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Phytochemical Characteristics and Pharmacological Properties Studies of the Powders of *Punica granatum* L. (Lythraceae) Leaves from Seven Regions of Burkina Faso with a View to Standardizing

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SO, TKT and BO initiated and elaborated the protocol. Authors TKT, BO and BCA carried out the work, performed the statistical analysis, interpreted the results and drafted the manuscript. Authors TKT, BO and BCA contributed to perform antioxidant and lipoxygenase inhibition tests. Authors SO, TKT, BO, BCA and AC wrote and proof read the manuscript. Authors RS, ML and SO contributed to analyze the results. All authors read and approved the manuscript.

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ABSTRACT

Introduction: *Punica granatum* is a plant used in traditional and alternative medicine for the management of several diseases.
Objective: The objective of the present work is to compare the phytochemical characteristics and the antioxidant properties of extracts of leaves powders of *Punica granatum* L. collected for standardization.
Methodology: We worked on seven samples of powders of leaves of *Punica granatum* L collected in seven different regions of Burkina Faso. Two types of extracts, aqueous and hydroethanolic were prepared with each sample. We performed a phytochemical screening by thin layer chromatography (TLC), then determined the content of the various extracts in total phenolic and flavonoids as well as a study of the antioxidant activity of the aqueous and hydro-ethanolic extracts of the plant.
Results: Fourteen extracts of *Punica granatum* are obtained and these contain secondary metabolites such as tannins, sterols, triterpenes, saponosides and flavonoids. The anti-free radical activities at the DPPH* were more important in the samples from the towns of Dedougou, Banfora and Fada. The anti-free radicals at ABTS of extracts from the towns of Manga, Banfora, Fada and Kaya were found to be the most active. The FRAP test shows better activity of samples from the cities, Manga, Dedougou and Banfora.
Conclusion: At the end of this work, the towns of Dedougou and Banfora may be the sites to be favored as harvesting sites because their samples were the richest in phenolic compounds and had the best antioxidant activities compared to the tests carried out.

Keywords: Punica granatum; powders of leaves; phytochemical profile; standardization.

1. INTRODUCTION

Throughout the ages, man has been able to rely on nature to provide for his basic needs: food, shelter, clothing and also for his medical needs [1] The use of plants remains one of the main remedies of a large majority of populations to solve their health problem, as it constitutes an important part of the cultural heritage. According to the World Health Organization, nearly 80% of populations depend on traditional medicine for primary health care [2]. The therapeutic property of plants varies depending on several parameters. The climate, the soil, the part of the plant used, the harvesting and extraction procedures are all parameters that can influence the properties of plants in traditional medicine.

Although much of the twentieth century was devoted to the development of synthetic molecules, the search for new pharmacological active agents through the screening of natural sources has made it possible to discover a large number of useful drugs which are beginning to play a major role in the treatment of many diseases [1].

Oxidative stress is defined as excessive oxidation due to an imbalance between the production of oxidative species or reactive forms of oxygen (ROS) and that of antioxidant systems. ROS are responsible for denaturing and degrading biological molecules and are produced during various biological processes by a large number of cells [2].

It therefore seems important to test the therapeutic effect of natural antioxidant molecules, which can act in the prevention of degenerative diseases, cancers and aging, on the condition of being introduced very early before the appearance of irreversible induced mechanisms, and in moderate doses because the basal production of free radicals is essential for many functions and should not be suppressed [3].

Antioxidants are groups of compounds that neutralize free radicals and reactive oxygen species (ROS) in the cell [4]. Antioxidants provide protection against damage caused by free radicals which have played an important role in the development of many chronic diseases, including cardiovascular disease, aging, heart disease, anemia, cancer, inflammation.

