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VALORIZATION OF WASTE FROM HYDRO-DISTILLATION OF *Lippia multiflora* LEAVES INTO SECONDARY METABOLITES AND EVALUATION OF ANTIOXIDANT ACTIVITY

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ABSTRACT

Maceration in an ethanol-water mixture (70-30: v/v) of fresh leaves of *Lippia multiflora* and their hydro-distillation waste leads to the corresponding hydroethanolic extracts with yields of 16.50% and 14.30%, respectively. Hexane, dichloromethane, ethyl acetate, ethanol, and water fractions resulting from the successive fractionation of hydroalcoholic extracts were obtained with yields from 7.48 to 47.07% and from 5.79 to 45.85% for fresh leaves and their hydro-distillation waste, respectively. Phytochemical screening of hydroethanolic extracts and fractions revealed the presence of almost all families of secondary metabolites sought in the two plant matrices except Leucoanthocyanins which were absent from the two samples. Total polyphenol contents decrease with the extraction of essential oil for ethyl acetate (74.93 to 51.2 mg GAE /g of dry extract) and aqueous fractions (51.2 to 49.6 mg GAE /g dry extract). Also, flavonoid contents of fractions gave higher values with waste from hydro-distillation (4.4 to 56.8 mg EQ/g of dry extract) except for ethyl acetate fractions where flavonoid content is higher in the fresh leaves (66.4 to 56.8 mg EQ/g of dry extract). Hydroalcoholic extract of hydro-distillation waste as well as fractions of fresh leaves have the highest antioxidant activities.

Keywords: Hydro-Distillation Waste, Antioxidant Activity, *Lippia Multiflora*, Total Polyphenols, Total Flavonoids, Secondary Metabolites.

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INTRODUCTION

Plants in general and aromatic plants in particular contain secondary metabolites responsible for their pharmacological properties and are widely used in therapy.[1,2] Thus, the leaves of *Lippia multiflora* are used in the African pharmacopeia for the treatment of hepatic insufficiency, fever, diarrhea, arterial hypertension, and malaria.[3,4] Preliminary phytochemical analyses of leaf extracts showed that they contain polyphenols, saponins, flavonoids, sterols and terpenes, alkaloids, and tannins.[5,6] Its hydroethanolic extract has antibacterial activities as well as positive inotropic and chronotropic effects.[7,8] Essential oil from its leaves has several activities such as anti-inflammatory, insecticidal, antioxidant, sedative, and analgesic activities.[9,10] However, after extraction of the essential oil, the hydro-distillation waste which represents more than 98% of the plant organ is very often neglected.[11] However, one of our recent studies showed that hydroethanolic extracts from hydro-distillation waste of *Lippia multiflora* leaves have good antibacterial activity.[7] Unfortunately, phytochemical studies on the hydro-distillation waste of *Lippia multiflora* leaves are almost non-existent despite many works of extraction of its essential oil. The present study therefore aims to valorize the waste from the hydro-distillation of *Lippia multiflora* leaves into secondary metabolites and to evaluate the antioxidant activity in comparison with fresh leaves.

EXPERIMENTAL

Plant Material

Fresh leaves of *Lippia multiflora* were collected in August 2018 in Yamoussoukro (6°54'24.158'' North and 5°19'25.66''West) in the center of Côte d'Ivoire. The Plant material was dried away from sunlight at

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room temperature (28±2°C) for 7 days and divided into 2 batches which were used to prepare the 2 samples.

Sample Preparation

The dry leaves of *Lippia multiflora* and their hydro-distillation waste, obtained after extraction of the essential oil using a Clevenger-type device for 3h, were ground using an IKA M20 electric grinder (France). The obtained powders were sieved using a 0.5 mm mesh sieve and were stored in colored jars at 4°C until further use.

Preparation of Hydroalcoholic Extracts

The hydroalcoholic extracts of the fresh leaves (EHA1) and their hydro-distillation waste (EHA2) were obtained following the protocol of Kassi *et al.*[12] The extractions were repeated three times.

Preparation of Fractions

Hydroalcoholic extracts (EHA 1 and 2) were successively fractionated with hexane, dichloromethane, ethyl acetate, ethanol and water following a protocol similar to that used by Bouamama *et al*. to afford the resulting hexane (FHE), dichloromethane (FDM), ethyl acetate (FAE), ethanol (FET) and aqueous (FAQ) fractions of the leaves and their hydro-distillation waste.[13] The fractionations were repeated three times.

