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B.Sc. PATHOLOGIE VÉGÉTALE

CHEMICAL AND BIOLOGICAL STUDY ON CONSTITUENTS OF *POPULUS TREMULOIDES* BUDS

ÉTUDE CHIMIQUE ET ACTIVITÉS BIOLOGIQUES DES CONSTITUANTS DES BOURGEONS DU *POPULUS TREMULOIDES*

DÉCEMBRE 2009

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ABSTRACT

Nowadays, a large number of compounds derived from plants are used in modern medicine and a majority of them are inspired by traditional applications. About 60% of anticancer drugs and 75% of compounds for infectious diseases are either natural products or their derivatives. However, it is estimated that only bioactive molecules of 5 to 15% of more than 250'000 plants species on earth have been investigated. Particularly, few scientific studies have been conducted on bioactive compounds from the medicinal plants of the boreal forest, wide-spread in Quebec and Canada. In recent years, LASEVE laboratory has evaluated the potential pharmacology of many plants and it was apparent that the buds of *Populus tremuloides* would represent an interesting subject of study in chemistry and pharmacology. In North America, leaf-buds of *P. tremuloides* were used in traditional medicine to treat numerous diseases. The aim of this research project was to isolate and characterize the polyphenolic constituents in buds of *P. tremuloides* and to evaluate the biological activities of the purified fractions and pure compounds. Phytochemical investigations of the EtOH extract of *P. tremuloides* leaf-buds led to the isolation of 24 phenolic compounds. Among them, one compound is a new molecule and was not reported in literature before; two compounds were not reported before for Salicaceae family; two compounds were not reported before for *Populus* genus; and ten compounds were identified for the first time in *P. tremuloides*. Three of the identified compounds have exhibited a moderate to strong cytotoxicity (5-20 μM) towards some of cellular tested lines (WS1, DLD-1, A-549). Anti-inflammatory activity of five phenolic compounds having a glycerol section has been evaluated using LPS-stimulated RAW 264.7 macrophages. Only one of the tested compounds significantly inhibited nitric oxide production (IC_{50}: 6.5 ± 0.4 μM), without cytotoxicity. Finally, none of the phenolic compounds isolated in the present work have exhibited a significant activity against *Staphylococcus aureus* and *Escherichia coli*.

RÉSUMÉ

De nos jours, un grand nombre de composés d'origine végétale sont utilisés dans la médecine moderne. Plusieurs d'entre eux ont été développés en s'inspirant des informations de la médecine traditionnelle. Environ 60 % des anticancéreux et 75 % des antimicrobiens de l'arsenal thérapeutique actuel sont des produits naturels ou des dérivés. Seulement 5-15 % des 250 000 plantes ont été étudiées afin d'en évaluer le potentiel pharmacologique. Les plantes de la forêt boréale québécoise ont été très peu étudiées au niveau de leur composition chimique et de leur potentiel pharmacologique. Au cours des dernières années, le laboratoire LASEVE a évalué le potentiel pharmacologique de plusieurs plantes et il en est ressorti que les bourgeons du *Populus tremuloides* représentaient un sujet d'étude intéressant au niveau chimique et pharmacologique. De plus, cette espèce a été utilisée en médecine traditionnelle pour traiter de nombreuses maladies. L'objectif de ce projet était d'isoler et de caractériser les composés phénoliques majoritaires des bourgeons de *Populus tremuloides* et d'en évaluer l'activité biologique (anticancéreuse, anti-inflammatoire et antibactérienne). Les travaux effectués sur l'extrait éthanolique des bourgeons de *Populus tremuloides* ont mené à l'identification de 24 composés phénoliques. Un de ces composés n'a jamais été rapporté auparavant dans la littérature scientifique et deux autres n'ont jamais été signalés dans la famille des *Salicacées*. De plus, dix de ces composés sont identifiés pour la première fois dans le *Populus tremuloides*. Trois des composés identifiés ont démontré une cytotoxicité de forte à modérée (5-20 μM) envers certaines des lignées cellulaires testées (WS1, DLD-1, A-549). L'activité anti-inflammatoire de cinq composés phénoliques constitués d'une section glycérol a été évaluée sur des macrophages de souris RAW 264.7 stimulé avec le LPS (lipopolysaccharide). Un seul des composés testés a significativement inhibé, sans cytotoxicité, la production d'oxyde nitrique avec une IC_{50} de 6.5 ± 0.4 μM. Finalement, aucun des composés phénoliques isolés dans ces travaux n'a démontré une activité importante contre *Staphylococcus aureus* et *Escherichia coli*.

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Special thanks to Dr. Vakhtang Mshvildadze and Mr. Serge Lavoie, for their kind help throughout this study and to my colleagues with whom I shared these years of research in LASEVE laboratory.

I am pleased to have participated in the master program offered by the Université du Québec à Chicoutimi, where I had the chance to develop my skills and find forward-looking friends.

Last, but not least, I am also most grateful to my husband, Alireza, for his never-ending support and understanding in all the situations and to my parents for unconditional support and encouragement.

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Chapter I

INTRODUCTION

1.1 General Introduction

The use of plants as medicines has a long history. Written proofs of man's ingenuity in using plants for treatment of various diseases are present by ancient Chinese, Indians, and North Africans [1]. These plants were initially used without modification, then as concentrated extracts to increase their effectiveness or as dried form to keep them for longer times with less degradation. In the 1900s, people learned how to isolate active compounds from medicinal plants.

Even today, natural products are important sources of drugs and almost 60% to 75% of the world's population use plant-based medicines for initial pharmaceutical cure [2]. Between 1981 and 2002, it is estimated that almost 50 % of new chemical entities authorized to initiate clinical studies were natural products or related natural products [3]. Moreover, natural products exhibiting various biological activities usually possess a complex and diverse structure leading to strong challenges in organic synthesis [3, 4]. About 60% of anticancer drugs and 75% of compounds for infectious diseases are either natural products or their derivatives [5, 6]. It is commonly accepted that less than 15 % of the plants were studied regarding their biological activities [7, 8].

In Canada, one of the most important natural resources is the boreal forest. The latter plays an important role in the traditional medicine of Canadian First Nations communities [9]. However, real effectiveness of these potential drugs is rarely studied in scientific ways. Few biopharmaceutical studies were undertaken on plant species of the boreal forest of Canada [9, 10]. Based on the knowledge acquired by First Nations communities, LASEVE

laboratory reported the identification of an antitumoral compounds from balsam fir [11]. Following this result, a large effort of this laboratory has been devoted to research on potential pharmaceutical contents in biomass of the boreal forest.

This project is inspired by previous research work performed on other *Populus* species (*Populus balsamifera*). The present research is focused on chemical and biological properties of bud constituents of the most widely distributed species of *Populus* in boreal forest, named *Populus tremuloides*.

1.2 Research objectives

The main goals of this research project are:

- Isolation and characterization of polyphenolic constituents from the buds of *P. tremuloides.*
- Investigation of crude extract, purified fractions and pure compounds for antibacterial, anti-inflammatory and cytotoxic activities (*in vitro*).
- Identification of the active compound(s) through bioassay-guided fractionation.
- Study of the relationship between the chemical structures and the biological activities.

This thesis is divided into five chapters. The first chapter is dedicated to the thesis outlines. Chapter II covers literature reviews about botany, applications and phytochemistry of the genus *Populus* and the species of *Populus tremuloides*. Chapter III presents a

scientific paper dealing with Cytotoxic polyphenols in leaf-buds of *P. tremuloides*. This article was accepted in *Canadian Journal of Chemistry*. Chapter IV describes the scientific work related to anti-inflammatory phenolic compounds from buds of *Populus tremuloides* and finally in Chapter V, conclusions and future directions are covered.

Chapter II

LITERATURE REVIEW: *Populus tremuloides* Michx

2.1 Principle Characteristics of Populus tremuloides

Populus tremuloides (Quaking aspen), from Salicaceae family is a decidous tree native to cooler areas of North America. It is a tall tree, usually 20 to 25 meters high at maturity, with a trunk diameter comprised between 20 to 80 cm [12, 13]. As shown in figure 1, this tree has a geographical distribution from Alaska to the east coast of Canada and is present down south in scattered populations (New Mexico state). However, It is absent from the central and south-eastern plains. It grows in humid calcium-rich soils.

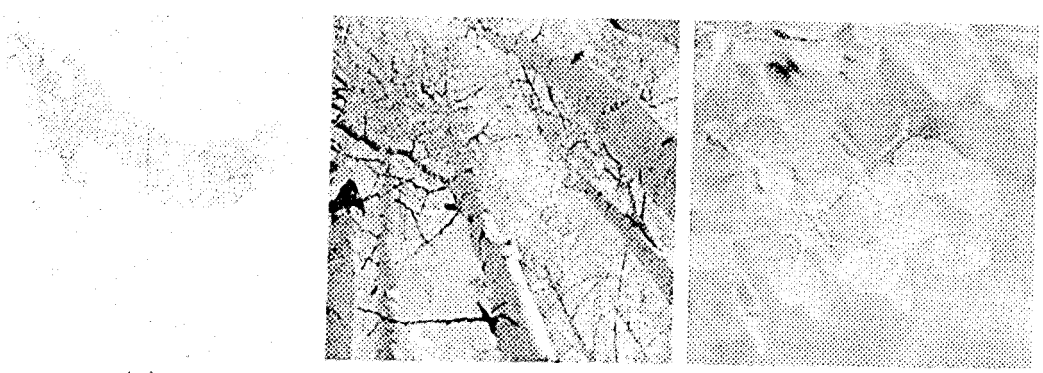


Figure 1: (a) Distribution of *P. tremuloides* in North America, (b) trees of *P.tremuloides*, (c) leaves of *P.tremuloides* [13, 19].

2.2 Applications by Amerindians

Quaking aspen has been used as herbal medicine for centuries. First Nations communities widely used this species especially for its antiseptic and analgesic properties for wounds healing and against skin complaints and disorders [9]. Now, it has similar applications in modern herbalism.

Its bark had applications in the treatment of rheumatism, arthritis, gout, lower back pains, urinary complaints, digestive and liver disorders, debility, and anorexia [14-16]. It also could reduce fevers and relieve the pain of menstrual cramps [16]. Infusion of inner bark was a remedy for coughs as well as a treatment for stomach pains, colds and fever [16-17].

Leaf-buds had applications as a nasal salve for children and adults to fight coughs, colds and irritated nostrils [9, 18]. They were also used against chronic bronchitis and rheumatism and externally for treating superficial wounds, external haemorrhoids, frostbite, sunburn and as an ointment for myalgia [19].

2.3 Chemistry of tne genus Populus

Having fast growth rates, the genus *Populus* attracted many economical interests, such as in the wood pulp industry and motivated longstanding investigations about its chemistry [20]. The genus tree family contains phenolic glycosides which are important secondary metabolites regarding plant resistance to both insect and mammalian herbivores [21, 22]. Finally, chemical principles from *Populus* species are known to have various biological activities such as fungicidal, antioxidant, antitumor, antiseptic and antiviral properties [23-25].

Several classes of natural products such as polyphenols, terpenoids, fatty acids, aliphatic alcohols and hydrocarbons are found in the genus *Populus* [26-29]. Due to difficulty in identification of Salicaceae species only based on morphological identification, secondary

phenolics were used as chemotaxonomic purposes. The chemotaxonomy of poplar (*Populus* spp) is related to a complex mixture of compounds in their bud exudates which include benzoic and phenolic acids and their esters, flavanoid aglycones, hydrocarbons and terpenoids [30, 31]. The presence of some phenolic compounds can be used as a 'fingerprint' to identify poplar species [32] since many phenolic components of buds exudates of European, Asian, and North American poplar species have been studied in recent years [33-38]. The analysis of poplar bud exudates have shown a high degree of complexity. The typical poplars of a particular section have a bud exudate composition which is characteristic of that section. For example, section Leuce, subsection Albidae, *e.g P. alba* L., exude only hydrocarbons [39] whereas those of subsection Trepidae, *e.g. P. tremuloides* Michx., secrete a phenolic exudate which includes only the flavanones produced by the first steps of flavonoid metabolism, such as naringenin (S,7,4'-trihydroxyflavonone) and its methyl ethers [29]. By contrast poplars of section Aigeiros, such as *P.deltoides* Marsh [40] and *P.nigra* L. [41], and of section Tacamahacha, such as *P.balsamifera* L. [42], produce a more complex bud exudate, which contains primarily flavonoids resulting from the further metabolism of naringenin. Phenolic compounds are also exuded onto the leaf surfaces of some poplars, such as *P. deltoides* [43] and *P. nigra* [44]. The bud of *P. tremuloides* is shown in figure 2.

Propolis, a resinous hive product collected by honeybees from parts of plants, especially buds and exudates of *Populus* species, has been used as folk medicines about 300 years before Christ (BC). Different biological activities, such as anticancer, antioxidant, anti-inflammatory, antibiotic and antifungal effects have been reported for propolis and its

constituents. It has also been extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer. Recently there is a renewed interest in the composition of propolis and related poplars [45-48]. Recently, it has been proven that the phenolic content of northern propolis is mainly inherited from *Populus* spp., a very attractive tree for honey bees of Canada. Thus, considering the promising biologically-active substances in propolis inheritated from poplars of section *Leuce*, subsection *Trepidae*, its plant source which is *P. tremuloides* is worth further investigation [49].

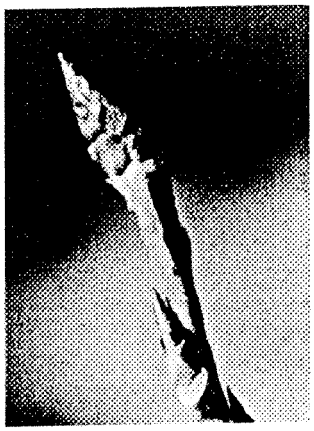


Figure 2: Bud of *Populus tremuloides*.

2.4 Populus tremuloides Michx

While *P. tremuloides* has a rather prominent importance as a model system for ecological and environmental research by the forest industries, few phytochemical or pharmacological studies were conducted so far on *P. tremuloides*. Fernandez *et al.* has identify 44 compounds (12 of them are shown in figure 3) such as phenolic compounds,

fatty acids, steroids, triterpenes and lipids in processing of *P. tremuloides* Michx. for pulp and paper, with a rapid gas chromatography–mass spectrometry (GC–MS) method, summarized in table 1 [50].

