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PHYTOCHEMICAL ANALYSES OF SUPERCRITICAL CO_2 EXTRACT OF TUNISIAN *PSIDIUM GUAVA* SEEDS

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ABSTRACT

This study aimed to determine the fatty acid profile and quantify the bioactive compounds present in tunisian guava seed powder (*Psidium guajava* L.). The research was conducted on the extracts obtained by supercritical fluid extraction (SFE) and compared with those obtained by maceration and soxhlet. The SFE with carbon dioxide (CO_2) and ethanol as co-solvent was applied using two modes of extraction. The SFE(A) direct mode at 20MPa and 333K and the SFE(B) mode in six steps using a pressure and temperature variation (10, 20 MPa / 313, 323, 333K). The SFE give biphasic extracts, ethanolic and lipid fractions. The ethanolic fractions were subjected to the scavenging tests of DPPH and ABTS radicals. The total phenolics content and flavonoids were measured using standard methods and the lipid fractions were analyzed by GC-MS. In SFE the yield was highest at high pressure and temperature (333K/20MPa) (5.9%). The polyphenols and flavonoid contained in ethanolic fractions obtained by SFE (B) were respectively 2.57 mg GAE.g^{-1}DM and 10, 19 mg EQ.g^{-1} DM. These values were twice higher than that obtained by the direct

mode SFE (1.19 and 6.23). The lipid profile of oils showed a predominance of unsaturated fatty acids (86.6 to 88.78%), especially linoleic acid (ω6) and oleic acid (ω9). The extraction method showed rich sample in phenolics compounds and fatty acids. The SFE (B) was efficient, selective and gave various extracts with different chemical compositions depending on temperature and pressure variations.

KEYWORDS: Psidium guava, Total phenolic, Flavonoïds, Antioxidant activity, Supercritical fluid extraction.

INTRODUCTION

The application of natural antioxidants in the pharmaceutical and food industries is a very promising and developing area. According to Menat, phenolic compounds in fruit and vegetables are considered the most important group of natural antioxidants.[1] The extraction of these high value-added active ingredients is currently attracting a lot of interest and raises several scientific researches.[2] However, the extractability of the antioxidant depends on several parameters which are directly related to the quality of the extracts and in particular the extraction technique used. Several authors have demonstrated the significant effect of the technique used to extraction polyphenols.[3]

Traditional techniques of solid-liquid solvent extraction are commonly used for the isolation of phenolic compounds of plant material.[4] However, these extraction techniques require significant adjustments to reduce the risks to health, safety and the environment. The trend of green analytical chemistry with these new extraction methods presents extraction by supercritical fluids (SFE) as a good alternative to conventional solid-liquid extraction techniques. SFE has a number of advantages in terms of product quality extract, cost and environmental pollution. This technique is well suited for the treatment of materials sensitive to heat which can be separated by conventional distillation. In this study the SFE was used to extract the antioxidants of guava with the objective to examine the influence of extraction conditions on the extractability of phenolic compounds from this fruit.

The guava *Psidium guajava* L. (Myrtaceae) is a native species of tropical America, possibly ranging from Mexico to Peru, where it still occurs in a naturally occurring state.[5] The species is widely distributed and can be found in all tropical and subtropical regions, as well as in Asia and Africa (South Africa, India, Algeria and Tunisia). This fruit has attracted attention of food industry due to nutritional and therapeutic characteristics. It's considered as

an excellent source of vitamin C, niacin, riboflavin and vitamin A. Several parts of the plant are used in traditional medicine for the treatment of various human diseases.[6, 7]

Given various scientifically confirmed guava uses and the broad potential of its applications, the seeds have been selected, as vegetable matrix, in our study that aimed to optimize the SFE of antioxidants. These tiny seeds (2-3 mm in diameter and length) scattered throughout the pulp guava often regarded as waste, have favorable attributes for various uses, ingested whole or chewed, these seeds serve as excellent laxatives,[8] their valorization is therefore necessary. They are considered as potential food byproducts, only few studies were performed about these seeds and their uses.

