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Metabolites profiling reveals for antimicrobial compositional differences and action mechanism in the toothbrushing stick "miswak" *Salvadora persica*

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ABSTRACT

Among many plant species suitable for preparing toothbrushing sticks, miswak (*Salvadora persica*, family Salvadoraceae) is found the most effective tool for oral hygiene. *S. persica* possesses antibacterial, antiviral and antifungal effects against oral microbes, mostly due to its benzyl isothiocyanate content. To provide insight into *S. persica* chemical composition, volatile constituents from roots and stems of *S. persica* grown in Egypt and Saudi Arabia were profiled using solid-phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC–MS). A total of 21 volatiles were identified with sulfur compounds amounting for the major volatile class. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) revealed for benzyl isothiocyanate (BITC) enrichment in roots versus stems. Primary metabolites contributing to *S. persica* taste *viz.* sugars and organic acids were profiled using GC–MS with silylation. Polyols (sugars) *viz.* arabitol, meso-erythritol, and mannitol were found to predominate sugars composition in *S. persica* stems being most enriched in meso-erythritol. The impact of saliva on *S. persica* aroma profile was further assessed and revealing for no enhancement in BITC production with salivation, and further not being detected in toothpaste preparation claimed to contain *S. persica* extract. This study provides the most complete profile of volatiles, sugars, and organic acids in *S. persica* organs and more rationalizing its use as a toothbrush.

1. Introduction

The WHO recommends the use of miswak as an effective oral hygiene tool, being available and of low-cost as evidenced by its use since ancient times [1]. Miswak, botanically known as *Salvadora persica* (family Salvadoraceae), is part of Greeko-Arab system of medicine and is regarded as the first ever known natural oral hygiene tool used as toothbrush. Several studies have confirmed *S. persica* beneficial effects against dental caries including its bacterial and fungal growth inhibition, anti-periopathic disinfectant action and anti-plaque properties. The vast oral benefits of miswak regarding its scientific, religious, and social evidences are well reported [2,3].

Abhary and Al-Hazmy showed that the aqueous extract of miswak contains many antimicrobial agents that has stronger antimicrobial activity than the alcoholic and nonpolar ones [4]. Chemical analysis in search of bioactive chemicals in *S. persica* mediating for its oral health effect has identified benzyl isothiocyanate (BITC) as a major antimicrobial volatile [5] in addition to inorganic elements *i.e.* fluoride, calcium and phosphorus [6]. Several other bioactive metabolites have been identified in that matrix including salvadourea, alkaloids, and fatty acids suggested to synergize the cleansing efficacy of miswak by leaching out in saliva [7]. Several of these chemicals also impart slightly bitter taste upon chewing which activates the flow of saliva acting as antiseptic [7].

Interest in BITC antimicrobial effect in miswak has prompted the search for other volatile forms with potential antimicrobial activity [8] and has led to the identification of 15 components *via* GC–MS including BITC (52.5%), benzyl nitrile (38.3%), carvacrol (3.3%), benzaldehyde (2.5%), aniline (0.7%) and naphthalene (0.6%) prepared using steam distillation. Compared to heat distilled oil, solid phase microextraction (SPME) emphasizes a revolution in the aroma pro-

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filing of herbal drugs, being more sensitive and less liable to artifact formation compared to distilled oil [9]. Headspace SPME coupled to GC–MS has been recently reported for the analysis of *S. persica* root, although monitoring only its two major volatiles BITC and benzyl nitrile [5]. Many toothpaste preparations are also currently marketed from *S. persica* extract which warrants the development of analytical methods for their quality control (QC) analysis. Currently, no defined monograph exists for *S. persica* QC to ensure consistency in its products bioactivity. Compared to root that has been a target for analysis in *S. persica*, much less is known on its aerial part chemical composition and its biological effects [10].

Metabolomics, metabolites characterization, and fingerprinting are modern approaches that are increasingly adopted for the investigation of herbal drugs QC analysis [11]. Metabolomics mostly procure the advantages of coupled techniques that depend on the isolation of metabolites either *via* gas or liquid chromatography coupled to mass spectrometry [12]. The objective of the current study was to apply metabolomics tools to investigate *S. persica* metabolism in the context of its both geographical origin and organ type as represented by 4 different *S. persica* accessions derived from different localities. Volatile constituents from root and stem samples were subjected to a comparative approach using (SPME) coupled to GC–MS for its volatile composition in parallel to direct GC–MS analysis of its silylated primary non-volatile metabolites. Owing to the complexity of acquired data, multivariate data analyses, principle component analysis (PCA), hierarchical clustering analysis (HCA) and orthogonal projection least squares discriminant analysis (OPLS-DA) were utilized to ensure good analytical rigorousness and classify samples. This study afforded a more complete profile for volatiles, sugars and organic acids distribution in *S. persica* stem, root and its commercial toothpaste product and further providing more evidence for the rationalization of its use as toothbrush.

Furthermore, the suggestion for an enzymatic activation to maximize miswak root antimicrobial effect with the release of BITC occurring when *S. persica* is chewed [5] prompted us to investigate the salivation effect on *S. persica* aroma profile and in its commercial toothpaste preparation.

2. Materials and methods

2.1. Plant material, SPME and chemicals

Four *S. persica* samples representing root and stem samples from different geographical origins were collected including: *S. persica* stem, Jeddah, Saudi Arabia (SPS1), *S. persica* root 1, Saudi Arabia (SPR1), *S. persica* root 2, Saudi Arabia (SPR2), *S. persica* root 3, Aswan, Egypt (SPR3). Miswak toothpaste was purchased from Dabur Egypt limited, 10th of Ramadan Egypt, BNEY0451 www.dabur.com. SPME holder and fiber coated with 50 μm/30 μm DVB–CAR–PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane) was supplied by Supelco (Oakville, ON, Canada). All other chemicals, volatile standards and sugars were provided from Sigma Aldrich (St. Louis, Mo., U.S.A.).

2.2. SPME volatiles isolation

Headspace volatiles analysis using SPME was adopted from [13,14] with few modifications. Briefly, roots and stems were ground, and 1 g was placed inside a 20 mL glass vials. (*Z*)-3-Hexenylacetate was spiked as an internal standard (IS) prepared in water and added to each vial at a final concentration of 1 μg/vial. Vials were then immediately capped and placed on a temperature controlled oven set at 50 °C for 30 min of volatiles adsorption with the SPME fiber inserted into the headspace above each sample. Adsorption was timed for 30 min. A system blank containing no plant material was run as a negative control.

