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## Original Article

# Multi-method determination of Antioxidant Capacity, phytochemical and biological investigation of four different solvent extractives of *Leucophyllum frutescens* (cenizo)

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# Abstract

The Four solvent extractives obtained from aerial parts of *Leucophyllum frutescens* were evaluated for their Total Antioxidant Activity (TAA) by ammonium molybdate method, scavenging potential by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox-Equivalent Antioxidant Capacity (TEAC) assays, metal-reducing potential by Cupric Reducing Antioxidant Capacity (CUPRAC) and Ferric Reducing Antioxidant Power (FRAP) assays, Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and their biological activities. The study concluded that BULE exhibited total antioxidant activity (226.235±1.222 mg AA.Eq.$gm^{-1}$ DE±S.D) by molybdate method, CHLE exhibited more scavenging potential (DPPH 209.589±8.500 mg trolox Eq.$gm^{-1}$ DE±S.D and TEAC 210.166±7.954 mg trolox Eq.$gm^{-1}$ DE±S.D) and reducing potential (CUPRAC 646.889±16.889 mg trolox Eq.$gm^{-1}$ DE±S.D & FRAP 472.981±15.625 mg trolox Eq.$gm^{-1}$ DE±S.D). Phytochemical quantification concluded high TPC by BULE (189.369±1.393 mg GA.Eq.$gm^{-1}$ DE±S.D) and high TFC by CHLE (232.458±1.589 mg Qu.Eq.$gm^{-1}$ DE±S.D). Strong inhibition of α-glucosidase and urease enzymes was observed by HELE ($IC_{50}$ 0.3321±0.007 mg.$ml^{-1}$±SD) and BULE ($IC_{50}$ 4.09±0.357 mg.$ml^{-1}$±SD) extractives, respectively. The hemolytic effect shown by hexane extract (HELE) was higher with $HA_{50}$ 25.545±0.927 ug.$ml^{-1}$±SD whereas methanol (MELE), chloroform (CHLE), and butanol (BULE) exhibited hemolytic effects at higher concentration with $HA_{50}$ 400.067±1.364, 321.394±1.332, and 332.957±0.465 µg.$ml^{-1}$±SD, respectively. GC-MS profiling of HELE of *L. frutescens* was performed for qualitative analysis. The principal phytochemicals tentatively identified by GC-MS analysis of HELE accounts for fatty acids (60.221%), lignans (17.687%), ketones (3.358%), phenols (2.584%), sesquiterpenes (1.265%), and aldehydes (0.345%).



**Keywords:**

*Leucophyllum frutescens*, Total Phenolic Contents (TPC), Total Flavonoid Content (TFC), Total Antioxidant Activity (TAA), DPPH, CUPRAC, FRAP, gas chromatography-mass spectrometry (GC-MS)

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# 1 INTRODUCTION

Natural drug molecules are sourced from plant, animal, micro-organism, and inorganic material. The primitive treatment approach was based on witchcraft, astrology, religion, and mysticism but the theme behind the success of treatment was the use of crude drugs mostly obtained from medicinal plants. Subsequently, successful treatment was recorded, documented, leading to the knowledge of drugs i.e. basis of pharmacognosy [1]. In the back 1980s, natural products have been the main source for the discovery of new molecule entities (NMEs) [2].

*Leucophyllum frutescens* is an autotrophic, open shape; perennial shrub with a height range of 1.2 to 2.4 meters. It is distributed in Rio Grande Valley Texas, New and North Mexico, and Asia. Ethnobotanically leaves decoction has been used with mild sedative effects for treating lung congestion, bronchitis, chills, fever associated with the common cold [3], dysentery, cough, cataract, asthma, and liver injury [4]. It also has been used for treating diseases caused by *S. aureus* [5]. The literature review revealed 90 percent methanolic extract of leaves of *L. frutescens* exhibited moderate inhibition of acetylcholine esterase inhibition ($IC_{50}$ 25.27±0.102 µg.ml$^{-1}$) [3]. In another study out of the 20 medicinal plants, the aqueous extract of *L. frutescens* reduced 80% TEAC radical formation, 50% reduction in lipid oxidation, <8% alpha-amylase inhibition and was declared one of the best aqueous extracts to inhibit maltase enzyme by 32% [3]. Phytotoxicity of lignans isolated from leave extract presented with phytotoxic effects on seed germination of *Agrostis stolonifera cv. Penncross* [6]. Another study reported, methanolic extract of leaves exhibited significant hepatoprotective activity on Wistar albino rats [7].

This study aims to prepare different solvent extractives from the aerial part of *L.frutescens* for investigation of total antioxidant activity, scavenging potential (TEAC, DPPH), and reducing antioxidant capacity (CUPRAC, FRAP) along with TPC, TFC, and their biological activities. HELE presented with significant inhibition of alpha-glucosidase enzyme and hemolytic activity. Therefore HELE was subjected to tentative identification of phytochemicals by GC-MS analysis.

# 2 Materials and Methods

## 2.1 Chemicals

Ammonium acetate ($NH_4CH_3CO_2$), ferric chloride ($FeCl_3$), methanol ($CH_3OH$), absolute ethanol ($C_2H_5OH$), hexane ($C_{10}H_{22}$), chloroform ($CHCl_3$), DMSO ($C_2H_6OS$), de-ionized water ($H_2O$), hydrogen peroxide ($H_2O_2$) purchased from Merck®-Germany. 2,2-diphenyl-1-picrylhydrazyl (DPPH), alpha-glucosidase, urease, quercetin, ascorbic acid, 2,2-azinobis(3-ethylbenothiazoline) 6-sulfonic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), potassium persulfate, neocuproine, aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), gallic acid ($C_7H_6O_5$), sodium hydroxide (NaOH), sodium phosphate buffer ($NaH_2PO_4$), ammonium molybdate $(NH_4)_2$ $MoO_4$, sodium carbonate ($Na_2CO_3$), sulphuric acid ($H_2SO_4$) were purchased from Sigma Aldrich®.

## 2.2 Study approval and plant specimen identification

The present study was approved by the Advanced Studies & Research Board-the Islamia University of Bahawalpur via letter No.673/AS&RB dated Bahawalpur 16th December 2019. The

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plant as a specimen was purchased from a local nursery for identification by a taxonomist of the Herbarium Department of Botany, Faculty of the Life Sciences-The Islamia University of Bahawalpur, and its voucher number was obtained (60/botany; dated 25th September 2018).

### 2.3 Plant Collection and Preparation of different solvent extractives

200 intact plants *of L. frutescens* were purchased from the local nursery city Bahawalpur-Pakistan and grown in the private land. The aerial parts were collected during the flowering season, shade dried, and pulverized. The Ten kilograms powder was macerated with 80 percent aqueous methanol for 15 days with occasional shaking. The methanol extract was separated from marc, filtered and condensed at 40 °C using a rotary evaporator yielding MELE 270 gm dry extract (2.7 % of powdered aerial parts). The dry extract was suspended in de-ionized water and successively extracted with hexane, chloroform, and butanol yielding HELE (5.56%), CHLE (44.44%), and BULE (35.18%) of methanol dry extract (Figure 1). All the extracts were stored in hermetically sealed containers in the refrigerator for further evaluation.

