

Chemical Profiling, Antioxidant and Lipoxygenase Enzyme Inhibition Activities of Wild Edible Truffle (*Terfezia boudieri*) from Northern Borders of Saudi Arabia

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ABSTRACT

Truffles are natural food product very famous for its health benefits for being significant biosource of essential fatty acids, proteins and other antioxidant and phenolic compounds. The current study was conducted to evaluate the phytochemicals, antioxidant and lipoxygenase inhibition activities of *Terfezia boudieri* of Saudi origin. Various phytochemicals were screened applying standard procedures. The total methanol extract (TME) of the truffle was subjected to several chromatographic procedures. The antioxidant activity was evaluated by DPPH antioxidant procedure, comparing results with trolox as standard. Results demonstrated that *Terfezia boudieri* chemically characterized by the availability of various constituents such as flavonoids, steroids, saponins, tannins and carbohydrates at different levels. Phytochemical investigation led to the isolation of β -sitosterol and gallic acid that were identified using ^1H , ^{13}C , DEPT, COSY, HMQC and HMBC NMR spectroscopic data. Results demonstrated high antioxidant activity with IC_{50} : 50.4 $\mu\text{g/ml}$ and 31.4 $\mu\text{g/ml}$ for TME and gallic acid, respectively. TME and gallic acid exhibited lipoxygenase inhibitory activity with IC_{50} values 4.59 and 0.53 $\mu\text{g/ml}$ for TME and gallic acid, respectively. The higher lipoxygenase inhibitory activity was presumably correlated to the high antioxidant activity. In conclusion, current investigation confirms the folklore use of *Terfezia boudieri* as anti-inflammatory food. Hence, the studied *Terfezia boudieri* may have a great potential as antioxidant and anti-inflammatory functional food and nutraceuticals products for pharmaceutical applications.

Key words: *Terfezia boudieri*, Truffle, β -sitosterol, Gallic acid, DPPH, Lipoxygenase inhibition.

INTRODUCTION

Globally, there is an increase in the search for, and the use of natural remedies including with about 25-50% of current pharmaceuticals. An increase in the discovery and use of plant origin remedies including food could lead to interesting possibilities in find unique anti-inflammatory therapeutic agent.¹⁻³ Moreover; these natural remedies are less toxic when compared to conventional drugs.⁴ Inflammation is defense response to hazardous factors such as allergens including free radicals or injury. Furthermore, an uncontrolled inflammatory response is considered as the main factor to serious disorders including allergies, cardiovascular issues and even leads to cancer consequently will lead to huge socio-economic burden on individuals and consequently on the country.⁵ Some mushrooms are valuable healthy foods, of low calories and fats and rich in essential unsaturated fatty acids, proteins and diversity of minerals.⁶⁻⁸ Traditionally, Desert truffles (*Terfezia*) as a member of mushrooms, are natural food sources that have significant nutritive value and reputed in folk medicine as antibacterial candidate.⁹ Bedouins used to apply filtered extract to reduce inflammation and pain of eyes, particularly during dust storms. Furthermore, they are used for their strong anti-inflammatory activity against eye and skin diseases as well as anti-oxidant properties.¹⁰ Truffles are bio-source of variety of bioactive molecules including flavonoids and steroids.¹¹ *Terfezia boudieri* (*T. boudieri*) is

considered as one of appreciated culinary species for its pleasant deep aroma. *T. boudieri* is seasonal and of a socio-economically importance in many countries including Saudi Arabia.¹²⁻¹⁴ It is edible and grown in certain arid and semiarid wild zones following the rainy season from March to May.¹⁵ Previous studies on *T. boudieri*, reported its high contents of fatty acids, amino acids, mineral, phenolic and flavonoids.^{16,17} *T. boudieri* exhibited antitumor, antidiabetic, antibacterial and antiviral activities.¹⁸⁻²¹ The current work on *T. boudieri* of Saudi origin is to investigate the phytochemical contents in order to isolate and elucidate the bioactive secondary metabolites and to assess antioxidant activity, besides, a view of providing further insight into its lipoxygenase inhibition activity.

MATERIALS AND METHODS

General procedures and chemicals

All NMR spectra were obtained using Avance 400 NMR spectrometer (^1H -NMR: 400 MHz and ^{13}C -NMR: 100 MHz, Bruker, Switzerland). Silica gel column chromatography (SCC) was carried on silica gel 60 (Sigma-Aldrich, Germany). Diaion-HP-20 (Sigma-Aldrich, Germany). Pre-coated silica gel 60 F254 plates (Sigma-Aldrich, Darmstadt, Germany) were used for thin layer chromatography (TLC). Visualization was detected using 10% vanillin/sulfuric acid in ethanol with a hotplate (150°C). 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-

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Aldrich, German). Lipoxygenase from soybeans (Sigma-Aldrich, Germany). The solvents: n-hexane, chloroform, and methanol were obtained from Sigma-Aldrich, Germany. All other chemicals were of analytical grade.