2.3. GC–MS volatile analysis

SPME fibers were desorbed manually at 210 °C for 1 min in the injection port of a Shimadzu Model GC-17A gas chromatograph interfaced with a Shimadzu model QP-5000 mass spectrometer (Kyoto, Japan). Volatiles were separated on a DB5-MS column 30 m length, 0.25 mm inner diameter, and 0.25 μm film (J&W Scientific, Santa Clara, CA, USA). Injections were made in the splitless mode to enhance detection level for a timing of 30 s. The gas chromatograph was operated under the following conditions: injector 220 °C, column oven 38 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min, and finally ramped at a rate of 40 °C min^{-1} to 220 °C and kept for 2 min, He carrier gas was set a flow rate of 1 mL min^{-1}. The transfer line and ion–source temperatures were both set at 230 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV with a scaning range set at *m/z* 40–500. Volatile components were identified using exact procedure described in [13,14] identified based on its retention indices (RI) relative to *n*-alkanes (C6–C20), mass spectrum matching to NIST, WILEY library database and with authentic standards when available. Relative percentile of metabolites was calculated by dividing each metabolite GC–MS peak area by the total detected peak areas within each sample.

2.4. GC–MS analysis of silylated primary metabolites

For analysis of primary metabolites, 100 μL of 50% aqueous extract (prepared by extracting 100 mg of roots or stems in 5 mL 50% MeOH with sonication for 30 min followed by centrifugation) was evaporated under N_2 till complete dryness. For derivatization, 150 μL of *N*-methyl- *N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was then added and incubated at 60 °C for 45 min. The samples were equilibrated at 28 °C and subsequently analyzed using GC–MS. Silylated derivatives were separated on a Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 μm film) column fitted in an Agilent HP 7890 quadropole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Injections were made in a (1:15) split mode and the GC was operated under the following conditions: injector 280 °C, column oven 80 °C for 2 min, then programmed at a rate of 5 °C/min to 315 °C, kept at 315 °C for 12 min. He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 280 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at 50–650 *m/z*. Silylated metabolites were identified using same parameters described for volatiles analysis, and their contents were determined based on peak areas relative to summed peak areas of identified metabolites within each sample.

2.5. Incubation of saliva samples with plant and analysis of volatiles

The study was performed on saliva samples obtained from clinically healthy subjects (n = 6). The study protocol was ethically reviewed and approved by Ethics Review Committee of the University of Cairo. Signed informed consent was obtained from all subjects prior to their inclusion in the study. All subjects were instructed to refrain from smoking, eating, drinking and toothbrushing for 12 h prior to the saliva collection. The mouth was rinsed with distilled water before saliva collection. Subsequently, saliva was allowed to accumulate on the floor of the mouth and the subjects were instructed to spit out into test tubes. Salivary pH was directly measured with pH paper (pH indicator strip, Merck,

pH = 2.0–9.0). Immediately, after collection, 1 g of powdered plant material was added to saliva at a final concentration of 1 g per 5 mL saliva sample using 20 mL headspace collection vial. Vials were then incubated at 37 °C for 15 min with shaking using orbital shaker and then subjected to volatiles collection using exact procedure used above for plant materials volatile collection (section 2.2.). Absolute quantification of BITC levels *in S. persica* with and without saliva exposure was calculated using standard calibration curve for BITC of serial dilutions spanning from (0.2, 2 & 20 μg) analyzed under same SPME conditions.

Equation used for calculation y = 6E + 09x + 1E + 09, R^2 = 0.97.

2.6. UPLC–MS analysis

The UPLC–ESI (electrospray ionization) were obtained from a LCQ Deca XP MAX system (ThermoElectron, San Jose, USA) equipped with a ESI source (electrospray voltage 4.0 kV, sheath gas N_2, capillary temperature 275 °C). The Ion Trap MS system is coupled with an Acquity Waters ultra performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA, USA) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 mm; Waters Corp.) applying the following gradient system at a flow rate of 150 uL min^{-1} starting from formic acid in water (0.1%):CH_3CN = 95:5–100% CH_3CN within 35 min, then isocratically for further 10 min; flow rate 70 μL/min.

2.7. Metabolites quantification & multivariate data analyses

Relative quantification and comparison of both the volatiles and primary metabolite profiles after GC–MS were performed using Met-idea software, which can be downloaded freely from (http://bioinfo.noble.org/download/). This software approach employs peak alignment, matching and comparison for principal component analysis (PCA), as described previously [15]. PCA and HCA were performed on the MS-data using custom script under the R 2.9.2 environment. Partial least squares-discriminant analysis (OPLS-DA) was performed with the program SIMCA-P Version 13.0 (Umetrics, Umeå, Sweden). Markers were subsequently identified by analyzing the S-plot, which was declared with covariance (p) and correlation (pcor). All variables were mean centered and scaled to Pareto variance.

3. Results & discussion

3.1. Volatiles analysis

The goal of this study was to investigate the diversity of volatile profile within *S. persica* stem (SPS1) and root derived from different places *viz.* Saudi Arabia (SPR1 & SPR2) and Egypt (SPR3) (see experimental Section 2.1.) using SPME GC–MS coupled to chemometrics. To assess for biological variance, three biological replicates for each specimen were extracted and analyzed in parallel under identical conditions. Headspace GC–MS analysis resulted in the identification of 21 volatile constituents (Table 1 & Fig. 1) with SPS1 stem specimen exhibiting the most distinct volatile profile compared to root specimens. The three root specimens SPR1, SPR2 and SPR3 showed one major volatile component *i.e.*, BITC accounting for *ca.* 99%. BITC is considered a vital component of the Crucifereae family plants' defense system, exhibiting an antibacterial effect against both gram positive and gram negative periodontal pathogens *i.e. Salmonella*, *Pseudomonas aeroginosa* in addition to antiviral effect against *Haemophilus influenza* [5]. Compared to root sample, stem sample was found less enriched in BITC amounting only for 25.6% of its volatile blend and more dominated by other nitrogen containing volatiles e.g. benzyl nitrile (33.7%). Benzyl nitrile has been reported as a dominant volatile constituent of *S. persica* leaf (54%) from Jordan with a considerable antibacterial effect on several oral aerobic bacteria, suggesting that it can also contribute to *S. persica* aerial stem part use as toothbrush [16]. Aliphatic and aromatic aldehydes constituted 22% of the stem volatile blend presented by benzaldehyde (12.2%) followed by nonanal (6.2%) and (*E*)-cinnamaldehyde (3.2%). Benzaldehyde is generally regarded as a safe food additive used worldwide and flavouring agent [17] and its antimicrobial effect [18] along with (*E*)-cinnamaldehyde [19] is likely to pose stem as potential toothbrush similar to the more commonly used root part. Three sesquiterpenes were found exclusively in stem at *ca.* 5% including zingiberene (1.1%), β-bisabolene (1.6%) and β-farnesene (1.9%). These sesquiterpenes are likely to contribute for the better aroma of *S. persica* stem compared to that of root. In stem sample, esters amounted for 4.2% of the total volatile blend presented by octyl acetate (3.8%), α-terpineol acetate (2.2%) and isopropyl myristate (0.4%), whereas aromatics *i.e.* naphthalene reached only 2% all of which might act to augment BITC antimicrobial effect [18]. Volatile organic compounds (VOCs) profiling in stem specimens extended the use of *S. persica* to include aerial part as a dental and buccal antiseptic and confirmed that in root part, the antiseptic activity is mainly due to the most prominent volatile component BITC.