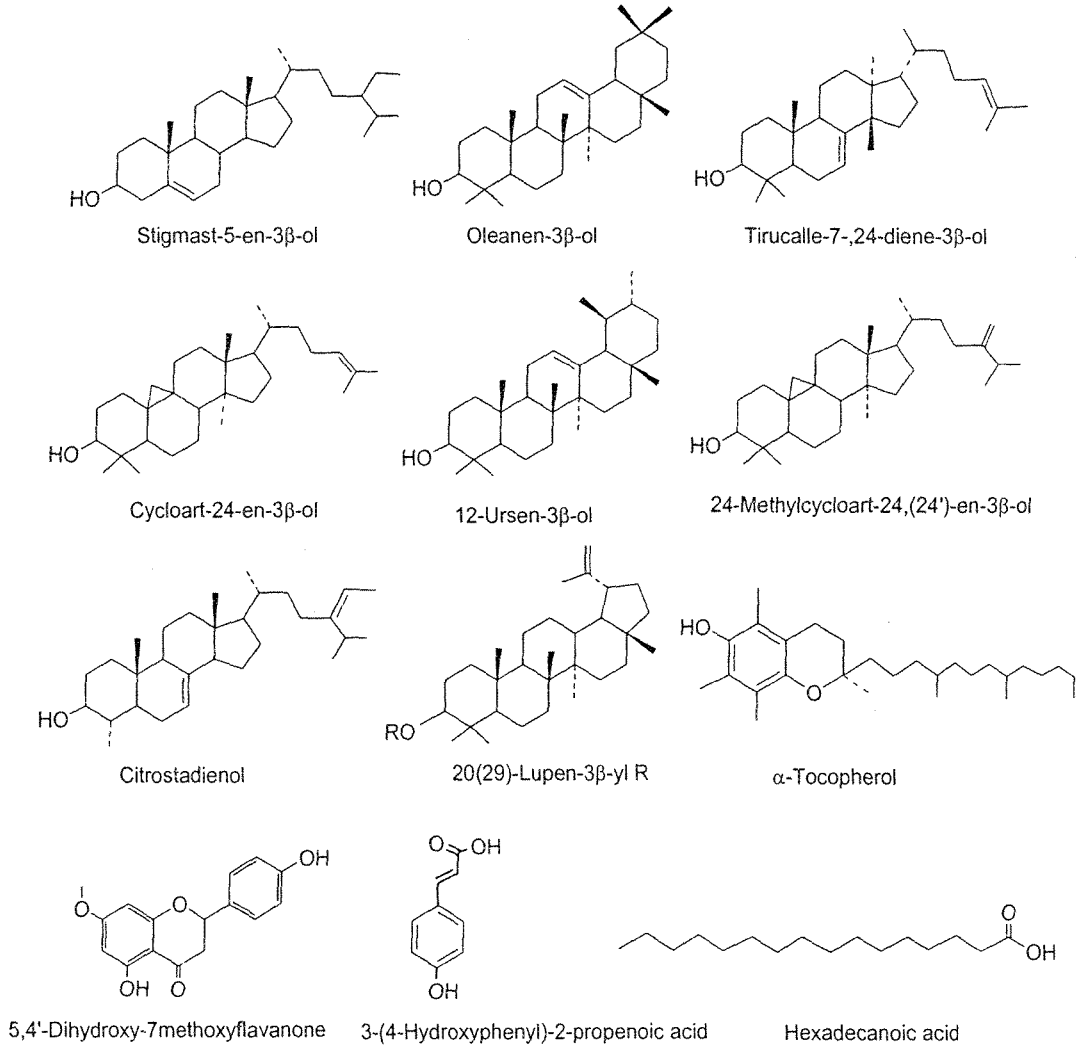


Figure 3: Molecular structure of some compounds described by Fernandez *et al* [50].

Table 1: GC–MS identification of the *P. tremuloides* pulp and paper constituents [1].

Compound family	Name of compounds
Monoaryl phenolics	benzoic acid
	1-Ethyl-4-hydroxybenzene
	3-(2-Hydroxyphenyl)-2-propenoic acid
	2-hydroxybenzyl alcohol
	4-Hydroxy-2-methylacetophenone
	4-Hydroxybenzoic acid
	3-(4-Hydroxyphenyl) propanoic acid
	3-(4-Hydroxy-3-methoxyphenyl)-2-propen-1-ol
	3-(4-Hydroxy-3-methoxyphenyl)-2-propenal
	3-(4-Hydroxyphenyl)-2-propenoic acid
	4-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid
	4-(3-Hydroxy-1-propenyl)-2,6-dimethoxyphenol
	3-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propen-1al
Fatty acids	9-oxononanoic acid
	Hexadecanoic acid
	(Z,Z)-9,12-Octadecadienoic acid
	Octadecadienoic acid
	Eicosanoic acid
	Docosanoic acid
	Tetracosanoic acid
	1-Docosanol
Flavonoids	4',5-Dihydroxy-7-methoxyflavanone
	4',5,7-Trihydroxyflavanone
	3,5,7-Trihydroxy-4'-methoxyflavone
	3,4',5,7-Tetrahydroxyflavone
Sterols / triterpene alcohols	Stigmast-5-en-3β-ol
	Oleanen-3β-ol
	Tirucalla-7,24-diene-3β-ol
	Cycloart-24-en-3β-ol
	12-Ursen-3β-ol
	24-Methylcycloart-24,(24)-en-3β-ol
	Citrostadeniol
Steryl / triterpene esters	Stigmast-5-en-3β-yl acetate
	Tirucalla-7,24-diene-3β-yl hexadecanoate
	12-Oleanen-3β-yl hexadecanoate
	20(29)-Lupen-3β-yl hexadecanoate
	12-Ursen-3β-yl hexadecanoate
	Tirucalla-7,24-diene-3β-yl octadecanoate
	12-Oleanen-3β-yl octadecanoate
	20(29)-Lupen-3β-yl octadecanoate
	12-Ursen-3β-yl octadecanoate
	Tiraculla-7,24-diene-3β-yl eicosanoate
Lipids	α-tocophérol
	(9Z.12Z)-Glycerol tri-9,12-octadecadienoate

The extractives of unsaturated alcohol fraction from *P. tremuloides* heartwood have been studied by Abramovitich and Micetich [51] and their work resulted in the identification of triterpenes and sterols, as shown in figure 4.

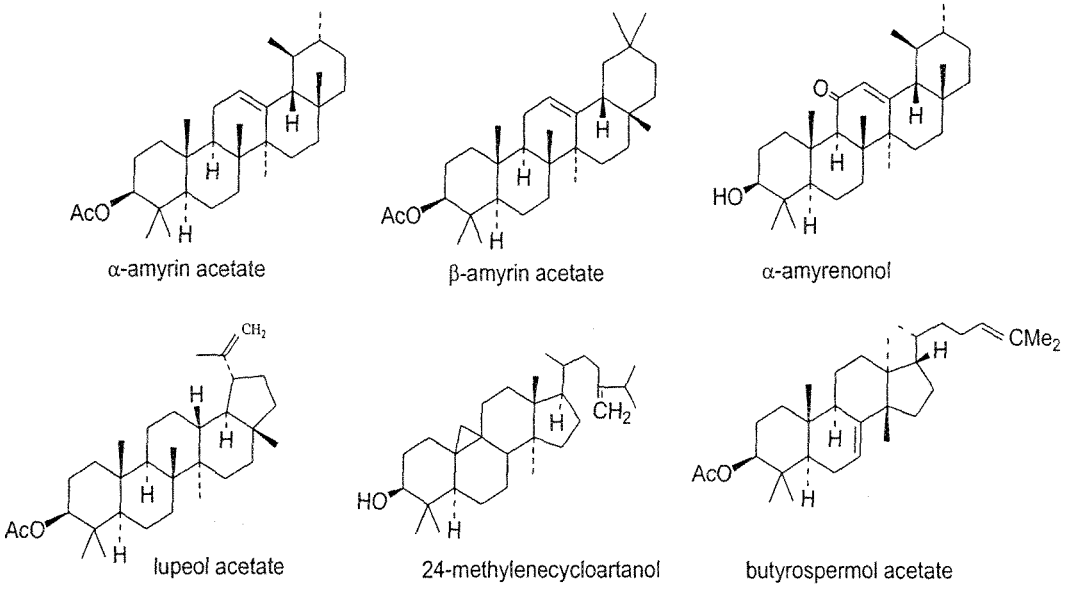


Figure 4: Triterpenes constituents from the heartwood of *P. tremuloides* [51].

Pearl *et al.* [52] showed that leaves of *P. tremuloides* provide a good source of the 6-monobenzoate of salicin, populin, while the bark of the same species provides a good source of the 2-monobenzoate of salicin, tremuloidin [52, 53]. Lindroth *et al.* [54] identified phenolic glycosides components, such as salicin, salicortin, tremuloidin, tremulacin from the crude extract of *P. tremuloides* leaves which exhibited differential toxicity against the insects such as *Papilio glaucus* subspecies.

Phytochemical investigation by English *et al.* [29] with GC-MS on buds of *P. tremuloides* revealed phenolic compounds, flavonoids and hydrocarbons listed in table 2.

Table 2: Components of buds of *P. tremuloides* [29].

Compound family	**Name of compounds**
Phenolic compounds	Benzyl benzoate
	p-Coumaric acid
	Ferulyl alcohol
	Ferulic acid
	Caffeic acid
	Benzyl *p*-coumarate
	Ferulyl benzoate
	Benzyl ferulate
	Benzyl caffeate
Flavonoides	Isosakuranetin
	Isosakuranetin chalcone
	Sakuranetin
	Sakuranetin chalcone
	Kaempferol methyl ether
Hydrocarbones (straight chain)	Tricosane
	Pentacosane
	Heptacosane
	Nonacosane
Hydrocarbon alcohol	1-Hexacosanol

Chapter III

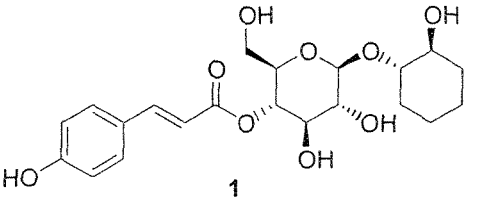
SCIENTIFIC PAPER:

Cytotoxic phenolic compounds in leaf-buds of *Populus tremuloides.*

Accepted in *Canadian Journal of Chemistry* (2009-10-05)

Graphical abstract

Bioassay-guided fractionation and separation of the EtOH extract of *Populus tremuloides* leaf-buds resulted in the isolation of a new compound, (1*S*,2*S*)-1-[4-*O*-(*E*)-coumaroyl-*β*-D-glucopyranosyloxy]cyclohexane-diol **1**, and 18 known products. *In vitro* cytotoxic and antibiotic activities were evaluated for 18 of them.



1

Cytotoxic phenolic compounds in leaf-buds of Populus tremuloides.

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Résumé

Les enquêtes phytochimique menés sur l'extrait éthanolique des feuilles-bourgeons du *P. tremuloides* ont conduit à l'isolement de 19 composés phénolique. Parmi eux, on y trouve est une nouvelle molécule, (1S, 2S) -1 - [4-O-E-coumaroyl β-D-glucopyranosyloxy] cyclohexanediol. La structure de cette dernière a été déterminée par spectroscopie (RMN et MS) et par des méthodes chimiques. Les autres Dix-huit composés isolés ont été testés pour l'activité cytotoxique en comparaison avec celle des lignées cancéreuses des poumons (A549) et celle du côlon (DLD-1) présent dans les cellules humaines affectés. L'activité antibactérienne a également été évaluée contre l'*Escherichia coli* et le *Staphylococcus aureus*.

Abstract

Phytochemical investigations of the EtOH extract of *Populus tremuloides* leaf-buds led to the isolation of 19 phenolic compounds. Among them, (1*S*, 2*S*)-1-[4-O-*E*-coumaroyl-*β*-D-glucopyranosyloxy]cyclohexanediol was reported for the first time and its structure was determined by spectroscopic (NMR and MS) and chemical methods. Seventeen of isolated compounds were tested for their cytotoxicity against lung carcinoma (A549) and colorectal adenocarcinoma (DLD-1) human cell lines. Antibacterial activity was also evaluated against *Escherichia coli* and *Staphylococcus aureus*.

Keywords

Quaking aspen; coumarate; flavonoids; NMR.

Introduction

The buds exudates of many plant species of *Populus* genus are known as raw material processed by bees into propolis (1). The latter product has been widely used in popular medicine as antibacterial (2-4), anti-inflammatory (5), antioxidant (6-7) and cytostatic treatments (8). The biological activity of propolis samples is mainly due to phenolic compounds like flavonoids, aromatic acids and diterpenic acids (9-10) which are the principal constituents of the buds of *Populus* species (11-13). Recently, studies in the northern-type propolis showed a potential source of biologically-active substances in *P. tremuloides* (14) which are widely spread across North America (15). In spite of its use as ointment by Amerindian traditional medicine to treat numerous diseases such as coughs, colds and irritated nostrils (16), few studies were carried out on the medicinal applications. In the present study, the isolation and structure elucidation of a new phenolic compound from *Populus tremuloides* Michaux, along with 18 known products are described.

Experimental

General

Optical rotations were measured with an automatic polarimeter Rudolph Research Analytical Autopol IV. FTIR spectra were recorded with a Perkin–Elmer SpectrumOne. High resolution electrospray ionization mass spectrum was conducted in positive mode with an Applied Biosystems/MDS Sciex QSTARXL QqTOF MS system. The 1D and 2D NMR spectra (^{1}H–^{1}H COSY, HSQC and HMBC) were performed using an Avance 400

Bruker spectrometer equipped with a 5 mm QNP-probe. Chemical shifts were expressed in δ (ppm) units relative to TMS as an internal standard and coupling constants were given in Hertz. Preparative HPLC was performed on an Agilent 1100 liquid chromatography system, equipped with a solvent delivery system, an autosampler and a UV-MWD detector. Samples were eluted in an Intertsil prep-ODS column C18 (20 × 250 mm; 10 μm) at room temperature with a flow rate of 10 mL min^{-1}. The GC-MS analysis were performed with an instrument (Agilent Technologies 6890N) fitted with a mass selective detector (Agilent Technologies 5973), a split-splitless injection port and an apolar capillary column DB-5MS (30 m × 0.25 mm × 0.25 μm).

