

Thin layer chromatography, high performance liquid chromatography and melting point for extraction and purification of cinnamic acid from cinnamon bark (*Cinnamom aromaticum*)

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Rec. 28 Aug, 2012 Accpt. 30 Sept, 2012

Abstract

The aim of this study to purified the cinnamic acid from the cinnamon bark. The partial purified cinnamic acid is obtained after column chromatography application. Thin layer chromatography (TLC) elution yielded pure crystals, which identified as cinnamic acid in comparison with the standard cinnamic acid 10.5 mg / 150 gm cinnamon bark powder obtain as a result of partial purification by TLC. Chemical tests for identification compound of extract crystals yield were included: general tests for phenolic compound, HPLC (High performance Liquid Chromatography) analysis and melting point estimation, in relation to standard cinnamic acid for tests. Concentration of cinnamic acid was 96%.

Keywords: Cinnamon, cinnamic acid, TLC, HPLC.

Introduction

In ancient Egypt, cinnamon was mainly used as medicine and as a flavoring for beverages. One main use of cinnamon was for embalming of the deceased as body cavities were filled with spices and preserves. In ancient Rome, Nero, an emperor in first century A.D., burned year's supply of cinnamon on his wife's funeral pyre (Gray & Miller, 1970). This was considered as extravagant gestures meant to signify the depth of his loss. Cinnamon was often paired with ginger in Medieval Europe, as most meals were prepared in a single cauldron and were casseroles containing both meat and fruit. It was also used as a bridge for flavors, and also mention in Bible. Cinnamon distributed through the world, but particularly suited to the warm, tropical locales of south India, Southeast Asia and Brazil. Most are evergreen but some, including the North America Sassafras, are deciduous (Robert *et al.*, 1996).

There are approximately between 50 to 250 different species of cinnamon. It's beautiful ornamental tree with golden red

bark that is dried and is the cinnamon spice. New foliage is deep red, and small white flowers are followed by dark purple fruit. The tree is small or medium sized, usually (15-20m), It will survive short frosts and temperatures to 32F, protect from hard freezes and prolonged cool weather, propagation by seeds, harvest after 1-2 years from planting (Al-Shamaa, 19890). the cinnamon is full with vitamins such as vitamins A and B₃, besides the minerals table (1). Cinnamon still known as "Kwai" in Chinese language. Its botanical name derives from the Hebraic and Arabic term "momon" meaning "fragrant spice plant". In ancient Egyptians known "cannon". Italians called it "canella" meaning "littletube", Germany known "Zimt", The name cinnamon comes from Phoenician and Hebrew through the Greek kinnámōmon. In Malayalam, it is called Karuva. In many other, particularly European, languages it has a name akin to French cannelle, diminutive of canne from its tube-like shape. In Persian, it is called darchin literary meaning taken, picked from tree (Dastur, 1977).

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	Nutrients	Amount
1.	Basic components	0.16gm, 3.6gm, 0.44gm, 0.16gm
	Protein, Carbohydrate, Water, Ash.	
2.	Calories	11.8gm, 1.2gm, 3.6gm, 0.16gm
	Total calories, Calories from fat, Calories from carbohydrate, Calories from protein.	
3.	Fats	0.16gm, 0.04gm, 0.04gm, 0.04gm
	Total fats, Saturated fats, Mono fats, Poly fats.	
4.	Vitamins	7.08 mcg, 0.04gm, 1.44 mcg, 1.32 mcg, 0.04 gm
	Vitamin A, Vitamin B3, Vitamin K, Folate, Niacin equid.	
5.	Minerals	55.64mg, 1.72mg, 2.52 mg, 0.76mg, 2.8mg, 22.68mg, 0.04mcg, 1.2mg, 0.08mg.
	Calcium, Iron, Magnesium, Manganese, Phosphorum, Potassium, Selenium, Sodium, Zinc.	

Table (1): Cinnamon Nutrition Facts: In 4.52 g of Cinnamon can find.