3.2. Multivariate data analysis of S. persica volatiles

To assess the effect of organ type and geographical origin on *S. persica* volatiles composition in an untargeted way, volatiles GC–MS dataset was subjected to multivariate data analyses *i.e.* PCA and HCA. These are unsupervised clustering methods, requiring no previous knowledge of the dataset and acting to reduce the dimensionality of multivariate data [20]. HCA was performed to define both similarities and differences among samples in a graphical way, showing two major clusters (Suppl. Fig. S1A). Volatiles collected from stem sample was found to cluster separately in one group from the other three root specimens, being clustered altogether as evident from their enrichments in BITC as revealed from the density of heatmap (data not shown). PCA was further performed to explore the relative variability within *S. persica* volatiles (Suppl. Fig. S1B). The metabolome clusters were located at different points in the two-dimensional space prescribed by two vectors, principal component 1 (PC1 = 86%) and principal component 2 (PC2 = 13%). The PC1/PC2 scores plot (Suppl. Fig. S1B) showed the segregation of stem samples in left lower quadrant along PC1 (negative score values). The metabolite loading plot for PC1 (Suppl. Fig. 3C), which exposes the most important components with respect to scattering behavior, revealed that BITC contributed the most in samples classification being generally found more enriched in root samples SPR1 and SPR3. No clear segregation based on *S. persica* root geographical origin could be observed with samples derived from Egypt being clustered close to that collected from Saudi Arabia and suggesting that this model cannot predict samples cultivation origin.

To confirm our hypothesis that root samples release more BITC than aerial portion, roots samples derived from the three origins were modelled as one class group against stem sample using supervised OPLS-DA. The derived score plot showed a clear separation between both samples (Fig. 2A). The model explains 97% of the total variance (R2 = 0.97) with the prediction goodness parameter Q2 = 0.94. The S-plot is a particularly useful tool to visualize an OPLS classification model. The S-plot results (Fig. 2B) showed that root samples were indeed much enriched in MS signals of BITC and confirming volatile results derived from the PCA loading plot (Suppl. Fig. S1C).

Table 1
Relative percentage of volatile compounds in *S. persica* accessions: SPS1, SPR1, SPR2 and SPR3 as analyzed *via* SPME-GC–MS. (n = 3).

	RT (min)	KI*	Identification	SPS1	SPR1	SPR2	SPR3
1	8.03	960	Benzaldehyde	12.26	0.01	0.06	0.17
2	10.22	1095	Nonanal	6.18	–	0.02	0.03
3	10.57	1119	Benzyl isocyanate	0.24	–	–	0.01
4	10.85	1139	Benzyl nitrile	33.69	0.04	0.33	0.15
5	11.55	1188	Benzoic acid	0.23	0.01	0.05	0.03
6	11.61	1192	α-Terpineol	0.63	–	–	0.01
7	11.63	1195	Naphthalene	2.05	–	0.05	0.01
8	11.66	1199	n-Octyl acetate	3.81	–	0.06	0.43
9	11.89	1214	2,2,4-Trimethyl-2,5-dihydrofuran	1.40	–	0.01	0.02
10	12.73	1277	(*E*)-Cinnamaldehyde	3.18	0.04	0.03	0.15
11	13.03	1300	3-Indolecarbonitrile	0.73	–	0.01	0.01
12	13.51	1339	α-Terpineol acetate	2.21	–	0.02	0.02
13	13.65	1351	Unknown nitrile	0.39	–	0.01	–
14	13.86	1368	Benzyl isothiocyanate (BITC)	25.61	99.81	99.26	98.85
15	13.99	1379	Oxalic acid, butyl propyl ester	1.61	0.06	0.01	0.02
16	14.00	1400	Dodecane	0.69	0.01	0.01	0.01
17	14.53	1422	Zingiberene	1.08	–	0.01	0.01
18	15.32	1495	(*E*)-β-Farnesene	1.90	0.01	0.01	0.02
19	15.43	1499	β-Bisabolene	1.59	–	0.01	0.02
20	15.83	1525	Butyl maleate	0.11	–	–	–
21	20.66	1798	Isopropyl myristate	0.40	–	–	0.01

*KI = Kovat Index.
-, absent.

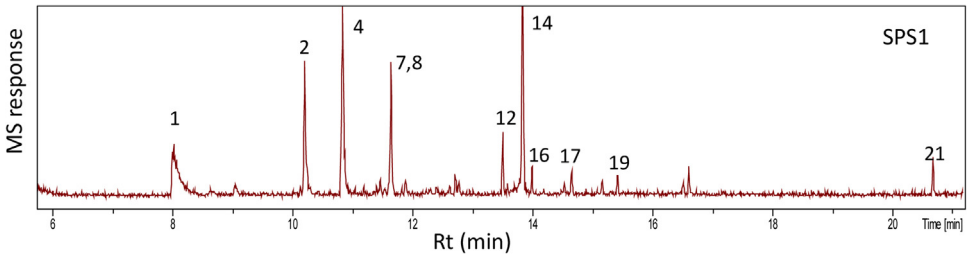

Fig. 1. SPME GC–MS chromatogram of *S. persica* stem SPS1. The corresponding compound names for volatile peaks follow that listed in Table 1.

3.3. *Effect of salivation on volatiles released from S. persica root, stem and its toothpaste*

To better evaluate the role of saliva on the release of BITC from *S. persica* root or toothpaste commercial preparation, volatile collection was attempted post incubation of plant material or toothpaste preparation with saliva for 15 min with gentle agitation (see experimental Section 2.5.). Release of BITC is known to occur also when *S. persica* is chewed on prior to cleansing of teeth [5]. Previous studies of volatiles analyses revealed that human saliva can impact positively or negatively aroma. Saliva has a complex composition of protein and ion components forming a viscoelastic solution capable of many effects among which affecting aroma release is one [21]. Results of the samples' volatiles analysis post incubation with human saliva showed the presence of one major peak for BITC quantified at a level of 0.5 and 5 ug g^{-1} from stem and root (Fig. 3A & B), respectively and lower than that detected in undigested root and stem samples ranging from 10 to 25 ug g^{-1}. The disappearance of other volatiles may be attributed to the ability of the saliva protein "mucin" to bind aroma compounds leading to reduction in aroma release or to enzymes *i.e.* α-amylase and lipase interaction with the aroma. Other scenario leading to VOCs reduction is *via* the formation of aggregates from the plant bioactive constituents *i.e.*, polyphenols and carbohydrates forming saliva-protein −polyphenol-carbohydrate complex which can encapsulate the hydrophobic aroma molecules [22]. Interestingly, toothpaste preparation claimed to contain *S. persica* extract from several commercial batches showed no peak of BITC either pre or post saliva incubation, being replaced by a major peak identified as (*E*)-anethole as detected *via* headspace SPME analysis (Fig. 3C). (*E*)-Anethole is a well-known flavoring agent in many dental preparations *i.e.* mouth washes and toothpastes [23]. Whether absence of BITC in toothpaste preparation is due to the source of *S. persica* extract or a loss during manufacture cannot be determined. Nevertheless, our results revealed that measurement of this major antimicrobial volatile should be taken in consideration for the QC analysis of *S. persica* dental products.

