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Research Article

GC–MS Characterization of the Ethanolic Extract of (*Ceratonia siliqua* L.) Fruit Pulp and Evaluation of Its Antibacterial Activity against *Stenotrophomonas maltophilia*

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Abstract

Background: *Ceratonia siliqua* contains compounds with potential antioxidant and antibacterial activities. **Objective:** This study aims to characterize the ethanolic extract of *Ceratonia siliqua* and to evaluate its DPPH radical scavenging activity and antibacterial activity against *Stenotrophomonas maltophilia*. **Methodology:** The methods included microwave assisted ethanolic extraction, GC–MS profiling and DPPH testing. Antibacterial activity was assessed using broth growth inhibition and agar well diffusion assays against *S. maltophilia* ATCC 13637. **Results:** Light brown viscous extract was obtained 18.0 % or 1.80g from 10.00g dried pulp. Some tentative GC–MS assignments indicated 1-methyl-5-fluorouracil (4.49%), a sulfur containing disulfide (3.08%), octamethylcyclotetrasiloxane (2.77%), oleic acid (2.34%) and n-hexadecanoic acid (2.02%). The DPPH scavenging ability of the extract rose from 14.63% at 25 µg/mL to 89.02% at 800 µg/mL with reported IC_{50} value of 132.0 µg/mL, while the estimated IC_{50} for ascorbic acid was 1.20 µg/mL. With a different dose range, comparator scavenging was also increased, ranging from 12.59% at 0.25 µg/mL to 92.33% at 8 µg/mL. Inhibition reached 74.00%, 81.68% and 82.17% at 64, 128 and 256 mg/mL, respectively. Growth inhibition was similar and ranged from 128 to 256 mg/mL only 0.49 percentage points apart. The small increase at the two highest concentrations indicates an apparent plateau; these measurements do not establish complete growth inhibition or bactericidal activity. **Conclusion:** This study represents the first approach to investigating ethanolic *Ceratonia siliqua* extract within a complete framework of a combined GC–MS profile and biological study.

1. Introduction

Considering the problems of bacterial resistance and the chemical diversity of plant secondary metabolites, the interest in an integrated analytical and biological assessment stems from these factors. Botanical extracts have phenolics, tannins and terpenoids that may interfere with the activity of microbes' membranes, proteins or enzymes and could, therefore, be useful as starting materials in in vitro testing but not yet as tested therapeutic alternatives [1]. For this investigation (*Ceratonia siliqua* L.) is a relevant plant matrix. Sugar content is high in its edible pulp, which also has a variety of polyphenols. The contents of the polyphenols vary with botanical source of the fruit, maturity and processing [2]. These characteristics warrant studying a defined pulp extract and the chemical constituents of the pulp in particular instead of seeds and other tissues. Chromatographic studies on carob fruits and products derived from these have shown that *Ceratonia siliqua*

fruit has a heterogeneous phenolic fraction containing tannins, flavonol glycosides and gallic acid [3]. But the extracted compounds are highly dependent on the extraction procedure. A study was done to make a comparison among different solvents of which ethanol is one and it was observed that the solvent composition, temperature, extraction time and loading of sample affected the recovery of phenolics from *Ceratonia siliqua* and ethanol should not be assumed to be the optimal solvent [4]. Higher levels of phenolics in separated pulp compared with commercial products were found in more recent profiling of both the separated pulp and commercial products and the effects of both geographical origin and thermal treatment were revealed [5]. Therefore, when correlating chemical profile to biological response, so must be preparation conditions.

There are some encouraging pieces of evidence in support of CPI to be analysed at an experimental level for antibacterial studies. Aqueous pulp extract was found to be active towards Bacillus subtilis and Klebsiella pneumoniae in agar diffusion testing while the ethanolic preparation was not found to be active towards either of the organisms, giving experimental evidence for the bacterial growth inhibition in case of aqueous extract of pulp [6]. Phenolic profiling of ***Ceratonia siliqua*** and carob gum macerates, together with antimicrobial screening, confirmed the importance of the study of these macerates and maintaining the distinction between both matrices [7]. Both antioxidant values and the presence of the same phenolics cannot be used separately to establish antibacterial activity, and activity against one bacterial species cannot be assumed for another. The fraction of an ethanolic extract that is volatile and thermally stable enough for GC to be performed can be characterized by GC–MS. Derivatisation can be used with the sample to allow detection of otherwise poorly volatile metabolites, but does not exhaustive coverage of a complex plant sample [8]. This may be especially pertinent when considering polyphenols as this class of compounds may necessitate additional analytical methods for the identification of larger or thermolabile analytes. In addition, the matches to the spectral library cannot be equated with confirmed identifications of compounds. Metabolomics reporting standards are geared toward full disclosure of sample preparation, conditions of analysis, quality control measures, and degree of confidence of identification [9]. Thus, chemical characterization needs to be carried out as an aid to, and in conjunction with, an independent biological assessment.

