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Short communication

An improved method for thin layer chromatographic analysis of saponins

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ABSTRACT

Analysis of saponins by thin layer chromatography (TLC) is reported. The solvent system was *n*-butanol:water:acetic acid (84:14:7). Detection of saponins on the TLC plates after development and air-drying was done by immersion in a suspension of sheep erythrocytes, followed by washing off the excess blood on the plate surface. Saponins appeared as white spots against a pink background. The protocol provided specific detection of saponins in the saponins enriched extracts from *Aesculus indica* (Wall. ex Camb.) Hook.f., *Lonicera japonica* Thunb., *Silene inflata* Sm., *Sapindus mukorossi* Gaertn., *Chlorophytum borivilianum* Santapau & Fernandes, *Asparagus adscendens* Roxb., *Asparagus racemosus* Willd., *Agave americana* L., *Camellia sinensis* [L.] O. Kuntze. The protocol is convenient, inexpensive, does not require any corrosive chemicals and provides specific detection of saponins.

1. Introduction

当前在保健品和功能性食品的研究重点是生物活性成分的鉴别和特征

Current focus of research on nutraceuticals and functional foods is identification and characterisation of their bioactive constituents (Kimura et al., 2006). Saponins are important constituents of nutraceuticals and functional foods (Güçlü-Ustündağ & Mazza, 2007; Raju & Mehta, 2009) and are credited with a number of bioactivities like anti-inflammatory, antimicrobial, immunostimulant, hypocholesterolaemic, anticarcinogenic and antioxidant (Francis, Kerem, Makkar, & Becker, 2002; Güçlü-Ustündağ & Mazza, 2007). In addition, saponins form complexes with cholesterol of erythrocytes membrane forming pits and holes, which leads to increase in permeability and haemolysis (Chwalek, Lalun, Bobichon, Plé, & Voutquenne-Nazabadioko, 2006; Hostettmann & Marston, 1995). Thin layer chromatography (TLC) has the advantage of speed of analysis and comparison of many samples simultaneously, versatility of supports, solvent systems and detection reagents (Stahl, 1969). These attributes make TLC an ideal classic tool for first stage of phytochemical analysis as well as for monitoring of column chromatography fractions during purification of natural plant products. The common methods for detection of saponins are colour reactions and haemolysis (Kerem, German-[illegible]noua, & Yarden, 2005; Muetzel, Hoffmann, & Becker, 2003). The former have the inherent disadvantage of lack of specificity. The capacity to haemolyse erythrocytes is one of the important properties of saponins (Gauthier, Legault, Girard-Lalancette, Mshvildadze, & Pichette, 2009). Haemolytic property has been used for detection of saponins on thin layer chromatograms (Muetzel et al., 2003). However, the protocol used did not provide sharp spots of saponins against a clear background. Analysis of saponins using high performance thin layer chromatography (HPTLC) has also been reported (Chaicharoenpong & Petsom, 2009). However, the HPTLC instrumentation is expensive. A simple procedure for detection of saponins would be of immense utility to natural products chemists and biologists for screening plant sources for saponins and for monitoring bioactivity guided purification. Here, we report a modification of the method for detection of saponins on thin layer chromatograms using the haemolytic property of erythrocytes (Gauthier et al., 2009; Muetzel et al., 2003). The method has been applied to saponin extracts from ten saponin-rich plants and compared with the common detection method based on acid based spray and heating (Kerem et al., 2005). The protocol is simple, convenient and inexpensive. It does not require any corrosive chemicals and provides specific detection of saponins. Unlike the previous studies, spots corresponding to saponins are visible with fine contrast against uniform background.