### 2.4 Determination of Plant extracts Yield

The percent yield of methanol extract was calculated as:

$$\% \text{ Percent yield} = \frac{weight\ of\ extractive}{weight\ of\ powder\ or\ main\ extract}\ x\ 100$$

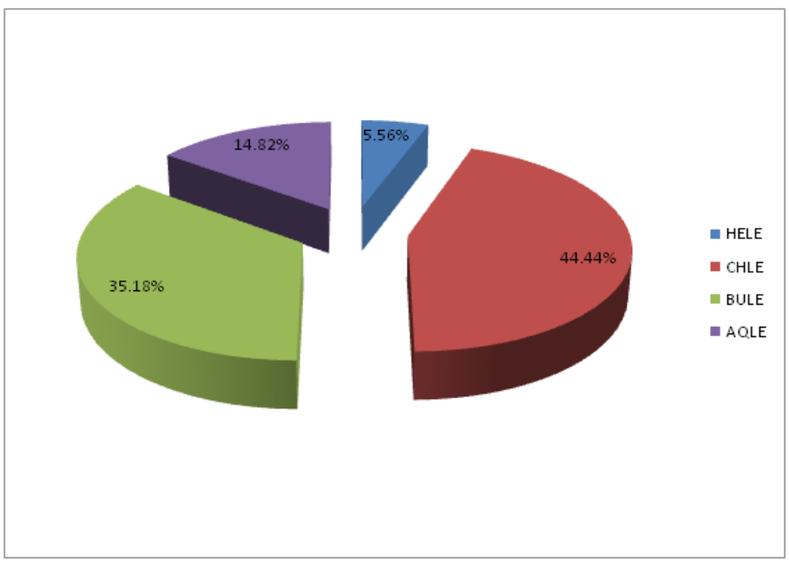


Figure 1 Percent yield of different solvent extractives from methanol extract (aerial)

### 2.5 Multi-method Estimation of antioxidant Activity

The antioxidant potential was estimated by phosphomolybdenum, DPPH, TEAC, CUPRAC, and FRAP methods.

#### 2.5.1 Total Antioxidant activity by phosphomolybdenum method

Total antioxidant activity was determined by adopting the method described in the literature with some modifications [8]. 200 µl of the extract solution (1 $mg.ml^{-1}$) was added to 2 ml eppendorf tube and mixed with 1.8 ml reagent [0.6M $H_2SO_4$ (sulphuric acid), 28 mM $NaH_2PO4$ (sodium phosphate), 4mM $(NH_4)_6MO_7O_{24}$ (ammonium molybdate)], the tube was capped and incubated in the water bath at temperature 95°C for about 90 minutes. The absorbance of the resulting solution was measured at λ 695 nm using the IRMECO U2020 UV-visible spectrophotometer. A

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calibration curve was drawn between 20-200 µg.ml$^{-1}$, and the total antioxidant activity of extracts was expressed as mg ascorbic acid equivalent per gram of dry weight of extract (mg AA.Eq.gm$^{-1}$ DE).

### 2.5.2 SCAVENGING POTENTIAL

Scavenging potential was determined by DPPH and TEAC assay (expressed in mg. trolox equivalent per gram of dried extract). Experimental procedure for DPPH [9] and TEAC [10] adopted from literature but with some modification are described below.

#### 2.5.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)

0.3127 mg.ml$^{-1}$ extract solution was prepared in methanol. 150 µl of 200 mM DPPH solution was added to the microtiter plate well followed by the addition of 50 µl extract solution. The mixture was incubated at room temperature for 30 minutes. A similar procedure was adopted for the positive control (trolox). For blank instead of extract or trolox solution, 50 µl of methanol was used. Absorbance was measured at 517nm by Biotek-Synergy HT. A calibration curve of trolox was plotted between 5-100 µg.ml$^{-1}$ for estimation of scavenging potential as mg trolox equivalent per gram of dried extract (mg trolox Eq.gm$^{-1}$ DE).

#### 2.5.2.2 Trolox Equivalent Antioxidant Capacity (TEAC)

1 ml extract solution (0.3127 mg.ml$^{-1}$) was added to a test tube containing 2 ml mixture of an equal volume of 2.5 mM 2,2-azinobis(3-ethylbenothiazoline) 6-sulfonic acid and 2.45 mM potassium persulfate, incubating test tube in the dark for 30 minutes, and measured absorbance at 734 nm. The calibration curve of trolox was plotted between 5-80 µg.ml$^{-1}$. Results were expressed in mg trolox equivalent per gram of dried extract (mg trolox Eq.gm$^{-1}$DE)

### 2.5.3 Reducing Antioxidant Potential

Reducing antioxidant potential was determined by CUPRAC (expressed mg. trolox equivalent per gram of dried extract) and FRAP (expressed mg. trolox equivalent per gram of dried extract). The procedures adopted are described below with minor modifications [10].

#### 2.5.3.1 Cupric Reducing Antioxidant Capacity (CUPRAC)

0.5 ml of extract solution (0.3127 mg.ml$^{-1}$) was mixed with a 3 ml reaction mixture of equal volumes of 10 mM $CuCl_2$, 7.5 mM neocuprion, 1M ammonium acetate buffer pH 7 incubated for 30 minutes at room temperature and measured absorbance at 450nm. A blank was prepared with methanol instead of the extract solution. The calibration curve of trolox was plotted between 2.5-100 µg.ml$^{-1}$. Results were expressed as mg trolox equivalent per gram of dried extract (mg.trolox Eq.gm$^{-1}$ of DE).

#### 2.5.3.2 Ferric Reducing Antioxidant Power (FRAP)

30 µl extract solution (0.3127 mg.ml$^{-1}$) was mixed with 2 ml reaction mixture with volume ratio 10:1:1 of 0.3 M acetate buffer pH 3.6, 20 mM ferric chloride and 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, respectively. The resultant reaction mixture was incubated for 30 minutes at room temperature. Similarly, blank was prepared without sample extract. The calibration curve for trolox was plotted between 2.5-100 µg.ml$^{-1}$. Results were presented in mg trolox equivalent per gram of dry extract (mg.trolox Eq.gm$^{-1}$ DE).

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## 2.6 Determination of Polyphenolic content

### 2.6.1 Total Phenolic Content (TPC)

Total phenolic content (TPC) of four extracts was estimated using Folin-ciocalteu (F-C) method described in the literature [11], with minor modification. Dried extract dissolved in methanol to get a stock solution 1 $mg.ml^{-1}$ and gallic acid dissolved in methanol to get its aliquots 10, 20, 40, 60, 80, 100, 200 $ug.ml^{-1}$ and calibration curve was drawn. 200 µl of extract solution or calibrators was added to 2 ml Eppendorf, 200 µl of Folin-ciocalteu reagent was added to it. The mixture was subjected to a vortex mixer followed by the addition of 0.8 ml of 700 µM sodium carbonate ($Na_2CO_3$) solution incubating at ambient temperature for 2 hours. Transferred 200µl of each assay mixture in 96 microtiter plate well and measured the absorbance at λ 765nm using Biotek-Synergy HT. Total phenolic content (TPC) of MELE, CHLE, and BULE was expressed as mg gallic acid equivalent per gram of dried extract (mg GA.Eq.$gm^{-1}$ DE).

**Note**: F-C reagent should be added before the addition of 700 µM sodium carbonate solution to avoid air oxidation of phenols.

