Plants Mixture Approach: Effect of Extraction Methods on Polyphenolic Content of Some Medicinal Plants Used against Oral Disorder

Hazim HAROUAK*, Jamal IBIJBIJEN, Laila NASSIRI

Environment & Valorization of Plant and Microbial Resources Unit, Moulay Ismail University of Meknes, Faculty of Sciences, B.P 11201, Meknes, Morocco

Abstract: The On the basis of an ethnobotanical survey with herbalists, who cites plants in a mixed way against dental disorders, comes the idea of this article, for tests of total phenolic compounds the colorimetric method Folin-Ciocalteu, for flavonoids content the standard catechin and for condensed tannins the acidified vanillin was used.

Three methods were also used for crude extractions; namely: infusion, decoction and Soxhlet techniques, the results were statistically analyzed and different correlations between samples and the inter samples were made; the results showed the antagonistic effect in these mixtures where their content decreases compared to plants alone, the phenolic profitability is observed in the mixtures containing less plants extracted by Soxhlet, contrary to the flavonoic compounds which are more in the mixtures containing more plants where their profitability is proportionally related to the extraction by infusion, but at condensed tannins level a specific plant in a specific mixture which can be richer in these compounds, the different correlations have approached these results which can be the object of a more interesting study at the level of the chemical reactions which is carried out from one family to another and from mixture to another.

Keywords: Plants mixture, Dental diseases, Polyphenol content, Aqueous extraction, Principal Component Analysis (ACP), Analytical correlations.

Corresponding author: hazim.harouak@gmail.com

Introduction:

Since oral cavity is the main route of entry into the human digestive system and subsequently the oral bioflux is home to a diverse group of microbial communities, the diversity quantity of these microorganisms is higher in this area than in any other area of human body (Costello et *al*., 2009). In fact; oral diseases have always been a serious and significant public health problem even in high-income countries; the problem is worsening in other, less low- and middle-income countries (Petersen et *al*., 2005).

On the other hand; The use of medicinal plants has always been present in cultural and traditional behavior. According to new statistics from the World Health Organization (WHO), 80% of the world's population uses herbal and traditional treatments to meet their primary health care needs (Bhar and Balouk, 2011).

Morocco's diverse climate and geographical position gives it the chance for almost all medicinal plants can grow and be cultivated easily (Faridi et *al*., 2013).

In dentistry, medicinal plants continue to enjoy a high reputation, especially in rural areas where there is a lack of dental care facilities and an attachment to a number of traditions and beliefs that favour the transmission of these traditional practices ((Harouak et *al*., 2019).

Commercial remedies used in worldwide contain plant extracts such as peppermint, myrrh, rosemary and miswak, which are the active ingredients in oral preparations, in the form of dental gels or mouthwashes (Sambawa et *al*., 2016).

According to our ethnobotanical survey carried out on the plants used against dental disorders; cited species are used in the form of a mixed solution of several cooked plants (Harouak et *al*., 2018a).

Indeed, the efficacy of medicinal plants is linked to its composition in bioactive molecules, polyphenols represent the most distributed and structurally diversified content, in fact the multiple biological activities is due to polyphenols, which are an excellent antioxidant (Lingua et *al*., 2016; Alamgir, 2018), antimutagens, anticarcinogens, anti-inflammatories and neuroprotectors (Skendi et *al*., 2017). In addition, these phytochemical compounds are among the useful solutions that control the induction of dental caries (Farkash et *al*., 2019) The polyphenols in tea leaves, for example, have been shown to be effective against cavities due to epigallocatechin gallate (EGCg) which is a potent inhibitor of glucosyltransferase (GTF) (Sakanaka et *al*., 1990; Otake et *al*., 1991), Cocoa polyphenols have been shown to reduce biofilm formation and acid formation by *Streptococcus mutans* and *Streptococcus sanguinis*,

also trigonelline, caffeine and chlorogenic acid found in coffee react with the adsorption of *S. mutans* on hydroxyapatite beads coated with saliva (Ferrazzano et *al.*, 2009), *Vaccinium macrocarpon* polyphenols, specifically flavonols and proanthocyanidins, showed significant inhibitory effects against specific elements of cariogenic virulence such as acidogenicity, acidity, glucan synthesis and hydrophobicity of bacteria (Philip and Walsh, 2019).

The present work aims to evaluate the antagonistic effect of polyphenolic content of seven mixture extracts, and the correlation between these quantities and the type of 3 hot aqueous extractions.

