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RESEARCH ARTICLE

# Utilization of wood industrial wastes for production of chemicals and pharmaceuticals

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## Abstract

**Objectives**: This study aims to isolate and identify valuable chemicals and pharmaceuticals from the waste of wood (*Pinus sylvestris* L.) industry and to determine their biological value. **Methods:** Chromatographic technique was used to isolate compounds from the waste. The chemical identity of the isolated compounds were evidenced by different spectroscopic analyses as well as comparative studies of reported data. The microbial transformed product of pinosylvin mono methyl ether was quantitatively determined using fluorimetric method**.** All the isolated compounds were tested for their antitoxoplasmosis activity (in vitro) against *Toxoplasma gondii*. **Findings/ Novelty:** Nine compounds were isolated and identified including three diterpenes : (1$\beta$-hydroxy dehydroabietic acid, 15-hydroxy dehydroabietic acid and Methyl-15-hydroxy dehydroabiet-18-ate) ; two aromatic aldehydes (coniferaldhyde and 3-hydroxy, 5-methoxy benzaldhyde) ; two stilbene derivatives; (Pinosylvin and Pinosylvin mono methyl ether) ; a flavan: (Pinocembrin) and a phytosterol ($\beta$-sitosterol). For the first time, the fluorimetric method was used for quantitative determination of the novel metabolite produced, E-Pinosylvin mono methyl ether 5-O-$\beta$-D-glucopyranoside, from the microbial biotransformation technique. All isolated compounds showed variable antitoxopasmosis activity, carried out for the first time, against *T. gondii* according to their % mortality and recorded $EC_{50}$.

**Keywords:** Pinus sylvestris L.; saw dust; antitoxoplasmosis; fluorimetry; microbial transformation

## 1 Introduction

There are many wastes which could be subjected to more treatment for more benefit, The toxicity of waste printed circuit boards (PCBs) to bacteria was considered as the major limitation in bioleaching of copper from PCBs. To reduce the toxicity of PCBs, copper extraction from PCBs was investigated

using bacteria-free cultural supernatant from some metallurgical microbial consortium[1]. Along with the rise of green chemistry, according to the biorefinery concept , lignocellulosic raw materials can be used for making valuable products that could replace petroleum derived products. The most important applications of lignocellulose biotechnology are in Bioenergy with the bioethanol and biohydrogen production and in Chemical Industry with some representative chemicals including acetone-butanol and polylactic acid (all obtained by glucose fermentation)[2]. There are many strategies that lead to high-value products from non-edible biomass waste streams also markedly increase the economic feasibility of lignocellulosic biorefineries[3]. Wood industrial wastes are also considered as a new source in the realm of nanocellulose[4]The Genus Pinus (Pinaceae) comprises up to ninety species spread over the northern hemisphere[5]. The plants of this genus are used in rosin and turpentine industry[6]. Wastes of this genus can be exploited to produce valuable compounds, essential oils isolated by different techniques from *Pinus pinaster* showed antioxidant activity[7,8] and also from other species[9,10]. Different species of genus Pinus showed anti-inflammatory, antitumor , antimicrobial, antioxidant and antiviral activities[11–14] . *Pinus sylvestris* L. (Scot's pine tree) was mentioned in different scientific fields , It was also reported to contain a versatile array of secondary metabolites, including mono-, di-, sesqui- and triterpenes, flavonoids, stilbenes and lignans amongst other secondary metabolites[14] and also in the effect of thermal modification process on the size distribution of dust particles of Scots pine generated during lengthwise milling[15]. The accumulation of the waste (saw dust) from the wood industries around Damietta governorate represents a serious hazards and environmental problem[16]. The biotechnology of industrial wastes for production of chemicals and pharmaceuticals has gained an interest recently to scientists[17] . The waste represents a valuable, priceless and renewable feed stock for production of useful products. Structure modification, through microbial transformation, of the major isolated compound was tried in this work in order to obtain more active or less toxic derivatives. Many compounds belonging to the same chemical classes of secondary metabolites previously identified in *P. sylvestris* showed antiprotozoal activity against *Plasmodium falciparum*, *Trypanosoma brucei* and *Leishmania donovani* [18,19]. Toxoplasmosis is a worldwide health problem that is caused by the intracellular parasite *Toxoplasma gondii*[20]. It is estimated that more than one billion people all over the world are infected with *T. gondii*[21]. The current anti *T. gondii* medication is not satisfactory. The first line therapy for the treatment or prophylaxis of toxoplasmosis is a combination of pyrimethamine and sulfadiazine, but unfortunately these drugs have serious side effects[22]. Herbal extracts as well as pure compounds of natural origin were shown to have anti toxoplasmosis activity in vivo and in vitro. Examples are the root extracts and fractions of Eurycoma longifolia Jack against virulent RH strain of *T. gondii*[23,24]; various molecular weight of chitosan nanoparticles[25] as well as lipophilic fractions from Sorghum bicolor[26]. This report describes the isolation and identification of nine compounds from wood industry wastes of scot's pine, the production of E-Pinosylvin mono methyl ether 5-O-$\beta$-D glucopyranoside from pinosylvin mono methyl ether (first time). The biological activity of the isolated compounds and the microbial transformed metabolite, were tested against *Toxoplasma gondii*.