This work focused on the study of seeds, valorize and propose possible uses of waste. The study permitted to determine the fatty acid profile and quantify bioactive compounds present in the Tunisian guava seed powder. The extraction was applied under different conditions and compared against conventional methods, such as maceration and Soxhlet extraction.

2. Materials and methods

2.1. Plant material

The fruits samples that are subject of this study were collected from the guava trees growing at Sousse (Tunisia 35°50′Nord 10°38′Est). The seeds samples were removed quantitatively from the flesh and dried in an air circulation oven (Tecnal, model TE-394 /L) at 50°C for approximately 12 hours. After dehydration, the seeds were ground to a fine powder using a domestic blender (Moulinex 180W). The particle size selected for extractions was from 0.2 to 0.3 mm. The sample was packaged in plastic bags and stored in the dark a dry place until that the extraction was performed.

2.2 EXTRACTION METHODS

Maceration extraction (ME)

The seeds powder (20 g) and the 100 mL of ethanol (Carlo Erba Reagents) were put in sterile erlenmeyer flask (125mL) wrapped in aluminum foil to avoid evaporation and exposure to light and was left for 3 days at room temperature with sporadic agitation. The mixture was filtered three times through Whatman N°2 filter paper and the solvent was evaporated in a vacuum evaporator (Buchi Rotavapor R-205) at 313K and 17.5 MPa. The recovered extracts were stored in domestic freezer until the analysis.

Soxhlet extraction (SE)

Extractions with Soxhlet method were performed using ethanol (EtOH) as solvent. The method consisted of 30 g of seeds powder placed inside a cartridge made by thick filter paper and loaded into the main chamber of the Soxhlet extractor. A 250 mL of solvent were used for extraction, with solvent continuously refluxing over the sample, the total extracting time was 10 h. After the extraction, the solvent was removed by evaporator, and the extraction yield was evaluated. The recovered extracts were stored in domestic freezer until the analyses.

Supercritical fluid extraction (SFE)

The experiments were carried out in a dynamic extraction unit conceived and assembled at the Reactions and Process Engineering Laboratory (LRGP, Nancy, France). The CO_2 used was 99.95% of purity (Messer France). The SFE unit is composed by a stainless steel cell of approximately 125 mL (300 x 23 mm) that supports pressures up to 25 MPa followed up by three cyclonic separators, a cold exchanger and a hot heat exchanger. The temperature was controlled thermostatically (Huber) and the pressure was regulated by means of a membrane pump (Dosapro Milton Roy – MilRoyal D), which enabled to reach the extraction pressure, with maximum mass flow rate 3.2 $kg.h^{-1}$, which was also connected to a cryostat in order to liquefy the CO_2. An electrical housing allows introducing the desired extraction pressure. The flow rate of the CO_2 was measured by means of a Coriolis force flow meter (Micro Motion) and hence, indicated the amount of CO_2 used during the extraction.

Experimental procedure

Based on results of the previous research[9,10], 2 modes of extraction was carried: SFE(A), mode direct (20MPa, 333K, 2h) and SFE(B), stepwise extraction in six steps (313K, 10MPa; 323K, 10MPa; 333K, 10MPa and 313K, 20MPa; 323K, 20MPa; 333K, 20MPa), each stage lasts 20 minutes. The extraction procedure consisted of placing 30 g of the sample (ground guava seeds) inside the extraction cell in using ethanol as co-solvents. The sample was added between two layers of glass beads (1.7 mm) which allow increasing the contact surface between the fluid and the solid matrix and promotes a uniform distribution, preventing the formation of preferential paths and agglomerations which may decrease the yield of the extraction. The CO_2 leaves the bottle in a gaseous state, the temperature being that of the atmosphere and the tank pressure of between 5 and 6 MPa, the gaseous CO_2 is then cooled to a temperature 276K, using a cold exchanger. The liquid is pumped and then heated to the

extraction temperature before being directed to the extractor, which is already at this temperature. At this level, it is supercritical. The unit was pressurized and the sample was kept in contact with SC-CO_2 with co-solvent for 30 min in static mode. After this time, the extractor valve was opened for complete depressurization of the extraction cell, and the intermediate valves between the separators were continuously adjusted in order to regulate the pressure and hence to keep a constant flow rate for each extraction. The temperature in the separators was 293K, and the pressures were respectively 5, 2 and 1 MPa in the separators (P1, P2 and P3). The supercritical CO_2 flow rate was comprised between 1.3 and 1.6 $g.min^{-1}$. Each one of the extractions was performed by triplicate.

