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Effect of Extraction Solvents on Polyphenol Content and Antioxidant Activity of Carob Tree (*Ceratonia siliqua* L.) Leaves

Siham BABA AHMED[1*], **Mohammed Adil SELKA**[2], **Ilham LAHFA**[3]

[1] Department of pharmacy, Pharmacognosy laboratory, Faculty of medicine, University Abou Bakr Belkaïd, 13000, Tlemcen, Algeria
[2] Department of pharmacy, TOXICOMED laboratory, Faculty of medicine, University Abou Bakr Belkaïd, 13000, Tlemcen, Algeria
[3] Department of medicine, TOXICOMED laboratory, Faculty of medicine, University Abou Bakr Belkaïd, 13000, Tlemcen, Algeria

*Corresponding author : sihem.babaahmed@univ-tlemcen.dz

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Abstract

Carob tree (*Ceratonia siliqua* L.) is widely used in traditional medicine and food. The aim of this study was to determine the most appropriate extraction solvent to optimize the extraction of polyphenols and flavonoids from the leaves, in order to obtain the highest antioxidant activity. In this context, the effects of six different solvents (100% methanol, 100% ethanol, 100% acetone, 50% (v/v) aqueous methanol, 50% (v/v) aqueous ethanol and 50% (v/v) aqueous acetone) with different polarities on the polyphenol content and antioxidant activity of carob tree leaf extracts were investigated. The total polyphenol and total flavonoid contents, as well as the antioxidant activity determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method of the extracts were evaluated. The results showed that carob tree leaves extracted with 100% methanol presented the highest values in terms of total polyphenol content (11.33± 0.08 mg EAG/g DM), total flavonoid content (8.55± 0.34 mg EC/g DM) and DPPH inhibition percentage (79.29 ± 3.75%). High positive correlations between total polyphenols, total flavonoids and antioxidant activity of carob tree leaf extracts were also observed. These results indicate that carob tree leaf extract obtained using an appropriate extraction solvent was able to enhance the protective effect against oxidative damage associated with free radicals.

Key Words: Antioxidants, Polyphenols, Flavonoïds, Extraction solvents, *Ceratonia siliqua* L. , Carob tree leaves.

1. Introduction

Carob tree (*Ceratonia siliqua* L.) is a typical Mediterranean species belonging to the Fabaceae family (Benmahioul et al., 2011). The fruit is a pod traditionally used as an antidiarrheal and in gastric ulcer, its seeds are natural appetite suppressants, and reduced to powder, they are used as an inexpensive alternative to cocoa in food, which is the reason why this tree is cultivated in its native countries. On the other hand, leaves have also pharmacological interest through their phenolic compounds content as: gallic acid, kaempferol, tannic acid, quercetin, rutin, biocanine, myricetin,

naringenin, genistein, catechin, taxifolin, epigallocatechin-3-gallate and epicatechin-3-gallate (Ouhaddou et al., 2014; Durazzo et al., 2014). Therefore, it is important to highlight the polyphenols and flavonoids as well as the antioxydant activity of carob tree leaves.

The solvent used for extraction is one of the most important factors influencing the efficiency of polyphenol extraction and the associated health benefits (Ngo et al. 2017). Polyphenols are often extracted from plant materials using polar solvents. Organic solvents such as methanol, ethanol and acetone, or aqueous mixtures, are generally preferred. Methanol is considered as a good solvent for extracting low-molecular-weight polyphenols, while aqueous acetone is more effective for extracting high-molecular-weight flavanols. Ethanol is also considered as a good solvent for polyphenol extraction overall (Do et al. 2014). The chemical composition of plant material varies from species to others. That's why, it is very difficult to propose a suitable extraction solvent for each plant material (Wijekoon et al. 2011).

