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A Preliminary Development of an Optimised Method for Analyzing *Allanblackia parviflora* Kernel and Seed Oils Using High-Performance Thin Layer Chromatography (HPTLC)

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Abstract

High-Performance Thin Layer Chromatography (HPTLC) is a reliable and rapid method useful for the detection of product quality and composition. In recent times, HPTLC instrumentation allows to obtain fingerprints suitable to determine composition and identity of plant raw materials. This study aimed at exploring the results of High-Performance Thin Layer Chromatography (HPTLC) analysis as a useful means for the authentication of *Allanblackia parviflora* kernel and seed oils using the fingerprint approach. Sixteen bulked kernel and seed oil samples from 16 different communities were analyzed by HPTLC method and their fingerprints were compared. The optimum experimental conditions established were: sample mass of 20 mg, hexane as appropriate dissolving solvent, toluene-ethyl acetate (85:15, v/v) as mobile phase solvent and p-anisaldehyde sulphuric acid as derivatization reagent. Using HPTLC fingerprinting, it was observed that no differences existed in fingerprints for all kernel and seed oil samples under illumination mode at 366 nm after derivatization. However, the seed oils showed clear, sharp and more intense bands indicating the presence of more phytochemicals. This suggests that there are phytochemicals in the seed shells/husks soluble in the seed oil.

Keywords: Kernel oils; Seed oils; *Allanblackia parviflora*; HPTLC; Authentication

Introduction

Allanblackia belongs to the *Guttiferae* family and is considered to be a wild, uncultivated plant species in the rainforests regions of some African countries such as Kenya, Tanzania, Nigeria, Cameroon, Angola, Uganda, Congo and Ghana [1,2]. It is an evergreen forest plant; which normally attains a height of 30 m [3] and has a spotted white stem and branches that are horizontal and crowded at the apex. Nine (9) species have been identified in Africa [4,5]. In Ghana *Allanblackia parviflora* is the only species found and mostly grows in the Western, Central, Eastern, Brong Ahafo and Ashanti regions [6,7]. The oval to sausage-shaped fruit is brown and corky with 40-80 seeds embedded in a pinkish-brown pulp [8,9]. Matured fruits that fall to the ground are often opened by animals in search of the seeds for food [10]. The seeds are red-brown, irregular in shape and have flattened suRfaces. The seeds have a thin coat which is brittle and can be easily removed. Seeds contain 67-73% oil (white fat at room temperature) consisting mostly of stearic and oleic acids [11-13]. Owing to this profile *Allanblackia* oil is useful in the production of margarine and in the manufacture of soaps, cosmetics and ointments. Many companies including Unilever PLC purchase the seeds form local communities, a situation which has created a guaranteed market for the product in Africa [4,5].

Increasing evidence of the nutritional and health benefits of virgin (non-refined vegetable) oils has generated consumer interest in virgin oils globally in recent times. The expression of oils with minimal heat application is advantageous and places higher commercial value on such oils. This is because oil expressed at low temperatures leaves naturally present bioactive compounds in the seeds un-deteriorated [14]. Additionally, *Allanblackia* oil is said to be non-refined especially those consumed mixed with palm kernel oil and costly hydrogenation step during margarine production is also skipped due to the solid nature of the fat [15]. The oil has a relatively simple fatty acid composition with the dominant fatty acid being the nutritionally essential stearic and oleic acid [9] and is reported to lower plasma cholesterol levels, therefore reducing the risk of heart attack [16]. *Allanblackia* oil remains a novel product recently sought and incorporated into Unilever Pty products (soaps and cosmetics) due to the oil's high quality oleic and stearic acids [4,5]. Spiking the product with synthetic materials is dangerous to consumers and noxious for future correct developing of the use of natural products. There have been instances where palm oil meant for both local and international markets were reported adulterated with lard [17] and Sudan IV dye, a category 3 carcinogen used as an industrial dye in the production of plastics and textiles to impact color [18]. *Allanblackia* oil is also liable and therefore to avert this situation, there is a need to profile the kernel and seed oils of *Allanblackia parviflora* in order to detect adulteration of the oils. Currently, TLC remains the immediate, simplest, reliable analytical tool to check the presence and the identity of known marker compounds, to follow the improvement of a synthesis, to test column

fractions' separation trend, and others. However, the current challenge of complex mixtures, as those present in the botanicals, need more effective proper analytical tools [19]. The study was aimed at using HPTLC analysis with particular reference to the fingerprint approach in classifying the composition of the *Allanblackia parviflora* kernel and seed oils.

