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Original article

Henna through the centuries: a quick HPTLC analysis proposal to check henna identity

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ABSTRACT

Henna leaves are the raw material of commercial body and hair dyes. According to historical and ethnobotanical information, henna was one of the first plants used for such purpose. However, differences can be observed between henna products by the origin of the raw material, the presence of other plants, or the addition of various contaminants that may cause allergies and permanent scarring. Nowadays henna is used everywhere but it lacks the necessary controls. We report a pharmacognostic study focused on quality control of henna's raw materials from different countries or based on other plants. The analytic approach based on High Performance Thin Layer Chromatography (HPTLC) was proposed as a reliable technique to evaluate natural products complex mixtures, as it is also the case of derived botanical marketed products.

Introduction

Henna through the centuries

The use of henna as medicinal plant and also for body painting is described in the Ebers Papyrus (Papyrus, 1912). Four hundred plants are mentioned in the Papyrus, used at that time to create 876 remedies. A lot of information can be found including the differences between the raw materials.

Concerning henna, at least seven different types are reported, describing where they were grown, its used parts and preparations using additional ingredients. It is also described that a fertile soil and moist conditions produce plants with low dyeing power which means a low active constituent content, whereas dry and hot conditions, and an iron rich soil give the opposite results. These indications have been confirmed by modern studies (Kumar et al., 2005). Therefore, we learnt from ancient Egyptians the fundamental information in pharmacognosy: the first piece of information relates to the origin of the plant, its growing conditions, crucial to define the quality of any raw material (Evans, 2009); second, the used part and third the preparation. In the case of henna, the latter means the procedure to obtain the dying substance by conversion of the plant's components. Finally various uses for henna were reported, including several medicinal uses as for hair dyeing. In fact, the study of the Royal Mummies revealed that henna was used for mummification in form of oilmen and to rejuvenate the mummy by colouring the hair, like in the case of Ramses III, who was blonde when he was young (Smith, 1912).

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The uses of henna lasted throughout the centuries, as most of the medicinal plants, across different civilisations. Therefore, we should split this story between European and Oriental countries. In Europe, henna was usually linked to aesthetic movements and the arts in general (Kortsch, 2009), in particular during English Orientalism in the XIX century, starting with the fashion of tinting the hair, against the English cultural tradition that considered red hair unattractive so as to denigrate Irish people. Thus, Pre-Raphaelites were irrationally devoted to the red hair (Loss, 1973). On the other side of the English Channel, the Impressionists made popular the connection between henna-red hair and bohemian life. Since that period a plethora of artists and singers used to dis hair with henna, until recent pop and rock stars (Sherrow, 2006).

In Orient the use of henna never declined and was always strictly linked to the culture and religion of the different countries, mainly for its ceremonial uses. Several examples have been documented. In the Middle East, muslim men have particular customs for dyeing their beards, whereas women are encouraged by religion to colour their nails and fingers red to display femininity. That leads us to the body art, the main utilization of henna. In many countries where henna naturally grows, the tradition is to use henna for honouring during special occasions, like victory in battle, births, circumcision, birthdays, even including the celebration of favourite animals. The most fascinating tradition is its use in weddings (Westermarck, 1972). Among Jews, Muslims, Sikhs, Hindus and Zoroastrians, on the Night of Henna the bride adorns her body, especially the hands, with sophisticated and artistic paintings (Brauer, 1993; Saksena, 1979). In all these cultures the message is the same: henna means joy.

Nowadays henna is commonly sold in packages available in grocery stores, as a cosmetic to dye hair and body (Dweck, 2002). Due to henna's worldwide popularity, in particular in the Middle East, Europe, Australia, Canada and the United States, all countries have their own production and different types, by request and tradition. Even if serious shops and trained artisans sell and use henna, in Europe most of the market and uses are completely unregulated, in particular the body art is carried out in the streets or on the beach under completely unsanitary conditions. Usually, henna raw material comes from distant countries, without any investigation of the constituents. Although health risks are evident and known, currently, controls are complex and difficult to be performed by the authorities. Origin, purity, preparation and utilization, are important aspects for safety and quality of henna, aspects similar to those analyzed for other botanicals, but with its peculiar problems. The aim of this paper is to examine each of these features and propose some solutions to problems posed.

To obtain the dying effect, henna products require a particular preparation consisting of a chemical reaction necessary to transform the herbal active constituents to obtain the true dye.