Material and Methods:

Plants materials:

Studied plants were collected and referenced (tab. 1); then dried at room temperature in the greenhouse for 15 days and then passed through a 0.5 mm sieve in a mill;

The obtained samples were stored in plastic bags in a dark, dry and cool environment:

Table 1: Studied plants

Scientific name	Reference code	Specimen number	Used part	Harvest point	Harvest date
Anacyclus pyrethrum L.	AP	MICSOLENV-AP/N°09	Roots	33°04'29.0"N 5°00'25.6"W	02-05-2019
Artemisia herba alba Asso.	AHA	MICSOLENV-AHA/N°13	Aerial part	33°14'43.6"N 5°02'53.0"W	16-06-2019
Marrubium vulgare L.	MV	MICSOLENV-PH/N°12	Aerial part	33°40'15.8"N 6°19'06.2"W	03-05-2019
Origanum compactum Benth.	OC	MICSOLENV-OC/N°10	Aerial part	34°04'28.5"N 5°30'50.9"W	23-06-2019
Thymus satureioides Coss.	TS	MICSOLENV-TS/N°08	Aerial part	33°24'53.8"N 5°10'39.8"W	16-06-2019
Mentha pulegium L.	MP	MICSOLENV-MP/N°14	Aerial part	34°04'07.7"N 5°30'42.9"W	23-06-2019
Lavandula multifida L.	LM	MICSOLENV-LM/N°16	Aerial part	33°53'14.7"N 5°54'53.0"W	05-05-2019
Pistacia atlantica Desf.	PA	1810/IS/N°38	Leaves	33°38'44.6"N 6°17'58.9"W	03-05-2019
Olea europaea L. subsp. *Sylvestris*	OE	MICSOLENV-OE/N°06	Leaves	34°03'52.9"N 5°30'09.5"W	23-06-2019
Juglans regia L.	JR	MICSOLENV-JR/N°07	Bark	33°52'10.8"N 5°32'35.3"W	07-07-2019
Tetraclinis articulata (Vahl) Masters	TA	MICSOLENV-TA/N°15	Leaves	33°52'39.4"N 5°54'54.9"W	03-05-2019

Mixture preparation and aqueous extraction:

Seven mixtures (tab. 2) of plants have been prepared on the basis of the criteria; autochthonous plants, same scientific family name, Mediterranean endemic, less work against oral diseases and more cited in our ethnobotanical research realized on the treatment of oral diseases by medicinal plants (Harouak et *al*., 2018b).

Table 2: Composition of plant mixtures

Mixture number	Mixture composition
M1	MV+AHA+PA+TA+TS+MP+OE+LM+AP+JR+OC
M2	TA+OE+JR+OC
M3	MV+AHA+TS+MP+LM+OC
M4	PA+TA+AP
M5	TA+AP
M6	PA+TA
M7	PA+AP

Tree aqueous extractions were prepared for each mixture using infusion, decotion and Soxhlet material at the rate of 10% in distilled water, then and after each extraction, the solution was filtered with a Wathman filter paper and evaporated in the oven at approximately 45 °C.

Yield of all samples was calculated as follow (Alara et *al*., 2018).

$$\% \; Yield \; of \; extracts = \frac{We}{Wt} * 100$$

Whit, W_e is the weight of extracts from plant sample (w) and W_t is the weight of dried plant sample.

Total phenolic content (TPC):

For the determination of the total phenol content, test tubes were used for each extract (with a concentration of 1 mg/ml in distilled water), 100 µl were added to 4.5 ml of distilled water and then 100 µl of Ciocalteu Folin reagent. This mixture was left to stand for 3 minutes at room temperature, then 300 µl of Na_2CO_3 (2% in water) was added. After 1h30 incubation in

the dark and at room temperature, absorbance was measured with a spectrophotometer (UV-2005) at 760 nm against a blank containing distilled water.

A calibration curve was plotted under the same operating conditions using gallic acid as the standard phenolic compound (100 to 1000 µg/ml); results were expressed in mg Galic acid equivalent (GAE)/1g dry extract and the analysis was averaged over three replicates ± the standard deviation (Slinkard and Singleton, 1977).

According to the calibration curve; TPC = (c*V)/m

c: is the TPC concentration of the calibration curve equation with gallic acid (y = 0.0008x-0.0145), R^2 = 0.998 (µg / ml) (Fig 1), V: is the total volume of solvent (distilled water) used in the test (ml), and m represents the weight of dried sample used (g) (Alara et *al*., 2018).