## 2 Material and Methods

### 2.1 Plant material

Saw dust (mesh size 30 mm) was collected from the waste of companies of carpentry industry at Damietta governorate (Egypt) in May 2015. Samples were identified as *Pinus sylvestris* L. family, Pinaceae by Prof. Dr. Ibrahim Kherallah, Forestry and Wood Technology Department, Faculty of Agriculture (El-Shatby), Alexandria University, Egypt. A voucher specimen of the waste was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

### 2.2 General

Column chromatography (CC) was performed on silica gel 70-230 mesh (Merck, USA) and TLC on silica gel 60 $F_{254}$ precoated plates (0.25 mm, Merck, USA), silica gel column (Merck, USA). $^{1}$H-, $^{13}$C-NMR and HMBC spectra

were determined on JEOL JNM-ECA500 (500 and 125 MHz for $^{1}$H- and $^{13}$C- spectra, respectively) (JEOL, Japan) and Bruker DRX 600 (600 and 150 MHz for $^{1}$H- and $^{13}$C- spectra, respectively) (Bruker, Germany). Components of $\alpha$-media[27] used in microbial biotransformation. Biological assay, the virulent RH strain of *Toxoplasma gondii* was obtained from Laboratory of medical parasitology department, Faculty of Medicine, Alexandria University), maintained by intraperitoneal passages in albino mice[28]. Phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO), trypan blue stain (0.2 % w/v) and clindamycin was obtained from Advanz pharma, Egypt. Fluorimetric assay was performed on Shimadzu RF-1501 spectroflurometer (Shimadzu, Japan), spectroscopic methanol (Merck, USA)Solvents were reagent grade.

## 2.3 Extraction and Isolation

About 3 Kg of *P. sylvestris* L. wood industrial waste was extracted with MeOH (3L x 6). The combined MeOH extract was evaporated under reduced pressure at 40° C to give 295 g of brown residue. The total residue was redissolved in 400 mL aqueous MeOH (50:50 v/v) and successively extracted with petroleum ether (1L x 3), $CH_2Cl_2$ (1 L x 3) and EtOAc (1 L x 2). Each of organic solvents extracts were evaporated under reduced pressure to obtain petroleum fraction **A** (77.7 g), fraction **B** (60.4 g) and fraction **C** (1.0 g), respectively.Each of fraction was separately chromatographed on silica gel columns according to the protocol in [ Figure 1] to obtain isolated compounds (1-9).

## 2.4 Microbial biotransformation

Microbial transformation of compound **5** was carried out adopting a two-stage fermentation protocol[29]. The fermentation was screened for potential metabolite using 22 microorganisms [ Table 1 ]. Only three microorganisms showed a single spot at R $_f$ value 0.15 on TLC developed in 5 % MeOH in $CH_2Cl_2$-EtOAc (50:50, v/v), *Asperigellus ochraceous* NRRL 398, *A. restricutus* NRRL 2869 and *Rhizopus sp*. ATCC6060 and made visible by spraying with vanillin/$H_2SO_4$ reagent followed by heating with a heating gun until maximum development of the spot colour. Preparative scale fermentation was performed utilizing *A. ochraceous* NRRL 398 due to ease purification of the product from secondary metabolites, using 200 mg of compound **5** and incubated for 2 weeks. The culture broth was filtered and extracted with EtOAc (0.5 L 3), the combined extracts were evaporated to dryness to obtain 230 mg of a yellow syrupy residue. The residue was chromatographed on silica gel column (40 x 0.8 cm) and isocratically eluted with a mixture of $CH_2Cl_2$-EtOAc (50:50 v/v) containing 5% of MeOH to yield 40 mg of metabolite **10** as a yellow amorphous powder.

