Food and Chemical Toxicology

Investigation into the biological properties, secondary metabolites composition, and toxicity of aerial and root parts of Capparis spinosa L.: An important medicinal food plant

--Manuscript Draft--

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Response to Reviewers:	To: Editor in Chief 12th June, 2021 Food and Chemical Toxicology Re: Revision of MS FCT-D-21-01389

First of all, I would like to pay thanks to you for granting me this opportunity to improve our manuscript, which we opine will benefit readers of your high-ranked journal.
Please find below our point-by-point response to the addressed comments. We have accepted all the suggestions made by the reviewers, and the manuscript has been revised accordingly.
We look forward to your positive response concerning the revision undertaken.
Thank you for your quick response.

Sincerely yours,

Dr. Hammad Saleem
Institute of Pharmaceutical Sciences (IPS),
University of Veterinary and Animal Sciences (UVAS),
Lahore, Pakistan.

Response to Comments

Reviewer #2:
We thanks the reviewer for his/her positive comments in order to improve the manuscript. All the major revisions as mentioned by the respected reviewer have been highlighted in yellow colour in the revised manuscript.

Comment: 1. Abstract, page 2, line 39: Should read "prpoperties, and in vitro toxicity.." Only in vitro tests were used.

Response: We have made the change as required.

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Comment: 9. Conclusions:
Page 13, line 293-294, Re-state as follows: "The tested extracts exhibited notable antioxidant and enzyme inhibition properties in vitro and also presented considerable toxicity against breast cells in vitro."

Response: We have made the change as required.

Comment: Reviewer Comments
While the paper adequately characterizes the chemical composition of the various extracts, it does not relate the in vitro antioxidant and enzyme inhibition properties and toxicity to in vitro breast cells to the biological role of these natural extracts to the medicinal and herbal remedy applications. The authors should relate the in vitro effects to the value of these preparations in vivo and also relate to the doses to which humans are exposed.

Response: Dear Reviewer, we thanks to you for your valuable suggestion. As in this study, we have performed only the preliminary toxicity evaluation of the crude extracts at a single concentration and not calculated the IC50 values, this is the reason we did nor related the in vitro bioassays with the toxicity.

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Food and Chemical Toxicology

To: Editor in Chief **Dated: 12-06-2021**
Greetings

Dear Professor,

We are pleased to submit our revised manuscript **(FCT-D-21-01389)** entitled **" Investigation into the biological properties, secondary metabolites composition, and toxicity of aerial and root parts of *Capparis spinosa* L.: An important medicinal food plant "** for possible publication in Food and Chemical Toxicology (**Special issue: Recent advances on Toxicological and ecotoxicological effects of natural products and its derivatives**).

The plant Capparis spinosa is a food plant belonging to family Capparidaceae, have been traditionally used for treating various common ailments. Nonetheless, this plant has not yet been explored in terms of its chemical and biological effects. We have investigated different methanol and DCM extracts of Capparis spinosa aerial and root parts for chemical composition (total bioactive contents, UHPLC-MS secondary metabolites, HPLC-PDA phenolic quantification) and biological activities. Antioxidant potential was appraised using a panoply of assays including DPPH, ABTS, FRAP, CUPRAC, phoshomolybdenum and metal chelating. Whereas, the enzyme inhibition activities of both extracts were tested against cholinesterases, α-amylase, α-glucosidase and tyrosinase. Moreover, in-vitro toxicity studies were also performed to highlight the safety parameters of this important medicinal plant.

We believe that our findings could be of interest to the readers of Food and Chemical Toxicology because this plant species can be further considered as a source of bioactive-functional agents for food industry and pharmaceutical applications.

We hope that the editorial boards and reviewers will agree on the interest of this study.

Sincerely yours,

Dr. H. Saleem

To:
Editor in Chief **12th June, 2021**
Food and Chemical Toxicology

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Selangor Darul Ehsan, Malaysia

[*] Corresponding Authors:

Hammad Saleem **(**hammad.saleem@uvas.edu.pk)

- Chemical, biological and *in-vitro* toxicological properties of *Capparis spinosa* extracts were studied.
- Methanol extracts of both the aerial and root parts were found to have higher total bioactive contents and comparatively higher antioxidant potential.
- UHPLC-MS and HPLC-PDA analysis revealed the presence of phenolic, alkaloid, glucosinolate, and flavonoid derivatives
- The plant was found to present weak to moderate toxicity against the tested cell lines.

Capparis spinose L. also known as Caper is of great significance as a traditional medicinal food plant. The present work was targeted on the determination of chemical composition, pharmacological properties, and in-vitro toxicity of methanol and dichloromethane (DCM) extracts of different parts of *C. spinosa*. Chemical composition was established by determining total bioactive contents and *via* UHPLC-MS secondary metabolites profiling. For determination of biological activities, antioxidant capacity was determined through DPPH, ABTS, CUPRAC, FRAP, phosphomolybdenum, and metal chelating assays while enzyme inhibition against cholinesterase, tyrosinase, α-amylase and α-glucosidase were also tested. All the extracts were also tested for toxicity against two breast cell lines. The methanolic extracts were found to contain highest total phenolic and flavonoids which is correlated with their significant radical scavenging, cholinesterase, tyrosinase and glucosidase inhibition potential. Whereas DCM extracts showed significant activity for reducing power, phosphomolybdenum, metal chelation, tyrosinase, and α-amylase inhibition activities. The secondary metabolites profiling of both methanolic extracts exposed the presence of 21 different secondary metabolites belonging to glucosinolate, alkaloid, flavonoid, phenol, triterpene, and alkaloid derivatives. The present results tend to validate folklore uses of *C. spinose* and indicate this plant to be used as a potent source of designing novel bioactive compounds.

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Abstract

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Keywords: *Capparis spinose*; antioxidant; secondary metabolites, enzyme inhibition, bioactive compounds

1. Introduction

Natural products have been utilized since time immemorial as curative agents for health management and treatment of common ailments because of their health-promoting properties and bioactive contents (Zhang and Ma, 2018). In consonance with the World Health Organization, the majority of the world's populations (about 80%) depends mostly on conventional/herbal drugs and in many countries, and the overall medicinal consumption is 30-50% that can be estimated from the preparation of conventional medicine (Locatelli et al., 2017; Zhang and Ma, 2018). For example, in Germany, approximately 90% of the population has utilized the old natural remedies for different health matters [2]. Hence, in industrial and developing countries, the use of traditional medicine is prevalent (Gunjan et al., 2015). The worldwide market for the use of traditional medicine is becoming very strong. Almost over $60 billion are covered from herbal medicine yearly, which is increasing progressively (Gunjan et al., 2015).

Capparis genus is from the family of Capparidaceae, which is in use widely for folk medicine from the distant past, particularly in countries of Western and Central Asia as well as the Mediterranean basin like Morocco, Spain, Tunisia, Italy and Turkey (Rivera et al., 2003). *C. spinosa* (also called as Caper) is a long-lasting shrubby plant that can grow in warm and dry weathers such as Middle and West Asia, the Mediterranean region and also numerous regions of Iran (Sultan and Çelik, 2009). The connection between capers and human beings is ancient that can be linked to the Stone Age. *C. spinosa* remains were discovered in archaeological areas like the inferior Mesolithic (9500–9000 b.p.) (Moufid and Farid, 2015). The remains of *C. spinosa* have been explored in China for the very first time and also in the eastern part of Central Asia which favors the use of caper as medicine from the last 2800 years (Jiang et al., 2007).

Caper is in use from ancient times in food preparation for fragrant and flavoring purposes, *C. spinosa* is also known for its use as an ordinary natural remedy because of its distinct properties for hypertension, poultice, tonic and diuretic problems (Duman and Özcan, 2014; Trombetta et al., 2005). *C. spinosa* is commonly found in hot and dry weathers and that its fruit, roots and barks are known because of their medicinal significance. It is traditionally used as the medicine for different health problems like diuretic, gout, rheumatism, hyperlipidemia, hyperglycemia, hypertension and also for liver and spleen disorders (Bonina et al., 2002; Lemhadri et al., 2007). In Morocco, this plant is usually used to control diabetes and its treatment and mostly used as a scented agent in Moroccan kitchens (Jouad et al., 2001). The parts of *C. spinosa*, such as fruits and roots, are known because of their beneficial properties on human health and are used as a herbal curative agent from the old times (Mansour et al., 2016). In earlier ages, The Egyptians and Arabs used the roots of *C. spinosa* for the treatment of kidney and liver disorders, and Romans used this plant as a therapeutic agent for paralysis. Moroccans also used it for diabetes treatment (Tlili et al., 2011). The root of *C. spinosa* is used for the treatment of enlarged spleen, mental problem and tubercular glands (Afzal et al., 2009). *C. spinosa* was also used as a medicine of rheumatoid arthritis and gout in China (Ao et al., 2007). It is also used in the treatment of hemorrhoids and gout in Iran (Mahboubi and Mahboubi, 2014).