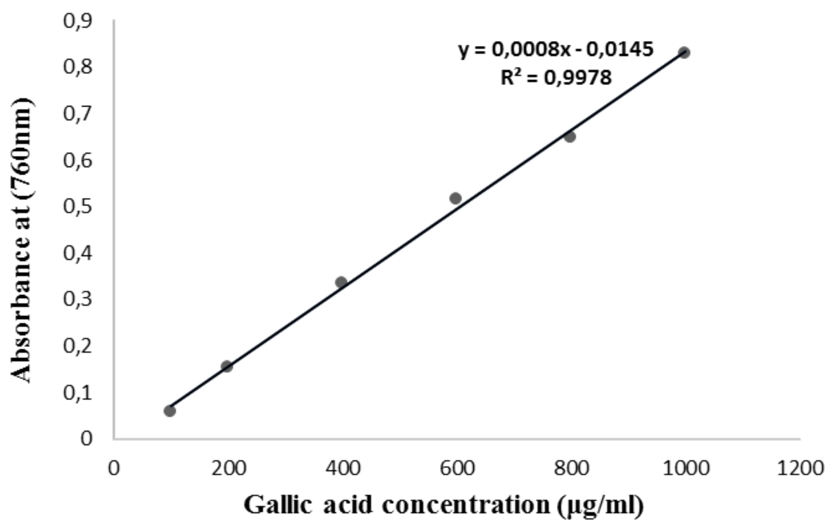


Figure 1: Calibration curve of gallic acid

Total flavonoids content (TFC):

The determination of TFC was performed according to (Zilic et *al*., 2011) method. A solution composed of (500 µl) of each extract (1 mg/ml) and 75 µl of $NaNO_2$ (5%) was prepared. After 6 min, 150 µl of an $AlCl_3$ solution (10%) was added and left to stand at room temperature for 5 min; then 500 µl of NaOH (1 M) was added and the total volume was adjusted to 2.5 ml with distilled water. The absorbance was measured at 510 nm against a blank of distilled water as extraction solvent. Under the same operating conditions, a calibration curve was plotted using catechin as the reference flavonoid (50 to 500 µg/ml); the

results were expressed in mg catechin equivalent (CE) / 1 g dry extract and the analysis was expressed as the mean of three replicates ± the standard deviation.

According to the calibration curve; TFC = (c*V)/m

c: is the TFC concentration of the calibration curve equation with catechin (y=0.0031x+0.0517), R^2=0.998 (µg/ml) (Fig 2), V: is the total volume of solvent (distilled water) used in the test (ml), and m represents the weight of dried sample used (g) (Alara et *al*., 2018).

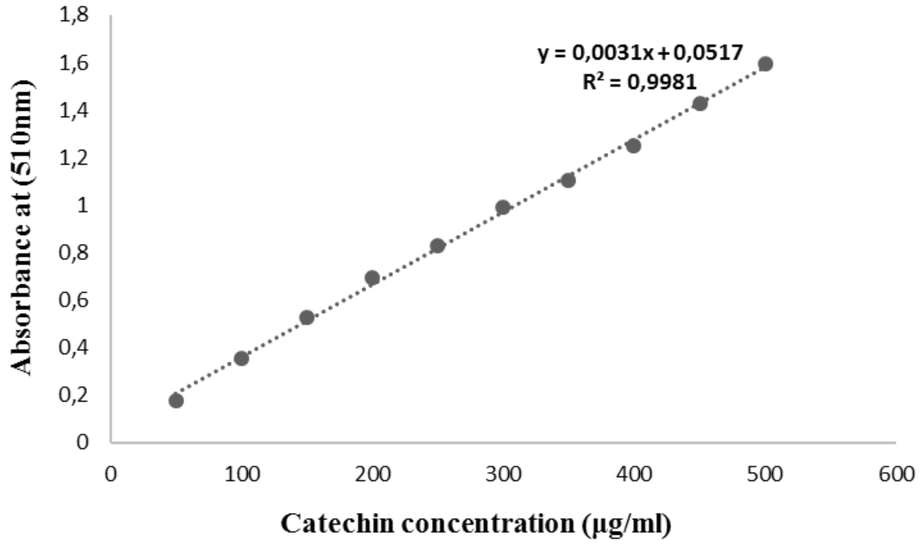


Figure 2: Calibration curve of catechin

Total condensed tannins content (TCT):

According to (Broadhurst and Jones, 1978) the TCT content was determined using acidified vanillin. In a test tube covered with aluminium foil, 0.5 ml of each aqueous extract (1mg/ml) was added to 1.5 ml of vanillin reagent (4%, w/v, vanillin in methanol). After vortex agitation, 750 µl of concentrated hydrochloric acid was added, followed by a second vortex agitation. The mixture was left in the dark at 20 °C for 15 minutes and then the absorbance was determined at 500 nm against a blank of distilled water as extraction solvent. Under the same operating conditions, a calibration curve was plotted using catechin as the reference condensed tannin (100 to 1000 µg/ml); the results were expressed in mg catechin equivalent (CE) / 1 g dry extract and the analysis was expressed as the mean of three replicates ± standard deviation.