After each extraction, the vessels collectors containing extracts were left for 5 min under ambient conditions to assure the complete removal of CO_2. The extracts obtained were in two phases: lipid phase (oil) and solvent phase (ethanolic fraction). The phases were separated and the high polarity phase was concentrated by rotavaporator (Buchi Rotavapor R-205) at 313K; 17.5 MPa for posterior reconstitution to a final volume of 25mL in ethanol. The results from all assays were evaluated considering the extraction yield (total extract, ethanolic fraction and oil). The ethanolic fractions (S10FE, S20FE and SDFE) were evaluated in term the extract quality (antioxidant activity, the total phenolics content (TPC) and total flavonoids (TF)). The lipid fractions (SD, S10 and S20) were analyzed by GC-MS for to determine the fatty acid profile of the oil seeds (Shimadzu GC-2010).

2.3 Antioxidant contents

The dry residue of the extracts (SE, ME, S10FE, S20FE and SDFE) was dissolved in methanol and the obtained solution was used to determine the content of total phenols, flavonoids and antioxidant activity. Folin-Ciocalteu phenol, Catechin, gallic acid (98%) and all other chemical reagents used were purchased from Sigma Co. (St. Louis, MO, USA).

2.3.1 The total polyphenol content was quantified in the seeds using the Folin–Ciocalteu reagent, according to the modified method previously reported by Singleton and Rossi.[11] A 1 mL of diluted extract was transferred to a 25 mL volumetric flask containing 9 mL of ultra pure water, the Folin–Ciocalteu reagent (1mL) was added and mixed. After 3 min, 1 mL of sodium carbonate (15 %) were added. After 30 min of incubation at 313K in the dark, the absorbance was measured at 700 nm using the Shimadzu UV-Vis spectrophotometer. The results are expressed as equivalents of gallic acid (mg $GAE.g^{-1}$ DM) using a calibration curve of the Gallic acid.

***2.3.2 Total flavonoid content* was measured** according to the modified colorimetric method of Zhishen et al.[12] Briefly, a 125µL of seeds methanol extract was added to a 75µL of $NaNO_2$ (5%). The mixture was incubated for 6 minutes. A 150µL of $AlCl_3$, 6 H_2O (10%) freshly prepared are added, after 5 minutes of incubation, 500µL of NaOH (solution 1M) added to the mixture, the final volume was adjusted to 2500µL with distilled water. The absorbance was measured at 510 nm in a spectrophotometer (Shimadzu UV-Vis spectrophotometer). The blank was prepared using the same procedure with ultra pure water without extract, and each measure was made in triplicate. A series of methanolic dilutions of quercetin were prepared and assayed; flavonoid amounts in extract were expressed in mg quercetin equivalent per g dry matter (mg QE.g^{-1} DM).

2.4 Antioxidant activity

2.4.1 DPPH scavenging method

The DPPH radical method was based on the procedure proposed by Burda and Oleszek[13] using 96-well microplates in a spectrofluorimeter (Flx-Genius, SAFAS-France) coupled with an automatic injector comprising two 1 mL syringes (SAFAS-France) and a water-bath (Fisher-France). DPPH "2,2-diphenyl-1-picryl-hydrazyl" (Ref. D -9132) was purchased from Sigma-Aldrich and ethanol from Carlo Erba. Briefly, it was used 220µL DPPH solution 46.7mg.L^{-1} in ethanol, with absorbance measured at 517 nm (A_0). Then, 80 µL of extract in ethanol at different concentrations (0.125 to 1 mg. L^{-1}) were added and the absorbance was measured after 1 h (A_f). The percentage of inhibition (%I) of free radical DPPH by extract sample was calculated using the formula given below: $\%I = ((A0 - Af) / A0) \times 100$

The results are expressed on the bases of IC50 values, defined as the concentration of the sample or the reference compound to decrease the absorbance at 515 nm (or concentration) of DPPH solution to half of its initial value.