Analytical thin-layer chromatography was performed with silica gel 60 F_{254}, 0.25 mm pre-coated TLC plates (Silicycle, Québec, Canada). Flash column chromatographies were performed on silica gel (40–63 μm with indicator F_{254}, Silicycle, Québec, Canada) and on C_{18} reversed phase silica gel (carbon 11 %, 40-69 μm, Silicycle, Québec, Canada). Polyamide CC-6 was purchased from Macherey-Nagel (Germany) and Diaion HP-20 from Supelco. Detection of the phenolic compounds was carried out by spraying TLC plates with polyethylene glycol (NP/PEG) reagent followed by heating at 110 °C and detected by UV absorption at 254 and 365 nm. TLC identification of monosaccharides were performed with CH_2Cl_2–MeOH–H_2O (50:25:5) solvent system. The compounds were visualized by spraying an orthophosphoric acid solution of naphtoresorcinol 5 % in EtOH, followed by heating at 110 °C.

The commercial samples used for biological tests, namely prunin (**3**), kaempferide (**13**) and *trans*-ferulic acid (**19**), were purchased from Indofine Chemical Company (USA). Rhamnocitrin (**10**) was purchased from Apin Chemicals Ltd (UK).

Plant material

Leaf-buds of *Populus tremuloides* Michaux were collected in the boreal forest to the south of Chicoutimi, Québec, Canada, in April 2006. Samples were identified by Patrick Nadeau (Département des sciences fondamentales, Université du Québec à Chicoutimi). A voucher specimen (QFA-A540466) was deposited at the Herbarium Louis-Marie of Université Laval, Québec, Canada.

Extraction and Isolation

The buds of *Populus tremuloides* (1 kg) were exhaustively extracted with EtOH (3 L, 60 °C, 3 times, 2 h each time) followed by EtOH-H_2O (7:2). The extracts were filtered and pooled. After evaporation of EtOH *in vacuo*, the aqueous phase was extracted successively with hexane (500 mL × 5) and saturated *n*-BuOH with H_2O (500 mL × 5). The *n*-BuOH phase was decanted and evaporated *in vacuo*. The residue (80 g) was fractionated using an open Diaion® column eluted with H_2O–MeOH with 30 %, 50 % and 80 % of MeOH. Three fractions were obtained: A (6.46 g), B (7.24 g) and C (58.96 g).

Fraction C was purified on silica gel CC, eluted with $CHCl_3$-MeOH gradient (60:1→5:1, v/v) and three fractions were obtained. Fr. C1 (16.73 g) was subjected to silica gel using a gradient of $CHCl_3$-MeOH (90:1→60:1, v/v) as eluent. Subfr. C1.1 (384 mg), obtained from $CHCl_3$-MeOH (90:1), was separated on silica gel CC with $CHCl_3$-MeOH (80:1) as eluent,

to give three fractions. Subfr. C1.1A was purified by preparative HPLC with a gradient elution of MeOH-H_2O (50:50→85:15, v/v) yielding compounds **15** (249 mg), **16** (5 mg) and **17** (41 mg). Subfr. C1.1B (2.36 g) was applied successively on a silica gel CC and a reversed-phase CC using gradients MeOH-H_2O (50:50→70:30, v/v) as eluent to give **11** (160 mg). Compound **9** (3 mg), **12** (18 mg) and a mixture of **10** and **13** (27 mg) were isolated after a silica gel CC eluted with $CHCl_3$-MeOH (90:1) and a preparative HPLC (isocratic CH_3CN-H_2O 40:60) of Fr. C1.2. Compound **18** (50 mg) was obtained from fraction C1.3 (294 mg) after repeated silica gel CC ($CHCl_3$-MeOH 75:1) and Polyamid flash column (MeOH–H_2O, 50:50→75:25). Fr. C2 was chromatographed on silica gel CC with a gradient elution of $CHCl_3$-MeOH (75:1 →15:1, v/v) to give eight fractions. Subfr. C2.7 (558 mg) was separated by preparative HPLC using an isocratic mobile phase of CH_3CN-H_2O-HCOOH (40:60:1) to afford **2** (19 mg) and **14** (3 mg).

Fr. C3 (5.4 g) was purified on silica gel using a gradient of $CHCl_3$-MeOH (25:1→7:1) for elution to give five subfractions. C3.2 was separated on silica gel CC with $CHCl_3$-MeOH (20:1) giving **6** (289 mg). Some purifications on different silica gel CC of subfr. C3.2.1 permitted to obtain **19** (2 mg). Subfr. C3.4 (418 mg) was separated by preparative HPLC using an isocratic mobile phase of CH_3CN-H_2O (30:70) to afford compounds **1** (20 mg), **3** (8 mg), **4** (14 mg) and **8** (37 mg). Subfr. C3.5 (482 mg) was separated by HPLC using a gradient of MeOH-H_2O (10:90 → 100:0) to give **5** (21 mg) and **7** (35 mg).

(1*S*, 2*S*)-1-[4-O-*E*-coumaroyl-β-D-glucopyranosyloxy]cyclohexanediol (1)

White amorphous powder; $[\alpha]_D^{25}$ -35.3° (c 1.0, MeOH); IR (neat) ν_{max} 3328, 2935, 1696, 1602, 1160, 1080, 1024, 982 and 833 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 2; HR-ESI-MS m/z 447.16225 $[M+Na]^+$ (calcd for $C_{21}H_{28}O_9Na$, 447.16310).

Acid hydrolysis of (1)

Compound **1** was dissolved in HCl 10 % and heated at 110 °C for 4 h. The resulting hydrolysate was extracted with $CHCl_3$. The organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. The presence of *p*-coumaric acid was confirmed with standard sample on TLC ($CHCl_3$-MeOH 10:1 as eluent and developing with NP/PEG reagent). The presence of cyclohexane-1,2-diol in the organic phase (α_D = + 3.4) was confirmed with a GC-MS analysis: Injector temperature 250 °C; ionization voltage, 70 eV (EI–MS); column temperature, 40°C for the initial 2 min followed by an increase of 15 °C min^{-1} up to 350 °C; carrier gas, He; column flow rate, 1 $ml.min^{-1}$. Cyclohexane-1,2-diol was detected at R_t 7.55 min. The aqueous phase was neutralized with N,N-dioctylmethylamine (10 % in $CHCl_3$) and the solvents were evaporated under reduced pressure. The residue contained the monosaccharide D-glucose (α_D = + 24.8).

Cell lines and culture conditions

Lung carcinoma (A549), colorectal adenocarcinoma (DLD-1) and normal skin fibroblast (WS1) human cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in minimum essential medium containing Earle's salts and L-glutamine (Mediatech Cellgro, VA), to which were added 10 % fetal bovine serum

(Hyclone), vitamins (1X), penicillin (100 I.U. mL^{-1}) and streptomycin (100 µg mL^{-1}), essential amino acids (1X) and sodium pyruvate (1X) (Mediatech Cellgro, VA). Cells were kept at 37 °C in a humidified environment containing 5 % CO_2. Antibacterial activity was tested on *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923E.

Cytotoxicity assay

Exponentially growing cells were plated in 96-well microplates (BD Falcon) at a density of 5×10^3 cells per well in 100 µL of culture medium (DMEM with 10 % SVF) and were allowed to adhere for 24 h before treatment. Increasing concentrations of each compound in MeOH or DMSO were then added (100 µL per well) and the cells were incubated for 48 h. The final concentration of MeOH or DMSO in the culture medium was maintained at 0.25 % (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using resazurin (17) on an automated 96-well Fluoroskan Ascent F1™ plate reader (Labsystems) using excitation and emission wavelengths of 530 and 590 nm, respectively. Fluorescence was proportional to the cellular metabolic activity in each well. Survival percentage was defined as the fluorescence in experimental wells compared to that in control wells after subtraction of blank values. Each experiment was carried out thrice in triplicate. IC_{50} results were expressed as means ± standard deviation.

Antibacterial assays

Antibacterial activity was evaluated using the microdilution method (42) but with some modifications: exponentially growing bacteria were plated in 96-well flat bottom microplates (BD Flacon) at a density of 5×10^3 gram-negative *E. coli* (ATCC 25922) or

40×10^3 gram-positive *S. aureus* (ATCC 25923) per well in 100 µL nutrient broth (Difco). The concentration of ethanol in the culture medium was maintained at 0.25 % (v/v) to avoid solvent toxicity. Fifty microliters of 4 % resazurin was added to each well and the microplates were incubated for 6 h at 37 °C. Fluorescence was measured after 6 h on an automated 96-well Fluoroskan Ascent FI™ plate reader (Labsystems) using excitation and emission wavelengths of 530 nm and 590 nm respectively.

Results and discussion

The leaf buds of *Populus tremuloides* were extracted with EtOH and EtOH-H_2O under reflux. After evaporation of EtOH *in vacuo*, the aqueous phase was successively partitioned with hexane and *n*-BuOH. The *n*-BuOH soluble extract was purified on an open Diaion® column with a gradient of decreasing polarity and three fractions were obtained. Each fraction was investigated for *in vitro* cytotoxic and antibacterial biological activities. Cytotoxic activity evaluations were carried out on human lung cancer (A549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) using the resazurin reduction test as previously described in the literature (17). Antibacterial activity was evaluated against *Escherichia coli* and *Staphylococcus aureus*. The results (Table 1), show that the last fraction C was found to exert a weak cytotoxic activity against A549 (IC_{50}, 96 ± 7 µg mL^{-1}) and DLD-1 (IC_{50}, 89 ± 6 µg mL^{-1}), but was inactive toward bacterial cell lines. Thus, bioassay-guided fractionation of fraction C was undertaken with a combination of different chromatographic techniques leading to the isolation of a new compound **1** together with 18 known compounds (figure 1): chaenomeloidin (**2**) (18), prunin (**3**) (19), echinaticin (**4**) (20),

echinacin (**5**) (21), tremulacin (**6**) (22), salicine (**7**) (23), tremuloidin (**8**) (24), genkwanin (**9**) (25), rhamnocitrin (**10**) (26), sakuranetin (**11**) (27), acacetin (**12**) (28), kaempferide (**13**) (29), aromadendrin (**14**) (30), phenylmethyl coumarate (**15**) (31), phenethyl *p*-coumarate (**16**) (32), cinnamyl coumarate (**17**) (33), phenylmethyl caffeate (**18**) (34) and *trans*-ferulic acid (**19**) (35). Known compounds were identified by comparison of their spectroscopic data with the values found in the literature. NMR spectroscopic data for phenethyl *p*-coumarate (**16**), which was also isolated from buds of *P. tremuloides*, were not available. Therefore, complete ^{1}H and ^{13}C NMR spectroscopic data for **16** are also displayed.

Table 1: *In vitro* cytotoxicity and antibiotic results of the Diaion® column's fractions[a]

Samples	IC_{50} ($\mu g\ mL^{-1}$)[b]			MIC[c]	
	A549	DLD-1	WS1	*S. aureus*	*E. coli*
Fraction A	> 100	> 100	> 100	> 100	> 100
Fraction B	> 100	> 100	> 100	> 100	> 100
Fraction C	96 ± 7	89 ± 6	80 ± 10	> 100	> 100
Etoposide[d]	2.8 ± 0.5	2 ± 1	> 50	NT[e]	NT[e]
Chloramphenicol[d]	NT[e]	NT[e]	NT[e]	> 5	0.37 ± 0.06

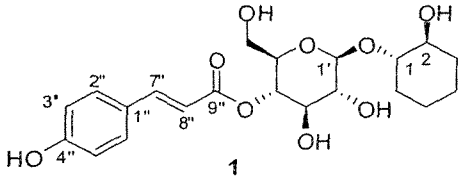
[a] Mean values (± standard deviation) for triplicate assays.

[b] Concentration of extract that caused 50 % inhibition of cell proliferation.

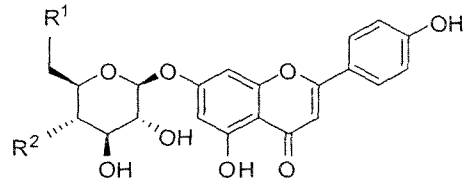
[c] Minimum concentration of extract that resulted in inhibition of visible growth

[d] Positive control.

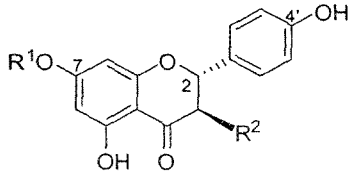
[e] Not tested.



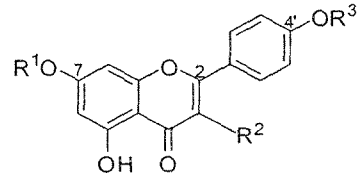
1



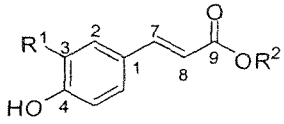
4 R^1 = OH; R^2 = Coumaroyl
5 R^1 = Coumaroyl; R^2 = OH



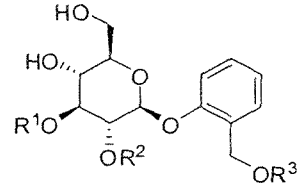
3 R^1 = Glc; R^2 = H
11 R^1 = CH_3; R^2 = H
14 R^1 = H; R^2 = OH



9 R^1 = CH_3; R^2 = H; R^3 = H
10 R^1 = CH_3; R^2 = OH; R^3 = H
12 R^1 = H; R^2 = H; R^3 = CH_3
13 R^1 = H; R^2 = OH; R^3 = CH_3



15 R^1 = H; R^2 = CH_2-Ph
16 R^1 = H; R^2 = CH_2-CH_2-Ph
17 R^1 = H; R^2 = CH_2-CH=CH-Ph
18 R^1 = OH; R^2 = CH_2-Ph
19 R^1 = OCH_3; R^2 = H



2 R^1 = CO-Ph; R^2 = H; R^3 = H
6 R^1 = H; R^2 = CO-Ph; R^3 =
7 R^1 = H; R^2 = H; R^3 = H
8 R^1 = H; R^2 = CO-Ph; R^3 = H

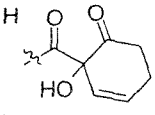


Figure 1: Polyphenols from *Populus tremuloides*

The molecular formula ($C_{21}H_{28}O_9$) of **1**, a white amorphous powder, was determined from its HR-ESI-MS spectrum (positive ion mode) on the basis of a quasimolecular ion peak at *m/z* 447.1623 $[M+Na]^+$ (calcd 447.1631). Infrared absorption bands at 3328, 1602, 1160, 982 and 833 cm^{-1} suggested the presence of hydroxyl groups, aromatic system and an

ester carbonyl groups. The ^{13}C NMR spectrum (Table 2) displayed 19 carbon signals separated by DEPT spectrum into five methylenes, seven aliphatic oxymethines, four unsaturated methines and three quaternary carbons (one for an ester carbonyl). Among them, six resonances could be assigned to a sugar moiety. The ^{1}H NMR spectrum confirmed the presence of a hexose moiety with anomeric proton at δ_H 4.45 (1H, d, J = 7.8 Hz). The ^{1}H NMR spectrum also shows the presence of two *trans*-olefinic protons at δ_H 7.66 (1H, d, J = 15.9 Hz) and δ_H 6.37 (1H, d, J = 15.9 Hz) and a 1,4-disubstituted aromatic rings with two protons at δ_H 7.47 (2H, d, J = 8.6 Hz) and 6.81 (2H, d, J = 8.6 Hz). HMBC correlations at δ_H 7.47 (H-2", H-6") and δ_C 147.3 (H-7"), δ_H 6.81 (H-3", H-5") and δ_C 161.5 (C-4") and δ_H 7.66 (H-7") and δ_C 168.6 (C-9") suggested the presence of a coumaroyl moiety. Analysis of the COSY, HSQC and HMBC spectra led to the identification of a third aglycone sytem: the cyclohexane-1,2-diol. HMBC correlation between the methine proton at δ_H 4.87 (H-4') and the carbonyl group at δ_C 168.6 (C-9") suggested the linkage between the glucose and the coumaroyl moiety. Finally, the correlation between δ_H 4.45 (H-1') and δ_C 79.4 (C-1), indicated the linkage site of the glucose moiety to the aglycone.

