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Phytochemical Constituent and Cumulative or Antagonistic Effects of Crops Plant Organ Combination on Free Radical Scavenging Capacity and Antioxidant Compound Content

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In Burkina Faso, the majority of plant-based recipes are a combination of plant organs from the same or different species. For these recipes' producers, the aim is to improve efficiency. However, our ethnobotanical surveys have revealed that for some species, efficacy is better when the species is not combined, or when it is combined in small proportions with other species. The aim of this study is to assess the effect of plant organ combination on free radical scavenging activity, phenolic compound content and flavonoid content.
Study Design: The work combined harvesting and processing of plant material from September to November 2023, and laboratories analysis from January to March 2024 at Natural Substances Departement of Institut de Recherche en Sciences Appliquées et Technologie, Ouagadougou.
Methodology: We prepared two batches of recipes, first combining two species from the same genus, then two species from different genera. The powder of each plant without combination was also used to compare data. Phytochemical screening was first carried out with TLC and LC-MS analysis on each plant extract. We then assessed the phenolic compound content (PCC) using Folin-Ciocalteu method, flavonoid content (FCC) using aluminum chloride test, antioxidant content using TEAC method and free radical scavenging activity using DPPH method, for different batches.
Results: Phytochemical constituent in the three species are phenolic compounds, terpenoids and nitrogen derivatives. The results of biological property evaluation suggest that for best efficacy and to obtain a high phenolic compound content, *Lippia multiflora* leaves should be used separately, or combined in 70% proportions with *Lippia alba*, then in 50% proportions if the combination is made with *Ocimum basilicum*.
Conclusion: This research has provided relevant results to guide players in the field of traditional medicine and users of herbal remedies.

Keywords: Phytochemistry; free-radical properties; plants organ combination; herbal recipes.

1. INTRODUCTION

Traditional medicine is currently the first choice for many different disease's treatment in low-income countries (Abdoulaye et al. 2020). Plants and their organs are used alone or in combination with organs of the same or different species (Constantin et al. 2015). In addition, the whole plant may be used separately or in combination with two or more other medicinal plants to improve the efficacy of the prepared product (Constantin et al. 2015). Our investigations in the field have shown that this use of plants is due either to the inaccessibility of pharmaceutical products, or to their ineffectiveness in certain diseases, or to a lack of financial resources. The association of several plants or plant organs would therefore be justified by the unequal distribution of bioactive compounds in the various organs due to different physiological and their biosynthesis processes (Constantin et al. 2015). However, interactions between chemical molecules are more often antagonistic than synergistic (Jianhua et al. 1995). Also, failure to master these combinations, in which the different organs or plants must be combined, can lead to a reduction in the efficacy sought. In fact, some molecules in high doses can have the opposite effect (Kailiu 2021). The aim of this study is to examine how the combination of these two medicinal plants (*Lippia multiflora/Lippia alba* or *Lippia multiflora/Ocimum basilicum*) affects free radical scavenging activity, phenolic compound content and flavonoid content. Despite the numerous studies carried out (from 2002 to 2024) on the Lippia genus, with most focusing on essential oils (Nébié et al. 2002, Hema et al. 2024), the first research on solvent extracts was carried out by our research team since 2015 (Constantin et al. 2015). This work is a continuation of previous work during which the phenolic compound content, DPPH radical scavenging activity and lipid peroxidation inhibition test of ethanolic crude extracts of *Lippia multiflora* and their fractions were evaluated. This work is part of a continuing effort to help the traditional practitioners we support to improve their recipes.

2. MATERIALS AND METHODS

2.1 Plant Material

Plant material included the leaves of *Lippia multiflora* (Gambia tea), *Ocimum basilicum* and *Lippia alba* (Verbena). These species were grown between September and November 2023 in an experimental garden (12°25'28.2'' N; 1°29'15.06'' W) of Institut de Recherche en Sciences Appliquées et Technologie (IRSAT)

(Ouagadougou). Young seedlings of each species were paid at the Centre National de Sémences Forestières (CNSF). Each plant leaves were dried separately in the shade at room temperature for 15-45 days. The individual organs were ground and the different powders were used for combination elaboration.

2.2 Formula Elaboration

The aim was to prepare different combinations of plants organs with the powders of both species in well determined proportions. The denomination of each combination and their combined proportions are grouped in Table 1. In all, 2 grams of each combination was prepared.

2.3 Chemicals Reagents

Chemical material was reagents, analytical grade solvents and chemical analysis equipment. For reagents, we have: Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenz-thiazoline-6- sulfonic acid (ABTS), Folin-Ciocalteu's reagent, aluminum chloride and ascorbic acid (all from Sigma-Aldrich (St. Louis, MO). Solvents were methanol (MeOH or CH_3OH), hydrochloric acid (HCl), formic acid (HCOOH), acetic acid (CH_3COOH), and water (H_2O). Chemical analysis equipment was as follows: BUCHI rotary evaporator, SAFAS spectrophotometer, CAMAG® automatic sample dispenser (LINOMAT 5) and silica gel TLC plates 60 F_{254}.

2.4 Extraction

Maceration extraction technique has been used (Constantin et al. 2015). This technique is based on free molecules diffusion through the vacuolar membranes due to their solubility or affinity with various solvents. In practice, 2 g of powder of each combination immersed in 15 mL of ethanol 96 ℃ acidified with 2 % acetic acid (14.7 mL of ethanol+0.3 mL of acetic acid 80 %) and placed under mechanical mixing for 24 h. After filtration, on Wathmann paper N°3, the residue is taken twice in the same solvent. The collected filtrate was concentrated under vacuum to the minimum solvent using a BUCHI rotary evaporator. The dry extracts obtained were kept in a refrigerator for further analysis.

2.5 Chemical Compounds Characterization

Phytochemical screening was carried out using TLC tests described by Hildebert et al. 2016 combined with LC-MS described by Hutabarat et al (2019) with minor modifications.

A **B** **C**

Fig. 1. *Lippia alba* (A) *Lippia multiflora* (B), *Ocimum basilicum* (C) species

Table 1. Abbreviations, proportions and masses of the different combinations

Combination Code		*L. multiflora* proportion in each combination (%)
Batch 1	**Batch 2**	
LV1	LB1	0
LV2	LB2	30
LV3	LB3	40
LV4	LB4	50
LV5	LB5	60
LV6	LB6	70
Lm		100

Batch 1: Combination of L. multiflora and Lippia alba or Verbena (Lippia/Verbena, (LV)) Batch 2: Combination of L. multiflora and Ocimum basilicum or Basilic (Lippia/Basilic, (LB))

For TLC, the HP-TLC was used and 10 µl of each extract at 20 mg/ml was applied to a silica gel plate in the form of a 5 mm long strip using a CAMAG Linomat 5 semi-automatic TLC autosampler. After application, the plates were developed upwards in a pre-saturated glass double-trough chamber for 30 minutes with a mobile phase consisting of the solvent system Ethyl Acetate/Acetic Acid/Formic Acid/H_2O (100:11:11:26). The length of the chromatographic run was 70 mm from the bottom edge of the plate. After development, the plates were dried using a hair dryer. Flavonoids were detected using Neu's reagent. Quercetin and rutin were used as standards.

For LC-MS analysis, the chromatograph used for separation is an Agilent Technologie HPLC 1290 system coupled to a binary pump, MS detector, autosampler and MassHunter workstation. Separation was performed on a ZORBAX 15 cm C18 column. An elution gradient was applied with a binary system composed of: phase A (H_2O-HCOOH 2% v: v) and phase B (acetonitrile 100 %). The flow rate was 0.8 ml/min at 25°C, and the gradient was programmed as follows: from 0-5 min, 5% B isocratic; 5-40 min, 5 to 95 % B; from 40-42 min, 95% B isocratic; and from 42-45 min, back to 5% B. Analysis time is therefore 45 mins.