2.4.2 ABTS scavenging method

Antioxidant activity of seeds extract was analyzed by investigating their ability to scavenge the $ABTS^{\bullet+}$ free radical using the modified method previously reported by Ozgen et al.[14] The stock solutions included 7 mM ABTS solution and 4.9 potassium persulfate solutions. Trolox "6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid" from Sigma-Aldrich (Ref. 56510). The working solution was prepared by mixing the two stock solutions in equal proportions and allowing them to react for 16 hours before use in order to produce ABTS radical ($ABTS^{\bullet+}$). This solution was stored in a dark place at room temperature. Before use,

the solution was diluted with ethanol to obtain absorbance between 700 nm and 800 nm. This solution was mixed with sample (5 to 40µg.mL^{-1}). A control containing methanol and ABTS$^{\bullet+}$ solution was also realized. The absorbance was read at 734 nm after 30 min incubation at 303 K. As unpaired electrons are sequestered by antioxidants in the sample the test solution turns colorless and the absorbance at 734 nm is reduced. All measurement was performed in triplicate. The percentage inhibition was calculated against a control and compared to a Trolox standard curve. The results are expressed in terms of TEAC (Trolox equivalent antioxidant capacity). This index is defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the extract.[15]

2.5 Fatty acid composition (GC/MS analysis)

Gas chromatograph coupled with mass spectrometer (GCMS QP-2010 Ultra (Shimadzu, Kyoto, Japan)) was used for the identification of each fatty acid methyl ester. The sample was injected through a Shimadzu auto sampler AOC-20s onto the Rt-2560 column (30 m length, 0.25 mm i.d., 0.25 m film thickness). Initially the oven temperature was 115°C, rising up with a ramp initial rate of 15°C min^{-1} to 180°C and once again at the rate of 1°C min^{-1} to 300°C. The injector temperature was held at 225°C. Helium was used as a carrier gas at a flow rate of 1.5 mL.min^{-1} and 1:30 was the split ratio. The MS scan parameters included a mass range of 50–500 m/z, a scan speed of 1666, operating in positive electron impact mode with an ionization energy of 70 eV. Ion source and interface temperature were 200°C and 245°C, respectively. For the identification of mass spectra, NIST11 library and NIST analysis program softwares were used. GCMS solution integrated software (Shimadzu Cat No. 225-21731-92) was also used for the chromatogram analysis.

2.5 Statistical analysis

All assays were performed in triplicate for each extracting condition. An analysis of variances (ANOVA) for each experiment (yield and quality evaluation) was carried out. The results are reported as standard deviation ±SD (standard deviation) obtained from the three measurements.

3. RESULTS AND DISCUSSION

3.1 Extraction yield

The results of the extraction yields obtained by SFE (A and B), SE (Soxhlet extraction) and ME (maceration extraction) are represented in table 1. They showed that during the SFE process small changes in the conditions may significantly influence process yield. In SFE the

highest yields were obtained with SFE (A) (oil fraction: 4%; total extract: 5.9%). It is noted that under these conditions the lipid fraction is twice as high as the ethanolic fraction (1.9%). The SFE (B) with progressive variation of pressure and temperature gave slightly lower global yields of total extracts and oily fractions. However the total yield in ethanolic fractions (S10FE + S20FE) was higher (2.8%). These results showed that at elevated temperature and pressure, more oil than phenolic compounds extracted. Indeed, the increased pressure increases the density of the supercritical CO_2, which increases its solvent power and its ability to extract bipolar compounds such as fatty acids. Under these conditions, the extraction is, however, less selective than SFE (B), and a large amount of non-phenolic compounds can be coextracted. According to the literature, the SFE applied to guava Colombia seeds gave higher yields of total extract (12.2 to 17.06%) and lower phenolic fractions (0.76 to 1.51%) to those the tunisian guava found in this study.[16] Conventional methods have led to larger quantities of extracts with yield 16.1% for SE (soxhlet) and 30% for ME (maceration) (Table 1). The yield of the SE is higher than the value given by Uchoa-Thomaz for the guava seed Brazil.[8] (13.93%).

