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Research Article

In vitro Antitubercular and Antibacterial activities of isolated constituents and column fractions from leaves of *Cassia occidentalis, Camellia sinensis* and *Ananas comosus*

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Background: Tuberculosis still remains a leading cause of death in the world. There is currently considerable interest in natural products and their derivatives in the area of drug research for multidrug resistant tuberculosis (MDR-TB). The present investigation focused on search for potent antitubercular and antibacterial natural leads from plants.

Objective: To evaluate isolated fractions and chemical constituents from leaves of *Cassia occidentalis, Camellia sinensis* and *Ananas comosus* for antibacterial and antitubercular activities.

Materials and Methods: Leaves were sequentially extracted with petroleum ether, benzene, chloroform, methanol and water. The obtained extracts were examined for the presence of various phytochemicals by thin layer chromatography, and then selected extracts were fractionated by column chromatography. A total of 15 column fractions were collected, and screened for antibacterial and anti-tubercular activities. The antibacterial activity was evaluated on American type cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* by broth microdilution method, whilst anti-tubercular screening was carried out against *Mycobacterium tuberculosis* $H_{37}R_V$ by microplate alamar blue assay (MABA) method.

Results: Several fractions (3, 6, 9) belong to *C. occidentalis, C. sinensis* showed good antibacterial activity (MIC: 2 – 8 µg/ml) and moderate antitubercular activity (MIC 25 - 50 µg/ml). Chemical constituents present in fractions 3, 6 and 9 were isolated and identified as cassiaoccidentalins, glucoronide soponin and catechin, respectively. Fractions of *A. comosus* showed MIC between 16 to 32 µg/ml for antibacterial activity and > 100 µg/ml for antitubercular activity.

Discussion: Among these plants, *C. occidentalis,*was found to more potential against antitubercular and antibacterial activity. *A. comosus* was relatively less potent against antibacterial and in effective against mycobacteria. Cassiaoccidentalin exhibited potent antibacterial activity and moderate antitubercular activity.

Key words: *Cassia occidentalis, Camellia sinensis*, *Ananas comosus,* anti-tubercular activity, antibacterial activity.

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1. Introduction

There is currently considerable interest in natural products and their derivatives in the area of drug research for multidrug resistant tuberculosis (MDR-TB). This is due to observations regarding importance of natural leads in current antitubercular therapy such as rifampicin and streptomycin in front-line treatment regime, where as

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second-line natural product or related drugs include capreomycin and cycloserine (Ormerod, 2005). As on present reports, tuberculosis still remains a leading cause of death in the world. The combination of a variety of factors like need for long treatment duration (6–9 months), increased incidence of (multi or extensive) drug resistance, co-morbidity with HIV-AIDS and lack of investment in anti-infectives drug discovery in pharmaceutical industries has led to a situation now where the discovery and development of newer agents in the treatments of tuberculosis is critical and needs more attention. Many researchers have highlighted the re-emerging interest in providing natural product based novel structures for the drug discovery effort for tuberculosis and being particularly effective as active leads for further design (Newman et al, 2000; Koehn and Carter, 2005; Butler and Buss, 2006). Several researchers continue to report antitubercular activity of natural product extracts, it is premature to speculate on chemical structure of the particular active constituents without further chemistry based studies (Copp, 2003; Graham et al, 2003; Jimenez-Arellanes et al, 2003; Leal et al, 2003; Al-Howiriny et al, 2005; Billo et al, 2005). Several reviews report on tuberculosis drug discovery, including information on developments in both *in vitro* and *in vivo* antituberculosis bioassays and natural product isolation techniques (Pauli et al, 2005), natural antimycobacterial metabolites (Okunade et al, 2004) plants and fungal products with activity against tuberculosis (De souza, 2005) and marine and terrestrial plants as sources of bacterial resistance modulators and anti-infective agents (Mayer and Hamann, 2005; Gibbons, 2005).

