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The HPTLC approach to metabolomic determination of neem products composition

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Abstract

Although seeds are the only part commercially used, determination of composition of neem marketed products is a complicated matter**.** Natural variability and different process methods play a key role in this variability, mainly derived from the enormous quantity of different constituents present.

The chemical complexity is the necessary pre-requisite for a great quantity of possible applications. An adequate method to determine the chemical composition, as complete as possible, must be performed. Here we report the application of HPTLC fingerprint method on analysis of different neem products and derived extracts.

KEY WORDS: NEEM CAKE, HPTLC, FINGERPRINT, AZADIRACHTA INDICA, AZADIRACHTIN

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Introduction

Ancient Indian universe of neem tree, testified by the millenarian texts, is full of indications of different applications, from the medicinal ones to agricultural and livestock care [1]. Technologic age attention was mainly focused on insecticidal action of neem's seed oil and limonoids as active constituents, with azadirachtins first for importance and quantity.

However, although azadirachtins are at the centre of the interest on the chemical and activity point of view, the real treasure of neem is the complexity of its chemical composition. This aspect means that chemistry of neem products changes according to the origin, the used part, the production method, influencing radically the resulting products and the utilization. Each of these aspects has important effects on the composition. Beside limonoids, other constituents could be responsible of the activities, as well as biodisponibility and stability of constituents. As for many other plant drugs, phytocomplex must the considered as the real active principle [2].

Therefore, it is important to perform an analytic metabolomic approach [3,4], in order to check *in toto* the chemical composition, that can not be limited to a single class of products, as so far performed mainly by HPLC analysis.

Metabolomic approach can be epitomized as the study of small molecules as many as possible. In order to face and solve the chemical complexity of neem, we selected HPTLC as the adequate analytic tool. HPTLC, High Performance Thin Layer Chromatography. HPTLC is the last evolution of planar chromatography [5]. HPTLC is tailored to separate and visualize organic substances in complex mixtures, in particular in nutraceuticals and botanicals [6]. Evolution of planar chromatography was achieved in chemical analysis by the transformation and automation of the old TLC into HPTLC, solving the limits of TLC, like capacity of separation, influence of environment changes, limit in storing data, repeatability and robustness. Against the usual analytic approach based on standard's comparison, HPTLC fingerprint is the typical expression of the untarget approach [7-11]. In HPTLC a fingerprint is the individual chromatographic track typical of the metabolic production of a species. HPTLC is able to generate a rapid, low cost and easily understandable chromatographic result in the form of an unique sequence of peaks, nearly corresponding to the analysed sample in its chemical fullness [12]. In HPTLC tracks of the same species, variations are generally quantitative, not qualitative. Therefore, a complex composition, that means a lot of visible and separated spots, can be used a resource. Being usually the difference in populations of the same species mainly quantitative, each species is reprehesented by its fingerprint. Possessing the adequate fingerprint, identity of a drug is restricted to a comparison. Also HPTLC densitometric profiles are used, converting spots into peaks and allowing a quantitative determination [13].

Materials and Methods

Chemicals, Reagents and Solutions. Methanol for analysis and HPLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). Azadirachtins, used as standards were obtained from previous researches. Detailed information on the analysed samples, i.e. producers, production conditions, storage method, etc., can be obtained by directly asking the correspondence author.

Chromatographic equipment. The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of (i) Linomat 5 sample applicator using 100 □'3fL syringes and connected to a nitrogen tank; (ii) chamber ADC 2 containing twin trough chamber 20 x 10 cm; (iii) Immersion device III; (iv) TLC Plate Heater III; (v) TLC visualizer; (vi) TLC scanner 3 linked to winCATS software. Glass plates 20 cm x 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 µm thickness). Before use, plates were prewashed with methanol and dried for 3 min at 100 °C.

Sample preparation and application. The samples

were weighted and dissolved in methanol (6 mg/mL). Name cake samples were grounded before extraction.

Filtered solutions were applied with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s μL^{-1} (100 nL s^{-1}); injection volume, 2 µL; band width, 6 mm; distance from bottom, 15 mm.