According to the calibration curve; TCT = (c*V)/m

c: is the TCT concentration of the calibration curve equation with catechin (y=0.0003x+0.0062), R^2=0.999 (µg/ml) (Fig 3), V: is the total volume of solvent (distilled water) used in the test (ml), and m represents the weight of dried sample used (g) (Alara et *al*., 2018)

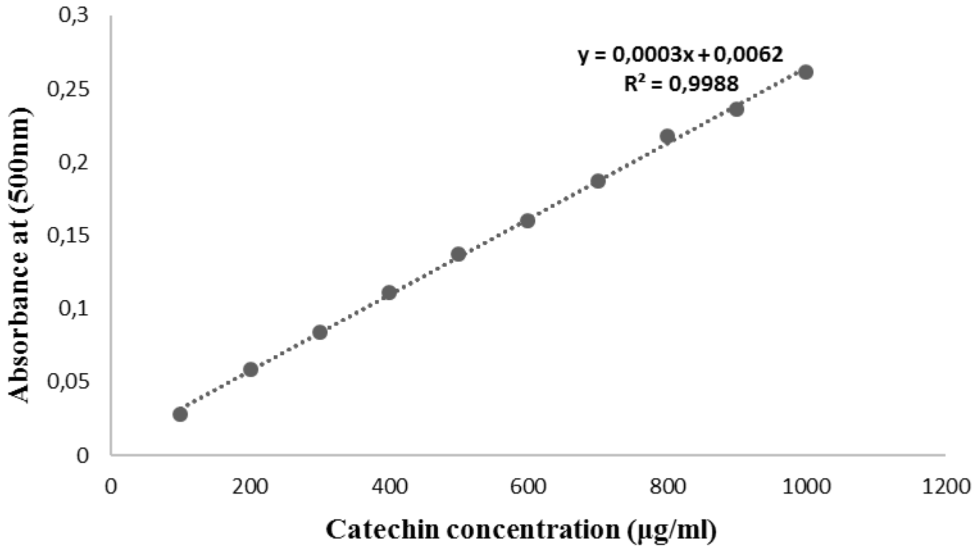


Figure 3: Calibration curve of catechin

Statistical analysis:

The statistical study of the results was carried out using GraphPad prism 8.2.263 and XLSTAT 2016 (18.02.01), the graphical representation and the significant difference of differente yields and polyphenol contents, also the different correlations (PCA) between variables (polyphenolic compounds assayed) and extraction type between individuals (samples studied) have been made (Harouak et *al*., 2021);

The study of significant difference of divers values was carried out by the two-way analysis of variance (ANOVA II), followed by a Tukey test to detect the degree of significance, this was taken at the probability of *p<0.05 for a significant difference, **p<0.001 for a very significant difference, ***p<0.0001 for a very highly significant difference (Harouak et *al*., 2021).

Results:

Extractions Yield percentages:

