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Silica Gel chromatographic methods for identification, isolation and purification of Gossypol acetic acid prepared in lab

Amro Abd Al Fattah Amara[1], Mohamed Hesham El-Masry[2†], Gamal Ali Salem[3] and Hoda Hassan Baghdadi[4]

[1] Protein Research Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt. (amroamara@web.de).
[2] Biotechnology Department, Graduate Studies and Research Institute, Alexandria University, Alexandria, Egypt.
[3] Granted PhD, Environmental Science Department, Graduate Studies and Research Institute, Alexandria University, Alexandria, Egypt. (gsalem662000@yahoo.com
[4] Environmental Science Department, Graduate Studies and Research Institute, Alexandria University, Alexandria, Egypt. (hodabaghdadi@yahoo.com)
Corresponding author : Amro Abd Al Fattah Amara (A. A. Amara) (Protein Research Department, Genetic Engineering and Biotechnology Research Institute [GEBRI], City of Scientific Research and Technological Applications [SRTA-City], Universities and Research Center District, New Borg El-Arab, P.O. Box: 21934 Alex, Egypt, Tel: +203-4593422; Fax: +203-4593407, Alexandria, Egypt, amroamara@web.de; aamara@srtacity.sci.eg).
ORCID: https://orcid.org/0000-0003-4629-5476
†Deceased author.

Abstract: Several cottonseed varieties are cultivated in different countries. Each variant produces a different amount of gossypol as a natural toxic compound. The raising interest in the cottonseed products (oil and feed) increase the demand for establishing simple methods for gossypol detection. Silica gel-based methods are ideal for its isolation, purification, and characterization. Silica gel is variants and can be used as simple methods for tracking plants' compounds. In this study, gossypol was isolated, characterized and purified as gossypol acetic acid in the form of yellow crystals. Methods were used for its characterization include TLC, preparative TLC, silica gel column, UV/IR spectrophotometer and HPLC (Robust spherical silica gel). A comparative study between its amount in both the Egyptian and Chinese varieties was performed. Under the experimental condition, the Egyptian's cottonseed contains 8.705 gm/kg, while the Chinese's cottonseed contains 5.395 gm/kg. TLC used in this study prove to be fast, accurate, and inexpensive. It can be used for gossypol acetic acid evaluation and quantification. Additionally, using TLC as a pre-purification step will give pre-judgment for the sample purity and quality. This step will protect expensive HPLC silica gel-based column from any unexpected impurities. During each step the silica gel itself could be simply removed by paper filtration. Collectively, the different silica gel-based methods as well as the other used method are recommended for better Gossypol acetic acid isolation, purification and characterization and for maintaining the HPLC column.

Keywords: Cottonseed; Silica gel, Silica gel column, Robust spherical silica gel, Gossypol; Gossypol acetic acid;

1. Introduction

Gossypol as poisonous pigment obtained its name from the cotton plant (*Gossypium barbadense*) family Malvaceae. Gossypol is a non-lactonic sesquiterpene. It has different physiological and biological activities. It is usually prepared as gossypol acetic acid (GAA). GAA shows a raising interest because of its biological properties such as: anticancer[1], antiviral [2,3], antimicrobial and antifertility properties [4-6]. It can inhibit cellular macromolecules [7]. The crude extract of the cottonseed (containing gossypol) can inhibit the formation of crown gall tumor formation in the potato disc bioassay which

were induced by *Agrobacterium tumefaciens* [8], and can inhibit hepatic microsomal benzo(α) pyrene hydroxylation and hydrogen peroxide production in mice treated with lindane [9].

Gossypol has shown a T/C of 150% at 10 mg/Kg in the PS system [10]. It has a unique chemical structure comprises the following formula: 1, 1', 6, 6', 7, 7'-Hexa hydroxy-3,3'-dimethyl- 5, 5'-bis (1-methylethyl) [2, 2'-binaphthalene] -8, 8'-dicarboxaldehyde; 1,1', 6, 6', 7, 7'-hexahydroxy- 5, 5'-diisopropyl-3, 3'-dimethyl[2, 2'-binaphthalene]-8, 8'-dicarboxaldehyde; 2,2'-bis[1, 6, 7-trihydroxy- 3 -methyl- 5 -isopropyl- 8- aldehyde naphthalene]; 2, 2'-bis[8-formyl-1, 6, 7-trihydroxy-5-isopropyl-3-methyl naphthalene]. It has a general structure of $C_{30}H_{30}O_8$, with molecular weight of 518.54 [11]. In case of GAA its general structure is $C_{30}H_{30}O_8.C_2H_4O_2$, with molecular weight of 578.62.

