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Original Article

Characterization of Bioactive Secondary Metabolites Produced by Streptococcus pyogenes Using Gas Chromatography-Mass Spectrometry (GC-MS) Evaluation of Its Antibacterial Activity

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Background: Metabolites are small molecules participating in metabolic reactions, which are necessary for cellular function, maintenance and growth. Typically, metabolites range from 50 to 1500 Da, while their concentrations span several orders of magnitude. The metabolome is highly dynamic, time-dependent, and metabolites are sensitive to many environmental conditions.

Aims and Objectives: The purpose of this laboratory study was to study the truly biologically active chemical compounds produced by *Streptococcus pyogenes* and to evaluate the antibacterial bioactivity of these products.

Method: *Streptococcus pyogenes* were isolated from pediatric patients with streptococcal pharyngitis, all swabs collected at Babylon Hospital for Women and Children, Samples were incubated in stationary culture at 37°C for 24 h, after which headspace collections were made. All bacterial species were grown on identical media with identical glassware. Concentration and analysis of volatile compounds : Volatile metabolites were concentrated and separated and analyzed via GC-MS.

In this research, (GC-MS) techniques were used to investigate the biochemical components that are commonly referred to as bioactive substances. Furthermore, the ethanolic extract of *Streptococcus pyogenes* was tested in an experimental laboratory to determine whether it actually has effective antibacterial properties.

Results: Using GC-MS analysis on Streptococcus pyogenes, the presence of the following bioactive components was experimentaly identified: Carvacrol, 1-(5(Methyl-2-furanyl)-1-buten-3-one, beta Sesquiphellandrene, Pentasiloxane, dodecamethyl, Pyrazine, 2,5-dimethyl-, Benzene, 2-ethyl-1,4-dimethyl, tumerone, AR- tumerone, ALPHA- tumerone, 6-Aza-5,7,12,14-tetrathiapentacene, β-HIMACHALENOXIDE, 3-Ethyl-o-xylene, Trimethylphenylsilane, 1,3-Hexadiene, 2,5-dimethyl, o-Mercaptoaniline, Benzonitrile, 4-amino, Cyclononasiloxane, octadecamethyl, 3-Decen-5-one, Cyclononasiloxane, octadecamethyl, Methyl linoleate, Gentisic acid, Shogaol, Isooctyl phthalate, 2,6,10,-Pentamethyl-2,6, 18-eicosapentaene, Heptasiloxane, hexadecamethyl, N-Methyl-1-adamantaneacetamide.

It was investigated whether the secondary metabolites produced by *Streptococcus pyogenes* have antibacterial properties against three dangerous pathogens. In the current research, the biological activity of the ethanolic extract of *Streptococcus pyogenes*, as well as the conventional antibiotics Rifambin and Cefotoxime, against three different pathogens were investigated. *Escherichia coli* (14.09±0.18, 20.17±0.25, and 17.37±0.23), *Proteus mirabilis* (12.91±0.16, 19.47±0.23, and 15.11±0.19), and *Staphylococcus aureus* (11.01±0.14, 20.07±0.25, and 17.00±0.21). *Streptococcus pyogenes* metabolites were shown to show remarkable activity against *Escherichia coli*, with a mean value of 17.37±0.23.

Keywords: Streptococcus pyogenes, Secondary metabolites, Antibacterial, GC/MS.

Introduction:

Metabolites are extensively exchanged with the environment, e.g., food intake, excretion, inhalation, secondary metabolites, such as medications, flavorings, and recreational drugs, which can be further processed by the gut microbiome or organs. Furthermore, metabolites are, chemically, very diverse (polarity, charge, pKa, solubility, volatility, stability, and reactivity), consequently no single method can capture and analyze the entire metabolome at once. Hence, many extraction methods were developed to identify and quantify specific classes of metabolites. The most commonly used approaches to explore the metabolome are gas chromatography (GC), liquid chromatography (LC) or, to a lesser extent, capillary electrophoresis (CE), online coupled to a mass spectrometer (MS), as well as nuclear magnetic resonance (NMR) spectroscopy [1]. Nevertheless, the contextual and chemical diversity makes the detection of a whole set of metabolites present in a biological sample an ambitious goal [1, 2]. Due to the complexity and chemical diversity of the metabolome with metabolite concentrations spread over a wide range of magnitudes, identification is still a challenge. In addition to the variety