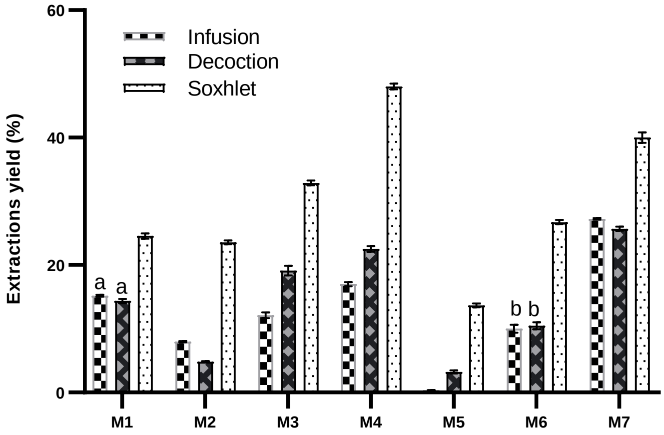
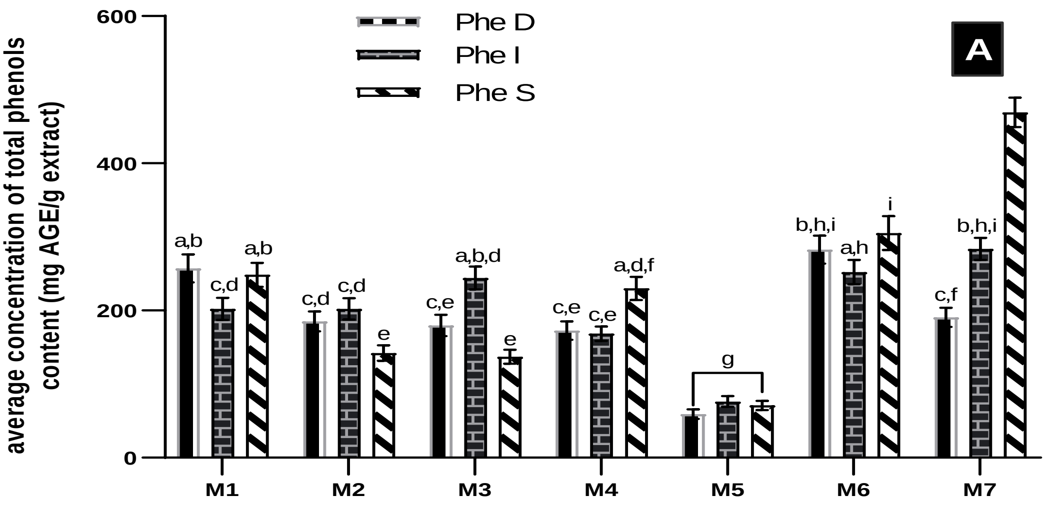
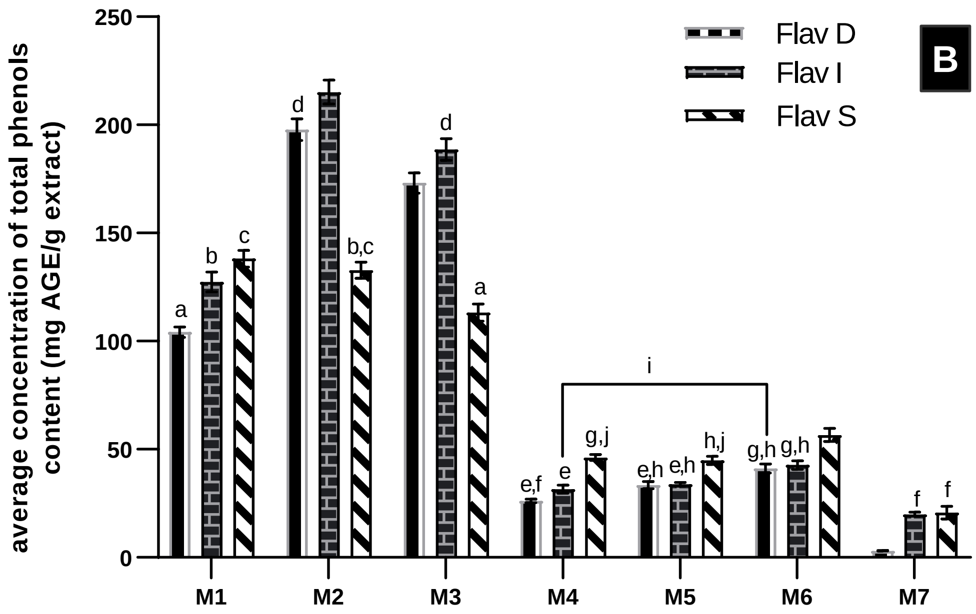


Figure 4: Yield extractions percentage, Values sharing same letters are not significantly different (p > 0.05)

All plants mixtures recorded significantly higher yields different p<0.0001 between the extraction by Soxhlet and infusion or decoction, the highest is noticed at the level of mixture 4 (48,02%), the comparison of these results with those of the plants alone gave a slight observation at the level of M3 where the extraction by Soxhlet reaches 32, 90% while for this mixture the plant with the highest yield was *Artemisia herba alba* (wormwood) 31.30% (Soxhlet extract), same note at M4 level 48.02% compared to 45.26% obtained from *Pistacia atlantica* Desf. leaf.

Quantitative distribution of polyphenols :