Plant material

T. boudieri was purchased from local market, Al-Hasa, Eastern Province, Saudi Arabia. Purchased *T. boudieri* was collected from the deserts of Hafar Al-Batin, of Northern borders of Saudi Arabia. *T. boudieri* was washed and sliced and subjected to air-drying and finally powdered. A voucher sample (TB-2019) was kept in Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University.

Extraction

Five hundred grams of dried powdered *T. boudieri* were thoroughly extracted three times with 10 L of 70% methanol for 1 week at 25 °C. The resulting extracts were evaporated to dryness using rotary evaporator to yield a dark reddish-yellow extract weighing 26.7 g and labeled as total methanol extract (TME).

Screening of secondary metabolites

Qualitative screening of the secondary metabolites was performed according to the standard protocols.^{22,23}

Flavonoid

Extract of 2 ml mixture was mixed with of 1 ml 2 % NaOH. Deep yellow color was developed, which disappeared on addition of 2 drops of diluted acid. This result refers to the presence of flavonoid.

Alkaloid

Extract of 0.5 ml was dissolved in 1.5 ml dilute Hydrochloric acid then filtered. The obtained filtrate was mixed with 2-3 drops of Dragendorff's reagent and observed for five minutes. Dragendorff's reagent produces an orange or orange to red precipitate refers to the existence of alkaloids.

Saponins

Extract of 1 ml was dissolved in 20 ml distilled water and mixture was shaken vigorously. The appearance of 1 cm froth for 15 minutes indicates the presence of saponins.

Steroid

Extract of 0.5 ml was dissolved in 5 ml chloroform followed by few droplets of acetic anhydride and concentrated sulphuric acid were added from the side of the test tube. The upper yellow layer with green/blue color indicates the presence of steroids.

Tannins/Phenolics

About 2-3 drops of 5% ferric chloride solution was added to 0.5 ml of extract gives intense blue-greenish indicating the presence of tannins/phenolics.

Glycosides

Extract of 2 ml was mixed equal portions of Fehling's solution of its both types (A) and (B) and mixture was heated on a water bath for about two minutes. After heating, it gives a brick-red color that refers to the presence of glycosides.

Anthraquinones

Extract 0.5 ml was boiled with dilute sulphuric acid then filtered and cooled. The filtrate was then partitioned with benzene. Next, dilute ammonia solution was added to it. The aqueous ammonia layer converted to pink to red refers to the presence of anthraquinones derivatives.

Cardiac glycosides

Extract of 10 ml was mixed with (4 ml) of solution of glacial acetic acid and 1 drop of 2% ferric chloride followed by 1 ml of concentrated sulphuric acid. A brown ring formed between the layers demonstrates the presence of cardiac glycosides.

Carbohydrates

Plant extract of 2 ml was mixed with a 10 ml Molisch reagent. Then, 2 ml conc. sulphuric acid was carefully dropped to side of the test tube. The presence of carbohydrates is indicated once a violet ring is formed at the interface of two phases.

Isolation of secondary metabolites

TME (26 g) was defatted using n-hexane, where TME was suspended in 500 ml of deionized water partitioned with n-hexane (5 times each with 500 ml). The resulting n-hexane fractions were concentrated to dryness to yield 7 g. The remaining mother liquor (19 g) was subjected to column chromatography using a Diaion HP-20 (500g) as stationary phase, then successively eluted with water to remove sugars and carbohydrates followed washing with 30% methanol and 100% methanol to obtain the fractions of water (9 g), 30% methanol (named, DTB1) (6 g) and 100% methanol (named, DTB2) (4 g). With the guidance of TLC patterns, the DTB1 fraction (6 g) was subjected to SCC (100 g, using 2 L of chloroform: methanol:water with ratio of (15:6:1) as mobile phase) to yield two compounds which were further purified using preparative RP-TLC to yield the pure Cpd1 (18 mg) and Cpd2 (23 mg).