of instruments and their different operation and acquisition methods, even more programs exist for data processing, typically for a specific class of data. For example, different instrument settings used in LC-MS, such as collision energy, resolution, targeted, non-targeted, and fragmentation methods, such as CID (collision induced dissociation), HCD (higher energy collision dissociation), ETD (electron-transfer dissociation), and PQD (pulsed-Q dissociation) [4-7], can give rise to different fragments species and intensities. Hence, it is a tough task to use or build metabolite-specific spectral databases for compound identification. One of the best, but time consuming, options is to create an instrument specific MRM method for a set of preselected metabolites. The advantage of using up to three MRMs and their MRM ion ratios, which are stable properties between transitions [8] for robust and unambiguous analysis of complex samples, was demonstrated in a screening approach of rotenone-treated HeLa cells [9]. In this study, a MRM method was developed using pure substances and the transitions had to co-elute at the same retention time with identical peak shapes. MRM ion ratios have been shown to be essential to avoid false-positive identifications in an example for inosine 51-monophosphate [10]. Schürmann and colleagues revealed the need of a third transition as well [11]. In their study, product ions from a co-eluting interfering matrix compound were consistent with MRMs of two sebuthylazine transitions. Following the European Union directive 2002/657/EC, which regulates the confirmation of suspected positive identifications, this would have resulted in a false-positive finding. To date there is an insufficient discussion about the relative importance of using certain transitions for compound identification. Fragment mis-assignments can originate from impure Q1 isolation, co-eluting isobaric compounds, wrong database entries, or incorrect peak picking. Due to its complex nature, non-targeted metabolomics has to be linked to advanced chemometric techniques, to reduce the data complexity into a smaller set of manageable signals [12]. Analytical methods and required improvements in non-targeted metabolomics are extensively described in a review of Alonso and colleagues. A METLIN search for the exemplary parent mass 136 Da reveals 131 isobaric, but unique metabolites, many of them with very similar structures and, thus, almost similar fragment spectra. As a result, a ranked list based on similarity scores is provided and cutoff values have to be used to verify the identification. It is debatable whether this is sufficient enough and how many false-positive identifications are actually included. A big effort is being done to improve spectral databases, but the development of accurate automatic identification algorithms is still subject to the availability of an exhaustive set of reference metabolite spectra As target identification is one of the most critical steps, in silico target identification methods, including chemical similarity database searches, are used, such as CSNAP (Chemical Similarity Network Analysis Pulldown) [13]. Several strategies exist on how unknown peaks can be deciphered and interpreted, but validation guidelines were missing for a long time. A collection of guidelines/minimum requirements for the validation of metabolite identification, were finally conceived by the Metabolomics Standards Initiative (MSI in 2005, in order to allow data to be efficiently applied, shared and reused. The initiative “COordination of Standards in MetabOlomicS” (COSMOS) is generating robust data infrastructures and exchange standards for metabolomics data and metadata. The objectives of this study were : Analysis of secondary metabolites produced by *Streptococcus pyogenes* Gas Chromatography-Mass Spectrometry (GC-MS). Evaluation of Its antibacterial activity.

Materials and Methods

Optimal environmental conditions for growth and identification of metabolites

Streptococcus pyogenes were isolated from pediatric patients with streptococcal pharyngitis, all swabs collected at Babylon Hospital for Women and Children, Samples were incubated in stationary culture at 37°C for 24 h, after which headspace collections were made. All bacterial species were grown on identical media with identical glassware. Concentration and analysis of volatile compounds : Volatile metabolites were concentrated and separated and analyzed via GC-MS. A rotary evaporator was used to evaporate metabolites at a temperature of 45 degrees Celsius. Metabolites were then extracted from the liquid culture and separated from the culture.