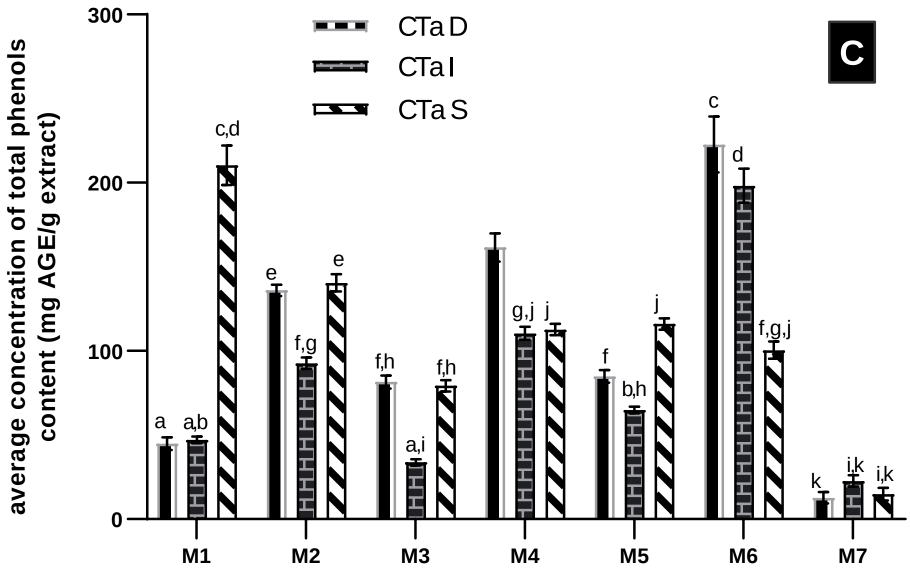


Fig 5: Average concentrations of polyphenolic compounds in plants mixture (fig A: Phe=Phenols, fig B: Flav= Flavonoids, fig C: CTa=Condensed tannins; I=Infusion, D=Decoction and S=Soxhlet extracts), Values sharing same letters are not significantly different ($p > 0.05$)

Total phenolic content (fig. 5.A):

The interaction between plants mixture and extraction type is considered very highly significant with: F = 56.38. DFn = 12, DFd = 42, The $P < 0.0001$.

The statistical analysis throught ANOVA II reaveal that the difference is very highly significant between plants mixture ($F = 270.15$. DFn = 6, DFd = 42, the P value is < 0.0001), also between the applied methods of extraction of total phenols, is very highly significant ($F = 39.18$. DFn = 12, DFd = 42, the $P < 0.0001$).

Static comparison between the phenolic content of the different mixtures revealed that the different phenolic content of the different mixtures differed from one mixture to the other; sometimes Soxhlet allows the extraction of more phenols (M1, M4, M6, M7), other times the infusion is profitable as in the case of M2 and M3, as well as it is noted that the higher contents have been registered at the level of the mixtures containing less plants (M6 and M7), these contents becomes less than for the plants alone (example of the highest Soxhlet M7 extract (469,38 ± 20,12 mg GA/g of extract) whereas the same extract of the pistachio tree plant alone gives 502,71 ± 19,94 mg GA/g of extract.

It can be said that higher phenolic compounds are more extractable in mixtures containing the minimum number of plants using the Soxhlet apparatus.

Total flavonoids content (fig. 5.B):

For the flavonoids compound, the interaction between plants mixture and extraction type is considered very highly significant with: $F = 180.45$. DFn = 12, DFd = 42, The $P < 0.0001$.

the statistical analysis shows that the difference between the investigated mixture as well as between the extraction type are significantly very highly different, with respectively ($F = 4068.99$. DFn = 6, DFd = 42, the P value is < 0.0001) and ($F = 129.91$. DFn = 2, DFd = 42, the P value is < 0.0001).

the highest contents in flavonoids were extracted by infusion in mixtures containing more or less specific plants (TA+OE+JR +OC).

Total condensed tannins content (fig. 5.C):

Both the plant mixture and the extraction method present an effect extremely significant in regards to the total condensed tannins content, respectively: ($F = 590.41$. DFn = 6, DFd = 42, the P value < 0.0001), and ($F = 136.49$. DFn = 2, DFd = 42 the P value is < 0.0001).

the interaction between plants mixture and extraction type is considered very highly significant with: $F = 188.21$. DFn = 12, DFd = 42, The $P < 0.0001$.

The contents of condensed tannins have revealed that the plant which contains more of these compounds (TA = 250.44 ±10.18 mg EC / g of extract) is the one responsible for the high value in mixture 1 (M1 = 210.44 ± 11.71 mg EC / g of extract) and 6 (M6 = 222.67 ± 16.67 mg EC / g of extract), and all the values were less than those of the plants alone.

