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ANTIOXIDANT AND α -AMYLASE INHIBITION ACTIVITIES OF MEDICINAL PLANTS ARE TRADITIONALLY USED FOR THE TREATMENT OF DIGESTIVE DISORDERS IN THE NEAR EAST REGION

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ABSTRACT

Medicinal plants have been used for longtime for the treatment of many illnesses. Many plants were described in folk heritage to have an anti-inflammatory activity to treat digestive diseases. In this study, thirteen plants commonly used by Jordanian folks to treat digestive disorders were investigated to find a connection between their contents and activities and their relationship with their assigned folk use. The ethanolic extracts of the plants were tested for their Total Phenolic Content (TPC), Total Tannin Content (TTC), Total Flavonoid Content (TFC) and their antioxidants activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Phenolic compounds were found in different amounts in all of the studied plants, *Terminalia chebula* contained the highest concentration and *Dracaena cinnabari* had the lowest concentration. TFC values are generally low, *Achillea millefolium* showed the highest concentration of flavonoids. *Illicium verum* showed the highest percentage of tannin. The lowest concentration of both flavonoids and tannin were found in *Dracaena cinnabari.* In addition, α-Amylase inhibitory activity of the plant extracts was determined, *Anethum graveolens* showed an excellent inhibition of α-Amylase while *Angelica sinensis* showed an excellent activation. Correlations between TPC, TFC, TTC, protein content with the antioxidant activities (DPPH and ABTS), and α-amylase inhibition were determined using Pearson correlation. The phenolic content and flavonoid content showed a high correlation with ABTS activity. The studied plants varied from activation to inhibition of α-Amylase, this may benefit people with hypoglycemia. This diversity of values directs people to which plant to use in case of multi-case disease.

Keywords: digestive disorders; antioxidant activity; secondary metabolites, α-amylase, near east

INTRODUCTION

Traditional medicine is the first treatment used in Jordan to treat illnesses and sometimes chronic diseases where most Jordanians consume medicinal plants as described in the folk heritage. Those plants are usually collected from the wild and mountains in spring or are cultivated in mostly each personal garden. Alternatively, people buy medicinal plants in the local market from "Al-Attar", a spice dealer or druggist, where medicinal plants are imported or collected locally. Recently, alternative medicine is the people's first choice as their primary health care. In Jordan, medicinal plants are considered a trusted substitute for medical treatment. When needed, a lot of people try to treat their illnesses with herbs. In addition, they use herbs when medicine prescribed by physicians is inefficient or very expensive (**Okoor *et al.*, 2019**).

As part of Arabian folk medicine, several preparations of local herbs and oils, like olive oil and water extracts of pomegranate, were used for the treatment of skin infections. Several studies showed that phenolic extracts exhibited potent antibacterial and anti-inflammatory effects **(Mohamed *et al.*, 2017; Radwan *et al.*, 2014**). These fractions contain high quantities of different phenolic acids including caffeic acid and ferulic acids which exhibited potent antimicrobial, antiseptic, preservative, and anti-oxidant activities **(Abu-Qatouseh *et al.*, 2019; Gadetskaya *et al.*, 2015; Tarawneh *et al.*, 2015**).

The characterization of many studied polyphenols in the Mediterranean area concerns foods and vegetables produced in countries with a strong tradition and currently placed in the European Union (France, Spain, Portugal, Italy, etc.), and also products of vegetable origin in nations with remarkable historical heritages and found in the Middle East or in the Maghreb area: Morocco, Algeria, Tunisia, Syria, Lebanon, Jordan, etc. For this reason, the description of the main phenolic compounds of interest (from the health and hygiene viewpoint at least) may be completed with the examination of certain vegetable varieties which are uncommon in Western-style countries **(Haddad *et al.*, 2020**).