薄层色谱(TLC)具有许多样品同时分析和比较的速度优势,用途广泛、溶剂体系和检测试剂的优势。。这是做天然产物想的经典工具薄层色谱的第一阶段分析以及植物监测的专栏

2. Experimental

2.1. Standards and samples

Standard samples of digitonin (Product # S4521) and saponin of Quillaja bark (Product # D5628) were purchased from Sigma–Aldrich, New Delhi, India. Shatavarin IV (3-*O*-{[β-D-glucopyranosyl (1 → 2)][α-L-rhamnopyranosyl (1 → 4)]-β-D-glucopyranosyl}-(25*S*)-5-β-spirostan-3β-ol), was isolated (98% pure) from the fruits of *Asparagus adscendens* and the structure was confirmed by spectroscopic analysis by NMR (^{1}H, ^{13}C) and mass spectrometer (Hayes et al., 2006). NMR experiments were performed on Bruker

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Table 1
Description of the plants, plant parts and the hR_{st} values in thin layer chromatographic analysis of saponins in the saponins enriched extracts (the hR_{st} value of shatavarin IV was taken as 100).

Name	Common Names	Plant part	hR_{st} values[a]
Asparagus adscendens Roxb.	Sansban, Saunspali	Fruit	110, 100, 57, 31
Asparagus adscendens Roxb.	Sansban, Saunspali	Root	119, 100, 81, 67, 57, 31
Camellia sinensis [L.] O. Kuntze	Tea	Seed	24
Aesculus indica (Wall. Ex Camb.) Hook.f.	Indian horse chestnut, Kanor, Bankhor	Fruit	52, 36, 24
Lonicera japonica Thunb.	Honeysuckle	Leaf	138
Silene inflata Sm.	Bigru	Root	7
Sapindus mukorossi Gaertn.	Soapnut, Reetha	Seed coat	186, 171, 155, 148, 131, 114, 98, 83
Chlorophytum borivilianum Santapau & Fernandes	Safed musli	Leaf	69, 48, 33, 24, 12
Asparagus racemosus Willd.	Shatavari	Root	100, 57, 31, 26
Agave americana L.	Agave, Bara Kunwar, Kantala, Ran Ban	Leaf	38, 29, 19

[a] Based of the thin layer chromatogram in Fig. 2a.

Avance-300 spectrometer. Mass spectra were recorded on QTOF-Micro of Waters Micromass.

For the validation of the method, saponins enriched extracts prepared from the plants listed in Table 1 were used. Saponins were isolated by protocol of Waller et al. (1996) with minor modifications. The plant samples were dried at 70 °C and 100 g dried sample was defatted with 1 L *n*-hexane using Soxhlet apparatus (5 h). The defatted sample (10 g) was extracted with methanol:water (1:1) thrice using 100 ml for each extraction. Methanolic extracts were pooled, the solvent was removed in vacuo and the remaining aqueous portion was partitioned three times with equal volume of *n*-butanol. The butanol layers were pooled, the solvent was removed in vacuo and the residue was used as saponins enriched extract for TLC analysis. Solutions of standard saponins and the saponins enriched extracts from plants listed in Table 1 were prepared as follows:

Quillaja bark saponin: 5 mg/ml in water, digitonin: 1 mg/ml in methanol, shatavarin IV: 1 mg/ml in methanol, saponins enriched extracts of different plants: each 20 mg/ml in methanol. The solvents were purchased from Merck (India) and were of analytical grade.

2.2. TLC analysis

TLC analysis was carried out on aluminium plates coated with silica gel 60 (Merck KGaA Darmstadt, Germany). A 10 µl aliquot of the solution of the standard saponins and the saponins enriched extracts of different plants (Table 1) was applied and the plates were developed in the solvent system *n*-butanol:water:acetic acid (84:14:7) to a distance of 15 cm from the origin (Kerem et al., 2005). The developed plates were air dried (Stahl, 1969).