Punica granatum L (pomegranate) is a deciduous shrub in the Lythraceae family. Pomegranate has been widely used as a source of traditional medicine [5]. Nowadays, besides its use as a fruit, its medicinal properties have aroused the interest of researchers in many countries. Pomegranate has medicinal properties such as anti-inflammatory [6] and antibacterial activities [7]. Pomegranate seed oil has an

inhibitory effect on skin and breast cancer. Ellagic acid is one of the main components of pomegranate with phenolic structure and antioxidant activity [8]. The therapeutic virtues of these compounds are well known. Indeed, protection for example against several diseases (cancer, Alzheimer's disease, cardiovascular disorders, etc.) is due to their antioxidant properties [9]. However, factors such as harvest area, temperature, time and time of collection, method of collection, drying, storage, age and part of the plant collected, etc. can greatly affect the quality and therefore the therapeutic value of herbal medicines. Thus, standardization of raw materials through phytochemical characteristics and pharmacological properties is necessary to ensure a permanent quality of the raw material. The objective of this work is to compare the phytochemical profile and the antioxidant properties of powders from the leaves of *Punica granatum* L. collected in seven different regions of Burkina-Faso with a view to standardizing plant raw materials.

2. METHODOLOGY

2.1 Plant Material and Extraction

The plant material consisted of *Punica granatum* L. leaves collected in seven towns, administrative centers of seven different regions of Burkina-Faso. It is in fact the city of Ouagadougou in the central region, the city of Dedougou in the Mouhoun loop, the city of Banfora in the Cascades region, the city of Ouahigouya in the Northern Region, the Kaya town in the North Center region, Manga town in the South Center region and Fada town in the East region.

Fifty (50) g of powder of leaves was mixed with 500 ml of distilled water and boiled under reflux for 30 min. After cooling, the mixture is centrifuged and the supernatant is frozen for lyophilization. Two hundred and fifty (250 g) of the powder of leaves was stripped with an ethanol: water (9: 1) mixture. A vacuum rotary evaporator was used to remove the solvent

2.2 Study Design

2.2.1 Thin-layer chromatography (TLC)

The aim of the chemical screening was to identify the main chemical groups present in the extracts by thin layer chromatography (TLC). It was carried out on chromatoplates (60 F250, 20 x 20 glass support, Fluka –Silica gel) and several specific reagents were used to reveal these groups of compounds.

2.2.2 Dosage of phenolics compounds the levels of phenolic compounds were determined by assaying total phenolics and flavonoids

2.2.2.1 Total phenolic compound

The total phenolic compounds were measured according to the Singleton method [10]. The reaction mixture consisted of 1 mL of extract, 1 mL of 2N Folin–Ciocalteu reagent (FCR) and 2 mL of a 20% sodium carbonate solution. A control solution referred to as white identical to the reaction mixture except that the extract was replaced with distilled water was used. The solutions were allowed to stand at room temperature for 40 min and then the absorbance was measured at 760 nm using the Visible UV spectrophotometer. A standard curve was plotted with gallic acid (1-5 µg / ml) of r^2= 0.999. The trials were carried out in triplicate. The results are expressed in mg gallic acid equivalents / mg extract (mg GAE/mg) with reference to the gallic acid calibration curve.

2.2.2.2. Total flavonoids content

The flavonoid dosage was realized according to the Kumaran method [9] adapted by Abdel-Hameed [11]. Two (2) mL of hydroethnolic or aqueousr extracts (1 mg / mL extract with methanol) were mixed with 2 mL of aluminium trichloride (2%). After 40 min of incubation at ambient temperature, the absorbance was measured at 415 nm using a spectrophotometer (Agilent 8453) against a quercetin standard curve of r^2= 0.999. The blank control tube consisted of 2 ml of methanol. The quantity of flavonoids in the plant extract was determined in microgram-equivalent quercetin (EQ) per mg dry matter (MS) (µg EQ / mg MS).

2.2.3 Antioxidants activities

The measurement of the antioxidant potential was carried out by determining the products resulting from the oxidation or by evaluating the radical scavenging capacity of the reaction models. Three methods (DPPH, FRAP and ABTS) were used to assess the antioxidant activity of the extracts.

2.2.3.1 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Methods

$DPPH^{\bullet}$ free radical scavenging of different extracts from *Punica granatum* was evaluated using method of Kim et al. [12]. Twenty (20) µL

of different concentrations of extracts or reference (Trolox) were mixed with 200 µL of a methanolic DPPH$^{\bullet}$ solution (0.08 mg/mL) in a 96-well microtiter plate. The absorbance was recorded at 490 nm with spectrophotometer BioRad model 680 (Japan), after 30 min incubation at room temperature. Each determination was carried out in triplicate. The percentage of residual DPPH was evaluated into the graph in function of quantity of antioxidant: % DPPHres = f (antioxidant quantity/ DPPH$^{\bullet}$ quantity)

Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50 % (IC_{50}).