Acidic hydrolysis of **1** and TLC analysis of the aqueous phase afforded identification of glucose as the sugar component. Absolute configuration of the glucose as D was determined by optical rotations in comparison with authentic standard. The presence of *p*-coumaric acid in the organic phase was confirmed by TLC in comparison with authentic standard. Cyclohexane-1,2-diol was also detected in the organic phase using GC-MS and NMR analysis (36). The absolute configuration of cyclohexane-1,2-diol could be determined directly from the organic phase since the other aglycon part, namely *p*-coumaric acid, is

optically inactive. The organic phase showed positive value in optical activity measurement meaning that (1*S*,2*S*)-cyclohexane-1,2-diol has been isolated (37). The structure of **1** was thus confirmed as (1*S*, 2*S*)-1-[4-O-*E*-coumaroyl-*β*-D-glucopyranosyloxy]cyclohexanediol.

Table 2: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for compound **1** in methanol-d_4.

Position	δ_C (mult.)[a]	δ_H (mult., *J* in Hz)
1	79.4 (*d*)	3.87 (*m*)
2	71.0 (*d*)	3.85 (*m*)
3	31.5 (*t*)	1.79 (*m*)
		1.55 (*m*)
4	22.4 (*t*)	1.65 (*m*)
		1.34 (*m*)
5	23.1 (*t*)	1.71 (*m*)
		1.31 (*m*)
6	27.6 (*t*)	1.82 (*m*)
		1.63 (*m*)
1'	102.2 (*d*)	4.45, (*d*, 7.8)
2'	75.1 (*d*)	3.35 (*dd, 9.3, 7.8*)
3'	75.6 (*d*)	3.65 (*t, 9.3*)
4'	72.5 (*d*)	4.87 (*m*)
5'	76.1 (*d*)	3.53 (*m*)
6'	62.4 (*t*)	3.62 (*dd, 15.0, 5.4*)
		3.54 (*m*)
1"	127.2 (*s*)	
2", 6"	131.3 (*d*)	7.47 (*d*, 8.6)
3", 5"	116.9 (*d*)	6.81 (*d*, 8.6)
4"	161.5 (*s*)	
7"	147.3 (*d*)	7.66 (*d*, 15.9)
8"	114.8 (*d*)	6.37 (*d*, 15.9)
9"	168.6 (*s*)	

[a] Multiplicities were deduced from DEPT experiments

Compound **16** has been identified by many authors but surprisingly, no complete NMR assignation was given (38). Therefore, complete ^{1}H and ^{13}C characterisation was accomplished using ^{1}H, ^{13}C and 2D spectra (Table 3). First, the same *p*-coumaroyl moieties as in **1** was identified with δ_H at 6.29 (1H, d, J = 15.9 Hz, H-8), 6.85 (2H, d, J = 8.1 Hz, H-3 and H-5), 7.42 (2H, d, J = 8.1 Hz, H-2 and H-6) and 7.62 (1H, d, J = 15.9 Hz, H-7) and δ_C at 115.5 (C-8), 115.9 (C-3 and C-5), 127.3 (C-1), 130.0 (C-2 and C-6), 144.6 (C-7), 157.7 (C-4) and 167.4 (C-9).

Table 3: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for compound **16** in methanol-d_4.

Position	δ_C (mult.)[a]	δ_H (mult., J in Hz)
1	127.3 (*s*)	
2, 6	130.0 (*d*)	7.42 (*d*, 8.1)
3, 5	115.9 (*d*)	6.85 (*d*, 8.1)
4	157.7 (*s*)	
7	144.6 (*d*)	7.62 (*d*, 15.9)
8	115.5 (*d*)	6.29 (*d*, 15.9)
9	167.4 (*s*)	
1'	137.9 (*s*)	
2', 6'	128.9 (*d*)	7.26 (*m*)
3', 5'	128.5 (*d*)	7.32 (*m*)
4'	126.6 (*d*)	7.25 (*m*)
7'	35.2 (*t*)	3.02 (*t*, 6.9)
8'	65.0 (*t*)	4.42 (*t*, 6.9)

[a] Multiplicities were deduced from DEPT experiments

Table 4: *In vitro* cytotoxicity results of isolated compounds (**1-19**)[a]

Compounds	IC_{50} (μM ± SD)[b]		
	A549	DLD-1	WS1
1	> 100	> 100	> 100
2	NT[c]	NT[c]	NT[c]
3	> 100	> 100	> 100
4	NT[c]	NT[c]	NT[c]
5	> 100	> 100	42 ± 4
6	> 100	> 100	> 100
7	> 100	> 100	> 100
8	81 ± 3	> 100	> 100
9	9 ± 3	9.2 ± 0.9	5.8 ± 0.3
10	31 ± 2	37 ± 3	87 ± 3
11	> 100	> 100	> 100
12	27 ± 3	23 ± 6	20 ± 2
13	60 ± 10	> 100	42 ± 5
14	> 100	> 100	> 100
15	19 ± 2	19.2 ± 0.9	26 ± 3
16	> 100	> 100	> 100
17	> 100	> 100	> 100
18	45.8 ± 0.9	39 ± 3	51 ± 7
19	> 100	> 100	> 100
Etoposide[d]	2.8 ± 0.5	2 ± 1	> 50

[a] Mean values (± standard deviation) for triplicate assays.

[b] Concentration that caused 50% inhibition of cell proliferation.

[c] Not tested.

[d] Positive control.

Additionally, five overlapped ^{1}H NMR signals between δ_H 7.20-7.30 along with two methylene triplets at 3.02 (2H, t, J = 6.9 Hz, H-7') and 4.42 (2H, t, J = 6.9 Hz, H-8') were attributed to a phenethyl moiety. The HMBC correlation between H-8' and C-9 confirmed the link between the phenethyl and the *p*-coumaroyl groups.

Compounds **10** and **13** were isolated as a mixture. Separation of each constituent was not performed due to their low amounts. Therefore, careful examination of NMR spectra (^{1}H, ^{13}C, DEPT and HSQC) and comparison with literature allowed the identification of these components as rhamnocitrin (**10**) (29) and kaempferide (**13**) (26). Moreover, the biological results were obtained using commercial pure products. Because of low isolated yields of compounds **3** and **19**, those compounds were also tested from commercial materials.

All isolated compounds, except compounds **2** and **4**, were evaluated using resazurin reduction test for their cytotoxicity against human lung cancer (A549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) (17). Results presented in Table 4 are expressed as the concentration of product inhibiting cell growth by 50 % (IC_{50}). Etoposide was used as positive control with IC_{50} of 2.8 and 2.0 µM against A549 and DLD-1 cell lines, respectively. The phenolic compounds were regarded as active when the IC_{50} was smaller than 100 µM (39). The compound **9** was found the most active with IC_{50} ranging from 5.8 to 9.2 µM. Moreover, compounds **10**, **12** and **15** were moderately active against cancer cells with IC_{50} ranging from 19 to 37 µM. In contrast to compounds **9**, **12** and **15**, the compound **10** was significantly selective toward cancer cells with IC_{50} of 31 µM for A549 and 37 µM for DLD-1 in comparison to 87 µM for normal cells, WS1. Although the

cytotoxicity of compounds **9** and **12** were known on A549 cells (40-41), the activity on human colorectal adenocarcinoma DLD-1 was never reported. Finally, compounds **13** and **18** were found weakly cytotoxic and all the other compounds tested were inactive. As far as structure-activity relationships are concerned, these *in vitro* results suggest that the addition of a double bond in C-2 position in molecule **9**, with regard to the compound **11**, increases the cytotoxic activity. Similarly, the presence of a methoxy group in C-7 position and a hydroxyl in C-4' position in the flavone **9**, seem to have a beneficial effect on the cytotoxic activity in comparison with the acacetin (**12**), where the inversion of these groups reduces the activity. On the other hand, the presence of hydroxyl group in R^2 of compounds **10** and **13** is detrimental for the activity in comparison with compounds **9** and **12**, respectively. In the case of compounds **15-19**, only molecules bearing a benzyl groups exhibited cytotoxicities (**15** and **18**). Moreover, hydroxyl group in R^1 of compound **18** reduces significantly the cytotoxicity in comparison to **15**. All compounds were also evaluated for their antibacterial activities against *S. aureus* and *E. coli* but no significant activity was observed.

Concluding remarks

In conclusion, the structure of a new compound **1** was described and 19 compounds were identified from *P. tremuloides*. Among them, compounds **2** and **5** were reported for the first time in *Populus* genus and compounds **3**, **4**, **9**, **10**, **12**, **16** and **17** for the first time in *Populus tremuloides*. Compound **9** was found the most cytotoxic against lung carcinoma cell (A549) and colorectal adenocarcinoma (DLD-1) human cell lines. Interestingly, the

compound **10** was selective toward both cancer cell lines in comparison to normal cells. Finally, all compounds tested do not possess antibacterial activity.

Acknowledgement

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Chapter IV

RESULTS AND DISCUSSION:

Other results related to phytochemical study of

Populus tremuloides

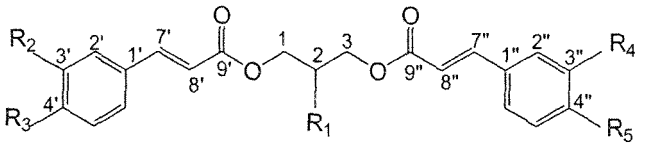
This chapter is devoted to the detailing of another five purified molecules found in this species which are not mentioned in chapter III. Thus, other processes of isolation and characterisation of interesting fractions will be discussed here. In addition, the performed biological assays which were not presented so far will be explained in the next sections.

4.1 Isolation and purification of phenolic compounds

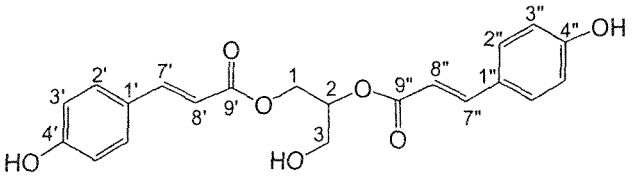
Fraction C2 (12.5 g) was chromatographed on silica gel column chromatography (CC) using gradient $CHCl_3$-MeOH (75:1 → 15:1, v/v) as eluent to give pure **22** (630 mg). Subfraction C2.3 was further separated on silica gel CC using $CHCl_3$-MeOH gradient (40:1 → 25:1) as eluent to yield compound **21** (16 mg). Subfraction C2.7 was purified by preparative HPLC using an isocratic mobile phase of CH_3CN-H_2O (40:60) to afford pures **20** (11 mg) and **24** (11 mg). Fraction C3 (10.37 g) was separated on silica gel CC using $CHCl_3$-MeOH gradient (15:1 → 7:1, v/v) as eluent to give 5 fractions. Then, subfraction C3.2 (1.19 g) was separated into four fractions by passage over silica gel, eluted with $CHCl_3$-MeOH (20:1) and pure **23** (11 mg) was obtained by preparative HPLC, gradient MeOH-H2O (50:50→70:30, v/v).

4.2 Identifiation of purified phenolic compounds

The phenolic compounds presented in figure 5 (**20-24**) have been identified mainly by their ^{1}H and ^{13}C NMR spectral data and their molecular formula. These compounds are newly identified products in the *P.tremuloides* and compounds **20** and **24** were never reported for family of Salicaceae.



20 R_1, R_3, R_5 = OH; R_2 , R_4 = H
21 R_1 = OAC; R_2 ,R_4 =H; R_3, R_5 = OH
22 R_1 = OAC; R_2 = H; R_3,R_4, R_5 = OH
23 R_1 = OAC; R_2 , R_3, R_4,R_5 = OH



24

Figure 5: Five phenolic compounds from buds of *P. tremuloides*.

4.2.1 Identification of compound 20

The ^{1}H and ^{13}C NMR spectra of compound **20** showed the presence of two equivalent *p*-coumarate moieties as one doublet at δ_H 7.44 (2H, d, *J*=8.6 Hz) and 6.80 (2H, d, *J*=8.6 Hz) for aromatic rings and δ_H 7.66 (1H, d, *J*= 15.9 Hz) and 6.35 (1H, d, J= 15.9 Hz) for alkene function (confirmed by ^{13}C NMR spectrum) of trans geometry. Remaining signals δ_H 4.28--4.16 (5H, m) in the ^{1}H NMR and δ_C 66.2 (t) and 68.4 (d) in the ^{13}C NMR spectra suggested the presence of a 1,3-0-di-trans-*p*-coumaroylglycerol. Spectroscopic data of compounds **20** were in agreement with the values reported in the literature [55].

Table 3: ^{1}H and ^{13}C NMR spectroscopic data for compound **20** in methanol-d4.