2.6 Free Radical Scavenging Properties Determination

Free radical scavenging properties of different tea formulations was measured using the DPPH method, first described by Brand-Williams et al. 1995 but modified for microplates (Rémy et al. 2023). This method is a colorimetric method based on the Beer-Lambert law. The 2,2-diphenyl-1-picryhydrazyl radical (DPPH) reduced by the antioxidant compound has a maximum absorption wavelength at 515 nm.

Protocol consists to mix 200 µL of DPPH reagent (methanolic solution with concentration 0.04 mg/mL) with 50 µL of standard (to establish the calibration curve) or each extract at different concentrations in microplates. After 10 min of incubation at room temperature, the absorbance of the yellowish colored mixture is read at 515 nm against a blank consisting of the different solvent used to prepare the extracts and the reagent. The antiradical capacity is determined by calculating the IC_{50} (concentration of a sample reducing 50 % of the DPPH radicals).

2.7 Phenolic Compounds Quantification

Phenolic compounds content (PCC) is measured according to Folin-Ciocalteu method modified for microplates (Rémy et al. 2023, Nihal et al. 2007). The method consists to mix 60 µL of sample (standard or extracts) with 60µL of Folin-Ciocalteu reagent (RFC) diluted tenfold. After 8 min, 120 µL of a 7.5% (w/v) aqueous Na_2CO_3 solution is added to neutralize the residual reagent (RFC). Absorbances are measured at 765 nm after 30 min incubation at 37°C. PCC values, determined from the equation for the gallic acid curve (used as a standard), are expressed as µg gallic acid equivalent per milligram of extract (µg GAE/mg).

2.8 Flavonoids Compounds Quantification

To assess total flavonoid content in extracts, a colorimetric method described by Zhishen et al (1999) was used, with some modifications for microplate applications (Rémy et al. 2023, Zhishen et al. 1999). In practice, a range of dilutions, from 2.5 to 0.019 mg/mL, were prepared with 50 µL of a 5 mg/mL solution of each extract in the microplates. Then 150 µL of bi-distilled water and 15 µL of an aqueous solution of sodium nitrite (concentration 0.05 mg/mL) were added to each well. After a waiting time of 5 minutes, 15 µL of an aqueous aluminum trichloride solution (concentration 0.1 mg/mL) is added to the previous mixture. This new mixture is kept at room temperature during 6 minutes before adding 50 µl of a 1 mol/L sodium hydroxide solution. A pinkish solution is formed and the absorbance of this mixture is read at 510 nm with a 96-well spectrophotometer (spectrophotometer MP96, SAFAS). The flavonoid content of extracts, expressed in mg quercetin equivalent per gram extract, was calculated by reporting absorbances on a reference curve established with quercetin under the same conditions as described in the previous section.

2.9 Antioxidant Compounds Quantification

Antioxidant compounds content (ACC) of extracts was evaluated using ABTS reagent method first reported by Miller et al. 1993. This method is a colorimetric method based on the radical-cation reduction by antioxidant compounds present in extracts. This radical-cation is first generated by mixing 10 mL of aqueous solution of potassium persulfate $K_2S_2O_8$

(39.2 mM) and 50 ml of aqueous solution of ABTS (7.01 mM). The mixture was placed in the refrigerator during 16 hours (minimum time to generate the radical-cation).

For the assay, the ABTS cation-radical solution must have an absorbance value between 0.3 and 0.7 ± 0.02 at 734 nm. If this is not the case, the solution is diluted with distilled water to get a good absorbance before use. In practice, to 50 µL of a range of extract solution or standard (concentration varying between 0.006 - 0.5 mg/mL) have been added to 120 µL of ABTS solution. After 10 min of incubation at room temperature; the absorbance was read at 734 nm. The standard used were Trolox. The absorbance was related to the calibration curve established with Trolox ($y = -19.41x + 0.7197$; $R^2 = 0.9953$). Total Antioxidant Contents (TAC) was expressed as µg Trolox Equivalent per mg of extract (TE/mg).

2.10 Statistical Analysis

Statistical analysis was performed using R software. The results are presented as mean ± standard deviation calculated at the probability limit less than or equal to 95%.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Chemical compounds characterization

The TLC profile (Fig. 2) shows blue, dark yellow, light yellow and red spots at 366 nm after spraying with Neu's reagent. These fluorescent spots could be flavanol and isoflavanone derivatives (blue), favonol derivatives (light and dark yellow) and terpenoids (red or blue) (Do et al. 2020, Hugues et al. 2011, Enot et al. 2022, Singh et al. 2021). We also observe that dark yellow spots have the same frontal reference as quercetin used as a standard. The results of phytochemical screening with TLC are summarized in the Table 2 and Table 3.

Data analysis shows that extracts from threes plants contain the major antioxidants recognized (flavonoids, terpenoids, etc.) (Constantin 2015, Rémy et al. 2023, Hugues et al. 2011, Singh et al. 2021). Extracts of the two species of Lippia genus contain mainly flavanol, isoflavanone and favonol derivatives. However, extracts of *O. basilicum* contain terpenoid or flavanol/flavanone derivatives. Molecular affinity with solvent and maceration time could explain the difference in chemical composition of the samples. HPLC analysis coupled with mass spectrometry was used to determine some molecular ions of the compounds contained in these samples.

Total ion chromatograms (TIC) (Fig. 2) show intense peaks at Rt= 31.49 min and 36.96 min in *Lippia multiflora* extract and at Rt= 4.47 min and 24.91 min in *O. basilicum* extract. Their mass spectra show that the majority peaks at Rt= 31.49 min and 36.96 min in *Lippia multiflora* extract are mono and diglucosilated flavonol derivatives. In contrast, the majority peaks at Rt= 4.47 min and 24.91 min in the *O. basilicum* extract are terpenoids LC-MS analysis of plant extracts confirms the TLC profile. Fig. 3 shows the mass spectra of the majority peaks in the chromatograms of the various extracts.

Only precursor ions were collected. Molecular ions and their suggested molecules are listed in Table 4 (*Lippia multiflora*); 5 (*Ocimum basilicum*); 6 (*Lippia alba*).

Table 2. TLC data (Front reference: Rf; Fluorescence under UV-365 nm) of the crude extract and standards

	Plants			Standards	
	Ocimum basilicum	***Lippia multiflora***	***Lippia alba***	**Quercetin**	**Rutin**
Numbers of spots	2	3	4	1	1
Front reference	0.83; 0.93	0.5; 0.83; 0.93	0.25; 0.5; 0.83; 0.93	0.83	0.25
Fluorescence under UV-365 nm after spraying	Blue and red	Blue; Dark yellow and Red	Blue, blue and dark yellow, pink	Light yellow	Dark yellow

Table 3. Chemical groups of plant leaves extracts

Plants	Flavonoids	Tannins	Saponins	Terpenoids	alkaloids
Lippia multiflora	+	+	+	-	-
Lippia alba	+	-	-	+	+
Ocimum basilicum	+	-	+	+	+

Table 4. Molecular or fragment ions of individual peaks of Total Ion Chromatograms (TIC) of *L. multiflora* extract