In past three decades, the minimum inhibitory concentration (MIC) of a crude extract was considered as reliable indicator of the chances for success in isolating a potent lead molecule from that extract. In fact, it may be reliable if an extract contains few moderately active major constituents at very low concentrations or moderately active crude extracts with minor compounds with high biological activity. In the area of drug discovery from natural products focused on a structural class that is consistently found to be active against pathogens or enzyme. Then the active compound shall be considered as more attractive for future developments than a single chemical constituent with high potency but no reported activity of natural analogues. Considering the above fact, it was felt that an unprejudiced antitubercular drug discovery effort should include a fractionation protocol which could able to isolate and to characterize minor constituents from the extract. Advantages of this protocol are likelihood for isolating significant quantities of structurally related natural products is greatly enhanced, greater chance for developing basic structure activity relationship after the identification/ elucidation of minor constituents, helps to assess the significance of the biological activity and to prioritize classes of active compounds for future studies (Pauli et al, 2005).

The present investigation focused on identification of significant chemical leads from leaves of three common plants - *Cassia occidentalis, Camellia sinensis* and *Ananas comosus.* Extensive literature revealed that there are several reports regarding biological activities of these plants but no investigation on antitubercular activity for these plants was reported. On the basis of considerable reports on antibacterial activities and abundant availability of these plants in our region, this study was designed.

Cassia occidentalis Linn, also called coffee senna in English, belongs to the family Caesalpiniaceae. Leaves of *C. occidentalis* plant have ethnomedicinal importance like paste of leaves is externally applied on healing wounds, sores, itch, cutaneous diseases, bone fracture, fever, ringworm, skin diseases and throat infection. Previous pharmacological investigations showed that *C. occidentalis* leaf extracts have antimicrobial (Jain et al, 1998; Saganuman and Gulumbe, 2006), antimalarial (Tona et al, 1999), antimutagenic (Sharma et al, 2000) antiplasmodial, (Tona et al, 1999), anticarcinogenic (Sharma et al, 2000) and hepatoprotective (Jafri et al, 1999) activity. Moreover, studies on this plant showed that the nature and amount of the phytochemicals varies according to the season and geographical location (Yadav et al, 2010).

Camellia sinensis, commonly called as tea plant, has astringent properties and often used for digestive ailments, to soothe insect bites, treat burns, and reduce swollen eyelids. Interestingly, black tea, green tea, white tea, and oolong tea are all harvested from *Camellia sinensis*, but they have different oxidation levels. The polyphenols found in tea are more commonly known as flavanols or catechins, and comprise 30-40 % of the extractable solids of dried green tea leaves. The main catechins in green tea are epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG), with the latter being the highest in concentration. Green tea polyphenols have demonstrated significant antioxidant, anticarcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties in numerous human, animal, and in vitro studies. (Alschuler, 1998; Raham, 1992).

The pineapple (*Ananas comosus*) is a tropical plant with edible multiple fruit consisting of coalesced berries, belongs to the family; Bromeliaceae (Coppens d'Eeckenbrugge., 2003). According to Harwell (1971) the pineapple fruit, peel, or juice is used in folk remedies for corns, tumors, and warts and also reported to be abortifacient, cholagogue, depurative, diaphoretic, digestive, discutient, diuretic, ecbolic, emmenagogue, estrogenic, hydragogue, intoxicant, laxative, parasiticide, purgative, refrigerant, styptic, and vermifuge. The substance bromelain known to possess biological properties like burn debridement, anti-inflammatory, smooth muscle relaxant, anti-ulcer, appetite inhibitor, enhanced fat excretion, sinusitis relief.

The present communication, reports the antitubercular activity of various column fractions obtained from leaf extracts of these plant and isolation and identification of active natural leads structure for future studies.

2. Materials and Methods

2.1 Collection of Plant Materials

Leaves of *Cassia occidentalis, Camellia sinensis* and *Ananas comosus* were collected from Rayalseema region of Andhra Pradesh, India during the month January 2013. Plant specimens were botanically identified and

authenticated by the botanist, Department of botany, Sri Krishnadevaraya University, Andhra Pradesh, India. . The voucher specimen of each plant was deposited at the department herbarium (Specimen no: p21/2013, p24/2013, p25/2013 respectively for *Cassia occidentalis, Camellia sinensis* and *Ananas comosus)*.