Free radicals play a main role in initiating inflammatory processes (**Cordaro *et al.*, 2020**). Antioxidants and radical scavengers can neutralize those processes. Most of the reactive oxygen species are produced in cells. Lipids and proteins are the main targets for oxidative attack. In the inflammatory response, leukocytes and mast cells are present in the damaged areas as a result of extra uptake of oxygen and thus rise the discharge of reactive oxygen species in the damaged area (**Rastogi *et al.*, 2018**). Several studies mentioned inflammation and digestive disorders and diseases, which are associated **(Engevik *et al.*, 2021; Jang *et al.*, 2018; Zebeli & Metzler-Zebeli, 2012**). In one study, saffron was used for its anti-inflammatory activity to treat digestive diseases **(Ashktorab *et al.*, 2019; Madkhali *et al.*, 2021**). Others used *Varronia multispicata* and *Nigella sativa* (black cumin) for their anti-inflammatory efficiencies in the treatment of digestive disorders **(Amin & Hosseinzadeh, 2016; Lopes *et al.*, 2019**).

Thirteen medicinal plants used in Jordanian folk medicine for the digestive disorder were included in this study to find a connection between their contents and activities and their relationship with their assigned folk use.

MATERIAL AND METHODS

All chemicals were purchased from Sigma Aldrich and used as received. Sodium carbonate (Na_2CO_3), anhydrous sodium carbonate, gallic acid, aluminium chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), Folin-ciocalteuphenol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dimethyl sulfoxide (DMSO), potassium persulfate ($K_2S_2O_8$), methanol (CH_3OH), ethanol (C_2H_5OH), α-Amylase, 3,5-dinitrosalicyclic acid, potassium sodium tartrate tetrahydrate, phenol, and sodium metabisulfite were used in various stages in this study.

Plants Material and extraction

All medicinal plants used in this study were purchased in the local market. 250 g of each plant were crushed and soaked with sufficient absolute ethanol, about 300 mL per plant, over 72 hours. The temperature was maintained within medium to low temperature (between 20 and 70 °C). They were subsequently filtered using a Whatman funnel and filter paper 110 mm. The ethanol was evaporated at a temperature of 40 ° C using a rotary evaporator (vacuum pump unit (vacuubrand

CVC 2000), IKA Werke Rotovapor RV06-ML + water bath HB4 basic and LAUDA Alpha RA 12 (Cooling thermostat 230 V; 50 Hz)) and then the extract was dried and kept at a temperature of -4 ° C. This process was repeated three times over 72 hours (24 hours gap between the repetitions).

Estimation of total phenolic content

The content of phenols in plant extracts was determined using the Folin-Ciocalteu method **(Izuegbuna *et al.*, 2019)**. A volume of 40 µl of the dissolved extract (0.1g in 1 mL of DMSO) was mixed with 1.0 mL of 10% Folin-Ciocalteu reagent and 0.8 mL of 7.5% Na_2CO_3 solution. The mixture was stored in the dark for 2 hours. Afterwards, the absorbance of the mixture was measured using a UV-Vis spectrophotometer at 760 nm. The TPC was determined using a standard curve with gallic acid solutions (10, 25, 50, 75, and 100 mg/mL). The TPCs in all the samples were calculated using the formula:

TPC = C × V/m

Where TPC = total phenolic content in mg GAE/g of dry extract, C = concentration of gallic acid obtained from calibration curve in mg/mL, m = mass of extract in gram, and V = volume of extract in mL.

Estimation of total flavonoid content

The flavonoid content was determined using an aluminium chloride colorimetric assay with a few adjustments **(Izuegbuna *et al.*, 2019**). 100 µl of the dissolved extract (0.1 g of the extract in 1 mL of DMSO) was mixed with 60 µl of 5 % $NaNO_2$ and 60 µl of 10 % $AlCl_3$ in a vial. 400 µl of 1M NaOH was added to the vial with 1.4 mL distilled water. All those additions were carried out according to a timetable. A set of reference standard solutions of rutin (100, 200, 300, 400, 500, and 600 mg/mL) were prepared in the same manner as described earlier for calibration. The absorbance for test and standard solutions were determined at 510 nm using a UV-Vis spectrophotometer. The TFCs in all the samples were calculated using the formula:
TFC = C × V/m
Where TFC = total flavonoid content in mg RU/g of dry extract, C = concentration of rutin obtained from calibration curve in mg/mL, m = mass of extract in gram, and V = volume of extract in mL.