Position	δ_C (mult.)	δ_H (mult., *J* in Hz)
1	66.2 (t)	4.28 (br d, 5.2)
2	68.4 (d)	4.16 (quint, 5.2)
1'	126.9 (s)	
2'	131.1 (d)	7.44 (d, 8.6)
3'	116.7 (d)	6.80 (d, 8.6)
4'	161.1 (s)	
7'	146.9 (d)	7.66 (d, 15.9)
8'	114.7 (d)	6.35 (d, 15.9)
9'	168.9 (s)	

4.2.2 Identification of compound 21

The ^{1}H and ^{13}C NMR spectra of compound **21** indicated the same structure as pure 20 (1,3-O-Di-trans-*p*-coumaroylglycerol) excepted for a difference in the triglycerol moiety. These spectra data (see table 4) confirmed the presence of an acetyl group; δ_H 2.08 (3H, s), δ_C 20.9 (COCH3), 172 (COCH3), at C-2 of 21 instead of hydroxyl group as in 20. This was ascertained by ^{1}H ^{1}H COSY, HMQC and HMBC correlations designed by arrows in figure 6. This molecule is named as 2-acetyl-1,3-dicoumaroylglycerol (Lasiocarpin A) and the spectroscopic data of compound **21** were in agreement with the values reported in the literature [56].

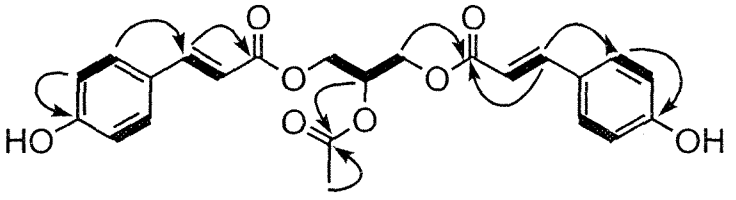


Figure 6: ^{1}H ^{1}H COSY, HMQC and HMBC correlations (**21**).

Table 4: ^{1}H and ^{13}C NMR spectroscopic data for compound **21** in methanol-d4.

Position	δ_C (mult.)[a]	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)
1	63.5 (t)	4.47 (dd, 12.1, 4.1)	4.35 (dd, 12.1, 5.9)
2	71.0 (d)	5.37 (m)	-
1'	127.1 (s)	-	-
2'	131.4 (d)	7.45 (d, 8.7)	-
3'	116.9 (d)	6.79 (d, 8.7)	-
4'	161.5 (s)	-	-
7'	147.3 (d)	7.63 (d, 15.9)	-
8'	114.5 (d)	6.34 (d, 15.9)	-
9'	168.7 (s)	-	-
Ac	-	-	-
CO	172.0 (s)	-	-
Me	20.9 (q)	2.08 (s)	-

4.2.3 Identification of compound 22

The ^{1}H and ^{13}C NMR spectra of compound **22,** with [α] D ^{25} 3.16° (*c* = 0.5, MeOH), were found to be similar to those of 21, except for a difference in one of the benzene rings with three protons as one singulet at C-2" δ_H 7.04 (1H, s), one doublet at C-5" δ_H 6.77 (2H, d, J= 8.4 Hz), and two other as one doublet at C-6" δ_H 6.95 (2H, d, J= 8.3 Hz). These spectral data (see table 5) indicated the presence of a hydroxyl group at C-3" of 22 instead of two *p*-coumaroyl groups in 21. Additionally, the presence of two double bound protons signals at δ_H 7.63 (1H, d, *J*= 15.9 Hz), δ_H 6.34 (1H, d, *J*= 15.9 Hz) and δ_H 7.57 (1H, d, *J*= 15.9 Hz), δ_H 6.27 (1H, d, *J*= 15.9 Hz) demonstrated that 2-acetyl-1-caffoyl-3-coumaroylglycerol (lasiocarpin B) was an asymmetrical triglyceride with acetic, *p*-hydroxycinnamic and 3,4-dihydroxy cinnamic acids (figure 6). The spectroscopic data of compound **22** were in agreement with the values reported in the literature [56].

Table 5: ^{1}H and ^{13}C NMR spectroscopic data for compound **22** in methanol-d4.

Position	δ_C (mult.)[a]	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)
1	63.5 CH_2	4.47 (ddd, 11.9, 4.0, 3.0	4.35 (ddd, 11.9, 5.7, 3.
2	71.1 CH	5.37 (m)	-
1'	127.1 (s)	-	-
2'	131.4 (d)	7.45 (d, 8.7)	-
3'	116.9 (d)	6.79 (d, 8.7)	-
4'	161.5 (s)	-	-
7'	147.3 (d)	7.63 (d, 15.9)	-
8'	114.5 (d)	6.34 (d, 15.9)	-
9'	168.7 (s)	-	-
1"	127.6 (s)	-	-
2"	115.2 (d)	7.04 (d, 2.1)	-
3"	146.9 (s)	-	-
4"	149.8 (s)	-	-
5"	116.5 (d)	6.77 (d, 8.4)	-
6"	123.2 (d)	6.95 (dd, 8.3, 2.1)	-
7"	147.7 (d)	7.57 (d, 15.9)	-
8"	114.4 (d)	6.27 (d, 15.9)	-
9"	168.7 (s)	-	-
Ac	-	-	-
CO	172.0 (s)	-	-
Me	20.9 (q)	2.08 (s)	-

4.2.4 Identification of compound 23

The ^{1}H and ^{13}C NMR spectra of compound **23** (see table 6) were similar to those obtained for 22. However, two olefinic protons at δ_H 7.57 (1H, d, J= 15.9 Hz) and at δ_H 6.27 (1H, d, J= 15.9 Hz) as well as the presence of aromatic rings with three protons as mentioned above for 22 confirmed a symmetric glycerol ester having an acetyl group at C-2 and two caffeoyl groups at C-1 and C-3 as 2-acetyl-1,3-dicaffoylglycerol (Lasiocarpin C) (see figure 5). The spectroscopic data of compound **23** were in agreement with the values reported in the literature [56].

Table 6: ^{1}H and ^{13}C NMR spectroscopic data for compound **23** in methanol-d4.

Position	δ_C (mult.)[a]	δ_H (mult., J in Hz)	δ_H (mult., J in Hz)
1	63.5 (t)	4.47 (dd, 12.0, 4.0)	4.34 (dd, 12.0, 5.8)
2	71.1 (d)	5.37 (m)	-
1'	127.6 (s)	-	-
2'	115.3 (d)	7.04 (d, 1.8)	-
3'	146.9 (s)	-	-
4'	149.8 (s)	-	-
5'	116.5 (d)	6.77 (d, 8.3)	-
6'	123.2 (d)	6.95 (dd, 8.3, 1.8)	-
7'	147.7 (d)	7.57 (d, 15.9)	-
8'	114.4 (d)	6.27 (d, 15.9)	-
9'	168.7 (s)	-	-
Ac	-	-	-
CO	172.0 (s)	-	-
Me	20.9 (d)	2.08 (s)	-

4.2.5 Identification of compound 24

The presence of two non-equivalent *p*-coumarate moieties was detected from the ^{1}H NMR spectrum of compound **24**. Additionally, this spectrum showed the presence of a 1,2-diacylglycerol moiety δ_H 5.24 (IH, m), δ_H 4.49 (1H, dd, J = 11.8 and 3.8 Hz) and 4.35 (lH, dd, J = 11.8 and 6.5 Hz) for acylated methylene protons and δ_H 3.79 (2H, m) for hydroxymethyl protons (see table 7). Based on the above spectral data, the structure of 24 was determined as 1,2-dicoumaroylglycerol. This was confirmed by ^{13}C NMR, ^{1}H ^{1}H COSY, HMQC and HMBC correlations (see figure 7) and the spectroscopic data of compound **24** were in agreement with the values reported in the literature [56].

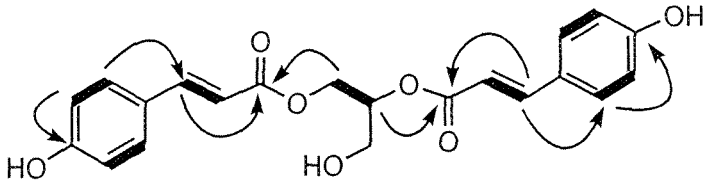


Figure 7: ^{1}H ^{1}H COSY, HMQC and HMBC correlations (**24**).

Table 7: ^{1}H and ^{13}C NMR spectroscopic data for compound **24** in methanol-d4.

Position	δ_C (mult.)[a]	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)
1	63.8 (t)	4.49 (dd, 11.8, 3.8)	4.35 (dd, 11.8, 6.5)
2	73.7 (d)	5.24 (m)	-
3	61.7 (t)	3.79 (br d, 5.1)	-
1'	127.2 (s)	-	-
2'	131.3 (d)	7.44 (d, 8.4)	7.04 (d, 1.8)
3'	116.9 (d)	6.78 (d, 8.4)	-
4'	161.4 (s)	-	-
7'	147.1 (d)	7.62 (d, 15.9)	-
8'	114.7 (d)	6.33 (d, 15.9)	-
9'	168.9 (s)	-	-
1"	127.1 (s)	-	-
2"	131.3 (d)	7.46 (d, 8.4)	-
3"	116.9 (d)	6.79 (d, 8.4)	-
4"	161.4 (s)	-	-
7"	147.1 (d)	7.66 (d, 15.9)	-
8"	115.0 (d)	6.37 (d, 15.9)	-
9"	168.7 (s)	-	-

The next section presents the evaluation of anticancer, anti-inflammatory and anti-bacterial activities of isolated compounds from *Populus tremuloides.*

4.3 Evaluation of cytotoxicity against human cell lines

Cytotoxicity of compounds **20-24** have been evaluated against human lung cancer (A549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) using resazurin assay as described by O'brien *et al.* [57]. Resazurin is a non-fluorescent dye reduced to fluorescent resorufin by living cells. The fluorescent is proportional to the quantity of cells. Consequently, this assay allows to determine inhibition of cell growth and cytotoxicity. Resazurin is easy to use as homogeneous assay substrate. It has a fast and robust read-out potential and usability for a wide range of cell types. On the other hand, there are possibilities of interaction with test reagents, spontaneous conversion to fluorescent resafurin and second step conversion of fluorescent resafurin to non-fluorescent metabolites. These disadvantages may cause conflicts in data interpretation [58]. The cytotoxicity was also assessed using Hoescht assay as described by Rago *et al.* [59], which measure cell DNA quantity, were also carried out to confirm the results of the resazurin tests. Hoechst stains are part of a family of fluorescent stains for labelling DNA It provides a linear relationship between fluorescence and DNA content over a broad range of DNA. This reaction is DNA-specific and does not work with other cellular components such as RNA, protein, or carbohydrate. This enables the rapid and accurate measurement of cell number involving minimal processing time, making this assay well suited for cell proliferation studies[60]. The results presented in table 8 are expressed as the concentration of compound inhibiting cell growth by 50 % (IC_{50}). The etoposide was used as positive control, exhibited IC_{50} of 3.4 ± 0.1, 27 ± 5 and 34 ± 4 μM against A549, DLD-1 and WS1

respectively for the resazurin test as well as 2.8 ± 0.5, 2 ± 1 and > 50 μM for Hoechst assay.

Compound **23,** never previously reported for anticancer activity, exhibited a significant cytoxicity against A549 and DLD-1 with IC_{50} of 9 and 20 μM respectively for the resazurin tests. Hoechst assays IC_{50} values were 9 and 12.8 μM. However, compound **23** was not selective towards cancer cell lines. IC_{50} values of compound **21** indicated that this molecule was weakly active on A549 and DLD-1 using both cytotoxic assays, whereas compounds **20**, **22** and **24** were found to be inactive (IC_{50} > 100 μM) against all cell lines tested.

Table 8: *In vitro* cytotoxicity results of phenolic compounds.

	IC_{50}(μM)[a]					
	Resazurin			**Hoechst**		
Compounds	A-549[b]	DLD-1[c]	WS1[d]	A-549	DLD-1	WS1
20	> 100	> 100	> 100	81 ± 2	> 100	> 100
21	49 ± 6	75 ± 5	60 ± 3	32 ± 4	42 ± 5	55 ± 4
22	> 100	> 100	> 100	45 ± 2	43 ± 6	77 ± 2
23	**9 ± 1**	**20 ± 1**	**10 ± 1**	**9 ± 1**	**12.8 ± 1**	**14 ± 7**
24	> 100	> 100	> 100	> 100	> 100	> 100
Etopside[e]	3.4 ± 0.1	27 ± 5	34 ± 4	2.8 ± 0.5	2 ± 1	> 50

[a] IC_{50} value of extract which was defined as a concentration that caused 50% inhibition of cell proliferation.

[b] Human lung carcinoma

[c] Human colorectal adenocarcinoma

[d] Human normal skin fibroblasts

[e] positive control.

4.4 Evaluation of the anti-inflammatory activity of isolated compounds on LPS activated RAW 264.7 macrophages

Exponentially growing cells were plated in 24-well microplates (BD Falcon) at a densisty of 2 x 10^5 cells per well in 400 µl of culure medium and were allowed to adhere overnight. Cells were then treated or not with positive control N(G)-nitro-L-arginine methyl ester (L-NAME), or growing concentrations of pure compounds dissolved in the appropriate solvents, and incubated at 37°C, 5% CO_2 for 24h. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 10 $\mu g.ml^{-1}$ lipopolysaccharide (LPS). After 24h, cell-free supernatants were collected and stored at -80°C until NO determination using the Greiss reaction [61] with 1% sulphanilamide and 50 µl of 0.1% N-1-naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4 at room temperature for 20 min. Absorbance at 540 nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron) and the presence of nitrite was quantified by comparison with an $NaNO_2$ standard curve. All the samples were tested at the highest concentration which presents no cytotoxicity. Anti-inflammatory activity was expressed as the concentration of drug inhibiting nitric oxide overproduction by 50% (IC_{50}).

The anti-inflammatory activity of phenolic triglyceride component **21** has been reported to be beneficial in the treatment of chronic inflammatory diseases [62] which prompted us to examine the activity of similar compounds. In the inflammation process, nitric oxide (NO) is related to inflammatory reaction and is produced from L-arginine by the inducible

NO synthase (iNOS) in certain cells activated by various pro-inflammatory agents like lipopolysaccharide (LPS). Although NO acts as a host defense, it contributes to tissue injury in inflammatory diseases [63]. Thus, effective inhibition of NO gathering by inflammatory stimuli represents a beneficial therapeutic strategy.

Anti-inflammatory activity was evaluated using NO inhibition on LPS-activated RAW 264.7 macrophages. NO released from cells is detected and quantified. L-NAME, a NO synthase inhibitor, prevented the formation of NO in LPS-stimulated RAW 264.7 macrophages [64] and as used as positive control. Resazurin test was also made to ensure that the reduced production of NO is not related to cytotoxicity. The resazurin assay was performed with growing concentrations of the extracts ranging from 0 to 40 µM.