Its toxicity was investigated on different cancer cell lines such as Her2 positive breast cancer cells [15]. It is able to cause up-regulation of somatostatin receptors 2 and 5 in DU-145 prostate cancer cells [16], and cultured murine erythroleukemia cells[17]. It can inhibit the growth of colorectal cancer and hepatocellular carcinoma [18] and the like. It has also been demonstrated that gossypol inhibits proliferating Ehrich ascites tumor cells [10]. (±)-Gossypol induced apoptosis and autophagy in head and neck carcinoma cell lines [19]. Gossypol decreased cell viability and down regulate the expression of some genes in human colon cancer cells [20]. Gossypol was demonstrated to be specific inhibitor of DNA synthesis [12]. Rosenberg et al. (1986) highlighted that gossypol affects nuclear DNA by upregulating DNA replication and mismatched proteins [13]. The mechanism of anti-cancer activity of gossypol is inducing apoptosis through the suppression of anti-apoptotic proteins of the Bcl-2 family [14].

Gossypol has antiproliferative and antimetastatic effects on MAT-lyla prostate cancer cells. It can be a potential therapeutic agent for androgen independent human prostate carcinoma [21]. Oral gossypol reported to be safely used on an outpatient basis for the treatment of metastatic adrenal cancer [22]. The genotoxic effect of GAA was evaluated by determining the frequency of micronuclei and mitotic index in male mice bone marrow cells *in vivo* [23]. The rate of DNA degradation by gossypol-Cu^{+2} complex was found to be the same both in the presence and absence of molecular oxygen [24,25]. It reported as inhibitor for Bcl-2, Bcl-XL, Bcl-W and Mcl-1 [26]. For more details refer to Fulda (2010) and the references within [26]. For that it and its derivatives are promising candidates for cancer treatment [1,26-28].

There is an increasing interest in using gossypol as antivirus [32-37]. Gossypol has many other important effects, where both racemic mixture and enantiomers of gossypol inhibit replicating human immunodeficiency virus-type 1(HIV-1) [29,30]. The *in vitro* anti-amoebic effects of gossypol were reported against axenic trophozoites from five *Entamoeba histolytica* strains [31].

This study is concerned with simplifying different silica gel chromatographic based methods including TLC, preparative TLC, column chromatography, HPLC (Robust spherical silica gel). Other simple analytical methods such as the UV/infrared spectrophotometric analysis are included. Authentic GAA sample was used in the entire study to assist quantifying and characterizing the prepared GAA. The silica gel-based methods and the other used methods are recommended for fast isolation, characterization, and purification. Silica gel different methods gives different choices. In the present study, the used methods ranging from so simple, handy, in-house, and inexpensive to a highly accurate and sophisticated.

2. Materials and Methods

1.1. Cottonseed sample

Gossypol was extracted and purified as GAA from Egyptian cottonseed. The used seeds were obtained from Alexandria Oil and Soap Company (Alexandria, Egypt).

2.1. Preparation of seed material

Three Kilograms of dried cottonseed were grinded in a blender. The grinded material was passed through a coarse sieve to remove greater parts of the lint and hulls. Two kg of the powdered seeds were transferred into a suitable closed clean and dry glass container.

2.2. Extraction with petroleum ether/diethyl ether

Two kg of the powdered seeds were first extracted with 3 liters of petroleum ether in a shaker (100 rpm) at room temperature. The resulting extract was filtered through filter paper (Whatman №1). The extraction process was repeated five times to remove the oily component of the powdered seeds. The powdered seeds were followed by air dried at room temperature to get rid of any solvent. The oil-free seed material was then extracted with 3 liters of diethyl ether. The extraction step was further carried out until no change in the color of diethyl ether is observed. The filtrates were concentrated under vacuum using rotatory evaporator at 40°C.

2.3. Precipitation of Gossypol as GAA

The concentrated extract obtained from 2 kg sample was transferred into a conical flask, then the mixture treated with 40 ml glacial acetic acid dropwise until pH 3 was obtained. The flask was well closed and placed in a dark static place (for one week), where yellow crystalline precipitate was obtained at the bottom of the flask. The supernatant liquid was discarded and the precipitate was taken and dried in a desiccator [38].

