antioxidant activity. Indeed, the antioxidative effectiveness of natural sources has been reported to be mostly due to the presence of phenolic and flavonoid compounds.They are a large group of ubiquitous molecules and possess antioxidant activity. Because of their hydroxyl groups, flavonoids react as radical scanvenger by donating hydrogen atom [24]. Their planar structure, number and position of hydroxyl groups as well as the presence of C2-C3 doubl bond can enhance the free-radical scanvenger capacities.

II.3. Lipid peroxidation inhibition of ethanol crude extract and fractions 6 and 7.

Ethanol crude extract and its fractions 6 and 7 are found to present higher TP and TF contents and also good antioxidant activity. Thus, these samples have been investigated for their lipid peroxidation inhibition.Results are presented in table 4.

Table 4 : Lipid peroxidation inhibition values

Extract	% of inhibition (%)		
	250 µg/mL	500 µg/mL	1000 µg/mL
Ethaol crude extr.	40,14	45,25	54,74
Fraction 6	29.19	33.57	47.15
Fraction 7	23.06	29.26	41.46
Quercetine	62,04	67,15	75,91

We note that ethanol crude extractand its fractions 6 and 7 inhibit lipid peroxydation between 23.06 and 54.74% at concentrations ranging from 250 to 1000 µg/mL, but ethanol crude extract is more potent than its fractions 6 and 7 at any concentration. This could be explained by the fact that inethanol crude extract, a synergetic effect of total flavonoids and other minority compounds could take place. Pure quercetine seems to be more potent than ethanol crude extract and its fractions.But taking in account the results of total flavonoids content, we can note that in 1000 µg of ethanol crude extract and 1000 µg of fraction 6 for example, there are respectively only 40.79 µg and 17.07 µg of QE. That support that flavonoids (quercetin in this case) are not only responsible of the activity of the extract.Some flavonoids, other than quercetin and perhaps more active than quercetin, may be present in *Lippia miltiflora* ethanolic extract.In their review on lippia species, M.E. Pascual et al. [25] mentioned that the phenolic compounds have been less studied ; only some flavonoids (6-hydroxylated and methoxyflavones) have been identified. In our next work, Fraction 6 could be used for isolation of some major flavonoids compounds of *Lippia multiflora* from Burkina Faso.

CONCLUSION

This research work was carried out to investigate total phenolics and total flavonoids contents of different crude extracts of *Lippia multiflora*(hexane, DCM, ethyl acetate and ethanol) as well as their antioxidative activities by DPPH and lipid peroxidation assay. Ethanol crude extract was found to contain higher content of total phenolic and total flavonoids. At 1000 µg/ mL, ethanol crude extract inhibits about 55% lipid peroxidation. A rude fractionation of this fraction afforded a fraction with good antioxidant activity. This fraction may contain some flavonoids which could be more potent than ascorbic acid.

A phytochemical screening showed that tannins, flavonoids, alkaloids,saponins are present in most of the extract.

These preliminary results suggest that *Lippia multiflora*, a medicinal plant largly spread in Burkina Faso, could be a source of natural antioxidant ; investigation on antifungal, anti-inflammatory and anti-cancer activities could be further undertaken on this medicinal plant.

Acknowledgement
Thanks to Government of India through CV Raman fellowship program for providing financial support for this research work.

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