Conducting a spectral study of the bioactive natural chemical components of *Streptococcus pyogenes* using (GC-MS).

An Agilent 789 A instrument was used to perform the examination, which was performed using a GC–MS approach. The gas chromatography column used was a DB-5MS column purchased from J&W Scientific in Folsom, California. The following measurements were made for this column: The film thickness is 0.25 µm and the diameter is 30 m with an internal diameter of 0.25 mm. Compared to the earlier experiment [10], the temperature in the furnace was kept at the same level throughout the process. The carrier gas used was helium and the flow rate was set at one milliliter per minute each time. Effluent from the gas chromatography (GC) column was directly injected into the mass spectrometer (MS) source via a transfer line that was heated to 250 degrees Celsius. 230 degrees Celsius (°C) was the temperature that was maintained at the ion source while the ionization process took place at a voltage of 70 electron volts (eV). A total of 41 atomic mass units (amu) were included in the measuring range, which reached up to 450.

Evaluation of the antibacterial efficacy of secondary metabolite chemicals against three pathogenic bacteria.

A sterile cork borer was used to create wells in the agar with a diameter of five millimeters. Then, 25 microliters of sample solutions containing metabolites generated by *Streptococcus pyogenes*, as well as the conventional antibiotics Rifambin and Cefotoxime, against three different pathogens were investigated *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* .

Statistical Analysis

If the p-value was less than or equal to 0.05, we used Student's t-test to find out whether the parametric data was statistically significant.

RESULTS and DISCUSSION

The fact that S. pyogenes remains a successful pathogen, despite its susceptibility to most modern therapies, reflects its exquisite ability to adapt its metabolism to exploit a variety of adverse environments and host tissues. Since metabolism is intimately linked to virulence, it is a burgeoning question and a matter of important discussion whether a virulence-promoting alteration to its metabolism comes at too high a cost in fitness and is a non-adaptive side effect of traits required for superficial symptomatic infection [11]. Cumulative evidence has provided unequivocal evidence that diverse metabolic activities enable S. pyogenes to survive successfully in the presence of a variety of stress conditions. The earlier interest in understanding the metabolism of S. pyogenes was driven by a need to investigate the biochemical basis for its growth requirements [12, 13]. The present chapter covers the knowledge obtained from subsequent reports of numerous investigators who used the established biochemical basis of metabolism to understand the underlying mechanisms of virulence as to how S. pyogenes, as a successful pathogen, senses its environment and changes its metabolic status to survive, persist, and proliferate in a broad range of host environments. It is also worth noting that early studies on S. pyogenes metabolism focused on various aspects of the pathogen’s carbohydrate and amino acid metabolism. However, its lipid metabolism and membrane transport have received relatively limited attention, despite the fact that these processes play a crucial role in the secretion of many cellular products, including many virulence factors.

The GC-MS chromatogram exhibited peaks that corresponded to the compounds that were previously identified. The following chemicals are mentioned: Carvacrol, 1-(5(Methyl-2-furanyl)-1-buten-3-one, beta Sesquiphellandrene, Pentasiloxane, dodecamethyl, Pyrazine, 2,5-dimethyl-, Benzene, 2-ethyl-1,4-dimethyl, tumerone, AR- tumerone, ALPHA- tumerone, 6-Aza-5,7,12,14-tetrathiapentacene, β-HIMACHALENOXIDE, 3-Ethyl-o-xylene, Trimethylphenylsilane, 1,3-Hexadiene, 2,5-dimethyl, o-Mercaptoaniline, Benzonitrile, 4-amino, Cyclononasiloxane, octadecamethyl, 3-Decen-5-one, Cyclononasiloxane, octadecamethyl, Methyl linoleate, Gentisic acid, Shogaol,

Isooctyl phthalate, 2,6,10,-Pentamethyl-2,6, 18-eicosapentaene, Heptasiloxane, hexadecamethyl, N-Methyl-1-adamantaneacetamide.