This evaluation can be done using a clinically relevant target, *Stenotrophomonas maltophilia*. This opportunistic pathogen has been found in respiratory and bloodstream infections, especially in immunocompromised patients and those who require intensive medical care without necessarily implying infection [10]. This is because it harbours resistance mechanisma such as chromosomal efflux systems, in addition to antibiotic inactivation, and acquired and adaptive resistance processes [11]. These properties make the assessment of antibacterial activity difficult and are a reason to conduct organism-specific testing of the candidate extracts. In addition to persistence and antibiotic tolerance, conventional planktonic susceptibility measurements do not necessarily reflect the phenomenon of biofilm formation [12]. From the prospective ANSELM study, it was seen that the formation of biofilm was observed to be high for the clinical isolates and that the biofilms that were formed by the clinical isolates were tolerant to the selected antibiotics under laboratory conditions [13]. Also, observational data from a meta-analysis of bacteremia studies found an association between inappropriate antimicrobial therapy and increased mortality, but this does not prove causation [14]. Controlled assays and endpoints are essential for reliable evaluation of extract, as diffusion zones cannot be compared to growth inhibition, or to be a comparable measure of potency, to killing the bacteria [15]. All of these factors contribute to the possibility of researching the *Ceratonia siliqua* via an integrated approach using chemical and microbiological methods and drawing conclusions on the basis of the specific experimental conditions examined.

The question is whether the extract of the CP prepared in ethanol and its other chemical entities as detected from the bio-efficacy experiment can inhibit *Stenotrophomonas maltophilia*, while the study problem is whether or not a defined ethanolic extract of the CP can inhibit *Stenotrophomonas maltophilia*. There is no current evidence available for other preparations or bacterial species to answer this question for the preparation being studied. For this purpose, a defined ethanolic extract of the carob fruit pulp (CFP) was assessed for its inhibitory activity against *S. maltophilia* and the GC–MS detectable constituents of the extract were characterized. In this study GC-MS profiling was performed followed by in vitro anti-oxidant and anti-bacterial assays to assess the tested preparation and its potential for further investigation.

2. Methodology

2.1. Collection and preparation of *Ceratonia siliqua*

Mature pods of *Ceratonia siliqua* L. were collected from three trees producing mature pods in the district of Abu Al-Khaseeb in Basra Governorate, Iraq and identified by a botanist from College of Science, University of Basra, Iraq. Pods were washed, deseeded, drained and peduncles were removed using distilled water. The pulp was approximately 1cm in size, dried at 40°C, mechanically ground and sieved through a 500µ sieve. The powder was kept in amber glass containers with a screwtop cap, at 4°C.

2.2. Microwave-assisted ethanolic extraction

The ethanolic extract was obtained using microwave-assisted extraction. Ten grams of powdered *Ceratonia siliqua* were placed in a conical flask containing 100 mL of 99% ethanol. Extraction was then performed using a dedicated microwave-assisted extraction system (model DKW-10, manufactured by Daewoo, South Korea) for 1–2 minutes at a temperature of 50°C [16]. The extract was left to cool for 10–20 minutes, then subjected to centrifugation at 3000 rpm for 15 minutes, and subsequently filtered using Whatman filter paper with a nominal pore size of 20 µm. The resulting liquid was then dried at room temperature (25°C) to ensure the complete removal of the ethanol. Finally, the crude extract was weighed prior to analysis.

2.3. Chemical profiling by GC–MS

The extract was subjected to analysis with a gas chromatograph (Agilent 7890B) in conjunction with a mass selective detector (Agilent 5977A) and operated with MassHunter software. The separation was on a 5% phenyl methyl siloxane column at 6.0799 psi. Initially, the oven was programmed to 40°C, and then ramping from 50 to 280°C at 10°C/min. Helium was used at 1 mL/min and temperature of the injector was 290°C with 4 min of the solvent delay. An aliquot (1 µL) was injected in pulsed splitless mode and spectra recorded in the scan range of m/z 35–650. Some evaluation of compounds was carried out by comparison with the NIST mass spectral library or by tentative

identification [17].

2.4. Antibacterial activity

Antibacterial activity was tested by broth growth-inhibition and agar well diffusion methods against *Stenotrophomonas maltophilia* ATCC13637. A new preparation of bacteria was used and was made to 0.5 McFarland standard, then diluted in double strength cation adjusted Mueller–Hinton broth. Dried extract was dissolved in 2% DMSO/saline and used at two times the desired concentration. The volumes of extract preparation and inoculum to be added into each of the microplate-wells were each 50 µL with the final extract concentrations ranging between 0.125 mg/mL and 256 mg/mL, full-strength broth and 1% DMSO and the inoculum density was 5 ± 10^5 CFU/mL. Plates were incubated at 35 ± 2°C for 24 h. Before and after incubation OD600 was measured. Growth inhibition was expressed as 100 × (1 - Gtest/Gcontrol), where G is the net growth obtained after deducting the respective uninoculated blank reading from the growth and the initial corrected reading. In addition, growth, vehicle controls and extract blanks at the same concentrations were added. The 0.5 McFarland saline suspension was uniformly applied to Mueller–Hinton agar plates, with a thickness of about 4 mm. Extract solution at 0.125 – 256 mg/mL was applied to a central 6 mm well. Plates were incubated at 35 ± 2°C for 24 h. Zone diameters were measured using digital callipers in two directions perpendicular to each other and the average was taken. Positive diameters were the well and the diffusion diameters and the percentage inhibition in the broth were reported independently [18, 19].