2.2 Extraction and Phytochemical Analysis

Leaves were cleaned with deionized water, air dried and powdered in a blender. The plant material (200 gm) was sequentially extracted with 2000 ml of different solvents according to their increasing polarity (petroleum ether, benzene, chloroform, methanol and water).

Soxhlet hot continuous extraction was carried out for 24 h at a temperature suitable to the boiling point of the respective solvent, except for water. Respective aqueous extract was obtained by cold maceration technique. The obtained extracts were vacuum filtered using Whatmann No. 1 filter paper and then concentrated under vacuum using a rotary evaporator. The extractive value for the extracts was calculated and displayed in **Table 1.**

Crude extracts obtained were characterized for colour and odour and then stored in refrigerator at 4 °C in sterile light protected container.

Preliminary chemical analysis for the presence of alkaloids, saponins, carbohydrates, glycosides, fixed oils and fats, aminoacids, flavonoids, anthraquinones, tannins and phenolic compounds were carried out by using the methods of Harbone (Jain et al, 1998) and Brindha (Saganuwan and Gulumbe, 2006). Results are shown in **Table 2.**

2.3 Column Chromatographic Separation

Extract for column chromatography was chosen on the basis of phytochemical analysis and thin layer chromatography (TLC) results that represent all possible chemical constituents in plant materials. Hence 5 g of petroleum ether extract of *Cassia occidentalis,* methanol extract of *Camellia sinensis* and aqueous extract of *Ananus comosus* were subjected to column chromatographic separation using n-hexane, ethyl acetate and a mixture ethyl acetate and methanol as eluting solvents. These gradient elution was started with 100% hexane, through 100% ethyl acetate to ethyl acetate:methanol (50:50 *v/v*). Different fractions of elution based on time were collected and tested by TLC. On the basis of TLC results regarding phytochemical constituents, obtained fractions were combined to 15 different fractions (Fraction no. 1 to 15 displayed in **Table 3**) and freeze dried.

2.4 Antibacterial activity

Antibacterial activity for column fractions was performed by broth micro-dilution method (Nagayama et al., 2008). Serial dilutions of the test fractions and reference drugs were prepared in DMSO to attain a final concentration of 1 mg/ml. Further progressive dilutions with Mueller-Hinton agar were performed to a concentration from 1 to 64 µg/ml. Tubes were inoculated with 10^5 cfu/ml (Colony forming unit/ml) of each organism, *Staphylococcus aureus ATCC 6538, Bacillus subtilis ATCC 6633, Proteus vulgaris ATCC 8427* and *Pseudomonas aeruginosa ATCC 9027* and incubated at 37 °C for 24 h. Minimum inhibitory concentration (MIC) was determined based on the absence of growth and comparison to the standard. Ciprofloxacin was used as standard and the obtained results were displayed in **Table 4** (Saganuwan and Gulumbe, 2006).

Table 1: Extractive values and organoleptic character for extracts

Extract	*Cassia occidentalis*			*Camellia sinensis*			*Ananas comosus*		
	% yield	Colour	Odour	% yield	Colour	Odour	% yield	Colour	Odour
Petroleum ether[a]	5.1	Greenish black	Sea weed	4.6	Brown	Pungent	3.3	Greenish black	Sea weed
Benzene[a]	2.2	Brownish black	Pungent	1.1	Dark black	Pungent	3.8	Brownish black	Pungent
Chloroform[a]	3.8	Dark black	Pungent	1.6	Greenish black	Pungent	3.2	Brown	Pungent
Methanol[a]	6.1	Brown	Alcoholic	3.4	Brown	Alcoholic	5.3	Dark	Alcoholic
Water[b]	8.8	Brown	Tobacco like	18.4	Greenish black	Pungent	7.5	Black Brown	Pungent

[a] *Extract obtained by Soxhlet extraction;*
[b] *Extract obtained by cold maceration*