Despite the plethora of studies related to the therapeutic uses of *C. spinosa*, data related to its chemical composition, antioxidant potential and enzyme inhibition activities related to most common human diseases is limited. Given the background regarding medicinal properties of *E. milii*, this work was conducted to probe into the enzymatic inhibitory activities of methanol and dichloromethane (DCM) extracts from aerial and roots of *C. spinosa* on key enzymes related to neurodegenerative ailments (acetylcholinesterase -AChE and butyrylcholiesterase -BChE),

diabetes (α-glucosidase and α-amylase) and skin hyperpigmentation (tyrosinase). Extracts were also appraised for their antioxidant potential utilizing free radical scavenging (2,2-diphenyl-1-picrylhydrazyl -DPPH and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) -ABTS), reducing power (ferric reducing antioxidant power -FRAP and cupric reducing antioxidant capacity -CUPRAC), phosphomolybdenum and metal chelation assays. The cytotoxicity was also performed against the MCF-7 and MDA-MB-231 breast cancer cell lines. All the extracts were chemically characterized by determining their total bioactive contents *via* spectrophotometric methods and individual secondary metabolic profiles by ultra-high-performance liquid chromatography- mass spectrometry (UHPLC-MS). Moreover, principal component analysis (PCA) statistical studies were performed to highlight possible interactions between the bioactive contents and tested biological assays.

2. Material and methods

2.1. Plant material and extraction

Aerial and root parts of *C. spinosa* were collected from Cholistan desert and identified by Mr. Hafiz Waris, Taxonomist, at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur. Additionally, a voucher specimen was deposited in the herbarium of Faculty of Pharmacy and Alternative Medicines, The Islamia University of Bahawalpur, for future reference. For extraction, powdered aerial and root parts were subjected for maceration (72 hrs) consecutively using DCM and methanol solvents and were kept at room temperature with intermittent shaking. The extracts obtained were made concentrated using a rotary evaporator and are abbreviated as CsA-M: *C. spinosa* aerial methanol extract; CsA-D: *C. spinosa* aerial DCM extract; CsR-M: *C. spinosa* root methanol extract; CsR-D: *C. spinosa* root DCM extract.

2.2. Total bioactive contents, UHPLC-MS analysis, and HPLC-PDA analysis

The standard Folin-Ciocalteu method was utilized to find out total phenolic content (Zengin et al., 2016c). The standard used for this purpose was gallic acid, and the amount of total phenolic content is expressed as mg GAE/g (gallic acid equivalents). Whereas to explore the total flavonoid content, the aluminum chloride colorimetric method was used (Chew et al., 2009), and quercetin was used as a standard. The results were expressed as mg QE/g (quercetin equivalent).

UHPLC-MS analysis of methanol and ethyl acetate extracts was performed (negative ionization mode) on Agilent 1290 Infinity LC system coupled with Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source as reported earlier (Saleem et al., 2019). The METLIN database was used for the tentative identification of different secondary metabolites in the tested samples. Moreover, a list of 22 different polyphenolic standards (including gallic acid, catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid, naringenin and carvacrol) was tested to be quantified in all the samples using HPLC-PDA analysis as reported previously (Locatelli et al., 2017).

2.3. Antioxidant assays

The standard methods were used to explore the free radical scavenging using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), reducing power by using FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity), total antioxidant capacity through phosphomolybdenum assay and metal chelating power as explained earlier in Grochowski et al. (2017) (Grochowski et al., 2017).

The results of all antioxidant assays were recorded as Trolox equivalents (except metal chelating assay for which EDTA was used as standard).

2.4. Enzyme inhibition assays

The enzyme inhibition studies of all the extracts against tyrosinase, acetylcholinesterase, butyrylcholinesterase, α-amylase, and α-glucosidase were exposed by utilizing the previous standard *in-vitro* methods (Grochowski et al., 2017). The AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) inhibition activity were expressed as standard galantamine equivalents (mg GALAE/g extract), while acarbose equivalent (mmol ACAE/g extract) for α-amylase and α-glucosidase and kojic acid equivalent (mg KAE/g extract) for tyrosinase were used.

2.5. MTT cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity activity of the tested samples was tested against two breast cancer cell lines, i.e., MDA-MB 231 and MCF-7 cells employing the previously described method (Nemudzivhadi and Masoko, 2014). The cell viability percentage (%) was determined as follows:

$$\text{Percentage cell viability} = \text{ABSs} - \text{ABSc} \times 100$$

Where ABSs: absorbance of the sample; ABSc: absorbance of control

2.6. Statistical analysis

The assays were carried out in a triplet, and independent experiment, and the results were calculated as a mean value ± standard deviation (SD). SPSS v.17.0 software was used for data analysis. One way analysis of variance via ANOVA followed by Tukey's test was done to find out the differences between means. A statistical value of $p < 0.05$ was considered significant. The principal component analysis (PCA) was carried out to identify the association between phytochemical content and biological properties.

3. Results and discussion

3.1. Total bioactive contents

In the present case, the extracts of *C. spinosa* were tested by the standard Folin-Ciocalteu and $AlCl_3$ methods for their total phenolic and flavonoid contents. The amount of total phenolic content was more in CsA-M (30.36 mg GAE/g extract) and CsR-M (23.53 mg GAE/g extract), as compared to the DCM extracts. Related results can be seen in the case of flavonoids as well (Table 1). Many studies have confirmed the presence of greater phenolic contents in methanolic extracts (Do et al., 2014; Murugan and Parimelazhagan, 2014).

The UHPLC-MS analysis of *C. spinosa* aerial methanol extract showed the presence of eleven different compounds (Table 2 and Figure 1). Most of these compounds were belonging to glucosinolate and flavonoid derivatives. The five flavonoids present were kaempferol 3-(2G-glucosylrutinoside), robinin, robinetin 3-rutinoside, luteolin 7-rhamnosyl (1->6) galactoside and tricetin 7-methyl ether 3'-glucoside-5'-rhamnoside. While glucoputranjivin, glucocochlearin and 4-Methoxyglucobrassicin were the present glucosinolates. Moreover, sarmentosin epoxide (cyanogenic compound), citric acid and gimgerol (phenol) were also detected. Similarity, *C. spinosa* root methanol extract identified the ten different compounds belonging to alkaloid and flavonoids (Table 3 and Figure 1). The alkaloids detected were calystegin B2, cadabicine, 3-O-acetylhamayne and michellamine B. Three flavonoids abruquinone B, melanoxetin and embigenin 2"-(2"'-acetylrhamnoside) were also identified. Moreover, one glucosinolate (glucoputranjivin), withanolide (withaperuvin H) and triterpene (licoricesaponin K2) were also present. The presence of these classes of secondary metabolites in *C. spinosa* is in agreement with previous studies (Moufid and Farid, 2015; Zhang and Ma, 2018).

Similarly, to have in-depth evaluation of the phytochemical composition, all the extracts of *C. spinosa* were studied by HPLC-PDA analysis for the quantification of 22 important phenolic compounds, and the results are presented in Table 4. The CsR-D extract was found to contain the maximum number of phenolics including vanillic acid, syringic acid, 3-OH benzoic acid, 3-OH 4-methoxy benzaldehyde, and 2.3-diMeO benzoic acid.

3.2. Antioxidant potential

A pathological activator of various diseases, such as Alzheimer's disease and Type II Diabetes, is oxidative pressure. Therefore, antioxidants are of great significance for the treatment of such oxidative stress. (Li et al., 2017). In this study, the antioxidant potential of *C. spinosa* aerial and root extracts was evaluated by utilizing six different protocols such as phosphomolybdenum, CUPTAC, FRAP, ABTS, DPPH, and metal chelating power, and the results can be seen in Table 5. The stable compound DPPH is free-radical, which shows the maximum wavelength at 517 nm and is commonly used for antioxidant determination (Loganayaki et al., 2013). All of the extracts were active against DPPH, showing activity in the following order CsA-M >CsR-M >CsR-D >CsA-D. This higher DPPH radical scavenging of aerial methanol (30.48±0.37 mg TE/g extract) and root methanol (28.45 mg TE/g extract) extracts shows correlation with their greater bioactive contents, and this is supported by the previous researcher who already explained that high DPPH scavenging activity was due to the presence of high phenolic content (Loganayaki et al., 2013; Piluzza and Bullitta, 2011). Another radical used for the determination of the antioxidant potential of plant extracts is ABTS and is a free blue/green radical with the maximum wavelength of 734nm (Zengin et al., 2018). In Table 5, it can be seen that the CsA-M and CsR-M extracts of *C. spinosa* actively scavenged ABTS radical, exploring the maximum Trolox equivalent values, i.e., 40.55 and 40.43 mg TE/g extract, respectively.

Other assays like FRAP and CUPRAC were utilized for the determination of the reducing capacity of the extracts. The reducing capacity can be quantified by observing the absorbance of ferric tripyridyltriazine to ferrous tripyridyltriazine while in the CUPRAC method, we can observe cupric reducing capacity to cuprous in the presence of copper(II)- neocuproine [Cu(II)-Nc] reagent (Al-Rimawi et al., 2016). It can be seen that both DCM extracts i.e., CsA-D (FRAP: 50.37 mg TE/g extract CUPRAC: 118.45 mg TE/g extract) and CsR-D (FRAP: 42.82 mg TE/g extract CUPRAC: 96.89 mg TE/g extract) has a potent reducing ability.

In the phosphomolybdenum method, Mo (VI) is reduced to Mo (V) in the presence of antioxidants (Chaouche et al., 2014). A reverse pattern can be observed as a trend for the phosphomolybdenum method, with CsA-D being the most active extract, in comparison with CsR-D and CsA-M extracts. The root methanol extract was not active. As this antioxidant assay measures the antioxidant potential of both phenolic and non-phenolic compounds, so the results recorded in phosphomolybdenum assay can be correlated to other non-phenolic compounds such as vitamin C or tocopherol in DCM extracts. These results are in agreement with the earlier studies (Albayrak et al., 2010; Llorent-Martínez et al., 2017) who reported the high antioxidant potential for DCM solvent.