To provide more insight of salivation effect on aroma release in *S. persica,* we attempted to analyze extract directly post salivation using ultra performance liquid chromatography coupled to mass spectrometry (UPLC–MS). Compared to SPME GC–MS, UPLC–MS can directly measure level of volatiles in solution, ruling out whether decrease in BITC levels was due to degradation or volatile saliva protein interaction affecting its volatility. Reconstructed ion chromatogram of *m*/*z* 149, molecular ion for BITC in *S. persica* root extracted either post water or saliva incubation showed no difference in their BITC peak area compared to that of uninucbated *S. persica* root with saliva (Suppl. Fig. S2). These results are not in agreement with previous report showing that saliva exposure enhance the release of BITC from *S. persica* upon chewing [5]. It should be noted that other volatiles monitored by GC–MS were not detected using LC–MS, either due to their low abundance and or lack of ionization potential to be ionized using electrospray ionization (ESI) conditions. BITC contain a heteroatom nitrogen and preferentially ionizes in positive ionization mode. Detailed analysis of non-volatile polar constituents *i.e.* glycosides and alkaloids in *S. persica via* UPLC–MS is underway and should be of interest to complement our current metabolite profiling in *S. persica*. Whether

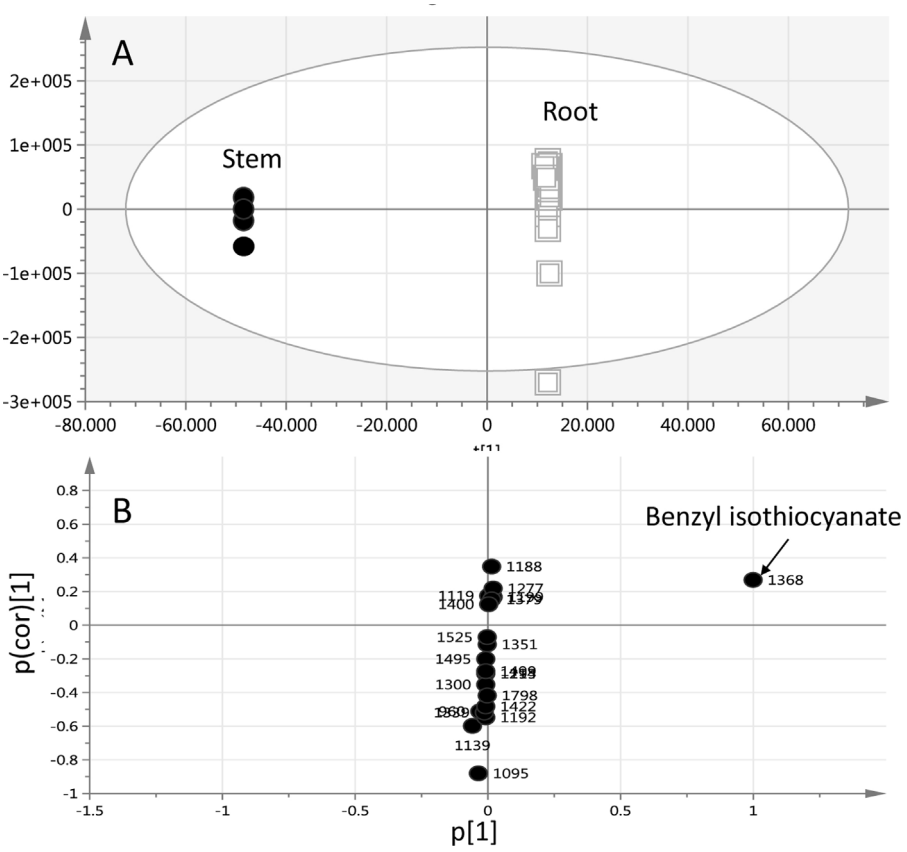

Fig. 2. (A) OPLS-DA score plot and (B) loading S-plot derived from modelling *S. persica* root samples in one group (□) against stem (●) .The S-plot showed the covariance p[1] against the correlation p(cor) [1] of the variables of the discriminating component of the OPLS-DA model. Cut-off values of $P < 0.01$ were used. Each volatile is denoted by its KI value listed in Table 1.

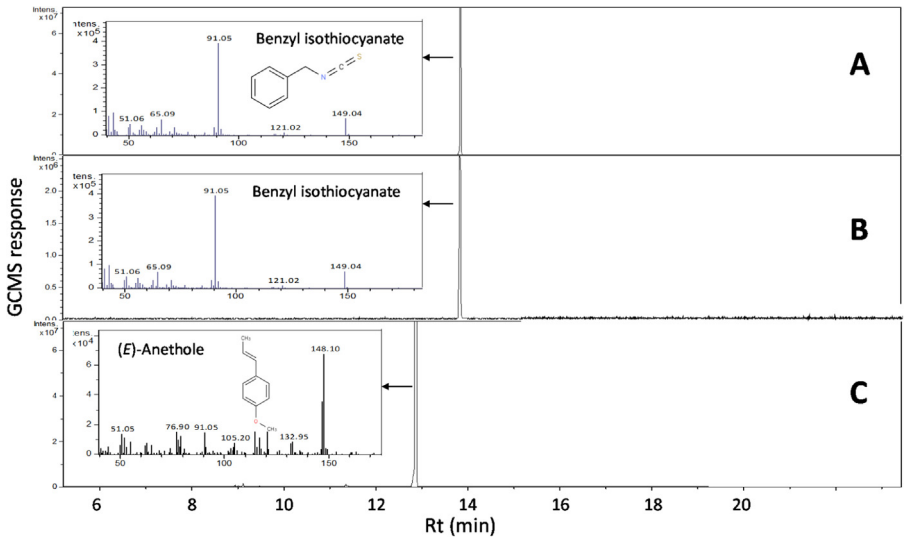

Fig. 3. SPME GC–MS chromatograms of volatiles collected from *S. persica* stem SPS1 (A), root SPR1 (B) and commercial toothpaste (C) stated to contain *S. persica* extract after incubation with saliva for 15 min prior to volatiles collection. Inside showed MS spectrum of BITC and (*E*)-anethole.

lack of antiseptic effect found in *S. persica* mouthwash reported by Saffari et al., [7] was also attributed to BITC depletion as observed in toothpaste preparation herein has yet to be examined. Assessing the antimicoribal effect of *S. persica* with and without saliva exposure shall provide a definitive role for saliva in affecting the plant oral health effects.