2.5. DPPH radical-scavenging assay

The antioxidant activity was assessed on the basis of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay based on the published procedure [20]. Equal volumes (1.5 mL) of extract dilutions and freshly prepared 0.06 mM DPPH in ethanol were mixed and kept in the dark for 30 min at room temperature. Absorbance was measured at 517 nm. Solvent and extract-only blanks and ascorbic acid as a positive control were added. The scavenging rate (%) was determined as 100 × (Acontrol - Asample)/Acontrol with Asample corrected by extract only blank absorbance, and Acontrol (absorbance of DPPH with solvent). Triplicates were done on measurements.

2.6. Statistical analysis

Descriptive tables were created for summarizing the data using IBM SPSS Statistics version (IBM Corp., Armonk, NY, USA). The DPPH scavenging, bacterial growth inhibition and inhibition-zone diameter were expressed as mean ± standard deviation (SD) for each concentration. The n = 3 mentioned in these summaries are technical readings/measurements not biological replicates on each concentration. IC_{50} values of carob extract and ascorbic acid were determined using linear log10-scale interpolations between the two mean values of the PPM response curve which were below and above 50%.

3. Results

3.1. Extract appearance and extraction yield

Ethanolic of *Ceratonia siliqua* extract was light brown in color, viscous in nature. The amount of dried crude extract (1.80 g) isolated from the dried *Ceratonia siliqua* was 18.0% of the extraction yield. The yield was expressed as mass of dry extract/mass of dry pulp × 100. The volume of extraction solvent 99% ethanol was 100mL and microwave irradiation conducted for 1 minute at 50°C and centrifuged and filtered. The results relate to the short-term irradiation time that corresponds to the fast active-heating phase, and to the yield which corresponds to the crude-extract recovery. The filtrate after the filtration was dried at 25°C to remove ethanol. Before analysis the crude extract produced was weighed. These observations are made with respect to short-duration microwave treatments and material recovery; they do not establish comparative energy efficiency or superiority to conventional extraction.

3.2. GC–MS profile of the extract

A total of 109 integrated peak entries were obtained from 4.134 to 35.870 min of the retention time from the GC – MS analysis Table 1. The sum of all TIC-area percentages is about 100% of the total reported. The largest integrated table entry was at 6.587 min with a normalized TIC area of 11.52%. The percentages of the normalized integrated TIC area for the oleic-acid and n-hexadecanoic-acid annotations were 2.34% and 2.02%, respectively. The chromatogram shows several signals, such as the high-signal peaks around 6.585, 22.854 and 24.535 minutes Figure 1. This dominant signal near 6.585 min is verified by the table entry at 6.587 min, but is not an independent confirmation of the identity of the compound.

Table 1: GC–MS peak list and tentative library assignments for the ethanolic *Ceratonia siliqua* extract