### 2.6.2 Total Flavonoid Content (TFC)

The total flavonoid content of MELE, HELE, CHLE, and BULE was determined by adopting a method available in the literature with minor modification [12]. A mixture of 1ml extract solution (1$mg.ml^{-1}$), 4 ml deionized water, 300 µl of 5% sodium nitrite solution, and 300 µl of 10% $AlCl_3$ solution added to glass test tube followed by the addition of 2 ml 1M sodium hydroxide solution. The mixture was incubated for 6 minutes. 2.4 ml deionized water was added to the final mixture and measured the absorbance at λ 510 nm using IRMECO U2020 UV-visible spectrophotometer. The calibration curve was plotted between 50-1000 µg.$ml^{-1}$. Total flavonoid content (TFC) of dried plant extract was expressed as milligram quercetin equivalent per gram of dried extract (mg Qu.Eq.$gm^{-1}$ DE)

## 2.7 Biological investigation

### 2.7.1 Alpha-glucosidase inhibition assay

α-glucosidase inhibition assay was performed according to method described in the literature with some modification [13]. A mixture of 10 µl of enzyme solution (1U/ml), 50µl of 50 mM phosphate buffer pH 6.8, and 20 µl of aliquots of the extract was added to 96 microtiter plate well and incubated for 15 minutes at 37 ºC. The absorbance of the mixture was measured at λ 405 nm (pre-read). 20 µl of 0.5 mM solution of p-nitro-α-D-glucopyranoside was added to the mixture as substrate followed by incubation for 30 minutes at 37˚C. The absorbance was measured again at 405 nm (after-read). The same procedure was adopted for the positive control (quercetin) and negative control (methanol). The percent inhibition of α-glucosidase was calculated by using the following formula. (net absorbance = after read – pre-read)

$$\text{Percent Alpha-glucosidase inhibition} = \frac{(1-abs.of\ sample)}{abs\ of\ control}\ x\ 100$$

Where Abs stands for absorbance recorded

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### 2.7.2 Urease inhibition assay (UIA)

The anti-urease activity of different solvent extractives was estimated by the method described in the literature [14]. A mixture of 20 µl of 0.025 % urease solution prepared in 1M phosphate buffer pH 7.0 and 20 µl of extract aliquot was added to the microtiter well followed by incubation for 15 minutes at 37 C. 60µl of 2.25 % aqueous urea added to the reaction mixture and kept in an incubator for 15 minutes at 37 ˚C. Measured the absorbance at λ 630 nm (pre-read). 60 µl of phenol reagent followed by 100 µl of sodium hypochlorite solution in alkali added to the above reaction mixture, again incubated for half an hour at 37 ˚C and measured the absorbance at λ 630nm (after-read). The same procedure was adopted for all aliquots of each extractive as well as hydroxyurea as a positive control. Note (net absorbance = after read – pre-read). Percent enzyme inhibition was estimated by the following equation

$$\text{Percent urease inhibition} = \frac{(1 - abs.of\ sample)}{abs\ of\ control}\ x\ 100$$

### 2.7.3 Hemolytic activity

The study protocol was in agreement with the Helsinki Declaration. The study approval was obtained from the Pharmacy Research & Ethics committee (PREC), The Islamia University of Bahawalpur. The written informed consent was obtained from the volunteer who participated in the study.

Hemolytic activity of MELE, HELE, CHLE, and BULE was estimated by the literature method [15]. The blood was withdrawn from a healthy volunteer and added to ethylenediaminetetraacetic acid (EDTA) tube. The EDTA tube containing blood was centrifuged. The plasma portion was discarded and the red portion was subjected to three times washing with phosphate buffer pH 7.4 and suspended in phosphate buffer (50:50). Stock solutions of MELE, HELE, CHLE, and BULE were prepared by dissolving 10 mg of each dry extract in dimethyl sulfoxide (DMSO). Different aliquots of these extracts were prepared by diluting the stock solution. Then, 975 µl of each aliquot of MELE, HELE, CHLE, and BULE was added to different test tubes with successive addition of 25 µl of red blood cell (RBC) solution mixed and centrifuged at 1000 rpm at ambient temperature. 200 µl of clear supernatant was separately added to wells of 96 microtiter plate. For negative and positive control 975 µl of phosphate buffer with sodium chloride and water were added respectively. Absorbance was recorded by Biotek Synergy plate reader at λ 575nm. Percent hemolytic activity of different solvent extractives was calculated as

$$\textbf{\textit{Percent hemolytic activity}}\ (\%) = \frac{Abs_{sample} - Abs_{negative\ control}}{Abs_{Positive\ control}}\ \textbf{x 100}$$

Where Abs stands for absorbance recorded.

## 2.8 GC-MS analysis

### 2.8.1 Instrument and chromatographic conditions

Molecular metabolomics study of HELE was performed by GC-MS analysis using the J& W HP-5MS GC capillary column with dimension 30 m x 0.25 mm with particle size 0.25 µm. Helium was used as the carrier gas in constant flow mode with a flow rate of 1 $ml.min^{-1}$. The inlet

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temperature was set to 280 °C. The oven temperature ramped to 280 °C at a rate of 5 °C.min$^{-1}$ starting from 60°C and then isothermal at 280 °C for 20 min. One micro-liter of HELE extract solution was injected and the split ratio was 20:1. The MS was set to scan mode from 40-550 amu and the EI voltage was 70 eV. The transfer line temperature kept at 280 °C and the ion source and quadrupole temperature were kept at 230°C and 150 °C, respectively.

### 2.8.2 Identification of components

The mass spectrum of each separated phytochemical on specific retention time was compared with the mass spectrum stored in the database of the National Institute Standard and Technology (NIST14). The name of each phytochemical with its retention time, molecular weight, and relative percent peak area was tabulated (Table 4).

### 2.9 Statistical analysis of data

For each experimental assay, three replicate values were expressed as the mean±standard deviation (n = 3). The quantification data was subjected to one-way ANOVA by Microsoft excel version 2016 using a data analysis tool, for determination of significant difference ($p<0.05$). The linear regression equation was used for calculation of TAA, TPC, TFC, scavenging potential by DPPH and TEAC, and reducing antioxidant potential by FRAP and CUPRAC. $IC_{50}$ was also calculated through a linear regression equation using Microsoft office 2016 through a data analysis tool.

## 3 Results and Discussion

### 3.1 Multi-Method Antioxidant activities

Reactive oxygen species such as $OH^{\bullet}$, $HO2^{\bullet}$–, and $OONO^{-}$, but not $O2^{\bullet}$ are normally produced during metabolic processes, and excessive accumulation of these ROS badly affects fatty acids, DNA, and proteins causing tissue injury and inflammation [16]. Therefore to enhance the defense system, these ROS need to get detoxified or scavenged by consuming antioxidants. To the best of our knowledge, the scientific literature review does not report the antioxidant activity of different solvent extracts of *L. frutescens* from Pakistan. A Mexican study reported the antioxidant activity of ethanolic extract on red blood cells using oxidant 2,2-azo-bis-(2-amidinopropane) dihydrochloride (AAPH). According to this study ethanol sub-fraction obtained from methanol extract of roots of *L. frutescens* (HF4) showed antioxidant activity at 100 µg.ml$^{-1}$ [17]. In another study, the scavenging potential of the methanolic extract by DPPH and TEAC method was reported 280.43±4.97 µM QE/g FW and 266.20 ±5.63 µM TE/g FW) respectively.