Iron is of vital importance for respiration, oxygen transportation, and enzyme activity, but it also plays a vital role in the redox reaction, hence playing a role in oxidative stress (Farina et al., 2013). The results of our study explained that the different extracts of *C. spinosa* could chelate iron (Table 5). Similar to reducing power results, both DCM extracts were found to be the most active metal chelators, followed by methanolic extracts. These findings show similarity with earlier studies which reported that there is no correlation between total phenolic and metal chelating capacity (Khorasani Esmaeili et al., 2015; Silva et al., 2008; Yerlikaya et al., 2017). At

this point, non-phenolic compounds like tocopherol, as previously isolated from *C. spinose* (Moufid and Farid, 2015), could be attributed to this activity. As presented in Figure 2, Pearson correlation analysis confirmed the tested antioxidant results and showed a significant relationship of total bioactive contents and radical scavenging capacities (DPPH and ABTS), while a moderate association was observed for FRAP, whereas a negative correlation was the recorder for phosphomolybdenum and metal chelation assays in relation with bioactive contents.

3.3. Enzyme inhibition assays

Similarly, α-amylase and α-glucosidase inhibitors are used as therapeutic agents in the case of DM. Tyrosine is the key enzyme used in melanin synthesis, and for the treatment of hyperpigmentation, tyrosinase inhibitors are used. According to this information, the enzyme inhibitors can be synthesized artificially. In this case, limited side effects can be observed, such as toxic properties and gastrointestinal problems (Kumar et al., 2011). So, many researchers are trying to isolate inhibitors from natural sources having no or minimal side effects. So, the enzyme inhibition studies were carried out on *C. spinosa* extracts against cholinesterases, tyrosinase, amylase and glucosidase. The results are expressed in Table 6. The CsA-M and CsR-M extracts revealed the highest cholinesterase inhibition on both AChE (4.06 and 5.58 mg GALAE/g extract) and BChE (4.71 and 4.13 mg GALAE/g extract). However, the CsR-D extract does not show inhibition against AChE. This observed activity of methanolic extracts can be linked to high levels of phenolic compounds in the extracts. These findings are supported by several researchers (Kennedy and Wightman, 2011; Mazlan et al., 2013; Roseiro et al., 2012), who reported a linear correspondence between phenolic content and cholinesterase inhibition. Moreover, as shown in Figure 2, a strong positive correlation was observed between total phenolic contents of the tested

extracts and their AChE and BChE inhibition (R values in the range of 1), whereas total flavonoids presented a strong positive correlation for BChE but moderate for AChE.

All extracts have the significant ability to inhibit tyrosinase enzyme, and the CsA-D extract showed great tyrosinase inhibition, which is 139.78 mg KAE/g extract. As for glucosidase inhibition, the methanolic extracts express maximum ability for inhibition as compared to DCM extracts. However, as indicated in Figure 2, a strong negative correlation was seen among total bioactive contents and tyrosinase inhibition (R values in the range of -1).

Glucosidase inhibition may be due to the presence of high phenolic contents. According to our study, the phenolic compounds were responsible for anti-diabetic activity (Etxeberria et al., 2012; Tundis et al., 2010). Though, the case for amylase was different because DCM extracts were found to be most active. Huseini et al. (Huseini et al., 2013) revealed those patients who were taking 1200 mg of *C. spinosa* fruit extracts in their daily routine expressed a significant low level of glycosylated hemoglobin and fasting blood glucose level as compared to the control group (p = 0.043 and 0.037, correspondingly) and it was also reported that there was an improvement in hyperglycemia and hypertriglyceridemia in diabetic persons. Likewise, it was also reported that *C. spinosa* is responsible for decreased absorption of carbohydrates, and another study reports that it decreases the rate of carbohydrate absorption and exerts the postprandial hypoglycemic effect on the gastrointestinal tract (Lemhadri et al., 2007). So, the molecular approaches can be more valuable to understand the interactions between enzymes and secondary metabolites. Our results are also supported by PCA analysis (Figure 2) which confirms a negative association among total phenolic and flavonoids with amylase inhibition, however, a strong positive correlation was observed for phenolic contents and glucosidase inhibition, while total flavonoid contents also showed a moderated correlation for glucosidase enzyme. According to our information, this is the

very first detailed study on *C. spinosa*. Altogether, this information can be beneficial for starting and designing unique functional products of natural origin.

3.4. Cytotoxicity assay

The cytotoxicity of all the four extracts of *C. spinosa* was also performed against two breast cancer cell lines including MCF-7 and MDA-MB-231 cells, and the findings of cytotoxicity activity are depicted in Table 7. From the results it is clear that, all the tested extracts presented low to moderate toxicity against the tested breast cell line. The CsR-M extract was noted to be most active against MDA-MB-231 cell line with a percentage viability of 73.81%. Likewise, the CsA-M extract was also found to be considerable active against both the cell lines. This is just a preliminary toxicity testing of the studied plant extract, and the detailed in-vivo toxicity studies are recommended.

4. Conclusion

The functional pharmaceutical products are of great interest in recent years. In this report, the current work describes the chemical profile and biological abilities of aerial and root parts of *C. spinosa*. The tested extracts exhibited notable antioxidant and enzyme inhibition properties and also presented considerable toxicity against breast cells. The plant was found to contain flavonoid, alkaloid, and glucosinolate derivatives as major secondary metabolites. The methanolic extracts exhibited higher phenolic and flavonoids as well DPPH and ABTS radical savaging activities. On the contrary, the DCM extracts were most active for reducing power, phosphomolypdenum and metal chelation assays. For enzyme inhibition, both methanolic extracts exerted considerable anti-cholinesterase, anti-tyrosinase and glucosidase inhibition. The expressed enzyme inhibition potential could be attributed to the higher levels of phenolic and flavonoid contents in methanolic extracts. The obtained results from the current work can provide new directions for the

bioprospecting of *C. spinosa* as a potential source of antioxidants and enzyme inhibitor bioactive molecules.

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List of abbreviations:

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CUPRAC: cupric reducing antioxidant capacity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; FRAP: ferric reducing antioxidant power; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA: principal component analysis; UHPLC-MS: ultra-high-performance liquid chromatography- mass spectrometry;

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Figure captions:

Figure 1. Total ion chromatograms (TICs) of *C. spinosa* aerial (A) and root (B) methanol extracts

Figure 2. Statistical evaluations, **A:** Correlation coefficients between total bioactive compounds and biological activities (Pearson Correlation Coefficient (R), $p < 0.05$); **B and D:** Distribution of the tested extracts on the factorial plan and representation of biological activities on the correlation circle based on PCA; **C:** Eigenvalues and percentage of variability expressed by the factors; **E:** Heat map of extracts in according to bioactive compounds and biological activities

Tables and Figures:

Table 1. Total bioactive contents in *C. spinosa* extracts

Extracts	Yield (%)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
CsA-M	13	30.36±0.65	31.58±0.17
CsA-D	11	18.88±0.17	3.09±0.08
CsR-M	15	23.53±0.23	8.78±0.08
CsR-D	09	12.44±0.34	1.22±0.08

CsA-M: *C. spinosa*aerial methanol; CsA-D: *C. spinosa* aerial DCM; CsR-M: *C. spinosa* root methanol; CsR-D: *C. spinosa* root DCM.

Data from three repetitions, with mean ± standard deviation; GAE: gallic acid equivalent; QE: quercetin equivalent.

Table 2. UHPLC-MS analysis of *C. spinosa* aerial methanol extract

S.no	RT (min)	B. peak *m/z*	Tentative compound identification	Comp. class	MFG formula	Mol. mass
1	0.92	290.09	Sarmentosin epoxide	Cyanogenic	$C_{11}H_{17}NO_8$	291.09
2	0.96	191.02	Citric acid	Organic Acid	$C_6H_8O_7$	192.02
3	1.12	360.05	Glucoputranjivin	Glucosinolate	$C_{10}H_{19}NO_9S_2$	361.05
4	2.05	374.06	Glucocochlearin	Glucosinolate	$C_{11}H_{21}NO_9S_2$	375.06
5	8.40	755.21	Kaempferol 3-(2G-glucosylrutinoside)	Flavonoid	$C_{33}H_{40}O_{21}$	756.21
6	8.61	477.07	4-Methoxyglucobrassicin	Glucosinolate	$C_{17}H_{22}N_2O_{10}S_2$	478.07
7	8.64	739.21	Robinin	Flavonoid	$C_{33}H_{40}O_{19}$	740.21
8	8.87	609.15	Robinetin 3-rutinoside	Flavonoid	$C_{27}H_{30}O_{16}$	610.15
9	9.20	593.15	Luteolin 7-rhamnosyl (1->6) galactoside	Flavonoid	$C_{27}H_{30}O_{15}$	594.15
10	9.26	623.16	Tricetin 7-methyl ether 3'-glucoside-5'-rhamnoside	Flavonoid	$C_{28}H_{32}O_{16}$	624.16
11	13.28	293.18	Gingerol	Phenol	$C_{17}H_{26}O_4$	294.18

RT: retention time; B. peak: base peak

Table 3. UHPLC-MS analysis of *C. spinosa* root methanol extract

S. no	RT (min)	B. peak *m/z*	Tentative compound identification	Comp. class	MFG formula	Mol. mass
1	1.18	360.05	Glucoputranjivin	Glucosinolate	$C_{10}H_{19}N\ O_9S_2$	361.05
2	1.66	174.08	Calystegin B2	Alkaloid	$C_7H_{13}NO_4$	175.08
3	8.87	434.21	Cadabicine	Alkaloid	$C_{25}H_{29}N_3O_4$	435.21
4	9.11	389.13	Abruquinone B	Flavonoid	$C_{20}H_{22}O_8$	390.13
5	9.24	328.12	3-O-Acetylhamayne	Alkaloid	$C_{18}H_{19}NO_5$	329.12
6	10.78	301.04	Melanoxetin	Flavonoid	$C_{15}H_{10}O_7$	302.04
7	10.79	647.20	Embigenin 2"-(2'"-acetylrhamnoside)	Flavonoid	$C_{31}H_{36}O_{15}$	648.20
8	11.47	577.25	Withaperuvin H	Withanolide	$C_{30}H_{42}O_9S$	578.25
9	11.58	755.34	Michellamine B	Alkaloid	$C_{46}H_{48}N_2O_8$	756.34
10	11.91	821.40	Licoricesaponin K2	Triterpene	$C_{42}H_{62}O_{16}$	822.40

RT: retention time; B. peak: base peak

Table 4. HPLC polyphenolic quantification of *C. spinosa* extracts (μg/g sample) (mean ± S. D).