Table 1: Yield obtained in different extraction processes.

<table>
<tr><th colspan="8">Supercritical fluid extraction (CO_2/Ethanol)</th></tr>
<tr><th colspan="3">Terms of extraction</th><th colspan="2" rowspan="2">Extracts</th><th rowspan="2">Yield (%) w/w</th><th rowspan="2">Global yield (%)</th></tr>
<tr><th>T(°C)</th><th>Time(mn)</th><th>P(MPa)</th></tr>
<tr><td rowspan="2">60</td><td rowspan="2">120</td><td rowspan="2">20</td><td rowspan="2">SFE(A)</td><td>SDFE</td><td>1. 9</td><td rowspan="2">5.9</td></tr>
<tr><td>SD</td><td>4</td></tr>
<tr><td rowspan="2">40/50/60</td><td rowspan="2">20/20/20</td><td rowspan="2">10</td><td rowspan="4">SFE (B)</td><td>S10FE</td><td>1.7</td><td rowspan="4">5.4</td></tr>
<tr><td>S10</td><td>1.5</td></tr>
<tr><td rowspan="2">40/50/60</td><td rowspan="2">20/20/20</td><td rowspan="2">20</td><td>S20FE</td><td>1.1</td></tr>
<tr><td>S20</td><td>1.1</td></tr>
<tr><th colspan="8">Other extraction methods (Ethanol)</th></tr>
<tr><th>Extraction</th><th>T(°C)</th><th>Time(h)</th><th>P(atm)</th><th colspan="2">Extracts</th><th colspan="2">Yield (%)</th></tr>
<tr><td>SE</td><td>69</td><td>8</td><td>1</td><td colspan="2">S2</td><td colspan="2">16.1</td></tr>
<tr><td>ME</td><td>25</td><td>72</td><td>1</td><td colspan="2">M2</td><td colspan="2">30</td></tr>
</table>

SFE(A): SFE direct mode, SFE(B): SFE mode by step;

SDFE, S10FE, S20FE: ethanol fractions; SD, S10, S20: oil fractions.

3.2 Antioxydants contents

Several studies have reported the wealth of antioxidants in the guava fruit, but little research has concerned the phytochemical study of the seeds of this fruit.

Phenolic compounds

The total phenolic content (TPC) expressed in mg gallic acid equivalent.g^{-1} DM are presented in table 2. The relatively high levels of phenolic compounds vary depending on the adopted extraction process. It should be noted that pure ethanol is a good solvent extraction of polyphenols, it dissolves properly moderately polar phenolic compounds and can also extract residual lipophilic substances.[3] The SFE (B) provides extracts (S10FE + S20FE) with a total amount of polyphenols (2.57 mg GAE.g^{-1}DM), twice higher than that obtained with SFE (A) in SDFE extract (1.19), therefore is noted that S20FE extract have the highest concentration of polyphenols (1.993). The change in operating conditions (temperature and pressure) causes the change in the CO_2 solvent power and it is allowing to selectively extract the molecules according to their chemical nature and molecular weight. According to the literature, TPC obtained in S10FE an S20FE extracts are twice as high as those obtained in SC CO_2 extract under the same conditions from guava Colombia seeds (1.15 mg GAE.g^{-1} DM).[9] On the other hand the TPC in the SDEF extract, is similar to that found in the SC CO_2 seeds extracts (1.31 mg GAE.g^{-1} DM) (30 MPa / 323K).[16] Several research studies showed that the total polyphenol content in plants are strongly influenced by the area and growing conditions.[17-19] Contrary to the results obtained for the polyphenols content in the leaves guava, the maceration and Soxhlet extracts provides a better contents of phenolic compounds for the seeds, compared to SFE extracts.[10]