3.4. GC–MS analysis of silylated primary metabolites

To provide an overview of *S. persica* primary metabolites *viz.* sugar and organic acids that can contribute to either its antimicrobial effect or organoleptic characters, GC–MS was adopted for primary metabolites profiling with silylation (Suppl. Fig. S3). The analysis resulted in the detection of 75 metabolites (Table 2). Identified primary metabolites included saccharides (mono- and di-)

Table 2
Relative percentage of silylated primary metabolites in *S. persica* accessions: SPS1, SPR1, SPR2 and SPR3 via GC–MS measurements.

RT (min)	KI*	Identification	Class	SPS1	SPR1	SPR2	SPR3
5.23	815	1,2-Propanediol-TMS	Alcohol	0.56	12.09	4.41	21.73
5.81	843	2,3-Butanediol-TMS	Alcohol	1.81	0.20	0.27	0.56
9.11	1025	Glycerol-TMS	Alcohol	10.88	0.10	1.94	1.09
		Total alcohols		13.25	12.39	6.62	23.38
6.64	884	L-Alanine-TMS	Amino acid	0.17	1.21	9.88	2.50
9.06	1022	L-leucine-TMS	Amino acid	0.18	0.06	0.27	0.80
9.42	1045	L-leucine isomer-TMS	Amino acid	0.28	0.19	1.12	0.86
10.31	1102	L-Serine-TMS	Amino acid	0.01	0.07	0.05	0.12
10.68	1128	L-threonine-TMS	Amino acid	0.01	0.01	0.02	0.03
12.36	1250	L-Aspartic acid-TMS	Amino acid	0.59	0.13	0.19	0.85
13.25	1319	L-Asparagine-TMS	Amino acid	0.04	0.13	0.08	0.14
		Total Amino acids		1.28	1.8	11.61	5.3
5.89	849	2-acetoxyacetate-TMS	Organic acid	9.88	1.19	1.13	1.03
6.05	855	Lactic acid-TMS	Organic acid	2.06	0.80	40.85	15.58
6.24	865	Acetic acid-TMS	Organic acid	0.92	0.14	0.12	0.12
6.44	874	Pyruvic acid-TMS	Organic acid	1.25	0.30	0.80	0.43
7.21	914	Propanoic acid-TMS	Organic acid	2.98	0.46	1.84	0.74
7.29	918	Pentanoic acid-TMS	Organic acid	0.50	0.06	0.07	0.07
8.08	963	Malonic acid-TMS	Organic acid	2.20	0.25	0.30	0.27
8.13	966	Acetoacetic acid-TMS	Organic acid	0.54	0.06	0.06	0.06
9.53	1052	3-oxobutanoic acid-TMS	Organic acid	0.33	0.05	0.12	0.05
9.59	1057	Succinic acid-TMS	Organic acid	0.18	0.07	1.43	0.40
9.71	1063	2-Ketoglutaric acid-TMS	Organic acid	1.78	0.23	0.27	0.19
9.77	1067	Butanedioic acid, methyl-, –TMS	Organic acid	0.24	0.03	0.04	0.02
9.91	1074	Glyceric acid-TMS	Organic acid	0.02	0.01	0.09	0.09
9.92	1076	Mevalonic lactone-TMS	Organic acid	0.79	0.08	0.09	0.07
10.01	1082	Fumaric acid-TMS	Organic acid	0.16	0.02	0.01	0.02
10.72	1130	Methylmaleic acid-TMS	Organic acid	0.05	0.01	0.01	0.01
10.87	1136	Glutaric acid-TMS	Organic acid	0.06	0.01	0.01	0.01
11.96	1219	Malic acid-TMS	Organic acid	0.36	1.60	0.04	0.18
		Total organic acids		24.31	5.37	47.28	19.34
12.29	1244	meso-Erythritol-TMS	Sugar	17.78	0.03	0.96	0.17
13.56	1343	D-Ribofuranose-TMS	Sugar	0.08	0.06	0.30	3.27
13.61	1348	D-Arabinopyranose-TMS	Sugar	0.04	0.03	0.16	1.21
13.98	1378	D-Arabose-TMS	Sugar	0.17	0.08	0.52	4.04
14.48	1418	D-Erythrotetrofuranose-TMS	Sugar	0.23	0.01	0.10	0.04
14.75	1441	meso-Erythritol isomer-TMS	Sugar	0.21	0.01	0.06	0.07
14.86	1450	D-Arabitol-TMS	Sugar	2.54	0.02	0.12	0.08
15.86	1529	D-Fructofuranose-TMS	Sugar	0.79	4.07	7.01	9.04
16.11	1550	D-Glucofuranoside-TMS	Sugar	0.30	1.96	1.05	1.95
16.58	1577	Ribonic acid-TMS	Sugar	0.60	0.91	0.93	1.21
16.70	1585	D-Allopyranose-TMS	Sugar	0.29	5.66	4.73	6.67
16.88	1598	Ribitol-TMS	Sugar	0.30	1.34	1.17	3.23
17.04	17.045	1,5-Anhydro-D-sorbitol-TMS	Sugar	0.22	1.37	0.33	0.98
17.14	1612	D-Mannitol-TMS	Sugar	0.14	0.02	0.96	3.58
17.53	1633	Lyxose-TMS	Sugar	0.33	5.94	5.33	7.08
21.11	1821	D-Glucuronic acid-TMS	Sugar	0.06	0.14	0.16	0.32
22.39	1888	Sucrose-TMS	Sugar	0.53	10.92	2.41	1.18
22.65	1901	3-α-Mannobiose-TMS	Sugar	2.81	11.23	0.19	0.10
23.08	1924	3-α-Mannobiose-TMS	Sugar	0.38	3.71	0.23	0.04
23.38	1939	Unknown disaccharide	Sugar	0.09	3.47	0.82	0.31
		Total sugars		27.89	50.98	27.54	44.57
8.68	997	Benzoic acid-TMS	Aromatic	0.45	0.17	0.33	0.16
		Total aromatics		0.45	0.17	0.33	0.16
8.29	975	*N*-benzylamine-TMS	Nitrogenous	0.02	3.14	3.85	4.97
8.49	986	Urea-TMS	Nitrogenous	2.82	0.05	0.07	0.07
13.35	1335	Benzyloxyamine-TMS	Nitrogenous	0.02	0.01	0.01	0.04
		Total Nitrogenous		2.86	3.20	3.93	5.08
8.86	1009	Octanoic acid-TMS	Fatty acid	0.05	0.01	0.01	–
10.18	1093	Nonanoic acid-TMS	Fatty acid	0.11	0.01	0.01	0.01
13.80	1362	Lauric acid-TMS	Fatty acid	0.25	0.02	0.03	0.02
15.93	1532	Myristic acid-TMS	Fatty acid	0.44	0.04	0.06	0.03
16.93	1601	Pentadecanoic acid-TMS	Fatty acid	0.18	0.02	0.05	0.04
17.65	1640	Hexadecenoic acid-TMS	Fatty acid	0.12	0.03	0.05	0.06
17.70	1642	Palmitelaidic acid-TMS	Fatty acid	0.07	0.01	0.04	0.03
17.88	1652	Palmitic acid-TMS	Fatty acid	9.74	0.38	1.10	0.83
18.61	1691	Heptadecenoic acid-TMS	Fatty acid	0.06	–	0.02	0.02
18.8	1701	Heptadecanoic acid- TMS	Fatty acid	0.26	0.01	0.05	0.05
19.43	1734	Linoleic acid-TMS	Fatty acid	0.87	0.02	0.16	0.12
19.47	1736	Oleic acid-TMS	Fatty acid	3.44	0.09	0.48	0.48
19.68	1747	Stearic acid-TMS	Fatty acid	1.81	0.10	0.12	0.10