2.4. Purification and Recrystallization

The precipitate was washed 3 times with 1 ml of glacial acetic acid, followed each time with 10 ml petroleum ether. In each time the mixture was filtered through filter paper (Whatman №1) and finally the precipitate was dissolved in 5 ml diethyl ether containing 1 ml glacial acetic acid. The ether was followed by evaporation until the purified crystals began to be readily separated. The crystals were dried in a desiccator at room temperature.

2.5. Detection and monitoring GAA by TLC

One milligram of each of the crystalline precipitate and reference GAA (Sigma) were separately dissolved in 50μl chloroform in a clean and dry glass container. Each sample was separately spotted at the starting point (baseline) across the chromatoplate [(20x20 cm) coated with 0.5 mm thick of 0.25 silica gel G60 containing 0.6% starch (Merk)]. The chromoplate was then activated. The samples were vertically migrated using solvent system; ether: petroleum ether (1:1) as a running solution. The plate was allowed to dry and the separated spots can be seen under the visible light as yellow spots (Figure 1) [39]. The TLC was used to monitor GAA in the different samples during the preparation and purification steps (Figure 1).

2.6. Preparative TLC for purification of GAA

Thirty milligrams of the crystalline precipitate were dissolved in 300μl chloroform in two clean and dry (as a duplicate replica) glass container. The solution was separately streaked in the form of a band across the baseline of the silica gel G60 chromatoplate (containing 0.6% starch as above) and then the chromatoplate was processed using the solvent system (ascending development) ether: petroleum ether (1:1).

The plate was air dried, where two separate bands (a and b) appeared under visible light. Another band (C) on the baseline was neglected (as in Figure 1). Images were taken for visualization. Each band was separately collected, and then eluted with ether, till no residue was obtained upon evaporating a small volume of the colorless eluate to dryness on a watch glass (clean and dry). Each eluted band was filtered where the filtrate was concentrated under vacuum to about 2 ml then left to crystallize. Finally, each crystallized

material (a and b as in 2.5.) was dried in a desiccator and then tested for the purity of the GAA using each of the spectrophotometer and HPLC (as below).

2.7. UV Spectrophotometer analysis for the spectra of GAA

The crystalline precipitate from petroleum ether-diethyl ether was dissolved in 95% ethyl alcohol, and then determined spectrophotometrically. 40 μg of both GAA precipitate and reference sample were diluted each to 3 ml ethyl alcohol and scanned at 500-188nm using quartz cuvette to determine the presence of GAA in the precipitate.[40]. The absorbance of the top of the best clear band (at 440nm) was used to measure GAA spectrophotometrically in the further investigations.

2.8. GAA Standard curve

Two milligrams of standard GAA (Sigma Co.) were weighed accurately in a clean and dry glass tube, and were then dissolved in 1 ml absolute ethanol. Volumes of 20, 40, 60, 80, 100, 120, 140, 160, and 180 μl of gossypol were used as a stock solution. Zero absorbance was adjusted against absolute ethanol blank at 440 nm. The absorbency of the prepared dilutions was determined with PYEUnicuan Spectrophotometer The absorbance of each preparation and the standard curve was generated.

2.9. Determination of the GAA (separated by TLC) using HPLC

Band A and B from TLC chromatoplate were removed and then separately treated by chloroform to dissolve the separated substance. The chloroform mixture was filtered to remove the silica-starch layer. The filtrate was further evaporated to obtain the purified substance in a crystalline form. 1.9 mg of the purified crystals obtained from band A and 1.5 mg of band B were separately dissolved in 10 ml 70% aqueous acetone. Five μl of each were separately injected in HPLC column (Column Art.720023 ET 250/814 NUCLEOSIL® 10 C18 MACHEREY. NAGEL, Japan) to detect GAA. The column contains Robust spherical silica gel, hydrophobic, weakly polar, end-capped phases for RP chromatography. The separation mechanism was based on hydrophobic (van der Waals) and slight residual silanol interactions. The following was the conditions in which the apparatus was adjusted; UV detector (365 nm), at 35°C, a mobile phase 0.15 M phosphoric acid in acetonitrile/water (4:1), and at a flow rate 1.5 ml/min and at a speed of 5 mm/ml.