Phytochemical Screening

Phytochemical screening of secondary metabolite families was carried out according to the method of Harbone.[14]

Polyphenols Assays

Total polyphenol contents were measured following the protocol of Wood *et al.*[15] The standard reference was Gallic acid and the total polyphenol contents were expressed in mg of gallic acid equivalent per gram of dry extract (DE) (mg GAE/g DE). The assays were repeated three times.

Flavonoids Assays

Total flavonoid contents were measured following the protocol of Marinova *et al.*[16] The standard reference was Quercetin and the total flavonoid contents were expressed in milligrams of quercetin equivalent per gram of dry extract (DE) (mg QE/g DE). The assays were repeated three times.

Antioxidant Assays

The antioxidant activity, by the 2,2-Diphenyl-1-picryl hydrazyl **(**DPPH) reduction test, was evaluated following the protocol of Sanchez-Moreno *et al.*[17] The standard reference antioxidant used was Ascorbic acid. The assays were repeated three times.

RESULTS AND DISCUSSION

Extraction and Fractionation Yields

Figure-1 presents the yields of the hydroalcoholic extracts (EHA) of *Lippia multiflora* leaves and their hydro-distillation waste as well as those of hexane (FHE), dichloromethane (FDM), ethyl acetate (FAE), ethanol (FET) and water (FAQ) fractions obtained from extracts.

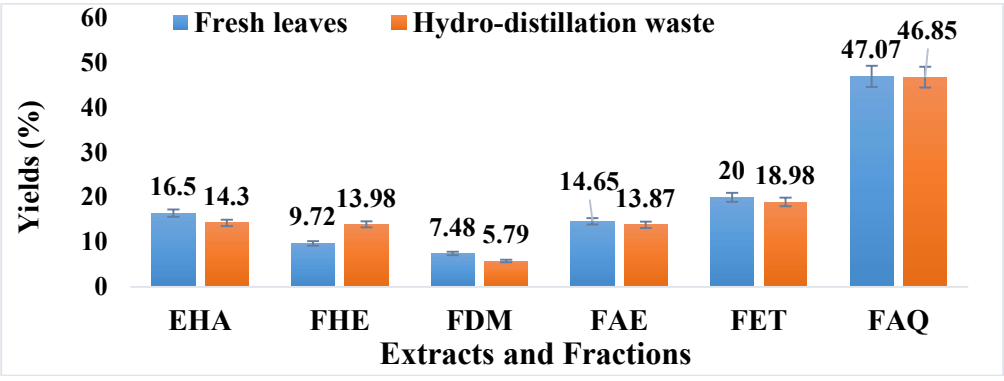

Fig.-1: Yields of Extracts and Fractions from *Lippia multiflora* Leaves and their Hydro-Distillation Waste

Fig.-1 shows that the yield of hydroalcoholic extract from hydro-distillation waste decreases slightly from 16.5 to 14.3%. This trend is similar to that of a previous study.[18] However, our yields, which are slightly higher than those previously reported, could be due to several factors such as the ratio volume of solvent per mass of ground material, the grain size of the ground material, and the maceration time as we reported in the literature.[19] The decrease in the yield of hydroalcoholic extract from hydro-distillation waste is contrary to the results of a similar study carried out on *Hyptis suaveolens* and could be explained by the loss of certain water-soluble molecules in floral water during hydrodistillation.[20] For fractions from hydroalcoholic extracts, only the hexane fraction of the fresh leaves is poorer in compounds than that of the hydro-distillation waste (12.72 and 16.98%). This could be explained by the rise in temperature during hydro-distillation, which transformed organic compounds into other compounds that were more soluble in hexane. Indeed, transformations of organic compounds under the effect of heat during thermal reactions are well known during experiments in gas chromatography coupled with mass spectrometry.[21] In general, the yields of secondary metabolites of the two plant matrices increase with the polarity of the solvents used from dichloromethane fractions (5.79 and 7.48%) to aqueous fractions (45.85 and 47.07%). Ethanol and water, both polar, extracted between 64.61% and 67.07% of the secondary metabolites. The similarity of these results to other studies reported in the literature shows the direct influence of solvent polarity on phytocompound extraction.[20,22] This could be explained by the possibility for water to extract a wide range of molecules including non-phenolic compounds such as carbohydrates and proteins.[23] Fresh leaves of *Lippia multiflora* and their hydro-distillation waste are therefore rich in hydrophilic polar compounds. Extraction of secondary metabolites is almost non-existent on the leaves of *Lippia multiflora* and their hydro-distillation waste despite numerous studies on the extraction of its essential oil. This did not allow us to compare our results with those of the literature.