In this study, the antioxidant potential has been estimated by total antioxidant activity using phosphomolybdenum, scavenging activity (DPPH and TEAC), and reducing potentials (CUPRAC and FRAP) methods (Figure 2). The antioxidant activity of each extract has been different from each other which is found in accordance with TPC and TFC.

Antioxidant activity by all the above-mentioned methods has been presented in Table 1. The total antioxidant activity of CHLE (226.235±1.222 mg AA.Eq.gm$^{-1}$ DE ± S.D) was recorded significantly higher ($p < 0.05$) than the total antioxidant activity of HELE (166.625±2.133 mg AA.Eq.gm$^{-1}$ DE ± S.D). Scavenging potential determined by DPPH and TEAC was found in order

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of CHLE>MELE>BULE>HELE. The results of the scavenging potential observed by DPPH and TEAC methods for each extract were not significantly different ($p > 0.05$) from each other but found significantly different ($p < 0.05$) between the extracts. CHLE reported higher scavenging potential by DPPH and TEAC (209.589± 8.500 and 210.166±7.954 mg. trolox Eq.gm$^{-1}$ DE ± S.D) compared to MELE (169.817± 2.935 and 178.109±5.179 mg. trolox Eq.gm$^{-1}$ DE ± S.D), CHLF (209.589± 8.500 and 210.166±7.954 mg. trolox Eq.gm$^{-1}$ DE ± S.D), and HELE (86.628± 9.614 and 66.941±5.364 mg. trolox Eq.gm$^{-1}$ DE ± S.D). High CUPRAC and FRAP were recorded by CHLE (646.889±16.889 and 472.981±15.625 mg. trolox Eq.gm$^{-1}$ DE ± S.D) contrary to MELE, CHLE and HELE. [18]. The results of reducing potential observed by CUPRAC and FRAP for each extract was not significantly different ($p > 0.05$) from each other but found significantly different ($p < 0.05$) between the extracts. It has been observed that scavenging and reducing potential observed by any of the extracts is directly proportional to polyphenolic content [19]. Hence the author concludes that for achieving maximum benefits CHLE should be utilized alternative to methanolic or ethanolic leave extract.

**Table 1. Determination of the antioxidant activity of four solvent extractives of *Leucophyllum frutescens (aerial)* through TAA, DPPH, TEAC, CUPRAC AND FRAP methods**

| Solvent Extract | TAA (mg AA.Eq.gm$^{-1}$ DE ± S.D) | DPPH (mg. trolox Eq.gm$^{-1}$ DE ± S.D) | TEAC (mg. trolox Eq.gm$^{-1}$ DE ± S.D) | CUPRAC (mg. trolox Eq.gm$^{-1}$ DE ± S.D) | FRAP (mg. trolox Eq.gm$^{-1}$ DE ± S.D) |
|---|---|---|---|---|---|
| MELE | 152.603±1.506$^{d}$ | 169.817± 2.935$^{b}$ | 178.109±5.179$^{b}$ | 466.222±13.556$^{b}$ | 343.219±7.202$^{b}$ |
| HELE | 166.625±2.133$^{bc}$ | 86.628± 9.614$^{d}$ | 66.941±5.364$^{d}$ | 235.111±3.778$^{c}$ | 184.891±0.488$^{c}$ |
| CHLE | 226.235±1.222$^{a}$ | 209.589± 8.500$^{a}$ | 210.166±7.954$^{a}$ | 646.889±16.889$^{a}$ | 472.981±15.625$^{a}$ |
| BULE | 194.046±1.150$^{b}$ | 155.243 ±1.518$^{c}$ | 160.166±0.925$^{c}$ | 436.445±3.3334$^{bc}$ | 358.113±20.142$^{b}$ |

**All values are mean of tripilicates vaules; AA.Eq means Ascorbic acid equivalent; $IC_{50}$ means 50 % inhibitory concentration; S.D** = standard deviation, **TAA** means total Antioxidant activity; **DPPH** means 2,2-diphenyl-1-picrylhydrazyl; **TEAC** means trolox equivalent antioxidant capacity; **CUPRAC** means cupric reducing antioxidant capacity; **FRAP** means ferric reducing antioxidant power; $^{abcd}$ Means with the different lowercase letters in the same column are significantly different ($p < 0.05$)

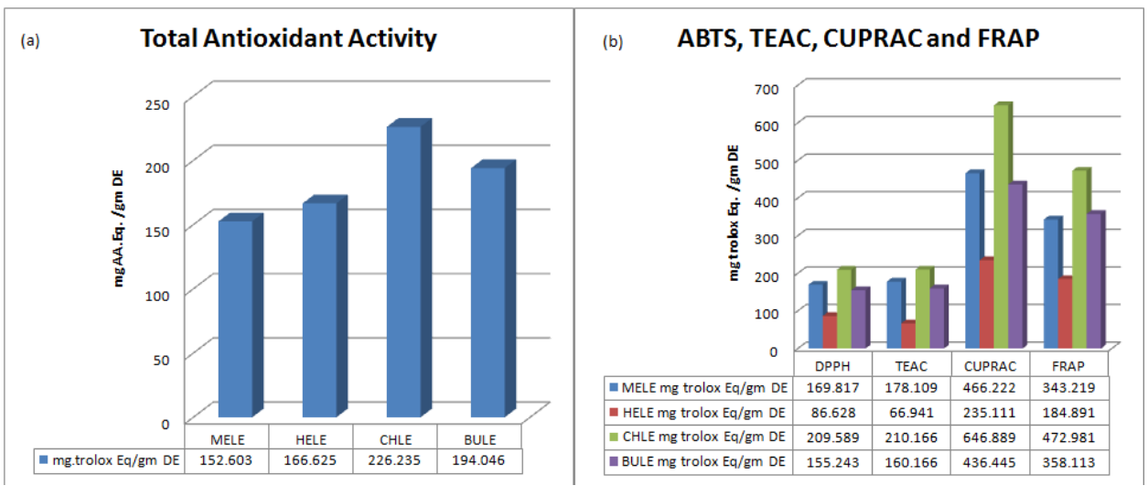


Figure 2. Graphical representation of (a) Total Antioxidant Activity (b) Antioxidant activity by DPPH, TEAC, CUPRAC, FRAP

### 3.2 Poly-phenolic contents

Polyphenols are biologically active compounds comprising chlorogenic acids, tannins, hydrolyzable tannins, and flavonoids mostly found in conjugation with sugar moieties.

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Polyphenols are considered as nutraceuticals having a variety of pharmacological effects on the body after their consumption e.g. antibacterial, antiviral, antiparasitic, antidiabetic, anticancer, and antioxidant [20].