Determination of antioxidant activity (DPPH assay)

This protocol is based primarily on the reduction of reagent DPPH dissolved in methanol and in presence of a donated proton from available molecules such as free radical scavenger substance, led to formation of a stable form. The reduction in amount of free radicals caused by antioxidant molecules present in media of reaction was observed by the decrease of values of absorbance at 515 nm. Consequently, the color was altered from violet to yellow indicating the presence of antioxidant. Different fractions were dissolved in 100 µl of methanol and placed in 96-well micro plate. The absorbance of was measured at 515 nm. Zero time was considered as Ab_{blank} . Then, 100 µl of DPPH solution (concentration of 200 µM) was inserted into every well. The plates were kept 25°C for 30 min. Then; quantification of the absorbance refers as Ab_{sample} . The percentage of inhibition was measured using this equation:

$$\% \text{ of inhibition} = [1 - [Ab_{sample} - Ab_{blank}] / [Ab_{control} - Ab_{blank}]] \times 100$$

Where: $Ab_{control}$ is the absorbance of mixture (with DMSO and all other reactant without tested extracts). IC_{50} was recorded as the sample concentration that is essential to produce inhibition of DPPH radical to be formed by 50%.²⁴

Lipoxygenase inhibitory activity assay

Lipoxygenase inhibitory activity was calculated using developed spectrometric method by Chun *et al.*²⁵ Mixture containing 160 µL of 0.1 mM sodium phosphate buffer with pH value of 7, then 10 µL of the serial concentrations of samples solution and 20 µL of lipoxygenase solution were mixed and incubated for 5 min at room temperature. Substrate of linoleic acid solution (10 µL) was then added to initiate the interaction and wait for 10 min. The test sample and the control were dissolved in 50% ethanol. % inhibition of lipoxygenase = $[(C - E)/C] \times 100$, where C ($Abs_{control} - Abs_{background}$) is the activity of the enzyme without sample and E ($Abs_{sample} - Abs_{background}$) is the activity of enzyme with sample. Where $Abs_{control}$ = absorbance of control, $Abs_{background}$ = absorbance of background and Abs_{sample} = absorbance of sample. IC_{50}

values were determined by the equation of nonlinear regression with the help of Excel Microsoft Office. All the reactions were performed in triplicate.

RESULTS AND DISCUSSIONS

Phytochemical screening of total methanol extract

The initial screening of phyto-constituents of TME demonstrated the availability of flavonoids, saponins, steroids, tannins/phenols, glycosides and carbohydrates and the absence of cardiac glycosides, anthraquinones and alkaloids.

Isolation and identification of secondary metabolites

The TME of air-dried *T. boudieri* (26 g) was subjected to various and repeated techniques of chromatography to yield two pure compounds; β -sitosterol (Cpd1) (Figure 1A) and gallic acid (Cpd2) (Figure 1B). The structure was elucidated by inspection of 1D- and 2D-NMR spectroscopic data including; ^1H , ^{13}C , DEPT, COSY, HMQC and HMBC. Results were compared with those available in literature. This study represents the first report on the isolation of β -sitosterol (Table 1 and Figures 2,3)^{26,27} and gallic acid (Table 2 and Figure 4)²⁸ from *T. boudieri*.

Table 1: ^1H and ^{13}C NMR spectral data of β -sitosterol (Cpd1) compared to literature (Lit.) (CDCl₃ at 400 and 100 MHz, respectively).

Position	Type	Chemical Shift δ (ppm)			
		$^1\text{H-NMR}$ (multiplicity)		$^{13}\text{C-NMR}$	
		Lit.	Cpd1	Lit.	Cpd1
1	CH ₂	1.46 (m)	1.46 (m)	37.28	37.25
2	CH ₂	1.56 (m)	1.55 (m)	31.69	31.54
3	CH-OH	3.54 (m)	3.55 (m)	71.82	71.81
4	CH ₂	2.32 (m)	2.32 (m)	42.33	42.32
5	C (quaternary)	-	-	140.77	140.73
6	CH=	5.37 (t)	5.36 (m)	121.73	121.72
7	CH ₂	2.04 (m)	2.01 (m)	31.93	31.91
8	CH	1.69 (m)	1.68 (m)	31.93	31.91
9	CH	1.55 (m)	1.55 (m)	50.16	50.13
10	C (quaternary)	-	-	36.51	36.50
11	CH ₂	1.52 (m)	1.50 (m)	21.11	21.08
12	CH ₂	1.51 (m)	1.48(m)	39.80	39.78
13	C (quaternary)	-	-	42.34	42.18
14	CH	1.50 (m)	1.50 (m)	56.79	56.76
15	CH ₂	1.58 (m)	1.58 (m)	24.33	24.30
16	CH ₂	1.85 (m)	1.86 (m)	28.27	28.24
17	CH	1.45 (m)	1.42 (m)	56.08	56.07
18	CH ₃	0.70 (s)	0.71 (s)	11.89	11.86
19	CH ₃	1.03 (s)	1.03 (s)	19.42	19.39
20	CH	1.60 (m)	1.65 (m)	36.17	36.14
21	CH ₃	0.94 (d)	0.94 (overlapped, d)	18.84	18.78
22	CH ₂	0.93 (m)	0.93 (m)	33.98	33.95
23	CH ₂	1.15 (m)	1.17 (m)	26.11	26.10
24	CH	1.38 (m)	1.36 (m)	45.86	45.84
25	CH	1.57 (m)	1.57 (m)	29.19	29.17
26	CH ₃	0.84 (d)	0.84 (overlapped, d)	19.84	19.81
27	CH ₃	1.86 (d)	1.86 (d)	19.06	19.04
28	CH ₂	1.10 (t)	1.11 (t)	23.10	23.07
29	CH ₃	0.82 (s)	0.82 (overlapped, t)	12.01	11.98