Phytochemical Screening of Hydroalcoholic Extracts and Fractions

Phytochemical screening of extracts and fractions of *Lippia multiflora* leaves and their hydro-distillation waste is presented in Table-1.

Table-1: Results of Phytochemical Screenings of Hydroalcoholic Extracts and Fractions from *Lippia Multiflora* Leaves and their Hydro-Distillation Waste

Secondary metabolites Families	Fresh leaves						Hydro-distillation waste from fresh leaves					
	EHA	FHE	FDM	FAE	FET	FAQ	EHA	FHE	FDM	FAE	FET	FAQ
Sterols and terpenes	+	+	+	+	+	-	+	+	-	+	+	-
Polyphenols	+	-	+	+	+	+	+	-	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Leucoanthocyanins	-	-	-	-	-	-	-	-	-	-	-	-
Catechic Tannins	+	-	-	+	+	+	+	+	-	+	-	+
Gallic tannins	+	-	-	+	-	+	+	-	+	+	-	+
Alkaloids	+	-	+	+	+	+	+	-	-	-	-	+
Saponins	+	-	-	-	+	+	+	-	-	-	+	+

Where, **+ :** Presence **- :** Absence

All the secondary metabolites families sought are present in hydroethanolic extracts from *Lippia multiflora* leaves and their hydro-distillation wastes except that of Leucoanthocyanins which is absent from both extracts. The presence of the Sterols and terpenes family in the hydro-distillation waste shows that essential oil did not recover all the compounds of this family as previously reported in the literature.[20] Leucoanthocyanins are absent from all fractions while flavonoids are present in all fractions. Polyphenols are present in all fractions except in hexane fractions. Tannins (gallic and catechic), alkaloids, and saponins are present in aqueous fractions while they are absent from hexane fractions except catechin tannins which are present in hexane fractions of hydro-distillation waste. Alkaloids, present in the dichloromethane, ethyl acetate, and ethanol fractions of the leaves, are absent from these fractions after extraction of essential oil. Gallic tannins, absent from dichloromethane fractions of the fresh leaves, are present in hydro-distillation waste. Saponins are present only in ethanolic and aqueous fractions of the two plant matrices. Overall, the results obtained differ slightly from those of Goly who did not detect any alkaloids in its leaves while

Leucoanthocyanins were present in the two plant matrices.[18] This difference could be due to the harvest area known for its impact on the chemical composition of plants.[9] The results obtained show that water extracts, used in the preparation of many traditional treatments, contain all compound families present in hydroalcoholic extracts except sterols and terpenes.

Phenolic Contents

Total phenol contents were determined from the gallic acid calibration line with an R^2 value of 0.9998 (regression coefficient). The results obtained are shown in Fig.-2.

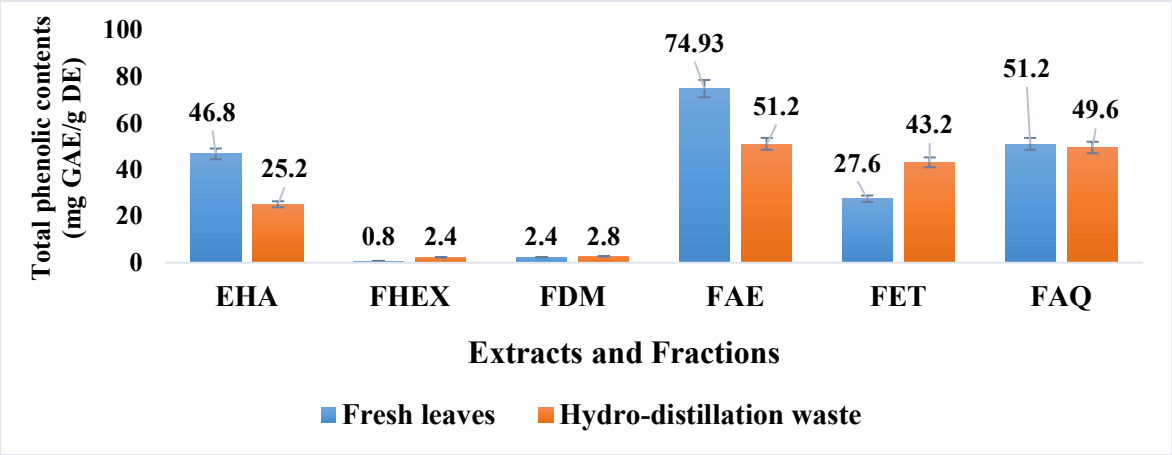

Fig.-2: Total Phenolic Contents of *Lippia multiflora* Leaves and their Hydro-Distillation Waste