Previous studies have shown that depending on the used plant material, the most suitable extraction solvent that can be used to determine polyphenol content and antioxidant capacity is acetone 50% for *Salacia chinensis* L. root (Ngo et al. 2017), acetone 60% for brewers' grains from *Hordeum vulgare* L. seeds (Meneses et al. 2013), acetone 100% for *Vaccinium myrtillus* L. leaf (Ceylan et al. 2017), ethanol 60% for *Cinnamomum cassia* (L.) J.Presl bark (Dvorackova et al. 2015), ethanol 100% for *Davidsonia pruriens* F.Muell fruit (Chuen et al. 2016), methanol 50% for *Allium sativum* L. husk (Kallel et al. 2014) and for *Ficus carica* L. seeds (Nakilcioğlu-Taş and Ötleş, 2021) and methanol 90% for *Helianthus annuus* L. florets (Ye et al. 2015).

While some studies have focused on optimizing the extraction of polyphenols from *Certanoia siliqua* L. seeds, none have focused on the leaves. There is therefore still no information on the effect of solvents of different polarities on the extraction of carob tree leaf polyphenols and their antioxidant activities. Consequently, this study aimed to determine, as a function of the extraction solvent (100% methanol, 100% ethanol, 100% acetone, 50% (v/v) aqueous methanol, 50% (v/v) aqueous ethanol and 50% (v/v) aqueous acetone), total polyphenol and flavonoid contents of carob tree leaves, and their relationships with their antioxidant activity (DPPH radical scavenging activity).

2. Material and Methods

2.1. Sample preparation

Carob tree leaves were harvested during the flowering period in October 2022 in Tlemcen (Algeria), geographic coordinates 35°04'06.6 "N 1°25'48.6 "W. They were thoroughly washed and then sent to the Pharmacognosy Laboratory of the Faculty of Medicine at Tlemcen for identification, leaf removal and fragmentation. They were then dried at room temperature for ten days, protected from light and humidity. After that, leaf fragments were ground using a mortar, in preparation for analysis.

2.2. Extraction process

Solid-liquid extraction process were used for the extraction of antioxidant compounds from carob tree leaves according to the protocol of Nakilcioğlu-Taş & Ötleş (2021) with slight modifications. Six different solvents were used: 100% methanol, 100% ethanol, 100% acetone, 50% (v/v) aqueous methanol, 50% (v/v) aqueous ethanol and 50% (v/v) aqueous acetone. 3 g of sample was mixed with 15 ml of solvent. The mixture was then stirred at 50°C for 90 minutes in a water bath (Memmert WNB 7, Germany) before filtering through filter paper. The volume of the extract aliquot was made up to 15 ml. Liquid extracts were stored at 4°C until analysis.

2.3. Determination of total polyphenols and total flavonoïds

Total polyphenols (TPs) and total flavonoids (TFs) determoination was carried out by spectrophotometric method in accordance with the protocol described and validated by Matić et al (2017). For total polyphenols, the standard used was gallic acid diluted in methanol (1, 10, 50, 100, 200, and 500 mg/L). 20 µL of standard or of the polyphenol sample, 1580 µL of distilled water, 100 µL of Folin-Ciocalteu reagent and 300 µL of Na_2CO_3 (200 g/L) were added to a glass tube. These solutions were vortexed (VWR® VV3, Germany) and incubated at 40°C for 30 min in a water bath. Absorbance was measured at 765 nm against a blank (containing 20 µL of distilled water instead of the prepared solution) using a UV-Vis spectrophotometer (Optizen 2120 UV, Korea). Results were expressed as milligram gallic acid equivalents per gram of dry matter (mg GAE /g DM). For total flavonoids, the standard used was catechin diluted in methanol (1, 10, 50, 100, 200, and 500 mg/L). 800 µL of distilled water, 200 µL of standard or sample and 60 µL of $NaNO_2$ (5%) were introduced into a glass tube. 60 µL of $AlCl_3$ (10%) were added after 5 min, then 400 µL of NaOH (1 mol/L) and 480 µL of distilled water were added after 6 min. The solution was stirred with the Vortex. Absorbance measurements were performed using spectrophotometer at 510 nm against a blank (containing 200 µL of distilled water instead of the prepared solution). Results were expressed as milligram catechin equivalents per gram of dry matter (mg CE/g DM).