δ (ppm); delta scale (part per million), Lit.; literature, s; singlet, d; doublet, t; triplet, m; multiplet.

Table 2: ^1H and ^{13}C NMR spectral data of gallic acid (Cpd2) (CD₃OD, at 400 and 100 MHz, respectively) compared to literature (Lit.) (CD₃OD at 600 and 150 MHz, respectively).

Position	Type	Chemical Shift δ (ppm)			
		$^1\text{H-NMR}$ (multiplicity)		$^{13}\text{C-NMR}$	
		Lit.	Cpd2	Lit.	Cpd2
1	C (quaternary)	-	-	122.04	121.99
2, 6	CH	7.06(s)	7.10(s)	110.37	110.44
3, 5	C (quaternary)	-	-	146.44	146.40
4	C (quaternary)	-	-	139.63	139.65
7	C (carboxylic acid)	-	-	170.45	170.54

δ (ppm); delta scale (part per million), Lit. ; literature, s; singlet.

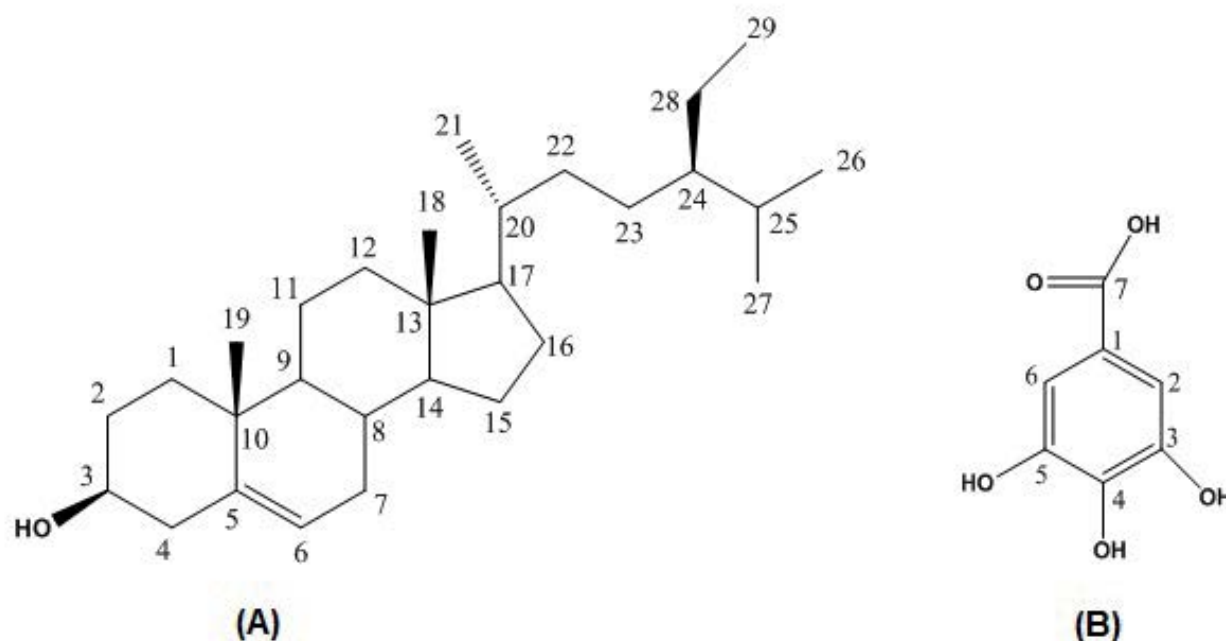


Figure 1: Structures of pure isolated compounds; β -sitosterol (A) and gallic acid (B).