For body art, the dried leaf powder should be mixed with a slightly acid solution, like lemon or orange juice, vinegar, making the final preparation useful for the finely detailed body painting. The resulting paste can be applied using many traditional and innovative tools, including syringes or applicator bottles or simply with the fingers on the basis of a preferred design. Several factors result in different nuances of the final colour that can range from orange to auburn.

Considering the mean quality of henna and its preparation, regardless of the hair original colour, the colouring paste is also influenced by the size of the particles of the raw material, defined by the degree of grind of the dried leaves. Henna powder suitable for body art is sifted to about 40 microns, little finer than silt and larger than most pollen.

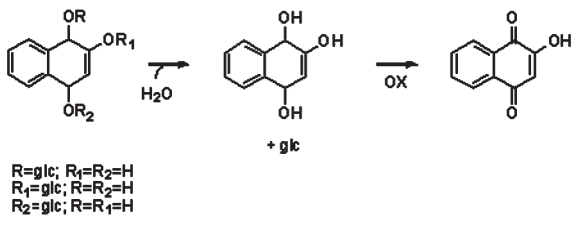
Chemistry

Henna will not stain hair or skin unless the molecules of its characteristic dye, lawsone, are released from the powdered henna leaf. This is obtained by the aforementioned preparation. In general, the available information regarding henna states that "the dying principle is lawsone, 2-hydroxy-1,4-naphtoquinone, that is contained in dried leaves in concentration of 0.5-2%". The same information has been previously reported in several publications, including scientific articles. Nevertheless, until now, our group was not able to identify free lawsone in the, tested henna samples (Gallo et al., 2008).

Lawsone is not present as a free molecule in the leaves, but it is derived from its precursors, the hennosides, during henna preparation. Hennosides are three isomers derived from the tautomeric forms of the keto-enol interconversion of the naphtoquinone structure. In this case the second ring is thrice oxygenated, that can give rise to three possible hydroxyl groups and consequently change to the diketonic form. Each of the hydroxyls can be glucosidated, giving rise to the three isomers. The aglycone, derived from their hydrolysis, is further converted by oxidation into lawsone that is the dying active compound (Scheme 1).

Such relation between glucosides and aglycone is common in natural products chemistry.

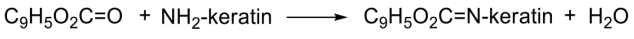
The integrity and stability of the active constituents of the herbal material is often preserved by glucosidation, since glucosides are solubilized and transported better in the cell or accumulated inside vacuoles, compared to the active but unstable aglycone. The aglycones need to be released by hydrolysis reaction to become active. Popular medicine performs this reaction in the traditional herbal preparations, usually using a hot aqueous infusion often acidified. This treatment favours the hydrolysis of the precursors.



Scheme 1. Conversion of the three isomerglucosides (hennosides) into the unique aglycone by hydrolysis in mildly acid conditions. Further transformation of the aglycone leads to the more stable lawsone by easy oxidation.

In the case of henna, hennosides are unavailable, in contrast with lawsone which is easily chemically produced or obtained from other sources, but they can be obtained by a careful phytochemical work. Other henna leaf constituents also occur during the dying process, like resins, flavonoids and tannins.

Because of the previous reasons, henna powder must rest in a mildly acid aqueous solution for 6 to 24 h before use to release the dye lawsone. Essential oils with high levels of monoterpene alcohols, such as tea tree, cajeput tree, eucalyptus or lavender oils, can improve and influence the skin staining. When the resulting paste is applied, the released lawsone gradually migrates from the paste into the outer layer of the skin/hair, and binds to the proteins in it by Michael addition, creating a fast stain (Dalglies, 1949).



Toxicity

All the regulatory attention is focused on lawsone, considered as the active ingredient (Nagwa et al., 2007), and also because the otherwise occurring addition of synthetic free lawsone could represent the main adulterant or pollutant.

However, in the case of lawsone there are some problems. The European Scientific Committee on Cosmetic Products and Non-Food Products Intended For Consumers, SCCNFP (2004), in its documented opinion SCCNFP/0798/04 an assessment and toxicological characterization of lawsone was provided and it was proposed to be considered class 2A of dangerous substances due to its potential genotoxicity/mutagenicity. The Scientific Committee on Consumer Products, SCCP (2005) in its published opinion SCCP/0943/05, highlighted that *L. inermis* was not listed yet in the Annex IV to Directive 76/768/EEC, among the dyes that can be contained in cosmetic products because investigation to assess its safety use was yet needed. In September 2013 the Scientific Committee on Consumer Safety, SCCS (2013), in its document SCCS/1511/13, confirmed the safety of the use of *L. inermis* for hair dyes with a maximum content of lawsone of 1.4 per cent. The presence of other species or other constituents is not allowed in products named as henna. The United States Food and Drug Administration (USFDA) has not approved henna for direct application to the skin. Henna imported into the U.S. intended for body art use is subject to seizure (FDA, 2013), but reported cases of prosecution are rare. On the contrary, henna use is unconditionally approved as a hair dye; therefore, it can only be imported for that purpose (FDA, 2012; Federal Register, 2009).