Estimation of tannin content

The tannin was determined by the Folin-Ciocalteu method.**(Izuegbuna *et al.*, 2019)** 10 µL of the plant extract (0.1 g in 1 mL of DMSO) and 750 µL of distilled water were mixed with 50 µL of 10 % Folin-Ciocalteu. Then, 100 µL of 35% Na_2CO_3 and 90 µL of distilled water were added. The mixture was left in the dark for 30 minutes. A set of reference standard solutions of gallic acid (10, 25, 50, 75, and 100 mg/mL) were prepared in the same manner as described for calibration. The absorbance for test and standard solutions were determined against the reagent blank at 725 nm with a UV-Vis spectrophotometer. The TTCs in all the samples were calculated using the formula:
TTC = C × V/m
Where TTC = total tannin content in mg GAE/g of the dry extract, C = concentration of gallic acid obtained from calibration curve in mg/mL, m = mass of extract in gram, and V = volume of extract in mL.

DPPH radical scavenging assay

DPPH radical scavenging activity of the plant extracts was determined according to the method described by Izuegbuna and others with some modifications.(**Izuegbuna et al., 2019)** 1.0 mL of 0.135 mM DPPH in methanol was mixed with 20 µL from the extract (0.1 g of the extract in 1.0 mL of DMSO). The mixture was left in dark at room temperature for 30 min after being centrifuged. The UV-Vis absorbance of the extracts was measured at 517 nm. Using the following equation, the DPPH radical scavenging activity was calculated:

DPPH radical scavenging activity = ((Abs control-Abs sample))/ ((Abs control)) ×100%

Where Abs control = absorbance of DPPH radical + methanol, Abs sample = absorbance of DPPH radical + sample extract.

ABTS radical scavenging assay

ABTS scavenging activity was determined using a method published by Rajurkar and colleagues with minor modifications **(Rajurkar & Gaikwad, 2012)**. First, an ABTS radical cation solution was prepared by adding 10 mL of 2.6 mM K2S2O8 to 10 mL of 7.4 mM ABTS solution and left to stand in the dark at room temperature for 15 hours. Working solutions were prepared by adding 1.0 mL of ABTS radical cation solution and then diluting it with methanol until the absorbance value reaches 0.7 ± 0.2 at 734 nm. 10 µL from the extract (0.1 g of the extract in 1.0 mL of DMSO) was mixed with 1.0 mL of the working solution for 30 minutes at room temperature. The UV-Vis absorption of the mixture was measured at 734 nm. The ABTS radical scavenging activity was calculated from the equation:

ABTS radical scavenging activity = ((Abs control-Abs sample))/ ((Abs control)) ×100%

Where Abs control = absorbance of ABTS radical + methanol, Abs sample = absorbance of ABTS radical + sample extract.

Estimation of Protein Content and Amino Acid composition

The protein concentration in the solution can be calculated by measuring the absorption at λmax. 0.1 g of the extract was dissolved in 1 mL of methanol. 10 µl of this solution extract was added to 3 mL of methanol. Using a UV-Vis spectrophotometer, the λmax of each plant extract was scanned over the range of 200-600 nm. To obtain the percentage concentration of protein contents, the percentage extinction coefficient (ε%) was used. In most proteins, extinction coefficients (ε percent) range from 4.0 to 24.0. The mean for a mixture of many different proteins will likely be around 10 (**Okoronkwo *et al.*, 2017**).
The percentage concentration = Absorbance/(ε percent)