Table 2 revealed TPC range from 2.372±0.129 to 189.369±1.393 mg GA.Eq.gm$^{-1}$ DE ± S.D. TPC values for MELE, HELE, CHLE, and BULE were calculated as 117.213±1.204, 2.372±0.129, 59.193 ± 1.122, and 189.369±1.393 mg GA.Eq.gm$^{-1}$ DE ± S.D, respectively. BULE showed the highest amount (189.369±1.393 mg GA.Eq.gm$^{-1}$ DE) of phenolic contents and HELE showed the lowest amount (2.372±0.129 GA.Eq.gm$^{-1}$ DE ± S.D) of phenolic contents. Only a single study has reported TPC of methanolic extract of stem of *L. frutescens* collected from different regions. According to this study, Sabinas Hidalgo and Apodaca stem extracts showed total phenolic contents 120.22 and 78.78 µg.mL$^{-1}$ of Gallic acid equivalent, respectively. TFC of MELE, HELE, CHLE, and BULE was determined as 135.259±1.102, 107.560±0.862, 232.458±1.589, 162.459 mg Qu.Eq.gm$^{-1}$ DE ± S.D respectively (Table 2). The highest amount of TFC was observed in CHLE and the lowest TFC was observed in HELE (Figure 3). It is concluded that the TPC of aerial parts of *L. frutescens* from the Pakistan region is not significantly different from the TPC of the stem of Spanish Sabinas Hidalgo. According to an extensive literature study, no scientific literature found reporting TPC and TFC of different solvent extractives of aerial parts of *L. frutescens* from Pakistan and rest of the Asian countries. In the present study, the results of TPC revealed that high polar solvents have more potential to extract phenolic compounds from *L. frutescens* compared to chloroform, hexane, and methanol [21].

**Table 2. Estimation of TPC AND TFC through regression equation, of four solvent extractives of *L. frutescens***

| Description | Fraction | Absorbance | B | A | $r^2$ | V (ml) | m (gm) | Amount (mg GA.Eq.gm$^{-1}$ DE ± S.D) | Amount (mg Qu.Eq.gm$^{-1}$ DE ± S.D |
|---|---|---|---|---|---|---|---|---|---|
| TPC | MELE | 1.863 | 0.0113 | 0.2078 | 0.9991 | 0.2 | 0.00025 | 117.213±1.204$^{b}$ | - |
| | HELE | 0.246 | | | | | | 2.372±0.129$^{d}$ | - |
| | CHLE | 1.041 | | | | | | 59.193±1.122$^{c}$ | - |
| | BULE | 2.863 | | | | | | 189.369±1.393$^{a}$ | - |
| TFC | MELE | 0.125 | 0.0014 | 0.0293 | 0.9892 | 1 | 0.0005 | - | 135.259±1.102$^{c}$ |
| | HELE | 0.103 | | | | | | - | 107.560±0.862$^{d}$ |
| | CHLE | 0.203 | | | | | | - | 232.458±1.589$^{a}$ |
| | BULE | 0.147 | | | | | | - | 162.459±2.638$^{b}$ |

**GA.Eq** means gallic acid equivalent**; Qu.Eq** means Quercetin equivalent**; S.D** means standard deviation**, TPC** means Total Phenolic Content**, TFC** means Total Flavonoid Content; $^{abcd}$ Means with the different lowercase letters in the same column are significantly different ($p < 0.05$)

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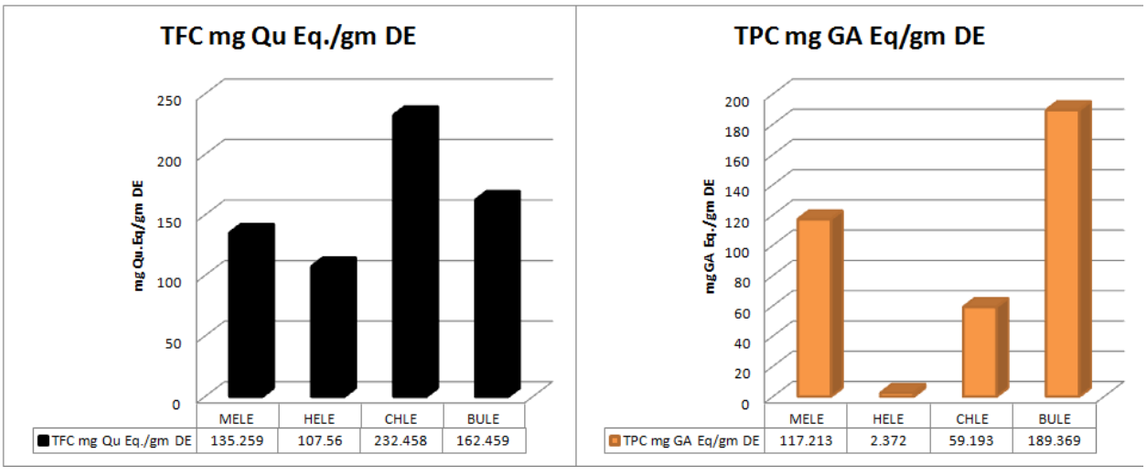


Fig 3. Graphical representation of TPC and TFC of four solvent extractives of *L. frutescens*

## 3.3 Biological Investigation

### 3.3.1 Hemolytic activity

Hemolytic activity of different concentrations of methanol (MELE), hexane (HELE), chloroform (CHLE), and butanol (BULE) extracts was determined (Table 3). Results revealed HELE showed significant ($p < 0.05$) hemolytic activity for human erythrocytes in comparison to BULE ($HA_{50}$ 332.957 µg.ml$^{-1}$) and MELE ($HA_{50}$ 400.067 µg.ml$^{-1}$), and CHLE ($HA_{50}$ 321.394 µg.ml$^{-1}$). Hemolytic effects of these polar extracts might be due to the presence of high amount of phytotoxic lignans, such as diayangambin and epiashantin [6]. GC-MS analysis of HELE validated presence of hemolytic compounds in high proportion such as methyl stearate and di-isooctyl phthalate [22].

### 3.3.2 Alpha-glucosidase inhibition

Diabetes is a chronic metabolic disorder both in low and high-income countries and is one of the leading causes of death. Among the two types of diabetes, Diabetes II is most commonly found due to insulin resistance or less amount of insulin secretion. According to an estimate by WHO about 442 million people are suffering from diabetes irrespective of the fact whether they are inhabiting low or high-income countries and about 1.6 million diabetic deaths are reported each year. Hence, for the survival of patients suffering from diabetes affordable medications including insulin is necessary. Different solvent extractives MELE, HELE, CHLE, and BULE obtained from aerial parts of *L. frutescens* were found to possess a significant amount of phenolic and flavonoid contents and therefore subjected to in-vitro anti-diabetic activity. HELE showed promising anti-diabetic activity with $IC_{50}$ 332.1±0.007µg.ml$^{-1}$ in contrast to quercetin $IC_{50}$ 8.625±0.002 µg.ml$^{-1}$ (Table 3 & Figure 4). The alpha-glucosidase inhibition activity of HELE is correlated with the presence of phytol, 9-octadecenoic acid, methyl ester, (E)-, eicosanoic acid, methyl ester, and gamma-sitosterol. The presence of these phytochemicals is validated by GC-MS analysis of HELE (Table 4).

### 3.3.3 Urease inhibition assay

In the US approximately 4.6 million people suffer peptic ulcers with 10% evidence of patients suffering from duodenal ulcers. About 70-90% of peptic ulcer and 90% duodenal ulcer is caused by helicobacter pylori. The helicobacter pylori can secrete urease extracellular, producing toxic effects on the epithelial lining of the gastric mucosa [23]. Urease converts transuded urea into

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ammonia which increases the pH of the surrounding environment resulting in a feasible environment for helicobacter pylori to grow and colonize. Four extractives obtained from the aerial part of *L. frutescens* were subjected to evaluation of anti-urease activity and it was observed that BULE exhibited maximum anti-urease activity with $IC_{50}$ 4.709 $mg.ml^{-1}$. The hydroxy urea was used as positive control and its $IC_{50}$ value was calculated as 0.960 $mg.ml^{-1}$ (Table 3).

