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Review

Recent advances in analytical approaches for the standardization and quality of polyphenols of propolis

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Analytical approaches utilized for the characterization of polyphenols from propolis useful for the determination of its quality is investigated in this study. A qualitative and quantitative evaluation of propolis bioactive molecules is of interest in medicine and nutraceuticals. Recent powerful analytical techniques are of great utility to separate and quantify polyphenols in extracts and finished products due to their capacity to produce typical fingerprints and a reliable identification of many components. According to this, an HPLC-UV-MS procedure was validated and applied for the characterization and quantification of bioactive substances in propolis and for an accurate assessment of their content in extract samples. By using this analytical approach, we obtained specific compositions related to brown propolis acquired from different geographic areas (and preparations and treatment). This is more important by considering the scientific opinion of European Food Safety Authority (EFSA) which provided a negative response related to health claims of propolis and its polyphenols. These results prove that HPLC-MS is an attractive tool for the standardization and quality control of propolis and may be realistically applied to screen raw material and to evaluate finished commercial preparations and nutraceutical benefits.

Key words: Propolis, polyphenols, flavonoids, phenolic acids, high-performance liquid chromatography-mass spectrometry (HPLC-MS), quality control, standardization

INTRODUCTION

Propolis is a resin-like material from the buds of poplar trees and is rarely available in its pure form. It contains bee products, and has a long history of medicinal use, dating back to 350 B.C. Propolis is collected by honeybees from various plants and trees, in particular from the poplar (*Populus*) genus but also from beech, horsechestnut, birch and conifer. This resinous material is mixed with wax and used in the construction and adaptation of bee nests (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). In spite of possible differences in the collected resins from various plant sources and their active molecular composition, most raw propolis looks quite similar consisting of resin, wax, essential oils and minor components such as pollen and organic compounds (Volpi, 2004; Volpi and Bergonzini, 2006). Resin is one of the most active

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fraction of propolis constituted of the polyphenolic component comprising flavonoids and related phenolic acids (Salatino et al., 2011; Viuda-Martos et al., 2008). Bees use propolis for mechanical aims and for its biological properties. In fact, its antibacterial capacity is an important property also used by human beings for therapeutic applications (Castaldo and Capasso, 2002; Sforcin, 2007; Watanabe et al., 2011). Moreover, many studies focused the attention on other possible beneficial activities of propolis such as anti-inflammatory, antiviral, antiulcer, local anaesthetic, hepatoprotective and immune-stimulating (Banskota et al., 2001; Castaldo and Capasso, 2002; Sforcin, 2007; Viuda-Martos et al., 2008; Salatino et al., 2011; Watanabe et al., 2011).

For the above-mentioned biological activities, propolis has been largely used in folk medicine due to the presence of more than 300 identified components, and many of them are biologically active, such as several aromatic compounds, flavonoids, prenylated *p*-coumarinic acids, acetophenone derivatives, caffeoylquinic acids, lignans, diterpenic acids, triterpenes as well as volatile compounds (monoterpenes, sesquiterpenes and aromatic compounds), sugars and derivatives (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011; Pasupuleti et al., 2017). Polyphenols are the most representative biomolecules possessing at least one aromatic ring with one or more hydroxyl functional groups. Flavonoids represent the most abundant group of phenolic compounds having their structures based on a C6-C3-C6 skeleton (Figure 1A) and classified into several classes such as chalcones, flavones, flavonols, flavanones, isoflavonoids, anthocyanidins and flavanols (catechins and tannins). Non-flavonoids species are composed of simple phenols, phenolic acids, coumarins, xanthones, stilbenes, lignins and lignans. Phenolic acids are further classified in benzoic acid derivatives, formed of a C6-C1 skeleton, and cinnamic acid derivatives, constituted of a C6-C3 skeleton (Salatino et al., 2011) (Figure 1B). Besides the presence of many of the above-mentioned compounds, the variability of propolis chemical composition is more complicated by several glycoside phenolic derivatives making the analysis of these molecules a very hard challenge.

Current applications of propolis include preparations mainly based on extracts (ethanolic, water, glygeric, glycolic, oily or mix of this) and specific components may be selected during the extractive and preparative processes (Galeotti et al., 2017; Galeotti et al., 2018). These processes remove wax and other inert material and enrich the polyphenolic active compounds. In fact, commercial and nutraceutical health care products contain propolis solubilized in various solvents such as organic, water, oily or mix (Galeotti et al., 2018).

As mentioned above, propolis is a complex and heterogeneous material possessing a variable physical consistence, color, fragrance and active components depending on many factors such as types of vegetable sources, geographic origin, season of collection as well as the type of collecting bees (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). Moreover, its biological properties and antibiotic activity are the result of completely different chemical composition and content of the actives. On this basis, many analytical approaches have been developed to analyse the raw propolis as well as the many derived finished products in an effort to standardize the collecting material, the derivatives generated during the preparative processes and the products dissolved in many solvents and matrices for commercial purposes. In particular, many analytical methods are available with the aim to standardize the polyphenol active propolis component by a chemical point of view as well as biological activity. Thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the most applied analytical methods. Moreover, the advent of mass detectors and soft ionization approaches such as electrospray ionization (ESI) combined with HPLC and further tandem mass spectrometry (MS/MS) has facilitated the study of polyphenols, their structural determination and their quantitation in low concentration (Cuyckens et al., 2001; Cuyckens and Claeys, 2004). Due to its powerful capacity to separate single components in complex mixtures and molecular structural identification, liquid chromatography (LC)-MS has gained large interest as a reliable and successful technique for the characterization of active compounds in biological products such as propolis.

For the utilization in the many healthcare finished products, propolis needs accurate standardization of its active compounds to assure quality, safety and efficacy to the consumers. Related to this, the aim of this paper is to present and discuss new recent analytical techniques useful for the determination of propolis polyphenols and of its quality with special attention to HPLC-MS characterization. This is more important by considering that in a recent scientific report (European Food Safety Authority - EFSA Journal, 2010), EFSA provided negative response to the health claims of propolis and its polyphenolic component. In fact, two main key points have been considered important by EFSA: A) propolis is a highly heterogeneous biological product and its active compounds polyphenols may change for composition and content also related to the different kind of extraction process, and B) there is no clear relationship between propolis structure/composition and health claims in the absence of its fine structural characterization.

SAMPLE PROPOLIS PREPARATION

As mentioned above, more than 200-250 compounds, mainly polyphenols and related derivatives may be present in propolis (Volpi, 2004; Volpi and Bergonzini, 2006).

Moreover, the content of polyphenols may vary depending on the origin and treatment of the raw samples and these differences are able to influence biological properties and clinical activities (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). In fact, propolis cannot be used as raw material but it needs to be purified by using different extractive procedures with the aim to purify the active plant molecules from inert and potentially dangerous material for further analytical evaluation and/or biological assays. Treatment of propolis with ethanol, usually at concentrations of 70-80%, is largely used for its capacity to produce wax-free extracts and tinctures very rich in polyphenols (Banskota et al., 2001; Castaldo and Capasso, 2002). Treatments with pure water (able to enrich the final extracts with phenolic acids that are highly soluble in water), methanol, acetone, hexane, chloroform and others have also been applied to the aim to purify propolis (Popova et al., 2004: Gómez-Caravaca et al., 2006; Stalikas, 2007). The quality control of raw propolis is essential to evaluate the characteristics of this material and, as a consequence, it is more analyzed than commercial finished products. The preparation of crude extracts is performed by dehydrating the propolis that can be obtained in a fine powder. Then, a weighted sample, at a frequently used concentration of 10 mg/mL, is dissolved in the solvent and left for 24 h at room temperature or at 70°C for 2-3 h under occasional mixer (Volpi and Bergonzini, 2006). After filtering, the procedure is repeated several times to assure a complete recovery of the active propolis component. The insoluble material is eliminated by filtration or centrifugation and the solvent is then evaporated to dryness under low pressure to reduce the extracted volume (Galeotti et al., 2017; Galeotti et al., 2018). The obtained solution is directly tested for molecular composition and biological activity.

SPECTROPHOTOMETRIC ASSAYS OF POLYPHENOLS

The increasing utilization of food supplements and nutraceuticals based on propolis preparations requires the application of reliable and reproducible techniques for the quantitation of their active compounds. Spectrophotometric assays are especially useful for the routine control of propolis products. In particular, quantitative methods are available for the determination of total flavonoids or total phenolics but also for specific classes of polyphenols such as for flavanones/ dihydroflavonols or flavones/flavonols (Popova et al., 2004: Gómez-Caravaca et al., 2006) (Figure 1A). Other advantages of these methods are rapidity, simplicity, good repeatability, acceptable accuracy and low costs of the reagents and equipment. On the other hand, these assays are unable to give a specific, reliable and complete characterization of the active species as well as to evaluate their possible changes due to working processes or intentional adulteration (Popova et al., 2004: Gómez-Caravaca et al., 2006; Galeotti et al., 2017).

The total polyphenols content is generally determined by using the Folin-Ciocalteu assay, that is the most largely used spectrophotometric method utilized for the total quantification of these compounds (Popova et al., 2004). Quantitative assay of flavonoids in propolis is carried out by two different colorimetric approaches. Flavone and flavonol quantification is performed by using the aluminium chloride assay based on the formation of a complex between the ion Al(III) and carbonyl and hydroxyl groups of the flavonoid (Bonvehi and Coll, 1994). On the contrary, flavanones and dihydroflavonols interact with 2,4-dinitrophenylhydrazine (DNP) forming in acidic media phenylhydrazones that are spectrophotometrically quantified (Nagy and Grancai, 1996; Popova et al., 2004). Finally, the real content of total flavonoids is obtained by the sum of the results obtained by the two above assays.

CAPILLARY ELECTROPHORESIS (CE) SEPARATION OF PROPOLIS SAMPLES

Different CE separations are available such as the capillary zone electrophoresis (CZE) able to separate the various molecules on the basis of their charge and size. The micellar electrokinetic chromatography (MEKC) is capable of also separating neutral compounds by using a differential partitioning between a surfactant added to the separation buffer (Volpi, 2004). This last approach is useful for the analysis of flavonoids due to their weakly acidic nature that permits their separation in the presence of buffer having basic pH values. However, flavonoids may be degraded under alkaline conditions making CZE the most suitable CE approach for the separation of polyphenols of propolis and of other biological products (Gómez-Romero et al., 2007). In fact, twelve different flavonoids, two phenolic acids and one stilbene derivative, resveratrol, were separated and quantified by CZE by means of a sodium tetraborate buffer on an uncoated fused-silica capillary using normal polarity (Volpi, 2004).

CHROMATOGRAPHIC DETERMINATION OF THE PROPOLIS POLYPHENOLS

The complete characterization of the active compounds of propolis requires both their identification and quantitation. Chromatographic techniques such as gas chromatography (GC) and in particular high-performance liquid chromatography (HPLC), assure specific profile, identification and quantification of the total as well as individual polyphenolic species (Gómez-Caravaca et al., 2006; Stalikas, 2007). Detection systems of polyphenols are of paramount importance due to their capacity to

quantify and identify these complex biomolecules. To this aim, polyphenols are mainly detected by ultraviolet (UV) absorption, often using a photodiode array detector (PAD) (Gómez-Caravaca et al., 2006; Stalikas, 2007). However, coupled off-line and recently on-line techniques, in particular with mass spectrometry (MS), are being routinely used for propolis analysis and standardization (Cuyckens et al., 2001; Cuyckens and Claeys, 2004).

Thin-Layer Chromatography (TLC)

TLC is able to analyze specific polyphenols by using suitable stationary phases and solvents depending on the structure of the molecular species needing to separate. Silica gel is a classical stationary phase widely used to separate the more apolar flavonoids such as flavonols and isoflavonoids (Figure 1A) by using different mobile phases, generally constituted of mixing of solvents such as ethanol/water, petroleum ether/ethyl acetate, petroleum ether/acetone/formic acid, chloroform/ethyl acetate, toluene/chloroform/acetone (de Rijke et al., 2006). Visualization and quantification of the separated molecular species are carried out by using short- and long-wavelength UV-light but also, in some specific application, by spraying on the plate different reagents. Due to its complexity and time-consuming characteristics along with its poor resolution, TLC is now rarely applied even if it may be of some utility in specific applications (Milojković Opsenica et al., 2016; Tang et al., 2014).

Gas Chromatography (GC)

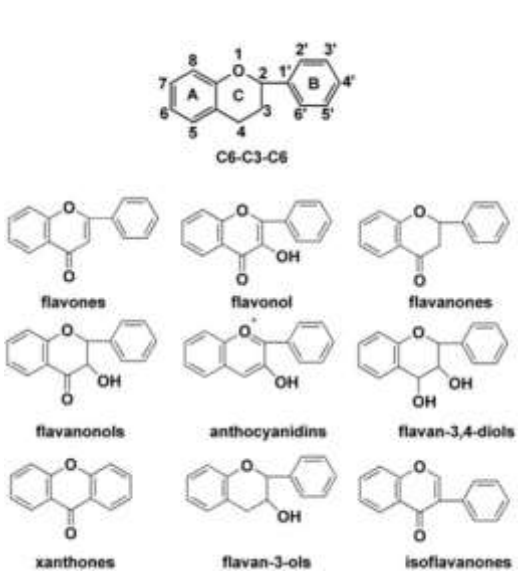
GC-FID (flame ionization detector) is applied for the analysis of polyphenols after their derivatization to make them volatile and suitable for chromatographic separation. GC coupled on-line with MS is now largely used thanks to the capacity of MS to detect the molecular mass values and structural information useful for the identification of unknown compounds. However, the majority of polyphenols from propolis are not volatile enough for GC-MS separation even after derivatization. As a consequence, GC-MS is generally used for the analysis of propolis volatile molecules that are mainly responsible for its specific aroma although their amount is generally very low (Mohtar et al., 2018; Pellati et al., 2013).