2.2.3.2 2,2'-Azino-bis(3-Ethylbenzothia- zoline-6- Sulfonic Acid) (ABTS) Methods

The method used is that described by Art et al., [13]. The antioxidant power in Trolox equivalent (Trolox Equivalent Antioxidant Capacity = TEAC) was determined by the ABTS test. A mass of 19.2 mg of ABTS was dissolved in 5 mL of distilled water and 3,312 mg of potassium persulfate are added thereto. The mixture is kept in the dark at room temperature for 12 to 16 hours. A volume of 4.5 mL of the mixture is then diluted in 220 mL of analytical ethanol. Twenty (20) µL of different concentrations of hydroethanolic, aqueous extracts or reference (Trolox) were mixed with 200 µL of a ABTS solution in a 96-well microtiter plate. The mixture was allowed to incubate for 30 min at room temperature. The absorbance was read at 415 nm with spectrophotometer BioRad model 680 (Japan) and the blank was the diluent solvent of the extract or standard. The curve of inhibition of the absorbance as a function of the concentration of the extract or Trolox was established for the determination of the 50% inhibitory concentration (IC_{50}).

2.2.3.3 Ferric ion reduction test (Ferric Reducing Antioxydant Power (FRAP))

The FRAP test was performed according to Fa et al. [14]. For the preparation of the FRAP solution, 10 mM TPTZ (2, 4,6-Tris (2-pyridyl) -s-triazine) and 20 mM of ferric chloride were diluted in 300 mM of sodium acetate buffer adjusted to a pH 3, 6 with a 01:01:10 ratio. Three milliliters of the FRAP solution was placed in a cuvette and then 100 µl of the sample were added thereto. The absorbance of the mixture was measured at 593 nm after 4 minutes of reaction. Ferrous sulfate heptahydrate $FeS0_4.7H_20$ was used as a standard at different concentrations. The AC was expressed in mM FRAP.

2.2.3.4 TLC screening for Anti-Radical Activity (AAR)

AAR screening was performed according to the method described by Takao et al. [15]. After migration of the extracts on a chromatography plate, we sprayed using DPPH as a developer.

2.3 Statistical Analysis

The experiments were carried out in triplicate and the results expressed as mean ± SEM. Analysis of the results was performed on the basis of statistical processing of Graph Prism version 6 software and unidirectional analysis of variance followed by Dunnett's test was used as statistical processing. The differences were considered significant when $p \leq 0.05$ compared to the control.

3. RESULTS

3.1 Phytochemical Screening

Several secondary metabolites were found in each of the samples subjected to chemical tests, including: sterols, triterpenes, and saponosides.

The results of phytochemical tests after thin layer chromatography are shown in the Table 1.

The results of the TLC carried out reveal the presence of tannins, flavonoids, saponosides, sterol and triterpenes in the aqueous and hydroethanolic extracts of *Punica granatum* from the different towns.

3.2 Dosage

The total polyphenol contents are determined by referring to a calibration curve produced with gallic acid. The concentrations of the total polyphenols obtained are presented in the Fig. 1 are expressed in mg EAG / g ES.

The content of the polyphenol extracts is higher with the hydroethanolic extracts from the towns of Banfora, Kaya, Fada and Dedougou with 457.1 417.9; 394.99 and 386.78 mgEAG / g respectively but also with aqueous extracts from the towns of Dedougou and Kaya, i.e., 395.75 and 340.09 mgEAG / g respectively.

Table 1. Phytochemical groups in hydroalcoholic and aqueous extracts of *Punica granatum* by TLC

Towns	Extracts	Tannins	Flavonoids	Saponosides	Sterols and Triterpenes
Ouagadougou	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Manga	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Kaya	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Dedougou	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Banfora	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Ouahigouya	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+
Fada	Aqueous	+	+	+	+
	Hydroethanolic	+	+	+	+

For the determination of the flavonoids a yellowish color is formed in all the extracts after the addition of the aluminum chloride solution ($AlCl_3$), this color reveals the presence of the flavonoids in the extracts analyzed. The results of the flavonoid assay are presented in the Fig. 2 and expressed in µgEQ / g ES.