2.4. Determination of antioxidant activity: Free radical scavenging method

The effect of antioxidant compound on radicals was assessed by DPPH according to the procedure described by Brand-Williams et al (1995), Sánchez-Moreno et al (1998), Kim et al (2015) and Selka et al. (2022). 0.1 ml standard ascorbic acid or sample diluted in methanol (1.8, 2.9, 4.9, 7.8, 12.7, 17.3 mg/L) was added to 3.9 ml DPPH freshly prepared in methanol (25 mg/L). Absorbances at 515 nm were measured after 30 minutes in the dark (until the reaction reached plateau). The Radical Scavenging Activity (RSA) was calculated as a percentage using the following equation:

$$\text{DPPH RSA (\%)} = [1 - (A_{sample}/A_{control})] \times 100$$

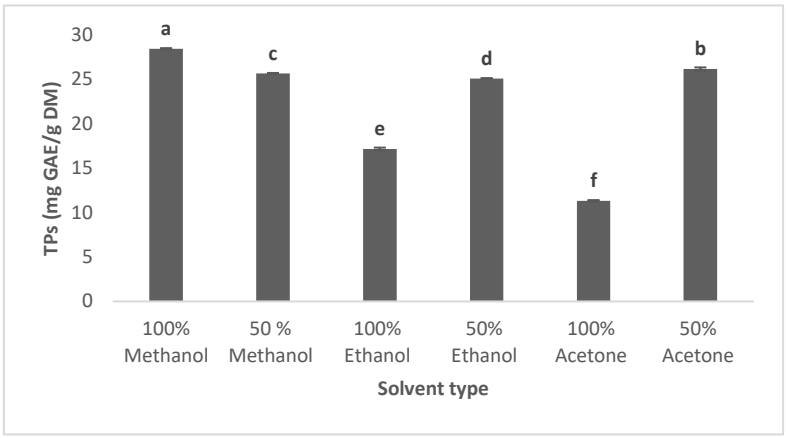
2.5. Analytical statistics

Results were expressed as mean value ± standard deviation, as each measurement was performed three times. To determine differences between values, ANOVA and Tukey test were used on IBM SPSS Statistics Trial Version 29.0.1.0 at a significance level of $p<0.05$. To determine the correlation between variables, Pearson correlation test was also used.