Name	Formula	RT	Area	Base Peak	TIC% Area
Silanol, dimethyl-	C_2H_8OSi	4.134	379487	61.02	0.402279
2-Amino-2-methyl-1,3-propanediol	$C_4H_{11}NO_2$	4.278	767150	74.01	0.813225
Ethanol, 2-(butylamino)-	$C_6H_{15}NO$	4.685	309536	86.01	0.328127
Benzenemethanol, .alpha.-[1-(ethylmethylamino)ethyl]-, [R-(R*,S*)]-	$C_{12}H_{19}NO$	4.764	506108	86.01	0.536505
Phenacylidene diacetate	$C_{12}H_{12}O_5$	5.185	366281	104.99	0.38828
2-Propenoic acid, ethenyl ester	$C_5H_6O_2$	5.363	190990	55.05	0.202461
2-Propenoic acid, ethenyl ester	$C_5H_6O_2$	5.481	1565071	55.05	1.659069
2-Propenoic acid, ethenyl ester	$C_5H_6O_2$	5.52	4234588	55.05	4.488916
Propanoic acid, ethyl ester	$C_5H_{10}O_2$	5.821	313233	57.04	0.332046
l-Alanine, N-methoxycarbonyl-, ethyl ester	$C_7H_{13}NO_4$	6.233	392464	102	0.416035
Propanoic acid, butyl ester	$C_7H_{14}O_2$	6.341	1642907	57.04	1.74158
Ethanol, 2-(trimethylsilyl)-	$C_5H_{14}OSi$	6.587	10871142	75.03	11.52406
Cyclobutane, 1,2:3,4-di-O-ethylboranediyl-	$C_8H_{14}B_2O_4$	6.854	172499	98.01	0.182859
Silanol, ethyldimethyl-	$C_4H_{12}OSi$	7.021	282421	75.03	0.299383
Silanol, ethyldimethyl-	$C_4H_{12}OSi$	7.091	234003	75.03	0.248057
Silanol, ethyldimethyl-	$C_4H_{12}OSi$	7.209	4014520	75.03	4.255631
Methylenecyclopropanecarboxylic acid	$C_5H_6O_2$	7.613	1669693	98.01	1.769974
1-Methyl-2-(1-piperidinyl)ethylamine, TMS derivative	$C_{11}H_{26}N_2Si$	7.87	120793	86.01	0.128048
Benzaldehyde diethylacetal	$C_{11}H_{16}O_2$	8.028	196478	55.05	0.208278
Ethanamine, 2-methoxy-N-(2-methoxyethyl) -N-methyl-	$C_7H_{17}NO_2$	8.087	2916395	102	3.091553
4-Cyclopentene-1,3-dione	$C_5H_4O_2$	8.181	902217	96	0.956404
1-Penten-3-one	C_5H_8O	8.507	554642	55.05	0.587954
Propanoic acid, propyl ester	$C_6H_{12}O_2$	8.575	1272529	75.03	1.348957
2(5H)-Furanone	$C_4H_4O_2$	8.811	571672	55.05	0.606006
1,2-Cyclopentanedione	$C_5H_6O_2$	9.008	2218511	98.01	2.351754
Silicon tetrafluoride	F_4Si	9.094	488921	85.01	0.518285
Cyclobutanecarboxylic acid, cyclopentyl ester	$C_{10}H_{16}O_2$	9.36	135626	68.04	0.143772
Silane, triethylmethoxy-	$C_7H_{18}OSi$	9.518	142506	116.98	0.151065
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	10.013	2155269	101	2.284714
Phenacylidene diacetate	$C_{12}H_{12}O_5$	10.244	440516	77.02	0.466973
2H-Pyran-2,6(3H)-dione	$C_5H_4O_3$	10.335	292234	55.05	0.309786
Maleic hydrazide	$C_4H_4N_2O_2$	10.41	1546841	58.04	1.639744
Cyclopentanecarboxylic acid, 2-fluorophenyl ester	$C_{12}H_{13}FO_2$	10.472	129571	112	0.137353
2-(Fluoromethyl)pyrrolidine	$C_5H_{10}FN$	10.794	234053	75.03	0.24811
2-Cyclopenten-1-one,3-hydroxy-2-methyl-	$C_6H_8O_2$	10.944	216725	112	0.229741
4-Methoxybenzoic acid, 2,3-dichlorophenyl ester	$C_{14}H_{10}Cl_2O_3$	11.104	191485	135.06	0.202986
Benzeneacetaldehyde	C_8H_8O	11.22	202264	91.04	0.214412
1,3-Dioxepane, 2-heptyl-	$C_{12}H_{24}O_2$	11.293	451029	55.05	0.478118
Nonane, 4,5-dimethyl-	$C_{11}H_{24}$	11.37	131828	71.06	0.139746
Pentanoic acid, 2-methyl-, ethyl ester	$C_8H_{16}O_2$	11.469	656336	61.02	0.695755
Furaneol	$C_6H_8O_3$	11.552	672496	128.01	0.712886
Thymine	$C_5H_6N_2O_2$	11.908	1596630	126	1.692523
Hexane, 3,4-bis(1,1-dimethylethyl)-2,2,5,5-tetramethyl-	$C_{18}H_{38}$	12.138	711380	57.04	0.754105
4-(Piperazin-1-ylsulfonyl)morpholine, N-acetyl	$C_{10}H_{19}N_3O_4S$	12.686	273076	85.01	0.289477
Methyl 2-[methoxy(methyl)amino]-2-methylpropanoate	$C_7H_{15}NO_3$	12.874	1497113	102	1.587029
1-Methyl-5-fluorouracil	$C_5H_5FN_2O_2$	13.014	4234341	144.01	4.488655
(S)-2-((S)-Pyrrolidine-2-carboxamido)pentanedioic acid, 3TMS	$C_{19}H_{40}N_2O_5Si_3$	13.245	109283	142.01	0.115847
Benzoic acid	$C_7H_6O_2$	13.27	267428	104.99	0.28349
N,4-Diethyl-4-heptanamine	$C_{11}H_{25}N$	13.632	361028	142.01	0.382711
5-(Hydroxymethyl)dihydrofuran-2(3H)-one	$C_5H_8O_3$	13.749	612741	85.01	0.649542
Catechol	$C_6H_6O_2$	13.868	736586	110.02	0.780825
Benzaldehyde, 3-ethyl-	$C_9H_{10}O$	14.09	247809	133.02	0.262692