The tree is small or medium sized, usually (15-20m), It will survive short frosts and temperatures to 32F, protect from hard freezes and prolonged cool weather, propagation by seeds, harvest after 1-2 years from planting (Al-Shamaa, 1989). the cinnamon is full with vitamins such as vitamins A and B₃, besides the minerals. Mainly there are two kinds of cinnamon: Ceylon cinnamon such as *Cinnamomum verum*, *Cinnamomum zeylanicum*. Many consider Ceylon cinnamon the true cinnamon. Ceylon variety is more refined and subtle, Implant in Srilanka and the west beach of India, it's called in markets cinnamon chips. The bark is light yellow, thin, rather creamy, aqueous extract is active, but alcoholic extract is more bio-activity. Volatile oil containing 75% of Cinnamaldehyde component and 8% from Eugenol, while found that the volatile oil contain 34 component, 78% Terpene-Hydrocarbons, 9% Oxygenated-Terpenoids and has suggested that volatile oil has an antioxidant activity rate (55.94% – 66.9%) in 100 -200 ppm on succession (Al-Zubaidi, 2005). and Chinese cinnamon which is the famous genus related to Chinese cinnamon is *Cinnamomum aromaticum*. Some of their synonyms are cassia cinnamon, cassia lignea, consist from the dried peel of cinnamon cassia blume, the original home is in China, but planting in India, Vietnam and Indonesia. The bark is peel off, dried and allows to form a roll-the cinnamon stick that known today, harder than the Ceylon cinnamon and containing cinnamaldehyde and eugenol with bitty amount and may be not (Al-Zubaidi, 2005).

Pure cinnamic acid is a white crystalline hydroxycinnamic acid and the most synonyms was phenyl acrylic acid, 3-Phenyl- propanoic acid, 3-Phenylacrylic acid, Cinnama. The chemical formula C₉H₈O₂. Cinnamic acid is slightly soluble in water and boiling at 300°C table (2) (Camarat *et al.*, 2006). It is obtained from oil of cinnamon, or from balsams such as storax. It is also found in Shea butter and is the best indication of its environmental history and post-extraction conditions. It can also be made synthetically. Cinnamic acid is used in flavors, synthetic indigo and certain pharmaceuticals, though its primary used in the manufacturing of the methyl, ethyl, and benzyl esters for the perfume industry. Cinnamic acid has a "honey, floral odor" and more volatile ethyl ester (ethyl cinnamate) that flavor components in the essential oil of cinnamon, in which related cinnamaldehyde is the major constituent. Cinnamic acid is also part of the biosynthetic shikimate and phenylpropanoide pathways. Cinnamic acid is soluble in diethyl ether, insoluble in hexane (Chen *et al.*, 2005).

Chemical formula	C ₉ H ₈ O ₂
Molar mass	148.17 gm / mol
Appearance	mono clinic crystals
Density	1.2475 gm/cm ³
Melting point	132 -135 °C
Boiling point	300°C
Solubility in	
Water	0.4 gm/L (20°C)
Chloroform	0.93M
Ethanol	0.86M
Methanol	1.1M
Acidity (PKa)	4.44
Molecular Weight	148.1586
Stability	Stable under ordinary condition

Table (2): Properties of cinnamic acid

In the last searches (Mousa *et al.*, 2011; Mousa *et al.*, 2011). cinnamic acid improved ability to be a vaccine as antioxidant and Hepatoprotectivity against pro-drug cytoxan. So, we looking for extraction and purification with the cheapest and easiest ways to recommendation in the future the dose from cinnamon to patients gain as much as could from cinnamon bark rich with.

Material & Methods:

The partial purified cinnamic acid happened after column chromatography application. Thin layer chromatography elution yield pure crystals, which identified as cinnamic acid in comparison with the standard cinnamic acid. Chemical tests for identification compound of extract crystals yield were included: general tests for phenolic compound, HPLC (High Performance Liquid Chromatography) analysis and melting point estimation, in relation to standard cinnamic acid for tests.

Collection of the samples:

Cinnamon plant were collected from the local market then kept in cool dark place until used to avoid losing some compound from the bark by vaporizing such as cinnamaldehyde (Jayaprakash *et al.*, 2002).