**Table 1.** Listof microorganisms used in biotransformation technique.

| Microorganisms | |
|---|---|
| *Asperigellus alliaceous* NRRL315 | *Coriolus hirsutus* ATCC MYA-828 |
| *Asperigellus flavipes* ATCC1013 | *Coriolus versicolor* ATCC48242 |
| *Asperigellus flavus* NRRL21882 | *Cunnighamela blackselena* NRRL1369 |
| *Asperigellus niger* NRRL 2995 | *Cunnighamela elegans* NRRL2310 |
| Asperigellus niger ATCC51049 | *Cunnighamela elegans* NRRL1395 |
| *Asperigellus niger* ATCC 51049 | *Daedalea malicola* NBRC 4978 |
| *Asperigellus ochraceous* NRRL 398 | *Penicillium chrysogenium* ATCC 9480 |
| *Asperigellus restricutus* NRRL2869 | *Penicillium chrysogenium* ATCC10002 |
| *Asperigellus versicolor* AUMC150201 | *Rhodotorula rubra* NRRL 41592 |
| *Ceriporia spissa* ATCC62024 | *Rhizopus sp.* ATCC 36060 |
| *Cunnighamela blackselena* MR198 | *Rhizopogen sp.* ATCC6060 |

Metabolite **10**: was obtained as a morphous yellow powder, R $_f$ 0.15, $CH_2Cl_2$- EtOAc (50:50, v/v) containing 5% MeOH. $^{1}$H-,$^{13}$C NMR and APT (600, 150 MHz, DMSO-$d_6$) ( Table 2 ), HRESI-MS (positive ion mode) m/z 411.1495 $[M+ Na]^+$ , integrated for the molecular formula is $C_{21}H_{24}O_7$ . Based on the previously reported data, it