Table 2: Preliminary phytochemical Analysis extracts

Phytochemical Constituents	*Cassia occidentalis* *(Petroleum ether)*	*Camellia sinensis* (Methanol)	*Ananas comosus* *(Aqueous)*
Carbohydrate	-	+	+
Tannins	-	+	+
Saponins	+	+	+
Terpenes	+	+	+
Sterols	+	+	+
Flavonoids	+	+	+
Alkaloids	-	+	+
Phenols	+	+	+
Resins	+	-	-
Balsam	+	-	-
Cardiac glycosides	+	-	+
Anthraquinones	+	+	+
Amino acids	-	+	+

Table 3: Characteristics of various Column fractions

Plant fractions	Fraction number	Volume (mL)	TLC (R_f Values)	Chemical constituents
C. occidentalis				
n-Hexane	1	120	0.68	Terpenens
	2	50	0.79	Sterols
Ethyl Acetate	3	180	0.59	Flavonoids (mix)
Ethyl Acetate : MeOH (50%)	4	200	0.48	Phenols
	5	250	0.67	Anthraquinone
C. sinensis				
n-Hexane	6	100	0.57	Saponins
	7	90	0.72, 0.65	Terpenens, Alkaloids
	8	70	0.82	Sterols
Ethyl Acetate	9	120	0.61	Flavonoids
Ethyl Acetate : MeOH (50%)	10	180	0.77	Phenols
A. comosus				
n-Hexane	11	250	0.44, 0.55	Tannins, Saponins
Ethyl Acetate	12	120	0.85	Steroids
Ethyl Acetate : MeOH (50%)	13	130	0.50	Flavonoids
	14	170	0.69	Phenols
	15	200	0.32	Amino acids

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2.5 Anti-tubercular activity

Screening of antitubercular activity was performed against *M. tuberculosis* $H_{37}Rv$ using microplate alamar blue assay (MABA). This methodology is a non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric methods (Collins et al., 1997). Briefly, 200 µL of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µL of the middlebrook 7H9 broth and serial dilutions of compounds were made directly on plate. Fractions were dissolved in DMSO and tested in the concentration range from 100 to 0.2 µg/ml. Same procedure was followed for standards and blank. Plates were covered and sealed with parafilm and incubated at 37 ºC for five days. At the end of 5th day, 25µL of freshly prepared 1:1 mixture of alamar blue reagent and 10% tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no mycobacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. (Maria et al, 2007). Results were displayed in **Table 4**.

2.6 Isolation and identification of chemical structure

Albino Fractions 3, 6 and 9 were subjected to further purification and fractionation by Flash chromatograph equipped with UV detector, silica gel packed column and binary pump. Elution was performed in gradient mode and resolution between components was monitored by Analyx software. Eluent was collected and vacuum dried. The purity of the obtained compounds was established by high performance liquid chromatographic technique using Agilent LC1200 equipped with C_{18} column and photo-diode array (PDA) detector. Compounds obtained were characterized by proton and carbon NMR, Mass Spectral Studies. Compounds spectral interpretation was carried out based on reported literatures (Hatano et al., 1999, Sagesaka et al., 1994 and Durre Shahwar et al., 2013). The obtained spectral data and molecular mass were similar to those reported structures in published reports (Hatano et al., 1999, Sagesaka et al., 1994 and Durre Shahwar et al., 2013). Structures were shown in (**Figure 1**).