α-Amylase inhibitory activity

α-Amylase inhibitory activity of the plant extracts was determined according to the method described by Alu'datt et al (**Alu'datt *et al.*, 2021**). First, mixing of 0.125 g of potato starch in 25 mL of phosphate buffer (pH 7.0) and stir at 65 °C for 20 min to prepare the starch solution. 30 mg of α-Amylase was mixed with 100 mL of distilled water. Mixing of 10.6 g 3,5-dinitrosalicyclic acid, 3.06 g potassium sodium tartrate tetrahydrate, 19.9 g sodium hydroxide, 7.6 g phenol, and 8.3 g sodium metabisulfite in 1416 mL of distilled water, then heating at 95 °C for 5 min, then cooling at ambient temperature in an ice bath to prepare the colorimetric reagent. Dilute the mixture in distilled water. control (80 µL of distilled water) or 80 µL test sample, 500 µL phosphate buffer (pH 7), 500 µL starch solution, and 500 µL α-amylase solution were mixed and incubated at 25 °C for 10 min. 1.0 mL of colorimetric reagent was added, the mixture was boiled for 5 min, then cooled at room temperature, and after that 7.4 mL of distilled water was added to the mixture. The UV-Vis absorbance of the extracts was measured at 540 nm. Using the following equation, the α-Amylase inhibitory activity was calculated:

α-Amylase inhibitory activity % = ((Abs control-Abs sample))/((Abs control)) ×100%

UV Instrument

UV data were obtained using a Shimadzu (UV-1800) double-beam spectrophotometer (Japan) with a quartz cuvette (1cm). Wavelength Range (nm):190.0 to 800. The UV spectrophotometer is linked to a Dell Compaq computer; all data were analyzed using UV-probe 2.33 software.

Statistical analysis

All calculations have been carried out using Microsoft excel 2016. All experiments were measured in triplicates and the results were documented as mean standard deviation (SD). Pearson correlation coefficients (r) were calculated using spss statistics viewer 23.0.

RESULTS AND DISCUSSIONS

Preparation of extracts

Method selection for phenolics, flavonoids and tannins extraction is essential for the best results in terms of contents and activities. Obtaining the highest percentage of extracted compounds is dependent on the choice of solvent and plant parts **(Mirjalili *et al.*, 2021**). Polar organic solvents have been proven to produce high yields in extraction compared to other solvents. Ethanol is the most famous solvent due to its polar properties and safe use in comparison to other solvents **(Monteiro *et al.*, 2020**). Hence, it was used as the best choice in this study. Also, the extraction process is affected by extraction temperature as it relates to the solvent's properties. High temperatures decrease the viscosity and increase the vapor pressure which results in extraction efficiency reduction (**Chaves *et al.*, 2020**). Therefore, the extracts in this study were extracted at a medium to low temperature (between 20 and 70 °C).

Total Phenolic Content

Phenolic compounds are of great importance; they relate to the antioxidants that scavenge free radicals. These compounds contain a hydroxyl group bonded to an

aromatic hydrocarbon group in their structure **(Nguyen *et al*., 2020)**. TPC values in Figure 1 show that phenolic compounds were found in different amounts in all of the studied plants. *Terminalia chebula* contained the highest concentration and *Dracaena cinnabari* had the lowest concentration. The disagreement of the results in this study with previous results can be attributed to several reasons including the solvent used and the method of extraction in addition to other parameters concerning the place of growth of the plant and the harvesting time (**Mayda *et al*., 2020**).

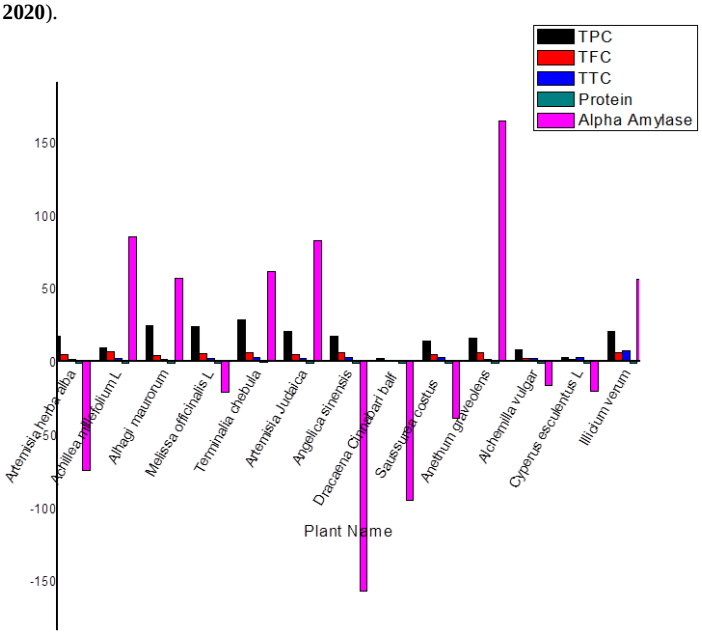

Figure 1 Total phenolic content (TPC), Total flavonoid content (TFC), Total tannin content (TTC), protein content and α-Amylase inhibition of the extracted medicinal plants.