Pic Nº	Rt (min)	Molecular or Fragment ions ($[M+H]^+$, $[M]^+$, $[M+H-H2O]^+$) $[M+Na]^+$	Suggested names of molecular (Formula)
1	5.35	955.4 ; 817.6 809.5 ; 644.3 ; 610.2 ; 503.0; 483.3 ; 448.1 ; 443.1 ; 427.1 ; 389.1 ; 373.1 ; 329.1 ; 278.9	Retigeric acid B ($C_{30}H_{46}O_6$) ; Isoorientin-7-O-[6-sinapoyl] -glucosid ($C_{21}H_{20}O_{11}$) ; Retigeric acid B -diglucosid-arabinosid ($C_{48}H_{74}O_{19}$) ; Cinnamic acid ($C_{10}H_{12}O_3$)
2	8.16	627.2 ; 609.4 ; 595.3 ; 464.1 ; 462.2 ; 448.1	Delphinidin-3,5-diglucosid ($C_{27}H_{31}O_{17}^+$) ; Malvidin 3,5-diglucosid ($C_{29}H_{35}O_{17}^+$) ; Peonidin 3-O-rutinosid ($C_{28}H_{33}O_{15}^+$) ; Cyanidin 3-rutinosid ($C_{27}H_{31}O_{15}^+$)
3	13.25	732.4 ; 537.2 ; 463.2	Peonidin 3-O-glucosid ($C_{22}H_{23}O_{11}^+$) Manassantin A ($C_{42}H_{52}O_{11}$)
4	16.84	671.2 ; 508.1 ; 495.2 ; 361.0 ; 346.0 ; 331.04	Gossypetin-3,8,3'-trimethylether ($C_{18}H_{16}O_8$) ; Malvidin-3-galactosid ($C_{23}H_{25}O_{12}$) ; Spinacetin-3-gentiobiosid ($C_{29}H_{34}O_{18}$)
5	20.09	975.5 503.3 459.3 ; 391.1 ; 313.1 ; 369.18 ; 291.1 ; 283.1	Madecassosid ($C_{48}H_{78}O_{20}$)
6	26.62	935.2 ; 788.1 ; 626.1 ; 611.1 ; 463 ; 465.0 ; 465.2 ; 463 ; 447.1 ; 441.1 ; 419.0 ; 420.2 ; 303 ; 302 ; 288.0 ; 288.1	Eriodictyol-7-glucuronid ($C_{21}H_{20}O_{12}$) ; Glucuronid-estriol ($C_{24}H_{32}O_9$) ; Quercetin-3-p-coumarylsophorosid-7-glucosid ($C_{42}H_{46}O_{24}$) ; Rutin ($C_{27}H_{30}O_{16}$) ; Grandidentosid or hydroxycinnamic acid ($C_{21}H_{28}O_{10}$)
7	31.49	565.1 ; 539.0 ; 397.3 ;	Cupressuflavon ($C_{30}H_{18}O_{10}$) ; Apigenin glucosid arabinosid ($C_{26}H_{28}O_{14}$) ; 2',4,4',6'-Tetramethylether-3'-prenylchalcon ($C_{24}H_{28}O_5$)
8	36.96	683.1 ; 615.2 ; 595.2 ; 593.2 ; 486.3 ; 437.2 ; 391.1 ; 355.2 ; 303.1	Apigenin-di-C-glucosid ($C_{27}H_{30}O_{15}$) ; Quercetin-dirhamnosid ($C_{27}H_{30}O_{15}$) ; Robinin ou Kaempferol-3-O-galactosid-rhamnosid-7-O-rhamnosid ($C_{33}H_{40}O_{19}$) ; Kaempferol-O-neohesperidosid ($C_{27}H_{30}O_{15}$) ;
9	40.89	637.1 ; 609.1 ; 593.1 ; 581.1 ; 449.1 ; 551.3 ; 461.2 ; 299.1 ; 286.1; 300.1 ; 391.2 ; 464.3 ; 302.1	Kaempferol-3, 5-diarabinosid-5''-acetate ($C_{30}H_{34}O_{18}$) ; Rhamnocitrin 3-rutinosid ($C_{28}H_{32}O_{15}$) ; Cyanidin 3-sambubosid ($C_{26}H_{29}O_{15}^+$) ;

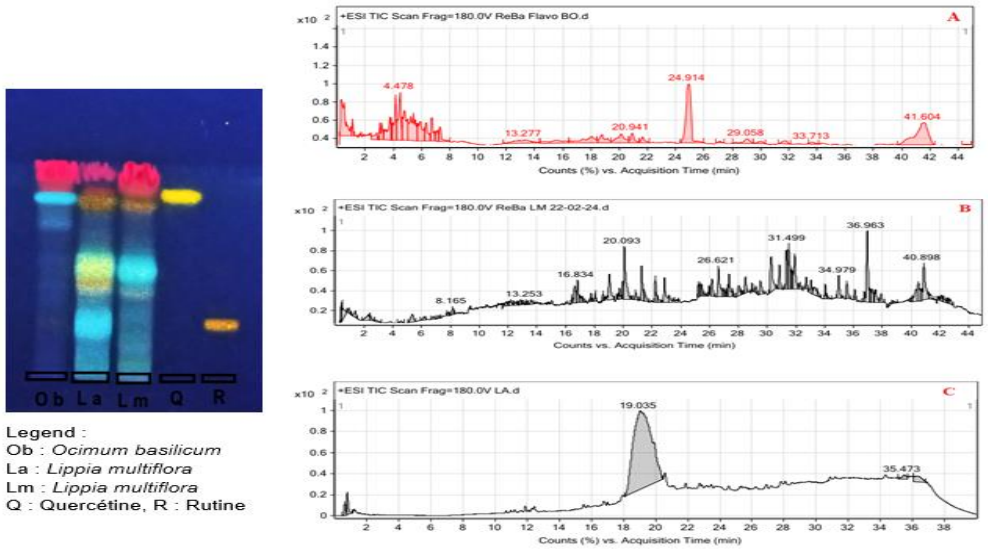

Fig. 2. TLC plate of three species extracts and TIC of *O. basilicum* extract (A), *L. multiflora* extract (B), *L. alba extract* (C)