Henna thrives outside arid regions. The countries of origin of the powder are several with very different climates thus the chemical constitution could be variable. Besides the natural henna that should contain only *L. inermis* leaves, some products sold as "henna" contain other natural dyes (Dweck, 2002) that give rise to other type of henna (Lemordant and Forestier, 1983), as is shown in Chart 1.

Attention must be focused on the recently popular "black henna temporary tattoo". The "black henna" may contain the synthetic product *p*-phenylenediamine (PPD). PPD is able to quickly stain skin black, but it can cause permanent scarring and severe allergic reactions that include blistering, intense itching and permanent chemical sensitivity (Van der Keybus et al., 2005; Stante et al., 2006). Estimates of allergic reactions range between 3 to 15%. The phenomenon of temporary tattoos is practiced in openly and is particularly common in tourist areas as beaches and city centres (Marcoux et al., 2002; Onder et al., 2001; 2003). However, by the time of the emergence of blisters from 3 to 12 days after the application, most tourists have already left and do not return to show the damage and complain. The artists deny the dangers encouraged by the high profits due to the high demand of "black henna" body art to emulate "tribal tattoos" (al-Sheikh, 1996). True henna does not cause these injuries (Jung et al., 2006; Muhterem et al., 2009). Para-phenylenediamine use on skin is illegal in Western countries. The USFDA (2006) specifically

Chart 1
"Henna" types and their different colour effects.

Type of henna	Utilised plant	Family	Common name	Effects
Henna	*Lawsonia inermis* L.	Lythraceae	Henna tree, or Egyptian privet or Mignonette tree	lighter or stronger shades of red
Neutral henna	*Cassia obovata* Collad and closely related *Cassia* and *Senna* species.	Fabaceae	Senna Italica	colourless
Black henna	*Indigofera tinctoria* L.	Fabaceae	True indigo	brown and black shades
Black henna	*Isatis tinctoria* L.	Brassicaceae	Indigo	brown and black shades
Mixed with henna	*Terminalia chebula* Retz.	Combretaceae	Myrabolan tree	a tannin mordant

forbids PPD to be used for that purpose and may prosecute "black henna" producers. Furthermore the presence of other ingredients, including metal salts, synthetic dyes, and solvents, like gasoline, kerosene, lighter fluid, paint thinner and benzene have been identified and linked to adult leukaemia (Ik-Joon and Mu-Hyoung, 2006; Hassan et al., 2009).

Among the above ingredients, metal salts can interact with other chemicals, oils and waxes may inhibit the dye and other chemical dyes are common allergens. Any product that comes in a cream, block or paste has some sort of additive. Some pastes have been noted to include silver nitrate, carmine, pyrogallol, disperse orange dye and chromium. These have been found to cause allergic reactions, chronic inflammatory reactions or late-onset allergic reactions to hair products and textile dyes (Dron et al., 2007).

Henna is known to be dangerous to people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which is more common in males than females. Infants and children of particular ethnic groups are especially vulnerable (Raupp et al., 2001).

In this paper an analytical method based on High Performance Thin Layer Chromatography (HPTLC) is described as a useful technique to reveal the provenience and possible substitution of henna raw material with other plants.

Materials and methods

Materials

Powdered dried leaves from samples of *Lawsonia inermis* L., Lythraceae, were obtained from suppliers of plant materials from different places of world: Morocco (two samples), India (one sample), Pakistan (one sample), Iran (twelve samples), Cameroon (one sample), Tunisia (one sample), Yemen (one sample), and Egypt (three samples). Plant material reference standard was *Lawsonia inermis* L. leaves identified and authenticated by M. Nicoletti, on the basis of morpho-anatomical and histochemical characters. Voucher samples were deposited into the Herbarium at Department of Environmental Biology, Sapienza University of Rome. Samples of *Cassia obovata, Indigofera tinctoria* and *Isatis tinctoria* were obtained from the market and confirmed by morphological analysis.