Materials and Methods

Reagents and solvents

All chemicals used were analytical reagent grade unless otherwise stated. Hexane, acetone, methanol from Fischer Chemical, toluene from Lab Scan Analytical Science (Australia), ethyl acetate, sulphuric acid, p-anisaldehyde from Aldrich Chemical Inc. (USA). Ethanol (96%) and formic acid were sourced from Univar, Australia. Petroleum ether and glacial acetic acid were purchased from Scharlau chemicals, Australia. Phosphomolybdic acid was purchased from Sigma-Aldrich, Australia. Silica gel 60 F254 HPTLC glass plates (20 cm × 10 cm and 10 cm × 10 cm) was purchased from Merck (Darmstadt, Germany).

The derivatization reagent, p-anisaldehyde sulphuric acid was prepared by measuring 85 mL of methanol into 100 mL Schott bottle and cooled in ice-cubes. Acetic acid, 10 mL was added slowly to the cooled methanol. Sulphuric acid, 5 mL was added to the resultant solution and was well mixed. The mixture was allowed to cool at room temperature (25ºC) and 0.5 mL p-anisaldehyde solution added. The second derivatization reagent, freshly prepared ethanolic phosphomolybdic acid was completed by adding 10 g phosphomolybdic acid to 100 mL ethanol (96%).

Study area and sample collection

The kernel and seed oils were sourced from *Allanblackia parviflora* fruits collected from 16 communities (10 trees per community) across three ecological zones (moist semi-deciduous, SD, moist evergreen, ME and wet evergreen, W) in Ghana. The oil was extracted using a manual press using the method described by Sefah et al. [12]. Bulked kernel and seed oils from the communities were stored in opaque plastic containers at room temperature (25ºC) until analyzed. Kernel and seed oil samples were named based on the communities trees were selected from: New Edubease (NEK, NES), Adansi Akrofuom (AAK, AAS), Fenaso (FK, FS), Anwona (ANK, ANS), Afosu (AFK, AFS), Atwereboana (ATK, ATS), Akoase (AKK, AKS), Wassa Akropong (WAK, WAS), Daboase (DK, DS), Sefwi Bodi (SBK, SBS), Samreboi (SK, SS), Benso (BK, BS), Kwansima (KSK, KSS), Asonti (ASK, ASS), Banso (BAK, BAS) and Nzema Akropong (NAK, NAS).

Sample preparation

Kernel oil of *Allanblackia parviflora* was used for the optimization process. Parameters varied for the HPTLC analysis included: sample mass (10 mg, 20 mg, 30 mg), extraction solvents (hexane, acetone, ethanol, petroleum ether), mobile phase solvents (toluene-ethyl acetate-formic acid, 25:15:10, v/v/v; toluene-ethyl acetate, 85:15, v/v) and derivatization reagents (p-anisaldehyde sulphuric acid and ethanolic phosphomolybdic acid). The various masses of the oil samples were prepared by dissolving in 1 mL of hexane in a sample vial. Two of the dissolving solvents (acetone and ethanol) were excluded from the analysis due to poor dissolution of oil samples. Sample concentrations ranging from 10 mg/mL to 30 mg/mL were analyzed with the HPTLC before choosing the optimal condition.