The result presented in table 9 show that the compounds **21**, **23**, and **24** were found cytotoxic against RAW 264.7 macrophages. Therefore, it is not possible to determine IC_{50} for these compounds. In contrast the compound **20** and **22** are not cytotoxic for macrophages. The compound **20** weakly inhibit NO overexpression induced by LPS with an IC_{50} of 62 µM. Interestingly, the compound **22** demonstrates a strong inhibition of NO production with an IC_{50} of 6.5 µM. In comparison, the IC_{50} of positive control L-NAME is about 1 mM.

Table 9: NO Inhibition induced by phenolic compounds on LPS-activated RAW 264.7 macrophages.

Compounds	$IC_{50}(\mu M)$[a]
20	62 ± 30[b]
21	ND[c]
22	6.5 ± 0.4
23	ND[c]
24	ND[c]

[a] IC_{50} value of extract which was defined as a concentration that caused 50% inhibition of cell proliferation.

[b] Data represent the mean ± standard deviation of three independent experiments.

[c] ND: not determined due to macrophage cytotoxicity.

4.5 Evaluation of antibacterial activity of compounds isolated from Populus tremuloides.

The antibacterial activity of compounds **20-23** was tested against two bacteria strains including gram-positive *Staphylococcus aureus* (ATCC 25923) and gram-negative *Escherichia coli* (ATCC 25922). The *S. aureus* and *E. coli* bacteria are implicated in various pathologies and often responsible for infections contracted in hospitals (nosocomial infections) [65, 66]. The results are expressed as IC_{50} (μM) (table 10). Chloramphenicol was used as positive control in this antibacterial assay with IC_{50} of >5 μM for *S.aureus* and 0.37 ± 0.06 μM for *E.coli*. The results presented in table 10 show that compound **20** and **23** was inactive against *S.aureus* whereas compound **21** and **22** was found weakly active with

IC_{50} of 34 and 59 μM, respectively. Interestingly, the compound **22** and **23** demonstrated significant antibacterial activity against *E.coli* with IC_{50} of 25 and 16 μM, respectively.

Table 10: Antibacterial activity of phenolic compounds against *S. aureus* and *E. coli*.

Compounds	**IC_{50}(μM)[a]**	
	S.aureus	*E.coli*
20	Inactive[c]	Inactive[c]
21	34 ± 4 [b]	Inactive[c]
22	59 ± 5[b]	25 ± 2[b]
23	Inactive[c]	16 ± 3[b]
Chloramphenicol[d]	> 5[b]	0.37± 0.06[b]

[a] IC_{50}: concentration of compound inhibiting fifty percent of bacterial proliferation.

[b] Data represent the mean ± standard deviation of three independent experiments.

[c] Pure compound was considered inactive when IC_{50} >100 μM.

[d] positive control.

Chapter VI

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Very few investigations have been carried out on bioactive constituents of *P. tremuloides* which were commonly used by Amerindian traditional medicine to treat numerous diseases. Some new information about chemical constituent of buds of *P. tremuloides*:

- The fraction C from *n*-BuOH extract showed an important cytotoxic activity against A549 and DLD-1 cell lines.
- Bioassay-guided fractionation of this fraction with a combination of different chromatographic techniques led to the isolation of 24 phenolic compounds, namely: (1*S*,2*S*)-1-[4-*O*-(*E*)-coumaroyl-*β*-D-glucopyranosyloxy] cyclohexane-diol (**1**), chaenomeloidin (**2**), prunin (**3**), echinaticin (**4**), echinacin (**5**), tremulacin (**6**), salicine (**7**), tremuloidin (**8**), genkwanin (**9**), rhamnocitrin (**10**), sakuranetin (**11**), acacetin (**12**), kaempferide (**13**), aromadendrin (**14**), phenylmethyl coumarate (**15**), phenethyl p-coumarate (**16**), cinnamyl coumarate (**17**), phenylmethyl caffeate (**18**), trans-ferulic acid (**19**), 1,3-0-di-trans-*p*-coumaroylglycerol (**20**), Lasiocarpin A (**21**), Lasiocarpin B (**22**), Lasiocarpin C (**23**) and 1,2-dicoumaroylglycerol (**24**).
- Compound **1** is a new molecule and was not reported in literature before.
- Compounds **20** and **24** were not reported before for Salicaceae family.
- Compounds **2** and **5** were not reported before for *Populus* genus.
- Compounds **3**, **4**, **9**, **10**, **12**, **16**, **17**, **21**, **22** and **23** were identified for the first time in *P. tremuloides*.

- Compounds **9**, **12**, **15**, **18** and **23** exhibited significant inhibitory activities against A549 and DLD-1 cells with IC_{50} values comprised between 8 and 24 μM.
- It was found, for the first time, that compounds **15** and **23** had anticancer activities.
- Compound **22** exhibited high anti-inflammatory potency towards RAW 264.7 cells.
- Complete ^{1}H, ^{13}C NMR spectroscopic data were performed for compound **15** and ^{13}C NMR spectroscopic data for compounds **22**, **23** and **24**. These data were not reported before.

For future studies, it would be valuable to evaluate the chemical structures and the corresponding biological activities of molecules in the bark, resin and root of *P. tremuloides* or other species of *Populus* of the boreal forest. Our experiment showed (TLC) that there were some differences in the materials of bark and resin of *P. tremuloides*. In addition, they are suggested to be effective in traditional medicine [9]. It would be interesting to investigate the synergic effects of isolated molecules for different biological activities.

It is certainly important to synthetize higher quantities of the bioactive molecules found in this research to test their other aspects in short and long term. Ultimately, *in vivo* studies on most active molecules are essential.

Chapter VI

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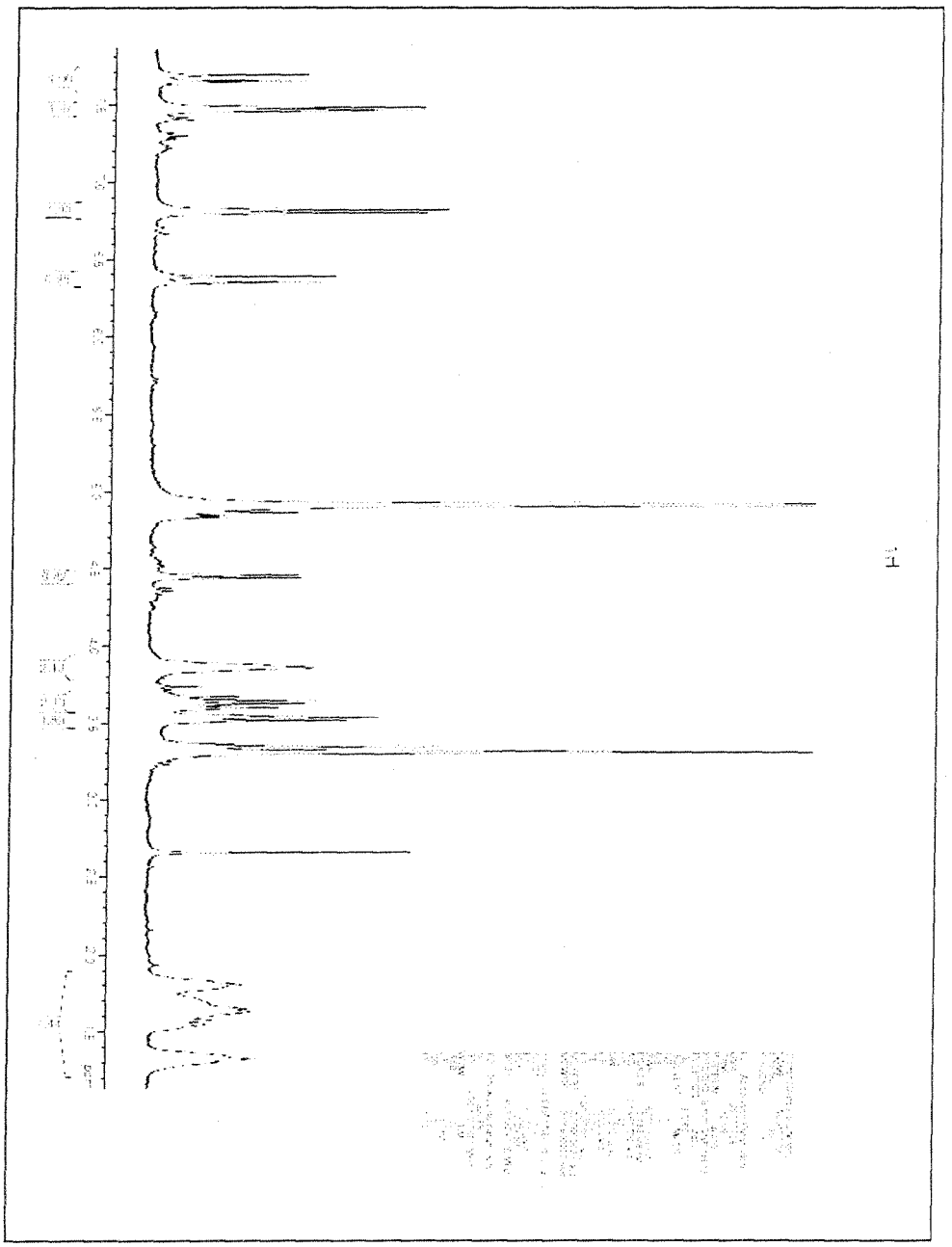
APPENDIX

NMR Spectra

^{1}H of compound (**1**)



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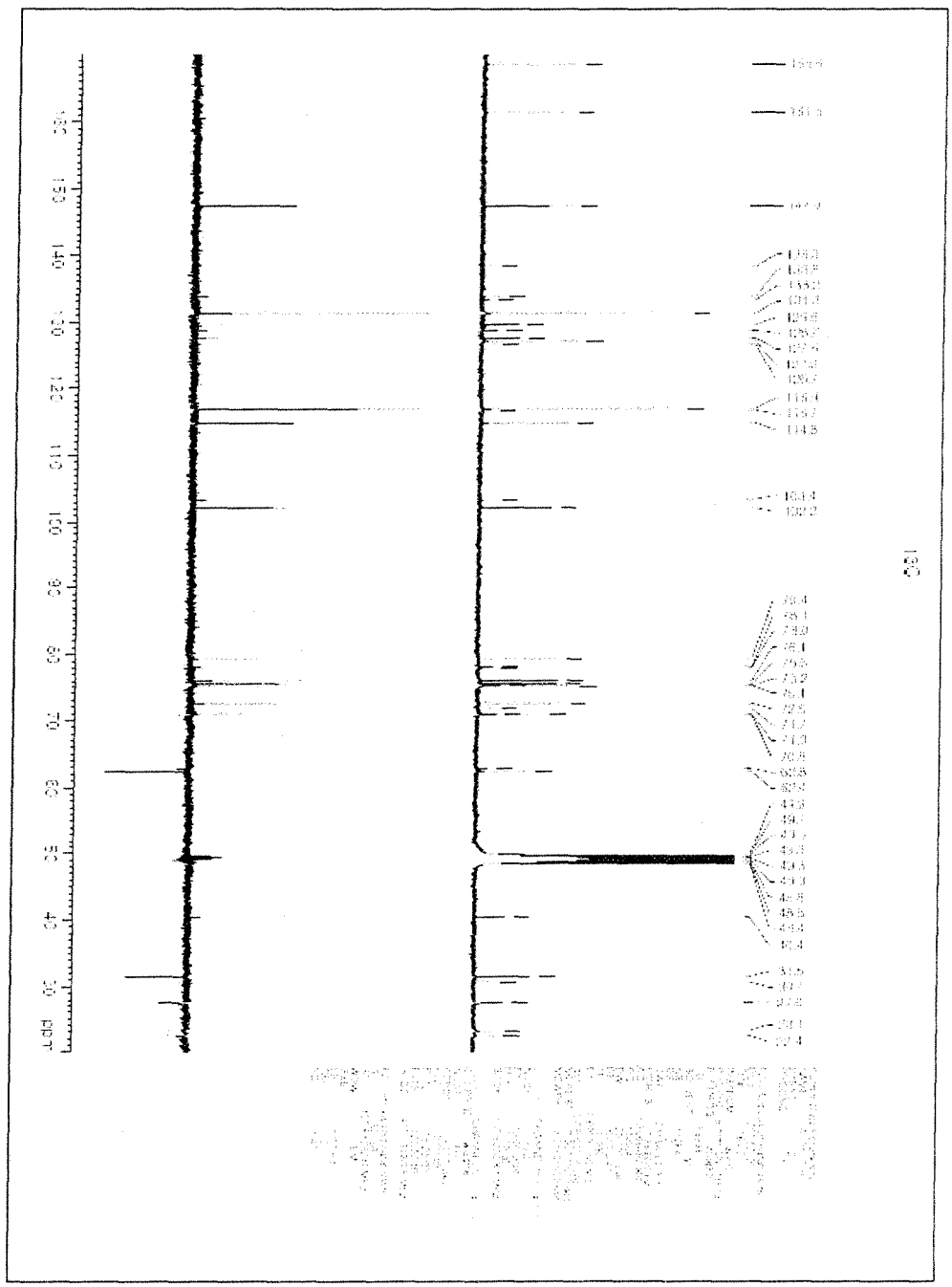