Table 2 (*Continued*)

RT (min)	KI*	Identification	Class	SPS1	SPR1	SPR2	SPR3
21.34	1833	Arachidic acid-TMS	Fatty acid	0.21	0.01	0.02	0.01
22.88	1914	Docosanoic acid-TMS	Fatty acid	0.47	0.02	0.03	0.02
		Total fatty acids		18.08	0.77	2.23	1.82
24.32	1988	Squalene	Sterol/triterpene	0.48	0.02	0.03	0.02
29.44	2256	Stigmasterol-TMS	Sterol/triterpene	0.16	0.02	0.02	0.01
30.33	2302	β-Sitosterol-TMS	Sterol/triterpene	0.83	0.17	0.11	0.14
		Total sterol/triterpenes		1.47	0.21	0.16	0.17
23.66	1954	Tetracosan-1-ol-TMS	Miscellanious	0.58	0.01	0.02	0.02
4.82	796	Carbonate-TMS	Miscellanious	5.90	0.30	0.16	0.14
11.74	1202	Unknown	Miscellanious	0.14	0.01	0.04	0.01
19.11	1717	Tetramethyl-2-hexadecene	Miscellanious	1.34	0.01	0.03	0.03
22.19	1877	1-Docosanol-TMS	Miscellanious	2.42	0.02	0.02	0.02
		Total miscellaneous		10.38	0.35	0.27	0.22

*KI = Kovat Index.

as well as fatty acids, amino acids and organic acids in addition to one inorganic compound *i.e.* carbonate. Carbonate is known to exert a bactericidal effect at high pH values (8.5) [24] and whether it contribute to the traditional use of *S. persica* as toothbrush is not determined. Sugars represented the major class (*ca.* 25–50% of the total ion count), and are likely to account for *S. persica* palatable taste, albeit with difference in their individual sugars composition. Whereas stem was found more enriched in sugar polyols *i.e.* erythritol and arabitol, cyclic monosacharides *i.e.* glucose and fructose amounted as the major sugar forms in root specimens. The enrichment of sugar polyols in stem sample was correlated by an abundance of its biosynthetic precursor glycerol [25] found at 10.8% in stem sample, albeit at trace levels in root (Table 2) which justify for the abundance of sugar alcohols in the aerial part. Stem appeared compromised in its BITC content compared to root as revealed *via* headspace SPME analysis (Table 1), the abundance of unfermentable sugar alcohols in stem (74%) favor more its use as toothbrush.

Several reports point to sugar polyols oral health effects including its antimicrobial action against several species of *streptococci*, plaque build-up as well as tooth decay prevention [26,27]. Similar accumulation pattern was observed in monitored nitrogenous metabolites found at comparable levels in root and stem specimens (3–5%), albeit with urea accounting for 98% of such class in stem versus *N*-benzyl amine 98% in root. Urea presence in aerial part is likely to serve as precursor for the urea derivative salvadourea known to accumulate in *S. persica* [28]. The lower abundance of *N*-benzyl amine in stem was found to negatively correlate with the accumulation of benzyl nitrile in that organ. Amino acids and sterols were both found at comparable levels in all specimens at *ca.* 0.2–2.0% for each class. Stem extract was more enriched in fatty acids (18%) compared to roots (1–2%), with palmitic acid (10%) and oleic acid (3%) as major forms. Oleic and linoleic acids have been suggested to contribute to miswak cleansing efficacy *via* leaching out in saliva, and for their antimicrobial effect [7].

One metabolite with negative impact on teeth health detected at high levels almost only in SPR2 accession was lactic acid found at *ca.* 40% and much lower levels in all other samples. Lactic acid creates extra acidity to decrease the pH to the extent of dissolving the calcium phosphate in the tooth enamel leading to the start of a cavity [29]. Considering, the negative impact of such metabolite, it should be considered in the QC evaluation of *S. persica* products.

3.5. Multivariate data analysis of silylated primary metabolites

Considering the relatively large number of peaks ca. 77 in 4 specimens with 3 biological replicates for each totaling 12 samples studied herein, multivariate data analyses viz. hierarchical clustering analysis (HCA) and principal component analysis (PCA) were applied for samples classification. The silylated GC–MS dataset was further subjected to PCA analysis as adopted for volatiles analysis (Suppl. Fig. S4). The PCA score plot (Fig. S4A) revealed 4 confined clusters, with stem samples positioned to the right lower quadrant of the vertical line representing PC1 (positive PC1 values) and the other ones corresponding to the three root samples being positioned on the three other quadrants along PC1 and PC2 to the right of the plot (positive PC1 values), with the main principal component to differentiate between samples, i.e. PC1, accounting for 47% of the variance. In general, better separation of samples was observed compared to that derived from volatile dataset PCA (Suppl. Fig. S1B) but still showing stem as the most distinct compared to root accessions. Examination of loading plots (Suppl. Fig. S4B) revealed that lactic acid and 1, 2-propanediol that contributed the most in samples segregation, being more abundant in *S. persica* root samples SPR1 and SPR2. To help identify variation in other metabolites, aside from lactic acid and its reduced product, 1, 2-propanediol showing the highest variance across samples; PCA (Suppl. Fig. S4C) was further performed for classification excluding lactic acid and 1, 2-propanediol MS signals from the data set. The main PC to differentiate samples in PCA, that is, PC1, accounted for 52% of the variance. Examination of the loadings plot (Suppl. Fig. S4D) revealed that variables corresponding to uncyclized sugar polyol "meso-erythritol" was found most enriched in stem sample SPS1, whereas cyclic sugars disaccharide *i.e.* sucrose, mannobiose and allopyranose (monoscharide) were more abundant in root samples. Meso-erythritol was detected at *ca.* 20–100 fold levels in stem sample versus roots (Table 2). Although *S. persica* stem sample was less compromised in its BITC content compared to root (Table 1), the enrichment of sugar alcohols in stem is considered favored and might enhance its antimicrobial efficacy. Meso-erythritol is a naturally occurring sugar alcohol with 60–70% of the sweetness of sucrose. It is used mainly as sucrose substitute due to its low caloric value (0.0–0.2 kcal/g), lack of off-taste, non-cariogenic, and is not fermented by oral bacteria compared to glucose being less prone to cause teeth decay [30]. To also confirm whether variant masses revealed from PCA analysis of primary metabolites in *S. persica* were sufficiently unique to be identified as markers for its organ type, OPLS-DA modelling was applied. A model was constructed with stem (Fig. 4A) modelled against all other three root specimens, present in one class group. The model showed one orthogonal component with R2 = 0.90 and Q2 = 0.98. The S-plot results (Fig. 4B) showed that stem was particularly enriched in meso-erythritol whereas lactic acid and 1, 2-propanediol amounted as major forms in root. It remains to be examined whether differential metabolite accumulation patterns are due to precursor limitations, growing habitat or to genetic differences in expression, regulation and enzymatic activity. Although, glycerol the direct precursor for the formation of meso-erythritol [25] was found at