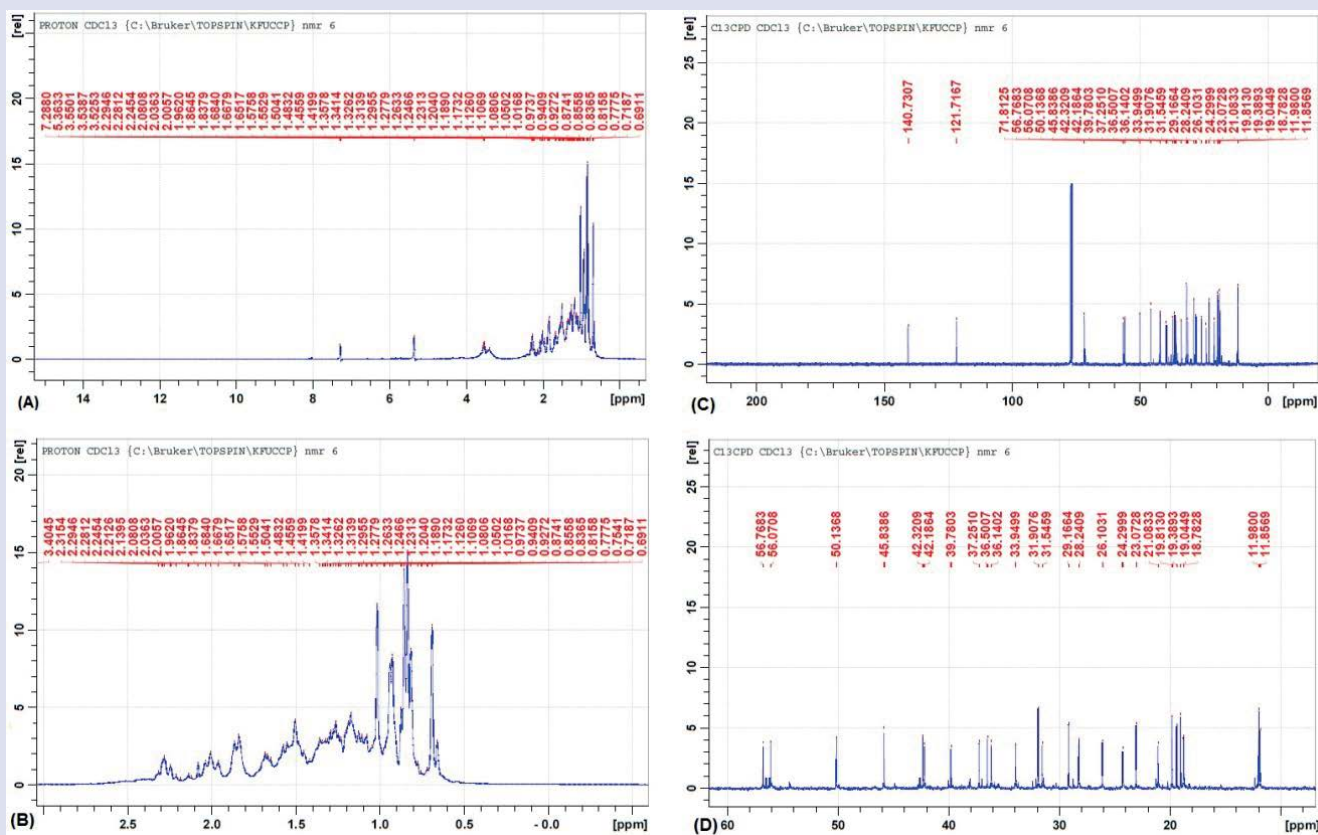


Figure 2: NMR spectral data of β -sitosterol (Cpd1); ^1H -NMR full spectrum (A), ^1H -NMR expanded spectrum (B), ^{13}C -NMR full spectrum (C) and ^{13}C -NMR expanded spectrum (D).