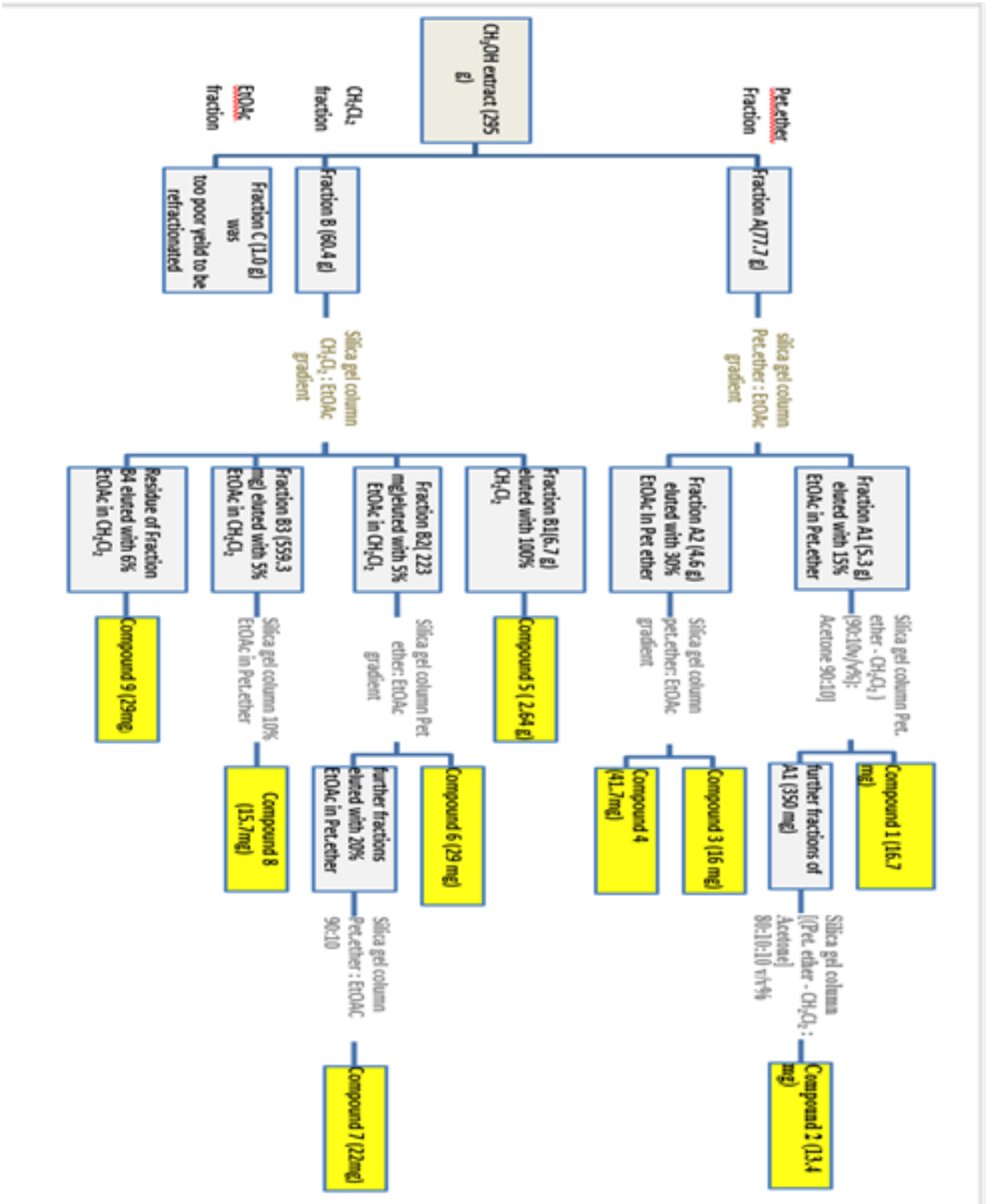


**Fig 1.** Protocol of isolation of compounds (1-9) from saw dust of *P. sylvestris* L. wood

can be concluded that metabolite **10** is E-Pinosylvin mono methyl ether 5-O-$\beta$-D glucopyranoside.

**Table 2.** $^{1}H$ and $^{13}C$ data (600 and150 MHz, DMSO-$d_6$) of metabolite **(10)** and the substrate **(5)**

| No. | E-Pinosylvin mono methyl ether 5-O-$\beta$-D glucopyranoside (10) | | | E-Pinosylvin mono methyl ether (5) | |
|---|---|---|---|---|---|
| | 1H | 13C | APT | 1H* | 13C* |
| 1 | ..... | 140.8 | C | ..... | 138.8 |
| 2 | 6.9s | 108.1 | CH | 6.66 brs | 106.2 |
| 3 | ..... | 159.9 | C | ..... | 158.6 |
| 4 | 6.63s | 103.2 | CH | 6.29(1H, dd, J=2.4, 2.4) | 100.9 |
| 5 | ..... | 161.8 | C | ..... | 160.6 |
| 6 | 6.8s | 107.4 | CH | 6.61 brs | 102.9 |
| A | 7.08(1H,d, J=16.5) | 128.9 | CH | 7.13(1H,d, J=16.5) | 128.3 |
| *B* | 7.15(1H,d, J=16.5) | 130.5 | CH | 7.17(1H,d, J=16.5) | 128.59 |
| 1' | ..... | 138.1 | C | ..... | 136.9 |
| 2',6' | 7.35 (1H,t, J= 7,7.5) | 127.36 | CH | 7.59 (2H,d, J= 8.4) | 126.4 |
| 3',5' | 7.50(1H,d, J=7.5) | 129.7 | CH | 7.37(2H,t, J=7.8,7.8 ) | 128.61 |
| 4' | 7.30(1H, d, J= 7.5) | 128.8 | CH | 7.27(1H, t, J= 7.2,7.2 ) | 127.5 |
| O-CH3 | 3.70 | 56.1 | CH3 | 3.75(3H,s) | 54.9 |
| OH | ..... | ..... | ..... | 9.4 | ..... |
| 1" | 4.90(1H, d, J= 7.2) | 102.0 | CH | ..... | |
| 2" | 3.34 | 74.5 | CH | ..... | |
| 3" | | 77.4 | CH | | |
| 4" | 3.60 to 3.70 m | 71.1 | CH | | |
| 5" | | 77.8 | CH | | |
| 6" | 3.80 | 61.8 | CH2 | ..... | ..... |