HPTLC operating conditions

A CAMAG High-Performance Thin Layer Chromatography (HPTLC) system was used to analyse the extracts. The extracts, and standard mixture were applied (2 µL unless otherwise stated) as 6 mm bands at 10 mm from the lower edge of a HPTLC plate (Silica gel 60F254 glass plate, 10 cm × 10 cm; Merck, Germany) at a rate of 150 µL/s using a semi-automated HPTLC application device (Linomat 5-CAMAG). The chromatographic separation was performed on the plates in an automated development apparatus (ADC2-CAMAG) using a mixture of toluene-ethyl acetate (85:15, v/v) as mobile phase. The plates were pre-saturated for 20 minutes with the mobile phase, automatically developed to a distance of 70 mm at room temperature and subsequently dried for 2 minutes by the apparatus. The obtained chromatographic results were documented using a HPTLC imaging device (TLC Visualizer-CAMAG) under white light, 254 nm and 366 nm respectively. The chromatographic images were digitally processed and analyzed using HPTLC software (vision CATS-CAMAG) which was also used to control the individual instrumentation modules.

After initial documentation of the chromatographic results, each plate was derivatized with p-anisaldehyde sulphuric acid reagent using an improvised spraying device. The plates were subsequently dried using a warm air fan until colors developed. Derivatized plates were then analyzed with a HPTLC imaging device (TLC Visualizer-CAMAG) under different illumination modes (white light, 254 nm and 366 nm).

Results and Discussion

Optimising extraction of phytochemicals from *Allanblackia parviflora* oils

To prepare samples for the optimization process, oil sample concentrations ranging from 10 mg/mL to 30 mg/mL were dissolved in 1 mL of dissolving solvents (hexane and petroleum ether) in a sample vial. The samples were applied to two different mobile phase solvent systems of varying compositions (toluene-ethyl acetate-formic acid, 25:15:10, v/v/v and toluene-ethyl acetate, 85:15, v/v).

Also, different application volumes (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 µL) were explored with two derivatization reagents (p-anisaldehyde sulphuric acid and ethanolic phosphomolybdic acid).

The 20 mg oil in 1 mL hexane and toluene-ethyl acetate (85:15) as mobile phase and p-anisaldehyde sulphuric acid as a derivatization reagent provided a good separation of compounds with bands that were both sharp and intense (Figure 1b), these conditions were ultimately adopted for the HPTLC analysis.

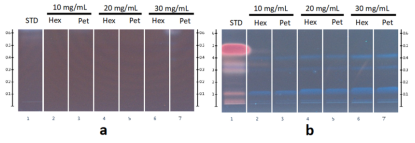

Figure 1: HPLTC plates showing the separation of phytochemicals in the samples using different oil mass (10 mg, 20 mg and 30 mg), solvents (hexane and petroleum ether), mobile phases (a) toluene-ethyl acetate-formic acid, 25:15:10, (b) toluene-ethyl acetate, 85:15 derivatized and monitored at 366 nm.

Derivatization reagents optimization

Two different derivatization reagents (p-anisaldehyde sulphuric acid and ethanolic phosphomolybdic acid) found in literature were tried [20]. In Figure 2 are displayed some results received from testing different derivatization reagents mentioned. Heating for 5 minutes after derivatization with ethanolic phosphomolybdic acid made the background more yellow. Tracks were not visible except the standard (track 1) which showed only one blurred band at Rf 0.6. A better separation was achieved by derivatising with p-anisaldehyde sulphuric acid solution. Under 366 nm, several bands were visible at different retention factors (Rf). Our results were in agreement with Gessler et al., [20] who also used similar derivatization reagents in essential oils HPTLC analysis.

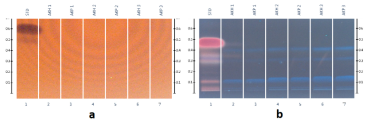

Figure 2: HPTLC plate image showing different derivatization reagents for the kernel oil (a) ethanolic phosphomolybdic acid (b) p-anisaldehyde sulphuric acid. Plates were monitored at 366 nm.

Optimising application volume of *Allanblackia parviflora* of kernel oils

The 20 mg/mL oil in hexane mixture was then applied to the plate at different volumes (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 µL). As shown in Figure 3 a application volumes 0.5-2.0 µL appeared to elute little phytochemicals and therefore showed weak bands on the plates at 3.0 Rf. However, 4 µL-5 µL appeared overloaded especially at retention factor (Rf) 0.6. Therefore, 3 µL was selected to be the optimal application volume because the bands were strong and sharp. The samples application was carried out using the same extract and applying five (5) times. Repeated application of this volume (2 µL, 5 times) onto the HPTLC plate showed excellent repeatability (Figure 3 b).