Phenolic compounds	**CsA-M**	**CsA-D**	**CsR-M**	**CsR-D**
Vanillic acid	nd	nd	nd	0.33±0.03
Epicatechin	0.59±0.06	nd	0.33±0.03	nd
Syringic acid	nd	nd	nd	0.27±0.02
3-OH Benzoic acid	BLD	0.45±0.04	5.67±1.03	0.56±0.04
3-OH 4-methoxy benzaldehyde	nd	nd	nd	0.24±0.02
Naringin	3.13±0.09	nd	nd	nd
2.3-diMeO benzoic acid	nd	nd	nd	1.02±0.09
Benzoic acid	nd	2.26±0.15	nd	nd
Carvacrol	0.33±0.03	nd	nd	nd

nd: not detected; BLD: below limit of detection (<0.1 μg/mL); Chlorogenic acid, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferullic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid were not detected in any of the tested extracts.

Table 5. Antioxidant properties of *C. spinosa* extracts

Extracts	Radical Scavenging activity		Reducing power		Total antioxidant capacity (TAC)	Ferrous chelating
	DPPH (mg TE/g extract)	**ABTS (mgT E/g extract)**	**FRAP (mg TE/g extr act)**	**CUPRAC (mgT E/g extract)**	**Phosphomolybdenum (mg TE/g extract)**	**Metal Chelating (mg EDTA/g)**
CsA-M	30.48±0.37	40.43±3.33	47.13±3.67	86.64±8.09	6.73±0.39	1.19±0.03
CsA-D	6.24±0.61	23.64±1.07	50.37±2.42	118.45±1.69	75.79±1.25	2.51±0.19
CsR-M	28.45±0.60	40.55±1.35	38.49±0.83	58.77±0.71	na	0.31±0.04
CsR-D	16.06±1.81	33.68±2.55	42.82±1.55	96.89±5.19	13.56±1.05	1.41±0.09

TE: trolox equivalent; EDTAE: EDTA equivalent; na: not active. All values expressed are means ± S.D. of three parallel measurements.

Table 6. Enzyme inhibition effects of *C. spinosa* extracts

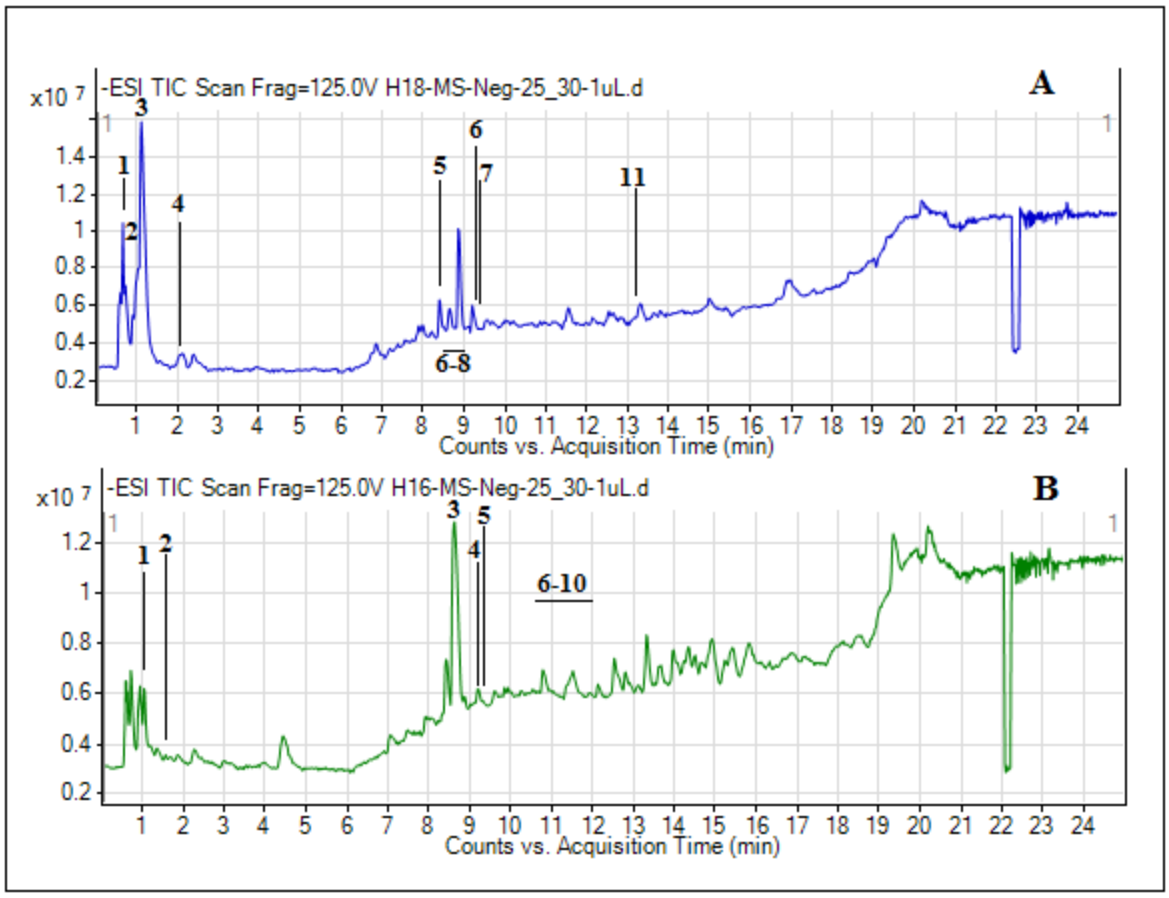
Extracts	AChE inhibition (mg GALAE/g extract)	BChE inhibition (mg GALAE/g extract)	Tyrosinase (mg KAE/g extract)	Amylase (mmol ACAE/g extract)	Glucosidase (mmol ACAE/g extract)
CsA-M	4.06±0.18	5.58±0.45	127.89±0.75	0.52±0.01	1.85±0.06
CsA-D	3.43±0.34	2.28±0.04	135.52±0.76	0.77±0.02	1.80±0.04
CsR-M	4.71±0.14	4.13±0.17	132.85±0.85	0.39±0.02	1.94±0.01
CsR-D	na	3.56±0.08	139.78±0.95	0.57±0.04	1.79±0.03

All values expressed are means ± S.D. of three parallel measurements. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; GALAE: galantamine equivalent; KAE: kojic acid equivalent; ACAE: acarbose equivalent; na: not active.

Table 7: Cytotoxicity of *C. spinosa* samples against breast cell lines.

Samples	% Viability (200 µg/mL)	
	MCF-7	MDA-MB-231
CsA-M	55.72	55.36
CsA-D	12.59	47.84
CsR-M	48.46	73.81
CsR-D	2.67	46.98

CsA-M: *C. spinosa*aerial methanol; CsA-D: *C. spinosa* aerial DCM; CsR-M: *C. spinosa* root methanol; CsR-D: *C. spinosa* root DCM.



781
782 **Figure 1.** Total ion chromatograms (TICs) of*C. spinosa* aerial (A) and root (B) methanol extracts
783