2.2.1. Detection

(a) By immersing in suspension of sheep erythrocytes

Fresh heparinized blood collected from sheep was centrifuged at 2300*g* for 5 min. The pellet was suspended in phosphate buffered saline (PBS), pH 7.0, to a concentration of 3% (v/v). The suspension of erythrocytes ($0.8 \times 10^6/mm^3$) was taken in a glass tray and the developed TLC plate was immersed for 20 s. The plate was taken out of the erythrocyte suspension and held vertically for 30 s. White spots against a pink background appeared. The plate was immersed in PBS for 30 s to remove excess blood on the plate surface and again held vertically for 30 min. A good contrast between the background and the spots developed during this period and the chromatogram was ready for documentation. The data were expressed as hR_{st} values:

$$\frac{\text{Distance of sample spot from the start point}}{\text{Distance of reference material from the start point}} \times 100$$

Fig. 1. TLC of Quillaja bark saponin, digitonin and shatavarin IV. Solvent system: *n*-butanol:water:acetic acid (84:14:7). Lane 1: saponin of Quillaja bark. Lane 2: digitonin. Lane 3: shatavarin IV. Detection: with haemolysis on the plate by immersion in erythrocytes suspension followed by washing.

The detection limit was determined by applying a series of concentrations of each of the samples.

(b) By spraying with ethanol:sulphuric acid (90:10)

TLC plate after development and drying was sprayed with freshly prepared solution of ethanol:sulphuric acid (90:10) followed by heating at 110 °C for 10 min (Kerem et al., 2005).

3. Results and discussion

The TLC chromatogram of the standard saponins detected by immersion in erythrocytes suspension is shown in Fig. 1. Saponins gave white spots against a pink background. The hR_{st} values (taking the value for shatavarin IV as 100) were nine for Quillaja bark saponin and 35 and 54 for the two main saponins in commercial sample of digitonin. The sensitivity of detection was 1 µg for shatavarin IV and digitonin and 5 µg for Quillaja bark saponin.

The validation of the method was done using the saponins enriched extracts from saponin-rich plants (Table 1, Fig. 2).

The saponins in the extracts of plants listed in Table 1 varied in polarity and number, the most complex profile was observed in *Sapindus mukorossi* (soap-nut seed coat) (Fig. 2a).外形