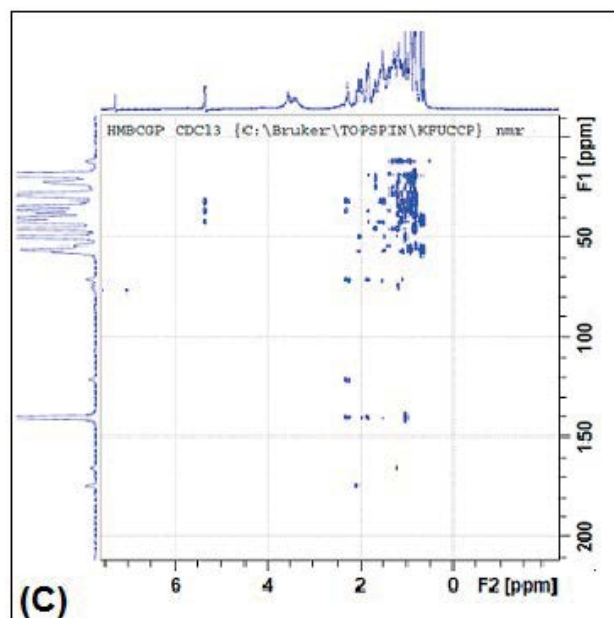
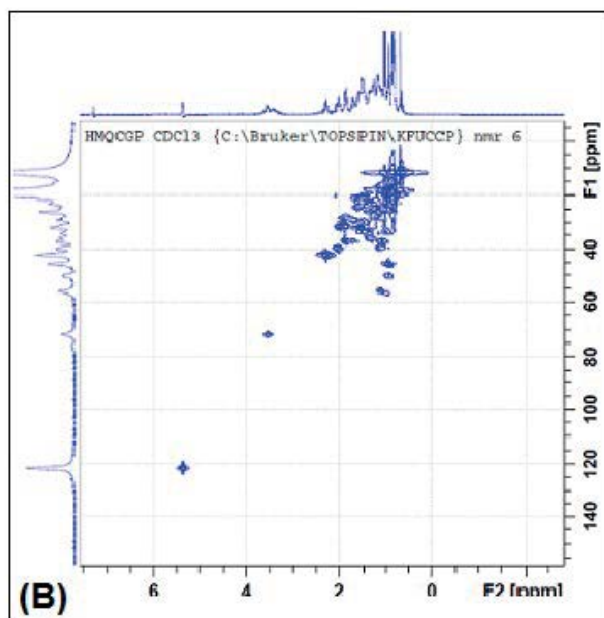
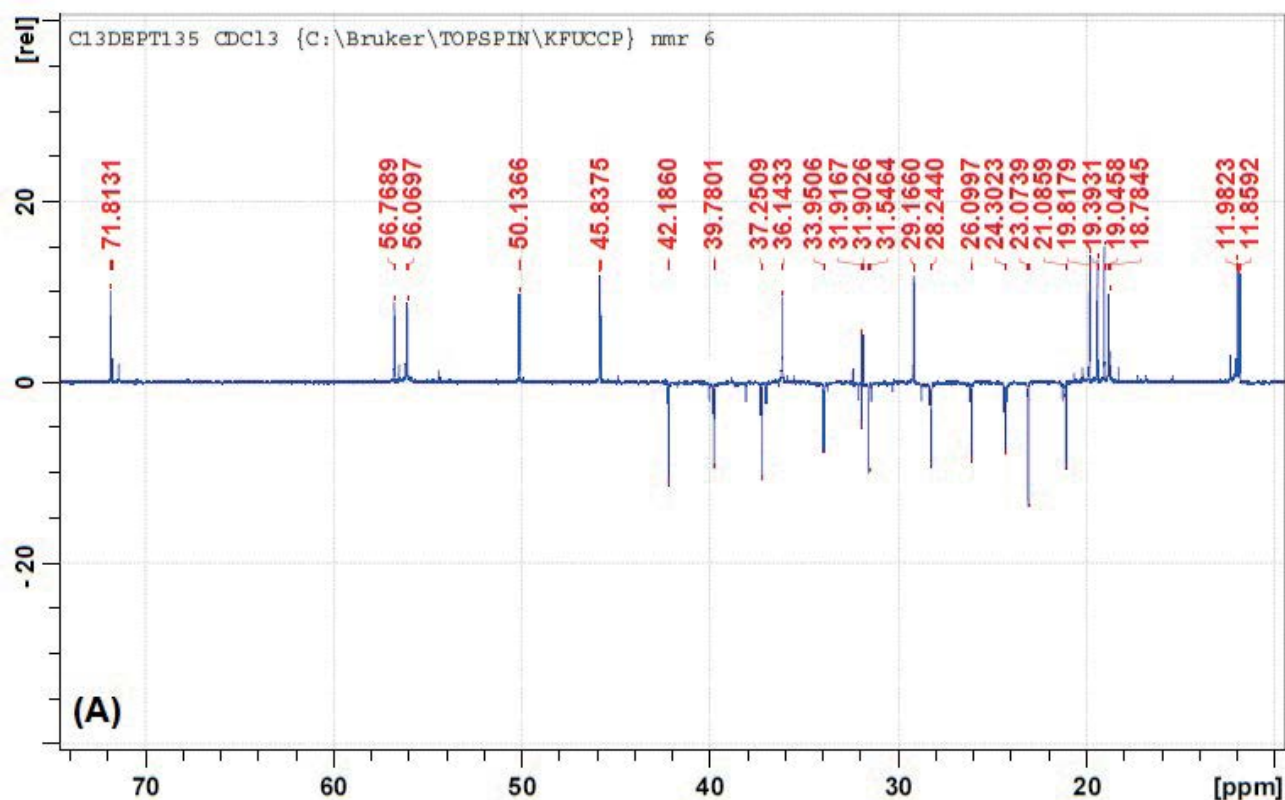