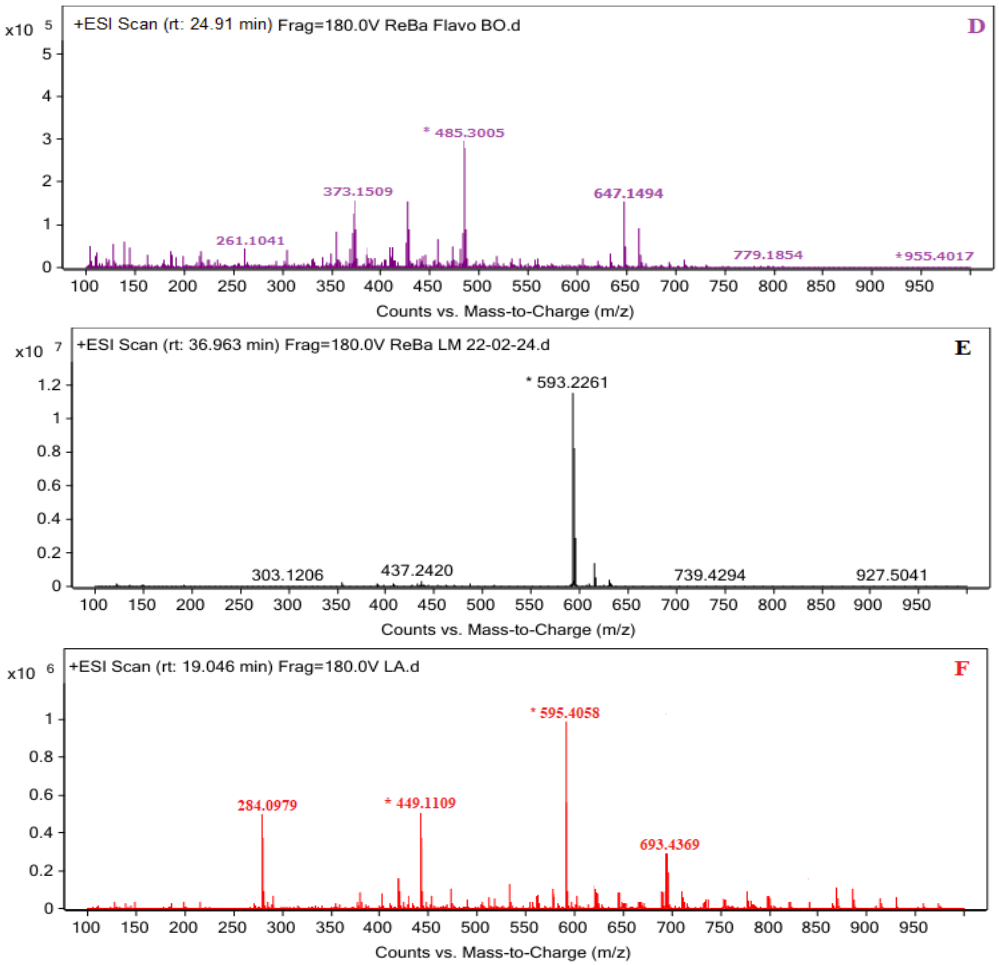

Fig. 3. Mass spectra of major peaks of *O. basilicum* extract (D), *Lippia multiflora* extract (E), *Lippia alba extract* (F)

Table 5. Molecular or fragment ions of individual peaks of Total Ion Chromatograms (TIC) of *O. basilicum* extract

Pic Nº	Rt (min)	Molecular or Fragment ions ($[M+H]^+$, $[M]^+$, $[M+H-H2O]^+$)	Suggested names of molecular
1	4.47	1223.6 ; 1060.5 ; 1089.5 ; 912.4 ; 897.3 ; 678.3 ; 633.3 ; 501.3 ; 472.3 ; 456.3	Sapogenins glycosides ($C_{58}H_{94}O_{27}$) ; Isocyclamin ($C_{58}H_{94}O_{27}$)
2	13.27	451.2 ; 439.2 ; 408.2 ; 376.1	6-Glucosyl-3,4',5,7-tetrahydroxyflavanon ($C_{21}H_{22}O_{11}$) ; Licorisoflavan A ($C_{27}H_{34}O_5$)
3	20.94	773.2 ; 627.1 ; 609.1 ; 464.0 ; 448.0 ; 301.0	Kaempferol-3-sophorosid-7-glucosid ($C_{33}H_{40}O_{21}$) ; Delphinidin-3,5-diglucosid ($C_{27}H_{31}O_{17}^+$) ; Kaempferol-3-glucosid ($C_{21}H_{20}O_{11}$)
4	24.91	955.4 ; 647.1 ; 485.3 ; 440.3	Chiisanosid ($C_{48}H_{74}O_{19}$) ; Ciisanogenin ($C_{30}H_{44}O_5$)
5	29.05	569.4 ; 551.4 ; 534.4 ; 429.3	Lutein or Zeaxanthin ($C_{40}H_{56}O_2$)
6	33.71	671.2 ; 595.2 ; 508.1 ; 448.1 ; 286.0 ; 346.0	Spinacetin 3-gentiobiosid ($C_{29}H_{34}O_{18}$) ; Luteolin-5-o-rutinosid ($C_{27}H_{30}O_{15}$)
7	41.60	ND	ND

Table 6. Molecular or fragment ions of individual peaks of Total ion chromatograms of *L. alba* extract

Pic Nº	Rt (min)	Molecular or Fragment ions ($[M+H]^+$, $[M]^+$, $[M+H-H2O]^+$)	Suggested name of molecular (Formula)
1	0.82	580.2 ; 550.1 ; 541.2 ; 425.6 ; 404 ; 391.2 ; 374.1	Digoxigenin ($C_{23}H_{34}O_5$) ; Cymarigenen ($C_{23}H_{32}O_6$), Fumitremorgin A ($C_{32}H_{41}N_3O_7$) ; 11-cétocyclopamine ($C_{27}H_{39}NO_3$)
2	11.88	611.6 ; 610.6 ; 464.4 ; 567.5 ; 370.2 ; 272.25	Neohesperidin ($C_{28}H_{34}O_{15}$) ; Quercetin 3-galactosid ($C_{21}H_{20}O_{12}$) ; Peonidin 3-rutinosid ($C_{28}H_{33}O_{15}$+) ; 6, 8 diarabinose-5, 7, 3'-trihydroxyflavone ($C_{25}H_{26}O_{15}$) ;
3	15.49	752.2 ; 593.4 ; 445.6 ; 336.2	Ginsenosid ($C_{30}H_{52}O_2$) ; Kaempferol 3-[6''-(3-hydroxy-3-methylglutaryl) glucosid] ($C_{27}H_{28}O_{15}$) ; 4',5,7-Trimethoxyflavanone ($C_{18}H_{16}O_5$)
4	16.72	683.2 ; 639.1 ; 611.1 ; 492.0 ; 447.1 ; 331.0 ; 284.0	Kaempferol-3,7-di-o-glucosid ($C_{27}H_{30}O_{16}$) ; Haploside D ($C_{30}H_{34}O_{18}$) ; Rutin ($C_{27}H_{30}O_{16}$) ; pentamethoxyflavone ($C_{20}H_{20}O_7$)
5	19.07	595.4 ; 464.5 ; 447.1 ; 452.3 432.0 ; 433.4 ; 372.4 ; 284.0	Kaempferol 3-O-arabinoside-7-O-rhamnosid ($C_{27}H_{30}O_{15}$) ; Quercetin 3-glucosid ($C_{21}H_{20}O_{12}$) ; epicatechin-O-glucosid ($C_{21}H_{24}O_{11}$) ; Apigenin 7-glucosid ($C_{21}H_{20}O_{10}$)
6	35.52	649.4 ; 239.1 ; 186.2; 138.08	ND
7	36.27	605.4 ; 186.2 ; 138.1	ND

Molecular ion analysis suggests that extracts from all three species contain phenolic compounds (flavonoids and phenolic acids), terpenoids and nitrogen derivatives. Indeed, analysis of LC-MS mass spectra (Fig. 3 and Tables 4, 5 and 6) shows molecular ions and/or fragments of known terpenoids and flavonoids. The different masses were assigned on the basis of spectral analysis and literature data. Although we didn't do MS/MS of individual molecules, the literature allowed us to give the position of some sugars on the aglycone. Indeed, during flavonoid biosynthesis, the order of attachment of sugars to the aglycone is well known (Singh et al. 2021, Fumi et al. 2012, Hutabarat et al. 2019, Latif et al. 2007, Wagner 1996). In addition, binding

considers the molecular weight, polarity and stability of the molecule (Qingguo et al. 2005). After chemical group identification, some compounds such as phenolic compounds in general and flavonoids in particular were quantified.

3.1.2 Phenolics and flavonoids compounds quantification (PCC and FCC)

Phenolic and flavonoid content were assessed by Folin Ciocalteux Reagent (RFC) and aluminum trichloride methods respectively. Values were calculated by plotting Optical Density (OD) against the equations of the etalon curves preestablished with gallic acid ($y = 7.0356x + 0.03$; $R^2 = 0.9999$) (Fig. 4A) (for phenolic compound content) and with quercetin ($y = 0,2911x + 0,025$; $R^2 = 0,9929$) (for flavonoid content) (Fig. 4B). For flavonoid content, values were expressed in µg Quercetin Equivalent (µg QE/mg) per mg extract, and for phenolic compound content, values were expressed in µg Gallic Acid Equivalent (GAE) mg extract (µg GAE/mg). All values were grouped together in Table 7. Data analysis showed that *Lippia multiflora* leaves were more phenolic (132.482±0.656 µg GAE/mg) than *Lippia alba* (62.131±3.695) and *Ocimum basilicum* (39.746±1.833). A similar observation was made with the values obtained for the quantification of flavonoids.