High-Performance Liquid Chromatography (HPLC)

To date, HPLC is the most suitable and reliable analytical approach for the identification and quantification of propolis active biomolecules. In fact, the relatively polar nature of polyphenols possessing several hydroxyl groups in the structure, combined with the UV adsorption of the aromatic rings and with the soft ionization techniques compatible with chromatography, make HPLC-UV(DAD) and HPLC-MS the most useful and reproducible methods for the characterization and determination of propolis active compounds (Table 1). As a consequence, HPLC coupled to UV and MS detectors has improved the analysis of non-volatile polyphenols allowing us to establish their quantification and structural identification (Table 1). The ion trap is the most recommended MS approach to characterize the propolis bioactives for its multiple fragmentation steps (MS^n) (Cuyckens et al., 2001; Cuyckens and Claeys, 2004). The structural identification of different classes and singular species of propolis polyphenols is obtained by comparing their chromatographic behavior and retention times, UV adsorption spectra and MS information to those of reference molecules. When standards are not commercially available, the structural nature of the unknown polyphenol can be obtained by comparing UV data and MS fragmentation pattern with those available in the literature (Cuyckens et al., 2001; Cuyckens and Claeys, 2004; Gardana et al., 2007). In fact, the pattern of fragmentation obtained by tandem mass belongs to a given molecule or class of molecules and, as concern flavonoids, their distinct classes differ in the presence of substitution groups strongly influencing the fragmentation pathway (Gardana et al., 2007). The interpretation of tandem mass and further MS^n data provides specific information permitting to identify the structure of the biomolecule of interest.

In a previous study, Volpi and Bergonzini (2006) developed a reliable HPLC-UV-ESI-MS analysis for the characterization of the chemical structure and therefore for the quality control of propolis applied to samples of various origin. We now report a further detailed characterization of brown propolis collected in different countries with the aim to observe or differentiate possible common features useful for the evaluation of its quality and to predict the biological activity.

We analyzed samples belonging to Europe from Italy, Spain, France, Romania, Bulgaria, Ukraine and Macedonia (Figure 2) while HPLC-MS profiles of brown propolis from Turkey and China are illustrated in Figure 3. Finally, samples collected from America, in particular from Uruguay and Brazil are shown in Figure 4. The total ion chromatograms (TIC) of ethanolic propolis extracts acquired in negative ion mode from the various European countries show quite the same profile for the presence of the same molecular species (Figure 2) identified by the same mass values and therefore the same ion species. HPLC-MS and MS^2 of the European brown propolis assured the identification of many compounds in each sample (Table 2) accounting for ~77-97% of the total molecular species. The remaining not identified percentage could be mainly represented by the family of triterpenoids and a small fraction of glycosylated derivatives (Galeotti et al., 2017). In fact, triterpenoids are



Hydroxybenzoic acids

Name	R1	R2	R3	R4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H

Hydroxycinnamic acids

Name	R1	R2	R3	R4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

B

Figure 1. Classification and structure of (A) flavonoids and (B) hydroxybenzoic and hydroxycinnamic acids derivatives.

abundant and very common in all vegetable forms and they are generally present in any kind extract (Connolly and Hill, 2010). Quantitative data reported in Table 2 were performed by MS detection versus a unique standard of galangin due to the absence of commercially available standard related to all polyphenol species identified. Anyway, this approach gives us the possibility to have a clearer picture of the different propolis samples along with the identification of the main polyphenols and quantification of the various classes useful to have a specific fingerprint. In fact, according to Figure 2 and Table 2, propolis from the various European countries are quite similar each other showing a common polyphenolic composition also with a comparable percentage. The ethanolic extracts of brown European propolis are rich in phenolic acid derivatives (in particular from caffeic acid), between ~5 and ~16%, and all samples show the presence of pinobanksin and derivatives (pinobanksin-3-*O*-acetate and pinobanksin-3-*O*-butyrate), chrysin, pinocembrin and galangin as main bioflavonoid species. Overall, flavones and flavonols are from ~22 and ~29%, flavanones and dihydroflavonols are between ~28 and ~48% while glycosilated species and terpenes are ~4 - 17%.

Propolis from Turkey and China (Figure 3) has a greater percentage of glycosilated derivatives and terpenes as also evident from the HPLC-MS profile accounting for ~20 - 21%. This is more evident for propolis from Uruguay very rich in glycosilated and terpenes species, ~34% with a lower percent of flavones and flavonols (13.6%) (Table 2).

Contrary to the brown propolis samples illustrated earlier, the two samples from Brazil show a different HPLC-MS profile (Figure 4) and composition (Table 3) accounting for a very low percent of the above identified polyphenols. In fact, we detected the presence of low percentages of chrysin, pinocembrin, galangin, pinobanksin-3-*O*-acetate and CAPE compared to the other brown propolis. Additionally, artepillin C, a molecular species specific for brown propolis from Brazil, was observed even if in percentages very lower than green propolis collected from Brazil (Cheung et al., 2011). Moreover, this peculiar composition was observed in two propolis samples of Brazil in different periods and seasons. This repeatability of propolis collected in various periods was also observed for different samples from China (Supplemental Figure S1) demonstrating that HPLC-MS is capable of giving a polyphenols fingerprint and composition specific for areas of production independently from the season of collection.

On the other hands, a simple HPLC profile acquired with UV/DAD detector (Figure 5) is unable to distinguish between samples from different geographic areas for the absence of UV chromophores in many of the molecular species present in propolis. This is evident also having standard commercially available due to the complexity of the propolis HPLC profile.

NMR ANALYSIS OF PROPOLIS

^{13}C- and ^{1}H-NMR and their bidimensional maps may provide specific fingerprints of propolis useful for obtaining global information in particular of complex samples. In fact, a recent study demonstrates that it is possible to use ^{1}H-NMR for the simultaneous recognition of propolis

Table 1. The most common HPLC conditions and detectors for the separation and characterization of polyphenols in propolis.

Column	Mobile phase	Detector	Application	Year	References
LiChrocart RP18	Gradient separation with water:formic acid 19:1 to methanol	DAD	Rosemary Propolis	1995	Gil et al. (1995)
LiChrospher 100 RP18	Gradient separation with formic acid to methanol	PAD	New Zealand Propolis	1996	Markham et al. (1996)
LiChrocart RP18	Gradient separation with water/formic acid 19:1 to methanol	DAD	Tunisian propolis	1997	Martos et al. (1997)
YMC Pack ODS-A	Isocratic separation with acetic acid/methanol/water 5:75:60	DAD	Brazilian Propolis	1998	Park et al. (1998)
YMC Pack ODS-A	Isocratic separation with water/methanol	DAD	Brazilian Propolis	2002	Park et al. (2002)
Capcell Pak ACR 120 C18	Gradient separation with 0.1% formic acid/water to B: 0.1% formic acid/acetonitrile	PAD	Korean Propolis	2004	Ahn et al. (2004)
Synergi Fusion-RP18	Gradient separation with 0.25% acetic acid and methanol	UV-VIS and ESI-MS	Propolis from Argentina, Azerbaijan, China, Ethiopia, Kenya, Italy, Spain	2006	Volpi and Bergonzini (2006)
Symmetry C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and triple quadrupole MS	Propolis form various geographic regions	2007	Gardana et al. (2007)
Symmetry C18	Isocratic separation with methanol/0.4% phosphoric acid 60:40	UV-VIS and PDA	Propolis form various geographic regions	2008	Zhou et al. (2008)
Ascentis C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Italian Propolis	2011	Pellati et al. (2011)
Zorbax SB-C18	Gradient separation with 0.05% acetic acid and acetonitrile	LTQ Orbitrap XL MS	Iraqi propolis	2011	Sulaiman et al. (2011)
Nucleosil C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Portuguese Propolis	2013	Falcão et al. (2013)
Sepax HP-C18	Gradient separation with 1% acetic acid and methanol	UV	Chinese Propolis	2014	Cui-ping et al. (2014)
Hypersil gold C18	Gradient separation with 1% formic acid and acetonitrile	Linear ion trap and Orbitrap hybrid MS	Serbian Propolis	2015	Ristivojević et al. (2015)
MultoHigh 100 RP18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Bolivian Propolis	2016	Nina et al. (2016)
LiChroCART Purospher StaR RP18	Gradient separation with 5% aseptic acid and methanol	DAD	Propolis from different geographic regions of Brazil	2016	Machado et al. (2016)
Zorbax Eclipse Plus	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS	Greek Propolis	2017	Kasiotis et al. (2017)
Tecnokroma C18	Gradient separation with 5% formic acid and acetonitrile	UV-VIS	Propolis form various geographic regions	2018	Escriche and Juan-Borrás (2018)

DAD = Diode Array Detector. PAD = Pulsed Amperometric Detector.

polyphenols by means of specific tools for spectra pre-treatment and analysis (Bertelli et al., 2012). However, the use of NMR requires very expensive equipment and highly expert operators in the field. In fact, in particular for propolis, the utilization of the NMR technique produces very complicated spectra that need to be further analyzed by chemometric methods. Finally, contrary to HPLC-MS, NMR is capable of just identifying a limited number of polyphenol species compared to the high complexity of propolis composition.

PROPOLIS FOR STANDARDIZATION AND QUALITY CONTROL

To obtain an accurate total polyphenols and flavonoids content, all assays need of specific calibration curves made of suitable standards. As a consequence, what are the most reliable standards to produce accurate and specific total polyphenols content of propolis? Many quality control (CQ) laboratories generally adopt a single commercially available polyphenol, such as galangin, pinocembrin, myricetin or others, or a limited mixture of these compounds. This is necessary due to the lacking of these compounds.

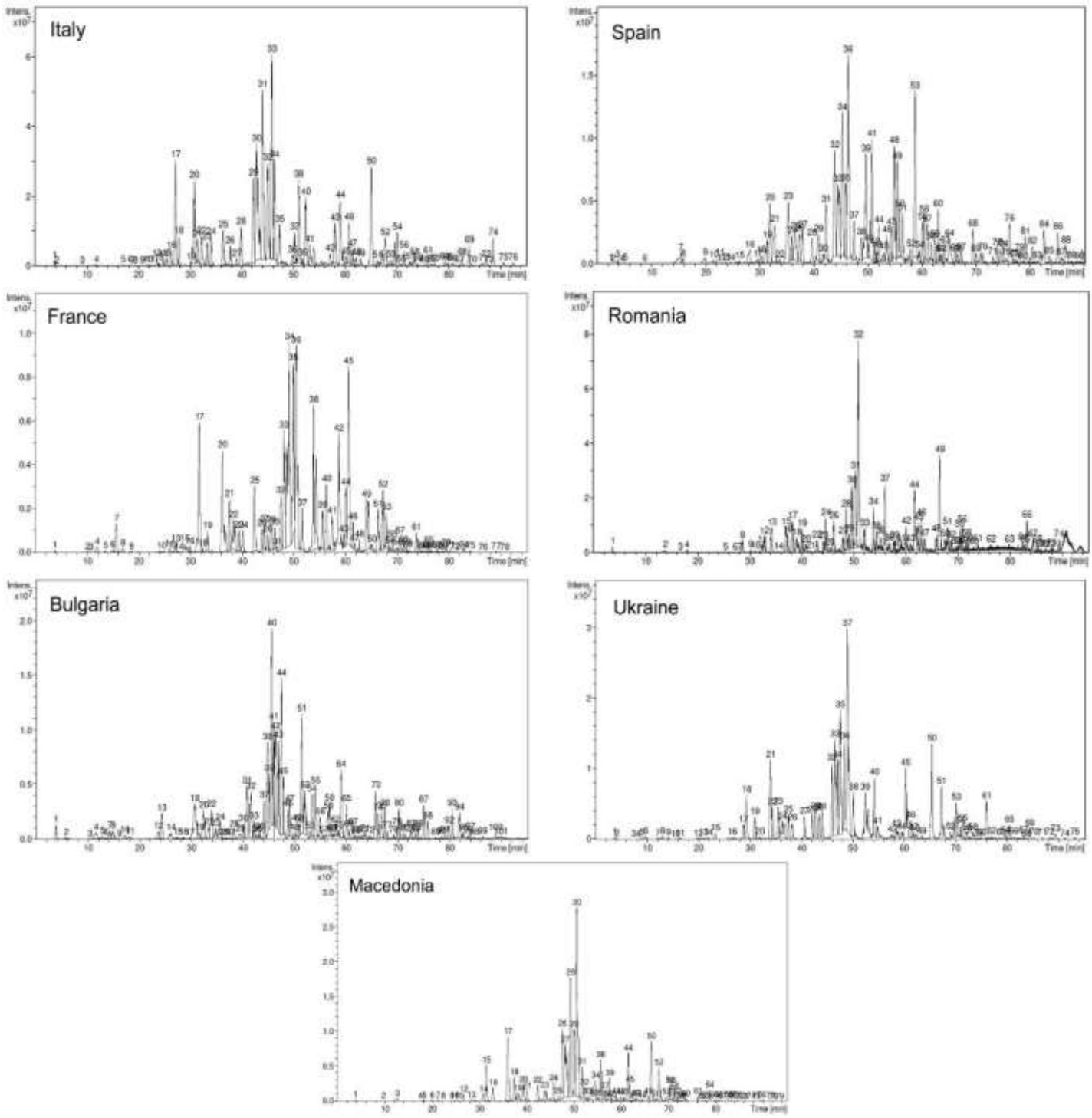


Figure 2. Total ion chromatograms (TIC) of ethanolic extracts of propolis of European origins.

This is necessary due to the lacking of a suitable propolis reference standard. However, as evident, the content of total polyphenols is highly underestimated by using a single standard due to the elevated complexity of propolis polyphenols and derivatives. The correct solution to this problem would be the adoption of “house propolis standards” constituted of highly purified and well characterized propolis samples. Finally, house standards possessing an overall structural composition and chemical profile similar to the samples to be analyzed

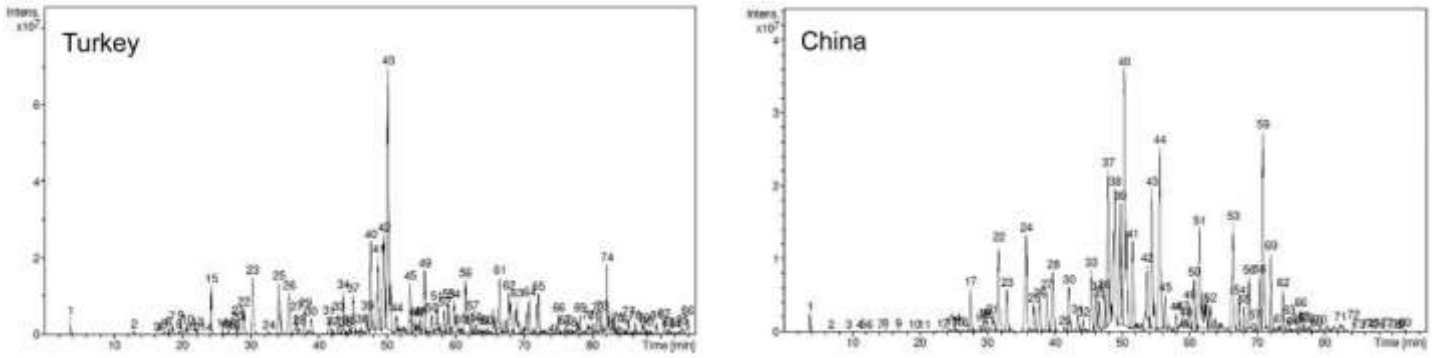


Figure 3. Total ion chromatograms (TIC) of ethanolic extracts of propolis from Turkey and China.