Flavonoïdes

The total flavonoids contents in extracts differed according to the used extraction method. It is noted that highest content was obtained with ME technique (11.08 mg EQ.g^{-1} DM) (Table 2). The total quantities of flavonoids contained in S10FE and S20FE extracts were 10.19 mg EQ.g^{-1} DM. This quantity decreases with SFE (A) (6.23 mg EQ.g^{-1} DM). The influence of operating conditions on the amount and quality of obtained extract is noted. The SFE with progressive variation of temperature and pressure gave extracts with, the best levels of flavonoids. This result was similar to SFE of guava leaves.[10] This operating mode in SFE is effective and more profitable than classical mode. The conventional extraction methods SE and ME allow the extraction of a large amount of flavonoids, the amount extracted by maceration (11.08) is almost twice than that extracted by soxhlet (5.36). The high levels of polyphenols and flavonoids obtained by maceration are due to the effect of temperature (25°C). Previous studies showed that the use of higher temperatures (50°C) decreases the total polyphenols yield, this is probably due to their degradation.[20, 21]

Table 2: Quality results in terms of antioxidant activity (by DPPH and ABTS methods) and TP, TF, for SFE, SE and ME.

Extract	TP mg GAE.g^{-1} DW	TF mg EQ.g^{-1}DW	TEAC(*ABTS*) mM.mg^{-1}	IC50(*DPPH*) µg mL^{-1}
SDFE	1.19 ± 0.20	6.23 ± 0.10	0.011	2089
S100FE	0.58 ± 0.05	5.10 ± 1.05	0.025	908.9
S200FE	1.99 ± 0.12	5.09 ± 0.01	0.033	686.26
M2(F2)	6.38 ± 0.35	11.08 ± 0.45	0.022	1016.2
S2	3.74 ± 0.20	5.36 ± 0.08	0.014	1553.5

TP: Polyphenols; TF: Flavonoids

S10FE, S20FE, SDFE: extract by SFE ; M2: extract by ME ; S2: extract by SE.

3.3 Antioxidant activity

In this study, two free radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ were used to assess the potential free radical-scavenging activities of guava extracts, due to their similar antioxidant mechanism and the high correlation observed between these techniques.[22, 23] The DPPH method is widely used to evaluate antioxidant capacity and to test the ability of compounds to act as free radical scavengers or hydrogen donors. The parameter IC50 is the concentration of substrate that causes 50% loss of the DPPH activity. The extracts were examined in relation to their IC50 value. These values are presented in table 2. The two fractions S10FE and S20FE obtained by SFE (B) presented a slight difference in terms of antioxidant activity. As the IC50 concentration and the antioxidant capacity have inversely proportional values, S10FE was established to have the lowest antioxidant capacity, while S20FE was found to be most active of all (IC50 = 686.26µg mL^{-1}). These fractions were more active than the extract obtained by SFE(A) (IC50 (SDFE) = 2089 mg mL^{-1}), these results are correlated with the contents of polyphenols found in these extracts.

The scavenging activity of the extracts on free radical ABTS was compared with a standard amount of Trolox and calculated as TEAC (Trolox equivalent antioxidant capacity). The results are showed in table 2. For all extracts SFE, the TEAC values ranged from 0.011 mM.mg^{-1} (for SDFE) to 0.033 mM.mg^{-1} (for S20FE). The results for DPPH assay expressed on the bases of IC50 values were in agreement with those of the ABTS assay, and those of TPC. According to the two tests, the S20FE extract has the best quality (IC50 = 686.26 µg mL^{-1}; TEAC =0.033 mM.mg^{-1}) and the highest amount of total polyphenols (TPC = 1.993 mg GAE.g^{-1} DM). Consequently SE and ME techniques gave rich extracts in polyphenols (3.7 and 6.3 mg GAE.g^{-1} DM for SE and ME) in comparison with SFE. However these traditional