2.10. Silica gel column isolation and HPLC quantification of gossypol from Egyptian and Chinese cottonseeds

One gram of fine powdered Egyptian and Chinese cottonseeds, each was separately extracted from 10 ml solution of 70% aqueous acetone in clean and dry glass container then incubated with shaking (150 rpm) at 25°C for 6 hrs. The extract was clarified by centrifugation at 4000 rpm where the supernatant was kept in a clean and dry glass container. Silica gel column chromatography was used for further purification (Merk G120 column 15 cm x 1 cm). The 70% aqueous acetone supernatant was allowed to be adsorbed on the silica gel column, and then washed with 100% petroleum ether to remove any impurities. The purified gossypol in the column was then eluted with 100 ml 70% aqueous acetone.

2.11. HPLC method for analysis of GAA in Egyptian and Chinese cottonseeds

One and half μl from the eluent were taken for detecting the gossypol using HPLC (under the same condition as above). Qualitative and quantitative results of GAA in different samples were made by the comparison between the peaks retention time and peak area of the standard solution and sample extract under the same conditions of operation (as above) [41-43].

2.11. Melting point of GAA

Electrically heated melting point apparatus was used to study the melting point of any crystalline substance. A few crystals of the crystalline precipitate were introduced into the melting point tube, and then exposed to an electrical heat. The reading of the thermometer was taken at the point at which the crystalline precipitate melt. The same procedure was used with a reference sample of GAA (Sigma Co.).

2.12. Infrared spectral measurements for GAA

Infrared spectra in a range of 600-4000 cm^{-1} were measured with a Perkin-Elmer Model 1420 spectrometer. The KBr pellets needed for IR analysis were prepared with the precautions recommended by O'Conner et al (1954) [44], to eliminate moisture. Each sample (0.8 mg) was mixed with 300 mg KBr. The mixture was placed in an agate mortar, and after being grinned manually for several minutes the mixture was transferred to the KBr disk press, which was evacuated for 15 minutes (pressure of 11 tons / cm^2) [44,45].

3. Results and Discussion

Cottonseed has economic importance as a source of oil and as animal feed. Even the seeds have high oil and nutritive components, unfortunately a natural toxic pigment is existed which named gossypol (related to the plant name (*Gossypium barbadense*). Thus, removing gossypol or degrading it is an important step [43,46-49]. So, there is a real need for a fast, cheap, and practical method(s) for its detection, isolation, purification, crystallization, identification, and quantification.

Additionally, there is an increasing interest concerning the different research done on gossypol, GAA and the other related and derived structures. In such a case, sensitive experiments such as *in vitro* cancer cell line, gossypol must be used in a highly purified form. Gossypol is usually isolated and detected as GAA. There is an interest in the research considering both gossypol and GAA. Simple silica gel methods could assist better purification. That will protect expensive columns from any unexpected impurities. In addition, using the described method as a model for isolating pure compound from plant source. This will assist isolating another interesting natural product, particularly those which could be isolated using solvents and purified using different silica gel methods.

Silica gel can be used in different forms and combinations with other molecules for isolating botanical compounds. One of its unique properties is its resistance to the chemical changes in presence of different solvents. In contrast to the other form of gels such as the gel groups that are used to isolate proteins; it is more stable and can be easily retrieved from column or from the glass plate surface; cleaned dehydrated and reused. Its chemical properties enable its coexistence with simple assistant compounds like the starch which used for preparing TLC. For its simplicity and efficacy, we aimed to select some of the simplest and most direct silica gel-based methods either from the research previously done on gossypol or from the other phytochemistry protocols. The study introduces a straightforward protocol for isolation, purification, and characterization of GAA. Generally, the methods described in the current study were collected based on their simplicity out of some others tested methods [data not shown] and based on our scientific group experiences in the field of phytochemistry.

Cottonseed from Egypt was collected, grained, and sieved to fine particles to enable better extraction. The grinding step is essential in removing debris and facilitates the extraction process. For defatting the seed materials, petroleum ether was used. After several extraction steps with the ether, the seeds were followed by air dried to remove any residual solvent. After that, diethyl ether was used to extract the gossypol. The diethyl ether extract was reduced to concentrate the sample.at 40^{o}C degree which was used during the entire steps to avoid degradation of gossypol. For that the solvent evaporation was done under vacuum.

