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A QUALITATIVE AND QUANTITATIVE ANALYSIS OF EXTRACTIVES FROM THE SPECIES Pinus pinea IN THREE DIFFERENT SOLVENTS

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Abstract:
This research deals with the quantitative and qualitative analysis of extractives of the species Pinus pine L. Samples were collected not only from the wood (separately heartwood and sapwood), but from the bark, needles and branches as well. Extractions were carried out with a Soxhlet device and three different solvents (water, ethanol, dichloromethane). Chemical analyses were conducted with gas chromatography and mass spectrometry. The results revealed significant amounts of the chemical compounds, such as D-limonene, myrtanol, phytol, megastigmatrienone, caryophyllene etc, found in the specimens, which have multiple applications in chemical, food and pharmaceutical industries.

Key words: *stone pine; extractions; gas chromatography; mass spectrometry.*

INTRODUCTION

All wood species contain many different organic compounds, which can be removed from the wood without transforming their structure. These compounds are called extractives. Extractives consist of gums, fats, resins, oils, alkaloids, tannins, etc. which don't participate in the structure of the cell walls, but are laid between them and in the cell cavities. They can be removed by using various solvents, such as hot water, alcohol, benzene, dichloromethane and others, without changing the structure of the material. Besides wood, extracts are found in other parts of the tree, like roots, bark, branches and foliage (Grigoriou 1992, Hillis 1962, Tsoumis 1991, Grigoriou 1992, Philippou 2014).

Pinus is a genus of great economic importance and includes species that are widely distributed all over the world. Some of these species are native in Greece, among them *Pinus brutia, Pinus, nigra, Pinus pinea* are included (Athanasiadis 1986). *Pinus* species are an economically important source of wood, paper, resins, charcoal, food and ornamentals (Gernandt et al. 2005; Le Maitre 1998). Moreover, *Pinus* forests are regarded to be very important sources of numerous useful products, including not only wood and cellulose but also non wood products used by the chemical, food and pharmaceutical industries (Rodrigues et al. 2012).

As far as the chemical composition of the species is concerned, *Pinus pinea* shows high content in oleic and linoleic acids in the wood as well as in the bark. In a research conducted by Hafizloglu (1989) it was discovered that alcanoiodide and hydroxy-alcanoiodide acids were the main group of souverin monomers, calculated to be approximately 20% of the bark. According to Nunes and others (1999), the average chemical composition of *Pinus pinea* bark is presented to table 1. The total extractives sum to 19,1% and consist mainly of polar compounds (14.0%). Half of the extractives (7.2%) are of phenolic character and correspond to tannins. De Simon and others (2001) identified, after ether extraction and chromatographic analysis of *Pinus pinea* needles, various compounds, showed at the following table (Table 1).

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Table 1

Chemical composition of Pinus pinea bark and chemical compounds of Pinus pinea needles (Nunes et al. 1999; De Simon et al. 2001)

Chemical composition of bark	
Component	Percentage of oven-dried weight
Ash	2.3
Total Extractives	19.1
$CHCl_2$	2.1
Ethanol	12.1
Water	4.9
Lipophilic compounds in needles	
Component	mg g^{-1} needles
Monoterpene	0.82
Sesquiterpene	0.23
Neutral diterpene	0.91
Fatty acids	0.61
Resin acids	3.30

Masendra et al. (2019) studied the hydrophilic content of extracts from six species of *Pinus*. Their results (Fig. 1) showed relatively high quantities of extractives in the bark of the species, with *Pinus oocarpa* to have showed the largest amount of all, whereas the species *Pinus elliotti* had approximately the smaller amount of all.