## 2.5 Fluorimetric assay

### *2.5.1 Fermentation and sampling procedures*

Quantitative determination of the produced metabolite was carried out using fluorimetric technique [30]. The substrate **5** was added separately to each of the stages, flasks containing one of the three microorganisms (*Asperigellus ochraceous* NRRL 398, *A. restricutus* NRRL 2869 and *Rhizopus sp*. ATCC6060 ) in presence of culture and substrate control, each of the culture broth was sampled daily (1 ml), extracted with EtOAc / MeOH (9:1 ,v/v) for 14 days. All samples were air- dried, diluted to10 mL in volumtric flasks with mixture of MeOH-$H_2O$ (60:40, v/v) and Sampling was done in triplicate. The intensity values of fluorescence for each sample broth was calculated using standard curve ( Figure 3) [30,31]. the unused concentration of substrate was determined fluorimetrically according to the reported procedure [32]. The fluorescence of each sample was recorded at 302.9 nm (excitation) and 402 nm (emission) at a scan speed of 2775 nm $min^{-1}$. Spectra of both samples and standards were corrected for background according recommendations reported in [33]. Corrections were made using the software (Denovix) facilities of the instrument.The maximum concentration of the substrate at day zero was estimated as 200 ng/ml. Concentration of the untransformed substrate was determined quantitatively by measuring intensity of its fluorescence. Concentration of the produced metabolite was indirectly calculated using the following formula:

Conc. of the product = maximum conc. of the substrate (200 ng/ml) - conc. of untransformed substrate (ng/ml).

### *2.5.2 Statistical analysis*

Calibration curves obtained from standard solutions in 9 points showed good linearity, and correlation coefficient was 0.985. All samplings were carried out in triplicate and experiments repeated at least 3 times. Data were expressed as mean + S.D statistically analyzed by using graph pad prism 6.0 (GraphPad Software, 2365 Northside Dr. Suite 560,

San Diego, CA 92108) and Excel 2016.

### 2.6 Anti toxoplasmosis assay

This assay was carried out according to the described method[34]. Serial dilutions of 100 $\mu$g/ml to 3.125 $\mu$g/ml of each test compound were prepared in DMSO (1%w/v)**.** Five $\mu$l of each dilution of test compounds were incubated with 45 $\mu$l of tachyzoites suspension containing $10^6$ cells/ml in a humidified $CO_2$ chamber at 37°c for 24 h. The mortality of tachyzoites was determined with trypan blue dye under light microscope and the results (mean value) were expressed as % mortality. Clindamycin was used as positive control.