Figure 3: NMR spectral data of β -sitosterol (Cpd1); DEPT spectrum (A), HMQC spectrum (B) and HMBC spectrum (C).

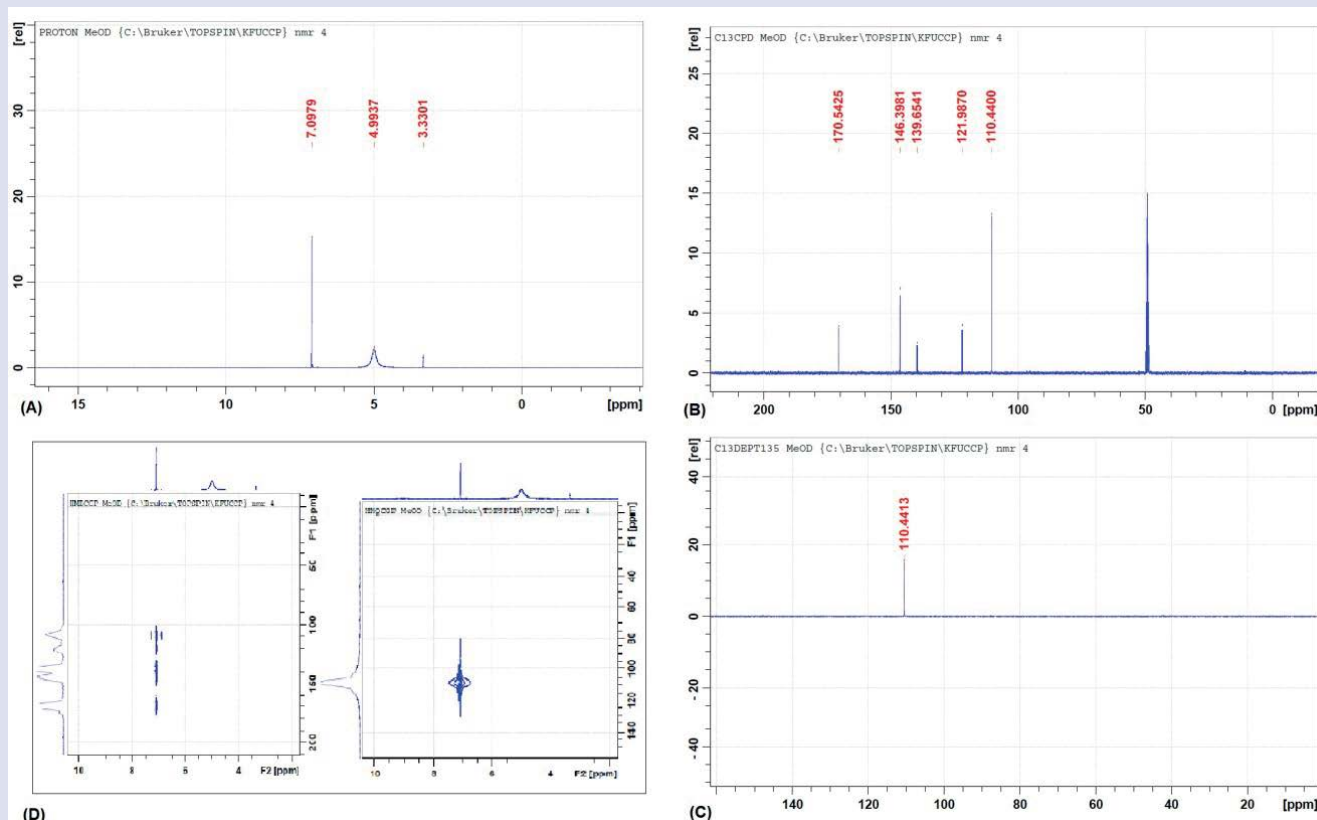


Figure 4: NMR spectral data of gallic acid (Cpd2); ¹H-NMR DEPT spectrum (A), ¹³C-NMR spectrum (B), DEPT spectrum (C) and HMQC and HMBC spectra (D).