Principal Component Analysis (PCA) :

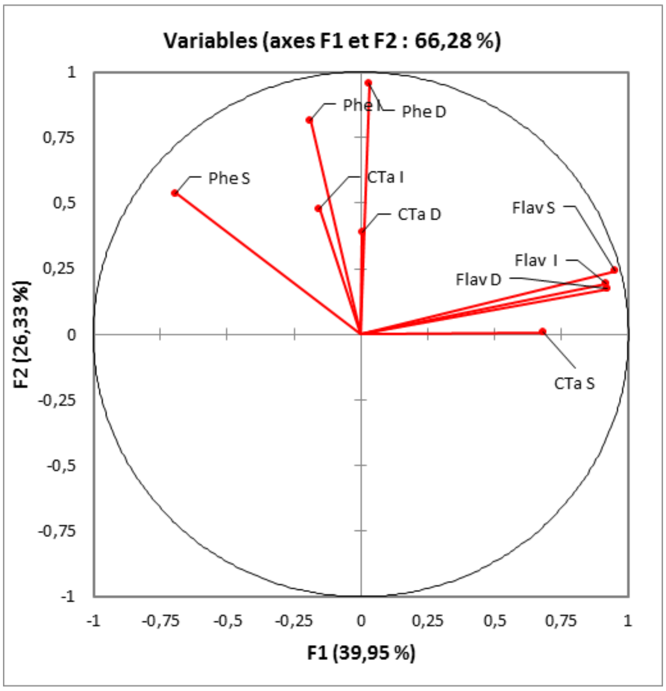


Figure 6: Correlation circle of variables (Phe: Phenols, Flav: Flavonoids, CTa: Condensed tannins, I: infusion, D: Decoction, S: Soxhlet)

The F1 axis represents (39.95%) and F2 (26.33%) of information; On the correlation circle, the variables are well presented on the F1 and F2 plane which explains 66.28% of the variability.

CTaS not correlated with PheD, FlavS isn't correlated with PheI, FlavI and FlavD aren't correlated with CTaI;

FlavI, FlavD and FlavS are strongly positively correlated and correlated negatively with PheS;

PheD, PheS and PheI are correlated positively.

The positive correlation between extraction types shows very clear that there is a small difference of phenols or flavonoids content using infusion, decoction or Soxhlet extractions, while for total condensed tannin content the extraction type has a very important effect on these phytochemical compounds.

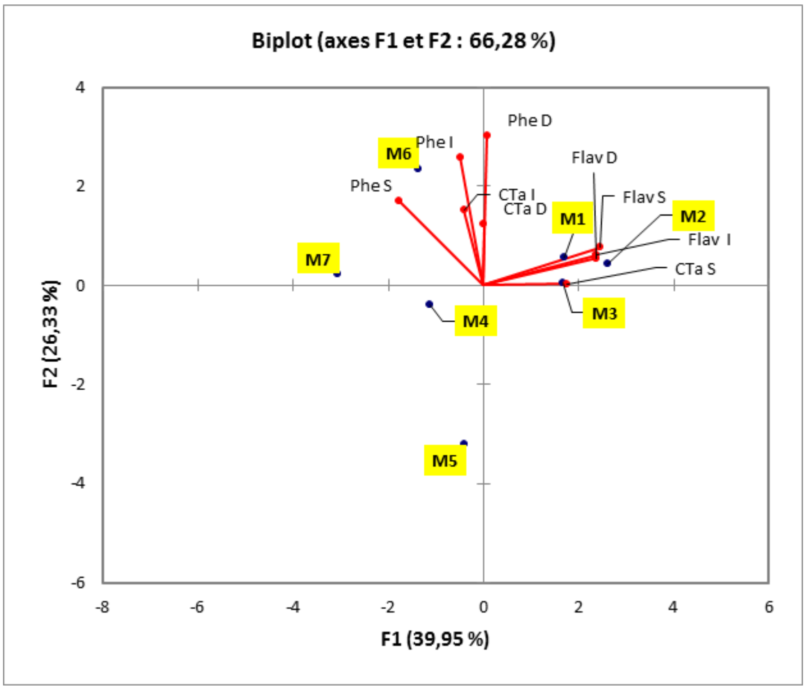


Figure 7: Projection of individuals on the factorial plane (F1 x F2)

According to the factorial plane (fig. 7); M1, M2 and M3 are richer in flavonoids compounds, M6 and M7 contain more phenols, while only M6 which is richer in condensed tannins, M4 and M5 are poor in polyphenolic content even they contain same plants richer in condensed tanins such as TA or phenols (PA); thoses results confirm the results graphs at the top (fig. 5).

Discussion:

The mixture of plants concerning extraction yields using Soxhlet technique was largely more bigger than that of the plants alone; In fact, because of its high performance compared to other conventional extraction methods, Soxhlet extraction is a well-applied technique (Luque de Castro and García-Ayuso 1998) and the most profitable way to obtain vegetable oils by extraction (Teixeira et *al.*, 2018), this difference observed in the extraction yields of mixed plants compared to single plants can be explained by an influence on the mixing by one or many plants (Elgndi et *al.*, 2017), it is mandatory to note that the performance of any extraction method depends on several parameters, including extraction time, type of solvents, volume and temperature used (Chew et *al.*, 2011; Costa et *al.*, 2012).