## 3 Results and Discussion

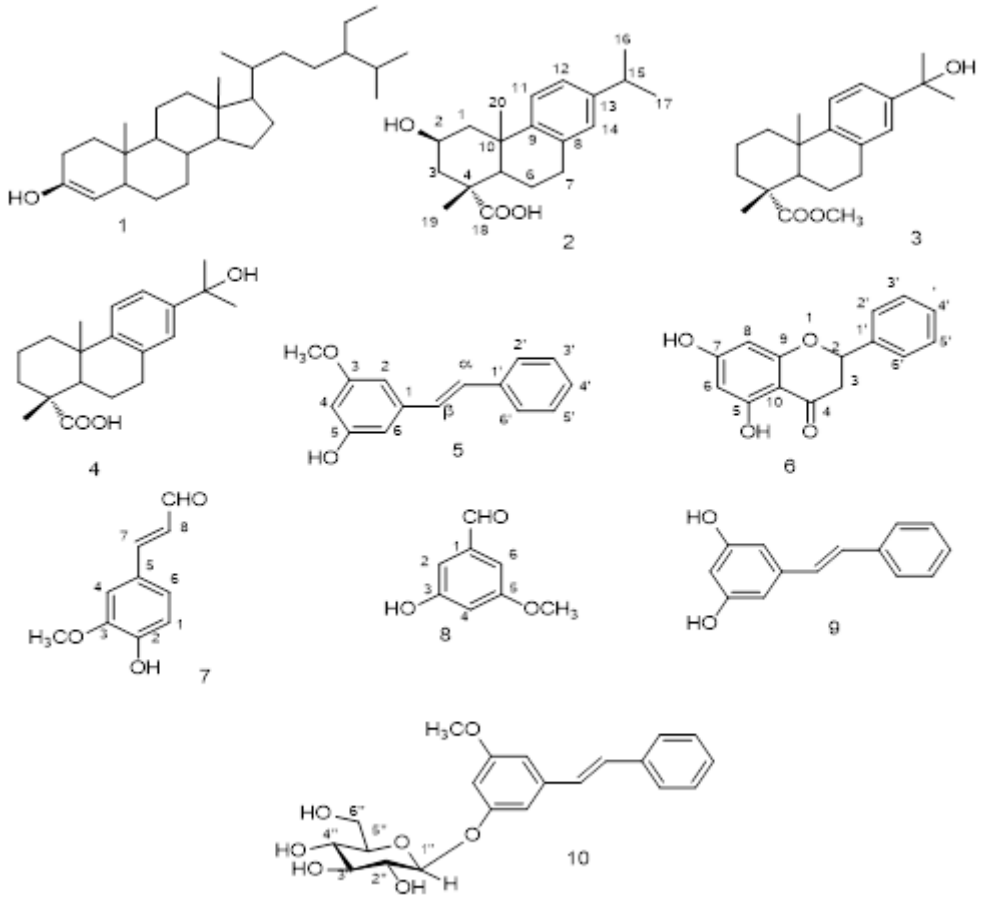


**Fig 2.** Chemical structures of compounds (**1**-**9**) and metabolite (**10**)

Nine compounds were isolated from the wood industry waste of *P. sylvestris* L. using different chromatographic techniques. The isolated compounds were identified using physical and spectroscopical data previously reportedas: $\beta$-sitosterol (**1**)[35], three abietane diterpenes including 1$\beta$-hydroxy dehydroabietic acid **2**, Methyl -15- hydroxy dehydroabiet-18-ate (**3**) and 15-hydroxy dehydroabietic acid (**4**)[36]; two stilbene derivatives, pinosylvin mono methyl ether (**5**) and pinosylvin (**9**)[37]; the flavan derivative, pinocembrin (**6**)[37], coniferaldhyde (**7**)[38] and 3-hydroxy, 5-methoxy benzaldhyde (**8**)[39] ( Figure 2 ). This is the first time to indicate the isolation of compounds (**2**) and (**7**) from this species. It is worth to mention the isolation of compound (**8**) for the first time from any natural

source, as it was known as a synthetic compound used in organic synthesis[39].

The major isolated compound **5** was screened for potential production of a new metabolite utilizing the biocatalytic activity of the selected 22 microorganisms including viz *Asperigellus ochraceous* NRRL 398, *A. restricutus* NRRL 2869 and *Rhizopus sp* ATCC 36060. Preparative scale fermentation was used for substantial preparation of the metabolite adapting the two- stage fermentation protocol utilizing *A. ochraceous* NRRL 398. The culture broth was extracted with EtOAc- MeOH (50:50, v/v). Concentrated residue (230 mg) was loaded on a silica gel column, eluted with mixture of $CH_2Cl_2$-EtOAc (50:50) containing 5% of MeOH. The isolated metabolite **10** was chemically identified as a new natural product, E-Pinosylvin mono methyl ether 5-O-$\beta$-D-glucopyranoside, using the chemical and spectroscopic techniques. HRESI-MS of a quasi molecular ion peak showed m/z 411.1495 $[M+Na]^+$, calculated value m/z 411.1512, corresponding to the molecular formula $C_{21}H_{24}O_7$ . The appearance of six new carbon signals in the aliphatic region of APT spectrum of metabolite **10** compared to that of the substrate **5** and the presence of a $\beta$-linked sugar was deduced from the large J value (7.2 Hz) of an anomeric proton doublet at d4.90 in the $^1$H-NMR spectrum of **10** which was correlated to an anomeric carbon signal at d102.0 in $^{13}$C-APT spectrum. The rest of signals in $^1$H- and$^{13}$C- APT spectra were comparable to those published for $\beta$-D-glucopyranose[40,41] **.**