Table 1 Continues

Name	Formula	RT	Area	Base Peak	TIC% Area
(1-Methoxy-pentyl)-cyclopropane	$C_9H_{18}O$	14.213	833443	85.01	0.883499
Furandimethanol	$C_6H_8O_3$	14.284	826781	97.02	0.876437
Adipicacid 3-methyl-1,5-pentandiol diester	$C_{12}H_{20}O_4$	14.358	471619	83.06	0.499944
Disulfide, propyl 1-(propylthio)ethyl	$C_8H_{18}S_3$	14.585	2905504	103.02	3.080008
1,2-Benzenediol, 3-methyl-	$C_7H_8O_2$	14.765	540782	124	0.573261
Nonane, 4,5-dimethyl-	$C_{11}H_{24}$	14.898	298268	85.01	0.316182
Ethane, iodo-	C_2H_5I	14.983	164078	156.02	0.173932
Ethanone, 1-(2,5-dihydroxyphenyl)-	$C_8H_8O_3$	15.004	123122	137.02	0.130517
Spiro(3.5)silanonanone	$C_8H_{16}Si$	15.173	484874	57.04	0.513995
l-Norvaline, N-ethoxycarbonyl-, heptyl ester	$C_{15}H_{29}NO_4$	15.287	1647472	55.05	1.746419
Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	15.471	334728	73.02	0.354832
1,2,4-Thiadiazole-3,5-diamine	$C_2H_4N_4S$	15.817	177972	73.02	0.188661
2,3-Oxiranedicarboxylic acid, 2-methyl-, dimethyl ester	$C_7H_{10}O_5$	15.974	601041	115.01	0.637139
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	$C_{10}H_{14}N_2$	16.082	124861	84	0.13236
Cyclobutanone, 4-isopropylidene-2,2,3,3-tetramethyl-	$C_{11}H_{18}O$	16.429	216656	55.05	0.229668
Nonane, 2,2,4,4,6,8,8-heptamethyl-	$C_{16}H_{34}$	16.529	509881	57.04	0.540504
2,2-Dimethylhexyl methylphosphonofluoridate	$C_9H_{20}FO_2P$	16.697	195266	57.04	0.206994
Cyclohexane, octyl-	$C_{14}H_{28}$	17.215	339071	83.06	0.359436
3-Ethyl-2,6,10-trimethylundecane	$C_{16}H_{34}$	17.327	371718	57.04	0.394043
Ethanone, 1-(2,4,6-trihydroxyphenyl)-	$C_8H_8O_4$	17.514	132055	69.05	0.139986
Dodecane, 1-iodo-	$C_{12}H_{25}I$	17.684	285770	71.06	0.302933
Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	17.728	115385	73.02	0.122315
Dodecane, 1-iodo-	$C_{12}H_{25}I$	17.766	579819	71.06	0.614643
Propane-1,1-diol dipropanoate	$C_9H_{16}O_4$	17.8	176229	57.04	0.186813
Alanine, N-methyl-N-ethoxycarbonyl-, octadecyl ester	$C_{25}H_{49}NO_4$	17.913	417123	130	0.442175
Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	$C_{19}H_{30}O_3$	18.041	520351	191	0.551603
3-Methylbut-2-enoic acid, 3-methylbut-2-yl ester	$C_{10}H_{18}O_2$	18.094	276164	83.06	0.29275
4,4-Dimethyl-2,5-dioxo-1-vinylimidazolidine	$C_7H_{10}N_2O_2$	18.31	588720	138.97	0.624078
Hexadecane	$C_{16}H_{34}$	18.996	310562	57.04	0.329214
Diethyl Phthalate	$C_{12}H_{14}O_4$	19.082	109477	149.02	0.116052
n-Butyric acid 2-ethylhexyl ester	$C_{12}H_{24}O_2$	19.416	1242475	71.06	1.317098
5,5-Dibutylnonane	$C_{17}H_{36}$	19.556	218184	57.04	0.231288
Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	19.715	218858	73.02	0.232003
Decane, 3-ethyl-3-methyl-	$C_{13}H_{28}$	19.99	198749	85.01	0.210686
5-Nonanol, trimethylacetate	$C_{14}H_{28}O_2$	20.125	1782852	87.03	1.88993
Dodecane, 1-iodo-	$C_{12}H_{25}I$	20.263	266922	71.06	0.282953
Dodecane, 1-iodo-	$C_{12}H_{25}I$	20.732	138438	71.06	0.146753
Tetradecanoic acid	$C_{14}H_{28}O_2$	20.799	151270	73.02	0.160355
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2, 8-dione	$C_{17}H_{24}O_3$	22.606	123039	205.03	0.130429
Palmitoleic acid	$C_{16}H_{30}O_2$	22.678	203889	55.05	0.216135
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	22.855	1902748	73.02	2.017027
Phthalic acid, pentyl trans-dec-3-enyl ester	$C_{23}H_{34}O_4$	22.984	127352	149.02	0.135001
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	23.166	518153	88	0.549273
1,5-Cyclooctadiene, 1,2-dimethyl-	$C_{10}H_{16}$	24.496	259189	67.02	0.274756
Oleic Acid	$C_{18}H_{34}O_2$	24.535	2208408	55.05	2.341044
Octadecanoic acid	$C_{18}H_{36}O_2$	24.718	274652	73.02	0.291148
Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	24.748	142782	67.02	0.151357
(E)-9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	24.788	446373	55.05	0.473182
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	27.656	207610	98.01	0.220079
9,17-Octadecadienal, (Z)-	$C_{18}H_{32}O$	29.045	485036	55.05	0.514167
Isophthalic acid, 2-formylphenyl propyl ester	$C_{18}H_{16}O_5$	31.04	506324	253.01	0.536734
Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	31.141	2609900	281.03	2.76665
3(2H)-Benzofuranone, 6-methoxy-2-[(2-methoxyphenyl)methylene]-, (E)-	$C_{17}H_{14}O_4$	31.196	830163	282.04	0.880022
4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, 2TMS derivative	$C_{24}H_{36}O_2Si_2$	31.32	3614975	207.02	3.83209
4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, 2TMS derivative	$C_{24}H_{36}O_2Si_2$	31.41	3901480	207.02	4.135802
5.alpha.-Stigmast-9(11)-en-3.beta.-ol	$C_{29}H_{50}O$	34.599	202419	414.15	0.214576
2-(4′-Methoxyphenyl)-2-(3′-methyl-4′methoxyphenyl) propane	$C_{18}H_{22}O_2$	35.87	1472826	255.02	1.561284
			94334303		100