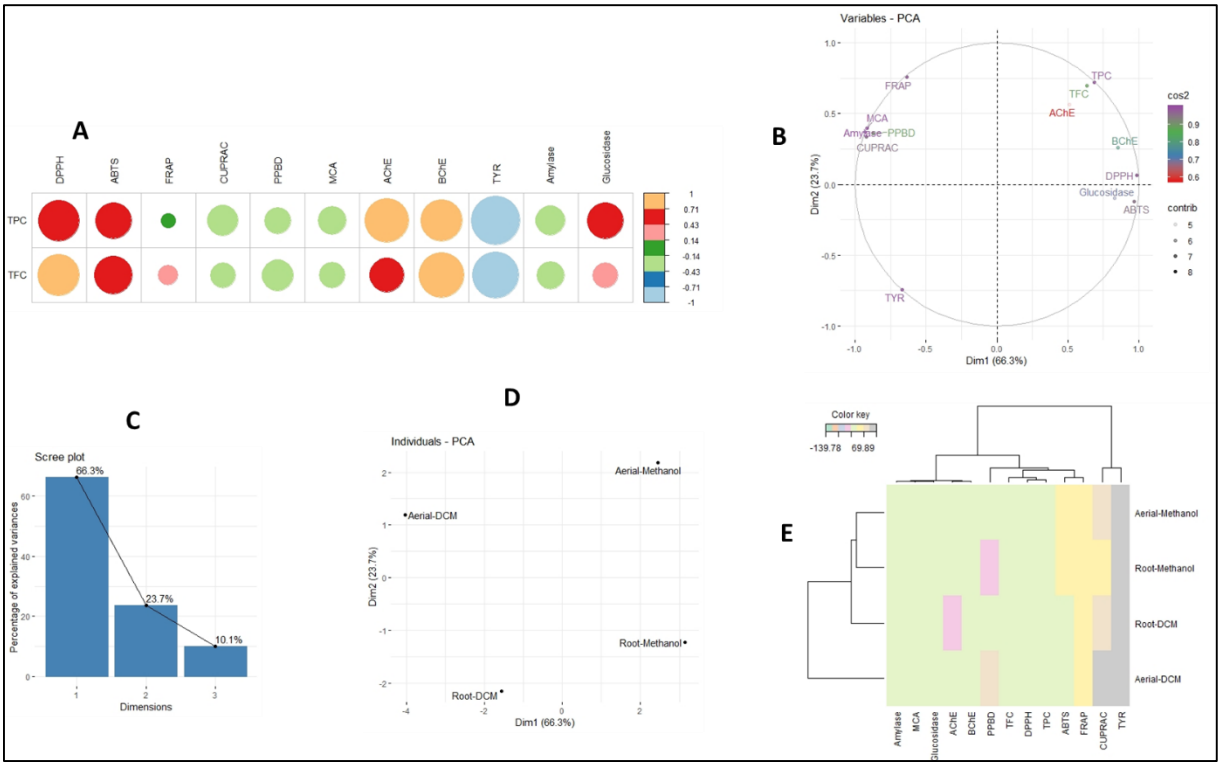


Figure 2. Statistical evaluations, **A:** Correlation coefficients between total bioactive compounds and biological activities (Pearson Correlation Coefficient (R), $p < 0.05$); **B and D:** Distribution of the tested extracts on the factorial plan and representation of biological activities on the correlation circle based on PCA; **C:** Eigen values and percentage of variability expressed by the factors; **E:** Heat map of extracts in according to bioactive compounds and biological activities

Investigation into the biological properties, secondary metabolites composition, and toxicity of aerial and root parts of *Capparis spinosa* L.: An important medicinal food plant

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Abstract

Capparis spinose L. also known as Caper is of great significance as a traditional medicinal food plant. The present work was targeted on the determination of chemical composition, pharmacological properties, and in-vitro toxicity of methanol and dichloromethane (DCM) extracts of different parts of *C. spinosa*. Chemical composition was established by determining total bioactive contents and *via* UHPLC-MS secondary metabolites profiling. For determination of biological activities, antioxidant capacity was determined through DPPH, ABTS, CUPRAC, FRAP, phosphomolybdenum, and metal chelating assays while enzyme inhibition against cholinesterase, tyrosinase, α-amylase and α-glucosidase were also tested. All the extracts were also tested for toxicity against two breast cell lines. The methanolic extracts were found to contain highest total phenolic and flavonoids which is correlated with their significant radical scavenging, cholinesterase, tyrosinase and glucosidase inhibition potential. Whereas DCM extracts showed significant activity for reducing power, phosphomolybdenum, metal chelation, tyrosinase, and α-amylase inhibition activities. The secondary metabolites profiling of both methanolic extracts exposed the presence of 21 different secondary metabolites belonging to glucosinolate, alkaloid, flavonoid, phenol, triterpene, and alkaloid derivatives. The present results tend to validate folklore uses of *C. spinose* and indicate this plant to be used as a potent source of designing novel bioactive compounds.

Keywords: *Capparis spinose*; antioxidant; secondary metabolites, enzyme inhibition, bioactive compounds

1. Introduction

Natural products have been utilized since time immemorial as curative agents for health management and treatment of common ailments because of their health-promoting properties and bioactive contents (Zhang and Ma, 2018). In consonance with the World Health Organization, the majority of the world's populations (about 80%) depends mostly on conventional/herbal drugs and in many countries, and the overall medicinal consumption is 30-50% that can be estimated from the preparation of conventional medicine (Locatelli et al., 2017; Zhang and Ma, 2018). For example, in Germany, approximately 90% of the population has utilized the old natural remedies for different health matters [2]. Hence, in industrial and developing countries, the use of traditional medicine is prevalent (Gunjan et al., 2015). The worldwide market for the use of traditional medicine is becoming very strong. Almost over $60 billion are covered from herbal medicine yearly, which is increasing progressively (Gunjan et al., 2015).

Capparis genus is from the family of Capparidaceae, which is in use widely for folk medicine from the distant past, particularly in countries of Western and Central Asia as well as the Mediterranean basin like Morocco, Spain, Tunisia, Italy and Turkey (Rivera et al., 2003). *C. spinosa* (also called as Caper) is a long-lasting shrubby plant that can grow in warm and dry weathers such as Middle and West Asia, the Mediterranean region and also numerous regions of Iran (Sultan and Çelik, 2009). The connection between capers and human beings is ancient that can be linked to the Stone Age. *C. spinosa* remains were discovered in archaeological areas like the inferior Mesolithic (9500–9000 b.p.) (Moufid and Farid, 2015). The remains of *C. spinosa* have been explored in China for the very first time and also in the eastern part of Central Asia which favors the use of caper as medicine from the last 2800 years (Jiang et al., 2007).

Caper is in use from ancient times in food preparation for fragrant and flavoring purposes, *C. spinosa* is also known for its use as an ordinary natural remedy because of its distinct properties for hypertension, poultice, tonic and diuretic problems (Duman and Özcan, 2014; Trombetta et al., 2005). *C. spinosa* is commonly found in hot and dry weathers and that its fruit, roots and barks are known because of their medicinal significance. It is traditionally used as the medicine for different health problems like diuretic, gout, rheumatism, hyperlipidemia, hyperglycemia, hypertension and also for liver and spleen disorders (Bonina et al., 2002; Lemhadri et al., 2007). In Morocco, this plant is usually used to control diabetes and its treatment and mostly used as a scented agent in Moroccan kitchens (Jouad et al., 2001). The parts of *C. spinosa*, such as fruits and roots, are known because of their beneficial properties on human health and are used as a herbal curative agent from the old times (Mansour et al., 2016). In earlier ages, The Egyptians and Arabs used the roots of *C. spinosa* for the treatment of kidney and liver disorders, and Romans used this plant as a therapeutic agent for paralysis. Moroccans also used it for diabetes treatment (Tlili et al., 2011). The root of *C. spinosa* is used for the treatment of enlarged spleen, mental problem and tubercular glands (Afzal et al., 2009). *C. spinosa* was also used as a medicine of rheumatoid arthritis and gout in China (Ao et al., 2007). It is also used in the treatment of hemorrhoids and gout in Iran (Mahboubi and Mahboubi, 2014).

Despite the plethora of studies related to the therapeutic uses of *C. spinosa*, data related to its chemical composition, antioxidant potential and enzyme inhibition activities related to most common human diseases is limited. Given the background regarding medicinal properties of *E. milii*, this work was conducted to probe into the enzymatic inhibitory activities of methanol and dichloromethane (DCM) extracts from aerial and roots of *C. spinosa* on key enzymes related to neurodegenerative ailments (acetylcholinesterase -AChE and butyrylcholiesterase -BChE),

diabetes (α-glucosidase and α-amylase) and skin hyperpigmentation (tyrosinase). Extracts were also appraised for their antioxidant potential utilizing free radical scavenging (2,2-diphenyl-1-picrylhydrazyl -DPPH and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) -ABTS), reducing power (ferric reducing antioxidant power -FRAP and cupric reducing antioxidant capacity -CUPRAC), phosphomolybdenum and metal chelation assays. The cytotoxicity was also performed against the MCF-7 and MDA-MB-231 breast cancer cell lines. All the extracts were chemically characterized by determining their total bioactive contents *via* spectrophotometric methods and individual secondary metabolic profiles by ultra-high-performance liquid chromatography- mass spectrometry (UHPLC-MS). Moreover, principal component analysis (PCA) statistical studies were performed to highlight possible interactions between the bioactive contents and tested biological assays.

2. Material and methods

2.1. Plant material and extraction

Aerial and root parts of *C. spinosa* were collected from Cholistan desert and identified by Mr. Hafiz Waris, Taxonomist, at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur. Additionally, a voucher specimen was deposited in the herbarium of Faculty of Pharmacy and Alternative Medicines, The Islamia University of Bahawalpur, for future reference. For extraction, powdered aerial and root parts were subjected for maceration (72 hrs) consecutively using DCM and methanol solvents and were kept at room temperature with intermittent shaking. The extracts obtained were made concentrated using a rotary evaporator and are abbreviated as CsA-M: *C. spinosa* aerial methanol extract; CsA-D: *C. spinosa* aerial DCM extract; CsR-M: *C. spinosa* root methanol extract; CsR-D: *C. spinosa* root DCM extract.

2.2. Total bioactive contents, UHPLC-MS analysis, and HPLC-PDA analysis

The standard Folin-Ciocalteu method was utilized to find out total phenolic content (Zengin et al., 2016c). The standard used for this purpose was gallic acid, and the amount of total phenolic content is expressed as mg GAE/g (gallic acid equivalents). Whereas to explore the total flavonoid content, the aluminum chloride colorimetric method was used (Chew et al., 2009), and quercetin was used as a standard. The results were expressed as mg QE/g (quercetin equivalent).