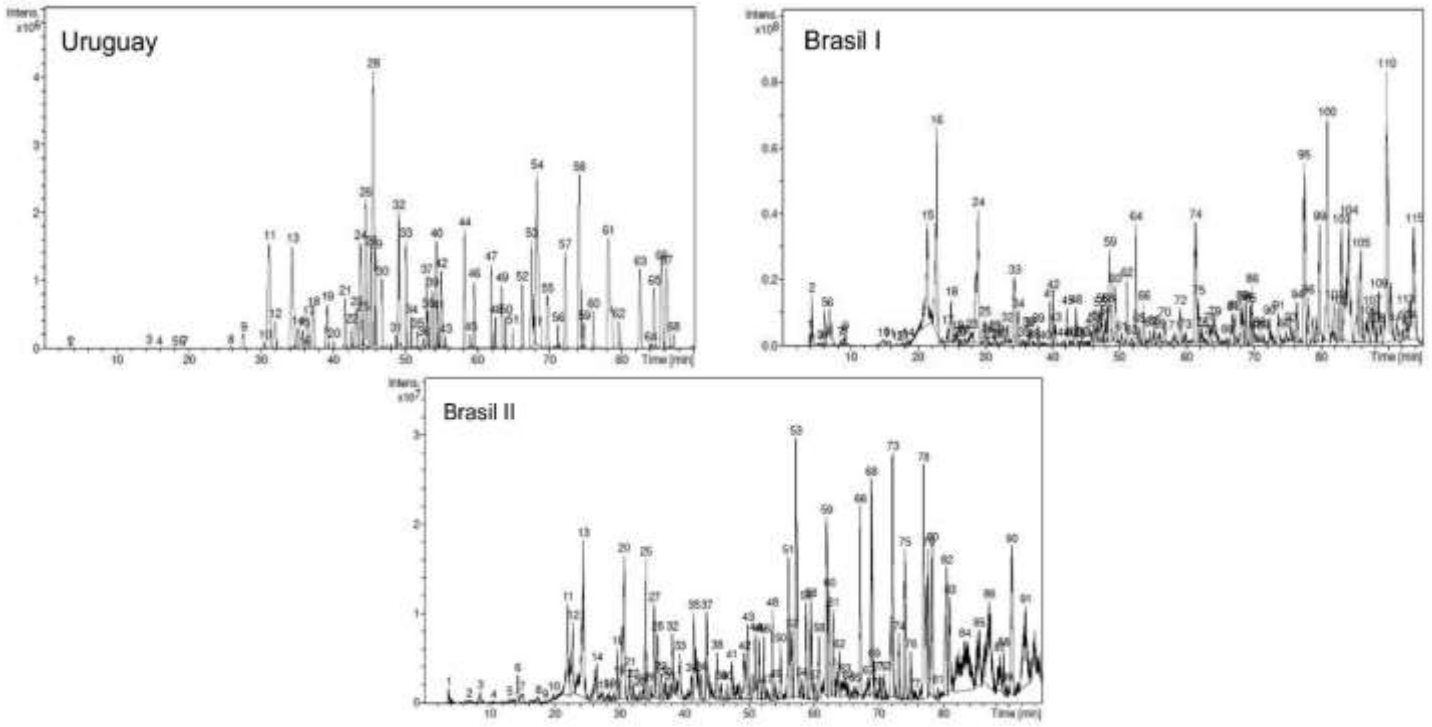


Figure 4. Total ion chromatograms (TIC) of ethanolic extracts of propolis from Uruguay and Brazil.

should be utilized to obtain more accurate and suitable quantitative determinations.

CONCLUSIONS

It is well known that polyphenols and flavonoids constitute the most important and active biomolecules of propolis responsible for the great part of its biological, nutraceutical and therapeutic effects. In this regard, HPLC-MS represents a powerful analytical technique capable of differentiating among various propolis samples. Thus, it could provide an effective alternative to classical analytical phytochemistry useful for the screening of commercial preparations of propolis and to evaluate their specific therapeutic benefits. In fact, due to its capacity to identify and quantify individual compounds from all the constituents present, also in the presence of overlapping signals, HPLC-MS method should be applied for the characterization of the main bioactives and for the evaluation of molecular markers useful for the identification of each propolis type.

By considering the results illustrated above and the many scientific studies available in the literature, we can

Table 2. Molecular composition of the propolis from different countries determined by HPLC-UV-ESI-MS.

Molecular Species		*m/z*	Italy	Spain	France	Romania	Bulgaria	Ukraine	Macedonia	Turkey	China	Uruguay
			European Propolis									
1	Dicaffeic acid	341	Np	np	np	np	np	np	np	np	np	np
2	Caffeic acid	179	Np	np	np	np	np	np	np	np	np	np
3	p-coumaric acid	163	Np	np	np	np	np	np	np	np	np	np
4	Ferulic acid	193	Np	np	np	np	np	np	np	np	np	np
5	Isoferulic acid	193	Np	np	np	np	np	np	np	np	np	np
6	3,4-dimethyl-caffeic acid (dmca)	207	Np	np	np	np	np	np	np	np	np	np
7	Quercetin	301	0.8	0.9	0.5	0.7	0.5	0.8	0.7	0.7	0,2	0,1
8	Pinobanksin-5-methyl-ether	285	1.5	3.0	5.5	1.4	1.7	2.6	3.4	1.4	3,1	4,1
9	Quercetin-3-methyl-ether	315	2.6	1.9	0.7	2.4	0.5	1.2	1.1	3.0	1,3	1,0
10	Cinnamic acid	147	Np	np	np	np	np	np	np	np	np	np
11	Chrysin-5-methyl-ether	267	Np	np	np	np	np	np	np	np	np	np
12	Apigenin	269	0.6	2.0	2.4	1.2	1.5	1.5	3.8	1.2	0,2	0,2
13	Kaempferol	285	2.2	0.9	1.7	2.7	1.7	1.7	1.8	2.3	0,8	0,5
14	Pinobanksin	271	1.6	1.1	3.0	2.3	2.0	4.9	3.0	1.9	4,1	3,9
15	Isorhamnetin	315	2.0	1.6	1.9	1.8	1.9	1.7	0.8	1.0	1,0	0,4
16	Luteolin-methyl-ether	299	1.2	1.0	0.6	1.8	0.5	1.1	1.3	1.2	1,3	0,8
17	Quercetin-dimethyl-ether	329	1.1	1.3	0.8	0.6	1.0	0.1	0.7	0.7	2,0	1,2
18	Galangin-5-methyl-ether	283	0.9	1.0	2.5	0.8	0.4	0.9	1.2	0.8	0,5	1,2
19	Pinobanksin-5-5methyl-ether-3-o-acetate	327	Np	np	np	np	np	np	np	np	np	np
20	Cinnamilidenacetic acid	173	Np	np	np	np	np	np	np	np	np	np
21	Quercetin-7-methyl-ether	315	0.7	0.9	0.7	0.5	0.6	1.2	1.4	1.0	0,3	0,1
22	Quercetin-methyl-x-methylether	329	2.6	1.0	0.7	1.8	0.6	1.3	1.3	1.7	2,1	1,3
23	Caffeic acid isoprenyl ester	247	0.9	2.1	1.7	0.8	1.6	3.7	5.5	0.8	nd	nd
24	Chrysin	253	5.3	4.3	5.5	2.4	3.7	6.7	6.9	4.0	5,3	2,8
25	Caffeic acid benzyl ester	269	0.2	4.6	0.1	0.8	0.9	1	0.3	0.2	0,3	0,2
26	Caffeic acid isoprenyl ester	247	0.1	0.1	0.2	0.1	0.8	2.1	0.1	0.1	0,1	0,1
27	Pinocembrin	255	4.5	5.5	10.3	4.8	3.0	6.7	10.4	3.6	5,9	4,5
28	Galangin	269	5.9	2.8	7.2	6.9	4.0	5.2	6.2	5.7	4,5	2,4
29	Caffeic acid phenylethyl ester (cape)	283	1.3	1.2	1.3	1.5	2.1	2.1	2.4	1.5	2,0	1,8
30	Pinobanksin-3-o-acetate	313	13.1	9.1	8.9	13.3	7.0	12.7	17.2	12.7	8,9	7,9
31	Methoxy-chrysin	283	1.0	0.8	0.6	1.2	0.9	4.8	0.8	0.6	2,9	1,6
32	p-coumaric prenyl ester	231	Np	np	np	np	np	np	np	np	np	np
33	p-coumaric benzyl ester	253	Np	np	np	np	np	np	np	np	np	np
34	Caffeic acid cinnamyl ester	295	0.4	4.6	1.0	0.5	0.5	0.7	2.9	0.4	5,2	3,5
35	p-coumaric prenyl ester	231	Np	np	np	np	np	np	np	np	np	np
36	Pinobanksin-3-o-propionate	327	4.3	4.8	3.1	5.4	5.2	5.1	3.2	3.1	6,8	2,6
37	p-coumaric cinnamyl ester	279	0.5	1.0	0.6	0.3	0.2	0.9	0.2	0.4	1,3	1,1

Table 2. Contd.

38	Pinobanksin-3-o-butyrate	341	Np	np	np	np	np	np	np	np	np	np
39	Pinobanksin-3-o-pentanoate	355	Np	np	np	np	np	np	np	np	np	np
40	Pinobanksin-3-o-hexanoate	369	Np	np	np	np	np	np	np	np	np	np
41	p-methoxy cinnamic acid cinnamyl ester	293	Np	np	np	np	np	np	np	np	np	np
Totally identified species			**86.0**	**91.0**	**90.2**	**91.6**	**77.2**	**94.9**	**93.7**	**82.2**	**97.4**	**93.6**
Phenolic acids derivatives			5.1	16.5	7.3	5.9	9.3	12.6	12.8	5.9	10.2	6.9
Flavones and flavonols (%)			28.9	22.4	25.8	25.2	23.8	28.2	28.0	23.9	22.4	13.6
Flavanones and dihydroflavonols (%)			35.5	35.5	39.8	43.4	28.0	44.0	48.5	31.6	44.7	39.0
Glycosilated species and terpenoids (%)			16.5	16.6	17.3	17.1	16.1	10.1	4.4	20.8	20.1	34.1

np = not present.

Table 3. Molecular composition of two propolis samples from Brazil determined by HPLC-UV-ESI-MS.

Molecular species	***m/z***	**Sample 1**	**Sample 2**
		Brazil	
	547	0.5	1.4
	515-353	2.2	3.4
	515-353	5.0	4.8
	487	0.6	0.9
	677-515	2.5	4.4
	301	1.2	2.0
	301	0.2	0.7
	331	1.1	0.7
Chrysin	253	0.6	0.4
Pinocembrin	255	0.4	0.5
Galangin	269	1.2	0.4
Caffeic acid phenylethyl ester (Cape)	283	1.0	0.4
Pinobanksin-3-o-acetate	313	1.7	2.5
	315	1.0	1.2
	393	0.8	1.1
	615-379	1.2	1.9
	333	2.9	2.0
	319	5.8	2.1
	452-315	3.7	2.9

Table 3. Contd.

	299	2.0	3.3
	485	1.6	1.5
	613	3.7	0.8
	613	0.5	1.2
	597-319	0.4	1.1
	405	1.0	0.9
	613	1.3	1.0
	471	2.6	0.8
	471	3.8	4.4
Artepillin C	593-471	1.6	2.3
	613-469	1.0	5.5
	559-471	6.0	2.2
	525	2.0	5.8
	513-305	4.7	3.5
	701-455-417	1.1	0.9
	657-547-453	0.6	1.1
	455-369-325	3.1	1.2
	561-527	3.7	8.7
	455-371-327	1.2	4.5