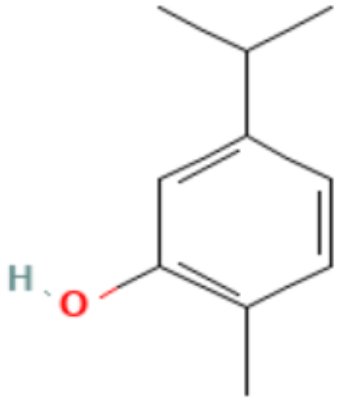
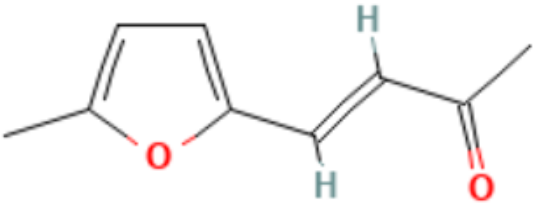
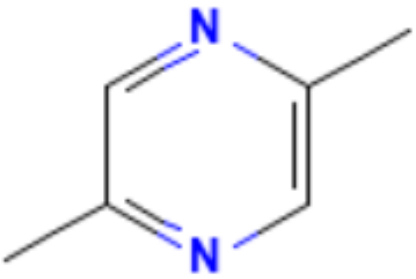
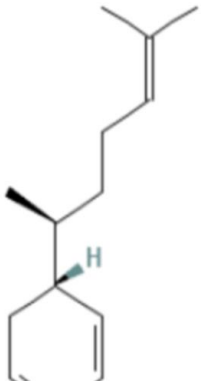
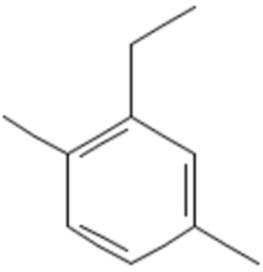
Our study provides evidence to pursue an ongoing study of volatile biomarkers in oropharyngeal streptococcal species. Several of the discriminant compounds, notably pyrazine compounds, are normally absent in healthy human exhaled breath but can be found in the Streptococcus pyogenes cultures [13, 14]. That these compounds are typically absent suggests that a number of these candidate biomarkers are not typically produced by other members of the oropharyngeal microbial community. Such S. pyogenes-specific volatiles thus show particular promise as volatile biomarkers of acute streptococcal pharyngitis. Ongoing studies are required to validate our candidate biomarkers in patients with and without naturally acquired acute bacterial pharyngitis. Multiple factors, such as metabolite availability and host interaction with the bacteria through inflammatory responses will likely alter bacterial volatile production in the setting of acute infection [15]. The ability to confirm the detection of pathogenic infection by S. pyogenes in the airways using breath analysis is a very exciting and non-invasive approach that will help guide immediate and appropriate antibiotic use upon infection confirmation [16].

It was investigated whether the secondary metabolites produced by *Streptococcus pyogenes* have antibacterial properties against three dangerous pathogens. In the current research, the biological activity of the ethanolic extract of *Streptococcus pyogenes*, as well as the conventional antibiotics Rifambin and Cefotoxime, against three different pathogens were investigated. *Escherichia coli* (14.09±0.18, 20.17±0.25, and 17.37±0.23), *Proteus mirabilis* (12.91±0.16, 19.47±0.23, and 15.11±0.19), and *Staphylococcus aureus* (11.01±0.14, 20.07±0.25, and 17.00±0.21). *Streptococcus pyogenes* metabolites were shown to show remarkable activity against *Escherichia coli*, with a mean value of 17.37±0.23.

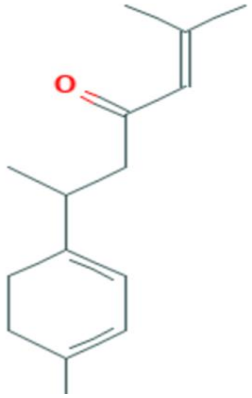
Table 1. Bioactive secondary metabolites produced by *Streptococcus pyogenes* using Gas Chromatography-Mass Spectrometry (GC-MS)