3. Results

The various extracts (petroleum ether, benzene, chloroform, methanol and water) for leaves of *Cassia occidentalis, Camellia sinensis* and *Ananas comosus* were examined for the presence of phytochemical constituents. Based on the results and extractive values, particular extract was selected for fractionation protocol by column chromatography. The petroleum ether, methanolic and aqueous extracts respectively for *Cassia occidentalis, Camellia sinensis* and *Ananas comosus* were selected and subjected to column elution according to increasing polarity of solvents in the order of n-hexane, ethyl acetate and a mixture of methanol and ethyl acetate (50:50% v/v). A total of 15 column fractions were collected for all three plants and tested for the presence of chemical constituents by TLC technique. The fraction results are displayed in **Table 3.** These isolated fractions were dried under vacuum and screened for antibacterial activity by using broth dilution assay method at a concentration range from 1 to 64 µg/ml. Among 15 column fraction from leaves extracts of *Cassia occidentalis, Camellia sinensis* and *Ananas comosus,* ethyl acetate fractions of *Cassia occidentalis* (fraction 3), *Camellia sinensis* (fraction 9) and n-hexane column fraction of *Camellia sinensis* (fraction 6) exhibited significant antibacterial activity against test species. The rest of the fractions exhibited moderate antibacterial activity. Results revealed that fractions 3, 9 showed significant antibacterial activity (MIC: 2 - 4 µg/ml) against all test species. Fraction 6 showed MIC of 4 µg/ml and 8 µg/ml respectively against gram-positive and gram-negative bacteria. Remaining fractions were exhibited antibacterial activity between 16 – 64 µg/ml. Detailed results are displayed in **Table 4.** None of the fraction of *A. comosus* was potentially active when compared to constituents of *C. occidentalis* and *C. sinensis.*

The anti-tubercular activity of the various column fractions were screened against *Mycobacterium tuberculosis* $H_{37}R_V$ strain in Middlebrook 7H9 (MB 7H9 broth) using microplate alamar blue assay (MABA) in serial concentrations from 100 to 0.2 µg/ml. Fraction 3 showed MIC of 25 µg/ml, whilst fractions 6 and 9 showed MIC of 50 µg/ml and rest of the fractions exhibited MIC of >100 µg/ml. Results are presented in **Table 4**.

Fraction 3 (*Cassia occidentalis*) and fractions 6, 9 (*camellia sinensis*) were further characterized and subjected to flash chromatography for isolation of chemical constituents. Isolated compounds purity was confirmed by RP-HPLC and structures were established by spectral studies. It was noted that none of the fractions of *Ananus comosus* were found to be active against mycobacteria. But exhibited considerable activity against bacteria.

The chemical constituents present in fractions 3, 6 and 9 were investigated by isolation and spectral studies. The respective constituents in fractions 3, 6 and 9 are identified as cassiaoccidentalins, glucuronide saponin and catechin. The chemical names are given in the following section and structures shown in **Figure 1**.

4. Discussion and Conclusion

Investigation on leaves part of three commonly available plants revealed that flavones of *Cassia occidentalis* and *Camellia sinensis* exhibited considerable antitubercular activity and potent antibacterial activity. These flavones structures were identified as cassiaoccidentalins and catechin. In addition to this, glucoronide saponin from *camellia sinensis* showed good antibacterial activity and mild antitubercular activity. These molecules may be considered for further development with appropriate structure modification in drug candidate search for tuberculosis. Further it was noticed from earlier literature that flavones from *camellia sinensis* exhibited potent enzyme inhibition properties, so these structures are expected to bind with protein of bacteria or mycobacterium and could be considered for development of new drug candidate in antitubercular and antibacterial drug research.

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Table 4: Antibacterial and antitubercular activity of fractions

Fraction no	Antibacterial activity (MIC in µg/ml)				Antitubercular activity (MIC in µg/ml)
	Staphylococcus aureus	*Bacillus subtilis*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	
1	16	16	32	32	>100
2	16	16	32	32	>100
3	2	2	4	4	25
4	8	8	16	32	>100
5	8	8	32	32	>100
6	4	4	8	8	50
7	16	32	64	64	>100
8	16	32	64	64	>100
9	2	2	4	2	50
10	16	16	32	>64	>100
11	16	16	32	32	>100
12	16	16	32	32	>100
s13	32	32	>64	>64	>100
14	>64	>64	>64	>64	>100
15	>64	>64	>64	>64	>100
Ciprofloxacin	8	8	8	8	---
Pyrazinamide	---	---	---	---	3.125
Streptomycin	---	---	---	---	6.25