Compared to the others, the aqueous extracts are richer in flavonoids and the towns of Banfora, Ouahigouya, Fada and Kaya and have a higher content, ie 70.1; 63.07; 56.93 and 55.97 µgEAG / mg respectively.

3.3 Antioxidant Activity

3.3.1 DPPH

After 30 minutes of incubation of the DPPH-extract solution (at different concentration), the purple color turns to a yellow color in the different extracts, this color change is due to the reduction of the DPPH radical. The results of the anti-free radicals by DPPH of the various extracts of Punica granatum are shown in Fig. 3.

The antioxidant activity evaluated for the various extracts of *Punica granatum* as well as the reference used is expressed in IC_{50} (inhibitory concentration 50) the concentration of extract which reduces 50% of free radical (DPPH), plus the IC_{50} is the lower the extract has a strong antioxidant potential.

In view of the results of the various extracts, all the extracts have an anti-free radical activity, but the aqueous and hydroethanolic extracts from the towns of Dedougou, Banfora and Fada have shown better anti-free radical activity. Better the aqueous extract of *Punica granatum* from the city of Dedougou has an anti-free radical activity almost identical to that of the reference which is Trolox, an inhibitory concentration of 4.19 µg / ml for the extract and 4.37 µg / mL.

3.3.2 ABTS

We used a 2nd test based on the proton trapping capacity of the cationic radical $ABTS^{\circ+}$ in order to show once again the anti-radical capacity of the extracts. The percentages of inhibition of *Punica granatum* extracts to ABTS are shown in Fig. 4.

These results indicate that the extracts from the towns of Manga, Banfora, Fada, Kaya exhibit a fairly remarkable inhibitory effect. Likewise, the hydroethanolic extracts of Manga and Kaya have respective inhibitory concentrations of 4.03 and 4.29 µg / mL. The extracts from these two cities have a more notable inhibition than the reference substance which has an inhibitory concentration of 5.75 µg / mL.

3.3.3 FRAP

The results shown in Fig. 5 illustrate the antioxidant activity against iron reduction by the extracts.

According to the values, it emerges that the hydroethanolic and aqueous extracts of cities such as Ouagadougou, Manga, Dedougou and Banfora are those which have shown great iron reduction capacities.