UHPLC-MS analysis of methanol and ethyl acetate extracts was performed (negative ionization mode) on Agilent 1290 Infinity LC system coupled with Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source as reported earlier (Saleem et al., 2019). The METLIN database was used for the tentative identification of different secondary metabolites in the tested samples. Moreover, a list of 22 different polyphenolic standards (including gallic acid, catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid, naringenin and carvacrol) was tested to be quantified in all the samples using HPLC-PDA analysis as reported previously (Locatelli et al., 2017).

2.3. Antioxidant assays

The standard methods were used to explore the free radical scavenging using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), reducing power by using FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity), total antioxidant capacity through phosphomolybdenum assay and metal chelating power as explained earlier in Grochowski et al. (2017) (Grochowski et al., 2017).

The results of all antioxidant assays were recorded as Trolox equivalents (except metal chelating assay for which EDTA was used as standard).

2.4. Enzyme inhibition assays

The enzyme inhibition studies of all the extracts against tyrosinase, acetylcholinesterase, butyrylcholinesterase, α-amylase, and α-glucosidase were exposed by utilizing the previous standard *in-vitro* methods (Grochowski et al., 2017). The AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) inhibition activity were expressed as standard galantamine equivalents (mg GALAE/g extract), while acarbose equivalent (mmol ACAE/g extract) for α-amylase and α-glucosidase and kojic acid equivalent (mg KAE/g extract) for tyrosinase were used.

2.5. MTT cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity activity of the tested samples was tested against two breast cancer cell lines, i.e., MDA-MB 231 and MCF-7 cells employing the previously described method (Nemudzivhadi and Masoko, 2014). The cell viability percentage (%) was determined as follows:

$$\text{Percentage cell viability} = \text{ABSs} - \text{ABSc} \times 100$$

Where ABSs: absorbance of the sample; ABSc: absorbance of control

2.6. Statistical analysis

The assays were carried out in a triplet, and independent experiment, and the results were calculated as a mean value ± standard deviation (SD). SPSS v.17.0 software was used for data analysis. One way analysis of variance via ANOVA followed by Tukey's test was done to find out the differences between means. A statistical value of $p < 0.05$ was considered significant. The principal component analysis (PCA) was carried out to identify the association between phytochemical content and biological properties.

3. Results and discussion

3.1. Total bioactive contents

In the present case, the extracts of *C. spinosa* were tested by the standard Folin-Ciocalteu and $AlCl_3$ methods for their total phenolic and flavonoid contents. The amount of total phenolic content was more in CsA-M (30.36 mg GAE/g extract) and CsR-M (23.53 mg GAE/g extract), as compared to the DCM extracts. Related results can be seen in the case of flavonoids as well (Table 1). Many studies have confirmed the presence of greater phenolic contents in methanolic extracts (Do et al., 2014; Murugan and Parimelazhagan, 2014).

The UHPLC-MS analysis of *C. spinosa* aerial methanol extract showed the presence of eleven different compounds (Table 2 and Figure 1). Most of these compounds were belonging to glucosinolate and flavonoid derivatives. The five flavonoids present were kaempferol 3-(2G-glucosylrutinoside), robinin, robinetin 3-rutinoside, luteolin 7-rhamnosyl (1->6) galactoside and tricetin 7-methyl ether 3'-glucoside-5'-rhamnoside. While glucoputranjivin, glucocochlearin and 4-Methoxyglucobrassicin were the present glucosinolates. Moreover, sarmentosin epoxide (cyanogenic compound), citric acid and gimgerol (phenol) were also detected. Similarity, *C. spinosa* root methanol extract identified the ten different compounds belonging to alkaloid and flavonoids (Table 3 and Figure 1). The alkaloids detected were calystegin B2, cadabicine, 3-O-acetylhamayne and michellamine B. Three flavonoids abruquinone B, melanoxetin and embigenin 2"-(2"'-acetylrhamnoside) were also identified. Moreover, one glucosinolate (glucoputranjivin), withanolide (withaperuvin H) and triterpene (licoricesaponin K2) were also present. The presence of these classes of secondary metabolites in *C. spinosa* is in agreement with previous studies (Moufid and Farid, 2015; Zhang and Ma, 2018).

Similarly, to have in-depth evaluation of the phytochemical composition, all the extracts of *C. spinosa* were studied by HPLC-PDA analysis for the quantification of 22 important phenolic compounds, and the results are presented in Table 4. The CsR-D extract was found to contain the maximum number of phenolics including vanillic acid, syringic acid, 3-OH benzoic acid, 3-OH 4-methoxy benzaldehyde, and 2.3-diMeO benzoic acid.

3.2. Antioxidant potential

A pathological activator of various diseases, such as Alzheimer's disease and Type II Diabetes, is oxidative pressure. Therefore, antioxidants are of great significance for the treatment of such oxidative stress. (Li et al., 2017). In this study, the antioxidant potential of *C. spinosa* aerial and root extracts was evaluated by utilizing six different protocols such as phosphomolybdenum, CUPTAC, FRAP, ABTS, DPPH, and metal chelating power, and the results can be seen in Table 5. The stable compound DPPH is free-radical, which shows the maximum wavelength at 517 nm and is commonly used for antioxidant determination (Loganayaki et al., 2013). All of the extracts were active against DPPH, showing activity in the following order CsA-M >CsR-M >CsR-D >CsA-D. This higher DPPH radical scavenging of aerial methanol (30.48±0.37 mg TE/g extract) and root methanol (28.45 mg TE/g extract) extracts shows correlation with their greater bioactive contents, and this is supported by the previous researcher who already explained that high DPPH scavenging activity was due to the presence of high phenolic content (Loganayaki et al., 2013; Piluzza and Bullitta, 2011). Another radical used for the determination of the antioxidant potential of plant extracts is ABTS and is a free blue/green radical with the maximum wavelength of 734nm (Zengin et al., 2018). In Table 5, it can be seen that the CsA-M and CsR-M extracts of *C. spinosa* actively scavenged ABTS radical, exploring the maximum Trolox equivalent values, i.e., 40.55 and 40.43 mg TE/g extract, respectively.

Other assays like FRAP and CUPRAC were utilized for the determination of the reducing capacity of the extracts. The reducing capacity can be quantified by observing the absorbance of ferric tripyridyltriazine to ferrous tripyridyltriazine while in the CUPRAC method, we can observe cupric reducing capacity to cuprous in the presence of copper(II)- neocuproine [Cu(II)-Nc] reagent (Al-Rimawi et al., 2016). It can be seen that both DCM extracts i.e., CsA-D (FRAP: 50.37 mg TE/g extract CUPRAC: 118.45 mg TE/g extract) and CsR-D (FRAP: 42.82 mg TE/g extract CUPRAC: 96.89 mg TE/g extract) has a potent reducing ability.

In the phosphomolybdenum method, Mo (VI) is reduced to Mo (V) in the presence of antioxidants (Chaouche et al., 2014). A reverse pattern can be observed as a trend for the phosphomolybdenum method, with CsA-D being the most active extract, in comparison with CsR-D and CsA-M extracts. The root methanol extract was not active. As this antioxidant assay measures the antioxidant potential of both phenolic and non-phenolic compounds, so the results recorded in phosphomolybdenum assay can be correlated to other non-phenolic compounds such as vitamin C or tocopherol in DCM extracts. These results are in agreement with the earlier studies (Albayrak et al., 2010; Llorent-Martínez et al., 2017) who reported the high antioxidant potential for DCM solvent.

Iron is of vital importance for respiration, oxygen transportation, and enzyme activity, but it also plays a vital role in the redox reaction, hence playing a role in oxidative stress (Farina et al., 2013). The results of our study explained that the different extracts of *C. spinosa* could chelate iron (Table 5). Similar to reducing power results, both DCM extracts were found to be the most active metal chelators, followed by methanolic extracts. These findings show similarity with earlier studies which reported that there is no correlation between total phenolic and metal chelating capacity (Khorasani Esmaeili et al., 2015; Silva et al., 2008; Yerlikaya et al., 2017). At

this point, non-phenolic compounds like tocopherol, as previously isolated from *C. spinose* (Moufid and Farid, 2015), could be attributed to this activity. As presented in Figure 2, Pearson correlation analysis confirmed the tested antioxidant results and showed a significant relationship of total bioactive contents and radical scavenging capacities (DPPH and ABTS), while a moderate association was observed for FRAP, whereas a negative correlation was the recorder for phosphomolybdenum and metal chelation assays in relation with bioactive contents.

3.3. Enzyme inhibition assays

Similarly, α-amylase and α-glucosidase inhibitors are used as therapeutic agents in the case of DM. Tyrosine is the key enzyme used in melanin synthesis, and for the treatment of hyperpigmentation, tyrosinase inhibitors are used. According to this information, the enzyme inhibitors can be synthesized artificially. In this case, limited side effects can be observed, such as toxic properties and gastrointestinal problems (Kumar et al., 2011). So, many researchers are trying to isolate inhibitors from natural sources having no or minimal side effects. So, the enzyme inhibition studies were carried out on *C. spinosa* extracts against cholinesterases, tyrosinase, amylase and glucosidase. The results are expressed in Table 6. The CsA-M and CsR-M extracts revealed the highest cholinesterase inhibition on both AChE (4.06 and 5.58 mg GALAE/g extract) and BChE (4.71 and 4.13 mg GALAE/g extract). However, the CsR-D extract does not show inhibition against AChE. This observed activity of methanolic extracts can be linked to high levels of phenolic compounds in the extracts. These findings are supported by several researchers (Kennedy and Wightman, 2011; Mazlan et al., 2013; Roseiro et al., 2012), who reported a linear correspondence between phenolic content and cholinesterase inhibition. Moreover, as shown in Figure 2, a strong positive correlation was observed between total phenolic contents of the tested

extracts and their AChE and BChE inhibition (R values in the range of 1), whereas total flavonoids presented a strong positive correlation for BChE but moderate for AChE.