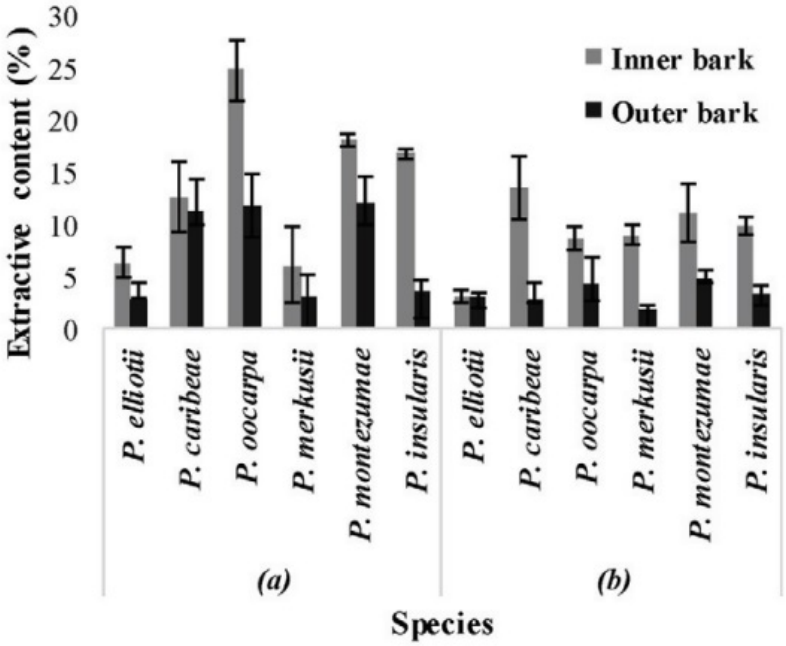

Fig. 1.
a. Ethanol extractive content; b. hot water extractive content of bark of six pine species (weight percentage of oven-dry wood, average from two measurements) (Masendra et al. 2019).

OBJECTIVE

The objective of this research was the extraction of important compounds from the species *Pinus pinea* using three different solvents, namely hot water, ethanol and dichloromethane (CH_2Cl_2). Moreover, the analysis was aiming in the identification of the differences in the extractives content between the samples that originated from various tree tissues (sapwood, heartwood, bark, needles, branches). The novelty of this research is the extraction of *Pinus pinea* compounds from different parts of the tree using the procedure mentioned above. It is a fact that there aren't data or essays available which are related to the species Pinus pinea and the extraction of its compounds from different parts of the tree.

MATERIALS AND METHODS

The under study material originated from two locations. Needles, branch, bark, sapwood, and heartwood samples were collected from trees of the Forest Research Institute of Thessaloniki (HAO-"Demeter" and from a forest located at the area of Strophilia (Pelloponisos, Greece), protected under the Ramsar Convention (confiscated illegal wood) (Fig. 2).

Fig. 2.
The stages of the collection of the samples.

From each tree, disks were taken from the chest height and then a longitudinal strip was cut from pith to bark. Afterwards, the bark, sapwood and heartwood were separated, so as to be treated apart from one another.

All samples - bark, heartwood, sapwood, needles, branches - were initially cut into smaller pieces by hand with a sharp blade and then were trimmed with a mill (Wiley's mill) (Fig. 3), in order to create particles with approximately the same dimensions, smaller than 0,1mm. The experiments were conducted on two or three samples in each case, based on the standards which were followed, in case a large difference in measurements was observed between two samples.

Fig. 3.
The stages of the extraction of the samples; starting material after trimming with Wiley's mill: a. wood after cut in sticks with knife; b. Wileys' Mill used for trimming; c. wood dust after the use of Wileys'Mill; d. wood dust in glass ready for Soxhlet device.

The quantitative estimation of extractives soluble in hot water, ethanol and dichloromethane were conducted according to ASTM Standards (ASTM D1107-96, D1108-96, D1110-84). The stages of the extraction of the samples are illustrated in Fig. 4.

a.  b.  c. 

d. 

Fig. 4.
a. Glass Soxhlet type device with a sample; b. Glass Soxhlet type device and rotary evaporator; c. branch extracts after extraction; d. gass chromatography.

For the extractions, a twin glass Soxhlet type device with the appropriate size was used, so as a 2,0g specimen and glass filter with medium porosity to be fit. Before each extraction, the dry weight (DW) of each specimen and of each glass filter was calculated by double weighing, after being dried in the oven at 103±2^{o}C for 24 hours (Table 2).

Each hot water extraction lasted at least 6 hours and at least 4 hours for each of the other two solvents and 4 full cycles of the solvent were repeated per hour, according to ASTM Standards. After the procedure, the specimens were removed from the Soxhlet device and left at normal conditions of temperature and humidity (approximately 25^{o}C and 55%) for 24h, before they were put in the oven at 103±2^{o}C for another 24 hours, until the total drying of the material. In the end, they were weighed to determine the dry weight of the extracted (wood) material, after the removal of the extractives (Chavenetidou 2009).