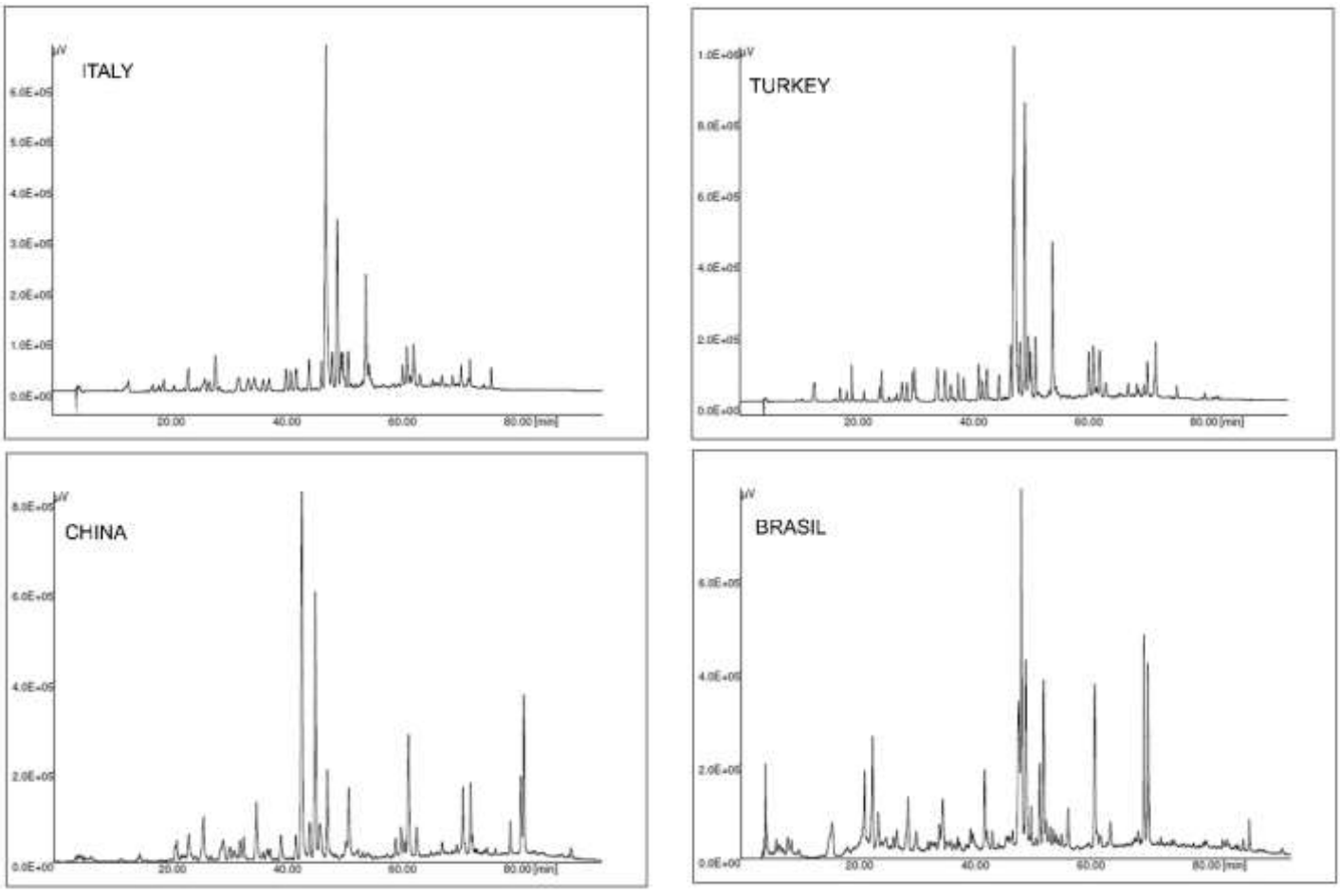


Figure 5. HPLC-UV chromatograms of ethanolic extracts of propolis from Italy, Turkey, China and Brazil.

affirm that, concerning the points provided by EFSA, it is possible to have a full and clear characterization of propolis and its main bioactive compounds and to obtain specific fingerprints of raw samples of different origin and finished preparations by using on-line HPLC-MS (and tandem mass when necessary).

Therefore, by the very numerous scientific studies on its activities, the total propolis extract is considered the “active principle” and biomolecular marker must be used for quality control applying the powerful analytical approaches discussed above. In the case of propolis, the most important bioactive compounds have been demonstrated to be polyphenols that are known to change depending on type and external factors. By considering this, it is possible to standardize propolis quality and final preparations used in medicine and nutraceuticals according to the corresponding chemical profile obtained by HPLC-MS.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

ABBREVIATIONS

CE, Capillary electrophoresis; **CQ,** quality control; **CZE,** capillary zone electrophoresis; **DAD,** diode array detector; **DNP,** dinitrophenylhydrazine; **ESI,** electrospray ionization; **FID,** flame ionization detector; **GC,** gas chromatography; **HPLC,** high-performance liquid chromatography; **MEKC,** Micellar electrokinetic chromatography; **MS,** mass spectrometry; **NMR,** nuclear magnetic resonance; **PAD,** pulsed amperometric detector; **TIC,** total ion chromatogram; **TLC,** thin-layer chromatography.

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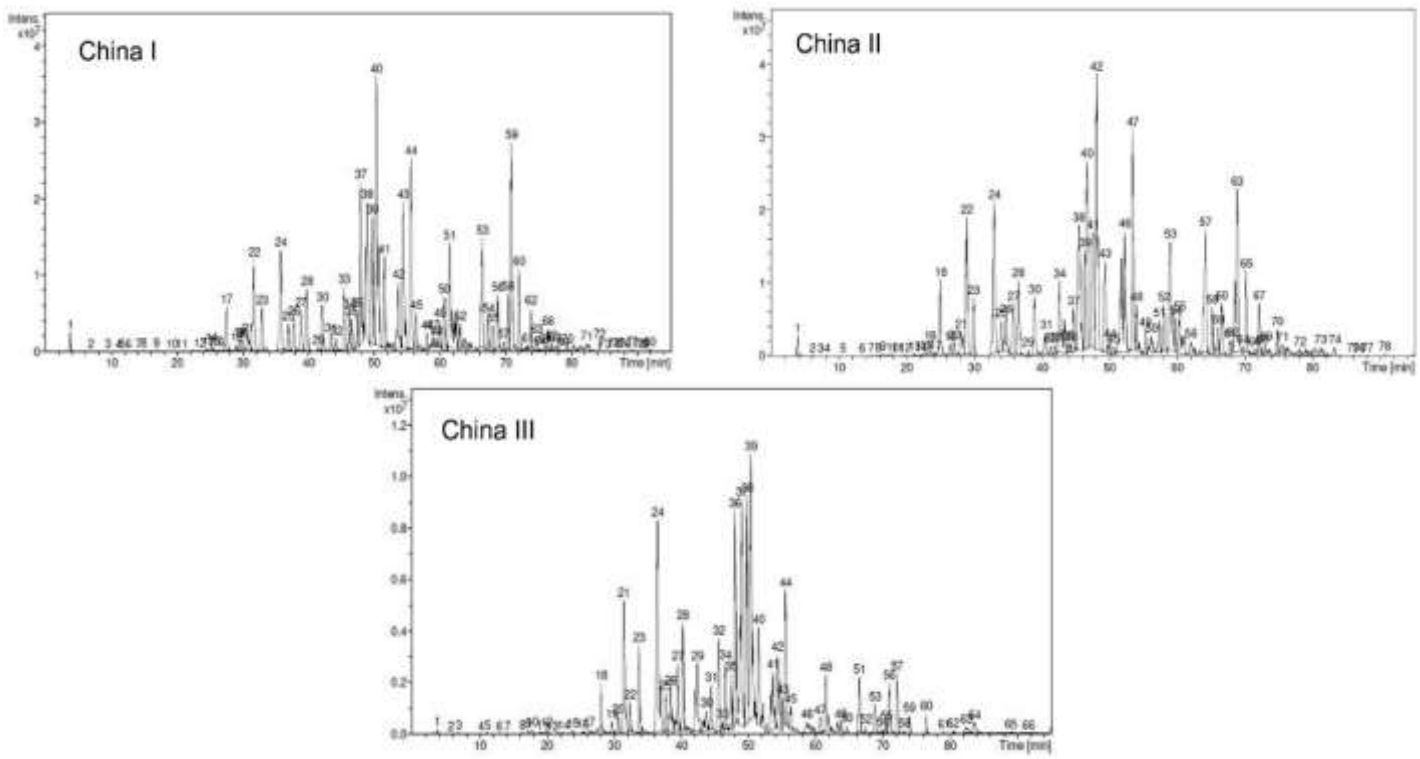
Supplemental Figure



Supplemental Figure S1. Total ion chromatograms (TIC) of three ethanolic extracts of different propolis samples from China.

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In vitro and *in vivo* antimalarial activity of *Nigella sativa* L. extracts

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The Arabs, Asians and, Traditional Health Practitioners in Mombasa county found in Kenya have been using *Nigella sativa* L. seeds to traditionally manage malaria associated symptoms that is, headache, fever, chills, loss of appetite among others. The present study investigated *in vitro* antiplasmodial, *in vivo* antimalarial activities and safety of different extracts of *N. sativa*. Five extracts obtained via aqueous extraction and sequential extraction using hexane, dichloromethane, ethyl acetate and methanol were tested against *in vitro* cultures of *Plasmodium falciparum*. The most active extracts (methanolic and ethyl acetate) were assessed for cytotoxicity and toxicity. The two active extracts were evaluated *in vivo* against *Plasmodium berghei* ANKA strain at 500, 250 and 125 mg/kg/day. On *in vitro* assay, methanolic and ethyl acetate extracts showed good activity with IC_{50} of 80.48±12.29 and 69.81±5.24 µg/ml against W2 strain and 31.93±4.31 and 53.79±6.02 µg/ml against D6 strain, respectively. The extracts exhibited weak cytotoxicity on Vero cells and high parasitemia suppression of 75.52 and 75.30% at 500 mg/kg dose of methanolic and ethyl acetate extracts respectively. Notably, there was significant decrease ($p<0.001$) in activity with lower doses of the extracts. The results explain the traditional use of this plant in the Middle East and Mombasa County.

Key words: *Nigella sativa* L. seeds extracts, *Plasmodium*, antimalarial activity.

INTRODUCTION

Malaria is one of the most vital parasitic infections common in many developing countries mostly affecting sub-Saharan Africa (Nkumama et al., 2017). Amongst the *Plasmodium* species affecting human, *Plasmodium*

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falciparum is the chief cause of morbidity and mortality (Maier et al., 2018). Approximately 219 million episodes of malaria were recorded in 2017 globally in comparison to 239 million occurrences in 2010 and 217 million cases in 2016. Notably, about 435, 000 malaria deaths were witnessed in 2017 worldwide, a decrease from 451, 000 approximated deaths in 2016, and 607, 000 in 2010 (WHO, 2018). In Kenya, malaria transmission is prevalent in the western region with the target population being children under the age of 5 years and pregnant women (Kepha et al., 2016). There has been significant reduction in morbidity and mortality due to control programs and scientific interventions (Mogeni et al., 2016). However, malaria remains an essential public health problem globally and in developing countries.

Chemotherapy plays a crucial role in malaria control (Mvango et al., 2018). Effective antimalarial drug should have therapeutic activity and no toxicity on human host (Na-Bangchang and Karbwang, 2009). However, the central drawback to treatment has been the rise of parasite resistance to most antimalarial drugs (Menard and Dondorp, 2017). Chloroquine (CQ) has been used since 1940s until resistance challenged its use (Ecker et al., 2012). However, it has remained the drug of choice for *Plasmodium malariae, Plasmodium ovale, Plasmodium vivax,* and the uncomplicated *P. falciparum* in some parts of the world where resistance to CQ is relatively low (Torres et al., 2013). A combination therapy of sulphadoxine and pyrimethamine was later introduced (Horn and Duraisingh, 2014). Afterwards, it incurred resistance associated with *dhfr, dhps* and *pfmdr* genes hence ineffective for *plasmodium* management (Jelinek et al., 1998; Jacques Le Bras and Durand, 2003).

A*rtemisinin*-based *combination therapies* (ACTs) remain the most effective *antimalarial* medicines available today (Sugiarto et al., 2018; White, 1999). Tolerance of the malaria parasite to ACTs is currently increasing in South-East Asia. Resistance associated with point mutations in the “propeller” region of a *P. falciparum* kelch protein gene on chromosome 13, also known as k13 or kelch 13 has been established (Ariey et al., 2014). There is a risk of artemisinin resistance spreading to Africa allowing resistant parasites to go global. Therefore, new products for malaria management are necessary to outcompete resistance (Ashley and Phyo, 2018).

N. sativa, also well-known as Black seed belongs to the family of *Rananculaceae*. The plant is extensively distributed especially in Middle Eastern Mediterranean region, South Europe, India, Pakistan, Syria, Turkey and Saudi Arabia. There has been rising concern to this plant since its wide discovery of therapeutic potential against many ailments (Ahmad et al., 2013; Kooti et al., 2016). Among the eminent clinical importance, *N. sativa* has been shown to possess antidiabetic, anticancer and anti-inflammatory activities (Heshmati and Namazi, 2015; Heshmati et al., 2015; Periasamy et al., 2016). It has also been demonstrated to possess antiparasitic activity in the Middle East folk medicine (Razavi et al., 2018).

The previous study conducted using ethanol, aqueous and chloroform extracts of *N. sativa* demonstrated higher percentages of parasitemia suppression against *P. berghei* PZZ1/00 strain. Parasitemia reduction of 86.19, 76.94 and 66.92% was recorded upon use of 100, 400 and 100 µL/kg doses of ethanol, aqueous and chloroform extracts respectively (Abdulelah and Zainal-Abidin, 2007). In Nigeria, methanolic and aqueous extracts’ activity of *N. sativa* against *P. berghei* (nk 65) infection in mice have been reported (Ashcroft et al., 2018). However, antiplasmodial and antimalarial potential of *N. sativa* extracts is yet to be documented in Kenya.

Mombasa County located in the Coast of Kenya, which principally was our geographical site is rich in ethnomedicinal knowledge. The region is highly inhabited by Arabs and Asians who apparently migrated from Asian countries where *N. sativa* plant originates (Silberman, 1950). This important group of society (Arabs and Asians) together with the Traditional Health Practitioners (THPs) have for a long time used *N. sativa* to traditionally treat malaria associated symptoms that is, headache, fever, chills, loss of appetite amongst others (Bahekar and Kale, 2013). Lack of affordability, limited access to pharmaceutical treatment and modern treatment amenities are the key reasons (Cunningham, 2001; Katz and Kimani, 1982). This study determined the effect of *N. sativa* seed extracts on *Plasmodium* parasite as a crucial step towards seeking effective drug for malaria. In addition, cytotoxicity and toxicity of the bioactive *N. sativa* extracts were evaluated in order to determine their selective indexes and lethal doses correspondingly. This will offer valuable information towards establishing lead compounds responsible for *N. sativa* antimalarial activity.

MATERIALS AND METHODS

Plant collection and authentication

N. sativa seed samples of Saudi Arabian origin were collected from Mwembe Tayari market in Mombasa County. The plant was positively identified by a taxonomist and indigenous Mombasa residents who use it traditionally. 2 kg of *N. sativa* seeds were packed in plastic bags (voucher specimen No; PAS0023) and transported to KEMRI Centre for Traditional Medicine and Drug Research, Nairobi for processing.