Figure-2 shows that the total phenolic contents of hydroalcoholic extract (EHA) from fresh leaves of *Lippia multiflora* (46.8 ± 0.01 mg GAE/g DE) is higher than that of their hydro-distillation waste (25.2 ± 0.01 mg GAE/g DE). This tendency has already been reported and could be linked to the extraction, during hydro-distillation, of certain phenolic compounds by floral water.[20,24] Total phenolic contents of the different fractions vary from 0.8 to 74.93 mg GAE/g DE and from 2.4 to 51.2 mg GAE/g DE for fresh leaves and their hydro-distillation waste respectively. In general, total phenolic contents of fractions increase after hydro-distillation except for ethyl acetate and water fractions. This increase in total phenolic contents agrees with that of Soumahoro et al. (2021) and could be justified by the formation, during hydro-distillation, of genins more soluble in these solvents.[20,25] In both plant matrices, polyphenol contents are higher in ethyl acetate, water, and ethanol fractions. Ethyl acetate is the best extraction solvent, with polyphenol contents for fresh leaves and their hydro-distillation waste of 74.93 ± 0.07 and 51.20 ± 0.01 mg GAE/g DE respectively, as previously reported.[20] Phenolic content of our fresh leaves is slightly higher than that of *Lippa multiflora* leaves harvested in Burkina Faso (71.17± 0.2 mg GAE/g DE) and this difference could be linked to edaphic factors.[26] Water is the second best solvent for extracting phenolic compounds after ethyl acetate with phenolic contents for fresh leaves and their hydro-distillation waste of 51.20 ± 0.01 and 49,60 ± 0.01 mg GAE/g DE respectively. The literature consulted does not mention studies that determine phenolic contents of hydro-distillation waste from the leaves of *Lippia multiflora* to compare them with our results.

Flavonoid Contents

Total flavonoid contents were determined from the Quercetin calibration line with an R^2 value of 0.9999 (regression coefficient). The results obtained are shown in Fig.-3.

Figure-3 shows that the total flavonoid contents of hydroalcoholic extracts from leaves of *Lippia multiflora* and their hydro-distillation waste are 20.4 ± 0.01 and 27.6 ± 0.02 mg QE/g DE, respectively. The increase in flavonoid content in hydroalcoholic extract from hydro-distillation waste could be explained by the release of free flavonoids following the destruction, during hydro-distillation, of the terpene-flavonoid compound complexes present in the essential oils.[20] Flavonoid contents could justify the antibacterial activities of alcoholic extract from hydro-distillation wastes of *Lippia multiflora* leaves as reported by our previous work.[18] Total flavonoid contents of the different fractions of *Lippia multiflora* leaves and their

hydro-distillation waste varies from 3.5 to 66.4 and 4.4 to 56.8 mg QE/g DE, respectively. In general, flavonoids are much more abundant in the hydro-distillation waste except in ethyl acetate fractions where their contents decrease.

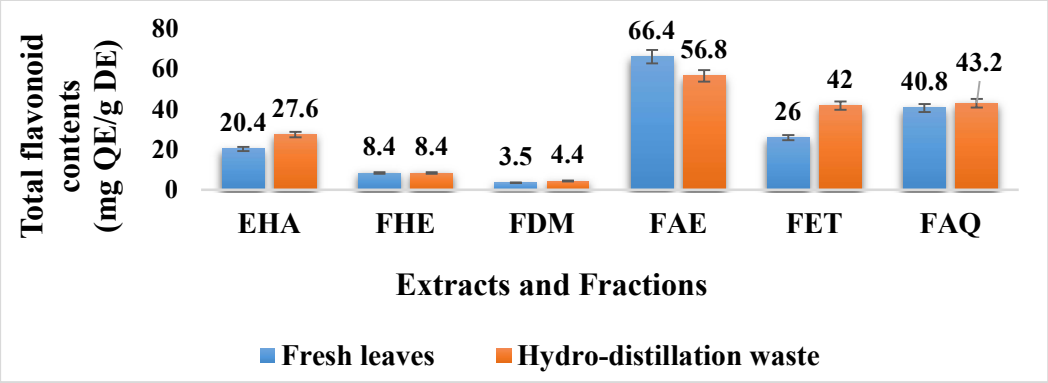

Fig.-3: Total Flavonoid Contents of *Lippia Multiflora* Leaves and their Hydro-Distillation Waste