Preparation of the cinnamon barks extraction:

Mixed 150 gm of cinnamon bark powder with 2.5 L methanol for 72 hrs. in cool dark place, then filtered and dried at (30-40°C) by rotary evaporator to obtain 1/10 from the original volume and stored at 20°C till the next steps. All these steps were alone away from direct light and extensive stress that led to oxidation of the plant extract (O'Neil, 2006).

Detection of polyphenol groups:

Phenolic group (C₉H₈OH) was detected after dissolving in NaOH 5%, by change the color from colorless to any color due to oxidation. Phenolic group in molecules can be determined by the following tests.

- I. Ferric chloride test: Phenolic group was detected by adding 1% aqueous or alcohol ferric chloride and change the color to green, purple, blue or black (O'Neil, 2006).
- II. Libermann reaction: Phenolic group with a free para position can be detected by libermann reaction, which included

addition 1ml conc. H₂SO₄ and a few crystal of NaNO₂, production of green or blue-violet color immediately indicates the presence of phenolic group (Moffat, 2004).

III. Phthalein test: Phthalein test was done by adding 2 drops of conc. H₂SO₄ to phenolic compound sample, then heated and poured to NaOH 10%, production of a red, blue, high green and colourless indicates the presence of phenol, catechol and hydroquinone, α and β Naphthol and p-cresol, respectively (Moffat, 2004).

IV. Specific Reaction of Benzene (Aromatic Ring) Aluminium chloride (AlCl₃) test (Friedle graft): Get 0.1gm of the unknown in 1ml of chloroform, then added a pinch of anhydrous AlCl₃ with a spatula along the sides of the test tube, crystals of AlCl₃ became yellow but turn dark orange within few minutes and the chloroform layer is colourless (Moffat, 2004).

V. Specific test for double bond: In order to find out unsaturated compound examined the following two tests (Moffat, 2004).

A. Bromine decolourisation test: 0.1gm of unknown was added with shaking to 2ml carbon tetrachloride contained a 5% solution of bromine, Disappear the reddish brown colour is a positive test for unsaturated compound.

B. The Baeyer test:

Baeyer reagent (alkaline KMnO₄ solution) was added to solution of 0.1gm compound and 2ml water or ethanol, with shaking. Disappearance of purple color indicates the unsaturated compound.

Isolation & purification of cinnamic acid:

Cinnamic acid can be oxidized and converted to other benzoic compounds (O'Neil, (Ed.). (2006). Fig (1) and losing by light, UV light, presence of heat, heavy metals and atmospheric oxygen (Bavaresco, 2003)So, this step to obtain as a much as could pure cinnamic acid during extraction in comparison with standard and steps for isolation cinnamic acid were:

Acid Hydrolysis:

Acid hydrolysis was done by using HCl 10% for (10-30) min in a water bath. This step led to hydrolysis glycosidic linkage and

obtain the glycone moiety, after cooling and filtering the product (O'Neil, 2006).

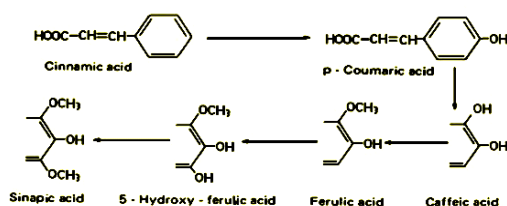


Figure. (1): Cinnamic acid when oxidized by light, UV or oxygen.

Liquid-Liquid portion:

The filtrate was transferred to separatory funnel. Chloroform was added in a quantity equal to the aqueous phase, shaking gently and repeating the process three times. The chloroform layers were collected and dried by rotary evaporator at 30°C, viscous green residue was stored in dark amber vessel at 20°C until use (O'Neil, 2006).

Column chromatography (partial purification):

A partial purification of the residue was proceeded by using open glass column (20x 20) cm packaged by silica gel F60. The residue was dissolved in (1-2) ml methanol and the mobile phase was benzene: acetic acid: water (6:7:3) (O'Neil, 2006).