Compound	Formula	M.W
Carvacrol	$C_{10}H_{14}O$	150.22 g/mol
1-(5(Methyl-2-furanyl)-1-buten-3-one	$C_9H_{10}O_2$	150.17 g/mol
beta.Sesquiphellandrene	$C_{15}H_{24}$	204.35 g/mol
Pentasiloxane, dodecamethyl	$C_{12}H_{36}O_4Si_5$	384.84 g/mol
Pyrazine, 2,5-dimethyl-	$C_6H_8N_2$	108.14 g/mol
Benzene, 2-ethyl-1,4-dimethyl	$C_{10}H_{14}$	134.22 g/mol
tumerone	$C_{15}H_{22}O$	218.33 g/mol
AR- tumerone	$C_{15}H_{20}O$	216.32 g/mol
ALPHA- tumerone	$C_{15}H_{22}O$	218.33 g/mol
6-Aza-5,7,12,14-tetrathiapentacene	$C_{17}H_9NS_4$	355.5 g/mol
β-HIMACHALENOXIDE	$C_{15}H_{24}$	204.35 g/mol
3-Ethyl-o-xylene	C10H14	134.22 g/mol
Trimethylphenylsilane	$C_9H_{14}Si$	150.29 g/mo
1,3-Hexadiene, 2,5-dimethyl-	C_8H_{14}	110.20 g/mol
o-Mercaptoaniline	C_6H_7NS	125.19 g/mol
Benzonitrile, 4-amino-	$C_7H_6N_2$	118.14 g/mol
Cyclononasiloxane, octadecamethyl	$C_{18}H_{54}O_9Si_9$	667.4 g/mol
3-Decen-5-one	$C_{10}H_{18}O$	154.25 g/mol
Cyclononasiloxane, octadecamethyl	$C_{18}H_{54}O_9Si_9$	667.4 g/mol
Methyl linoleate	$C_{19}H_{34}O_2$	294.5 g/mol
Gentisic acid	$C_7H_6O_4$	154.12 g/mol
Shogaol	$C_{17}H_{24}O_3$	276.4 g/mol
Isooctyl phthalate	$C_{24}H_{38}O_4$	390.6 g/mol
2,6,10,-Pentamethyl-2,6, 18-eicosapentaene	$C_{25}H_{42}$	342.6 g/mol
Heptasiloxane, hexadecamethyl	$C_{16}H_{48}O_6Si_7$	533.1 g/mol
N-Methyl-1-adamantaneacetamide	$C_{13}H_{21}NO$	207.31 g/mol

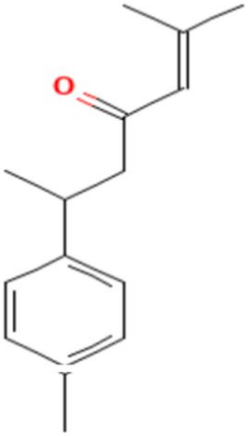
Table 2. Structures of metabolites produced by Streptococcus pyogenes

Compound	Structure
Carvacrol	
1-(5(Methyl-2-furanyl)-1-buten-3-one	
Pyrazine, 2,5-dimethyl-	
beta.Sesquiphellandrene	
Benzene, 2-ethyl-1,4-dimethyl	