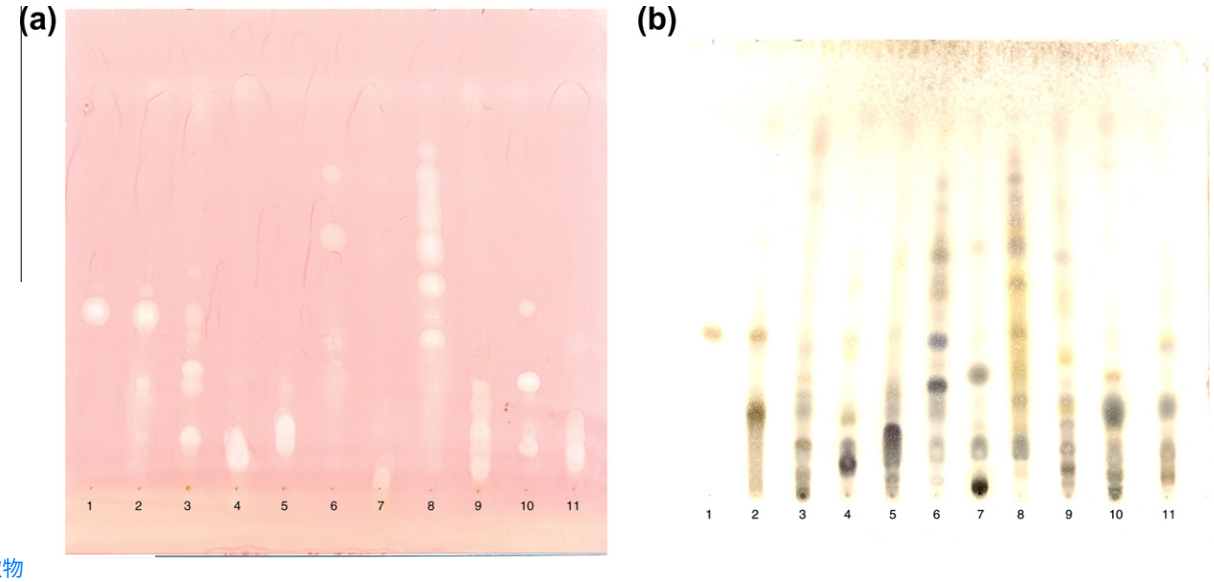

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Fig. 2. TLC of saponins enriched extracts from different plants: Lane 1: shatavarin IV. Lane 2: *Asparagus adscendens* Roxb. (fruit). Lane 3: *Asparagus adscendens* Roxb. (root). Lane 4: *Camellia sinensis* [L.] O. Kuntze (seed). Lane 5: *Aesculus indica* (Wall. ex Camb.) Hook.f. (fruit). Lane 6: *Lonicera japonica* Thunb. (leaf). Lane 7: *Silene inflata* Sm. (root). Lane 8: *Sapindus mukorossi* Gaertn. (seed coat). Lane 9: *Chlorophytum borivilianum* Santapau & Fernandes (leaf). Lane 10: *Asparagus racemosus* Willd. (root). Lane11: *Agave americana* L. (leaf). Solvent system: *n*-butanol:water:acetic acid (84:14:7). Detection: (a) with haemolysis on the plate by immersion in erythrocyte suspension followed by washing and (b) spraying with ethanol:sulphuric acid (90:10) followed by heating at 110 °C for 10 min.

喷涂乙醇：硫酸90：10再100度加热10分钟　　红血球溶解　　红血球跟着悬浮

The hR_{st} values for the saponins from different plants are shown in Table 1. The data show the diversity in the number of constituents as well as in the range of hR_{st} values (7–186). A comparison of the detection of saponins by the method we report and the common method based on ethanol–sulphuric acid spray followed by heating (Kerem et al., 2005) is shown in Fig. 2a and b. The detection by haemolytic method showed clear well defined spots (Fig. 2a). In addition, the erythrocytes suspension could be reused at least three times with satisfactory results. On keeping the plates at ambient temperature (~25 °C) for 4 h, the background turned off-white and the white spots corresponding to saponins could still be discerned. These chromatograms could be preserved at ambient temperature without any further change in the background up to a month, which was an added advantage over the detection by spraying. Detection of the TLC chromatograms of different saponin extracts along with shatavarin IV as a standard using ethanol: sulphuric acid (90:10) spray is shown in Fig. 2b. A comparison of the chromatograms in Fig 2a and b shows that detection by ethanol–sulphuric acid based sprays detects a number of additional compounds, presumably non-saponins. The detection by haemolysis of erythrocytes is not only specific but the spots are more prominent and sharp (Fig. 2a). The sulphuric acid based spray reagents commonly used for detection of saponins (Kerem et al., 2005) are not specific and are common for the detection of steroids, triterpenoids and bile acids (Stahl, 1969). Earlier, the haemolytic property of saponins has been used for detection on thin layer chromatograms by spraying with erythrocyte suspension. This method did not give a smooth background and there was not proper contrast between saponin spots and the background on thin layer chromatograms (Muetzel et al., 2003). Another protocol for detection of saponins on TLC plates by haemolysis of erythrocytes involved overlaying the chromatogram with blood–gelatin solution (Stahl, 1969). To prevent the blood–gelatin solution from spillage, an adhesive tape is stuck all round the edges. This protocol is cumbersome as compared to the method we report here. Moreover, the blood–gelatin solution can be used only once unlike the reuse of erythrocyte suspension in our method.

4. Conclusions

Saponins are a very diverse group of compounds with variations on account of sapogenin, the sugar length, the sugar linkage and the substituents on sugars (Hostettmann & Marston, 1995). So, the choice of the solvent system for TLC would depend upon the compound(s) under investigation. Oleszek and Bialy (2006) have given a comprehensive list of solvent systems which can be used for optimum resolution of saponins under investigation. Further, the haemolytic activity of saponins is also dependent on their structural features like sugar length, linkage, the substituents on the sugar and the aglycone (Chwalek, Ple & Voutquenne-Nazabadioko, 2004; Wang et al., 2007). Accordingly, the working concentrations of different saponins for TLC and detection by haemolysis would vary and are required to be ascertained by pilot experiments. The method we report is simple, specific, convenient and time saving for analysis of saponins by TLC for purification, chemoprofiling of plants, and nutraceutical applications.

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