Antioxidant activity (DPPH)

DPPH is considered as one of the most effective methods for assessment of amount of antioxidant metabolites where, the significance of sample as antioxidant is correlated with its capacity to reduce the stable violet DPPH radical to yellow DPPH-H.²⁹ TME, β -sitosterol (Cpd1) and gallic acid (Cpd2) were inspected for their radical scavenger potential using the DPPH radical scavenging assay. Gallic acid (Cpd2) exhibited significant activity (IC_{50} : 31.4 μ g/ml) while TME demonstrated marked scavenging activity (IC_{50} : 50.4 μ g/ml). This is in consistency with previous reports of *T. claveryi* (IC_{50} : 52 μ g/ml).³⁰ On the other hand, β -sitosterol (Cpd1) expressed weaker potential with IC_{50} above 100 μ g/ml. Results were comparable to the standard trolox (IC_{50} : 22.7 μ M).

Lipoxygenase inhibitory activity assay

Lipoxygenases enzymes are responsible for the conversion of polyunsaturated fatty acid into biologically active markers associated with the inflammatory and other immune responses. Lipoxygenase inhibitors are considered as promising therapeutic candidates against wide range of inflammatory-related diseases.^{31,32} IC_{50} values of lipoxygenase inhibition activity was calculated using the regression equation: $y = 0.4167x - 16.245$, $R^2 = 0.9723$ for TME, regression equation: $y = 0.0076x + 0.152$, $R^2 = 0.9066$ for gallic acid (Cpd2) and regression equation $y = 0.0063x - 0.0415$, $R^2 = 0.955$ for baicalein (positive control). Based on obtained results, the IC_{50} values were 4.59 and 0.53 μ g/ml for TME and gallic acid (Cpd2) respectively. On the other hand, β sitosterol (Cpd1) did not show activity. Results were compared with the positive control baicalein (IC_{50} : 0.27 μ g/ml).

CONCLUSION

The present investigation demonstrated the isolation of two pure constituents namely; β -sitosterol and gallic acid from *T. boudieri* of

Saudi origin for the first time. Besides, the results of this study confirm the folklore use of *T. boudieri* extract as a natural anti-inflammatory agent through significant antioxidant activity and the promising anti lipoxygenase properties of TME and the pure isolated gallic acid. Therefore, present study encourages and supports the practice of *T. boudieri* extract as functional food and nutraceuticals remedy in the pharmaceutical industry.

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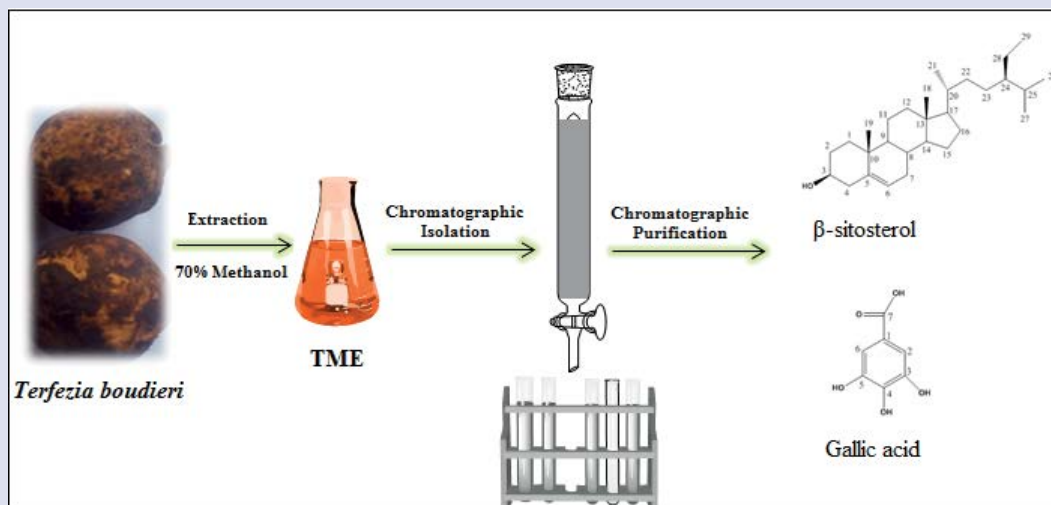
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GRAPHICAL ABSTRACT



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