The concentration of the biotransformation product **10** was determined indirectly (by difference) by measuring the concentration of the untransformed substrate in culture broth. This is because the substrate showed higher fluorescence intensity than the product, $\lambda_{excitation}$ and $\lambda_{emission}$ of the substrate **5** were determined at 302.9 and 402 nm, respectively and the intensity of fluorescence was linear at concentrations range (5- 400 ng/mL). The intensity of fluorescence (I) of the untransformed substrate in culture broth had been recorded as (Mean + S.D) for the three different microorganisms viz.; *A. ochraceous* NRRL 398, *A. restricutus* NRRL 2869 and *Rhizopus sp*. ATCC 36060 for 14 days. Concentrations of the untransformed substrate and the product were calculated by using the calibration curve of the substrate **5** ( Figure 3 )**.** According to these results, the decrease in the intensity of fluorescence of the substrate indicated its consumption in favor of the formation of the biotransformation product. *Rhizopus sp*. ATCC 36060 showed the highest yield followed by *A. ochraceous* NRRL398 and finally *A. restricutus* NRRL2869 at earlier time (day 7) ( Table 3 ). Although *Rhizopus sp*. had the best results in quantitative estimation of the yield (195 ng/mL), *A. ochraceous* was preferred and used in scaling up the microbial biotransformation because its yield was easier to isolate and purify of metabolite **10** from its culture broth.

**Table 3.** Concentrations (ng/ml) of the biotransformation product determined in culture broth of the three different fungi.

| Time (day) Fungi | *A.ochraceous* NRRL398 | *A. restricutus* NRRL 2869 | *Rhizopus sp.* ATCC36060 |
|---|---|---|---|
| **1** | 0+0 | 0+0 | 0+0 |
| **2** | 36.37+21.4 | 46.34+17.99 | 74.27+11.13 |
| **3** | 82.75+22.5 | 54.32+20.08 | 103.61+33.86 |
| **4** | 118.17+19.04 | 100.21+16.16 | 150.58+11.10 |
| **5** | 125.65+17.1 | 121.79+12.78 | 170.04+3.49 |
| **6** | 134.62+15.1 | 133.13+17.70 | 192.48+0.50 |
| **7** | 140.61+5.5 | 139.61+21.73 | 195+0.13 |
| **8** | 155.57+6.83 | 142.1+19.86 | 195+0.13 |
| **9** | 156.07+0.87 | 144.6+20.44 | 195+0.13 |
| **10** | 156.0+0.87 | 148.1+17.45 | 195+0.13 |
| **11** | 156.0+0.87 | 148.1+17.45 | 195+0.13 |
| **12** | 156.0+0.87 | 148.1+17.45 | 195+0.13 |
| **13** | 156.0+0.87 | 148.1+17.45 | 195+0.13 |
| **14** | 156.0+0.87 | 148.1+17.45 | 195+0.13 |

All results arerepresented as mean + S.D

The experimental results indicated that the isolated compounds and the new microbial metabolite of wood industry wastes of *P. sylvestris* have in vitro antitoxoplasmosis activity against *T. gondii* Rh virulent strain. This strain