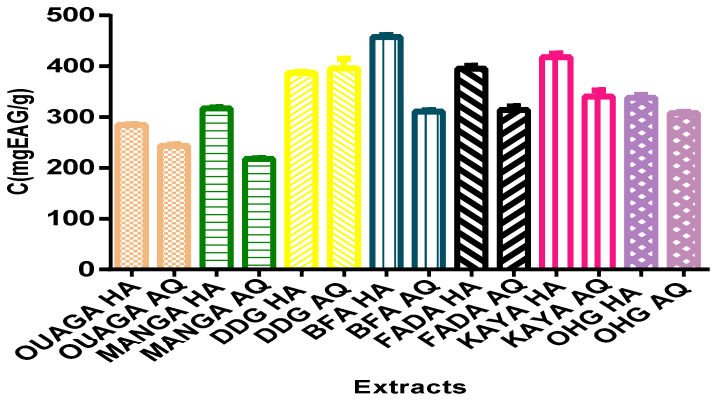

Fig. 1. Total phenolic content of *Punica granatum* extracts

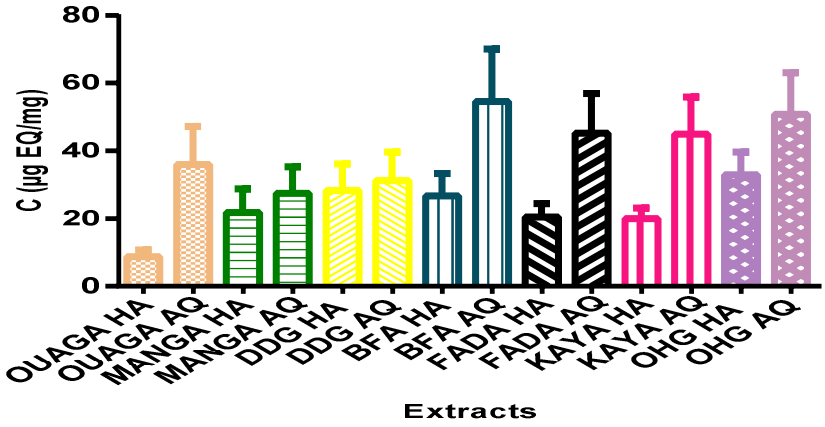

Fig. 2. Flavonoids content of *Punica granatum* extracts

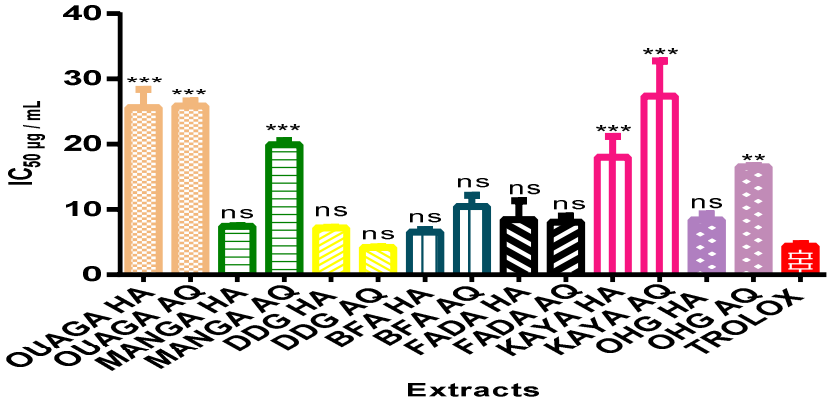

Fig. 3. Evaluation of the anti-free radicals DPPH of the extracts of *Punica granatum* ** *P* = .05 Vs control

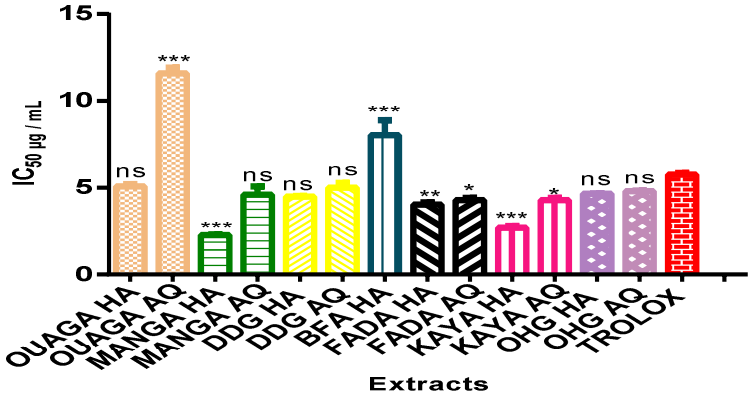

Fig. 4. Evaluation of the anti-free radical power of extracts of *Punica granatum* by the ABTS method ** *P* = .05 Vs Control

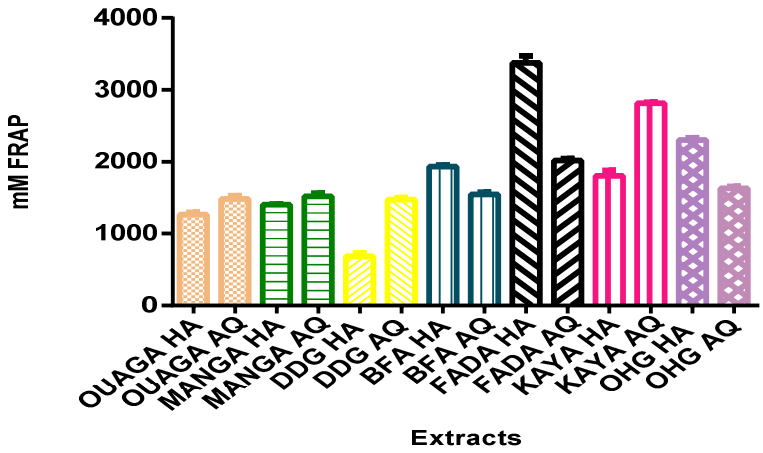

Fig. 5. Evaluation of the anti-free radical power of extracts of *Punica granatum* by the FRAP method

3.3.4 DPPH Activity on plate

The results of the Anti-Radical Activity (AAR) screening allowed to dismantle the extracts from the different towns and showed an AAR which consists of compounds that trap DPPH (Fig. 6).

3.4 Corrélations

The correlation between the content of phenolic compound and the antioxidant activity made it possible to obtain the following curves:

The results of correlations between the antioxidant activity and the content of the polyphenol of extracts give correlation coefficients (r^2) that vary between 0.84 and 0.88.