Simple methods for purification and recrystallization were used. Multiple runs represent washing with petroleum ether followed by filtration and then, re-dissolving in diethyl were conducted. Finally, acetic acid was used for precipitating the gossypol as GAA. For further purification, TLC preparative chromatography was used. TLC (silica gel G60) preparative chromatography ensures an additional purification step to obtain suitable quantity in a short time. Samples were taken and tested for the presence or for the purity of the GAA in any of the above-described steps as in Figure 1 (a and b).

Acetic acid was used to precipitate the gossypol as GAA. To our knowledge the perception is succeeded after putting the sample in a tightly closed bottle under static condition in a dark chamber. Apparently, darkness was an essential factor in precipitating gossypol as GAA. The GAA was separated on the TLC for two bands, A and B. Another band, C appeared in the bottom of the TLC beside the starting point (was neglected) as in Figure 1 (b). The concentration of GAA present in the spot A and B after preparative TLC chromatography using HPLC analysis (Figure 4) indicate that spot A contained 17.5% of GAA while spot B contained 1.02% GAA.

The precipitated gossypol as GAA was subjected to a series of washing steps. The GAA which was obtained from the precipitation and recrystallization process was about 2.6 g/kg cottonseed, and it was in the form of brilliant yellow micro crystals (0.26% based on powdered cottonseed). The difference in the calculated amount between the TLC preparation steps and that obtained from the HPLC data (as below) prove the efficacy of the HPLC in accurate determination of the GAA quantity. Meanwhile, during the crystallization, purification/recrystallization steps loss in the quantity is expected. One should differentiate between the quantitative determination of the existed amount which need a sophisticated instrument like the HPLC which need amount in μg (or less) and the need for purifying suitable amount of gossypol (as GAA) to conduct certain experiment.

The silica gel column chromatography is another alternative purification method was used. Even the gossypol is dissolved in 70% aqueous acetone but upon loading the sample in the silica gel column and because of its absorbency, it enables the sample to be trapped and adsorbed in the column. The silica gel column will do more than one function to purify the gossypol. First it will prevent passing any solid existed material. Second it will enable using a concentrated sample. Third it will enable fast washing using minimum amount of 100% petroleum ether. Fourth, the sample could be simply eluted using the same used solvent which used to dissolve the gossypol (70% aqueous acetone).