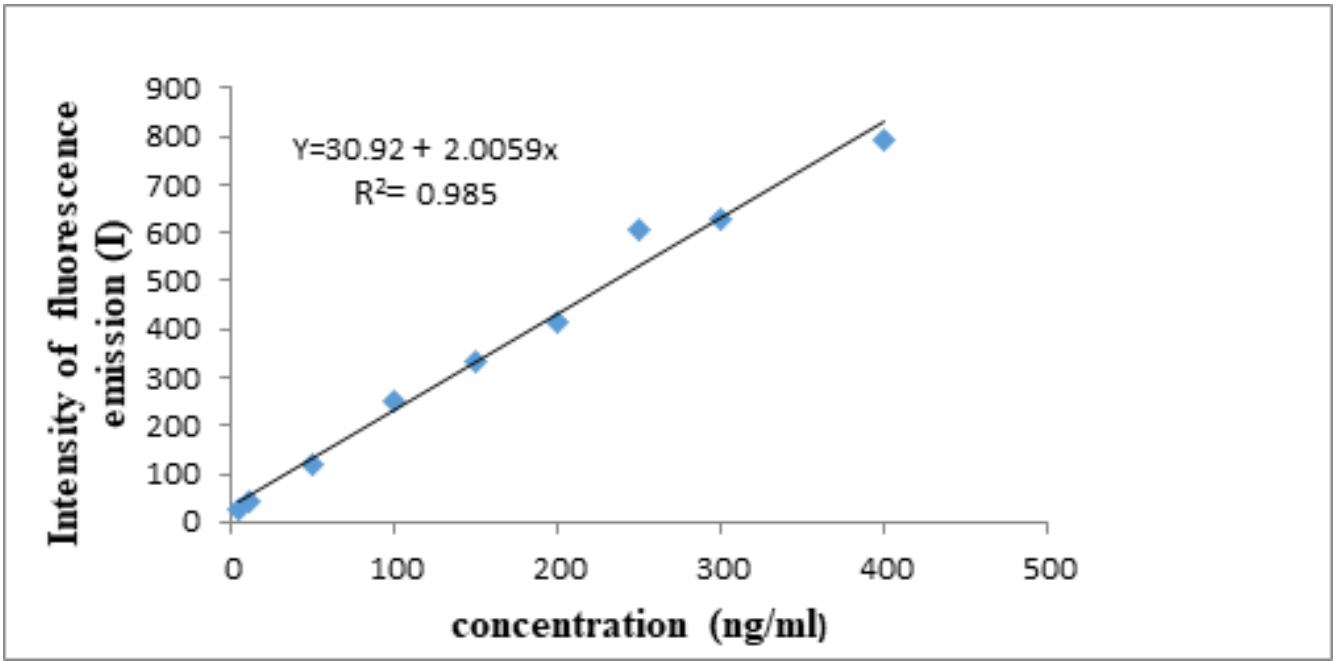


**Fig 3.** Calibration curve of E-pinosylvin mono methyl ether (5)

belongs to type I *T. gondii*, which is the most virulent and is usually associated with acute infections and congenital toxoplasmosis[42] .

Compounds **(2-10)** were tested for their antitoxoplasmosis activity against tachyzoites of *T. gondii* ( Table 4). In vitro screening of these compounds revealed that all test compounds had considerable antitoxoplasmosis activity at concentrations of 100 and50 $\mu$g/mL with % mortality ranging from 85.6-50.2. Compounds **2**, **5** & **10** showed the highest activity at both concentrations (83.7, 83.1; 85.6, 84.4; 81.8, 81.2) ( Table 4 ) which is slightly lower than that of clindamycin used as positive control in this assay (90.3 & 87.1). $EC_{50}$ of the test compounds **2**, **5** &**10** were comparable to that of clindamycin, with those of **2, 5** even slightly lower ( Table 4).

**Table 4.** Results of in vitro screening of anti toxoplamosis activity (% mortality)

| Conc. ($\mu$g/ml) | % Mortality | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Clind. | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 100 | 90.3 | 83.7 | 52.5 | 66.6 | 85.6 | 56.0 | 60.0 | 58.4 | 62.7 | 81.8 |
| 50 | 87.1 | 83.1 | 50.9 | 59.1 | 84.4 | 50.2 | 58.0 | 55.6 | 60.3 | 81.2 |
| 25 | 84.5 | 81.4 | 39.2 | 42.2 | 81.5 | 45.9 | 52.2 | 48.2 | 51.8 | 80.0 |
| 12.5 | 81.8 | 78.7 | 38.6 | 41.6 | 80.1 | 39.9 | 40.3 | 36.1 | 40.1 | 73.6 |
| 6.25 | 75.2 | 72.5 | 30.5 | 34.1 | 73.6 | 31.0 | 37.0 | 32.4 | 36.2 | 71.0 |
| 3.125 | 72.4 | 70.9 | 27.1 | 25.2 | 71.1 | 25.7 | 26.2 | 24.8 | 30.4 | 69.1 |
| EC50 ($\mu$g/ml) | 1.008 | 0.9805 | 47.863 | 34.674 | 0.9462 | 47.863 | 21.878 | 30.200 | 22.38 | 1.12 |

## 4 Conclusion

This study revealed that wood industry waste from *P. sylvestris* could be exploited for the production of valuable products showing variable anti toxoplasmosis activity in vitro for the first time. In addition to production of a new biotransformed metabolite, E-Pinosylvin mono methyl ether 5-O-$\beta$-D-glucopyranoside, that also showed high antitoxoplasmosis activity. The used material, *P. sylvestris*, showed to have the same chemical profile as that of the

genus. Moreover, the Fluorimetric assay described in this work was very sensitive and selective for quantitative determination of the produced metabolite based on the intensity of the fluorescence.

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