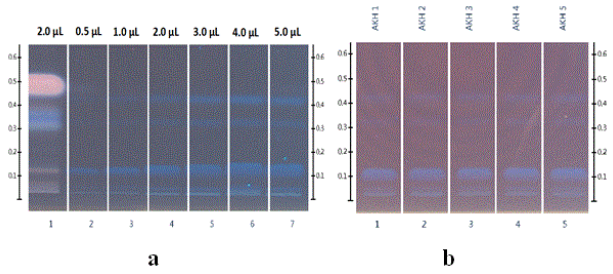

Figure 3: (a) HPTLC plate image showing different sample application volumes of kernel oil and (b) tracks of 5 repeated 3 µL application kernel oil samples visualized under UV at 366 nm.

Application

The optimised HPTLC method was used to separate kernel oils of *Allanblackia parviflora*. The optimal condition was therefore applied to both 16 kernel and seed oils from the 16 communities sampled. The HPTLC separation of the oils from the different communities is provided in Figures 4 (a, b). The consistency of the standard profile across the samples is demonstrated with the consistent bands at 0.45, 0.35, 0.3 and 0.1 Rf for HPTLC plate derivatized and monitored at 366 nm. The HPTLC plates monitored at the same illumination mode revealed the most sharp and intense bands at 0.1 and 0.4 retention factor (Rf) for samples AFK, AKK, SK, KSK and NAK. However, it is also evident that there are subtle differences between the samples which include the varying intensity of the weak band at 0.35 Rf for the same samples. Most noticeable are the less intense bands for the NEK, AAK, FK, ATK, DK, SBK and BK oil samples from the various communities. It was evident that most of the bands at 0.35 Rf were missing from these samples.

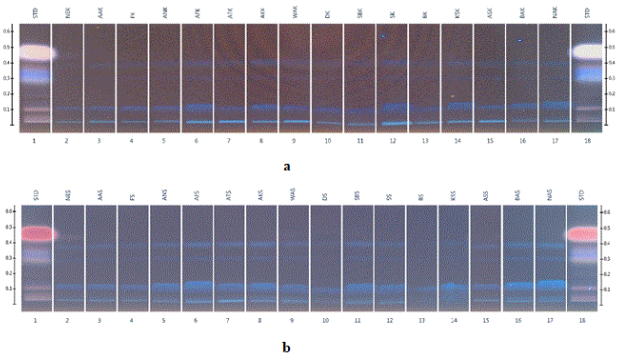

Figure 4: HPLTC separations of (a) kernel and (b) seed oils monitored at 366 nm, derivatized with p-anisaldehyde sulphuric acid.

The seed oil samples from the same 16 communities were also separated by HPTLC and again, there was consistency across the communities. This is evidenced by the consistent bands visible for the plate monitored at 366 nm (Figure 4 b) at retention times 0.1, 0.35 and 0.4. Samples ANS, AFS, ATS, AKS, WAS, SBS, SS, BAS and NAS.

Again, the less intense bands recorded for the samples NES, AAS, FS, DS, BS and ASS at the same band lengths.

The seed HPTLC plate produced sharp and intense bands compared to kernel oil HPTLC plate for all the communities, indicating the key components present in shells/husks. Interestingly, in a separate HPTLC analysis, the distinct differences in the HPTLC plates for the extracts from various communities are evident in the kernel cake samples. This suggests that the shells/husks contained phytochemicals which are more soluble in oil.

Conclusion

A HPTLC method has been developed for the analysis of phytochemical extracts of *Allanblackia parviflora*. The method provides a characteristic profile for the tree across 16 communities. The intensity of the bands reflected the phytochemicals recorded for the samples. Therefore, HPTLC may provide an alternative or complementary method for the determination of phytochemicals in *Allanblackia parviflora*. This method can be adopted to compare the phytochemical profile of different species of *Allanblackia* for authentication.

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