4. DISCUSSION

The phytochemical study shows the presence of several secondary metabolites in extracts from different regions. The phytochemical screening shows that the aqueous and hydroethanolic extracts of *Punica granatum* are identical in all the regions where the harvest was carried out. Tannins, flavonoids, saponins and triterpene sterols are the metabolites highlighted and the differences extracts. Several previous studies have confirmed these results, including those of Trabelsi et al. [16] also dealing with the leaves of *Punica granatum*. The presence of these secondary metabolites could later explain the antioxidant activities of the *Punica granatum* plant [15].

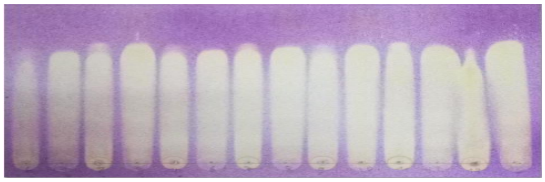

Fig. 6. Anti-radical activity on plate

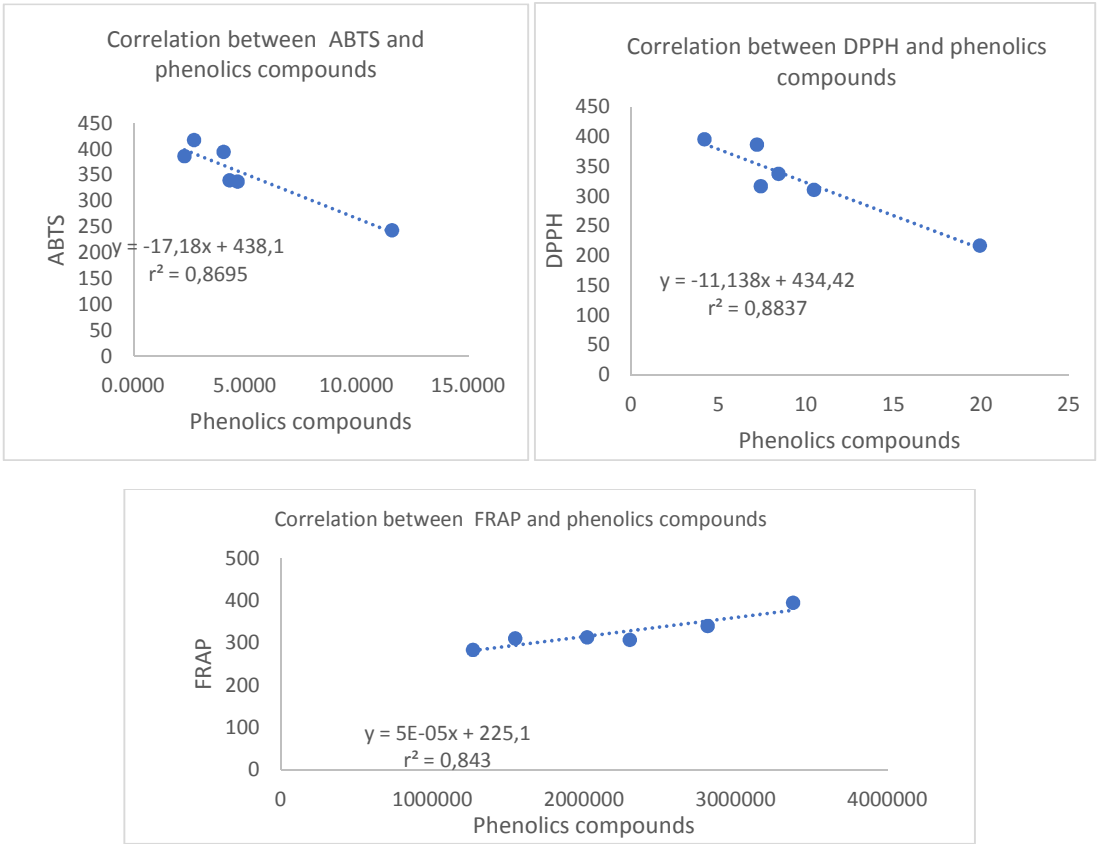

Fig. 7. Correlations between antioxidant activities and the content of different extracts