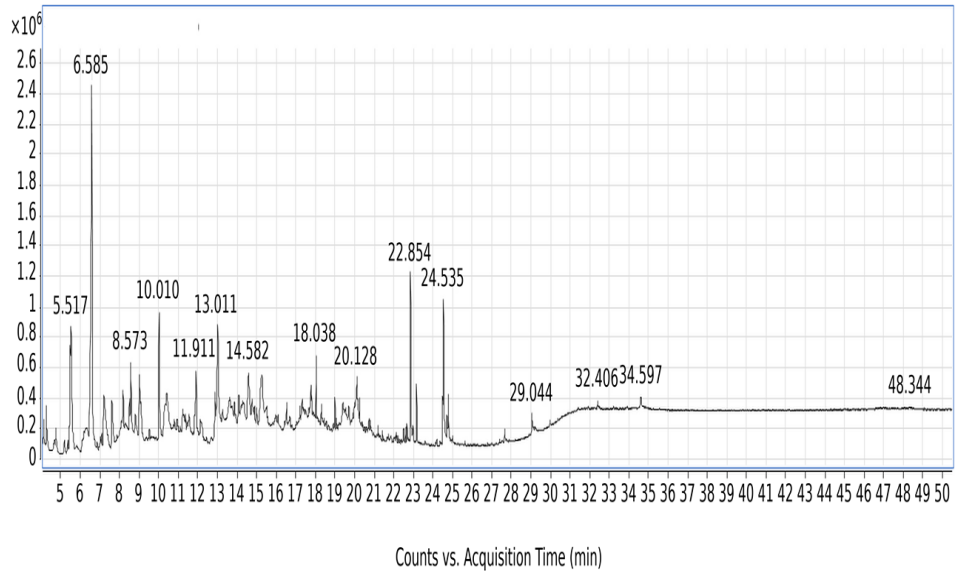


Figure 1: GC–MS total ion chromatogram of the ethanolic *Ceratonia siliqua* extract

3.3. DPPH radical-scavenging activity

According to the DPPH dataset, there is a concentration dependent radical scavenging ability of the ethanolic extract of *Ceratonia siliqua* Table 2; Figure 2A. Activity rises from 14.63 ± 1.14% at 25 µg/mL to 60.70 ± 1.79% at 200 µg/mL, reaching 89.02 ± 1.85% at 800 µg/mL. Smaller increases in scavenging activity at higher concentrations can be explained by a flattening out of the DPPH response. Ascorbic acid is presented in a separate dose range, increasing from 12.59 ± 0.77% at 0.25 µg/mL to 92.33 ± 1.40% at 8 µg/mL Figure 2B. IC_{50} was estimated by linear interpolation of scavenging percentages between the two values of mean response on the graph between scavenging and log10 concentration that enclosed 50% scavenging.

Table 2: DPPH radical-scavenging activity of carob extract and ascorbic acid

Ethanolic Ceratonia siliqua extract		**Ascorbic acid comparator**	
Final concentration (µg/mL)	**DPPH scavenging (mean ± SD, %)**	**Final concentration (µg/mL)**	**DPPH scavenging (mean ± SD, %)**
25	14.63 ± 1.14	0.25	12.59 ± 0.77
50	25.93 ± 0.33	0.5	24.39 ± 0.62
100	42.85 ± 2.43	1	43.19 ± 3.44
200	60.70 ± 1.79	2	69.43 ± 4.37
400	78.46 ± 2.21	4	87.00 ± 2.10
800	89.02 ± 1.85	8	92.33 ± 1.40
IC_{50} (µg/mL)	**132.0**	**IC_{50} (µg/mL)**	**1.20**