Sample preparation

Powdered dried leaves of each sample were sieved (355) (20 g) and were separately mixed with methanol (100 ml) and sonicated in a model 3210E-MTH (Branson, Danbury, CT) ultrasonic bath for 45 min (Jin et al., 2006; Putzbach et al., 2007). The supernatant was filtered through Albet (Barcelona, Spain) qualitative analysis filter paper (43-38 µm) and the filtered extracts were concentrated under vacuum to dryness to obtain solid amorphous residues. The yield of the obtained dried extracts was about 18% (w/w). The obtained extracts were dissolved in methanol (5 mg/ml) for analysis.

HPTLC analysis

The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of (I) a Linomat 4 sample applicator using 100 µl syringes connected to a nitrogen tank; (II) an Automatic Developing Chamber 2 containing twin trough chamber of 20 x 10 cm; (III) a chromatogram immersion device III; (IV) TLC Plate Heater III; (V) TLC visualizer; (VI) TLC scanner 3 linked to winCATS software.

Solvents for extraction and HPTLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). Glass plates 20 cm x 10 cm with glass-backed layers silica gel 60 (2 µm thickness) were purchased from Merck (Darmstadt, Germany). Before use, plates were prewashed with methanol and dried for 3 min at 100 °C.

Sample application

Methanol filtered solutions of the dried extracts were applied to a plate with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s/µl (100 nl/s); injection volume, 6 µl; band width, 6 mm; distance from the bottom, 15 mm.

Development

The HPTLC plates were developed in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at room temperature. The developing solvents were ethyl acetate:formic acid:water (82:9:9, v/v/v). The length of the chromatogram run was 80 mm from the point of application. The developed layers were allowed to air dry for 5 min and then were derivatised with Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 ml of ethyl acetate), afterwards they were allowed to dry in the open air and were dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 ml of dichloromethane). Finally, the plates were warmed for 5 min at 120 °C before inspection. All treated plates were then inspected under a UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively, using a Camag TLC visualizer, before and after derivatisation. For the densitometric analysis the scanner was set at 337 nm, after a multi-wavelength scanning between 190 and 800 nm in the absorption mode had been preliminarily tried. Minimum background compensation was performed on the *x*-axis during the scanning. The sources of radiation were deuterium and tungsten lamps. The slit dimension was kept at 6.00 x 0.45 mm and the scanning speed used was 100 mm/s. A CAMAG DigiStore2 digital system with winCATS software 1.4.3 was used for the documentation of derivatised plates.

Sample solution stability

Sample solutions of the extracts were prepared and were stable for three days stored at room temperature (25 ± 2 °C) and for at least one month at 4 °C. They were applied on the same HPTLC plate, and the developed tracks were evaluated for possible additional band in comparison to the chromatogram at time zero. Similarly the band stability was evaluated by keeping the developed layers and inspecting them at intervals of 12, 24 and 48 h. Sample solutions were stable for at least two days on the HPTLC plates.

Validation: specifity and repeatability

Overlapping of bands is a typical analytical challenge for complex mixtures like botanical products. HPTLC allowed a good separation and visualisation of the constituents. Repeatability was determined by running a minimum of three analyses. Rf values for principal selected compounds varied ± 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison.

Results and discussion

Quality control of henna and its derived products should consider three main aspects: a) the provenience of the raw material; b) the presence of other plants; c) the addition of adulterants.

In this paper we will focus on the first two aspects, proposing a simple original analytic approach based on a HPTLC,that could be useful also for the third point. HPTLC is the latest tool for planar chromatography (Reich and Schibli, 2007; Nicoletti et al., 2012). Allowing the capacity to elucidate natural products, including those in very low concentrations, HPTLC is used to perform metabolomic studies, like the determination of most of constituents of an extract (Ram et al., 2011; Nicoletti, 2011; Gallo et al., 2011). Plates can be visualised and derivatised in several ways to obtain a great amount of information, and convert it into a series of peaks that represent the "fingerprint" (Gallo et al., 2008) by densitometric analysis. In such way, the comparison between samples is reliable and facilitated by the visual inspection so samples can be analysed side-by-side in exactly the same conditions (Nicoletti et al., 2012). HPTLC, born as an analytic tool for the identification of single substances, is now revealing important applications in pharmacognostic studies, as evidenced in this paper (Reich and Widmer, 2009; Agatonovic-Kustrin and Loescher, 2013).