Table 3 Alpha-glucosidase and urease inhibition assays of four solvent extractives of *L. frutescens (aerial)*

| Extract | Hemolytic activity $HA_{50}$ µg.$ml^{-1}$ | Alpha-glucosidase $IC_{50}$ mg.$ml^{-1}$ | Urease $IC_{50}$ mg.$ml^{-1}$ |
|---|---|---|---|
| MELE | 400.067±1.364[a] | - | - |
| HELE | 25.545±0.927[c] | 0.3321±0.007 | - |
| CHLE | 321.394±1.332[b] | - | 4.711±0.147[a] |
| BULE | 332.957±0.465[b] | - | 4.709±0.357[a] |
| Quercetin | N/A | 0.00863±0.002 | - |
| Hydroxy urea | N/A | - | 0.960±0.118 |

All values expressed are mean of triplicate. SD means standard deviation; $IC_{50}$ means amount which causes 50% enzyme inhibition; $HA_{50}$ means amount which causes 50% hemolytic effects; N/A means not applicable; [abcd] Means with the different lowercase letters in the same column are significantly different ($p < 0.05$)

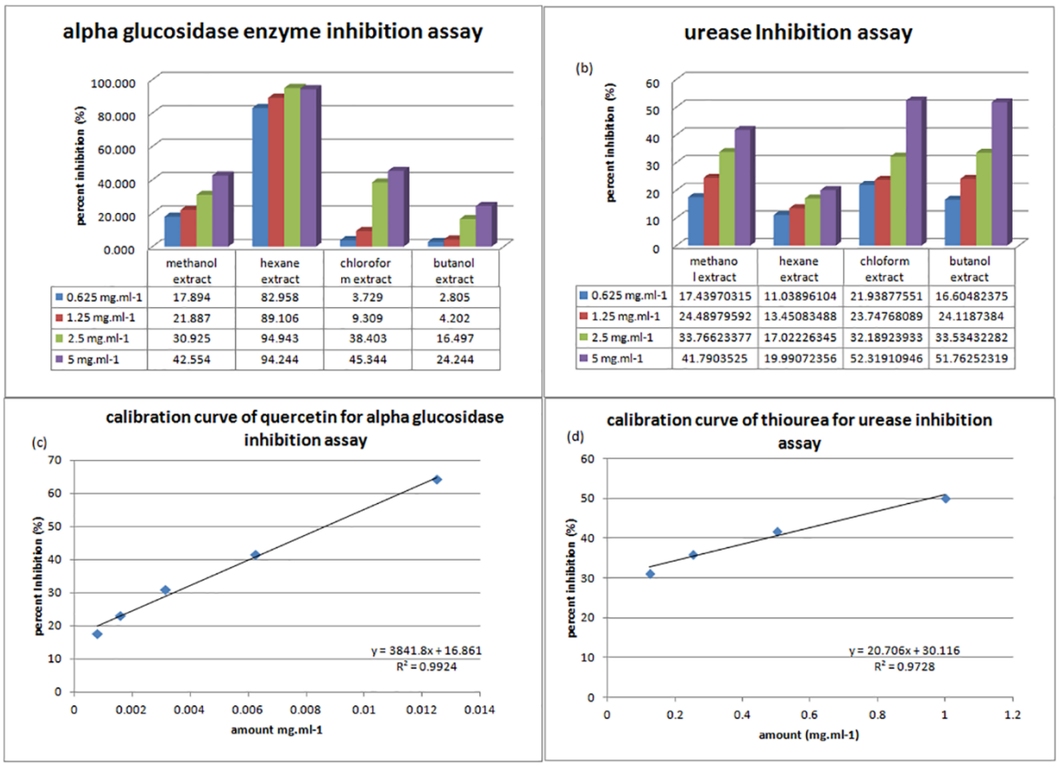


Figure 4. (a) alpha-glucosidase inhibition (b) urease enzyme inhibition (c) calibration curve of quercetin (d) calibration curve of thiourea

### 3.4 GC-MS analysis

HELE was subjected to gas-chromatography mass spectrometry (GC-MS) analysis (Figure 5) and tentatively identifying 39 different phytochemicals (Table 4). A phytochemical study revealed the presence of fatty acids, coumarin, sesquiterpenes, flavonoids, alcohols, lignans, phenols, aldehyde, and ketone, etc (Table 5). The principal leading compounds identified were 9-octadecenoic acid (Z)-, methyl ester (14.909 %), pentadecanoic acid, 14-methyl-, methyl ester (11.537 %), 2,6-

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bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0) octane (9.279 %), (+)-sesamin (7.126%), 9,12-octadecadienoic acid, methyl ester (7.848 %), 9,12,15-octadecatrienoic acid, ethyl ester, (Z,Z,Z)- (6.506 %), n-hexadecanoic acid (4.529 %), hexadecanoic acid, ethyl ester (3.745 %), phytol (2.584 %), methyl stearate (2.251%), cyclohexanone (2.036%), 1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4-dimethoxy phenyl)tetrahydro-, [1R-(1.alpha.,3a.alpha.,4.alpha.,6a.alpha.)]- (1.283 %), and squalene (1.040 %). n-hexadecanoic acid has strong antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, hemolytic inhibitor, anti-androgenic activity. [24]. Gamma-sitosterol has been reported to possess anti-diabetic and anticancerous properties [25]. Di-isooctyl phthalate showed toxic effects [22] and was experienced during shade drying, individuals living in that area suffered from a severe cough and sneezing due to the volatile components of *L. frutescens*. Alpha-amyrin and beta-amyrin exhibited an anti-inflammatory effect [26]. Methyl or ethyl ester of fatty acids also have been identified e.g. pentadecanoic acid 14-methyl-, methyl ester, 9-octadecenoic acid, methyl ester, (E)-octadecanoic acid, n-hexadecanoic acid, and tetradecanoic acid belongs to the class of fatty acids and they have been reported to possess antibacterial, antimycobacterial and low antioxidant activity [27-29]. Butanaldehyde has also been identified and scientific literature reported its antimycobacterial activity [30]. Another study conducted on roots of *L. frutescence* confirmed antimycobacterial effects against resistant strains of mycobacterium tuberculosis [31]. Leading phenolic compound such as catechol was also identified which have been reported to have antifungal activity [32]. *L. frutescens* also possess cytotoxic activity due to furofuran lignans [6] but cytotoxic fatty acid methyl stearate is also found in GC-MS analysis [29]. Vitamin E and vitamin A precursor phytol part of the chlorophyll side chain which belongs to a class of acyclic diterpene unsaturated alcohol, has been identified as a major compound with strong antidiabetic effects [33]. GC-MS analysis confirmed the presence of various pharmacologically active compounds and ascertained in Table 5.