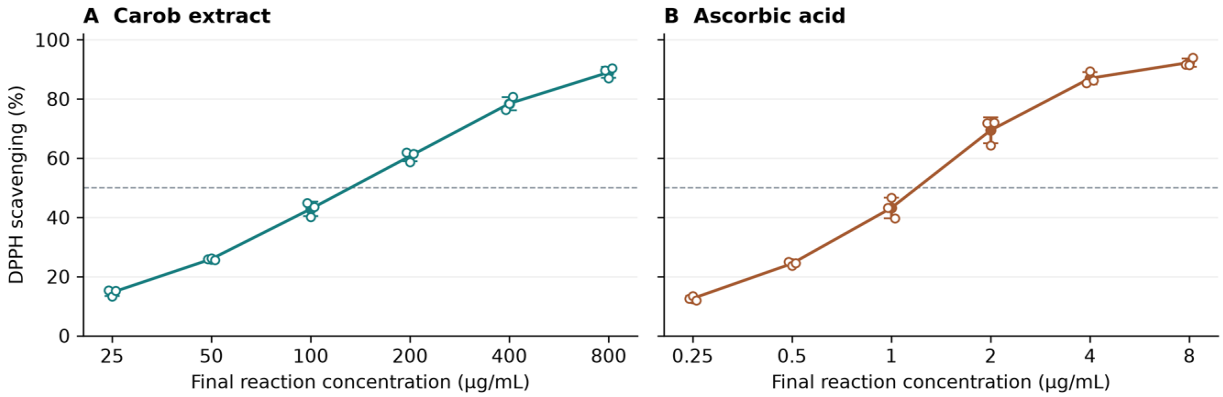


Figure 2: DPPH dose–response profiles: (A) carob extract and (B) ascorbic acid. Open points show technical readings; filled means and error bars show mean ± SD. The dashed line marks 50% scavenging. Dose ranges differ between panels; connecting lines are not fitted curves

3.4. Antibacterial activity across the concentration series

Increased growth inhibition is apparent in the concentration-series and there is an apparent plateau at the upper end of the series Table 3. Inhibition rises from 74.00 ± 1.15% at 64 mg/mL to 81.68 ± 1.14% at 128 mg/mL and 82.17 ± 1.36% at 256 mg/mL. The last increase is just 0.49 percentage points. Inhibition-zone diameters were 22.26 ± 0.19, 23.18 ± 0.42 and 24.03 ± 0.53 mm at these three concentrations, respectively. The mean broth growth inhibition (MGI) was only 0.49 percentage points higher at 256 mg/mL compared to 128 mg/mL. Instead, this flattening caused by a description is not biological saturation, total growth inhibition or elimination of bacteria.

Table 3: Growth inhibition and diffusion-zone diameters across 0.125–256 mg/mL

Extract concentration (mg/mL)	Growth inhibition (mean ± SD, %)	Inhibition-zone diameter (mean ± SD, mm)
0.125	4.28 ± 0.81	0.00 ± 0.00
0.25	8.56 ± 0.93	0.00 ± 0.00
0.5	14.73 ± 1.07	7.50 ± 0.17
1	23.91 ± 1.54	9.84 ± 0.11
2	35.68 ± 1.79	12.36 ± 0.42
4	47.26 ± 1.88	15.50 ± 0.45
8	57.84 ± 1.47	17.89 ± 0.19
16	65.39 ± 1.31	20.31 ± 0.60
32	70.62 ± 1.19	21.67 ± 0.35
64	74.00 ± 1.15	22.26 ± 0.19
128	81.68 ± 1.14	23.18 ± 0.42
256	82.17 ± 1.36	24.03 ± 0.53

n = 3 technical values per concentration.

4. Discussion

The light-brown viscous ethanolic extract and its yield value of 18.0% (1.80 g from 10.00 g of dried pulp) suggest that it is more of a crude fraction, and as such it contains a heterogeneous group of constituents of potential antibacterial activity. Carob has been used because it contains carbohydrates, fibre and phenolics and visual appearance is not enough for composition [21]. The use of 99% ethanol as extractant is also selective, extraction studies indicate that for the extraction of tannin, the composition of the extractant affects the recovery rate [22]. The results obtained by microwave heating for 1 min at 50°C signify short active heating, but not necessarily more energy-efficient overall when compared by no measurement [23]. Carob powder is also subject to processing variation, which, again, hinders comparisons with differently processed materials [24]. A total of 109 integrated chromatographic peaks (ICPs) were observed over the retention time range of 4.134-35.870 min for the GC–MS analysis Table 1. The relative peak areas (RPA) were referred to the total integrated ion current and the cumulative percentage of the RPA was around 100%. The largest integrated peak on the chromatogram was at 6.587 min with a proposed NIST library matched response to 2-(trimethylsilyl)ethanol from Figure 1 with 11.52% of the normalized TIC area. A peak at 13.014 min was tentatively matched by the library to 1-methyl-5-fluorouracil (1-methyl-5-FU) and made up 4.49% of the area of the normalized TIC. Altogether these data describe the chemical profile of the extract of *Ceratonia siliqua* in ethanol which is accessible by GC–MS.