techniques showed low antioxidants activities (IC50 (ME) = 1016.00 and IC50 (SE) = 1553). This can be due to the fact that these extractions can cause a large number of non-antioxidant compounds. The SFE extracts obtained in this study have a higher antioxidant activity compared with those reported Castro-Vargas et al. for SC CO_2 extracts (TEAC = 1.30 mmol.g^{-1} at 30MPa/ 323K), for SE extracts (TEAC = 2.24 mmol.g^{-1}) from Colombia guava seeds.[16] They are also higher than those cited by Hernandez et al. for SC CO_2 extract obtained under the same conditions (with gradual increase in pressure and temperature) (TEAC = 1.49 mmol.g^{-1}) for the Colombia guava seeds.[9] The results obtained on guava seeds were compared with methods described in literature for extraction of leaves. They presented a significant difference at the antioxidant potential between the leaves and seeds as showed in figure 2.[10] This result is expected since the seeds are more rich in lipidic fraction than phenolic one.

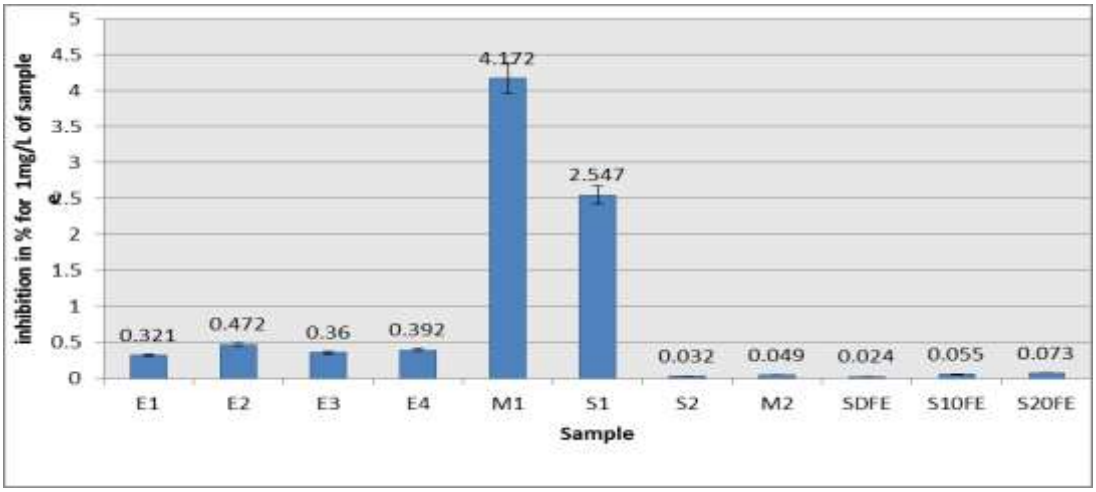

Figure 1: Percent inhibition of DPPH by extracts of leaves and seeds guava[10]

E1(10MPa),E3(20MPa): extract of leaves recovered separator S1 (mode by steps).

E2(10MPa): extracts of leaves recovered separator S2(mode by steps).

E4(20MPa): extract of leaves recovered separator S1(direct mode).

M1: extract of leaves by maceration; S1: extract of leaves by soxhlet.

2.5 Fatty acid composition

The obtained oil fractions with SFE extracts were analysed by GC MS. The analysis data are shown in table 3. The main components identified in all the SFE extracts were fatty acids methyl esters (FAMEs) with a predominance of unsaturated fatty acids (86.6 - 89.3%) especially linoleic acid ω6 (76.85 – 82.8%) and oleic acid ω9 (6.5 -7.8%), palmitic acid and