**Table 4. Identification of bioactive phytochemicals by GC-MS screening of hexane extract of *Leucophyllum frutescence***

| Sr. No. | Retention time | Name of compound | Quality | Molecular weight | Molecular formula | Relative % peak area |
|---|---|---|---|---|---|---|
| **1.** | 4.139 | Cyclohexanone | 94 | 98.073 | $C_6H_{10}O$ | 2.037 |
| **2.** | 11.074 | Dodecane | 95 | 170.203 | $C_{12}H_{26}$ | 0.253 |
| **3.** | 13.704 | Tridecane | 97 | 184.219 | $C_{13}H_{28}$ | 0.316 |
| **4.** | 16.271 | Tetradecane | 97 | 198.235 | $C_{14}H_{30}$ | 0.426 |
| **5.** | 18.734 | Pentadecane | 96 | 212.250 | $C_{15}H_{32}$ | 0.233 |
| **6.** | 22.147 | Benzene, (1-propyloctyl)- | 95 | 232.219 | $C_{17}H_{28}$ | 0.216 |
| **7.** | 22.641 | aR-Turmerone | 97 | 216.151 | $C_{15}H_{20}O$ | 1.102 |
| **8.** | 23.186 | 2-Propenoic acid, tridecyl ester | 91 | 254.225 | $C_{16}H_{30}O_2$ | 1.013 |
| **9.** | 23.873 | Methyl tetradecanoate | 95 | 242.225 | $C_{15}H_{30}O_2$ | 0.289 |
| **10.** | 23.995 | Benzene, (1-pentylheptyl)- | 97 | 246.235 | $C_{18}H_{30}$ | 0.259 |
| **11.** | 26.266 | Neophytadiene | 89 | 278.297 | $C_{20}H_{38}$ | 0.163 |
| **12.** | 26.394 | 2-Pentadecanone, 6,10,14-trimethyl- | 99 | 268.277 | $C_{18}H_{36}O$ | 0.219 |

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| | | | | | | |
|---|---|---|---|---|---|---|
| **13.** | 28.011 | Pentadecanoic acid, 14-methyl-, methyl ester | 99 | 270.256 | $C_{17}H_{34}O_2$ | 11.537 |
| **14.** | 28.691 | n-Hexadecanoic acid | 99 | 256.24 | $C_{16}H_{32}O_2$ | 4.529 |
| **15.** | 28.8 | Heptacosane | 90 | 380.438 | $C_{27}H_{56}$ | 0.689 |
| **16.** | 29.326 | Hexadecanoic acid, ethyl ester | 99 | 284.272 | $C_{18}H_{36}O_2$ | 3.745 |
| **17.** | 31.205 | 9,12-Octadecadienoic acid, methyl ester | 99 | 294.256 | $C_{19}H_{34}O_2$ | 7.848 |
| **18.** | 31.327 | 9-Octadecenoic acid (Z)-, methyl ester | 99 | 296.272 | $C_{19}H_{36}O_2$ | 14.908 |
| **19.** | 31.533 | Phytol | 99 | 296.308 | $C_{20}H_{40}O$ | 2.584 |
| **20.** | 31.783 | Methyl stearate | 99 | 298.287 | $C_{19}H_{38}O_2$ | 2.252 |
| **21.** | 31.879 | 9,12-Octadecadienoic acid (Z,Z)- | 99 | 280.24 | $C_{18}H_{32}O_2$ | 1.505 |
| **22.** | 32.411 | 9,12-Octadecadienoic acid, ethyl ester | 99 | 308.272 | $C_{20}H_{36}O_2$ | 3.037 |
| **23.** | 32.527 | 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)- | 99 | 306.256 | $C_{20}H_{34}O_2$ | 6.506 |
| **24.** | 32.97 | Octadecanoic acid, ethyl ester | 99 | 312.303 | $C_{20}H_{40}O_2$ | 0.469 |
| **25.** | 34.753 | Heneicosane | 98 | 296.344 | $C_{21}H_{44}$ | 0.719 |
| **26.** | 34.811 | 11-Eicosenoic acid, methyl ester | 99 | 324.303 | $C_{21}H_{40}O_2$ | 0.348 |
| **27.** | 35.241 | Methyl 18-methylnonadecanoate | 98 | 326.318 | $C_{21}H_{42}O_2$ | 0.849 |
| **28.** | 38.442 | Docosanoic acid, methyl ester | 99 | 354.35 | $C_{23}H_{46}O_2$ | 0.469 |
| **29.** | 38.756 | Phthalic acid, di(2-propylpentyl) ester | 91 | 390.277 | $C_{24}H_{38}O_4$ | 0.853 |
| **30.** | 40.95 | Tricosane | 97 | 324.376 | $C_{23}H_{48}$ | 0.433 |
| **31.** | 41.412 | Tetracosanoic acid, methyl ester | 99 | 382.381 | $C_{25}H_{50}O_2$ | 0.461 |
| **32.** | 42.811 | Squalene | 99 | 410.391 | $C_{30}H_{50}$ | 1.041 |
| **33.** | 43.728 | Tetracosane | 99 | 338.391 | $C_{24}H_{50}$ | 0.411 |
| **34.** | 44.184 | Hexacosanoic acid, methyl ester | 99 | 410.412 | $C_{27}H_{54}O_2$ | 0.397 |
| **35.** | 46.121 | 2-Methyltriacontane | 95 | 436.501 | $C_{31}H_{64}$ | 0.738 |
| **36.** | 46.769 | Stigmasta-3,5-diene | 93 | 396.376 | $C_{29}H_{48}$ | 0.495 |
| **37.** | 47.654 | 2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane (asarin or D-seasamin | 96 | 354.11 | $C_{20}H_{18}O_6$ | 9.279 |
| **38.** | 47.808 | (+)-Sesamin | 99 | 354.11 | $C_{20}H_{18}O_6$ | 7.126 |
| **39.** | 48.559 | 1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4 dimethoxyphenyl)tetrahydro-, [1R-(1.alpha,3a.alpha,4.alpha,6a.alpha)]-Eudesmin | 96 | 386.173 | $C_{22}H_{26}O_6$ | 1.282 |

**Table 5. Description of class and biological activities of bioactive phytochemicals identified in GC-MS analysis of *Leucophyllum frutescens***

| Sr. No. | Compound name | Class | Biological activities |
|---|---|---|---|
| 1. | butanedioic acid, monomethyl ester | Fatty acid methyl ester | Insulin Secretagogue |