As it has been already reported (Chart 1), commercial products named "Henna" instead of *Lawsonia inermis* L., can contain other plants as *Cassia obovata* Collad., *Isatis tinctoria* L. and *Indigofera tinctoria* L. In Fig. 1 the tracks of each raw material are presented. It is possible from the fingerprint obtained to elucidate the plant contents in the product and to confirm its identity by comparison with the corresponding plant reference standard. Fig. 2 reports the densitograms of the most frequently used plants in henna commercial products (Gallo et al., 2008), evidencing the differences in main constituents as well as minor ones better.

According to marketing indications, the best quality henna, known for its color richness and content purity, is grown and harvested in Northwestern India. However, *L. inermis* is cultivated and manufactured in several other countries (Kumar et al., 2005). As far as we know, this is the first study that compares the chemical composition of marketed henna leaves from different countries. In particular, the high sensitivity of the fingerprint approach is very useful to ascertain minimum differences. In fact the HPTLC approach is not based on a single constituent considered responsible of the activity, but on the whole metabolome, according to the principle to study "as many small molecules as possible". Therefore, untargeted analysis can be performed with reliable results instead of targeted ones, usually reported in Pharmacopoeias.

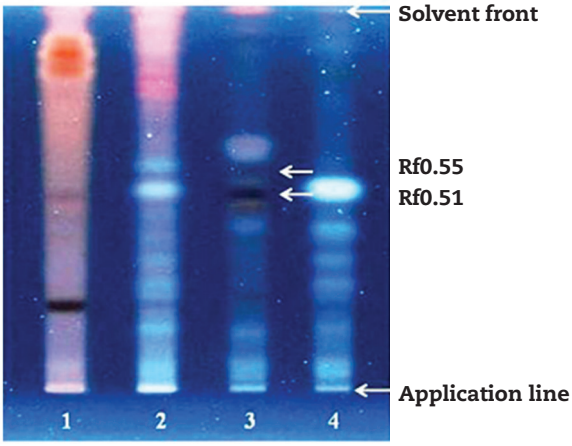


Figure 1 - HPTLC fingerprints of representative Henna commercial products based on tracks of *Cassia obovata* (1), *Isatis tinctoria* (2), and *Indigofera tinctoria* (4), in comparison to *Lawsonia inermis* reference standard (3). Mobile phase: ethyl acetate: formic acid: water (82:9:9). Derivatisation: NPR and Macrogol. Visualization: UV 366 nm.

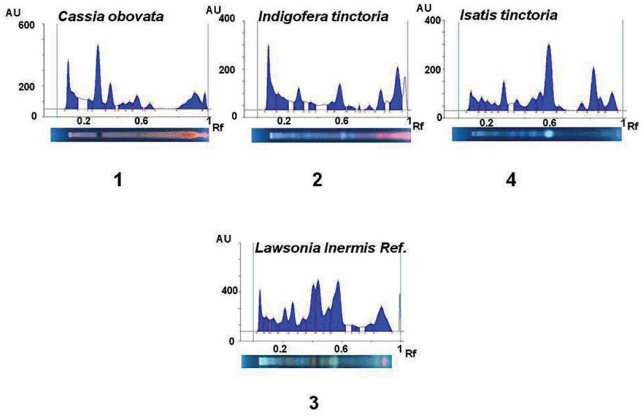


Figure 2 - Densitometric conversion of tracks reported in Fig. 1.

Samples of henna leaves were provided by manufacturers of raw plant materials from eight different countries: Morocco, 1, (two samples), India, 2, (one sample), Pakistan, 3, (one sample), Iran, 4, (twelve samples), Cameroon, 5, (one sample), Tunisia, 6, (one sample), Yemen, 7, (one sample), and Egypt, 8, (three samples).

Their methanol extracts were analysed by HPTLC method. The resulting fingerprints were compared to *L. inermis* reference standard plant material extract. The obtained fingerprints generally were in accordance with the standard and samples coming from the same country of origin showed its own fingerprint (Figs. 3 and 4). Some differences were better evidenced in the densitometric profiles, allowing the identification of the origin of the tested samples (Fig. 3).