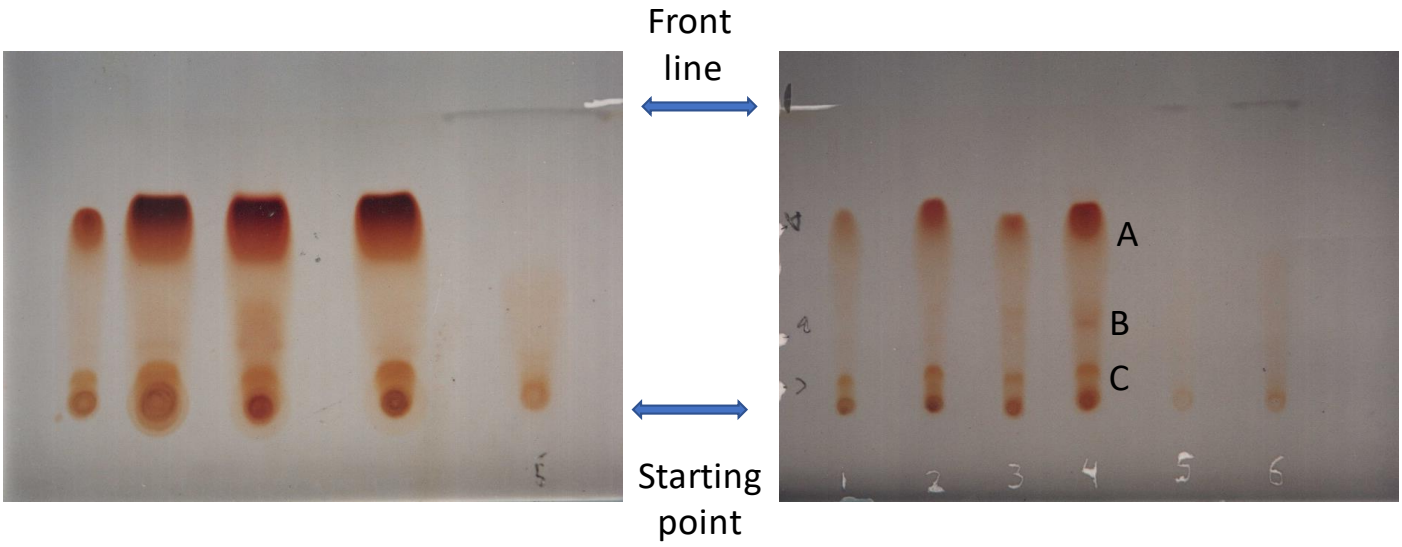

Figure 1. (a); Different TLC sample showing different amounts of gossypol; acetic acid (samples collected during the purification steps), 1(b); purified samples show 2 bands A and B.

The HPLC, a method was adjusted to quantify GAA using spectrophotometer. Authentic sample from Sigma was used as reference. GAA spectra were detected using wavelengths in the range of 188 and 500 nm. The best wavelength in the peaks obtained from UV spectra was used (440 nm) as in Figure 2. Standard curve was generated from the authentic sample and the purified gossypol was evaluated [data not shown]. Furthermore, the spectra of the authentic sample were compared with that of the purified GAA and proved to be identical as in Figure 2 (a and b). The results obtained either from the standard curve or from the spectra prove that we have a well-purified compound. IR analysis was further used to compare the purified GAA with the authentic sample as in Figure 3. The spectra of both are identical.

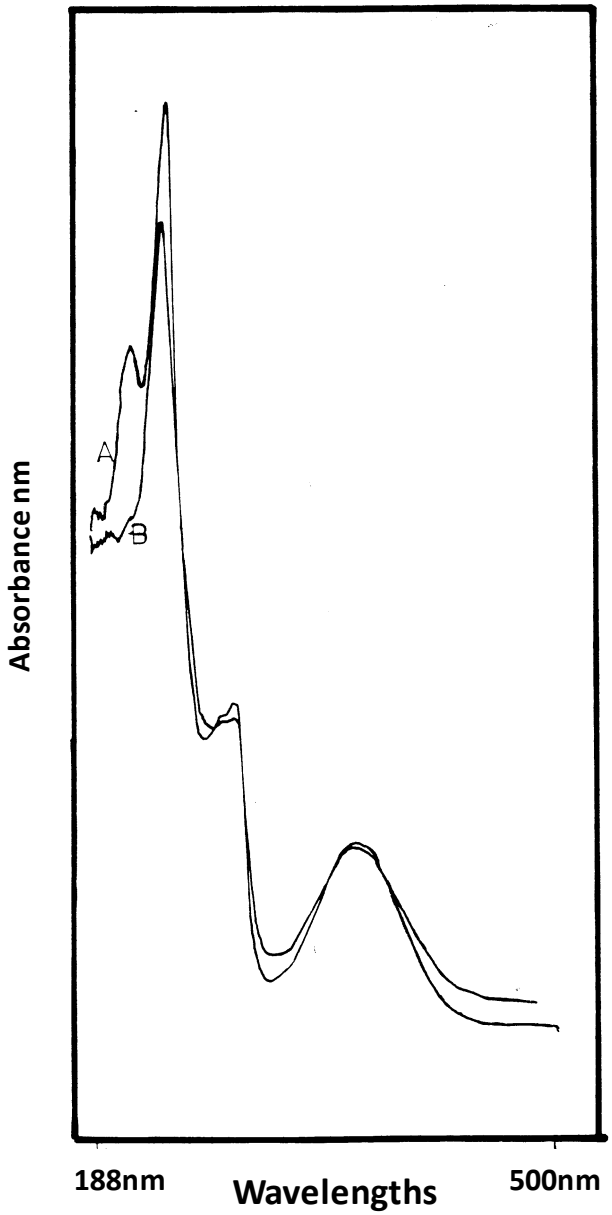

Figure 2. UV spectrum of GAA reference sample (A); Isolated crystals (B).