Total flavonoid content (TFC)

Flavonoids provide plants with defense against pathogens, UV protection, antioxidants, coloration of flowers and fruits (**Baskar *et al*., 2018**). Rutin was used as a standard in the TFC study. UV-Vis spectrophotometer was used to measure the percentage of flavonoids in plants. $AlCl_3$ addition, in this method, caused shifting the wavelength to the visible region. TFC values are generally low. The highest concentration of flavonoids was found in *Achillea millefolium*, while the lowest concentration was found in *D. cinnabari*. This latter founding correlates with the TPC value of the same plant. Compared to previous studies, there were discrepancies in the results. Those discrepancies may be connected to previous reasons. The flavonoid content was the second most abundant family in those studied plants.

Total tannin content (TTC*)*

Tannin is a polyphenol that is water soluble. It is found in all parts of plants and has antioxidant properties (**Singh *et al.*, 2021**). Tannin was found in plants in different proportions using ethanol. *Illicium verum* showed the highest percentage of tannin, and again *D. cinnabari* showed the lowest. These results were not in agreement with the results of previous studies that can also be attributed to the previously mentioned reasons. TTC came in third after TPC and TFC. The results of Total flavonoid content (TFC), Total tannin content (TTC) are described in figure 1.

Total protein content

Proteins' UV absorption is mostly due to the presence of tryptophan because of the indole ring linked to a methylene group. Tyrosine and phenylalanine follow tryptophan, and to a lesser extent cysteine. Table 1 lists the UV absorption of some amino acids present in plants **(Singh *et al*., 2021).**

Table 1 Amino acids identified at different wavelengths

Wavelength Range	Amino acid
204 – 220	Cysteine
240 – 265	Phenylalanine
274 – 300	Tyrosine
275 – 312	Tryptophan
> 312	Histidine

Cysteine and histidine among other amino acids were found on the studied plants through the UV absorption that covered the range from 215-369 nm as shown in Table 2.

Table 2 Amino acids identified at different wavelengths from the plant extracts

Medicinal plant	Observed Wavelengths (nm)	Identified amino acid
Artemisia herba-alba Asso L	292 332	Tyrosine and Tryptophan Histidine
Achillea millefolium L	254 291 337	Phenylalanine Tyrosine and Tryptophan Traces of Histidine
Alhagi graecorum Boiss	263 291 331	Phenylalanine Tyrosine and Tryptophan Histidine
Melissa officinalis L	263 288 331	Phenylalanine Tyrosine and Tryptophan Histidine
Terminalia chebula Retz. L	264 310 369	Phenylalanine Tyrosine and Tryptophan Histidine
Artemisia Judaica L	215 252 286 353	Cysteine Phenylalanine Tyrosine and Tryptophan Histidine
Angelica sinensis (Oliv.) Diels L	295	Tyrosine and Tryptophan
Dracaena Cinnabari balf f	252 276	Phenylalanine and Tyrosine Tryptophan
Saussurea costus (Falc.) Lipsch. DC	264 283	Phenylalanine and Tyrosine Tryptophan
Anethum graveolens L	284 326	Tyrosine and Tryptophan Histidine
Alchemilla vulgaris L	287 321	Tyrosine and Tryptophan Histidine
Cyperus esculentus L	224 256 282	Cysteine Phenylalanine Tyrosine and Tryptophan
Illicium verum Hook f	241 263 291	Phenylalanine Phenylalanine Tyrosine and Tryptophan

In this estimation, *T. chebula* was found to have the most protein concentration, while *D. Cinnabari* showed the lowest of proteins. The protein concentrations in percentages of the extracted medicinal plants are listed in Table 3.