Other assignments mentioned are: propyl 1-(propylthio)ethyl disulfide (3.08 %), oleic acid (2.34 %) and n-hexadecanoic acid (2.02 %). The signals at around 22.854 and 24.535 minutes are a wide-band alignment to the listed fatty-acid peaks. However, reliable identification by GC–MS requires authentic standards and the corresponding retention index. This is especially true in the cases of fluorinated and trimethylsilylated assignments but were not documented as such [25]. Analytical blanks are also important for siloxanes and phthalates as they can create false signals from contamination [26]. For poorly volatile components, complementary LC–MS can be beneficial and with proper quality control [27]. The percentages of the areas under the peaks do not represent the mass fraction of extracts nor the effective amount of antibacterial substance administered. DPPH scavenging increased consistently across the extract series: 14.63 ± 1.14%, 25.93%, 42.85%, 60.70 ± 1.7%, 78.46% and 89.02 ± 1.85% at 25, 50, 100, 200, 400 and 800 µg/mL, respectively. This type of behavior can be indicative of a concentration dependent chemical radical scavenging, as is the basis of the reduction based chemistry involved in this assay [28]. The reduction in increments at higher concentrations indicates the approach to response plateau, but is not an indicator of compounds responsible for the responses. Ascorbic acid likewise increased from 12.59 ± 0.77% at 0.25 µg/mL through 24.39%, 43.19%, 69.43% and 87.00%, reaching 92.33 ± 1.40% at 8 µg/mL. These curves have to be visible when they are compared within different concentration ranges.

The scavenging per unit mass of the crude preparation under similar assay conditions is very much lower from the reported descriptive IC_{50} values; 132.0 µg/mL (extract) and 1.20 µg/mL (ascorbic acid). To make meaningful comparisons between materials which differ in purity, standardized measurements are necessary [29]. But, DPPH IC_{50} is an operational concentration end-point, which is not a kinetic constant or biological antioxidant protection demonstration [30]. Light exposure and sample interference, solvent and reaction time can affect this end-point [31]. Therefore, the antioxidant results cannot be used alone to conclude antibacterial activity or clinical value. Inhibition rose from 4.28% at 0.125 mg/mL and 8.56% at 0.25 mg/mL to 14.73% at 0.5 mg/mL, then increased through intermediate concentrations, reaching 47.26% at 4 mg/mL, 65.39% at 16 mg/mL and 74.00 ± 1.15% at 64 mg/mL. Additional inhibition values were 23.91%, 35.68%, 57.84% and 70.62% at 1, 2, 8 and 32 mg/mL, respectively. Assay technique and organism selection are presently important factors that limit plant-extract screening, and such graded responses show the dependence of effects on concentration [32]. These relatively high crude-extract concentrations also necessitate careful assessment of the potency and subsequent selectivity testing, and not therapeutic claims [33].

At 128 and 256 mg/mL, inhibition reached 81.68 ± 1.14% and 82.17 ± 1.36%, respectively. Little additional inhibition was observed over this range (128 to 256 mg/mL); at this level, growth was not stopped and bactericidal activity was not established. Diffusion zones were not observed at the two lowest concentrations, did occur at 0.5 mg/mL (7.50-mm diameter), and increased with concentration to 4 mg/mL (15.50 mm diameter) and 16 mg/mL (20.31 mm diameter). Upper-dose diameters (22.26 ± 0.19, 23.18 ± 0.42 and 24.03 ± 0.53 mm) did not

appear to differ significantly in the same general direction, although they measure different phenomena: diffusion versus broth endpoint. Positive diameters take into account the diameter of the 6mm well. The remaining zone diameters were 9.84, 12.36, 17.89 and 21.67 mm at 1, 2, 8 and 32 mg/mL, respectively. Do not view the parallel antioxidant and antibacterial trajectories as mechanistically connected: there are different endpoints and concentration units and no compound-level correlation was observed. The activity of polyphenols towards the effects on bacterial membranes or enzymes may be a mechanism by which this extract acts, although this could not be confirmed, and free fatty acids may be responsible for the other active mechanism [34, 35]. Tentative chemical assignments do not establish either explanation. Activities and synergy must be reported with an appropriate fractionation and direct test. Organism specific assessments will be useful due to the capacity of Stenotrophomonas to survive in the environment [36]. It has intrinsic resistance mechanisms such as efflux and antibiotic-inactivation mechanisms [37] and the resistance in the biofilm may differ from the resistance in planktonic form [38]. Genomic heterogeneity also means that an ATCC strain is not necessarily a good representative of populations in the clinic [39].

5. Conclusion

The ethanolic extract resulted in concentration-dependent scavenging of DPPH radical and growth inhibition of *Stenotrophomonas maltophilia* ATCC 13637 under in vitro conditions. Interpolated DPPH IC_{50} was found to be 132.0 µg/mL. The broth growth inhibition % was 82.17 at 256 mg/mL, and the agar-well diffusion zone diameter was 24.03 mm; a confirmed identification of active constituents was not obtained, but rather their tentative GC–MS spectral-library annotations. These findings offer additional evidence of the in vitro activity of the preparation tested but are not a composition–activity relationship, bacterial killing, or proof of clinical efficacy. Independent experimental replication and confirmatory chemical analysis are necessary.

Article Information

Ethical Approval: Not applicable.

Data Availability: Data availability is not applicable to this article because no datasets were generated or analyzed.

Conflict of Interest: The authors declare that they have no conflict of interest.

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