stearic acid are also present but in smaller amounts. The fatty acid compositions of the SFE extracts obtained with both modes A and B are not significantly different, they were similar and their relative percentages were very close. However these extracts showed the presence of other saturated fatty acids like heptadecanoic acid in SFE (A) extracts. These results can add value to Tunisia guava seeds, due to the abundance and predominance of linoleic acid. This latter is an essential fatty acid which cannot be synthesized by the body and must be supplied in the diet. Its importance lies in the fact that it is essential for the body because it is a constituent of cell membranes.[24] On the other hand unsaturated fatty acids are highly relevant since they have an important role in reducing blood cholesterol level and in the treatment of atherosclerosis.[25] The values obtained were similar to those given by Opute et al. for SE extracts from guava Brasilia seeds.[26] (linoleic acid: 77.3%; palmitic acid: 8.0%; oleic acid: 9.42%; stearic acid: 4.48%). However these values are in contrast to those given by Castro-Vargas for SC CO_2 extracts obtained from the guava Colombia seeds, the fatty acid composition is different and the predominance of unsaturated is not noticed (linoleic acid: 50.85%; palmitic acid: 6.33%; oleic acid: 2.7%; stearic acid: 40%).[16] The difference between these results could be due either to the technique employed or the geographical origin which could account for differences in chemical composition of the same plant species.[26] Note that the fatty acids present in the extracts of guava seeds are cis form, this is the general conformation of unsaturated fatty acids of vegetable origin. Trans fatty acids are either of animal origin (ruminants and dairy products) or industrial origin or the result of processing of vegetable oils during cooking. The general classification of fatty acids in all samples can look as follows (Tab.3): *PUFA>MUFA and PUFA>SFA*. The ratio PUFA/SFA varies between 6.32and 8.03, these values are higher a minimum threshold of 0.45 advocated by the by HMSO.[27]

Table 3: Fatty acid profile Tunisian guava seeds extract S10FE, S20FE and SDFE obtained with SC-CO_2/EtOH.

Fatty acid	N° Carbon	Relative content [%]		
		SFE extract		
		SD	S10FE	S20FE
Palmitic ac.	C16:0	7.63	6.38	7.4
Heptadecanoic ac.	C17:0	0.07	-	-
Stearic ac.	C18:0	4.68	4.30	2.9
Oleic ac.	C18:1 cis-9	7.80	6.98	6.5
	C18:1 cis-11	0.52	-	-
Linoleic ac.	C18:2 cis-9,cis-12	76.85	81.89	82.8
	C18:2 cis-12,cis-15	1.44	-	-

SFA[a]		12.38	10.68	10.3
MUFA[b]		8.32	6.98	6.5
PUFA[c]		78.29	81.89	82.8
USFA[d]		86.61	88.87	89.3
PUFA / SFA		6.32	7.66	8.03

[a]*SFA= saturated fatty acids;* [b]*MUFA= monounsaturated fatty acids;*
[c]*PUFA= polyunsaturated fatty acids;* [d]*USFA= unsaturated fatty acid*

II.3 CONCLUSION

In this work, the supercritical fluid extraction (SFE) from the Tunisia guava seeds using the supercritical CO_2 with ethanol as co-solvent was realized. Two processes of extraction were conducted, SFE (A) at 60°C/20 MPa and SFE(B) with a progressive variation of temperature and pressure. The extracts obtained have remarkable antioxidant activities (686.26µg mL^{-1}) and were rich in unsaturated fatty acids, mainly linoleic acid (ω6) and oleic acid (ω9). The highest global yield was observed in direct SFE (A) mode. However the SFE (B) in stepwise mode provides the best results in terms of quality of the extract (antioxidant activity, PTC and TF). This method of extraction has led to amounts of polyphenols and flavonoid twice as high as those obtained with the direct mode and provides more oils rich in unsaturated fatty acids.

The SFE results were compared with those of two conventional extraction methods, ME and SE. ME gives a better yield than SFE with a higher content of phenolic compounds but leads to the extraction of a large amount of non-antioxidant compounds. This study showed that the SFE with a progressive variation of temperature and pressure is a soft extraction technique. It is a selective technique since it product various extract with different chemical composition. The selective separation of different chemical families is indeed one of the main challenges facing chemists, hence the need to develop this kind of study.

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