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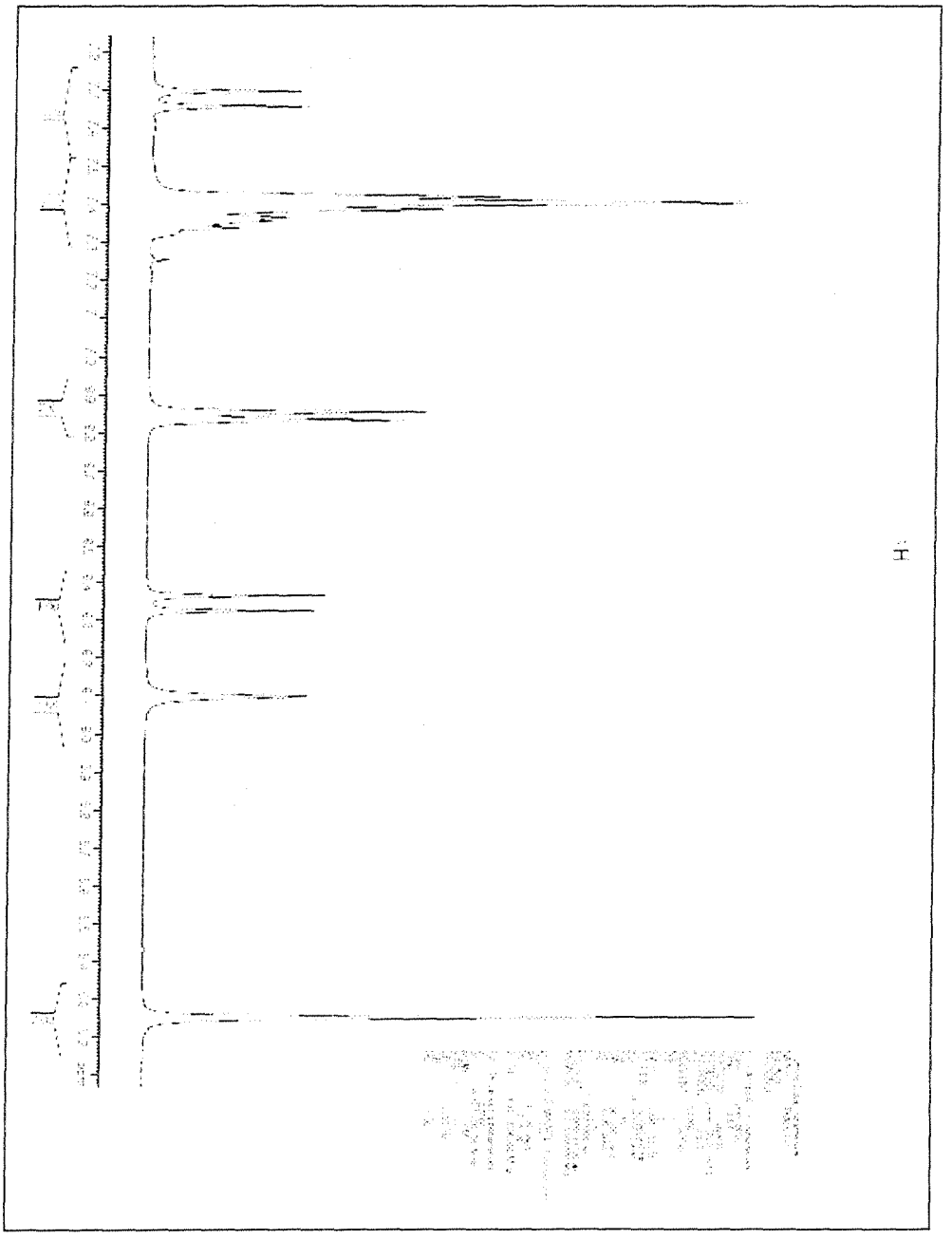

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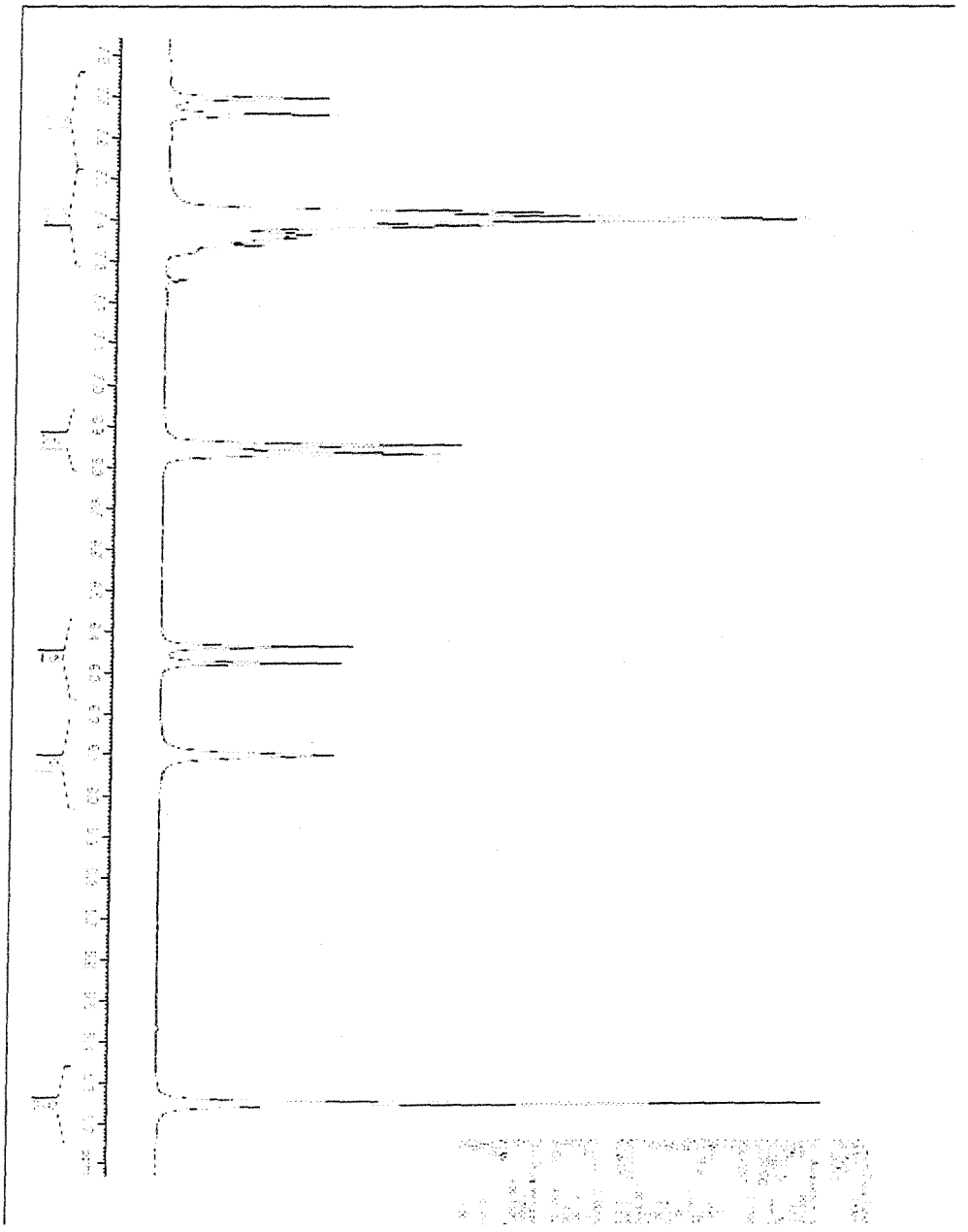

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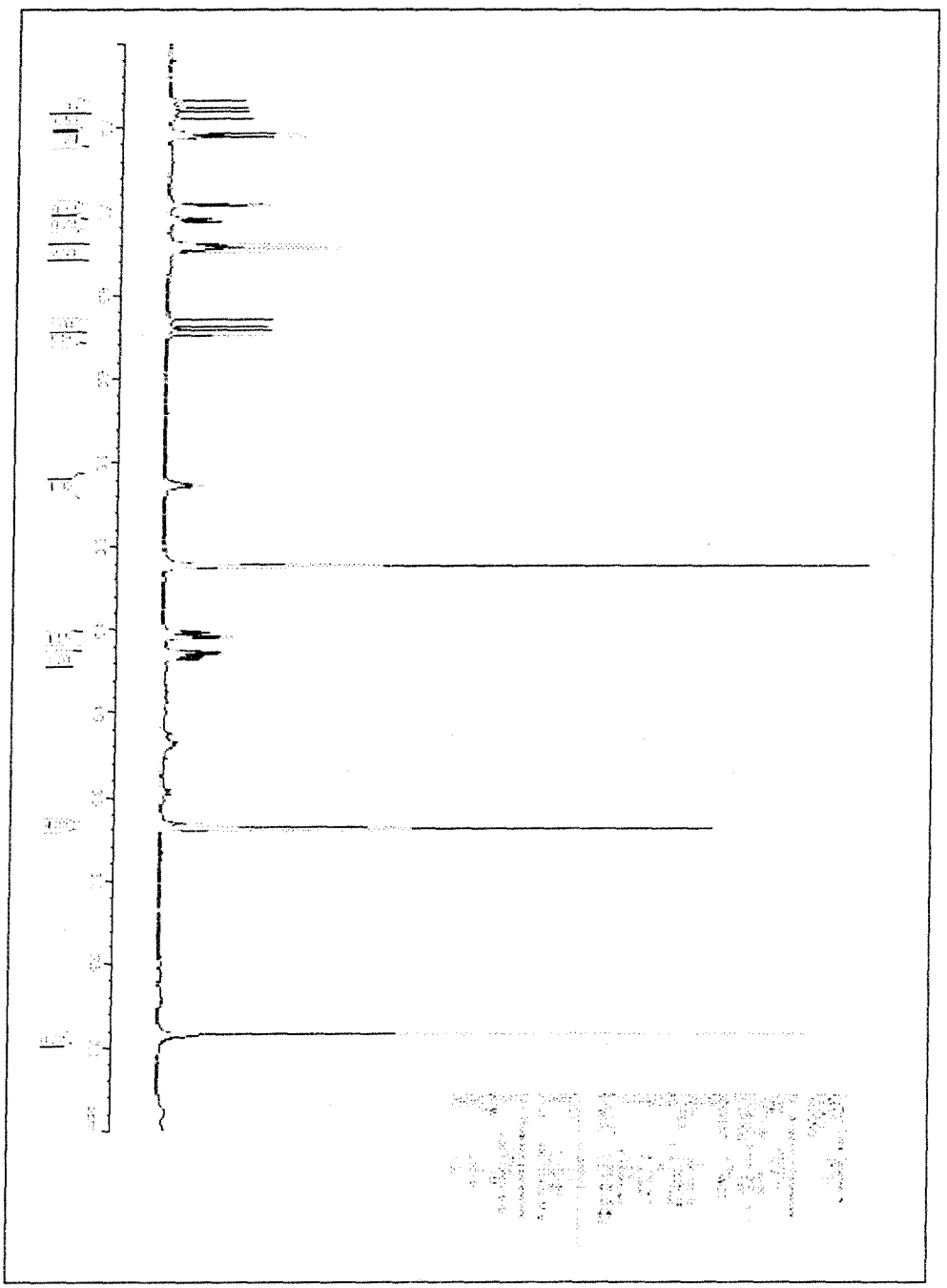

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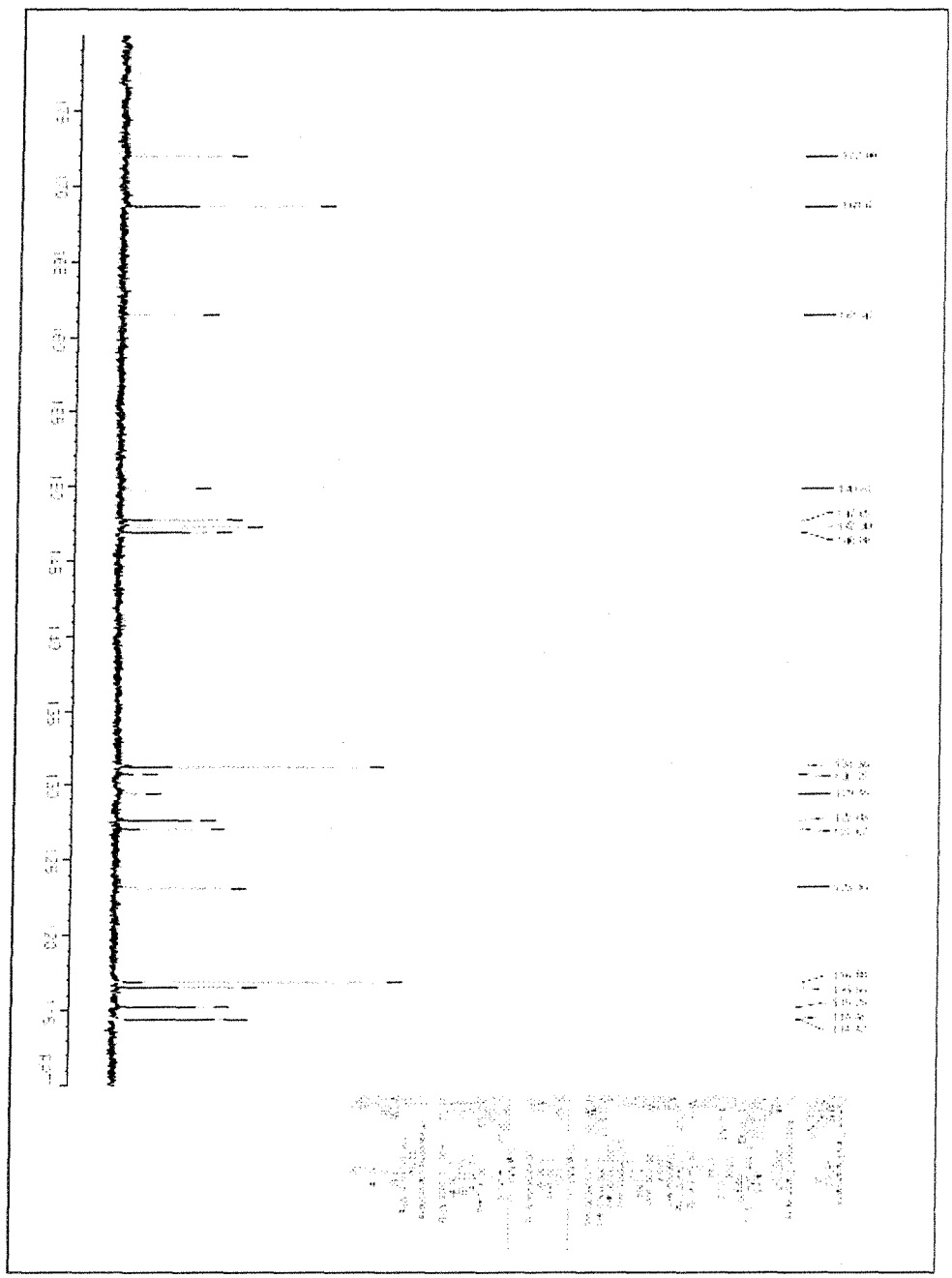
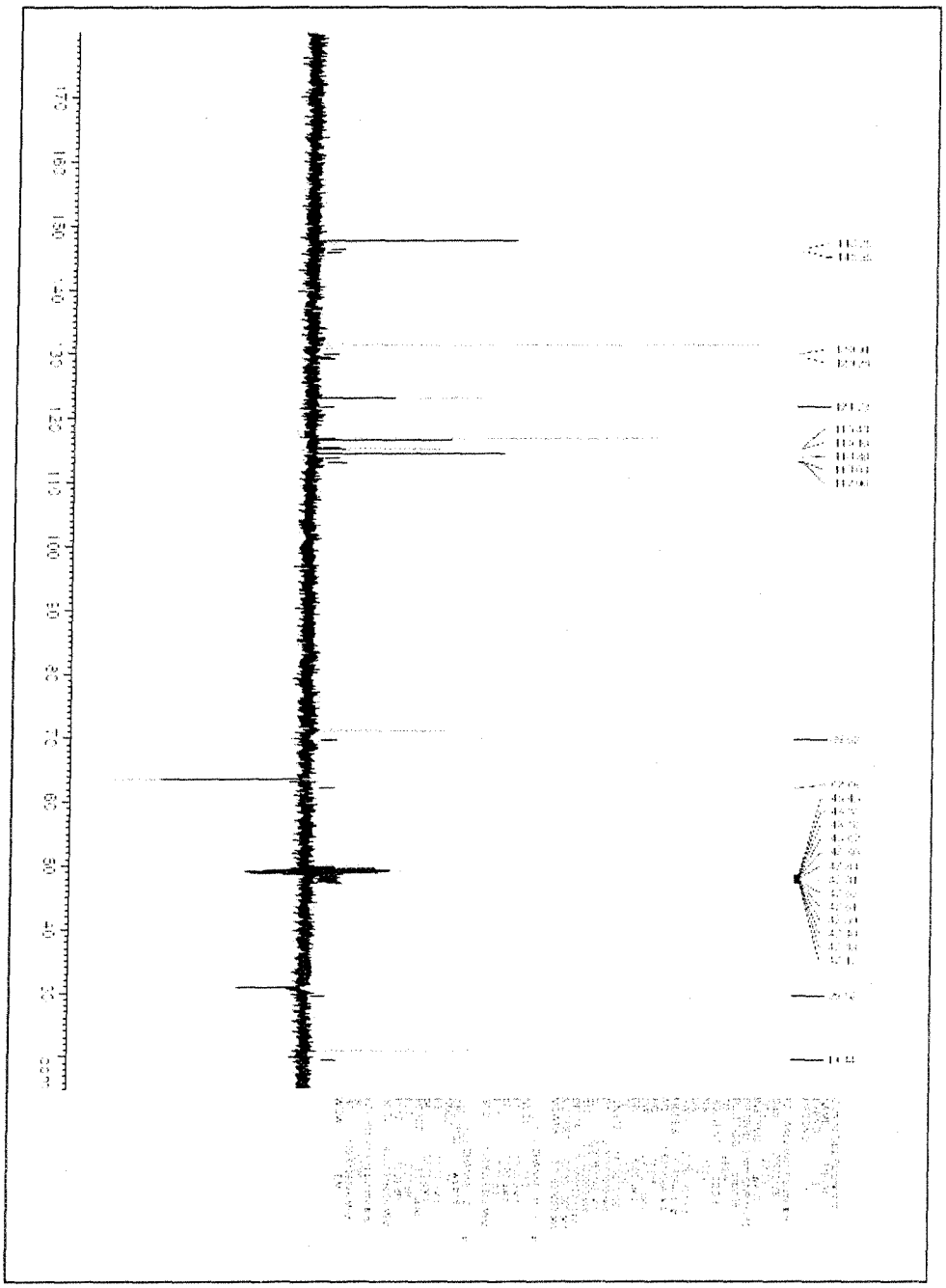