Polyphenols have shown several effects against dental diseases, as an example these compounds have the power on orthodontic surfaces to inhibit the formation of mixed biofilms (Farkash et *al.*, 2019), in fact polyphenols tea mixed with the nutrition or water of tested rats

have shown a significant reduction in total cracking lesions (Sakanaka et *al.*, 1992), catechin and epigallocatechin gallate, which is a major constituent of plant leaf, inhibited tumor promotion and carcinogenesis (Fujiki et *al.*, 1992), separate saponins from this plant are effective against gastric ulcers and have anti-inflammatory and leukotriene antagonistic activity (Sagesaka et *al.*, 1996), almost all catechins studied in oolong tea have shown antialgesic activity (Ohmori et *al.*, 1995).

Luteolin extracted from the seed of *Perilla frutescens Britton* var. *Japonica Hara* showed a very remarkable antimicrobial effect compared to other phenolic compounds tested against oral cariogenic *streptococci* and periodontopathic *Porodyromonas gingivalis* (Yamamoto and Ogawa, 2002), Moreover, although this compound and others (morin, naringin, quercetin and rutin) have significant antibacterial and antifungal activity, morin is apparently the most active flavonoid (Gutiérrez-Venegas et *al.*, 2019).

Also, it was revealed that tannins extracted from *Phyllanthus columnaris* bark were active against the growth of all oral pathogens tested (Othman et *al.*, 2019).

Indeed, treatments with polyphenolic compounds compared to synthetic compounds (fluoride) depth of artificial carious lesions and mineral loss was less when treated with flavonoids (Epasinghe et *al.*, 2016).

For thoses reasons, the thought of increasing polyphenols content in the herbal extracts is highly recommended by synergic effect for example, in fact to guarantee this effect of medicinal compounds; some recently studies have revealed that blends of natural herbs can be a good and innovative method to optimize the individual effects of the components, and to bring all the benefits that come from their mixture (Kang et *al.*, 2011), this synergistic effect is very much in demand in modern medicine, it is the case of the synergy between antioxidants (Johansson et *al.*, 2010, Valgimigli et *al.*, 2013), and in the case of antibiotics against multi resistant bacteria (Sanhueza at *al.*, 2017), but these effects of phenolic compound accumulation can be influenced by environmental factors such as nitrogen, temperature and light (Løvdal at al., 2010).

The quantity of total pehnols obtained in our study is more extractable sometimes using soxhlet and other by infusion which are two different techniques; that can be explained by the fact that the increased temperature helps the mass transfer and consequently improves the extraction yield (Wang et *al.*, 2008), or the opposite when the temperature rises above 60°C

this last one can accelerate the degradation of phenolic compounds (Ruenroengklin et al., 2008; Benmeziane et *al.*, 2014).

The degradation of total flavonoids observed in the high temperature extracts can be explained by the decrease of these compounds and also of their particular structure (Sharma et *al.*, 2015).

Concerning condensed tannins content; the higher value of *Tetraclinis articulata* (Vahl) Masters (TA) was obtained from infused extract, which was higher than that for the mixtures M1 and M6; the values were at the level of soxhlet and decoction extracts respectively, this may be due to the different chemical reactions from one mixture to another in different reaction environments (high and continuous temperature or stable pressure).

we can therefore say that the content of condensed tannins does not depend on the number of plants contained in a mixture, it depends more on specific plants richer in these compounds, and the antagonistic effect is well present which is perhaps due to the chemical reactions that are manifested by a mixture of the plants.

The antagonistic effect observed in this study when mixing plants can be explained by previous research which revealed that mixtures of quercetin and caffeic acid are slightly antagonistic in the linoleic acid system; this has been justified by the reason that the more effective molecule replaces the less effective one; in this situation, caffeic acid is replaced by quercetin (Peyrat-Maillard et *al.*, 2003).

According to our PCA the correlation found between the content of total phenolic compounds and flavonoids is normal since these compounds are very related to each other (Maisuthisakul et *al.*, 2008).

Conclusion:

Traditional practices are not always correct, as the synergistic or antagonistic effect is always an issue when molecular mixing.

These results show that plants alone can give a higher content of polyphenolic compounds than their mixtures, because the most effective molecules can replace the less effective ones according to some previous research.

But, the specific content of total phenols, total flavonoids and condensed tannins is relatively related to the number of plants mixed, the extraction procedure and the nature of the plants used.

So, the plant alone gives more polyphenolic compounds, and its mixture with other plants may extract more specific compounds.

To confirm this, it is necessary to make quantitative studies of each compound of the mixtures and to compare it with the individual plants, since the research in this field is almost absent.

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