Extraction

The seeds were air dried for 72 h and ground using an electric laboratory mill (Christy and Norris Ltd., Chelmsford, England). 400 g of the ground seed material was used for aqueous extraction whereas 1 kg of the material underwent sequential extraction method with different solvents of increasing polarity; hexane, dichloromethane, ethyl acetate and methanol. Briefly, ground plant material (400 g) was extracted in 1.2 L of double distilled water in a water bath at 60°C for 1 h, cooled and filtered using Whatman No 1 filter paper (Whatman, England). The filtrate was then concentrated to dryness by lyophilization.

One kilogramme (1 kg) of the plant was extracted using 1.5 L of the solvents in an order of increasing polarity by maceration at room temperature for 48 h. The soaked plant material was first filtered using gauze followed by Whatmann's No 1 filter paper (Whatman, England). The residue was re-macerated for another 48 h and filtered. The filtrates were then pooled together and concentrated to dryness under reduced pressure using rotary evaporator. The filtered plant residue was dried and the next solvent introduced as per the order of increasing polarity. Finally, the collected crude extracts were further dried in a vacuum drier to remove any possible traces of solvents and later stored at -20°C until use.

In vitro anti-plasmodial studies using *P. falciparum* strains

Two different isolates of laboratory adapted *P. falciparum* cultures were used in the study. These were; Chloroquine Sensitive Sierra Leone 1 (D6) and Chloroquine-resistant Indochina 1 (W2) strains. *In vitro* anti-plasmodial study with *P. falciparum* strains was performed according to Trager and Jensen (2005) method with slight changes. The parasites were maintained in a continuous culture in RPMI 1640 medium supplemented with 10% human serum and hematocrit of O+ red blood cells under a gas mixture of 92% nitrogen, 5% carbon dioxide and 3% oxygen.

All crude extracts of the plant were initially diluted in DMSO (Sigma Chemical Co., St Louis, MO, USA) and then dilution to a concentration of DMSO was reduced to <1% in the final working solution. Distilled water was used to dissolve CQ (positive control). Semi- automated micro-dilution technique that measures the ability of extract to inhibit the incorporation of [G-^{3}H] hypoxanthine into the parasite was utilized (Desjardins et al., 1979; Le Bras and Deloron, 1983). Briefly, 200 µl of 2% (v/v) suspension of parasitized erythrocytes composed of 1-2% parasitemia was added to all wells except the last well H that was the negative control having un-parasitized erythrocytes. The contents in the plate were then incubated at 37^0 C supplied with a gas mixture of 3% CO_2, 5% O_2, and 92% N_2 for 48 h. After 48 h, 25 µl of culture medium comprising 0.5 µCi of [G-3H] hypoxanthine (Amersham International, Burkinghamshire, UK) was added to each well and incubated further for 18 h. Harvesting of the plates was done onto glass fibre filters, washed using distilled water and dried. The glass fibre filters were smeared with 25 µl liquid scintillation fluid. The filters were measured for radioactivity using micro-beta plate scintillation counter (Wallac MicroBeta TriLux).Counts per minute (CPM) were obtained and then used to compute the IC_{50} values (Sixsmith et al., 1984). Non-linear regression analysis enabled calculation of the concentration of test samples that inhibited 50% (IC_{50}) growth. This calculation was carried out using Chemosen Program 2 according to the following formula;

IC_{50}=antilog (logX1+ [(logY50-logY1) X (logX2-logX1)]/logY2-logY1

Where, Y50 is the Counts per minute (cpm) value midway between parasitized and non-parasitized control cultures, and X1, Y1, X2, Y2 are the concentrations and cpm values respectively for the data points above and below the cpm midpoints (Sixsmith et al., 1984).

In vivo assay

Host, environment and parasites

Male Swiss albino mice, 6-8 weeks old, weighing 20±2 g bred in the KEMRI animal facility Nairobi were used as subjects. Test mice were housed in experimental rooms in standard microloan type II cages, well labeled with experimental details in air-conditioned rooms at room temperature and 60-70% relative humidity. They were well nourished with commercial rodent feed and water *ad libitum*. Five mice were used for every cage per test sample. *P. berghei* ANKA parasites were used to determine parasite reduction in mice.

Four days parasite suppression test

Four days parasite suppression test study was conducted according to Peters (1975) protocol with slight adjustments. *P. berghei* strain ANKA infected blood was obtained by heart puncture from donor mouse and mixed with 1% (w/v) heparin. The infected blood was diluted in physiological saline to approximately 10^8 parasitized erythrocytes per ml. The test animals were infected by intraperitoneal injection with 0.2 ml (2×10^7 parasitized erythrocytes) and randomly grouped. The experimental groups were treated orally with 0.2 ml single dose of 125, 250 and 500 mg/kg of the test sample, 2 h post infection (D0) (Gessler et al., 1995). Only, extracts active as per *in vitro* assay were used (methanolic and ethyl acetate extracts). Two control groups comprising five mice each were treated with a placebo (vehicle; 3% dimethyl sulfoxide, 10% tween 80 in PBS) and 5 mg/kg of CQ for negative and positive controls respectively. The mice were consecutively treated orally for 3 days (D1, D2, and D3) with equivalent doses. Blood films were taken on the fifth day (D4) from tail snips, fixed in methanol and stained with 10% Giemsa stain (Abdela et al., 2014). Microscopic examination of thin blood film provided parasitemia counts in 4 fields of ~100 erythrocytes per view. The difference between mean numbers of parasites per view in the negative control group and those of the test groups were calculated and expressed as percentage suppression (chemo suppression) according to the formula (Tona et al., 2001):

$$PS = \frac{A-B}{A} \times 100$$

Where; A=Mean parasitemia in the negative control group on day 4, B=Corresponding parasitemia in the test group.

The mean survival time (days) for each group was determined over a period of 30 days post infection.

Toxicity evaluation

Cytotoxicity evaluation

The MTT assay that is based on the principle of conversion of yellow tetrazolium MTT to purple formazan dye by mitochondrial dehydrogenases of living cells was used for cytotoxicity determination (Van Meerloo et al., 2011). Vero cells obtained from kidney epithelial cells of the African green monkey, stored in liquid nitrogen in KEMRI; Centre for Traditional Medicine and Drug Research were used. The Vero cells were grown in Eagle's minimum essential medium, supplemented with 5% foetal bovine serum (FBS) in 25 ml cell culture flasks. They were incubated at 37°C in 5% CO_2 incubator (Kurokawa et al., 1995). The cells were sub-cultured three times a week to achieve 80% confluence. Afterwards, the cells were seeded with 5×10^4 cells per well in 96-well plates. They were then incubated at 37°C for the next 2 days. Fresh MEM (GIBCO, Grand Island, NY) containing test extracts at different concentrations was added to replace the culture medium and later incubated for another 2 days. Tripsinization of the cells for each sample was done in triplicate wells to allow detachment. A hemocytometer was used to count viable cells. Data from inhibition was then plotted as dose-response curves and CC_{50} (concentration able to cause visible alterations in 50% of intact cells) was determined. Selectivity index (SI) was used as parameter of clinical significance of the test samples by comparing general toxins and

Table 1. *In vitro* antiplasmodial activity of five extracts.

Extract/drug	Extract yield (%)	W2 IC_{50} (Mean±SD) (µg/ml)	D6 IC_{50} (Mean±SD) (µg/ml)
H_2O	7.50	>200	>200
Hexane	6.00	>200	151.25±22.96
DCM	3.00	138.25±14.31	141.34±10.23
MeOH	2.75	80.48±12.29	31.93±4.31
EtOAc	3.00	69.81±5.24	53.79±6.02
Control (CQ)		65.13±17.17 ng/ml	13.54±1.20 ng/ml

The results are expressed as mean ± SD of mean of IC_{50}s of the extracts and control (CQ).

selective inhibitory effect on *P. falciparum* calculated as (Wright and Phillipson, 1990);

$$SI = \frac{CC_{50}\ (Vero)}{IC_{50}\ (\textit{Plasmodium falciparum})}$$

In vivo acute toxicity

6-8 weeks old, male Swiss albino mice weighing 20 ± 2 g were used to determine acute toxicity of the active extracts (methanolic and ethyl acetate) (Lorke, 1983; Tona et al., 2001). Precisely, groups of mice (5 per group) were accustomed for 5 days to laboratory conditions. 6 groups were used to determine extracts toxicity while the other group for negative control. The mice were starved of food but provided with clean water *ad libitum* for 24 h before oral treatment. The negative control group was treated once with 0.2 ml of the vehicle while the other 6 groups were subjected to 1000, 1500 and 2000 mg/Kg of body weight per extract. Mortality within the first 24 h and subsequent behavioral changes were monitored for the next 14 days.

Ethical consideration

Permission to conduct the study was granted by *Scientific and Ethics Review Unit* (*SERU*), (Study KEMRI/SERU/CBRD/191/3803). The study was performed according to KEMRI guidelines on animal care and use. Furthermore; the internationally accepted principles for laboratory animal use and care as per WHO guidelines were considered. Gauge 23 needles were used to carry out IP injection. Animal-handling was in a humane way and experimental terminations were through sacrificing the mice in a chamber of carbon (iv) oxide gas. All sacrificed mice as well as carcass in the course of the experiment were bagged for disposal and incinerated.

Statistical analysis

All *in vitro* experiments were carried out in duplicate measurements and data were analyzed by Microsoft Excel 2013 using the nonlinear regression analysis aided determination of IC_{50}. *In vivo* assay analysis was done using Windows SPSS Version 25. One-way analysis of variance (ANOVA) followed by Tukey's honest significant difference post-hoc test were employed to determine statistical significance for comparison of parasitemia suppression and survival time among groups. p value of <0.05 was considered statistically significant.

RESULTS

In vitro assay

The results from the *in vitro* antiplasmodial assay of five different extracts of *N. sativa* against the two strains of *P. falciparum* and respective extract yields are listed in Table 1. The ethyl acetate and methanolic extracts showed good activity against D6 and W2 strains. However, best activity was exhibited in methanolic extract followed by ethyl acetate extract against D6 strain. Notably, ethyl acetate extract demonstrated better activity against W2 strain when compared to methanolic extract. Water extract had IC_{50} >200 µg/ml thus no activity against the two strains of *P. falciparum*. Hexane and DCM extracts proved inactive against D6 and W2 strains with IC_{50} >130 µg/ml. Adopted categorization of anti-plasmodial activity was as follows; high ($IC_{50} \leq$ 10 µg/ml), moderate (IC_{50} 10 -50 µg/ml, low (IC_{50} 50 -100 µg/ml) and inactive (IC_{50} >100 µg/ml) (Gathirwa et al., 2008).

Cytotoxic activities

The two active extracts (methanolic and ethyl acetate) were tested for cytotoxicity and exhibited relatively weak cytotoxicity against Vero cells with CC_{50}s of 164.13 and 73.66 µg/ml respectively with low selectivity index indicating mild cytotoxicity as shown in Table 2.

In vivo antimalarial activity of methanolic and ethyl acetate extracts of *N. sativa* against *P. berghei* ANKA infected Swiss albino mice.

The antimalarial activity of the two potent extracts (methanolic and ethyl acetate) against *P. berghei* ANKA infected Swiss albino mice by the 4-day suppressive method is presented in Table 3. Chemosuppresion was observed in a dose dependent manner after four days of antimalarial screening of *N. sativa* extracts (125-500 mg/Kg). The mean parasitemia in the groups treated with methanolic extract ranged from 4.03±0.67 to 7.18±0.73 while that of animals treated with ethyl acetate extract

Table 2. Cytotoxicity (CC_{50}) of methanolic and ethyl acetate extracts to Vero cells.

Extract	CC_{50} (µg/ml)	SI[a] (W2)	SI[a] (D6)
MeOH	164.13	2.04	5.14
EtOAc	73.66	1.06	1.37

[a] SI-selectivity index calculated as CC_{50}/IC_{50}.

Table 3. Mean of parasitemia, % parasitemia suppression and survival time after treatment termination.

Drug/ extract	Dose (mg/Kg)	Mean ± SD parasitemia (%)	% suppression of parasite	Mean survival time (days)
MeOH	125	7.18±0.73	56.29	10.50±0.58
	250	5.58±0.39	66.05	11.25±0.96
	500	4.03±0.67	75.52	16.25±0.96
EtOAc	125	6.75±0.31	59.02	11.00±0.82
	250	4.72±0.19	71.27	12.40±1.14
	500	4.04±0.87	75.30	15.60±1.52
CQ		1.20±0.23	92.59	28.25±1.50
Vehicle (3% dimethyl sulfoxide, 10% tween 80 in PBS)		16.45±3.08		5.00±0.82

The results are expressed as mean ± SD.

varied from 4.04±0.87 to 6.75±0.31. The mean parasitemia in the negative control group was 16.45±3.08. There was a significant percentage parasitemia difference between the test groups when compared with the untreated control group ($p< 0.001$). The extracts demonstrated highest percentage parasitemia reduction at 500 mg/Kg. However, there was a slight difference in percentage parasitemia reduction between the positive control and the two extracts. The extracts were able to prolong survival of the animals after treatment termination ($p < 0.001$) in comparison to the negative control.

Acute toxicity study

The acute toxicity study results indicated that the extract caused no mortality at a dose of 1000-2000 mg/Kg within the first 24 h as well as subsequent 14 days. There were no physical and behavioral signs of over-toxicity like; loss of appetite, inability of movement, diarrhea amongst others. This suggests that LD_{50} of the extracts is greater than 2000 mg/Kg.