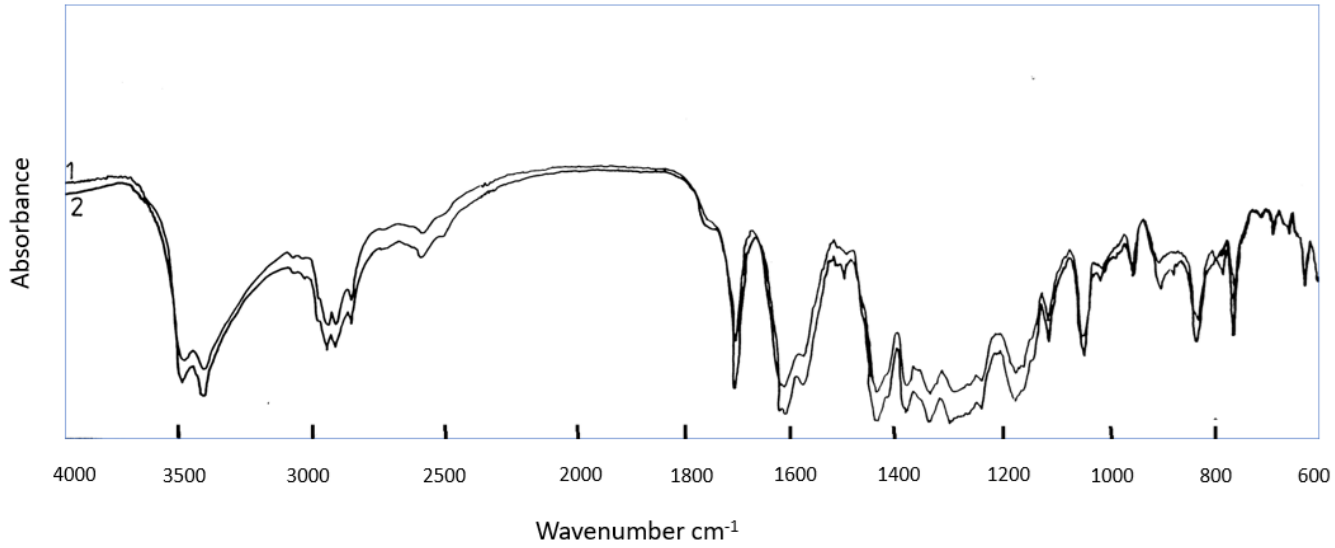

Figure 3. IR spectrum for (1) GAA reference sample; (2) GAA purified samples.

The infrared spectrum of gossypol using potassium bromide disk showed the following absorption bands (Figure 3): the broad band of strong intensity at 3500 and at 3420 cm^{-1}-are attributed to OH stretching vibration. Bands of moderate intensity at 2960, 2920 and 2860 cm^{-1} are ascribed to CH stretching vibration. The band of strong intensity at 1700 cm^{-1} is because of CHO stretching vibration. Two bands at 1440 and at 1380 cm^{-1} are because of C-H banding. The band at 1380 cm^{-1} is because of CH_3 symmetrical deformation. The band at 1340 cm^{-1} is because of OH bend. Bands characteristic for C-OH appeared at 1240 and at 1180 cm^{-1}. Bands below 1120 (1100, 1050, 960, 840, and 700 cm^{-1} represent the "finger print'' of GAA.

The melting points of the crystalline substance and the reference sample of GAA were the same. The melting points were 184°C. Even it is a simple experiment but could prove or disprove the identity of two compared samples. For comprising the gossypol obtained from the Egyptian cottonseed and the Chinese cottonseeds, HPLC was used. Silica gel column was used to absorb the gossypol following by a washing step by ether to remove any impurities. Then the gossypol was eluted using diethyl ether. The results indicated that the yield of gossypol from the Egyptian cottonseed is 8.705 gm/kg while the yield produced from the Chinese cottonseed was 5.293 gm/kg.

The methods used for identifying gossypol and GAA were simplified in this study. We recommend the use of the different silica gel methods and the analyses described in this study to be used for evaluating the gossypol and GAA in the cottonseed oil and animal food products. It might be useful to use cottonseed varieties have less gossypol content in one side. In other side high gossypol content cottonseed is more resistance against different infections.