Eluted sample were collected in 50 separated tubes and collected as 3ml per each tube. All fractions were tested for FeCl₃ 1% solution as a colorimetric method for polyphenols detection (O'Neil, 2006; Moffat, 2004) only the positive results were collected and dried under vacuum by a rotary evaporator. The cinnamic acid spots were detected on a TLC aluminum sheet silica gel (60F₂₅₄) in comparison with standard spot using the same mobile phase in the column chromatography. Cinnamic acid spots were examined with blue color spot under UV 254 lamb (O'Neil, 2006). The collected elution after drying was referred as "partial purification" for cinnamic acid.

Preparation of Thin Layer Chromatography (TLC) (partial purification):

TLC is a commonly used technique for the separation and identification of illicitly manufactured drugs. It is inexpensive, rapid, sensitive (sub-milligram quantities of analyze required), flexible in the selection of both the stationary and mobile phase and amenable to a wide variety of substances, in

base and salt form, ranging from the most polar to non-polar materials(United Nations Office on Drugs and Crime, 2012).

The dried samples obtaining were subjected to another extraction such as hexane: dichloromethane solvent (1:9) to obtain red color according to solubility of phenolic compound and insolubility of cinnamic acid. The layers dichloromethane which collected and dried under vacuum at 30°C in darkness with rotary evaporator were ready for TLC analysis.

The TLC method was described by (Cannell, 1998; Moffat, 2004). The application of the extract was done after resolving in 3ml of solvent system, benzene: methanol: water (6:7:3). The chamber was saturated with vapors for one hour before running the samples. Silica gel glass plates without fluorescence were activated for 10 minutes at 115°C.

It is done by using a micropipette for the sample application as a continues straight time to be loaded in a width rang (2-4)mm wide. The procedure was running on in a dark cool place, when the solvent system reached to the upper edge of the plate, removed and sprayed with:

- I. Diphenylboiloxetilamine is a detector for cinnamic acid, examined after dried for few minutes, the pure cinnamic acid line appeared as a dark straight line (Gulab *et al.*, 2005).
- II. AlCl₃ 1% ethanol solution get dark spot for both standard and extract cinnamic acid, then sprayed with starch 1% to fix the unsaturated compound with blue dark spots(Huiping & Huang, 2002).
- III. To visualize the fraction, sprayed small edge of the TLC plates with phosphomolibinin acid, *then heated for 5min* (Huiping & Huang, 2002).

Th line was scraped and eluted with 10 ml of methanol for three times and stored at 20°C for 2 days. An amorphous off yellow green crystal formed and collected rapidly in cool dark place, then kept in amber reservoir at 20°C, these crystals were refer as "pure cinnamic acid ". The fraction that did not react was scraping off and extract with dichloromethane for two hours with constant stirrer (Gulab *et al.*, 2005), then filtered and evaporated in a rotary evaporator under vacuum, weight and stored at 10°C until used.

The crystals were examined with the following tests:

I. Thin Layer Chromatography: Spots were made from crystals and standard samples on plate of silica gel 60 with fluorescence and using benzene: methanol: water (6:7:3) as a mobile phase.

II. High performance Liquid Chromatography (HPLC):

A. Dissolved 4gm of standard cinnamic acid 99.9% in 1litter methanol and made the concentrated (1, 2, 3.2, 6.75, 12.5, 25, 50, 100 and 200) ppm to get the standard curve (Huiping & Huang, 2002).

B. Dissolved 4gm cinnamon extract in 1litter methanol and diluted to (1, 2, 3.2, 6.75, 12.5, 25, 50, 100 and 200) ppm.

Both standard and cinnamon extract were examined by HPLC with condition (Huiping & Huang, 2002).

- The column was hypersil C18 (4.6X 250 mm, 5 μ m).
- The mobile phase was: methanol: acetic nitrile : water : acetic acid (25:26:49:0.3).
- The flow rate was 0.1ml / min.
- The UV detector was set at 270nm.