Ethyl acetate fractions (FAE) of the two plant matrices are the richest in flavonoids (66.40 ± 0.03 and 56.8 ± 0.02 mg QE/g DE) followed by aqueous fractions (FAQ) (40.80 ± 0.01 and 43.20 ± 0.01 mg QE/g DE) and ethanol (FET) (26 ± 0.02 and 42 ± 0.1 mg QE/g DE). Indeed, ethyl acetate is known for its great capacity to extract flavonoids from plants. Also, apolar fractions (Hexane and Dichloromethane) are poor in flavonoids and this confirms the influence of the polarity of solvents during the extraction of flavonoids.[20] The literature consulted does not mention studies that determine flavonoid contents of hydro-distillation waste from the leaves of *Lippia multiflora* to compare them with our results.

Antioxidant Activity

Only hydroalcoholic extracts and polar fractions, with the best polyphenol and flavonoid contents, were evaluated for antioxidant activities during the DPPH test. The results obtained are presented in Fig.-4.

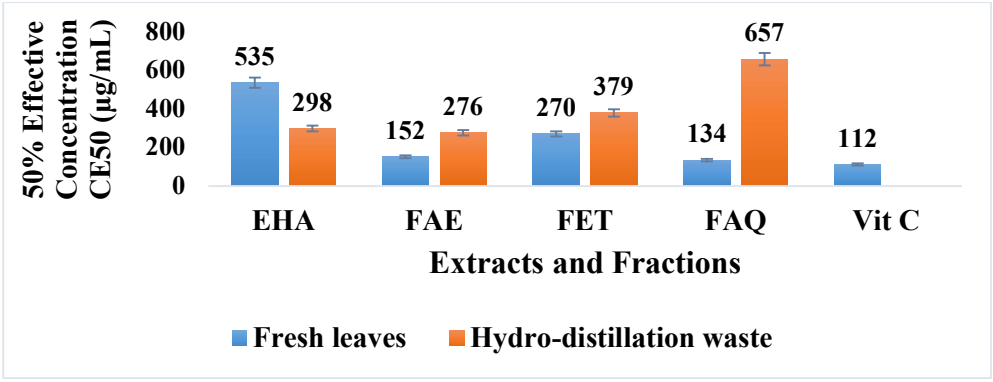

Fig.-4: EC_{50} Values of Hydroethanolic Extracts and Fractions from Fresh Leaves of *Lippia Multiflora* and their Hydro-Distillation Waste

Figure-4 shows that hydroalcoholic extract (EHA) from hydro-distillation waste, with a lower EC_{50} value (298 µg/mL) than that of the fresh leaves (EC_{50} = 535 µg/mL), has higher antioxidant activity. This activity is consistent with its higher flavonoid content and confirms the role of flavonoids in antioxidant activities. The EC_{50} value of the different fractions from fresh leaves of *Lippia multiflora* and their hydro-distillation waste vary from 134 to 270 µg/mL and from 276 to 657 µg/mL, respectively. All fresh leaf fractions were more active than those from hydro-distillation wastes despite the abundance of flavonoids in ethanol and water fractions of hydro-distillation waste. This result could be due to the nature of flavonoids present in hydro-distillation waste which could influence their action mode on DPPH. Indeed, the evaluation of an antiradical property by the DPPH method is based on the ability to give a hydrogen atom or electrons in various compounds and extracts.[27] The antioxidant activity of ethanolic fraction from our fresh leaves is

lower than that of ethanolic extract from *Lippia multiflora* leaves harvested in Burkina Faso.[6] This difference is probably due to the harvest area and extraction method. In all cases, hydroalcoholic extracts and fractions of fresh leaves from *Lippia multiflora* and their hydro-distillation waste are less active than Vitamin C taken as a reference. The literature consulted does not mention studies that determine the antioxidant activity of the hydro-distillation waste from the leaves of *Lippia multiflora* to compare them with our results.

CONCLUSION

A phytochemical study and comparative antioxidant activity of secondary metabolites from fresh leaves of *Lippia multiflora* and their hydro-distillation waste were carried out. Compared to fresh leaves, hydro-distillation waste still contains secondary metabolites particularly polyphenols and flavonoids probably responsible for their antioxidant activities. This waste can therefore be recovered as a source of antioxidants in the food and pharmaceutical industries. The structures of secondary metabolites are currently being determined.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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