All extracts have the significant ability to inhibit tyrosinase enzyme, and the CsA-D extract showed great tyrosinase inhibition, which is 139.78 mg KAE/g extract. As for glucosidase inhibition, the methanolic extracts express maximum ability for inhibition as compared to DCM extracts. However, as indicated in Figure 2, a strong negative correlation was seen among total bioactive contents and tyrosinase inhibition (R values in the range of -1).

Glucosidase inhibition may be due to the presence of high phenolic contents. According to our study, the phenolic compounds were responsible for anti-diabetic activity (Etxeberria et al., 2012; Tundis et al., 2010). Though, the case for amylase was different because DCM extracts were found to be most active. Huseini et al. (Huseini et al., 2013) revealed those patients who were taking 1200 mg of *C. spinosa* fruit extracts in their daily routine expressed a significant low level of glycosylated hemoglobin and fasting blood glucose level as compared to the control group (p = 0.043 and 0.037, correspondingly) and it was also reported that there was an improvement in hyperglycemia and hypertriglyceridemia in diabetic persons. Likewise, it was also reported that *C. spinosa* is responsible for decreased absorption of carbohydrates, and another study reports that it decreases the rate of carbohydrate absorption and exerts the postprandial hypoglycemic effect on the gastrointestinal tract (Lemhadri et al., 2007). So, the molecular approaches can be more valuable to understand the interactions between enzymes and secondary metabolites. Our results are also supported by PCA analysis (Figure 2) which confirms a negative association among total phenolic and flavonoids with amylase inhibition, however, a strong positive correlation was observed for phenolic contents and glucosidase inhibition, while total flavonoid contents also showed a moderated correlation for glucosidase enzyme. According to our information, this is the

very first detailed study on *C. spinosa*. Altogether, this information can be beneficial for starting and designing unique functional products of natural origin.

3.4. Cytotoxicity assay

The cytotoxicity of all the four extracts of *C. spinosa* was also performed against two breast cancer cell lines including MCF-7 and MDA-MB-231 cells, and the findings of cytotoxicity activity are depicted in Table 7. From the results it is clear that, all the tested extracts presented low to moderate toxicity against the tested breast cell line. The CsR-M extract was noted to be most active against MDA-MB-231 cell line with a percentage viability of 73.81%. Likewise, the CsA-M extract was also found to be considerable active against both the cell lines. This is just a preliminary toxicity testing of the studied plant extract, and the detailed in-vivo toxicity studies are recommended.

4. Conclusion

The functional pharmaceutical products are of great interest in recent years. In this report, the current work describes the chemical profile and biological abilities of aerial and root parts of *C. spinosa*. The tested extracts exhibited notable antioxidant and enzyme inhibition properties and also presented considerable toxicity against breast cells. The plant was found to contain flavonoid, alkaloid, and glucosinolate derivatives as major secondary metabolites. The methanolic extracts exhibited higher phenolic and flavonoids as well DPPH and ABTS radical savaging activities. On the contrary, the DCM extracts were most active for reducing power, phosphomolypdenum and metal chelation assays. For enzyme inhibition, both methanolic extracts exerted considerable anti-cholinesterase, anti-tyrosinase and glucosidase inhibition. The expressed enzyme inhibition potential could be attributed to the higher levels of phenolic and flavonoid contents in methanolic extracts. The obtained results from the current work can provide new directions for the

bioprospecting of *C. spinosa* as a potential source of antioxidants and enzyme inhibitor bioactive molecules.

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List of abbreviations:

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CUPRAC: cupric reducing antioxidant capacity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; FRAP: ferric reducing antioxidant power; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA: principal component analysis; UHPLC-MS: ultra-high-performance liquid chromatography- mass spectrometry;

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Figure captions:

Figure 1. Total ion chromatograms (TICs) of *C. spinosa* aerial (A) and root (B) methanol extracts

Figure 2. Statistical evaluations, **A:** Correlation coefficients between total bioactive compounds and biological activities (Pearson Correlation Coefficient (R), $p < 0.05$); **B and D:** Distribution of the tested extracts on the factorial plan and representation of biological activities on the correlation circle based on PCA; **C:** Eigenvalues and percentage of variability expressed by the factors; **E:** Heat map of extracts in according to bioactive compounds and biological activities

Tables and Figures:

Table 1. Total bioactive contents in *C. spinosa* extracts

Extracts	Yield (%)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
CsA-M	13	30.36±0.65	31.58±0.17
CsA-D	11	18.88±0.17	3.09±0.08
CsR-M	15	23.53±0.23	8.78±0.08
CsR-D	09	12.44±0.34	1.22±0.08

CsA-M: *C. spinosa*aerial methanol; CsA-D: *C. spinosa* aerial DCM; CsR-M: *C. spinosa* root methanol; CsR-D: *C. spinosa* root DCM.

Data from three repetitions, with mean ± standard deviation; GAE: gallic acid equivalent; QE: quercetin equivalent.

Table 2. UHPLC-MS analysis of *C. spinosa* aerial methanol extract

S.no	RT (min)	B. peak *m/z*	Tentative compound identification	Comp. class	MFG formula	Mol. mass
1	0.92	290.09	Sarmentosin epoxide	Cyanogenic	$C_{11}H_{17}NO_8$	291.09
2	0.96	191.02	Citric acid	Organic Acid	$C_6H_8O_7$	192.02
3	1.12	360.05	Glucoputranjivin	Glucosinolate	$C_{10}H_{19}NO_9S_2$	361.05
4	2.05	374.06	Glucocochlearin	Glucosinolate	$C_{11}H_{21}NO_9S_2$	375.06
5	8.40	755.21	Kaempferol 3-(2G-glucosylrutinoside)	Flavonoid	$C_{33}H_{40}O_{21}$	756.21
6	8.61	477.07	4-Methoxyglucobrassicin	Glucosinolate	$C_{17}H_{22}N_2O_{10}S_2$	478.07
7	8.64	739.21	Robinin	Flavonoid	$C_{33}H_{40}O_{19}$	740.21
8	8.87	609.15	Robinetin 3-rutinoside	Flavonoid	$C_{27}H_{30}O_{16}$	610.15
9	9.20	593.15	Luteolin 7-rhamnosyl (1->6) galactoside	Flavonoid	$C_{27}H_{30}O_{15}$	594.15
10	9.26	623.16	Tricetin 7-methyl ether 3'-glucoside-5'-rhamnoside	Flavonoid	$C_{28}H_{32}O_{16}$	624.16
11	13.28	293.18	Gingerol	Phenol	$C_{17}H_{26}O_4$	294.18

RT: retention time; B. peak: base peak

Table 3. UHPLC-MS analysis of *C. spinosa* root methanol extract

S. no	RT (min)	B. peak *m/z*	Tentative compound identification	Comp. class	MFG formula	Mol. mass
1	1.18	360.05	Glucoputranjivin	Glucosinolate	$C_{10}H_{19}N\ O_9S_2$	361.05
2	1.66	174.08	Calystegin B2	Alkaloid	$C_7H_{13}NO_4$	175.08
3	8.87	434.21	Cadabicine	Alkaloid	$C_{25}H_{29}N_3O_4$	435.21
4	9.11	389.13	Abruquinone B	Flavonoid	$C_{20}H_{22}O_8$	390.13
5	9.24	328.12	3-O-Acetylhamayne	Alkaloid	$C_{18}H_{19}NO_5$	329.12
6	10.78	301.04	Melanoxetin	Flavonoid	$C_{15}H_{10}O_7$	302.04
7	10.79	647.20	Embigenin 2"-(2'"-acetylrhamnoside)	Flavonoid	$C_{31}H_{36}O_{15}$	648.20
8	11.47	577.25	Withaperuvin H	Withanolide	$C_{30}H_{42}O_9S$	578.25
9	11.58	755.34	Michellamine B	Alkaloid	$C_{46}H_{48}N_2O_8$	756.34
10	11.91	821.40	Licoricesaponin K2	Triterpene	$C_{42}H_{62}O_{16}$	822.40

RT: retention time; B. peak: base peak

Table 4. HPLC polyphenolic quantification of *C. spinosa* extracts (μg/g sample) (mean ± S. D).

Phenolic compounds	**CsA-M**	**CsA-D**	**CsR-M**	**CsR-D**
Vanillic acid	nd	nd	nd	0.33±0.03
Epicatechin	0.59±0.06	nd	0.33±0.03	nd
Syringic acid	nd	nd	nd	0.27±0.02
3-OH Benzoic acid	BLD	0.45±0.04	5.67±1.03	0.56±0.04
3-OH 4-methoxy benzaldehyde	nd	nd	nd	0.24±0.02
Naringin	3.13±0.09	nd	nd	nd
2.3-diMeO benzoic acid	nd	nd	nd	1.02±0.09
Benzoic acid	nd	2.26±0.15	nd	nd
Carvacrol	0.33±0.03	nd	nd	nd

nd: not detected; BLD: below limit of detection (<0.1 μg/mL); Chlorogenic acid, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferullic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid were not detected in any of the tested extracts.