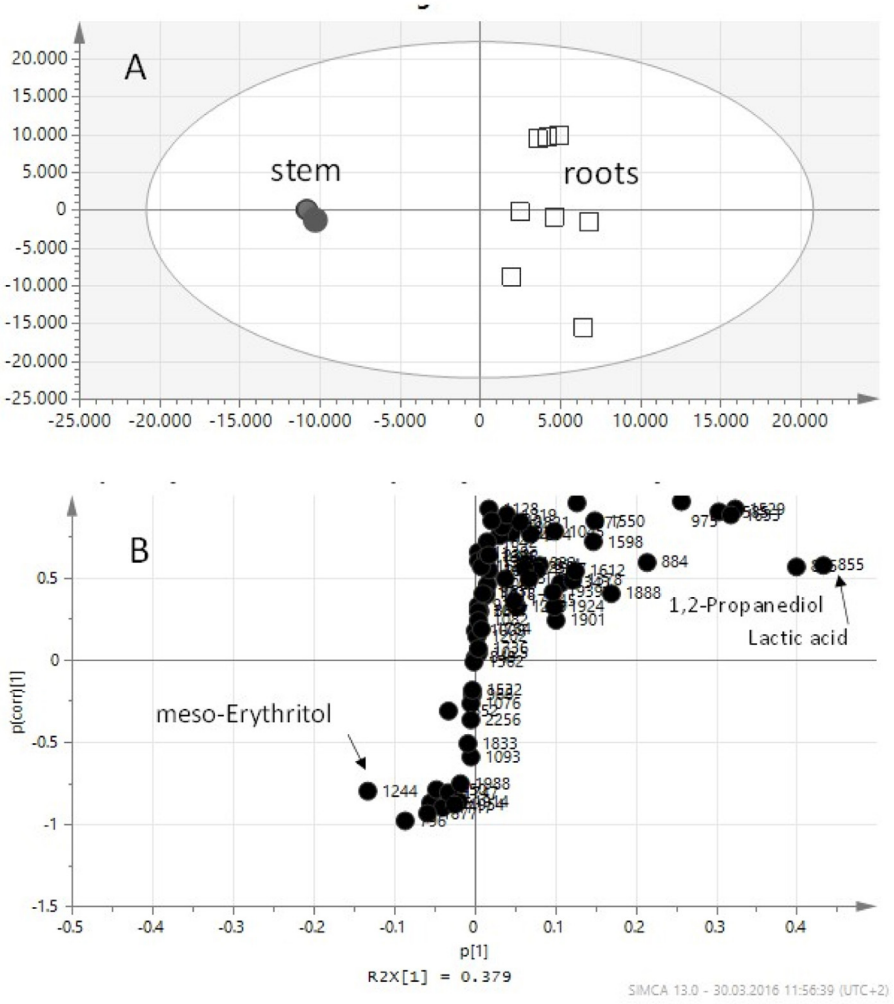

Fig. 4. OPLS-DA score plot (A) and loading S-plot (B) from modelling *S. persica* root samples in one group (□) against stem (⬭) of its silylated primary metabolites. The S-plot showed the covariance p[1] against the correlation p(cor)[1] of the variables of the discriminating component of the PLS-DA model. Cut-off values of $P < 0.01$ were used; variables selected are highlighted in the S-plot with KI values and identifications are discussed in the text.

10 to 100 folds level in stem sample compared to root, suggesting that glycerol pool limitation in root that might account for its absence in that organ.

4. Conclusion

In conclusion, results presented herein provide better insight on metabolites based rational for the antimicrobial effect of *S. persica* root and its aerial part used as toothbrush. Additionally, results present an additional evidence for the efficacy and complementarity of headspace SPME and GC–MS volatiles profiling and primary metabolites analysis in this plant. While stem appeared compromised in BITC content, its sugar content and primary metabolite profile was more favored being enriched in sugar alcohols *i.e.*, meso-erythritol versus cyclic sugars in root. In the field of quality control analysis, this example demonstrates the potential of modern metabolomics to provide better measures for QC analysis of *S. persica* based on its metabolite profile instead of targeting just one chemical. We forecast that the implementation of other spectroscopic fingerprinting techniques *i.e.* NMR, applied to *S. persica* crude extracts will be increasingly adopted for its quality control analysis. It has yet to be examined, if other constituents found in *S. persica* matrix *viz*, sulphur glycosides, alkaloids, sugar alcohols or flavonoids, can additively or synergistically enhance the antimicrobial effect of BITC. The absence of BITC in a commercial toothpaste preparation warrants the development of better quality control measures for *S. persica* dental preparations QC analysis. Additionally, the presence of lactic acid in one of the root accessions warrant it monitoring upon evaluating *S. persica* dental products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.11.018.

References

[1] B.H. Aboul-Enein, The miswak (Salvadora persica L.) chewing stick: cultural implications in oral health promotion, Saudi J. Dent. Res. 5 (2014) 9–13, http://dx.doi.org/10.1016/j.ksujds.2013.06.002.