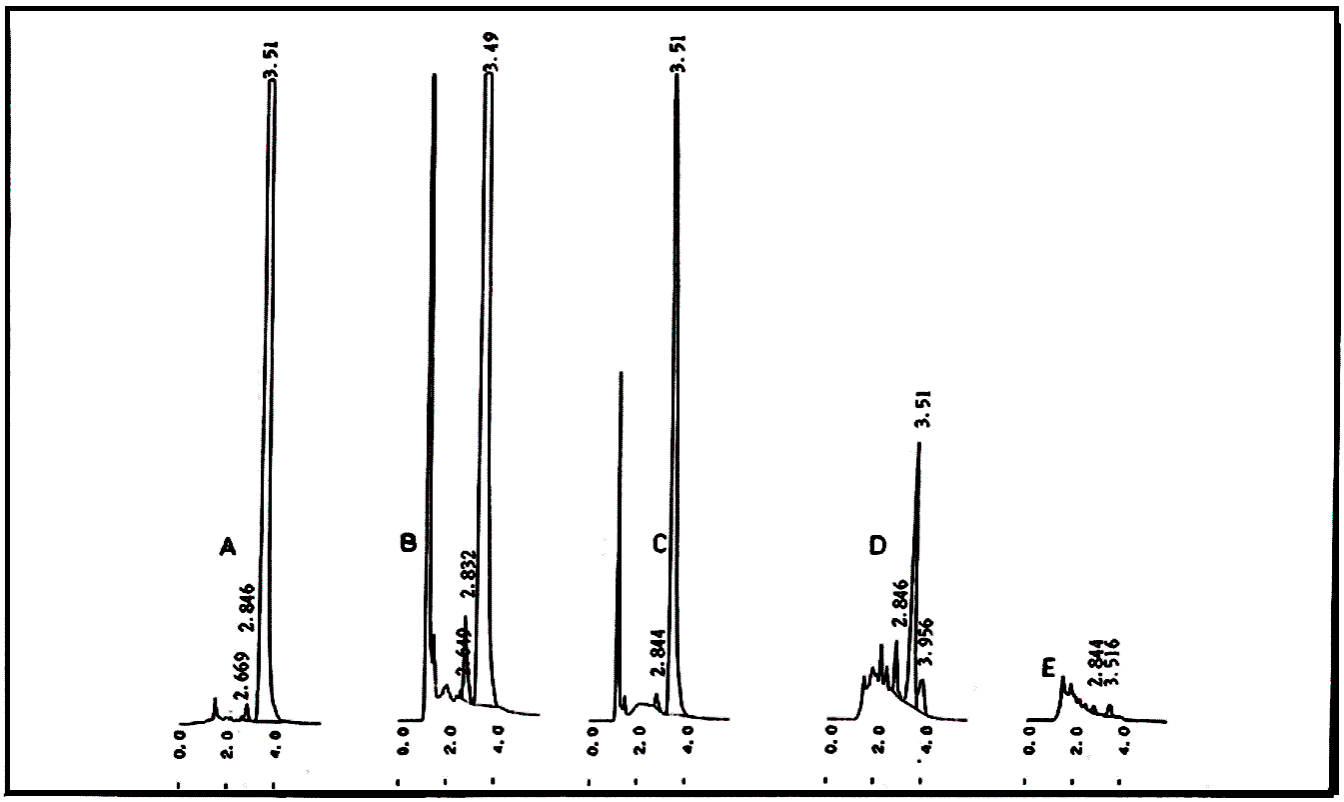

Figure 4. HPLC pattern of A-Authentic sample; B-Egyptian GAA sample; C-Chinese cottonseed gossypol; D-Crystalline purified precipitate from TLC spot (A) and E-Crystalline precipitate gossypol from TLC spot (B).

4. Conclusions

The paper summarizes many experiences in different fields. It combines between a real use of the Silica gel to produce gossypol acetic acid in quantity that can assist the research concerning the it. Different kind of silica gel-based methods are used.

Gossypol was extracted, crystallized, and purified as GAA. Simple silica gel methods were used such as TLC preparative TLC, Silica gel column, and simple spectrophotometric assays beside more sophisticated analysis represented by HPLC. This study showed that the use of TLC could obtain purified GAA and observed quantitatively. The main aim of this study is to enable scientists from purifying the gossypol as GAA using simple and efficient tested silica gel-based methods to use it in different experiments. What is the most important issue in the present investigation? It is the up-front method for isolating the gossypol till its characterization and quantification, which could stand alone as a protocol for preparing gossypol as a GAA in lab. This will enhance its investigation against many cancers' cell lines and other biological interests.

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