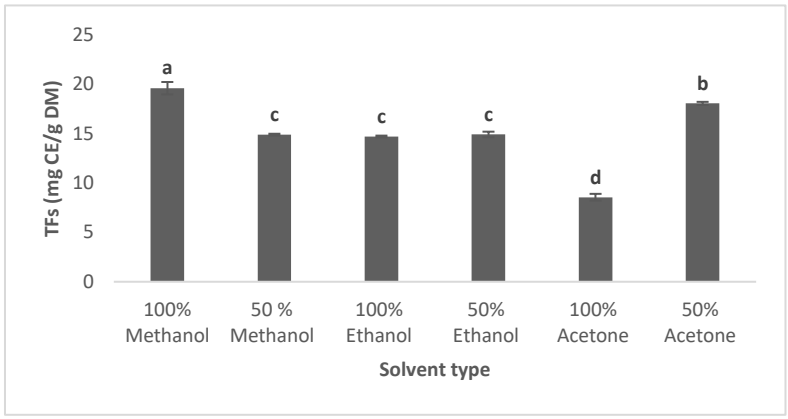
3. Results and Discussion

3.1. Total polyphenol, total flavonoid and antioxidant activity of the carob tree leaf extracts

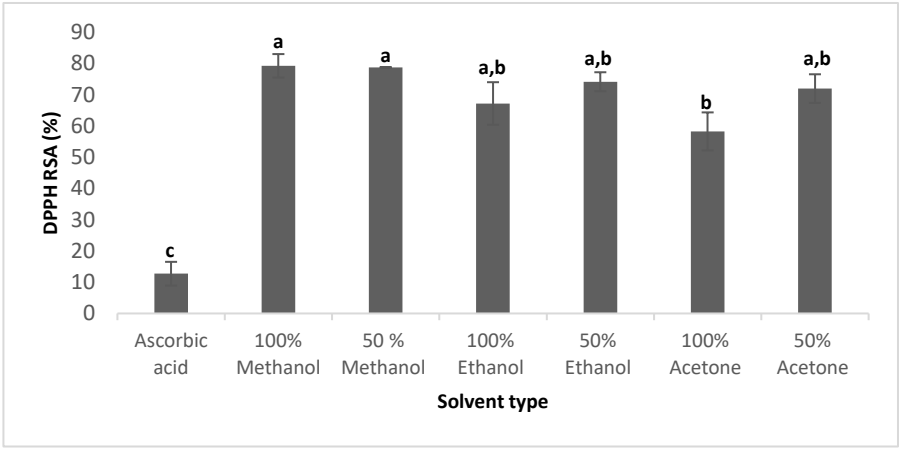
In this study, solvents of different polarities were used to extract polyphenols and antioxidant compounds from carob leaves. As shown in Figure 1 A, B, total polyphenol and flavonoid contents varied between extracts ($p<0.05$). For TPs, the linear trend curve for gallic acid followed this formula $y = 0.001 x - 0.0002$ with a correlation coefficient $R^2 = 0.999$, the deduced contents vary in the following ascending order: 100% acetone < 100% ethanol < 50% ethanol < 50% methanol < 50% acetone < 100% methanol ($p<0.05$). Whereas for TFs, the linear catechin trend curve followed the following formula $y = 0.0008 x + 0.0175$ with $R^2 = 0.991$ and contents varied in the following ascending order: 100% acetone < 100% ethanol =50%ethanol=50% methanol



A



B



C

Figure 1. TPs **A**, TFs **B** and DPPH radical scavenging activity (% inhibition) **C** values of carob tree leaves extracted with various solvents (n = 3 ± S.D.). Values marked with the different lowercase letters (a–f) are significantly different from each other at $p<0.05$.

< 50% acetone < 100% methanol ($p<0.05$). It can be said that the variations in TPs and TFs content between extracts showed the same general trend. Being the least polar solvent, 100% acetone was the least efficient in extracting this group of molecules, these same results were found in Nakilcioğlu-Taş & Ötleş's (2021) study on the influence of solvents on the TPs and TFs of fig kernels. But by adding distilled water, its extraction efficiency increased considerably becoming better than that of

wetted alcohols, indeed 50% acetone was the solvent that extracted the most TPs and TFs from bunga kantan in the study of Wijekoon et al. (2011) and from various vegetables in the study of Xu & Chang (2007), and 60% acetone for brewer's spent grains in the study of Meneses et al. (2013). For carob leaves, it was the 100% methanol that allowed to have the optimal content of PTs and FTs, these results approaching those reported in the study by Çelebi Sezer et al. (2017) on dried mushrooms where the best solvent was 80% methanol and those reported in the study by Ye et al. (2015) on sunflower florets where the best solvent was 90% methanol. The chemical composition of these plant parts made this solvent the preferred choice.

In this study, carob tree leaves were obtained from north-west Algeria, their TPs ranged from 11.33± 0.08 mg GAE/g DM in 100% acetone to 28.43± 0.08 mg GAE/g DM in 100% methanol, and their TFs ranged from 8.55± 0.34 mg CE/g DM in 100% acetone to 19.59± 0.63 mg CE/g DM in 100% methanol. The study of Elbouzidi et al (2023) revealed that TPs of carob tree leaves ethanolic obtained from northeastern Morocco were 96.98 ± 1.15 mg GAE/100 g DW and TFs were 5.92 ± 0.06 mg RE/100 g DW. Environmental factors, including harvesting season, extraction method and storage conditions, had a significant impact on phenolic composition.