III. Melting point process: Using melting point apparatus (Glascoo, U.K.) especially for this process. Cinnamic acid melting point ranges (133–135) °C (Budavari, 2001).

Results and Discussion:

The cinnamic acid extraction:

Cinnamon bark were kept in a cool dark place, then 150gm of dried powder was hydrolyzed by hydrochloric acid and extracted with chloroform, then cinnamic acid separating by liquid-solid adsorption chromatography technique (silica gel column) to yield partial purified of cinnamic acid which eluted and isolated as crystals by TLC method. Pure crystals were about 10.5mg result from 150gm bark powder. Cinnamic acid was oxidized and converted to benzoic as Fig (1), by light, UV light, presence of heat, heavy metals and atmospheric oxygen (Bavaresco, 2003; O'Neil, 2006). The bark powder of cinnamon aromatic is rich with cinnamic acid derivatives compounds (85%) and bitty amount of eugenol (Al-Zubaidi, 2005).

The procedure for extraction was concluded different studies, all these processes have been carried out in the dark

(O'Neil, 2006). The bark powder was extracted with 2.5 liters methanol, because it's a good in many purpose solvent for preliminary extraction (Huiping & Huang, 2002). The cinnamic acid was hydrolyzed with 10% HCl (v/v) in water bath for (10-30) min (Budavari, 2001).

Purification of Cinnamic acid:

The partial purification was done by the silica gel G₆₀ column chromatography technique with a mobile phase benzene: acetic acid: water (7: 6: 3), then eluted fractions according to their affinity to mobile phase. The resultant fractions that gave positive ferric chloride test 1% solution were detected by TLC with silica gel 60 F₂₅₄ plate and the same mobile phase (Cannell, 1998; Arce *et al.*, 1998; Brandolini *et al.*, 2002). as indicated in Fig (2).

Spot 1 and 3: 1 μ l of cinnamic acid standard solution 0.1%.

Spot 2: 1 μ l of partial purified cinnamic acid gave positive results with FeCl₃ solution 1% after column chromatography.

Spot 4: 1 μ l of extract after column chromatography gave negative results with FeCl₃ solution 1%.

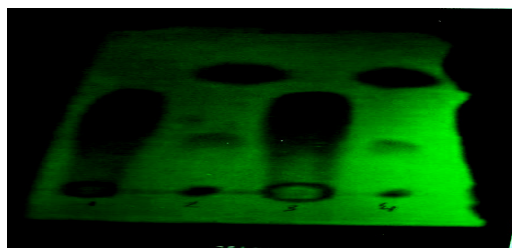


Figure (2): TLC chromatogram for detection partial purified cinnamic acid under UV lamp (254nm) explained the positive and negative results with FeCl₃ 1% test.

Figure (2) appeared fractions of positive result and standard fluorescence spots with (Retention factor) R_f value = (0.75), while the negative fraction were not. The dried collection was designed as "partial purified cinnamic acid. The cinnamon bark contains numerous amounts of chemical compounds, therefore, the extraction up is necessary by Liquid-Liquid partition technique for separation compounds according to the different distribution coefficients and the solvent affinity to solvents (Cannell, 1998). Dichloromethane is a good solvent for cinnamic acid taking up is viewing in many studies.

TLC method can achieved separation less than microgram of material, while for large scale, TLC was considered as a good technique for isolation and purification to obtain purified components for grams amount, by using benzene : acetic acid : water (7: 6: 3) as a mobile phase with silica G 60 glass plate (0.75 mm). Pure cinnamic

acid line appeared as a dark straight line (the silica gel 60 was using without fluorescence).

Chemical Identification of Cinnamic acid:

Cinnamic acid (partial and pure crystals) was tested for general phenolic tests Table (3).