Development and derivatization. The HPTLC plates were developed in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase, toluene/acetato di etile 4:6 (*v*/*v*), for 20 min at room temperature. The developing solvents (i.e. type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 80 mm from the point of application. The developed layers were allowed to dry in air for 5 min and then derivatised with a selected solution, including anhysaldehyde (1.5 ml *p*-anisaldehyde, 2.5 ml H_2SO_4, 1 ml AcOH in 37 ml EtOH) and/or Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate), dried in the open air and then dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 mL of dichloromethane). Finally, the plates are warmed for 5 min at 120 °C before inspection.

Inspection. All treated plates were then inspected under a UV light at 254 or 366 nm or under reflectance and transmition white light (WRT), respectively, at a Camag TLC visualiser, before and after derivatisation.

Densitometric analysis. For the densitometric analysis the scanner was set at 366 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 [12]. 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^{-1}.

Documentation. CAMAG DigiStore2 digital system with winCATS software 1.4.3 was used for the documentation of derivatised plates.

Stability and Validation. Sample solution of the extracts were prepared and stored at room temperature for 3 days and then applied on the same HPTLC plate and the chromatogram evaluated for additional band. Similarly band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 49 h. Overlapping of bands is a typical analytical challenge for complex mixtures like multi-ingredient products. HPTLC allowed a good separation and visualization of the constituents. Sample solutions of the extracts were found to be stable at 4 °C for at least 1 month and for at least 3 days on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. RF values for main 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. On the contrary, the results were critically dependent on prewashing of HPTLC plates with methanol.

Results

Beside the seed oil, that is by far the most marketed neem product, attention was focused on neem cake, in consideration of its potentiality. Neem cake is the waste by-product remaining after extraction processes. The quality of the oil, as that of the cake, strictly depends from the quality of seeds as well as from the type of extraction processes used, which strongly influences the chemical composition of the product. In neem cake a residual oil content is still present, up to 6%. Currently, based on this content the different types of commercial neem cake on the market are roughly identified as oiled and deoiled cake, but several other differences can be detected. The differences are relevant and must be determined, to obtain the necessary correlation between chemical constitution and biological activities.

We already reported on the composition differences of six different samples of neem cakes. The analytic method, developed using the HPTLC

devices, resulted adequate and reliable to evidence the main constituents of neem products and the differences therein.

Now neem cake samples were extracted with different solvents in order to evidence the different constituents. The comparison between the total methanolic extract with the ethyl acetate extract (Fig. 1) evidenced the presence of interesting compounds very fluorescent at 366 nm after derivatisation.

Preliminary analysis on these substances evidences a common highly unsaturated polycyclic skeleton actually under investigation. Again the main difference between two examined marketed samples was the content in fatty acids.

Differences in composition can be better evidenced by extraction or repartition of the raw material with solvents at different polarity, intensifying the evidence of minor components.

Therefore, a further analysis (Fig. 2) was performed on samples obtained by repartition of the total methanolic extract in solvents at increasing polarity (EtOAc, n-BuOH, H_2O) and the corresponding fingerprints reported in comparison with apolar (EtOAc) and polar total extracts.

The analysis performed evidenced three aspects: a) the changes in fatty acids predominance starting from the effect of oil production; b) differences in limonoids composition; c) accumulation of secondary constituents during the transformation process. Therefore examining the chromatographic plates of marketed neem products, three main classes of compounds could be attributed.

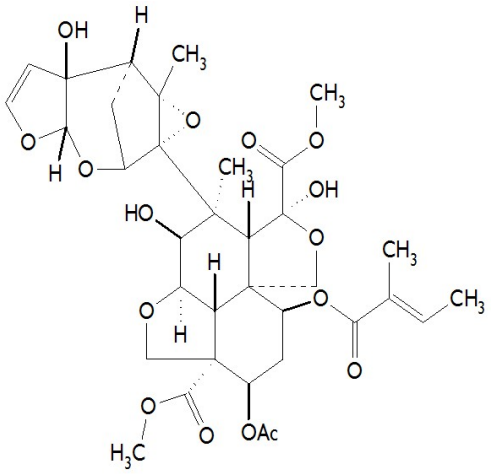
A -the lipophylic components, usually by far the most abundant and evident by the red intense colour when the plate is derivatised by NPR+Anhysaldeide regents. In the reported plate, they can be divided into two set of spots: the fatty acids and their methyl ester derivatives, placed on the front, and the glycerides, evidenced by a strong spot up near to the middle of the plate.