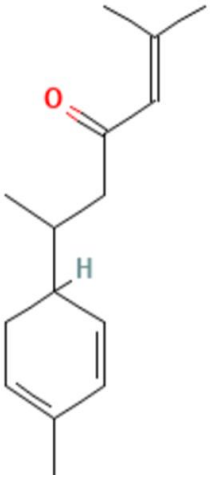
TUMERONE



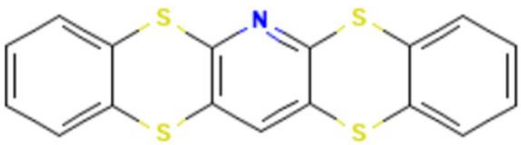
AR-TUMERONE



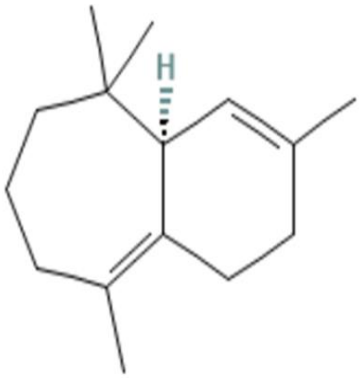
ALPHA.-TUMERONE



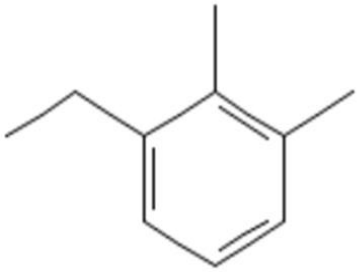
6-Aza-5,7,12,14-tetrathiapentacene



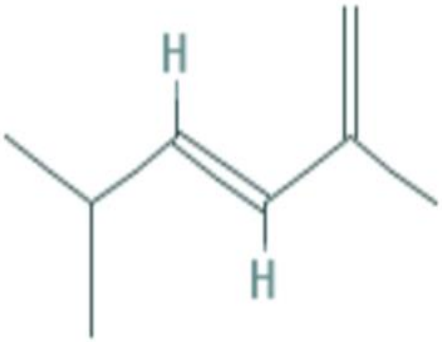
β-HIMACHALENOXIDE



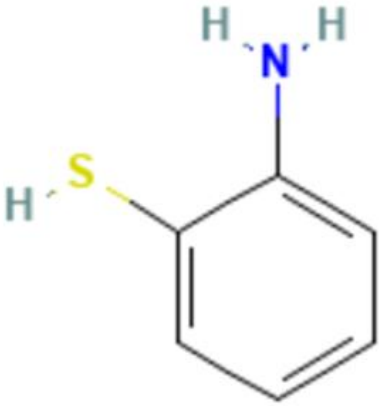
3-Ethyl-o-xylene



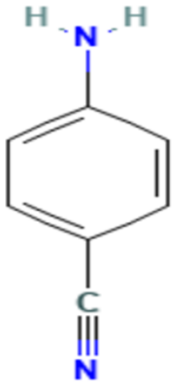
1,3-Hexadiene, 2,5-dimethyl-



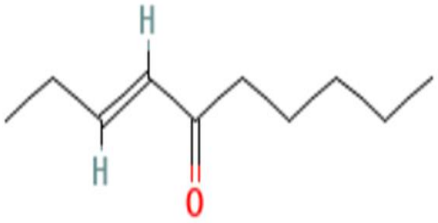
o-Mercaptoaniline



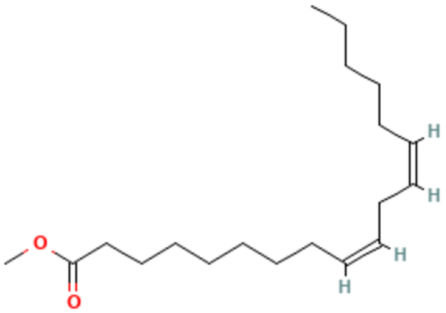
Benzonitrile, 4-amino-