Percentage of extractives (%)=
(Dry weight before extraction-dry weight after extraction)/Dry weight before extraction

Qualitative analysis of extractives was conducted with gas chromatography and mass spectrometry. The solvents containing the extractives after extraction was reduced with the use of rotary evaporator instrument (Bùchi Rotavapor R-215, Bùchi Heating bath B-491) up to 1-2mL. (the rotation speed of the vial ranged between 80 and 120 rpm and the temperatures of heating bath water ranged between 15-20^{o}C above the boiling point of each solvent), in order to trace very small amounts of the chemical compounds of interest. In most cases, at the final stage of the procedure the reduction was reached with the use of gentle pure nitrogen gas stream until the optimum maximum volume of 1 mL was reached.

In all cases, specimens were cleaned up by passing through a glass chromatographic column of 1cm inner diameter, in order to remove any microparticles and also any moisture traces that may be present and they could cause damage to the gas chromatography column, with the use of the following properly activated materials: Florisil (MgO_3Si) 2.5g, Al_2O_3 3.5g and Na_2SO_4 1.5g to absorb moisture.

For the identification of the compounds and the quantification of the results gas chromatograph Agilent 7890A was used, provided with non-polar capillary column DB-5ms, 30m length and 0.25mm internal diameter, film thickness 0.25μm and as a filler 5% phenyl polysiloxane, 95% methyl polysiloxane, using Helium as a carrier gas (flow rate 0.99333 mL/min, pressure 11.656 psi) and mass spectrometer with quadrupole Agilent 5975C). Finally, the mass spectrometer with quadrupole Agilent 5975C was also implemented and the substance 1-bromo-2-nitrobenzene was used as Internal Standard (IS) for the estimation of the quantity (Tziouvalekas 2011).

Two temperature programs were applied, in order to succeed better analyses. The temperature programs which were applied where:

1. Initial temperature: 60^{o}C – for 4 minutes, raising rate 50^{o}C/min - final temperature: 240^{o}C for 5 minutes.
2. Initial temperature: 70^{o}C – for 4 minutes, raising rate 50^{o}C/min - final temperature: 280^{o}C for 10 minutes.

Identification was based on mass spectrometry and the results came from the substances recognized at each peak of diagrams conducted by mass spectrometer.

RESULTS AND DISCUSSION

Quantitative analyses

A variation in the amount of extractives is observed, related to the origin of the samples from the various parts of the tree. More specifically, the quantity of extractives isolated are higher at the needles/leaves and the branches of the species, lower at the bark and even lower at heartwood and at sapwood. Table 2 as well as Fig. 5 show the percentage of hot water, ethanol and dichloromethane soluble extractives from sapwood, heartwood, bark, leaves and branches of *Pinus pinea*, as estimated with the abovementioned procedure. Furthermore, in Table 2 the dry weight (DW) of the samples before and after extraction is presented.

The amount of extractives found in *Pinus pinea* species, more specifically in the bark, is similar to the results of Masendra et al. (2019) essay as well as of Masendra et al. (2018a), besides the fact that the researchers studied different kinds of *Pinus* species.

These quantities appeared to have the same range published for ethanol soluble extracts from the bark of *P. pinea* (Miranda et al., 2017) and *P. taeda* (Eberhardt, 2012).

Table 2

Hot water, ethanol and dichloromethane soluble extractives from sapwood, heartwood, bark, needles and branches of Pinus pinea

Pinus pinea	***Extractives, %***
Hot water	
Bark	20.566
Needles	35.213
Branches	23.850
Ethanol	
Sapwood	1.797
Heartwood	5.700
Bark	21.386
Needles	27.983
Branches	27.579
Dichloromethane	
Sapwood	2.366
Heartwood	8.740
Bark	4.532
Needles	9.600
Branches	14.805