The determination of the contents of the extracts in total phenolic and in flavonoids confirms the phytochemical screening. While the presence of secondary metabolites does not vary from region to region, the level however depends on the region. Indeed, these results show that the extracts from the cities of Banfora, Kaya, Fada, Ouahigouya and Dedougou are richer in phenolic compounds and flavonoids than the other cities. It is interesting to note that the concentration of polyphenols and flavonoids in plants, which determines a large part of their activity, depends on many factors such as climatic conditions, time of harvest, mode of storage [17] or part of the plant used [18]. Similarly, the activity and the polyphenol content of plants can also vary depending on the maturity of the plant or the part of the plant harvested [19].

In this study, the harvest being done in the same period, on the same part of the plant (leaves) and stored under the same conditions, it could be that the factor that may be responsible for the diversity of the content is the climatic factor. Indeed, the cities where the crops of the plant were made are located in different regions of the country and therefore the climate and the type of soil are different.

According to these results, all the extracts, whatever the city, have an anti-free radical activity. However, this anti-free radical activity is

more important in the towns of Dedougou, Banfora and Fada. The difference between the anti-radical activity against DPPH of different extracts is probably due to their composition and their content of different phenolic compounds due to the diversity of climate and soil in different regions. The reduction of the DPPH radical is generally not due to the action of a single compound but to the interactions between several compounds, these interactions can exist in one extract not in another, thus leading to this difference in activity between the extracts of the same species.

With regard to the anti-free radical capacity by the ABTS method, extracts from the towns of Manga, Banfora, Fada and Kaya have proved to be more active.

These results would be compatible with the concentration of polyphenols determined where a predominance was clearly identified in the extracts from these cities which would explain these relatively high anti-radical powers in these cities.

The hydroethanolic extracts of Manga and Kaya have a significantly higher inhibition than the reference substance, i.e. an inhibitory concentration of 4.03 and 4.29 µg / ml for the towns manga and Kaya respectively while the reference substance has an inhibitory concentration of 5.75 µg / mL.

The nature of phenolic compounds such as flavonoids and tannins make them relatively hydrophilic and very soluble in water and polar organic solvents such as methanol, ethanol and acetonitrile, or their mixture of water. The hydroalcoholic extraction was carried out in order to improve and enrich the traces of phytochemicals in the aqueous extract [20]. This difference in activity may therefore be due to the high contents of the various metabolites contained in the ethanolic extracts compared to those contained in the aqueous extracts. Too, aqueous extracts contain hydrophilic secondary metabolites while hydroethanolic extracts contain not only hydrophilic but also non-hydrophilic secondary metabolites. This difference in activity may also be due to the presence of non-hydrophilic compound in the hydroethanolic extracts having an anti-free radical effect or potentiating the effect of hydrophilic metabolites by virtue of their presence.

Finally, the FRAP test and screening for anti-radical activity confirm the antioxidant activity of these extracts. The FRAP test shows a better reducing power of the ferric ion of the leaves of *Punica granatum* from the different cities, but as in the previous tests, the cities, Manga, Dedougou and Banfora stand out with better activity. Thus by these 4 tests, we see that the antioxidant activity of the extracts could be explained by the content of flavonoid and polyphenol [21].

The correlations carried out are evidence of the involvement of polyphenols in the anti-free radical activity of the extracts [22]. These correlations between the content of the extracts in total phenolics and the inhibition of the DPPH radical, ABTS and ferric iron give correlation coefficients close to 1.

5. CONCLUSION

This study made it possible to carry out a comparison of the vegetable raw materials obtained from the leaves of *Punica granatum* collected in seven different regions of Burkina Faso. Phytochemical analysis demonstrated the diversity by harvest site of secondary metabolites in the leaf powders of *Punica granatum*. The results of the determination of the total phenolics and flavonoids of the 14 extracts (aqueous and ethanolic) studied showed that the hydroethanolic extracts had high contents compared to the aqueous extracts. This difference in content also impacted the results of antioxidant activities. Indeed, the antioxidant power is proportional to the phenolic and flavonoid content of the extracts. In addition, the antioxidant power varied depending on the city. At the end of this work, the raw materials from the harvesting sites in the localities of Dedougou and Banfora were the richest in phenolic compounds and exhibited the best antioxidant activities with regard to all the tests carried out.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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