DISCUSSION

There is need to seek improved antimalarial drug substitutes in order to fight resistance against *P. falciparum* (Mueller et al., 2000). The most dependable source of remedy is possibly medicinal plants (Gasquet et al., 1993; Wright and Phillipson, 1990). The world is a rich source of medicinal plants and more research needs to be conducted to exploit their uses. Quinolones and artemisinin derivatives, the most widely effective antimalarials were principally obtained from traditional plants (Waako et al., 2005). *N. sativa*, having been used traditionally to heal many ailments, it has been mentioned to contain antiparasitic activity although underutilized in antimalarial studies (Kooti et al., 2016). This study employed *P. falciparum in vitro* culture and *P. berghei* ANKA; *in vivo* model which takes into account the prodrug effect and involvement of the immune system in infection eradication for prediction of treatment outcomes. Many conventional antimalarial agents such as chloroquine, mefloquine and lately artemisinin derivatives have been established using rodent malaria model (Waako et al., 2005). The four day suppressive test mostly assesses the antimalarial activity of candidates on early periods of infection (Verma et al., 2011). The best antimalarial drug should be safe without adverse effects (Saito et al., 2018). As a result, cytotoxicity and toxicity studies were appropriate to identify potential application of *N. sativa* in malaria management. The results indicate that the concentrations of the extracts used to carry out the *in vivo* experiments were nontoxic with no mortality recorded during the first 24 h and subsequent 14 days at 1000-2000 mg/Kg dosage. Experimental animals survived

for the entire 4 days of the 4 day suppressive test without mortality. Usually, when the mice die in the course of the 4' Day suppressive test or before the fifth day, the death is attributed to the safety of the test drug rather than the animal parasitemia levels (Satayavivad et al., 1998). Therefore, cytoxicity and toxicity studies of the plant were important to determine selective indexes and lethal doses correspondingly. However, a weak cytotoxicity of IC_{50}s of 164.13 and 73.66 µg/ml with low selectivity index for methanolic and ethyl acetate extracts respectively was recorded. Al-Sheddi et al. (2014) noted earlier that *N. sativa* is weakly cytotoxic at higher concentrations. Additionally, our findings agree with those of Zaoui et al. (2002) that demonstrated relatively low toxicity levels and organ stability upon use of *N. sativa* extracts. This suggests that at lower concentrations, *N. sativa* extracts can be used safely to manage malaria. Therefore, the cytotoxicity and toxicity study justifies the safety of the plant in treating malaria.

The extracts demonstrated significant ($p<0.05$) parasitemia reduction activity in all the antimalarial evaluations. The *in vitro* study showed antiplasmodial activity of the five different extracts enabling the selection of the most effective extracts. Methanolic and ethyl acetate extracts showed the best activity with IC_{50}s of 80.48±12.29 and 69.81±5.24 against W2 strain and 31.93±4.31 and 53.79±6.02 against D6 strain respectively. These two active extracts also demonstrated good activity *in vivo.* The *in vivo* assay showed significant reduction in percentage parasitemia on the test groups compared to negative control group ($p<0.001$). These indicates the need for more studies on the use of the two solvents for extraction of active antimalarial constituents from *N. sativa* as shown by Mzena et al. (2018) in a different antimalarial plant study. The presence of single or diverse bioactive compounds possibly present in the seed extracts might have played a greater role in the antimalarial activity portrayed by the plant. Our results concurred with the findings of Ashcroft et al. (2018), who recorded much higher parasitemia suppression (>90%) upon use of methanolic extracts of *N. sativa* seeds. However, their results upon using aqueous extracts differed significantly with our findings as they reported up to 88.18% parasitemia reduction. Nonetheless, their seeds origin was different from ours. As a result, this may influence the bioactive compounds present in the seeds thus the activity. Mahmood et al. (2003) explained that the possible constituents of *N. sativa* have the ability to obstruct production of nitric oxide (NO) in macrophages. This in turn leads to escalation of tryptophan degradation via indolamine deoxygenase stimulation in human peritoneal macrophages. Consequently, the parasite dies as it is deprived of a vital amino acid. The immunomodulatory properties of *N. sativa* seeds that have been exhibited in past work done by Haq et al. (1999), might largely contribute to host-parasite interaction as noted earlier on by Anthony et al. (2005). Consistent with this model, Salem (2005) attributed anti-microbial effects in his study to the immunomodulatory properties of *N. sativa* seeds constituents. The abovementioned factors might have singly or together achievably supported the antimalarial effects of the seed extracts exhibited in this study. The limitation of the study is that identification of specific compounds responsible for antimalarial activity was not attained as this was beyond the scope of the current study. We therefore recommend that further analysis should be done on *N. sativa* seeds to identify the specific antimalarial compounds present.

Conclusion

The results in this study provide room for future comprehensive investigations on the plant through bioassay-guided fractionation, isolation and characterization of the bioactive compounds leading to establishment of novel antimalarial compounds fighting against drug resistant malaria. This study explains the use of *N. sativa* plant traditionally in the Middle East folk medicine and in Mombasa County.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Preliminary study to identify anti-sickle cell plants in Niger's traditional pharmacopoeia and their phytochemicals

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The management of sickle cell disease is a major challenge at the international level. In many African countries, sickle cell anemia is one of the major causes of mortality and is a critical public health problem in Niger. In this part of the continent, the estimated prevalence is around 18 to 22%, which is amongst the highest in Africa. Nowadays, despite the existence of some ways to improve the prognosis of sickle cell anemia as allograft, it turns out that these resources are expensive and out of reach of underdeveloped countries. The purpose of this study was to identify the preliminary anti-sickle cell plants in Niger's traditional pharmacopoeia. To do this, an ethnobotanical survey was conducted among the patients consulting the National Reference Center for Sickle Cell Disease (CNRD) and the traditional healers in the city of Niamey. At the end of this survey, 29 plant species were identified. The phytochemical study of 12 plants showed the presence of large chemical groups known for important biological properties (polyphenols, alkaloids, gallic tannins, sterols and polyterpenes).

Key words: Phytochemical study, sickle cell disease, Niger, medicinal plants, ethnobotanical survey.

INTRODUCTION

Sickle cell disease is a genetic, hereditary disease that attacks red blood cells and deforms them (Pliya, 1994). It is due to a point mutation of the gene coding for the synthesis of the β chain of hemoglobin. This results in the replacement of glutamic acid by valine (André and Marc, 1988). Abnormal hemoglobin (Hbs) deforms red blood cells that take the form of a lunar crescent (sickle cell). These sickle cells block the fine vessels of certain organs: lungs, eye, brain, etc., hence the clinical manifestations of sickle cell disease (Pliya, 1994). Sickle cell anemia modifies the membrane flexibility of erythrocytes making them more sensitive and fragile against free radicals. In black Africa, sickle cell disease is one of the leading causes of infant mortality; almost 95%

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of children die before the age of 4 (Tshilolo et al., 2019; Mamounata et al., 2006). In Niger, the prevalence of sickle cell trait is between 18 and 22% (Issaka, 2012). This justifies the creation of the National Reference Center for Sickle Cell Disease (CNRD) in August 2009 (Issaka, 2012).

Recently conducted survey on the screening and management of medicinal plants in four localities of Niger through the traditional practitioners revealed that 110 species in 89 genera and 47 families are used in the tradItional pharmacopoeia, among them plants with potential effect against sickle cell disease (Mounkaila et al., 2017). The work of Souchet and Louis (2013) reported that in West Africa, 93 species are potential plants with effect against sickle cell disease. Among them *Cajanus cajan*, *Carica papaya*, *Piper guineense*, *Pterocarpus osun*, *Sorghum bicolor*, *Syzygium aromaticum*, *Zanthoxylum zanthoxyloides*, *Justicia secunda*, *Moringa oleifera* and *Vinga unguiculata* seem to be the most promising species for sickle cell disease treatment.

Plants are naturally sourcing of various bioactive compounds thought with various health benefits such as prevention of blood sickle cell disease. Plant metabolites can be divided into primary metabolites associated with nutrition and secondary metabolites, which perform several functions like interaction between a plant and its environment, and also involves in many drug formulation or preparation (Uko et al., 2019). Secondary metabolites included a group of compounds known as phenolic (Raji et al., 2019). In Africa, medicinal plants have a good reputation, as nearly 75% of the population uses these plants for their medical care (Grosse et al., 2011; Mamounata et al., 2006). Nigeriens use traditional medicine more than the modern, because of the low monthly income of parents of sickle cell patients and the high cost of modern medicine (Karl Rachid, 2013). This study aimed to identify, through an ethnobotanical survey in the region of Niamey (Niger), the medicinal plants used for the treatment of sickle cell disease and carry out the phytochemical screening of these plants.

MATERIALS AND METHODS

Niamey region is located at the extreme West of Niger Republic where the National Reference Center for Sickle Cell Disease created in 2009 is located; and at the same time the place where the survey was conducted with the patients or their parents of CNRD but also with traditional healers (Issaka, 2012). The data collection was carried out using an ethnobotanical survey through a direct interview. To carry out this survey, two questionnaires were prepared for sickle cell patients and traditional healers. The questions have been translated into local languages, Hausa and Zarma (the two most spoken languages in Niamey region). The inventory survey questions were mainly focused on the anti-sickle cell plants, the manifestations of the disease, the parts of the plants used and the methods of drug preparation. The names of the plants listed are given in vernacular languages and for each plant species, a sample was collected for identification and phytochemical study. The identification of the plants species was carried out by the Biology Laboratory of the Faculty of Science and Technology, Abdou Moumouni University of Niamey, with help of Niger Republic national plant nomenclature and Professor Mahamane Saadou. In total, fifty-three (53) sickle cell patients (28 female and 25 male) from the CNRD and seven (7) all-male traditional healers were questioned for ethnobotanical survey. As for the patients, the survey focus more on the age of the patient, sex, origin, electrophoresis, type of hemoglobin, age of diagnosis of the disease, major symptoms of the disease, names of plants used, parts of plants, method of preparation, and locality of plants.

Chemical characterization

The plants organs depending on the part needed were collected from 15 to 30th December, 2013 during the day in the Tillabery (Niger) region at 115 km far from Niamey and 527 km away from Ouagadougou Burkina Faso. The plants were authenticated by a botanist professor Mahamane Saadou, Department of Biology at the Faculty of Science and Technology, Abdou Moumouni University of Niamey, Niger and were given voucher numbers from HI/011 to HI/039. After harvesting the different parts of the plant's materials, they were then cleaned and dried in an aerated area at room temperature for two weeks. The dried plant materials were grinded, sieved and the given powder were pulverized followed by extraction of the secondary metabolites using three different solvents petroleum ether, methanol and distilled water. Different chemical characterizations were performed as described in the work of Ronchetti and Russo (1971). The sterols and polyterpenes were characterized by the reaction of Liebermann, and the polyphenols content was determined through the reaction with ferric chloride ($FeCl_3$). The cyanidine reaction was used to characterize the flavonoids. The tannins, quinones and alkaloids content were determined using Stiasny, Bornstraëgen and Dragendorff's reagents, respectively. Picrosodium and foam test were used to characterize the cyanogenic glycosides and saponosides respectively.

RESULTS AND DISCUSSION

Plant organs and prescription

The survey revealed 29 plant species distributed among 20 families used in the treatment of sickle cell disease. The results of the ethnobotany survey are summarized in Table 1. Capparidaceae and Rubiaceae are the most represented (10.34%), followed by Bombacaceae, Meliaceae, Combretaceae, Convolvulaceae and Anacardiaceae (6.89%). At the end, the least-mentioned families are Lilliaceae, Vitaceae, Cyperaceae, Asclepiadaceae, Cochlospermaceae, Malvaceae, Acanthaceae, Bignoniaceae, Scrophulariaceae, Caesalpiniaceae, Mimosaceae, Sterculiaceae and Poaceae (3.45%). Depending on the size of the organs used, the leaves are the most recommended (41%), followed by the leafy stems (17%), the barks of the trunk (14%), the roots (10%), root barks (7%), fruits and seeds (4%) and finally, pods (3%). Figure 1a shows the percentages of utilized plant organs. The massive use of leaves is explained by their richness in primary and secondary metabolites, through photosynthesis. Indeed,

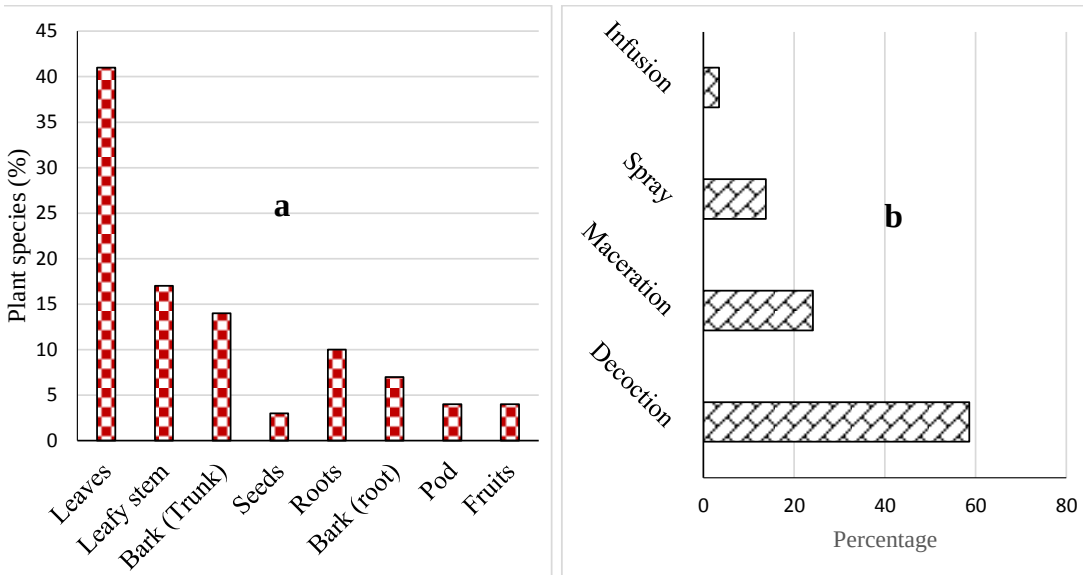

Figure 1. Percentages of plants species and methods of drug preparation from ethnobotanical survey of anti-sickle cell plants

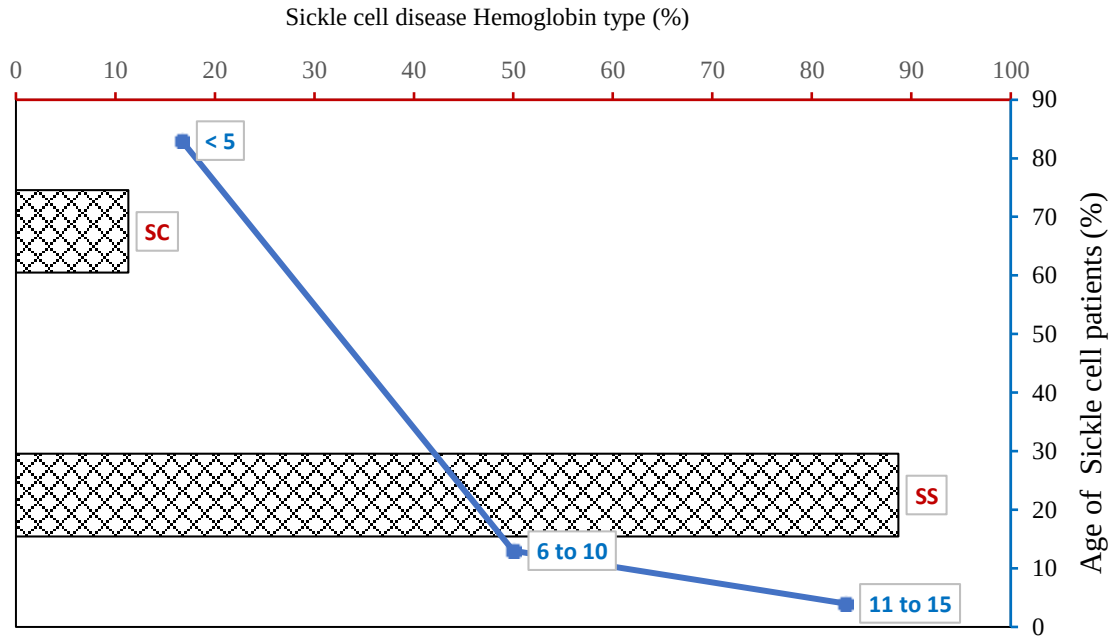

Figure 2. Sickle cell disease hemoglobin type and age of patients.

plants ensure the synthesis and storage of these metabolites with their chlorophyllian pigment. Secondary metabolites have very important biological interests (N'Guessan et al., 2009) which justifies in general, the use of leaves by the traditional healers in the treatment of sickle cell disease.