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| | | | |
|---|---|---|---|
| | | | [34] |
| 2. | 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one | Pyrans | Strong antioxidant [35], anti-proliferative and pro-apoptotic acid [36] |
| 3. | Thymol | Monoterpene | Antibacterial, antifungal, and antioxidant, anti-inflammatory, anti-nociceptive activity, vasorelaxant, anticancer [37] |
| 4. | 2-Naphthalenemethanol, decahydro-alpha, alpha, 4a-trimethyl-8-methylene-,[2R (2.alpha,4a.alpha,8a.beta.)] | Cyclic Alcohol | Hypotensive [38] |
| 5. | Methyl tetradecanoate | Fatty acid methyl ester | Antioxidant, Cancer-preventive, Hypercholesterolemic, Nematicide activities. [28] |
| 6. | 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol | Methoxy- phenol | Antioxidant, antimicrobial, and anti inflammatory [39] |
| 7. | (1R,4aR,7R,8aR)-7-(2-Hydroxypropan-2-yl)-1,4a-dimethyldecahydronaphthalen-1-ol | Cyclic alcohol | Antispasmodic [40] |
| 8. | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | Spirolactone | Diuretic and antihypertensive, anti-hirusitism, acne in female [41] |
| 9. | Pentadecanoic acid, 14-methyl-, methyl ester | Fatty acid methyl ester | Antioxidant, Antifungal, Antimicrobial activities [28] |
| 10. | 9,12-Octadecadienoic acid, methyl ester, (E,E)- | Fatty acid methyl ester | Antiinflammatory, Nematicide, Insectifuge, Hypocholesterolemic [29] |
| 11. | 9-Octadecenoic acid, methyl ester, (E)- | Fatty acid methyl ester | Inhibitor α-glucosidase [33] |
| 12. | Phytol | Acyclic diterpenoids | Antidiabetic [33] |
| 13. | Ethyl 9,12,15-octadecatrienoate | Fatty acid ethyl ester | Cell viability [42] |
| 14. | 2-Butenedioic acid (E)-, bis(2-ethylhexyl) ester | Fatty acid Ethylhexyl ester | Antidermatitic, antioxidant, anti-hepatocarcinogenic, antioxidant, antitumor, flavor [43] |
| 15. | Eicosanoic acid, methyl ester | Fatty acid methyl ester | Alpha-glucosidase inhibitor activity [28] |
| 16. | Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)- | Cyclic hydrocarbons | Antibacterial and Anticancer activity [44] |
| 17. | Diisooctyl phthalate | Benzene derivative | Toxic [22] |
| 18. | alpha-Tocospiro A and alpha-Tocospiro B | Tocopherol | Antioxidant [25] |
| 19. | gamma-Sitosterol | Phytosterol | Antidiabetic, anticancerous properties [25] |
| 20. | beta-Amyrone | Triterpene | Moderate anti-acetylcholinesterase activity, Antifungal [45] |
| 21. | alpha-Amyrin and beta-Amyrin | Triterpene | Anti-inflammatory [26] |
| 22. | Betulinaldehyde | | Antimycobacterial [30] |
| 23. | aR-Turmerone | Sesquiterpene | (acetylcholinesterase) inhibitor Antivenom, Antidepresseur, Anti-inflammatory, Neuroprotective activities. [46] [47] |

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| | | | |
|---|---|---|---|
| 24. | 2-Propenoic acid, tridecyl ester | Carboxylic acid | No activity reported 3-Eicosene, (E)- Antimicrobial, Antihyperglycemic, Cytotoxic activity, Antioxidant, Insecticidal activity [48] |
| 25. | Neophytadiene | Sesquiterpenes | analgesic, antipyretic, anti-inflammatory, antimicrobial, and antioxidant [49] |
| 26. | 9-Octadecenoic acid (Z)-, methyl ester | Fatty acid | Antimycobacterial [50] |
| 27. | Methyl stearate | Fatty acid | Cytotoxic [29] |
| 28. | 9,12-Octadecadienoic acid (Z,Z)- | Fatty acid | Anti-inflammatory, Nematicide, Insectifuge, Hypocholesterolemic, Cancer preventive, Hepato-protective, Antihistaminic, Antiacne, Antiarthritic, Antieczemic, [29] |
| 29. | Octadecanoic acid, ethyl ester | Fatty acid ethyl ester | Antimycobacterial [50] |
| 30. | Phthalic acid, di(2-propylpentyl) ester | Flavonoids | oral toxicity during pregnancy and sucking in the Long-Evans Rats [24] |
| 31. | Squalene | Triterpene | Antibacterial, antioxidant, antitumor, anticancer, immunostimulant, lipoxygenase inhibitor, [51] |
| 32. | (+)-Sesamin | Lignan | Larvicidal, anti-cholestermic, antihypertensive, antioxidant [52-54] |
| 33. | 1,2-Cyclopentanedione | Cyclic ketone | Antioxidant [55] |
| 34. | Catechol | Phenol | Antifungal [32] |
| 35. | Phenol, 2,6-dimethoxy- | Phenol | Anti-termite [56] |
| 36. | Benzoic acid | Aromatic carboxylic acid | Antifungal, antimicrobial, and preservative [57] |
| 37. | 2-Methoxy-4-vinylphenol | Methoxy phenols | Antioxidant, antimicrobial, and anti-inflammatory [58] |
| 38. | 3-Methoxy-4,5-methylenedioxybenzaldehyde | Myristin aldehyde | Anti-neoplastic activity [59] |
| 39. | (E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol | Methoxy phenol | Antioxidant, antimicrobial, and anti-inflammatory [39] |
| 40. | 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one | Essential oil | Anti-diabetes [33] |
| 41. | Oxacyclopentadecan-2-one | Macrocyclic lactone | Flavoring agent [60] |

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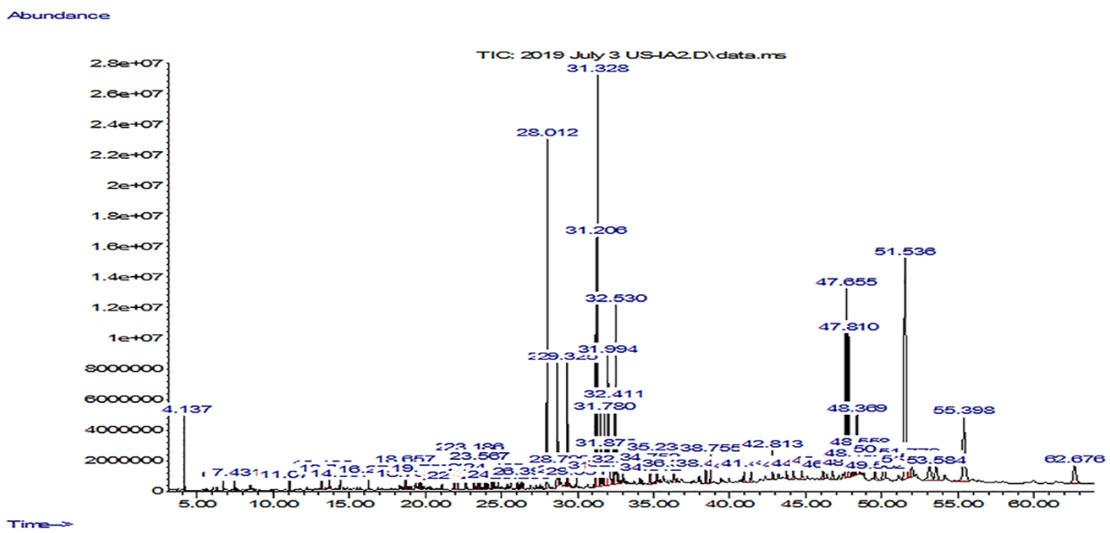


Figure 5. GC-MS chromatogram for hexane extract of aerial parts of *L. frutescence*

## 4 Conclusion

GC-MS analysis concluded that principal phytochemicals tentatively identified in HELE by GC-MS analysis belong to the class of fatty acid-ester (60.221%), lignans (17.687%), sesquiterpenes (1.265%), ketone (3.358%), aldehyde (0.345%), and phenols (2.584%). The study also concluded that different solvent extractives obtained from aerial parts of *L. frutescens* demonstrated promising enzyme inhibitory activity (HELE strongly inhibited alpha-glucosidase and CHLE inhibited urease) with a strong antioxidant activity validated by multi-method approach. Conclusively, the results obtained provide the rationale for further extensive studies on biochemical analysis as well as the pivotal therapeutic potential of *L. frutescens* through in-vivo studies essentially focusing on isolation of antioxidants, antidiabetic, antibacterial, and phytotoxic compounds.

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## 6 Conflict of interest

Authors declare that they have no competing financial, professional, or personal interests that might have influenced the performance or presentation of work described in the manuscript.

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**Data Availability statement**

All relevant data are within the manuscript.