Table 7 data analysis shows also, for Batch 1, that total phenolic content varies from 62.131±3.695 µg GAE/mg (LV1; 100 % *Lippia alba*) to 159.205±2.709 µg GAE/mg (LV6). Flavonoid content ranged from 57.775±3.331 µg QE/mg to 124.086±1.025 µg QE/mg. The highest flavonoid (124.086±1.025 µg QE/mg) and total phenolic compound (159.205±2.709) contents were obtained in the blend containing 70 % *Lippia multiflora* (LV6). Except LV4 (Combination containing 50 % *Lippia multiflora*), the evolution of the different contents followed the proportion of *Lippia multiflora* in the formulations. Indeed, the LV4 blend showed a lower phenolic compound content (118.193±7.228) than the LV3 proportion (123.304±3.359) containing 40 % *Lippia multiflora.*

For batch 2, data show that phenolic compound values range from 39.746±1.833 to 138.042±0.153 µg GAE/mg, and flavonoid values from 40.433±1.001 to 143.33±8.528 µg QE/mg (Table 7). The best phenolic compound value (138.042±0.153) was obtained with LB6 (70 % *Lippia multiflora*). This represents an increase of 4.54 % compared with Lm (100 % *Lippia multiflora*). In terms of flavonoids, contents were improved with LB6 (143.33±8.528) and LB4 (119.635±3.624) combinations.

A curve of phenolic compound and antioxidant content variations has been established.

An analysis of these curves shows that, depending on the proportions in which species of the same or different botanical genera are combined, PCC values are generally lower than initial levels, although PCC values are higher in some cases (LB4, LB6 and LV6).

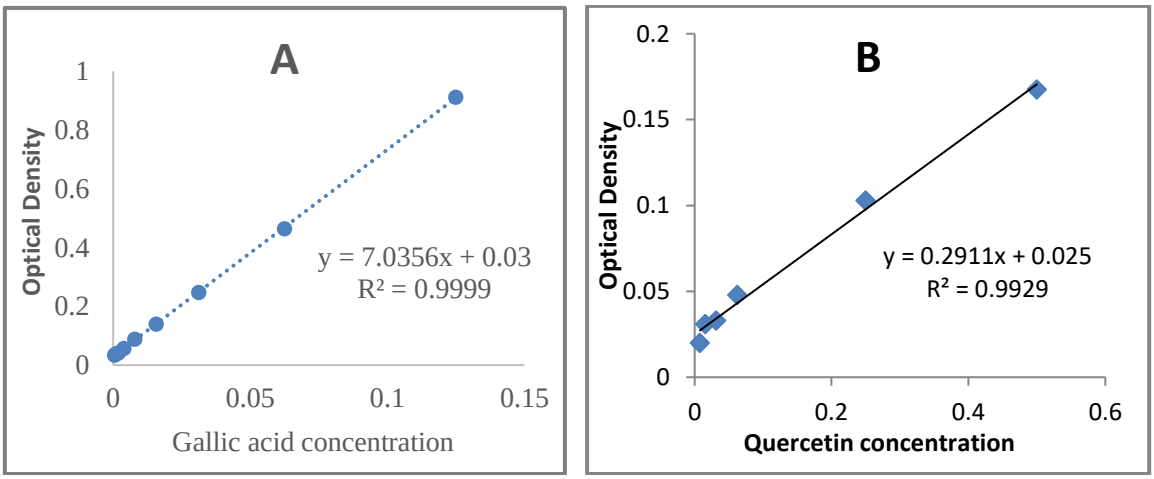

Fig. 4. Gallic acid standard curve (A) Quercetin standard curve (B)

Table 7. Data of FRSP, PCC, ACC and FCC

Extracts		FRSP (mg/ml)	PCC	ACC	FCC
Batch 1	LV1	5.605±0.876	62.131±3.695	14.213±0.082	57.775±3.331
	LV2	2.504±0.007	106.85±4.408	27.021±0.565	90.563±2.562
	LV3	1.758±0.005	123.304±3.359	31.169±0.162	93.916±2.306
	LV4	1.903±0.005	118.193±7.228	30.166±0.113	100.983±3.971
	LV5	0.254±8E-04	129.285±2.164	33.843±0.716	106.872±2.690
	LV6	0.208±6E-05	159.205±2.709	36.612±0.47	129.086±1.025
Batch 2	LB1	0.494±0.027	39.746±1.833	23.771±0.376	40.433±1.001
	LB2	0.887±0.003	50.530±0.769	27.579±0.345	75.712±1.595
	LB3	1.127±0.014	74.257±1.106	17.456±0.642	87.298±1.007
	LB4	0.682±0.023	93.130±1.617	100.445±5.422	119.635±3.624
	LB5	0.783±0.047	71.176±0.939	23.662±1.175	102.171±4.540
	LB6	1.441±0.031	138.042±0.153	41.384±1.504	143.33±8.528
Lm		0.896±0.020	132.482±0.656	34.536±0.565	113.938±3.203
Trolox		0.012±3.82E-05	ND	ND	ND
scorbic acid		0.0783±0.096	ND	ND	ND

Free Radical Scavenging Properties (FRSP); Phenolic Compounds Content (PCC); Antioxidant compound content (ACC); Flavonoids compounds content (FCC); No Determined (ND)

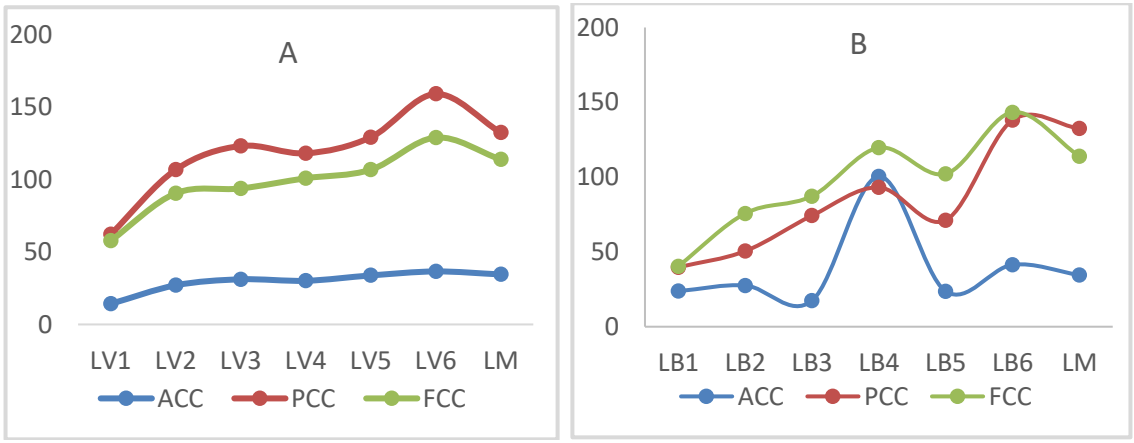

Fig. 5. Phenolic and antioxidant compound content trends for Batch 1 (A) and Batch 2 (B)

For batch 1, results showed that a blend containing 30 % *Lippia multiflora* (LV2) increased phenolics and flavonoids by an average of 60 % over *Lippia alba* values (Fig. 5A and Table 7). In contrast, 30% *Lippia alba* produced an average increase of 15 % at all levels compared with 100 % *Lippia multiflora* (Fig. 5A and Table 7). Thus, phenolic content evolved in line with the proportion of *Lippia multiflora* in the mixture. However, following the *Lippia multiflora* values, only the association of 30 % *Lippia alba* leaves with *Lippia multiflora* leaves resulted in a 20.72 % increase in phenolic compound content (Fig. 5A and Table 7).