The majority of prescriptions against sickle cell disease are decoctions (58.62%) (Figure 1b). The most requested method of preparation has to do with efficiency in the extraction of a sufficient quantity of secondary metabolites (Detemmerman et al., 2018; Seca and Pinto, 2018). This result is close to that established by N'Guessan et al. (2009) who showed that the decoction is mainly used (42.30%) (Figure 2). It was found that the detection of sickle cell disease was early in most of the sickle cell patients (83%), which explains the importance of creating

Table 1. Different plant species cited by traditional healers and sickle cell patients.

N°	Botanical names	Family	Vernacular (Hausa)	Vernacular (Zarma)	Plant part	Preparation Method	Frequency
01	*Adansonia digitata*	Bombacaceae	Kuuka	Kôgna	F	Macération	01
02	*Allium sativum*	Lilliaceae	Tafarnua	Tafarnua	G	Décoction	01
03	*Ampelocissus africana*	Vitaceae	Kabakura	komnitanda	F	Décoction	01
04	*Anogeissus leiocarpus*	Combretacea	Marké	Gonga (kodjal)	F	Décoction	01
05	*Azadiracta indica*	Meliaceae	Bédi	Turiforta	F	Macération	02
06	*Bombax costatum*	Bombacaceae	Bagaye	Forgo	ER	Décoction	01
07	*Boscia angustifolia*	Capparidaceae	Agajini	Hasukoirey	F	Décoction	01
08	*Boscia senegalensis*	Capparidaceae	Anza	Anza	F	Décoction	01
09	*Cajanus cajan*	Cyperaceae		Dobudobugna	F	Pulvérisation	03
10	*Calotropis procera*	Asclepiadaceae	Tunfafiya	Sagaye	ER	Décoction	01
11	*Capparis corymbosa*	Capparidacea	Bagaye	kubi nya	F	Décoction	03
12	*Cochlospermum tinctorium*	Cochlospermaceae	Lawaga	Bagarbey	R	Pulvérisation	01
13	*Combretum micranthum*	Combretaceae	Géza	Kubu	F	Décoction	02
14	*Crossopteryx febrifuga*	Rubiaceae	hincin'morgo	hincin'morgo	F	Décoction	01
15	*Faidherbia albida*	Mimosaceae	Bagarbey	Kokoye	ET	Macération	01
16	*Gardenia sokotensis*	Rubiaceae	Gaud'in geza	tondifara	F	Décoction	01
17	*Hibiscus sabdarifa*	Malvaceae	Yakwa	waraou	Gr	Décoction	03
18	*Hygrophila senegalensis*	Acanthaceae	Djan tanki	banguizé	TF	Décoction	01
19	*Ipomoea asarifolia*	Convolvulaceae	Dumankada	Talhana	TF	Infusion	06
20	*Ipomoea batatas*	Convolvulaceae	Dankaly	Kudaku	TF	Pulvérisation	01
21	*Khaya senegalensis*	Meliaceae	madachi	farré	R	Macération	01
22	*Kigelia africana*	Bignoniaceae	Yawirya	Combey	Fr	Décoction	01
23	*Lannea microcarpa*	Anacardiaceae	Malga	Falunfa	ET	Macération	01
24	*Mitragyna innermis*	Rubiaceae	giayia	kabey	ET	Macération	01
25	*Sclerocarya birrea*	Anacardiaceae	dânia	Diney	ET	Décoction	01
26	*Striga hermontica*	Scrophulariaceae	kujiji	mâlu	TF	Pulvérisation	01
27	*Tamarindus indica*	Caesalpiniaceae	Tsamiya	bôsey	F	Décoction	01
28	*Walteria indica*	Sterculiaceae	Ankufuwa	Nunebasi	R	Décoction	01
29	*Zea mays*	Poaceae	Massara	kolgoti	TF	Macération	03

TF = Leafy stem: ER = bark of the roots; F = leaves; Gr = seed; G = pod; Fr = fruit; ET = bark of the trunk; Org. = Body; No. = number; Freq: frequency of the species mentioned.

the CNRD in Niamey. Similar findings by Gernet (2010) stated that the neonatal diagnosis was the major (59%) method of sickle cell disease screening. At the CNRD Reference Center, it was revealed that the SS-type sickle cell disease was the highest (88.70%) recorded (Figure 2). Concomitantly, the work of Gernet (2010) and Dreux (2012) on the sickle cell patients are similar to these results in which the SS-type were respectively 90 and 71%, although these show the predominant nature of hemoglobin S in Africa (Tshilolo et al., 2019).

Phytochemical screening of the plants

The phytochemical screening carried out during this work revealed the presence of a set of chemical groups in these identified plants as shown in Tables 1 and 2. In addition, the laboratory analysis revealed the presence of secondary metabolites either isolated or in combination such as tannins, sterols and polyterpenes, alkaloids, saponosides, flavonoids, quinones, polyphenols and cyanogenic glycosides (Tables 1 and 2). However, the literature review on the phytochemical study of some of these plants in the survey showed the presence of other chemical groups, namely, cardiotonic glycosides, anthocyanins and coumarins (Salma et al., 2018). The numerous therapeutic properties of secondary metabolites may justify the use of these plants in the treatment of sickle cell disease (Raji et al., 2019). Indeed, alkaloids and derivatives are used as painkillers and have vasodilator properties (Raji et al., 2019). They also show an anti-edematous action, causing a marked increase in blood pressure with a strong diuresis (Nurain et al., 2016; N'Guessan et al., 2009). Moreover, the anthocyanins promote antifalcemic action of sickle cells (Gbolo et al.,

Table 2. Phytochemical screening of the twelve (12) plants.

Plants			Secondary metabolites								
			Sterols and polyterpenes	Polyphenols	Flavonoids	Tannins		Quinones	Alkaloids (Dragendorff)	Saponosides (Cm)	Glycosides cyanogenic
Names	Org.	Extracts				Gallic	Catechin				
Bombax costatum	ER	EMA	++ - +++	+ ++ ++	++ - +++	- + ++	- + -	- - -	+++ - ++	4.5	++ ++
Boscia angustifolia	F	EMA	+ - +++	+ +++ +++	++ - ++	- +++ +	- +++ -	- - -	+++ ++ +++	0.0	- -
Calotropis procera	ER	EMA	++ - +++	++ - +	+ + -	- - +	- + +	- - -	++ +++ +++	5	- -
Capparis corymbosa	F	EMA	+++ - ++	++ ++ +++	+ + -	- + ++	- +++ ++	- - -	+++ +++ +++	5.5	- -
Hygrophila senegalensis	TF	EMA	+++ +++ +++	+ +++ +++	++ - +++	- +++ +++	- ++ ++	- - -	+++ +++ +++	2.5	- ++
Ipomea asarifolia	TF	EMA	++ + +++	++ +++ +++	+ - -	- +++ +++	- ++++ -	- - -	+++ + ++	2	- -
Ipomea batatas	TF	EMA	+++ +++ +++	- +++ +++	- + -	- +++ +	- +++ -	- - -	+++ +++ +++	00	- -
Kigelia africana	Fr	EMA	+++ - ++	+++ +++ +++	+ - -	- +++ +++	- - +++	- - -	+++ + ++	3	- -
Mitragyna innermis	ET	EMA	+++ - +++	++ +++ +++	++ +++ +++	- - ++	- - -	- ++ ++	+++ - ++	2.5	+ +++
Striga hermontica	TF	EMA	- +++ ++	+++ +++ +++	- - -	- + +++	- +++ -	- - -	+++ +++ +++	3.5	+++ -
Walteria indica	R	EMA	+++ - +++	++ +++ +++	- +++ -	- +++ +++	- +++ +++	- - -	+++ +++ +++	0.0	- -

Table 2. Contd.

Zea mays	TF	EMA	+++	-	-	-	-	-	+++	1.5	-
			+++	++	-	++	++	-	+++		
			+++	+	-	-	-	-	+++		-

Org.: Body; EMA: Aqueous methanolic ether; - = absent; + = present in small quantity; ++ = present in average quantity; +++ = present in large quantity.

2017); and the cyanogenic glycosides exhibit very high toxicity (Oluseyi, and Cohall, 2018). The combination of tannins and saponosides resulted to antibacterial properties (N'Guessan et al., 2009). As for the tannins, they have anti-inflammatory properties through their scavenging capacity on free radicals (Biseko et al., 2019; Amadou et al., 2012). Sudasinghe and Peiris (2018) reported in their phytochemical screening the presence of alkaloids, unsaturated sterols, triterpenes, saponins, flavonoids, tannins and proanthocyanidins present in the leaves of *Passiflora suberosa* L; and showed that the herbal preparations are capable of exercising glycaemic regulation with no significant toxic effects.

Generally known, the health benefits associated with polyphenols may be related to their various roles as scavenging agent and their ability to combat cell damage (Table 3). In addition, they possess hepatoprotective activity by preventing lipid peroxidation (Xing et al., 2019; Traoré, 2006), though polyphenols may also impact genes and gene expression, which make it an important candidate for combating sickle cell disease (Osei-Hwedieh et al., 2016; Amadou et al., 2012). Recent researches reported many health benefits through cell signaling pathways and antioxidant effects from flavonoids which are a group of plant metabolites. They are compounds that strengthen the inner lining of blood vessels, helping to help prevent circulatory disorders (Raji et al., 2019; N'Guessan et al., 2009). Traditionally used, naturally occurring substances around the world contain the coumarins which exhibit very important pharmacological activity such as an anti-oedematous, antithrombic, antioxidant and vasodilatory action (Traoré, 2006). In addition, it was established that the presence of phenolic compounds such as flavonoids, and tannin in the plant extracts can be considered to be responsible for inhibition of free radicals formed as a result of lipid peroxidation (Medini et al., 2014). The anthocyanin antioxidant efficacy can also be responsible for reduction of haemolysis of erythrocytes. Furthermore, compound with potent antioxidant efficacy and thus capable of preventing generation of free radicals (Mpiana et al., 2013) could be used as an antisickling drug. Bandara et al. (2018) revealed the presence of anthocyanin in the aqueous extract of *Passiflora suberosa* with potent antihaemolytic activity. Thus, antihaemolytic activity was found to be an important feature of antisickling agents.

The therapeutic effects of these different plants used may be related to their composition in secondary metabolites (polyterpenes, sterols, polyphenols, flavonoids, tannins, saponosides, alkaloids, quinones and anthocyanins). Previously, the antifalcemic activity of several plants such as *Fagara xanthoxyloïds* Lam and *Zizyphus mucronate* was demonstrated on *in vitro* blood sickle cell SS study, focusing on anthocyanins effect (Mpiana et al., 2009). The work of Baraka et al. (2018) showed the capacity of *Sterculia setigera* in preventing haemoglobin oxidation to form methaemoglobin, thus suitable for the management of sickle cell anaemia according to some traditional healers in northern Nigeria.

Conclusion

The study thus revealed that the ethnobotanical surveys carried out among sickle cell patients and traditional healers has enabled identifying of many ways of handling sickle cell cases, based on twenty-nine (29) plant species distributed among twenty (20) families. Several organs of these plant species have been used in the drug preparation to cope with sickle cell crises. These are manifested through several symptoms such as joint pain, headache, stomach, edema and jaundice. The plant parts analysis highlighted compounds like sterols and polyterpenes, polyphenols, flavonoids, tannins, saponosides, alkaloids, quinones, cyanogenic glycosides and anthocyanins. Natural substances also provide a basis for potential new drugs used against several diseases, including sickle cell anemia. Moreover, herbal medicine can currently be considered as a way of relieving anemia in general and sickle cell disease in particular.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 3. Phytochemical screening of seventeen (17) other plants.