All studied solvents had significantly higher antioxidant activity than ascorbic acid ($p<0.05$). The results of the DPPH test followed the same trend as for polyphenols and flavonoids, except for 50% methanol, which with 100% methanol gave the highest inhibition percentages :78.74± 2.99% and 79.29 ± 3.75% respectively ($p<0.05$) (Figure 1C). Methanol at 50% enabled the extraction of compounds with strong antioxidant activity, since the latter depending not only on the concentration of polyphenols, but also on their structure and their nature in the extract. On the other hand, this solvent was found to have the strongest antioxidant activity in the study of Kallel et al. (2014) on garlic husk and in that of Nakilcioğlu-Taş and Ötleş (2021) on fig kernels. Like TPs and TFs, antioxidant activity was stronger with 100% methanol, as reported in various studies on the same plant part (Mimoun et al. 2022, Madi et al. 2023). Carob tree leaves extracted with 100% acetone showed the lowest value (58.28%) which was positively related to TPs and TFs, this was also reported in the literature (Ngo et al. 2017, Nakilcioğlu-Taş & Ötleş 2021). Finally, there was no statistical difference with 50% acetone, 100% ethanol and 50% ethanol extracts in terms of the antioxidant capacities ($p<0.05$), which moreover, they remained close to those of methanolic extracts by their close polarities.

3.2. Correlation analysis between polyphenols and antioxidant activity

Correlation analyses were carried out between polyphenols contents and the antioxidant activity (Table I). Significant linear correlations were observed between TPs, TFs and DPPH values ($p<0.05$). Although flavonoids were a sub-group of polyphenols, the correlation between TPs and TFs ($r = 0.537$) was significant only at $p<0.05$, providing evidence of the presence of polyphenols other than flavonoids. TPs values were strongly positively correlated with DPPH values ($r = 0.892$) ($p<0.01$). This showed that polyphenols contributed to antioxidant capacities of extract samples. This indicates that most polyphenols in carob tree leaves were capable of reducing H+ ions. Statistically significant correlations were determined between TFs and DPPH values ($r=0.623$) ($p<0.01$). This proved that flavonoids significantly affected antioxidant capacities of the extracts. Several studies in the literature had also clearly established a close relationship between polyphenol content and antioxidant capacity (Xu &Chang 2007, Ghasemzadeh et al. 2015, Ye et al. 2015).

Table I. Correlation between TPs, TFs and antioxidant activity of carob tree leaf extracts.

Pearson's correlation (N=18)	TPs	TFs	DPPH RSA (%)
TPs	1		
TFs	0,537*	1	
DPPH RSA (%)	0,892**	0,623**	1

* The correlation was significant at 0,05
** The correlation was significant at 0,01

4. Conclusion

The results obtained in this study showed that carob tree leaves were a natural source of bioactive compounds. It was also found that extraction with solvents of different polarities affects the TPs, TFs and antioxidant capacity of carob tree leaf extract. Polyphenols and antioxidant compounds extractability from carob tree leaves was optimized by pure methanol. Furthermore, significant positive correlations were determined between TPs, TFs and antioxidant capacities of these extracts. Carob tree leaves could be considered as a source of important phytochemicals with antioxidant properties that could have beneficial effects on health. These results also indicated that carob tree leaf extract obtained using an appropriate extraction solvent could enhance the protective effect against oxidative damages associated with free radicals.

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Author Contribution

Concept, design, resources, analysis, interpretation and editing were carried out by Siham BABA AHMED. Monitoring and critical reviews were led by Mohammed Adil SELKA. Supervision was provided by Ilham LAHFA.

Conflicts of Interest

All authors declare no conflict of interest.

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