Table 3 Percentage concentration of protein in the plant

Medicinal plant	Concentration (%)
Artemisia herba-alba Asso L	0.24
Achillea millefolium L	0.24
Alhagi graecorum Boiss	0.11
Melissa officinalis L	0.19
Terminalia chebula Retz. L	0.39
Artemisia Judaica L	0.18
Angelica sinensis (Oliv.) Diels L	0.09
Dracaena Cinnabari balf f	0.01
Saussurea costus (Falc.) Lipsch. DC	0.03
Anethum graveolens L	0.04
Alchemilla vulgaris L	0.05
Cyperus esculentus L	0.07
Illicium verum Hook. f.	0.24

Antioxidant activity

The free radical scavenging activity of the ethanol extract has been tested by DPPH radical and ABTS radical methods. The results are shown in Figure 2.

DPPH assay depends on using the DPPH radical that is a stable free radical molecule which has an odd electron in the outer orbital of a nitrogen atom. When DPPH is protonated by an antioxidant, it becomes a non-radical molecule changing its color from violet, when dissolved in a polar solvent, to light violet or pale yellow, reducing its absorbance value **(Adam *et al*., 2021**). The results of the radical scanning activity of DPPH ethanol extracts indicate that all extracts possess antioxidant activity (Figure 2). *T. chebula* extract showed the highest antioxidant activity while *A. vulgaris* extract had the lowest activity.

ABTS●+ radical cation is used in the determination of the antioxidant activity. It is obtained by oxidizing ABTS with $K_2S_2O_8$ that produces a blue-green color (**Konan, Le Tien and Mateescu, 2016**). All plant extracts have high antioxidant activity as in Figure 2. Six of them have values more that 99%; *T. chebula* extract has the highest antioxidant activity and *D. Cinnabari* has the lowest. The negative value of DPPH inhibition of *A. vulgar* extract can be attributed to the fact that DPPH spectrophotometric method is unable to measure colored samples (**Flieger**

& Flieger, 2020). Also, it could be that this extract activates the oxidation does not inhibit it. The latter finding can be easily excluded for the same extract exhibits an ABTS inhibition with a value about 97%.

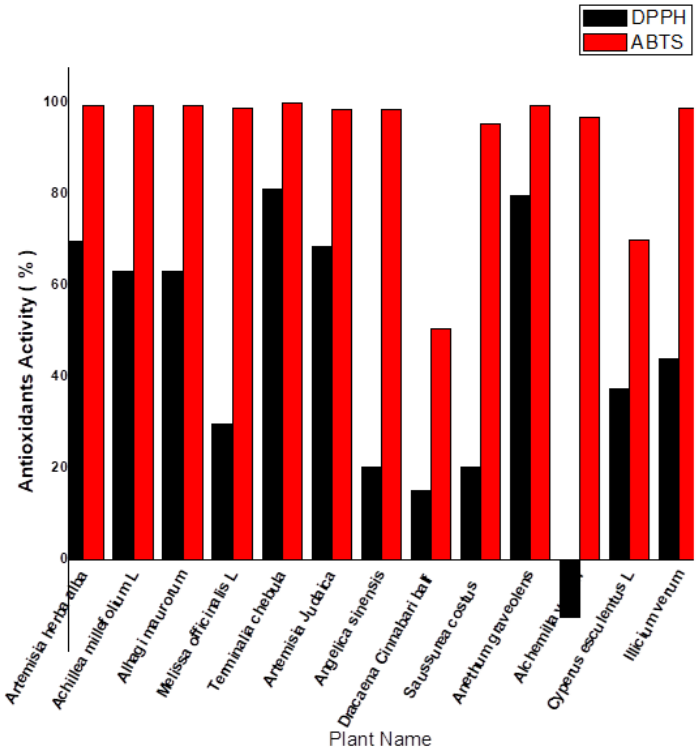

Figure 2 Antioxidant activities of the extracted medicinal plants: DPPH inhibition, ABTS inhibition