[2] F. Niazi, M. Naseem, Z. Khurshid, M.S. Zafar, K. Almas, Role of Salvadora persica chewing stick (miswak): a natural toothbrush for holistic oral health, Eur. J. Dent. 10 (2016) 301–308, http://dx.doi.org/10.4103/1305-7456.178297.
[3] H. Deswal, Y. Singh, H.S. Grover, A. Bhardwaj, S. Verma, Miswak As an alternative treatment modality in medicine and dentistry: a review, Innovare J. Life Sci. 4 (2016) 6–8.
[4] M. Abhary, A.-A. Al-Hazmi, Antibacterial activity of Miswak (Salvadora persica L.) extracts on oral hygiene, J. Taibah Univ. Sci. 10 (2015) 513–520, http://dx.doi.org/10.1016/j.jtusci.2015.09.007.
[5] A. Sofrata, E.M. Santangelo, M. Azeem, Benzyl Isothiocyanate, a major component from the roots of Salvadora persica is highly active against gram-negative bacteria, PLoS One 6 (2011) e23045, http://dx.doi.org/10.1371/journal.pone.0023045.
[6] S. Kumar, C. Rani, M. Mangal, A critical review on Salvadora persica: an important medicinal plant of arid zone, Int. J. Phytomed. 4 (2012) 292–303.
[7] H. Ahmad, K. Rajagopal, Biological activities of Salvadora persica L. (Meswak), Med. Aromat. Plants 2 (2013) 1–5, http://dx.doi.org/10.4172/2167-0412.1000129.
[8] E. Noumi, M. Snoussi, N. Trabelsi, H. Hajlaoui, R. Ksouri, E. Valentin, A. Bakhrouf, Antibacterial, anticandidal and antioxidant activities of Salvadora persica and Juglans regia L. extracts, J. Med. Plants Res. 5 (2011) 4138–4146.
[9] S. Koning, H.-G. Janssen, U.a.T. Brinkman, Modern methods of sample preparation for GC analysis, Chromatographia 69 (2009) 33–78, http://dx.doi.org/10.1365/s10337-008-0937-3.
[10] M. Kamil, F. Ahmad, A.F. Jayaraj, C. Gunasekhar, S. Thomas, M. Habibullah, K. Chan, Isolation and identification of a flavonol glycoside using high speed counter current chromatographic technique from the leaves of Salvadora persica, Pakistan, J. Sci. Ind. Res. 43 (2000) 255–257.
[11] T.K.T. Do, F. Hadji-Minaglou, S. Antoniotti, X. Fernandez, Authenticity of essential oils, Trends Anal. Chem. 66 (2015) 146–157, http://dx.doi.org/10.1016/j.trac.2014.10.007.
[12] J.B. Coble, C.G. Fraga, Comparative evaluation of preprocessing freeware on chromatography/mass spectrometry data for signature discovery, J. Chromatogr. A. 1358 (2014) 155–164, http://dx.doi.org/10.1016/j.chroma.2014.06.100.
[13] M.a. Farag, L.a. Wessjohann, Volatiles profiling in medicinal licorice roots using steam distillation and solid-phase microextraction (SPME) coupled to chemometrics, J. Food Sci. 77 (2012) C1179–C1184, http://dx.doi.org/10.1111/j.1750–3841.2012.02927.x.
[14] M.a. Farag, D.M. Rasheed, I.M. Kamal, Volatiles and primary metabolites profiling in two Hibiscus sabdariffa (roselle) cultivars via headspace SPME-GC–MS and chemometrics, Food Res. Int. 78 (2015) 327–335, http://dx.doi.org/10.1016/j.foodres.2015.09.024.
[15] C.D. Broeckling, I.R. Reddy, A.L. Duran, X. Zhao, L.W. Sumner, MET-IDEA. data extraction tool for mass spectrometry-based metabolomics MET-IDEA: data extraction tool for mass spectrometry-based metabolomics, Anal. Chem. 78 (2006) 4334–4341, http://dx.doi.org/10.1021/ac0521596.
[16] F. Alali, T. Al-Lafi, GC–MS analysis and bioactivity testing of the volatile oil from the leaves of the toothbrush tree Salvadora persica L, Nat. Prod. Res. 17 (2003) 189–194, http://dx.doi.org/10.1080/1057563021000040790.
[17] M. Fiume, Final report on the safety assessment of benzaldehyde, Int. J. Toxicol. 25 (2003) 11–27, http://dx.doi.org/10.1177/1091581803022S203.
[18] I. Ullah, A.L. Khan, L. Ali, A.R. Khan, M. Waqas, J. Hussain, I.J. Lee, J.H. Shin, Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by Photorhabdus temperata M1021, J. Microbiol. 53 (2015) 127–133, http://dx.doi.org/10.1007/s12275-015-4632-4.
[19] N. Didry, L. Dubreuil, M. Pinkas, Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria, Pharm. Acta Helv. 69 (1994) 25–28, http://dx.doi.org/10.1016/0031-6865(94)90027-2.
[20] R. Goodacre, B. Shann, R.J. Gilbert, Adaoin M. Timmins, A.C. McGovern, B.K. Alsberg, D.B. Kell, N.a. Logan, Detection of the dipicolinic acid biomarker in Bacillus spores using curie-point pyrolysis mass spectrometry and fourier transform infrared spectroscopy, Anal. Chem. 72 (2000) 119–127, http://dx.doi.org/10.1021/ac990661i.
[21] G.H. Carpenter, The secretion, components, and properties of saliva, Annu. Rev. Food Sci. Technol. 4 (2013) 267–276, http://dx.doi.org/10.1146/annurev-food-030212-182700.
[22] C. Muñoz-González, G. Feron, E. Guichard, J.J. Rodríguez-Bencomo, P.J. Martín-Álvarez, M.V. Moreno-Arribas, M.Á. Pozo-Bayón, Understanding the role of saliva in aroma release from wine by using static and dynamic headspace conditions, J. Agric. Food Chem. 62 (2014) 8274–8288, http://dx.doi.org/10.1021/jf503503b.
[23] T.S.C. Poon, S. Freeman, Cheilitis caused by contact allergy to anethole in spearmint flavoured toothpaste, Australas. J. Dermatol. 47 (2006) 300–301, http://dx.doi.org/10.1111/j.1440-0960.2006.00300.x.
[24] G.N. Jarvis, M.W. Fields, D. a. Adamovich, C.E. Arthurs, J.B. Russell, The mechanism of carbonate killing of Escherichia coli, Lett. Appl. Microbiol. 33 (2001) 196–200, http://dx.doi.org/10.1046/j.1472-765X.2001.00976.x.
[25] W. Rymowicz, A. Rywińska, M. Marcinkiewicz, High-yield production of erythritol from raw glycerol in fed-batch cultures of Yarrowia lipolytica, Biotechnol. Lett. 31 (2009) 377–380, http://dx.doi.org/10.1007/s10529-008-9884-1.
[26] A. Al Loman, L.K. Ju, Inhibitory effects of arabitol on caries-associated microbiologic parameters of oral Streptococci and Lactobacilli, Arch. Oral Biol. 60 (2015) 1721–1728, http://dx.doi.org/10.1016/j.archoralbio.2015.09.004.
[27] N.M. Alamoudi, A.G. Hanno, H.J. Sabbagh, M.I. Masoud, A.S. Almushayt, D.A. El Derwi, Impact of maternal xylitol consumption on mutans streptococci, plaque and caries levels in children, J. Clin. Pediatr. Dent. 37 (2012) 163–166.
[28] A. Ray, L. Chand, S. Dutta, Salvadourea: a new urea derivative from Salvadora persica – linn, Chem. Ind. (1975) 517–518.
[29] A. Shimada, M. Noda, Y. Matoba, T. Kumagai, K. Kozai, M. Sugiyama, Oral lactic acid bacteria related to the occurrence and/or progression of dental caries in Japanese preschool children, Biosci. Microbiota Food Health 34 (2015) 29–36, http://dx.doi.org/10.12938/bmfh.2014-015.
[30] H.J. Moon, M. Jeya, I.W. Kim, J.K. Lee, Biotechnological production of erythritol and its applications, Appl. Microbiol. Biotechnol. 86 (2010) 1017–1025, http://dx.doi.org/10.1007/s00253-010-2496-4.