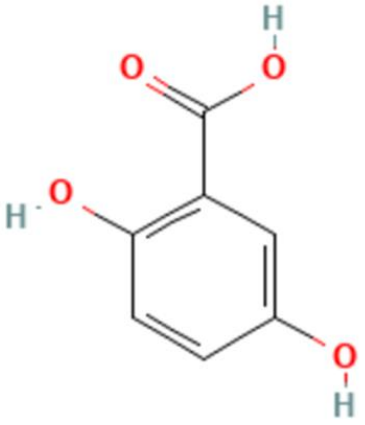
3-Decen-5-one



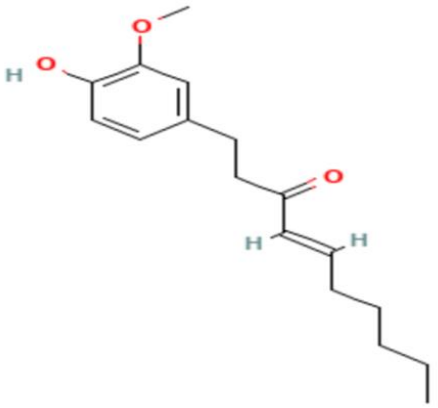
Methyl linoleate



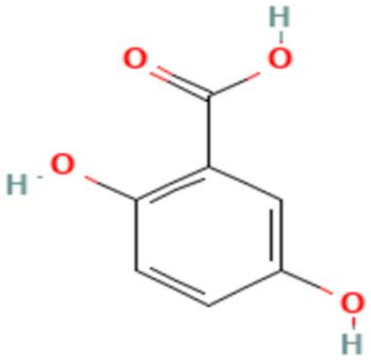
Gentisic acid



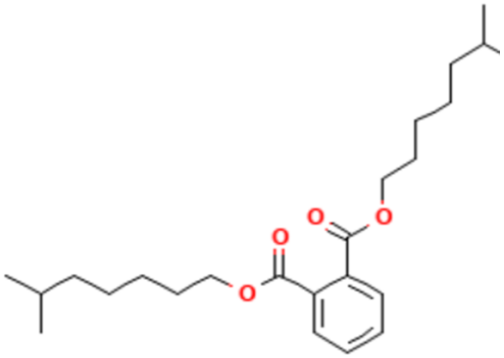
Shogaol



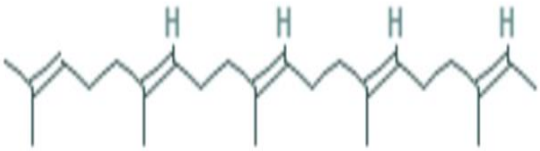
Gentisic acid



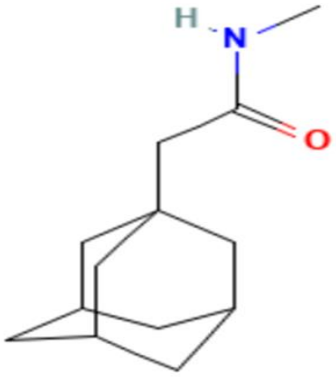
Isooctyl phthalate



2,6,10,14,18-Pentamethyl-eicosapentaene



N-Methyl-1-adamantaneacetamide



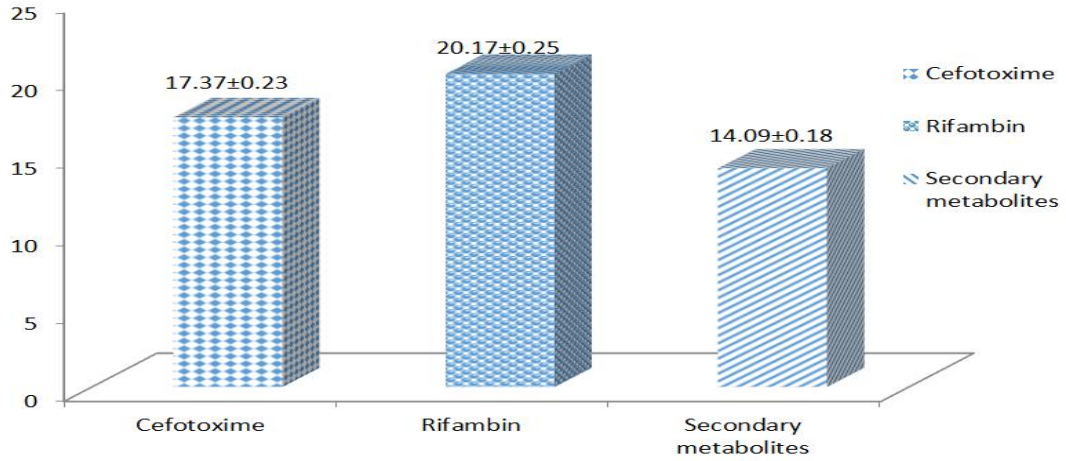


Figure 1. Metabolite products of *Streptococcus pyogenes*, Rifambin and Cefotoxime as anti-bacterial activity against *Escherichia coli*.