Dept135 of compound (**22**)



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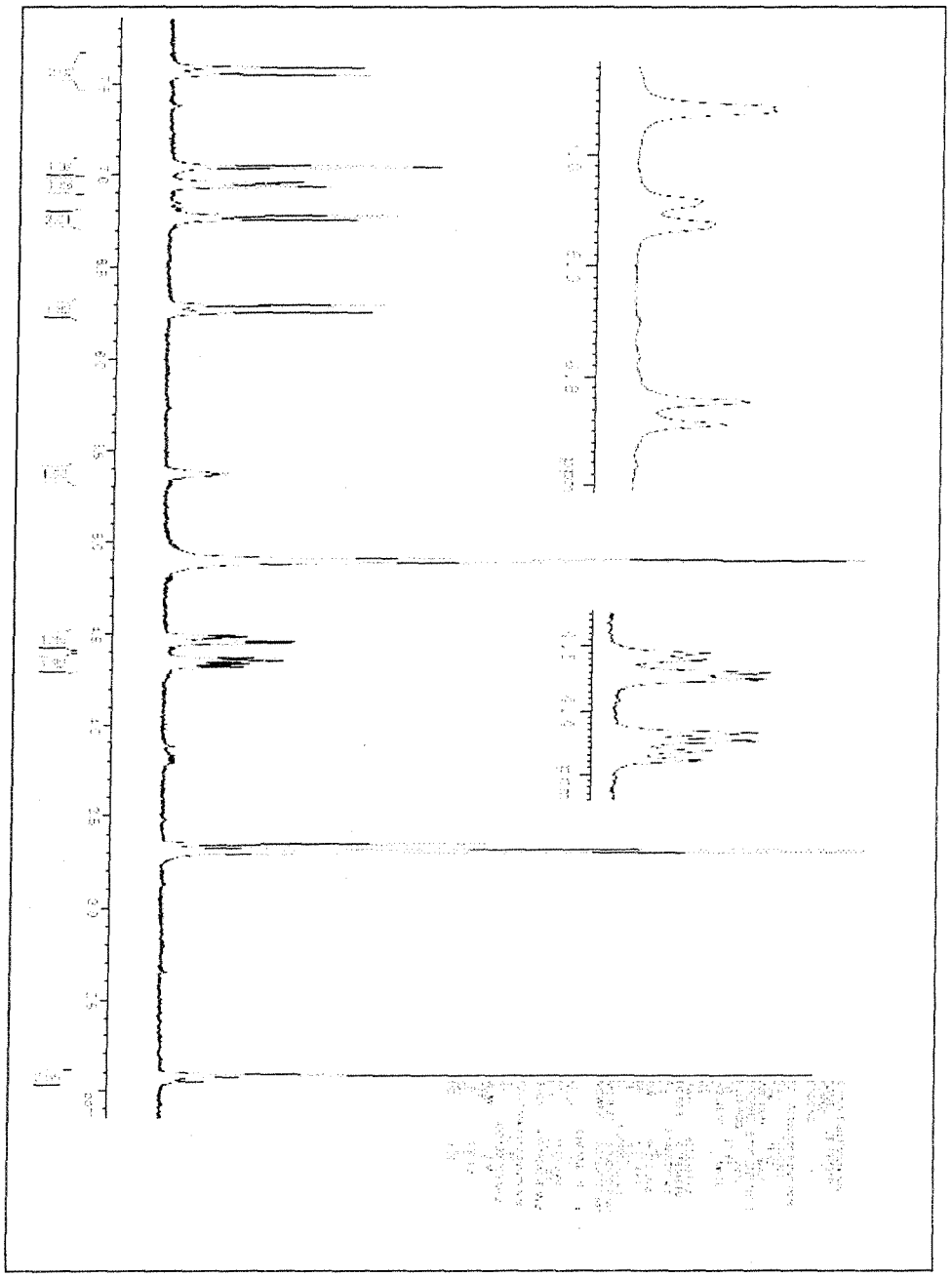

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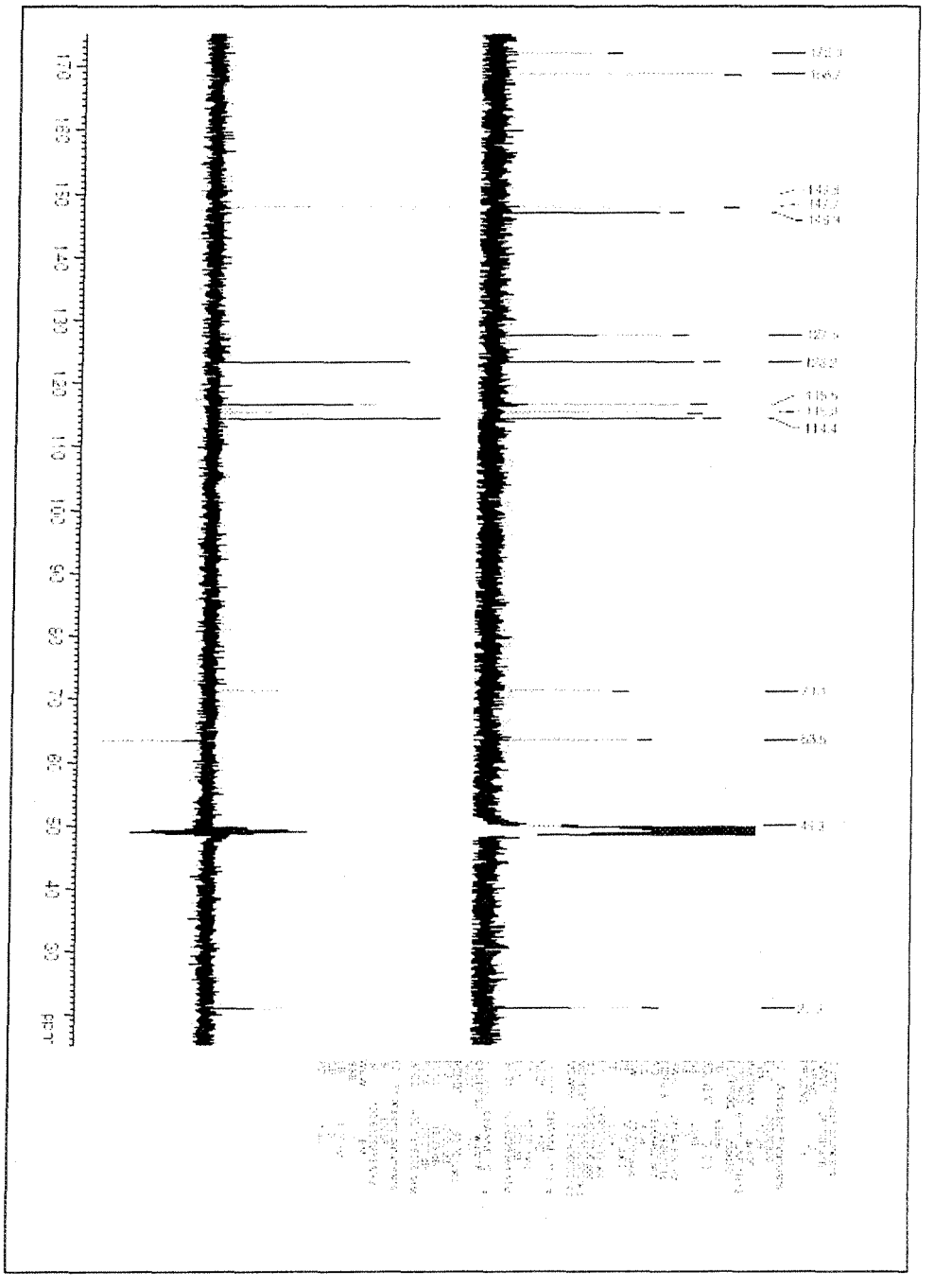

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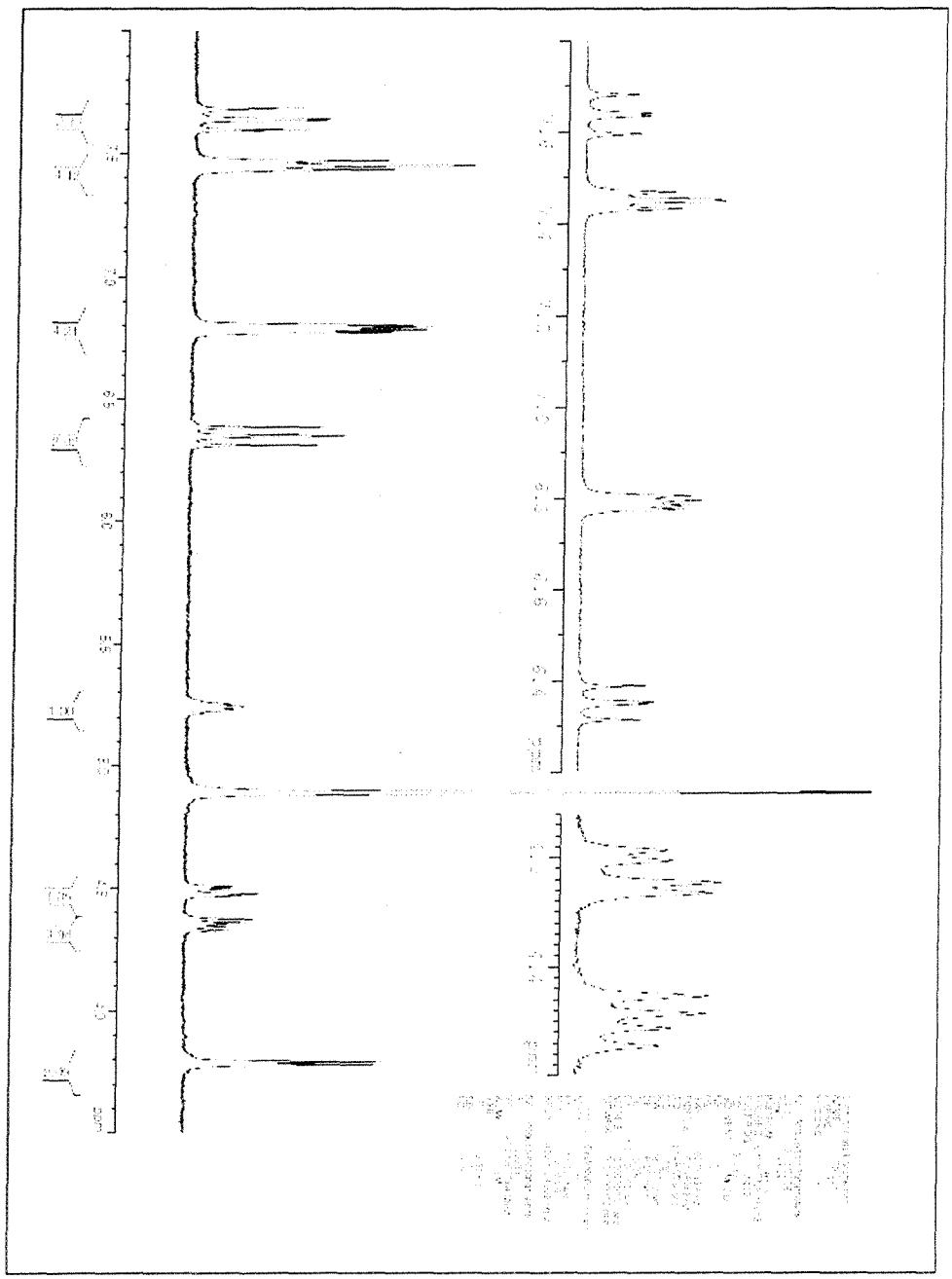

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