Plants		Secondary metabolites								
Names	**Org.**	**Sterols-polyterpenes**	**Polyphenols**	**Flavonoids**	**Tannins**	**Quinones**	**Alkaloids**	**Saponosides**	**Glycosides cyanogenic**	**Anthocyanes**
Adansonia digitata	F	-	NT	+	-	+	-	+	+	+
	Gr	-	NT	+	+	+	-	-	-	+
	R	-	NT	-	-	-	-	-	+	-
	pulpe	-	NT	+	-	-	-	-	-	-
Allium sativum	G	-	+	-	+	-	+	+	NT	-
Ampelocissus africana	PA	+	NT	-	+	+	+	+	NT	NT
Anogeissus leiocarpus	F	-	NT	-	+	-	-	+	NT	NT
	ET	+	NT	-	+	-	-	+	NT	NT
	ER	+	NT	-	+	-	-	+	NT	NT
	Fle,F,Fr, Tg		NT	NT	+	NT	NT	NT	NT	NT
	PE	+	NT	-	+	-	-	-	NT	NT
Azadiracta indica	F	-	NT	+	-	-	-	-	NT	NT
	ET	-	NT	+	-	-	-	+	NT	NT
Boscia senegalensis	F	+	NT	-	+	-	-	+	NT	NT
	Gr	-	NT	-	+	-	+	+	-	-
Cajanus cajan	ER	-	NT	+	+	-	-	+	NT	NT
	ET	-	NT	+	+	-	-	+	NT	NT
	F	-	NT	-	-	-	-	-	NT	NT
Cochlospermum tinctorium	F	+	NT	-	+	+	-	-	NT	NT
	R	+	+	+	+	NT	NT	+	NT	NT
Combretum micranthum	TF	NT	NT	NT	NT	NT	+	NT	NT	NT
	F	+	+	+	+	+	+	NT	NT	NT
Crossopteryx febrifuga	F	+	+	+	+	-	+	+	NT	NT
	ER	+	+	+	+	-	+	+	NT	NT
	F	+	NT	-	+	-	-	-	NT	NT
	ET	-	NT	-	+	-	-	-	NT	NT
	ER	+	NT	-	-	-	-	-	NT	NT
Faidherbia albida	F	+	+	-	+	-	-	+	-	NT
Gardenia sokotensis	F	+	NT	-	+	-	+	+	NT	NT
Hibiscus sabdarifa	Gr	+	NT	-	-	+	+	+	-	-
		+	NT	-	-	-	+	+	+	-
	T	+	+	+	+	NT	+	+	-	+
	F	+	+	+	+	NT	+	+	+	+
	R	+	+	+	+	NT	+	+	+	-

Table 3. Phytochemical screening of seventeen (17) other plants.

	Gr	+	+	+	+	NT	+	+	-	-
	Gr	+	+	+	+	NT	+	+	+	+
Khaya senegalensis	F	+	NT	+	+	-	-	+	-	+
Lannea microcarpa	ET	+	-	-	NT	-	-	+	-	+
	Fle, F, T, Fr	NT	NT	NT	+	NT	NT	NT	NT	NT
Sclerocarya birrea	F	+	NT	+	+	-	-	+	NT	NT
	PE	+	-	+	+	-	+	+	-	-
	Fle, F, T, Fr	NT	NT	NT	+	NT	NT	NT	NT	NT
Tamarindus indica	Fle, F, T, Fr	NT	NT	NT	+	NT	NT	NT	NT	NT

Org.: Body; - = absent; + = present; NT = not tested; Fle = Flower; PE = whole plant; F = sheet; Fr = fruit; R = root; ER = root bark; ET = bark of the trunk; T = stem; Gr = seed; G = pod; PA = aerial part. Source: Mounkaila et al. (2017).

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Community pharmacists' knowledge and perspectives regarding the medicinal use of *Nigella Sativa* Seeds (*Ranunculaceae*),: A qualitative insight from Dubai, United Arab Emirates

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The seeds of *Nigella* sativa (NS) (family: *Ranunculacea*e), (NS) are widely used as medicine throughout the world. It is very popular in various traditional systems of medicine like Unani and Tibb, Ayurveda and Siddha. Seeds and oils have a long history of folklore usage in various systems of medicines and food. The objective of this study was to investigate the knowledge of community pharmacists in Dubai, United Arab Emirates about the medicinal use of NS and the challenges facing the dispensing and/or the prescribing process. A qualitative research methodology was adopted in this study. The participants were licensed pharmacists recruited using Dubai health authority database. A sample of 36 pharmacists (19 male, 17 female) was interviewed using the semi-structured interviewing technique. Based on the content analysis of the interviews, four major themes have emerged. Future research might attempt to compare the knowledge and perceptions of different healthcare providers of the medicinal use of NS seeds. In conclusion, respondents are aware of the potential effect of NS products available at their pharmacies on several diseases.

Key words: *Nigella sativa*; black seeds; traditional medicine; community pharmacists; Dubai.

INTRODUCTION

Nigella sativa (NS), which is often called black seeds or black cumin, is an annual flowering plant in the family Ranunculaceae, native to south and southwest Asia (Heiss and Oeggl, 2005). Since early 1970s, this herbal remedy has been extensively explored and studied for its therapeutic properties such as analgesic, anti-hypertensive, diuretic, antidiabetic, anticancer and immunomodulatory, antimicrobial, anthelmintics, analgesics and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective and antioxidant properties, all attributed to its quinone constituents in the seeds (Kanter, 2008a, b; Anwar, 2005; Zafeer et al., 2012; Ulu et al., 2012; Ahmad et al., 2013). Besides that, there are new promising

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therapeutic properties of NS that have been recently explored like its positive role in treating the central nervous system (CNS)-related ailments (Sahak et al., 2016).

Nevertheless, NS is an important component of many civilizations' folklore. This is contributed to its ability to treat various diseases and assist the body in its own natural healing process (Goreja, 2003; Abel-Salam, 2012). However, the real re-exploration of NS was by Muslim scientists who extensively studied and observed its therapeutic effects on patients like what the Muslim physician and philosopher IbnSina, commonly known by scientists as Avicenna, had mentioned in his famous medical reference book "Canon of Medicine," which was used as the primary medical text book until the 17th century in Europe. In his writings, he stated that NS has preventative features as it stimulates the body's energy and helps in the recovery from fatigue. Ibn Sina also recommended NS as a remedy for fever, common colds, headache, toothache, skin diseases, wounds, fungus, parasites, and worms as well as against bites and stings by poisonous animals (Luetjohann, 1998; Sahak et al., 2016).

Due to its several therapeutic uses that have been discovered by great ancient civilizations, a lot of researchers endeavored to study NS from different angles. In addition, the availability of NS products in pharmacies as Complementary and Alternative Medicine (CAM) and higher patients' recognition and awareness of the comparatively lower risk of consuming CAM especially for chronic illnesses (Brink-Muinen and Rijken, 2006) elevated the need to conduct a study on the role of pharmacists in educating their patients on the therapeutic uses of NS. To date, there has been no single research exploring into the community pharmacists' knowledge and perspectives with regard to the medicinal use of NS seeds in Dubai, United Arab Emirates (UAE).

MATERIALS AND METHODS

Semi-structured interviews were used to collect data in this research after extensive literature review and depending on a study done in Qatar (Kheir et al., 2014). The application of previously validated instruments to address pharmacy practice issues was found to be useful to ensure the instrument's validity and reliability (Felicity, 1997).

The participants were licensed pharmacists recruited using Dubai health authority (DHA) registered pharmacists' list that was adopted as the sampling frame. The sample of 36 pharmacists (19 male, 17 female) was randomly selected via a predetermined numbering system from a current list of approximately 2000 registered pharmacists in the software system of DHA within the control zone of the Government of Dubai, UAE. Selected candidates were then emailed a letter of invitation describing the study and their expected role. The study took place between January 10, 2017 and March 11, 2017. The appointed research team experts managed the arrangements for the time and place of interviews during the initial contact and obtained written consents from the participants prior to each interview.

The interviews mainly focused on exploring into the professional knowledge and attitudes of licensed pharmacists related to the medicinal use of NS in Dubai, UAE. Probing questions were used where necessary by the research team and the participants were given the freedom to express their views at the end of the interview session. Each interview was conducted by the researcher at the place and time convenient for the pharmacists and it lasted approximately twenty to thirty minutes. The research team conducted all the interviews in English, audio taped and transcribed them verbatim. The author verified the transcripts for their accuracy by listening to the tapes. Then, the transcripts were analyzed line by line, by reading them repeatedly and thematically analyzing their content (Creswell et al., 2004).

Table 1. Participants' demographic data.

Description	N
Age range	
Under 30	2
30-40	11
41-50	13
51-60	7
60 +	3
Gender	
Male	19
Female	17
Nationality	
UAE national	7
Expatriate	29

RESULTS

Thirty six interviews were conducted by an independent research team. Among the 36 participants, 19 were males and 17 were females. The demographic characteristics of the respondents are shown in Table 1. The thematic content analysis yielded four major themes: 1) Perceptions about NS use, 2) Sources of NS knowledge, 3) CAM rules and regulations and 4) Challenges when dispensing and/or prescribing NS products.

Perceptions about the use of NS

To investigate the way pharmacists in Dubai perceive the use of NS, they were asked about their personal experiences in dispensing and/or prescribing NS products to their patients. Almost all respondents (n=34) pointed to the use of NS products in hypertension. More than half of the participants (n=20) added other uses of NS oral-consumed products like being liver tonic, diuretic, digestive, anti-diarrheal, appetite stimulant, analgesic and anti-bacterial. Moreover, NS oil products were mentioned (n=6) to be helpful in skin disorders.

Table 2. Main indications of NS products in a selection of studies.

N	Indication	Study
1	Antidiabetic	Al-Awadi et al. (1991), Al-Hader et al. (1993), Matira and Zesmin (2008) Mohamed et al. (2009)
2	Antimicrobial	El-Kamali et al. (1998), Salem and Hossain (2000)
3	Analgesic and Anti-inflammatory	Abdel-Fattah et al. (2000), Al-Ghamdi (2001)
4	Antihyperlipedemic	Bahram et al., 2009; Ghanya et al. (2010)
5	Effect on gastro-intestinal tract	Gilani et al. (2001), Abdel-Sater, 2009)
6	Contraceptive and anti-fertility activity	Keshri et al., 1995, Agarwal et al. (1990)
7	Anxiolytic activities	Gilhotra and Dhingra (2011).
8	Activities in Neuroinflammation Model	Velagapudi et al. (2017)
9	Against Traumatic Brain and Spinal Cord Injuries.	Üstün et al. (2014), Jakaria et al. (2018).
10	Antipsychotic-like activities	Khan et al. (2014), Jakaria et al. (2018).
11	Antioxidant defenses	Hamdy and Taha (2009).
12	Antifungal activity	Rogozhin et al. (2011).

Sources of NS knowledge

When asked about the main source of knowledge related to the medicinal use of NS, many respondents (n=24) connected it to their cultural backgrounds. The second category of knowledge was related to the undergraduate pharmacy studies (n=7). The third category was gained from their activity of surfing through the Internet (n=5).

CAM rules and regulations

Most of the respondents (n=32) were not very sure about the rules and regulations related to dispensing and prescribing NS products in the UAE. They were aware about having a procedure of CAM registration by the health authorities; however, they were not sure about its steps. In addition, participants stated that the rules and regulations in different pharmacy sector control zones of the UAE do not require a prescription to dispense CAM.

Challenges when dispensing and/or prescribing NS products

Almost half of the respondents (n=17) mentioned the trust concern while suggesting CAM to their patients. They pointed out that they notice that some pharmacy customers underestimate the role of pharmacists especially when it comes to prescribing a drug therapy or correcting a mistake in a prescription provided to them. Another challenge related to CAM is to stay up-to-date with all the necessary information required about its use, side effects, and drug-drug interactions (n=15).

DISCUSSION

Based on the literature, hypertension is one of the most important indications of NS seeds (El-Tahir et al., 1993, Zaoui et al., 2002, Yar et al., 2008). From the results of this qualitative study, most of the samples pooled mentioned hypertension as the first indication for NS seeds. Over the last two decades, other important indications of NS products have been suggested elsewhere by many researchers. Many of these indications were mentioned in this study by more than half of the pharmacists interviewed (n=20). Table 2 shows a selection of studies proving NS seeds' effectiveness in some diseases. In this table, indications mentioned by the respondents of this study are highlighted.

According to the results extracted from this study, many respondents connected their knowledge about NS with their cultural backgrounds. Black Seeds products are very famous traditional remedies in Muslim countries. The actual importance of NS to the Muslims came from the holy saying of the Prophet Mohammed about the Black Seeds where he named it as the medicine for every disease except death (Ghaznavi, 1991).

Despite being aware about having a procedure for registering CAM products in UAE; respondents were not very sure about its steps. They asserted that there are no binding rules and regulations which organize dispensing and/or prescribing CAM products in the country. Federal law number 4 of 1983 of pharmaceutical professions and institutions in the UAE describes the procedure of licensing pharmacists, pharmacy technicians and pharmaceutical institutions and organizations (MOH, 1983). Through the law's 97 articles, there are no guidelines regulating dispensing items in pharmacies in the UAE. However, the UAE Ministry of Health (MOH) published a booklet describing guidelines and minimum acceptable standards for good pharmacy practice in UAE pharmacies (MOH, 2003). In this booklet, MOH shed light on the general guidelines followed by the aspect of dispensing medicines in UAE pharmacies.

World Health Organization (WHO) issued a guideline

which defines the basic criteria for the evaluation of quality, safety and efficacy of herbal medicines with the goal of assisting national regulatory authorities, scientific organizations, and manufacturers in assessing documentation, submissions, and dossiers in respect of such products (WHO, 2006). Based on the results from the respondents, the main challenge while dispensing and/or prescribing NS products is the lack of trust from some pharmacy customers. A research paper about the role of community pharmacists in Dubai, UAE pointed to this particular issue of underestimation by some pharmacy customers especially when it comes to prescribing over-the-counter medicines (Rayes et al., 2015).

Conclusions

In conclusion, respondents are aware of the potential effects of NS products available at their pharmacies on several diseases such as hypertension. Their knowledge about NS is basically derived from their cultural backgrounds. They stated that there are no clear guidelines controlling the dispensing and/or prescribing of CAM in UAE. In addition, some participants felt underestimated by some pharmacy customers when they tried to prescribe CAM products to them. Future studies might compare the knowledge and perception of pharmacists on the medicinal use of NS in particular and CAM in general in different countries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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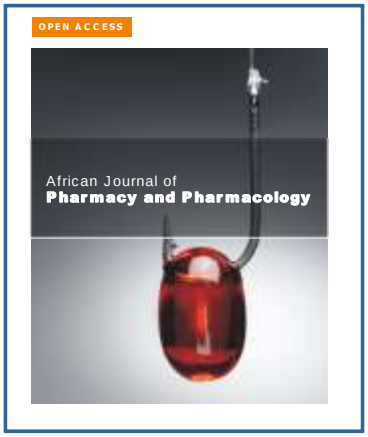

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