Our data shows, for batch, that an addition of 30 % *O. basilicum* increases *Lippia multiflora* phenolic compound content to 4.54 %, flavonoid content to 20.58 % and antioxidant compound content to 26.54 % while equally mixing both plants results in a 20% increase in flavonoid content (Fig 5A and Table 7).

3.1.3 Free Radical Scavenging Properties (FRSP) determination

FRSP of different combinations was evaluated by calculating IC_{50}s. IC_{50} is the concentration of an extract capable of inhibiting 50% of DPPH free radicals. The values, expressed in units of mass concentration (mg/mL), are shown in the table (Table 7).

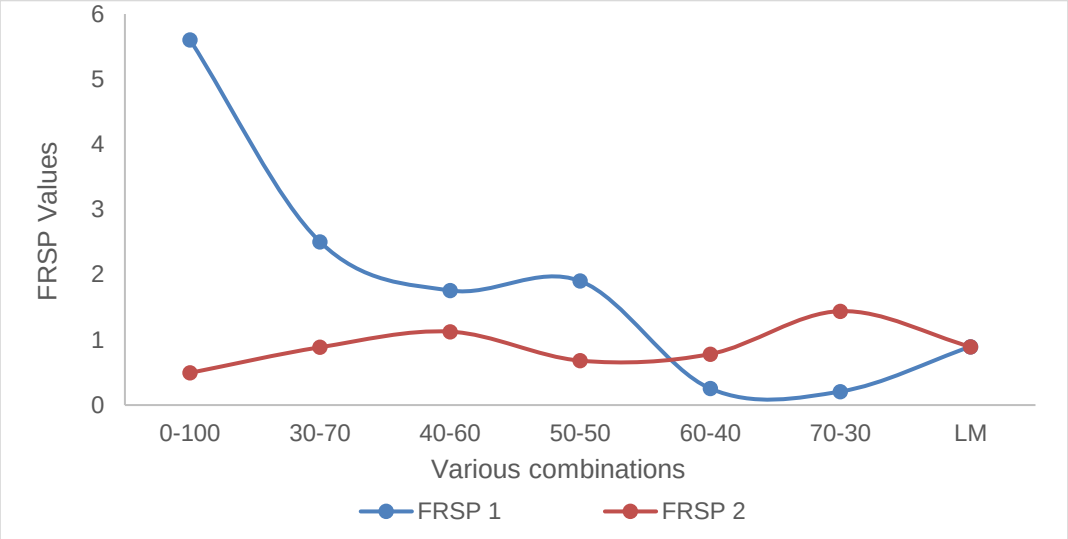

Fig. 6. Free radical scavenging activity trends for combinations
Free Radical Scavenging Properties (FRSP) of batch 1 (FRSP 1) and batch 2 (FRSP 2)

Data analysis shows that FRSP values range from 0.208±6E-05 mg/mL to 5.605±0.876 mg/mL for batch 1 and from 0.494±0.027 to 1.441±0.031 mg/mL for batch 2. As a reminder, progressively lower IC_{50} values indicate higher extract activity.

Thus, the LV6 combination showed the best free radical scavenging activity (0.2084±6E-05 mg/mL). This value is relatively close to that of LV5 (0.254±8E-04 mg/mL). However, LV6 is 2 times less active than ascorbic acid and 20 times less active than Trolox (0.012±3.828E-05 mg/mL), two reference antioxidants. Overall results show that FRSP increases with the proportion of *Lippia multiflora* in formulation for batch 1 (Fig. 4). The lowest value was observed in the LV1 combination (100 % *Lippia alba*). An excellent antiradical activity could be observed for a combination LV5 and LV6 (Fig. 6).

3.1.4 Antioxidant compounds quantification

Antioxidant levels assessed using the TEAC method ("ABTS" radical cation method) are presented in Table 7. Values are expressed in µg Trolox Equivalent per mg (µg TE/mg). Analysis of the data shows that levels range from 14.213±0.082 µg TE/mg for LV1 to 36.612±0.47 (µg TE/mg) for LV6. The combinations which showed the best antioxidant content with ABTS test also showed better free radical scavenging activity. However, with the ABTS method, the variation seems small from one combination to another compared with the DPPH test, where the variation is large. Nevertheless, both tests (DPPH and ABTS) showed similar results in terms of the evolution of values with the proportion of *L. multiflora*. These results are in line with the literature. Indeed, numerous studies have shown that antioxidant activity varies with the type of test used (Baliyan et al. 2022, Fukumoto et al. 2000, Laila et al. 2017).

3.2 Discussion

Phytochemical screening (Table 2 and Table 3) has shown that, except flavonoids, extracts from each Lippia species present opposite chemical profiles. Many factors, such as soil and environmental conditions, can influence enzymatic reactions for secondary metabolites synthesis (Izadi et al. 2022, Iris et al. 2006, Amdoun 2009). In addition, many research studies on secondary metabolites have shown that chemical solubility differences are also a factor explaining some compounds' absence in some extract (Jianhua et al. 1995, Kailiu et al. 2021, Hildebert et al. 2016, Hugues et al.; 2011). We can also add to this time spent by the solvent in contact with the plant material.

Then, phenolic and flavonoids compounds, antioxidant compounds and free radical scavenging properties (FRSP) was quantified. This study showed that anti-radical activity increases inversely with the proportion *Lippia alba* and *O. basilicum* for batch 1 and batch 2 respectively. In other words, activity improves when we progress to low *Lippia alba* and *O. basilicum* proportions. These observations seem

to contradict consumer expectations. Indeed, a preliminary local survey of consumers and herbal tea sellers on the purpose of combining plant organs revealed that the aim was to improve the effect. In the case of *Lippia multiflora* and *Lippia alba* combinations, some consumers emphasized their desire for a systematic increase of *Lippia multiflora* free radical scavenging activity with the addition of *Lippia alba* (verbena).

The results suggest using *Lippia multiflora* leaves separately, or combining them in proportions of 70 % *L. multiflora*. For combinations involving *Ocimum basilicum*, the data suggest mixing *Ocimum basilicum* with *Lippia multiflora* at (50-50) proportion.

Several factors such as the reaction mechanism involved, the structure, the maceration and content of the molecules in the samples could explain the differences observed (Hugues et al. 2022, Marie et al. 2021).

On the maceration side, many studies have shown that the maceration time influences the quality of the extracts (Stephanie et al. 2009). Long maceration times improve extract quality. In addition, maceration accompanied by physical phenomena such as mechanical agitation or sonication has been shown to have a positive influence on the chemical composition of extracts (Stephanie et al. 2009).