B – the nortriterpenes, mainly limonoids, differing in the position in the plate, scattered between Rf 0.7 and 0.3, and in the colours obtained after revelation. Their identities were performed by comparison with authentic samples. In this way it was possible to evidence the different content of these constituents into the different extracts and the marketed compounds. Therefore, if the oil, as expected, showed the prevalence of azadirachtins, whereas on the contrary in the neem cake salannin resulted the main constituent.

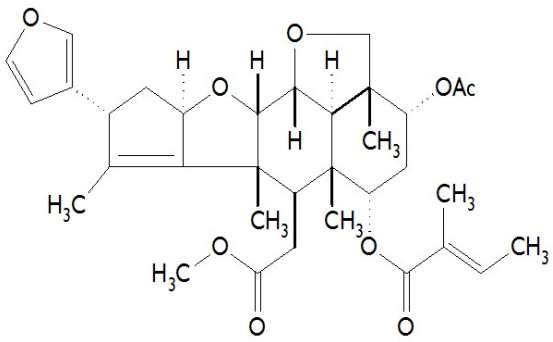
C – a set of high fluorescent spots, placed at 2/3 of the plate, between the two sectors of part A, whose intensities and evidence change in consideration of the extraction and purification process.

In accordance, if the oil evidences clearly its chemical nature by the dominance of the spots at the top of the plate, the neem cake shows the presence of several other spots, that become progressively more evidenced, including in particular the high fluorescent spots. Finally, the region at low Rf values present several compounds the start line, again in very different concentrations. Usually these spots are assigned to poliphenols, like tannins and similar constituents, all characterized by low solubility in the utilized mobile phase.

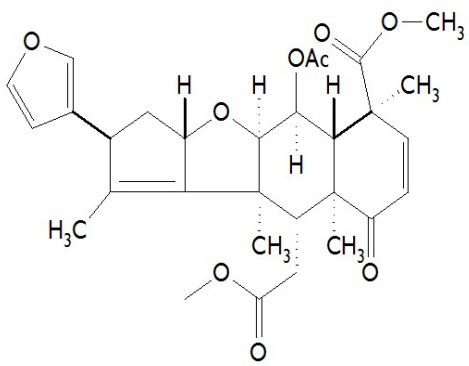
In conclusion, the HPTLC data showed a variation of composition in the neem oils and other products, according to the origin and the process, as well as the a prevalence of different constituents in relation to the extraction of the raw material.