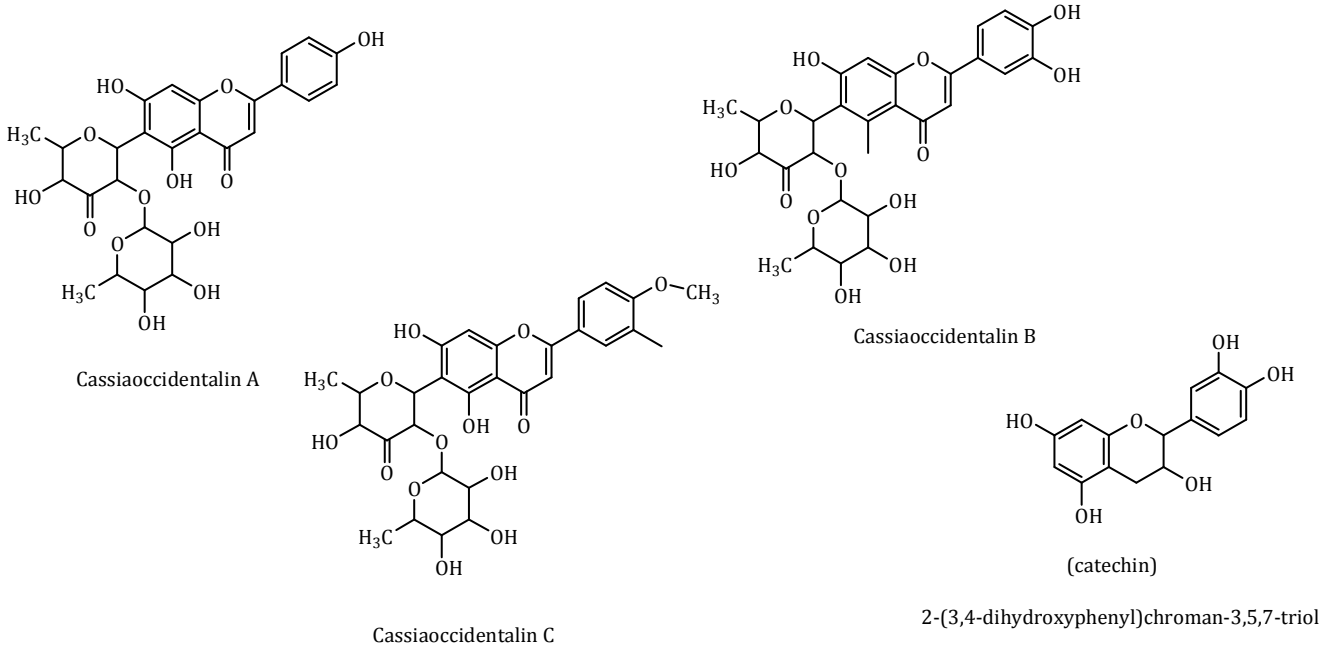

Figure 1: Structure of cassiaoccidentalin A, B, C (Fraction 3) and Catechin (Fraction 6)

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Cassiaoccidentalin A: *5,7-dihydroxy-6-(5-hydroxy-6-methyl-4-oxo-3-(3,4,5-trihydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)- tetrahydro- 2H-pyran-2-yl)-2- (4-hydroxyphenyl)- 4H- chromen - 4-one.*

Cassiaoccidentalin B: *2-(3, 4-dihydroxyphenyl)-5,7-dihydroxy-6-(5-hydroxy-6-methyl-4-oxo- 3-(3,4,5-trihydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-tetrahydro- 2H-pyran-2-yl)-4H-chromen-4-one.*

Cassiaoccidentalin C: *5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-6-(5-hydroxy-6-methyl-4-oxo-3-(3,4,5-tri hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-tetrahydro-2H- pyran-2-yl)-4H-chromen-4-one.*

Glucuronide saponin*: (3-O-[beta-D-galactopyranosyl(1-->2)-[beta-D- xylopyranosyl(1-->2)-alpha-L-arabinopyranosyl(1-->3)] -beta- D- glucuronopyranosyl]-21-O-cinnamoyl -16,22-di-O-acetylbarr ingtogenol C).*

Catechin*: 2-(3, 4-dihydroxyphenyl) chroman-3,5,7-triol.*

Conflict of Interest declaration

The authors declare no conflict of interest

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