About the reaction mechanism, as the 1,1-diphenyl-1,2-picryl hydrazine molecule is a free radical (possessing an unpaired free electron), an electron transfer mechanism (SET) is required. This suppose that the molecules involved in free radical scavenging are those capable of electron transfer. This excludes antioxidants whose mechanism is hydrogen atom transfer (HAT). Also, the structure of the 1,1-diphenyl-1,2-picryl hydrazine molecule does not favor reactions involving complex molecules. A steric gene effect may be required (Hugues et al. 2022, Marie et al. 2021, Sushama 2011). It would be necessary to quantify antioxidants using other methods. Although several methods exist for the determination of antioxidants in a sample, we chose to use the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation radical ABTS, which is likely to combine two mechanisms and whose reaction sites seem more accessible. This method, known as the Trolox Equivalent Antioxidant Capacity (TEAC) method, is based on the ability of a molecule to inhibit the $ABTS^{\bullet+}$ cation radical compared with 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Tolox), taken as the reference antioxidant. Trolox is the water-soluble form of vitamin E. Data for this test are reported in Table 7. Similar to the DPPH method, we note that antioxidant content of *Lippia multiflora* increases with small proportion of *L. alba* or *O. basilicum*.

Chemical composition may also explain the differences observed. In general, an extract containing high levels of both phenolics and diterpenes has better free radical scavenging properties than one containing only one of the three families (Constantin et al. 2015, Baliyan et al., 2022, Laila et al. 2017, Sushama et al. 2011, Xu et al. 2014). While this rule was successful for some of our combinations, particularly those from same genus species, it was not for combinations from different genus species. Indeed, some studies have demonstrated that at high doses, some phenolic compounds exhibit prooxidant activity (Fukumoto et al. 2000, Xu et al. 2014, Yen et al. 2002, Okezie et al. 1993).

To these literature explanations, we can add intermolecular competition between different molecules in an extract to explain the differences observed in our data. This new explanation could also be very possible. Indeed, a reaction is possible between molecules with labile protons, such as flavonoids, phenolic acids, and alkaloids known for their basic character. The results of these reactions are shown in figure (Fig. 7). Alkaloids' free nitrogen doublets can interact with flavonoid hydroxyl protons, alpha-carbonyl protons for flavones and acid protons for phenolic acids, inhibiting their free radical scavenging activity. Also, certain molecules such as anthocyanins are unstable in basic environments (Fig. 8).

Although phenolic compounds are known for their antioxidant power, the mechanism of action, the often-complex structure of these compounds, the synergy of action or the antagonistic effect can influence this antioxidant power (Laila et al. 2017, Okezie 1993, Honghai et al. 2009, Saha et al. 2008, Kaboré et al. 2021). In the literature, numerous studies show that phenolic compounds use several mechanisms to exert their antiradical activities (Do et al. 2020, Marie et al. 2021, Leopoldini et al. 2011). Among these mechanisms, free radical scavenging and metal chelation are the most common in vitro mechanisms of action used by phenolic compounds in free radical scavenging reactions (Figs. 9 and 10).

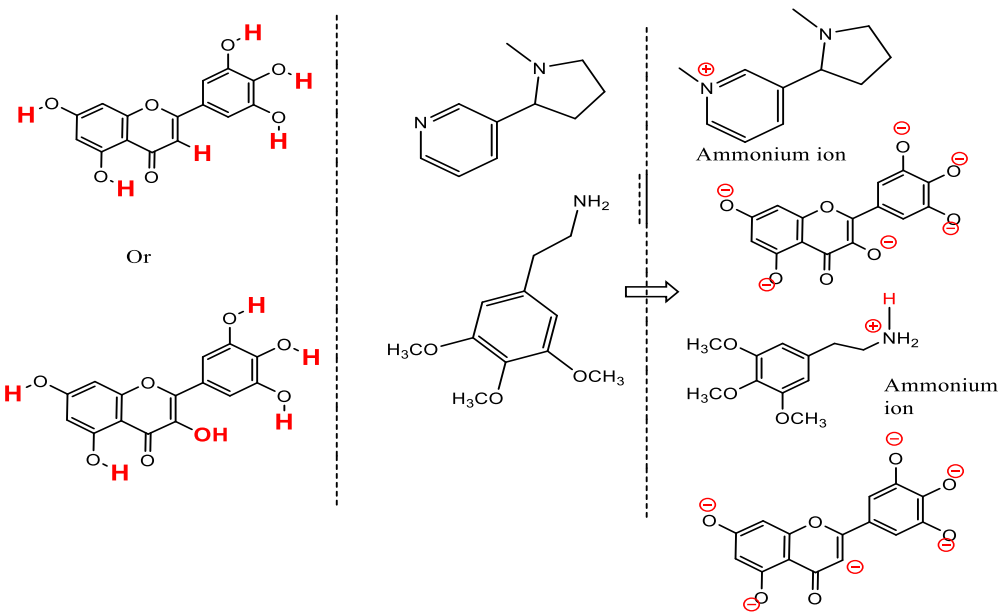

Fig. 7. Interaction between molecules in the same extract

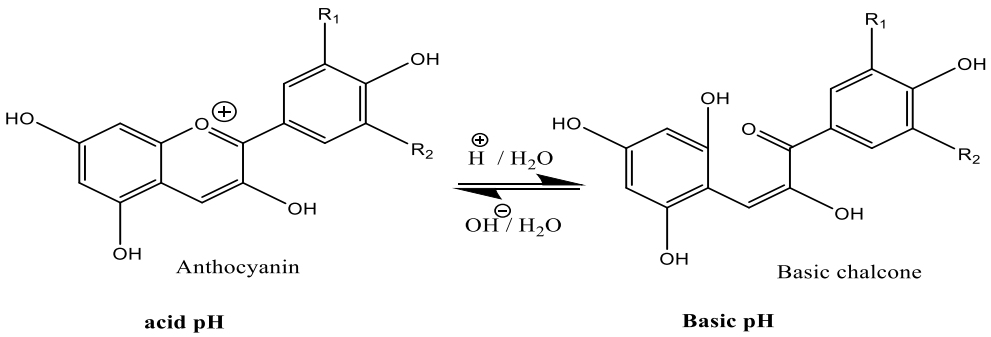

Fig. 8. Anthocyanin structures depending on pH

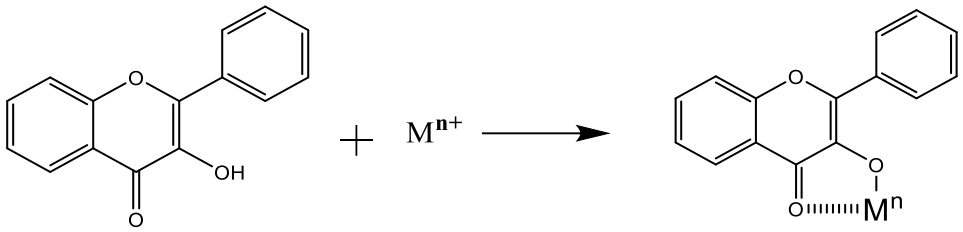

Fig. 9. Metal chelation mechanism of phenolic compounds

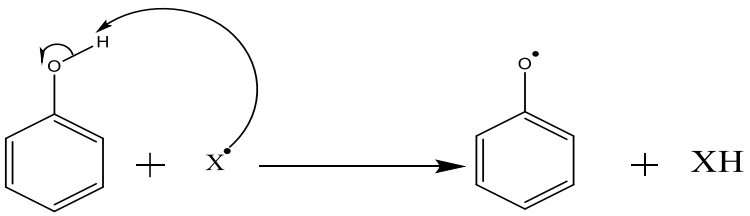

Fig. 10. Radical capture mechanism of phenolic compounds

However, these mechanisms can be influenced by the structure of the core and the number of hydroxides on the core (Brand-Williams et al. 1995, Fumi et al. 2012, Lee et al. 2013, Hodek et al. 2002). In general, phenolic compounds with flavonol, catechol and resorcinol cores possess excellent antioxidant activity. Trihydroxybenzenes have also been shown to have better activity than dihydroxybenzenes. This is due to the availability of the free doublets of the oxygens in positions 3, 4, 5, 4' and 5' of the flavonoid cores (Fig. 11).