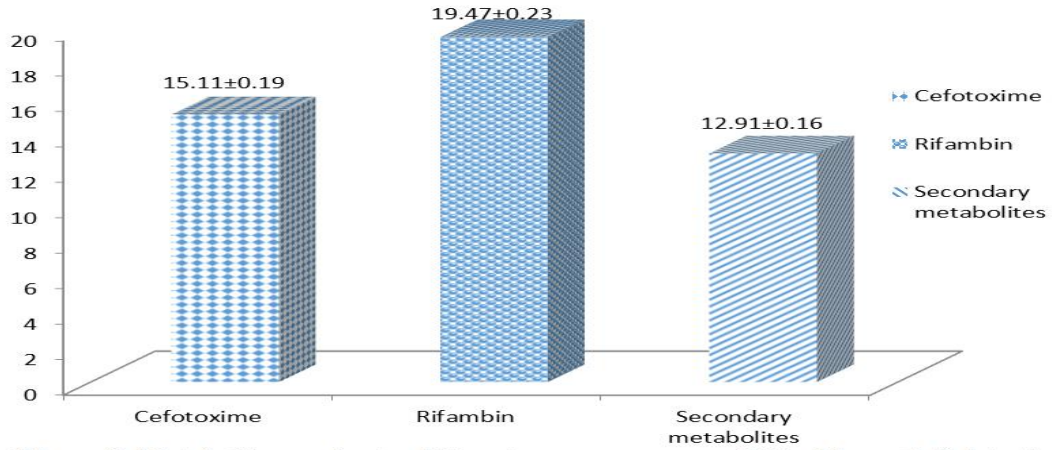


Figure 2. Metabolite products of *Streptococcus pyogenes*, Rifambin and Cefotoxime as anti-bacterial activity against *Proteus mirabilis*.

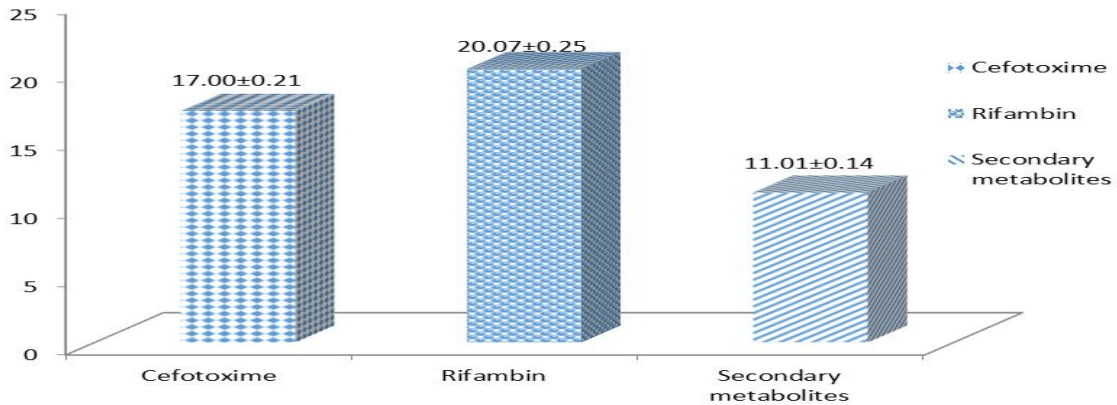


Figure 3. Metabolite products of *Streptococcus pyogenes*, Rifambin and Cefotoxime as anti-bacterial activity against *Staphylococcus aureus*.

CONCLUSION

Our study provides evidence to pursue an ongoing study of volatile biomarkers in oropharyngeal streptococcal species. Several of the discriminant compounds, notably pyrazine compounds, are normally absent in healthy human exhaled breath but can be found in the *Streptococcus pyogenes* cultures. That these compounds are typically absent suggests that a number of these candidate biomarkers are not typically produced by other members of the oropharyngeal microbial community. Such S. pyogenes-specific volatiles thus show particular promise as volatile

biomarkers of acute streptococcal pharyngitis. Based on the results of the antibacterial activity test, it was determined that the metabolites of *Streptococcus pyogenes* show remarkable activity against *Escherichia coli*, with a mean value of 17.37±0.23.

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