It is obvious here that the ABTS assay gives higher values for those studied plants extracts. Thermodynamically, a compound can reduce ABTS•+ if it has a redox potential lower than that of ABTS. Hence, many phenolic compounds have low redox potentials and can thus react with ABTS•+. This could be the reason why ABTS and DPPH inhibition values are not correlated. On the other hand, the DPPH-radical may have a poor reactivity with antioxidants due to its stability and steric inaccessibility which limits its efficiency. Thus, small molecules have higher apparent antioxidant activity with this assay. And since plants extracts contain varying sizes of molecules, this assay cannot cover all of its spectrum. Also, the ABTS assay is applicable to both hydrophilic and lipophilic antioxidants which broadens its range in detecting antioxidants (**Prior *et al.*, 2005**).

α-Amylase inhibition

Several plants in this study showed their effectiveness in inhibiting the α-amylase enzyme as shown in Figure 1. The studied plants varied from activation to inhibition of α-Amylase. *A. millefolium*, *A. graecorum*, *T. chebula*, *A. Judaica*, *A. graveolens*, and *I. verum Hook*. f. inhibited α-Amylase. *A. graveolens* showed an excellent inhibition of α-Amylase with values of 166% followed by *A. illefolium L* and *A. Judaica* with values of 86% and 84% respectively. On the other hand, *A. herba*, *M. officinalis*, *A. sinensis*, *D. Cinnabari*, *S. costus*, *A. vulgaris*, and *C. esculentus* activated α-Amylase. *A. sinensis* showed an excellent activation of α-amylase (-156%) followed by *D. Cinnabari* (-94%), and *A. herba* (-73%). This may benefit people with hypoglycemia. This diversity of values directs people to which plant to use in case of multi-case disease.

Correlations between TPC, TFC, TTC, protein content with the antioxidant activities (DPPH and ABTS), α-amylase inhibition

Pearson correlation is an indicator to the dependency of one activity/parameter on the other. To find out whether phenols, flavonoids, and tannins contribute to the antioxidant capacity, and α-Amylase inhibition (Table 4).

Table 4 Pearson correlation between, DPPH, ABTS, α-Amylase, TPC, TFC, TTC, and total protein content

	TPC	TFC	TTC	Total protein content
DPPH inhibition	0.525	0.537	0.055	0.601
ABTS inhibition	0.716	0.826	0.350	0.542
α-Amylase inhibition	0.309	0.392	0.189	0.337

Pearson correlation coefficients were analyzed. The phenolic content showed a low correlation with α-Amylase inhibition values, a moderate correlation with DPPH, and a high correlation with ABTS inhibitions. Flavonoid content showed a low correlation with α-Amylase, a moderate correlation with DPPH and a very high correlation with ABTS inhibitions. Tannin content shows a very low correlation with DPPH and α-Amylase inhibitions and a low correlation with ABTS. The protein content is highly correlated to DPPH inhibition, moderately correlated to ABTS, and slightly correlated to α-Amylase. The degree of correlation depends on the values of Pearson correlation: ($0< r \leq 0.19$, Very Low Correlation), ($0.2 \leq r \leq 0.39$, low Correlation), ($0.4 \leq r \leq 0.59$, Moderate Correlation), ($0.6 \leq r \leq 0.79$, High Correlation) and ($0.8 \leq r \leq 1.0$, Very High Correlation).

CONCLUSION

In this work, ethanolic extracts of some medicinal plants that are used in traditional therapeutic in near east region for the treatment of digestive disorders were experimentally studied for their antioxidant activity and inhibitory action toward alpha amylase enzyme. Our study has reported phenols, flavonoids, and tannins contribution to the antioxidant capacity and α-Amylase inhibition by using Pearson correlation. The association of phenols, flavonoids, and tannins with ABTS is higher than DPPH and way higher than α-Amylase. The correlation here might not necessarily mean causation in which for example the presence of flavonoids in high concentrations in a plant will provide its extract of antioxidant activity. Those activities can be caused by a sum of variables. And since the composition of an extract is not totally known, this cause-and-effect cannot be guaranteed.

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Conflicts of interest: The authors declare no conflict of interest.

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