Activity can also be partly attributed to the mesomeric effects of electron delocalization, which takes place on several conjugated doublets, making the aryloxy radicals formed stable (Fig. 10). Activity could also be influenced by the Mac Lafferty rearrangements that some aryloxyls derivatives can undergo (Fig. 12). Indeed, compounds such as catechol rings, due to their rearangements, are highly stable after radical-neutralizing reactions (Leopoldini et al. 2011, Jovanovic et al. 1994).

As the reactions involved in free radical scavenging are based on the oxidation-reduction principle, the pH and redox potential of the Ox/Red couples are decisive factors in the reaction kinetics. Indeed, high-potential nuclei are less oxidizing than low-potential nuclei, and in a neutral environment, certain nuclei such as rutin and catechin see their redox potential decrease. This makes them more active.

As extracts are complex mixtures containing several molecules, competition between the synergistic action of certain molecules and the antagonistic effect of others may also be at the root of the differences observed in the values. Indeed, many authors have highlighted the pro-oxidant activity of certain phenolic compounds, notably propylgallate, gallic acid and certain highly reducing polyphenols (Fukumoto et al. 2000, Yen et al. 2002). A low content of gallic acids or hydrolyzable tannins (Fig. 13) and of moderately reducing compounds would therefore be an asset for good antiradical activity.

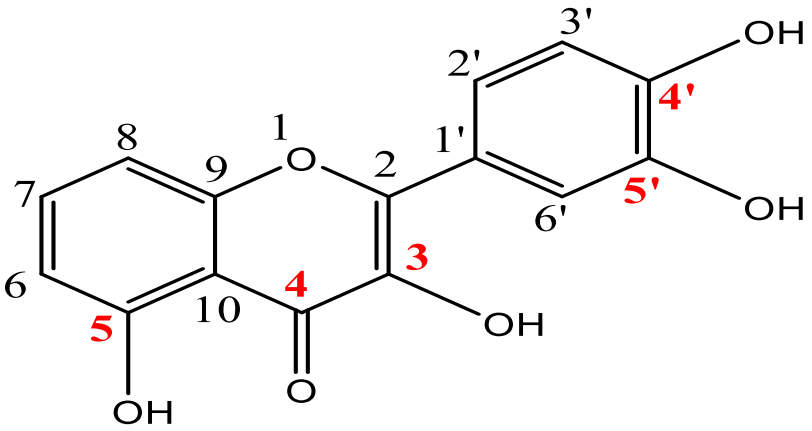

Fig. 11. Flavonoids basic structure

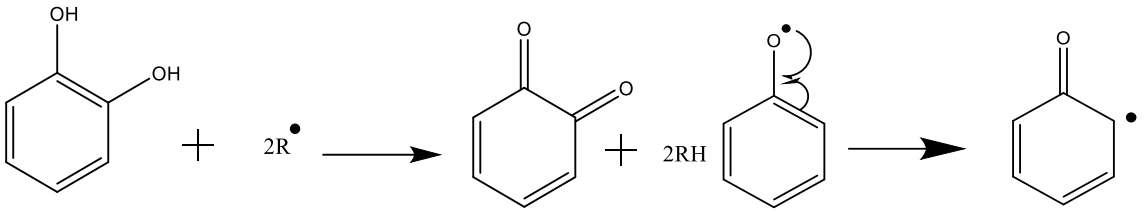

Fig. 12. Stability effects of aryloxyls by mesomerization

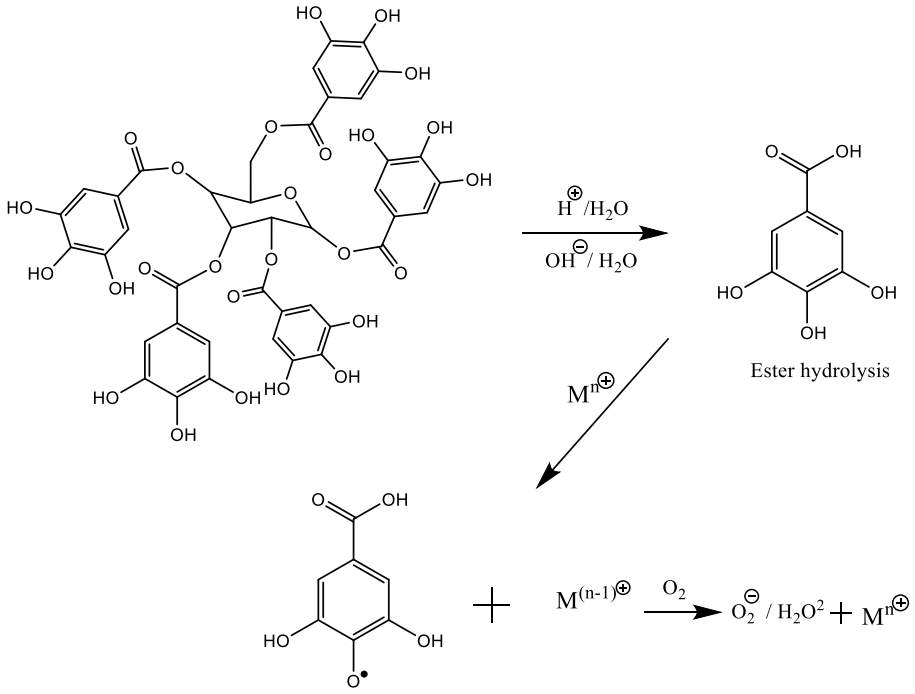

Fig. 13. Hydrolyzable tannin structure

Although plant or plant organ combination in recipe preparation is an empirical practice, scientific data on the benefits of organ or plant combination in the treatment of pathologies is lacking (Stephanie 2009, Chun-Tao et al. 2013, Vi et al. 2023). However, numerous studies on the biological activities of plant extracts *in vitro* have shown a strong correlation between the biological activity of plant extracts and their antioxidant and phenolic compound content (Stephanie et al. 2009, Chun-Tao et al. 2013, Vi et al. 2023, Hossain et al. 2023, Emadeldin et al. 2023). Any combination which results in a reduction in anti-free radical activity could have a weak pharmacological property. Molecular interaction can produce compounds whose site of attack on the pathogen differs from the initial molecule. In addition, molecules derived from interaction reactions may be inactive on the pathogen. The large number of attack sites for plant extracts, due to their complex mixtures, was an important factor in combating microorganisms at several sites. This study recommends that, for maximum efficacy, traditional healers should formulate their herbal recipes by mixing plants in moderate proportions. However, this study's results, together with data from surveys of traditional healers on medicinal plant combinations, show that *in vivo* animal research and clinical follow-up are needed to control the quality of plant-based recipes derived from plant or plant organ combinations.

4. CONCLUSION

This work, which assessed the effect of plant association on free radical scavenging activity and phenolic compound content, is a first in our country. Two batches of mixtures were used, one combining species from the same genus (*Lippia multiflora*/*Lippia alba*) and the other combining species from different genera (*Lippia multiflora*/*Ocimum basilicum*) in the respective proportions of (30:70); (40:60); (50:50); (60:40); and (70:30). The results showed that the plants individually contain considerable levels of phenolic compounds, flavonoids and antioxidants, and have good anti-free radical activity. However, in some cases, their combination resulted in an approximate 30% reduction in content and a 23% reduction in anti-free radical activity compared with the initial values of the individual plants. This shows that, to improve anti-free radical efficacy and the content of anti-free radical compounds, the combination of species from the same genus would be recommended, but at a lower proportion (20%) than that of species from different genera. In fact, antagonistic effects were more pronounced when involving different

species than when involving species of the same genus.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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