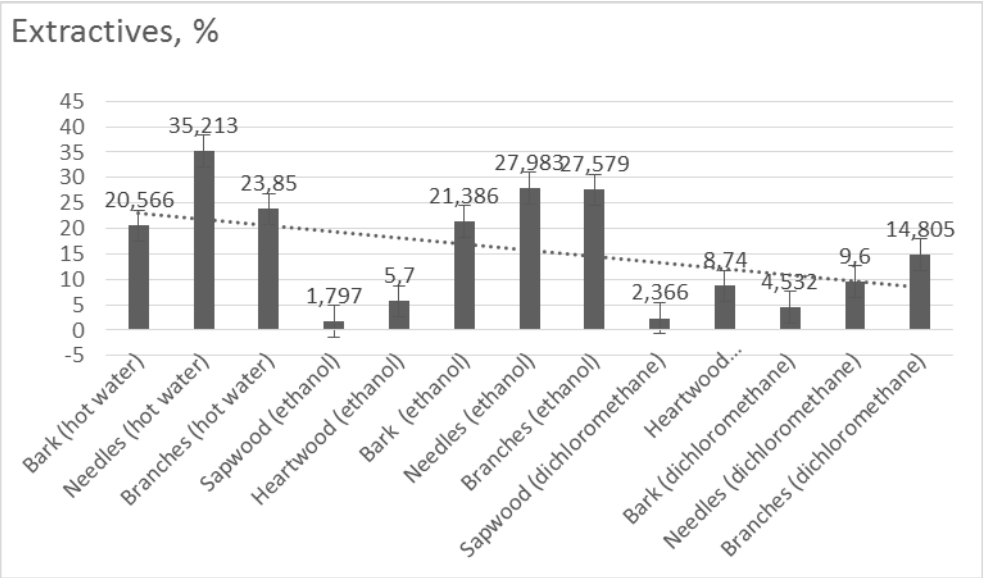

Fig. 5.
Hot water, ethanol and dichloromethane soluble extractives of Pinus pinea.

According to Fig. 5, needles contain the highest amount of extractives, especially when extracted with water as a solvent (35.21%), followed by branches (23.85%). In all cases, needles and branches contained high quantities of extractives, in comparison to the rest of the samples from other parts of the tree. In most cases, water as a solvent is more effective than ethanol and dichloromethane. Sapwood appeared to have the lowest quantity of extractives (1,797%, 2.366%), a fact which was expected, based on literature (Grigoriou 1992, of Masendra et al. 2018a, Eberhardt 2012).

Qualitative analyses

The results of the analysis are presented in detail in the following tables (Tables 2, 3, 4, 5 and 6), which contain data from all the three solvents applied, since the interest of this essay was to discover the chemical compound found in the species at first and then at a following research to discriminate and quantify in more detail. From the detailed processing of the data it is obvious that:

- Specimens from different parts of the tree appeared to contain some widely used chemical compounds, some of them in significant amounts, such as **D-Limonene** (heartwood-1.823), **1,4-Methanoazulene** (heartwood-0.281, branches-0.510), **Caryophyllene** (hearwood-0.669), **squalene** (branches-0.135), **methyl abietate** (branches-1.881, needles-1.120), **choladienol** (needles-2.037) and others.
- After analyzing the data, an attempt to interpret the results was made. The chemical analysis revealed that *Pinus* species contained various phenolic compounds with different isomers.
- Needles contained greater amount of **phenol, 2,4-bis (1,1-dimethylethyl)**, **homovanillyl alcohol** and **methyl abietate** than branches.
- **Squalene** appeared in larger quantity at needles than branches, and the smallest at hearwood.
- Branches contained a larger amount of **thiophene** compared to needles and a greater quantity of **longipinane** than heartwood.
- **D- limonene** appeared in larger amounts at heartwood than needles and higher percentage of **caryophyllene** than branches.

Table 3

Chemical compounds found at Pinus pinea heartwood specimens

Pinus pinea heartwood chemical compounds	Integration area/internal standard area	*Pinus pinea* heartwood chemical compounds	Integration area/internal standard area
.alpha.-Pinene	0,007	.alpha.-Caryophyllene	0,118
Benzaldehyde	0,011	Longipinane, (E)-	0,011
.beta.-Myrcene	0,012	Caryophyllene oxide	0,205
D-Limonene	1,823	Ylangene	0,037
Borneol	0,106	Longifolenaldehyde	0,017
Benzenamine, 2-bromo-	0,170	Pyrimidine, 2,4,5-triamino-	0,125
Benzene, 1-bromo-2-nitro-	1,000	Phenanthrene	0,006
1-(2-Vinylphenyl) ethanone	0,011	Tributylacetylcitrate	0,358
(-)-Isosativene	0,015	Methyl abietate	0,055
Naphthalene, 2,6-dimethyl-	0,037	Phenol, 2,2'-methylenebis	0,338
1,4-Methanoazulene	0,281	Squalene	0,058
Caryophyllene	0,669	5-Methyl-2-phenylindolizine	0,088
3,5-Dimethoxybenzaldehyde	0,055		