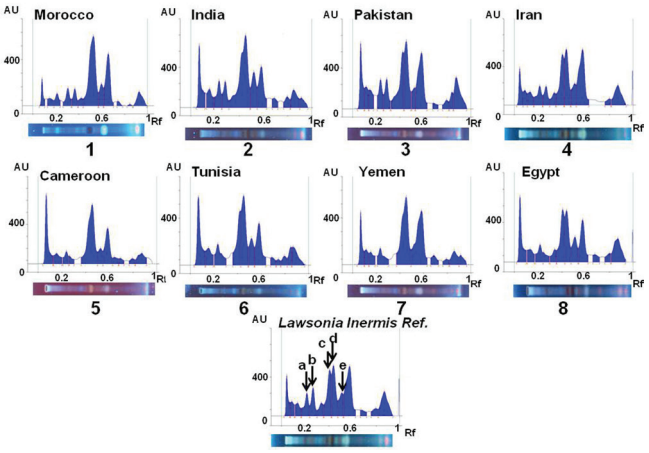


Figure 3 - Fingerprints (1-8) of representative *Lawsonia inermis* samples from different countries in comparison to the sample of plant material reference standard.

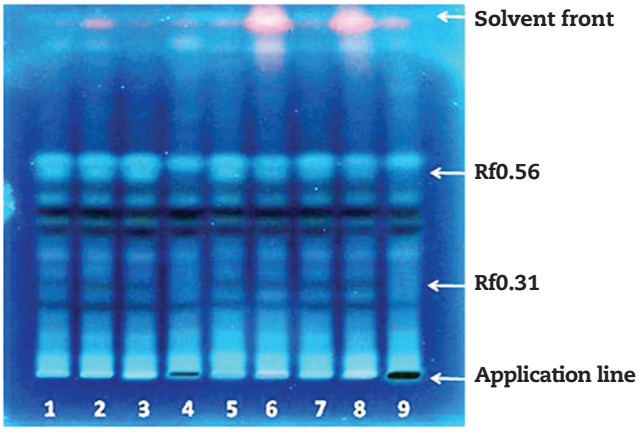


Figure 4 - Tracks of other samples from different countries. (7) *Lawsonia inermis* reference standard; (1-3; 6, 8) Iran; (4, 9) Egypt; (5) Pakistan. Mobile phase: ethyl acetate:formic acid:water (82:9:9). Derivatisation: NPR and Macrogol. Visualization: UV 366 nm. Tannins are evident at the starting line in samples (4, 9) from Egypt.

Samples coming from Morocco, India, Pakistan and Iran showed the same fingerprint characterised by two main evident peaks at Rf 0.23 and 0.29, respectively (peaks a, and b in Fig. 3). All samples presented three more or less separated peaks at Rf 0.42, 0.45 and 0.52, respectively (peaks c, d, e in Fig. 3). It is noteworthy to focus the attention on the starting line of the plate in Fig. 4, that shows further samples from different countries, where polar polyphenols, like tannins (polarity and Folin-Ciocalteu's test are in accordance), are present as black spots particularly in tracks 4 and 9, both from Egypt. In the same tracks the spots at Rf 0.56 and 0.31 were lacking (Fig. 4). Tannins correspond to a high percentage (5-10%) of the henna extract and participate along with lawsone in the reaction with skin proteins (Musa and Gasmelseed, 2012). Furthermore, three samples from Iran evidence an anomalous red spot at the top of the layer (more evident in tracks 6 and 8) that requires isolation and identification, albeit to our experience they could be degradation products of chlorophylls. Similar considerations can be deduced from the plates of Figs. 1 and 4 when visualised by other derivatisating agents (data not reported).

Conclusions

Body art and decoration is a necessity present in ancient and modern civilizations, due to different motivations and perceptions. In the last years the use of tattoo and piercing increased, mainly in adolescents and young people (Laumann et al., 2006), as a popular form of self expression, evidencing the need of identification and body control against modern alienation (Carroll and Anderson, 2002). People prefer temporary and natural tattoos because they are considered safer than the synthetic and permanent ones. However, reports on allergic and chronic inflammatory reactions are increasing that ask for higher quality controls. In fact, the case of henna shows that also the cosmetic sector, usually considered safe, requires adequate controls in order to avoid adulteration and polluting with toxic substances in order to enhance activity. Thus it is important to verify state, origin and identity of the utilised raw material as well as of the marketed products.

The reported data based on HPTLC fingerprint approach can be proposed as a quick and reliable analytic model for the pharmacognostic study on raw plant materials used in commercial products.

Authors' contributions

SMMZ (MSc student) and PCNB (undergraduate student) contributed in collecting plants and sample identification. FRG, GM, GP, GP (PhD student) contributed to sample processing and chromatographic analysis and to critical reading of the manuscript. MN contributed to in plant identification, designed the study, supervised the laboratory work and contributed to draft the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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