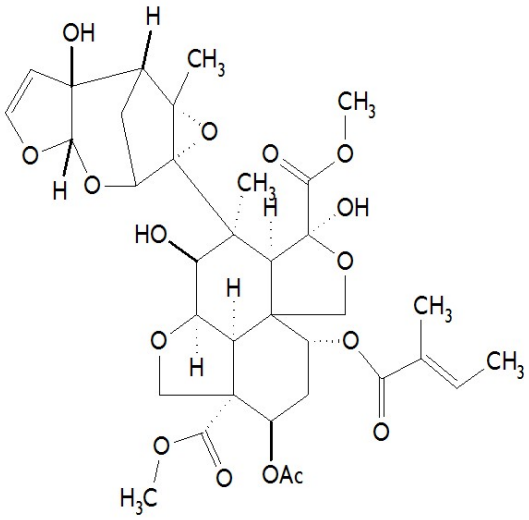


azadirachtin A



salannin





azadirachtin B

a

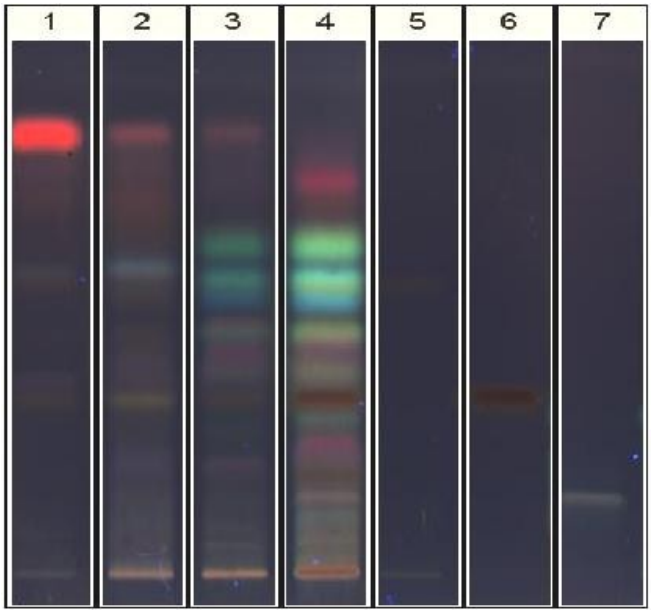


Fig. 1. HPTLC analysis of neem products in comparison with selected standards. Mobile phase: toluene/ethyl acetate 6:4. Derivatisation: Anhysaldehyde + NP Reagent. Visualisation: 366 nm. Tracks: 1, neem oil; 2, marketed sample n.1 of neem cake (ethyl acetate extract); 3, marketed sample n. 2 of neem oil (ethyl acetate extract); 4, marketed sample n. 2 of neem oil (ethyl acetate extract deoiled); 5, azadirachtin B; 6, salannin; 7, azadirachtin A.

b

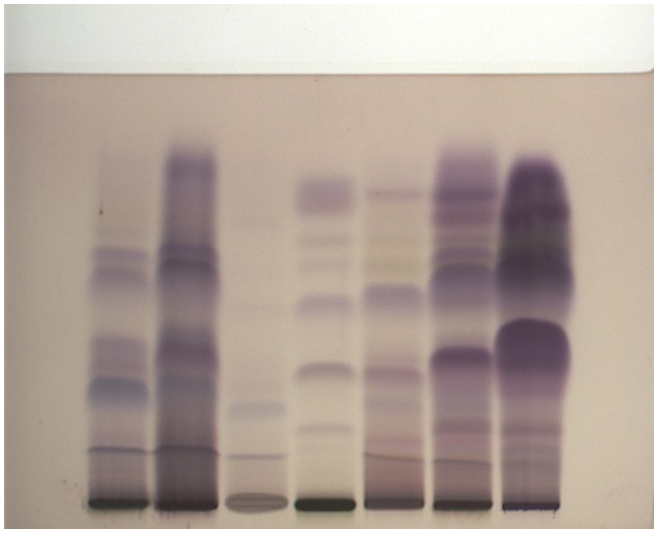


Fig. 2. HPTLC analysis of neem cake products. Mobile phase: toluene/ethyl acetate 6:4. Derivatisation: Anhysaldehyde + NP Reagent. Visualisation: a, white light, b, 366 nm. Tracks: 1, total methanolic extract of neem cake sample 1; 2, NCE; 3, NCB; 4, NCW; 5, ethyl acetate extract of neem cake sample n.1; azadirachtin B; 6, total methanolic extract of neem cake sample 2; 7, ethyl acetate extract of neem cake sample n.2

Conclusion

Actually HPTLC is reported as reliable analytic method by several international authorities [14-16]. The reported results are the necessary basis to give interpretation to the biological effects of the different products and their complete validation. Utilization of natural products appears in this moment the unique solution to an eco-friendly approach.

So far economic aspect, mainly the cost, was dominant and still must first considered, in particular in emerging countries. For this reason, the utilization shift from oil to neem cake is imposed. Chemistry, pharmacology and industry are called for tailoring new products for next environmental care solutions.

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