Test	Result	
NaOH 5% solution (detected oxidation phenolic compounds)	+ve	Soluble
Sodium carbonate 5% solution (detected antioxidation phenolic compounds)	+ve	Insoluble
Ferric chloride 1% solution (detection phenolic compounds)	+ve	Green color
Liebermann reaction (detection of phenolic compound with para position)	+ ve	Red
Phthalein reaction (phenolic compounds)	+ ve	Red colour
Aluminum chloride test (Friedle graft) (for the aromatic ring)	+ve	Yellow to orange colour
Bromine decolourisation test (for the double bond)	+ ve	Discharge of reddish – brown colour
Baeyer test (for the double bond)	+ve	Disappears of the purple colour

Table (3). General phenolic compound tests.

*+ve= positive.

High Performance Liquid Chromatography (HPLC) for Cinnamic acid:

HPLC is another major separation technique commonly used in forensic drug analysis. For ease of sample preparation, best reproducibility and detectability, reversed phase chromatography is recommended for the analysis (O’Neil, 2006).

External or internal standard calibration may be used. The use of peak area for HPLC quantitation is recommended, because peak

broadening (decreases in peak height) may occur as a result of deterioration of the stationary phase, rendering peak height unsuitable for quantitation (United Nations Office on Drugs and Crime, 2012).

For cinnamic acid detection, HPLC method according to (Huiping & Huang, 2002) was used with wave length: 280 nm for both cinnamic acid extract and the standard under the same conditions. Table (4), appeared peak area and retention area and the corresponding retention time in both cinnamic acid standard and extraction.

Sample	Wave length	Concentration (ppm)	Retention area	Peak area
Standard Cinnamic acid	280 nm	100 ppm	8.40	2653179
		25ppm	8.91	6214651
Extraction Cinnamic acid	280 nm	100 ppm	8.13	3614220
		25ppm	8.88	5910090

Table (4). HPLC results for pure extracted and standard cinnamic acid (128 min).

Results emphasized that the conditions for extraction and purification occur in dark place, the study focus on qualitative specification of the cinnamic acid which is the proper way for extraction and application. Steps followed can be assumed for cinnamic acid qualitative study, such as

TLC method for purification and proved to be a high efficiency method, then the isolated substance was determined by the specific λ_{max} and HPLC technique. Measuring both standard and extracted cinnamic acid at maximum wave length absorption and the retention time explained

the selectivity and accuracy of the applied method (O'Neil, 2006).

Melting point of cinnamic acid:

The melting point was provided information about the purity of the substance. Pure crystalline solids have a sharp melting point temperature, but the mixture tends to melt at temperature below the melting point pure solids (Budavari, 2001). The melting point of cinnamic acid and the extracted are between the (133-135) °C that reflects the purity of extraction cinnamic acid which gain from partial purification (United Nations Office on Drugs and Crime, 2012).

Conclusion:

1. The partial purification by column chromatography and TLC obtain white crystal of cinnamic acid yield 10.5 mg from 150 gm cinnamon bark powder in comparison with standard.
2. All general chemical tests of phenols showed positive results.
3. The purification of extraction cinnamic acid in HPLC was 96% in comparison with standard.
4. The melting point of extraction cinnamic acid showed 96% in comparison with standard.

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الملخص العربي

كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا السائل العالي الأداء ودرجة الانصهار استخلاص لاستخلاص وتنقية حامض السيناميك من قلف نبات الدارسين (*cinnamon aromaticum*)

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الخلاصة

تهدف الدراسة إلى تنقية حامض السيناميك من قلف نبات الدارسين حيث أجريت التنقية الجزئية بعد كل من كروماتوغرافيا العمود كروماتوغرافيا الطبقة الرقيقة (TLC) والتي أعطت طبقة كرسالية رقيقة لحامض السيناميك بمقدار ١٠.٥ ملغم مسحوق لكل ١٥٠ غم من قلف نبات الدارسين المطحون بالمقارنة مع حامض السيناميك القياسي كنتاج من التنقية الجزئية. أجريت اختبارات كيمائية للتعرف على الطبقة الكرسالية كانت تتضمن اختبارات عامة للمركبات الحلقية، كروماتوغرافيا السائل العالي الأداء (HPLC) وتقييم درجة الانصهار بالمقارنة مع حامض السيناميك القياسي وكانت درجة النقاوة ٩٦%.