Table 5. Antioxidant properties of *C. spinosa* extracts

Extracts	Radical Scavenging activity		Reducing power		Total antioxidant capacity (TAC)	Ferrous chelating
	DPPH (mg TE/g extract)	**ABTS (mgT E/g extract)**	**FRAP (mg TE/g extr act)**	**CUPRAC (mgT E/g extract)**	**Phosphomolybdenum (mg TE/g extract)**	**Metal Chelating (mg EDTA/g)**
CsA-M	30.48±0.37	40.43±3.33	47.13±3.67	86.64±8.09	6.73±0.39	1.19±0.03
CsA-D	6.24±0.61	23.64±1.07	50.37±2.42	118.45±1.69	75.79±1.25	2.51±0.19
CsR-M	28.45±0.60	40.55±1.35	38.49±0.83	58.77±0.71	na	0.31±0.04
CsR-D	16.06±1.81	33.68±2.55	42.82±1.55	96.89±5.19	13.56±1.05	1.41±0.09

TE: trolox equivalent; EDTAE: EDTA equivalent; na: not active. All values expressed are means ± S.D. of three parallel measurements.

Table 6. Enzyme inhibition effects of *C. spinosa* extracts

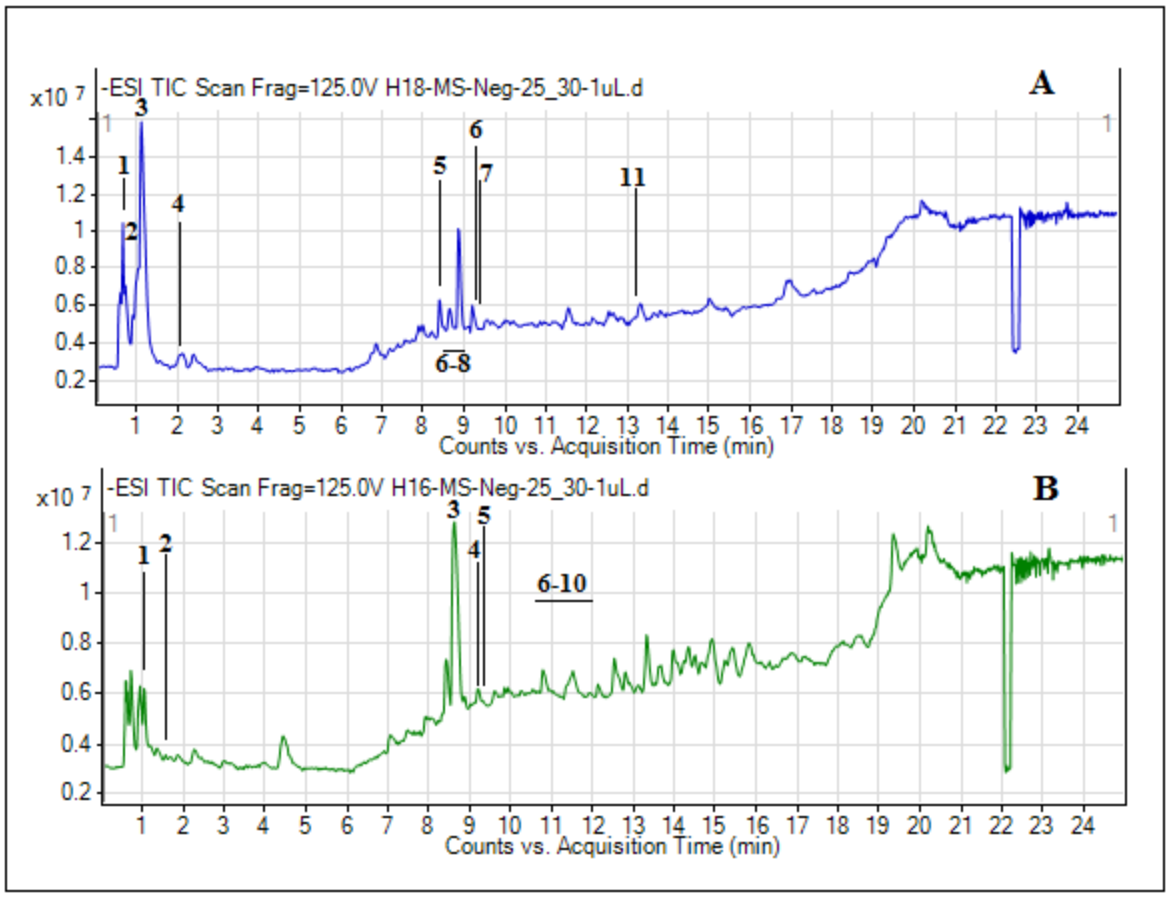
Extracts	AChE inhibition (mg GALAE/g extract)	BChE inhibition (mg GALAE/g extract)	Tyrosinase (mg KAE/g extract)	Amylase (mmol ACAE/g extract)	Glucosidase (mmol ACAE/g extract)
CsA-M	4.06±0.18	5.58±0.45	127.89±0.75	0.52±0.01	1.85±0.06
CsA-D	3.43±0.34	2.28±0.04	135.52±0.76	0.77±0.02	1.80±0.04
CsR-M	4.71±0.14	4.13±0.17	132.85±0.85	0.39±0.02	1.94±0.01
CsR-D	na	3.56±0.08	139.78±0.95	0.57±0.04	1.79±0.03

All values expressed are means ± S.D. of three parallel measurements. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; GALAE: galantamine equivalent; KAE: kojic acid equivalent; ACAE: acarbose equivalent; na: not active.

Table 7: Cytotoxicity of *C. spinosa* samples against breast cell lines.

Samples	% Viability (200 µg/mL)	
	MCF-7	MDA-MB-231
CsA-M	55.72	55.36
CsA-D	12.59	47.84
CsR-M	48.46	73.81
CsR-D	2.67	46.98

CsA-M: *C. spinosa*aerial methanol; CsA-D: *C. spinosa* aerial DCM; CsR-M: *C. spinosa* root methanol; CsR-D: *C. spinosa* root DCM.



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782 **Figure 1.** Total ion chromatograms (TICs) of*C. spinosa* aerial (A) and root (B) methanol extracts
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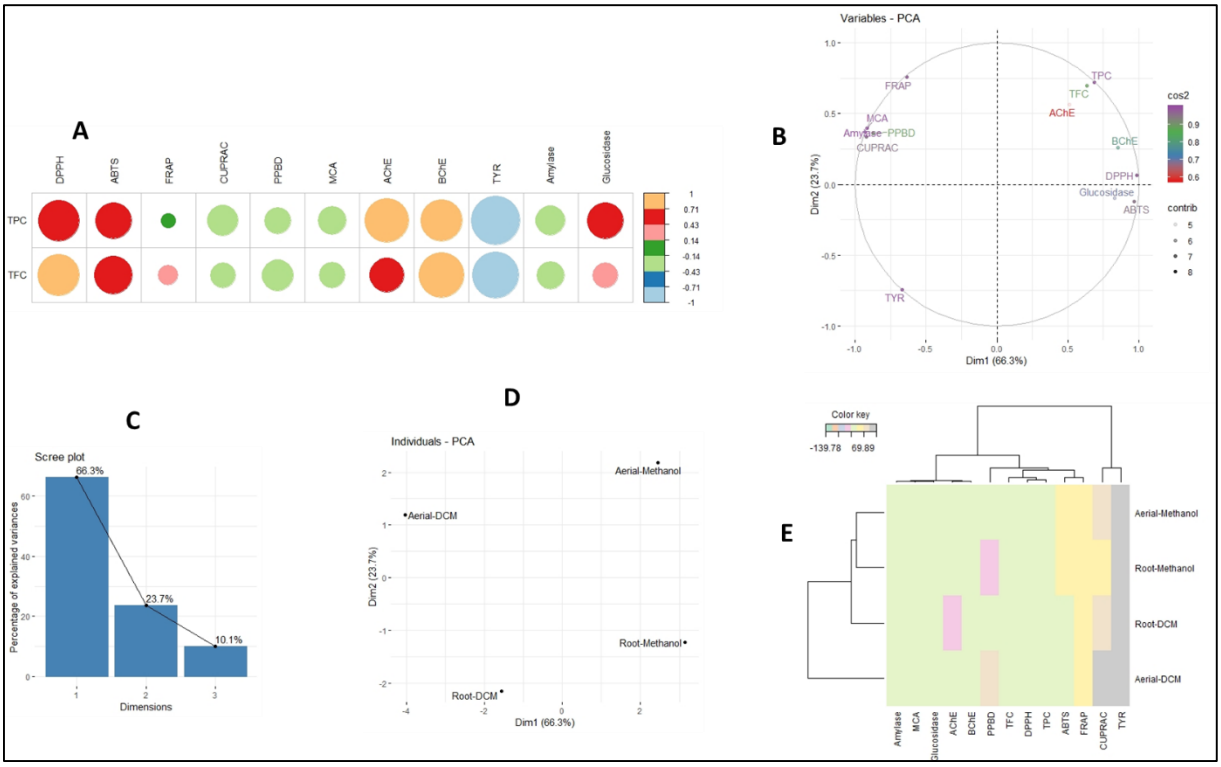

Figure 2. Statistical evaluations, **A:** Correlation coefficients between total bioactive compounds and biological activities (Pearson Correlation Coefficient (R), $p < 0.05$); **B and D:** Distribution of the tested extracts on the factorial plan and representation of biological activities on the correlation circle based on PCA; **C:** Eigen values and percentage of variability expressed by the factors; **E:** Heat map of extracts in according to bioactive compounds and biological activities

To Whom It May Concern

I **Hammad Saleem** is submitting a manuscript entitled for possible publication in Food and Chemical Toxicology. It is stated that there is no Conflict of Interest for the submitted paper.

Sincerely Yours,

Hammad Saleem

Credit Author Statement

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