Table 4

Chemical compounds found at Pinus pinea specimens from branches

Pinus pinea branches chemical compounds	Integration area/internal standard area	*Pinus pinea* branches chemical compounds	Integration area/internal standard area
Phenol, 2,4-bis(1,1-dimethylethyl)	0,208	5-thio-D-glucose	0,068
Homovanillyl alcohol	0,088	2-furancarboxaldehyde	0,738
Benzophenone	0,218	Silane	0,157
Thiophene	0,134	Benzene, 4-ethyl-1,2-dimethyl	0,019
Pyridine, 2-pentyl-	0,874	Benzyl alcohol	0,072

Kaur-16-ene	0,037	Isopinocarveol	0,043
Ethyl oleate	0,235	Benzeneamine, 2-bromo-	0,164
Tributylacetylcitrate	0,136	Benzene, 1-bromo-2-nitro-	1,000
Methyl abietate	1,881	1,4-methanoazulene	0,510
Cedran-diol	0,127	Caryophellene	0,111
Chol-7-en-12-ol	0,377	.alpha.-farnesene	0,029
squalene	0,135	Phenol, 2-methoxy-4-propyl-	0,044

Table 5

Chemical compounds found at Pinus pinea specimens from needles

Pinus pinea needles chemical compounds	Integration area/internal standard area	*Pinus pinea* needles chemical compounds	Integration area/internal standard area
5-amino-1-ethylpyrazole	0,051	2(4H)benzofuranone	0,169
Benzaldehyde	0,038	Homovanillyl alcohol	0,212
1R-alpha-pinene	0,016	Diethyl phthalate	0,583
Isoxazolidine	0,063	Megastigmatrienone	0,160
Phenol	0,022	Longipinane (E)	0,030
Allantoic acid	0,064	Benzeneacetic acid	0,596
Pyridine	0,069	Camphene	0,229
m-ethylaminophenol	0,096	Phytol	0,011
D-limonene	0,026	Tributylacetlcitrate	0,492
Benzoic acid, ethyl ester	0,425	Thiophene	0,429
Benzene, 1-bromo-2-nitro-	1,000	Phenanthrene	2,292
Phenol, 2-methoxy-3-(2-propenyl-)	0,066	Methyl abietate	1,120
3-pyridinecarboxamide	0,084	Squalene	0,180
Benzene-2-fluoro-1,3,5-	0,150	Quinazolin-4(3H)-one (mercaptoacetic acid)	0,133
Piperidine, 1,2-dimethyl-	0,097	Myrtanol, 2-mercapto-	0,085
Phenol, 2,4-bis(1,1-dimethylethyl)	0,490	Chola-5,22-dien-3-ol	2,037

Table 6

Chemical compounds found at Pinus pinea specimens from bark

Chemical compounds of Pinus pinea bark	
Phenol, 2,4-bis (1,1-dimethylethyl)	Benzoyl bromide
Pyridine	Silane, trimethyl
Benzophenone	1-benzoyl-2-piperonylhydrazine
Benzaldehyde	Acetophenone
Benzothiazole	2-furaldehyde diethyl acetol
Cynamide, dibutyl-	Naphthalene

Similar chemical compounds, such as Pyrazine, propylamine, triazine, D-glucose, were found by other researchers as well (Masendra et al. 2019, Miranda et al. 2017).

CONCLUSIONS

Pinus pinea contains a significant amount of extractives, which appear to have notable variation and, as a result, multiple applications in various sectors of industry. This research showed a large amount of water, dichloromethane and ethanol soluble extractives especially in the bark, needles and branches with a tendency to diminish in the heartwood and then in the sapwood.

Needles contain the highest amount of extractives, especially when extracted with water as a solvent (35.21%), followed by branches (23.85%).

In all cases, needles and branches contained high quantities of extractives, in comparison to the rest of the samples from other parts of the tree. In most cases, water as a solvent is more effective than ethanol and dichloromethane. Sapwood appeared to have the lowest quantity of extractives (1,797%, 2.366%), a fact which was expected, based on literature (Grigoriou 1992, of Masendra et al. 2018a, Eberhardt 2012).

- Specimens from different parts of the tree appeared to contain some widely used chemical compounds, some of them in significant amounts, such as D-Limonene (heartwood-1.823), 1,4-Methanoazulene (heartwood-0.281, branches-0.510), Caryophyllene (hearwood-0.669), squalene (branches-0.135), methyl abietate (branches-1.881, needles-1.120), choladienol (needles-2.037) and others.
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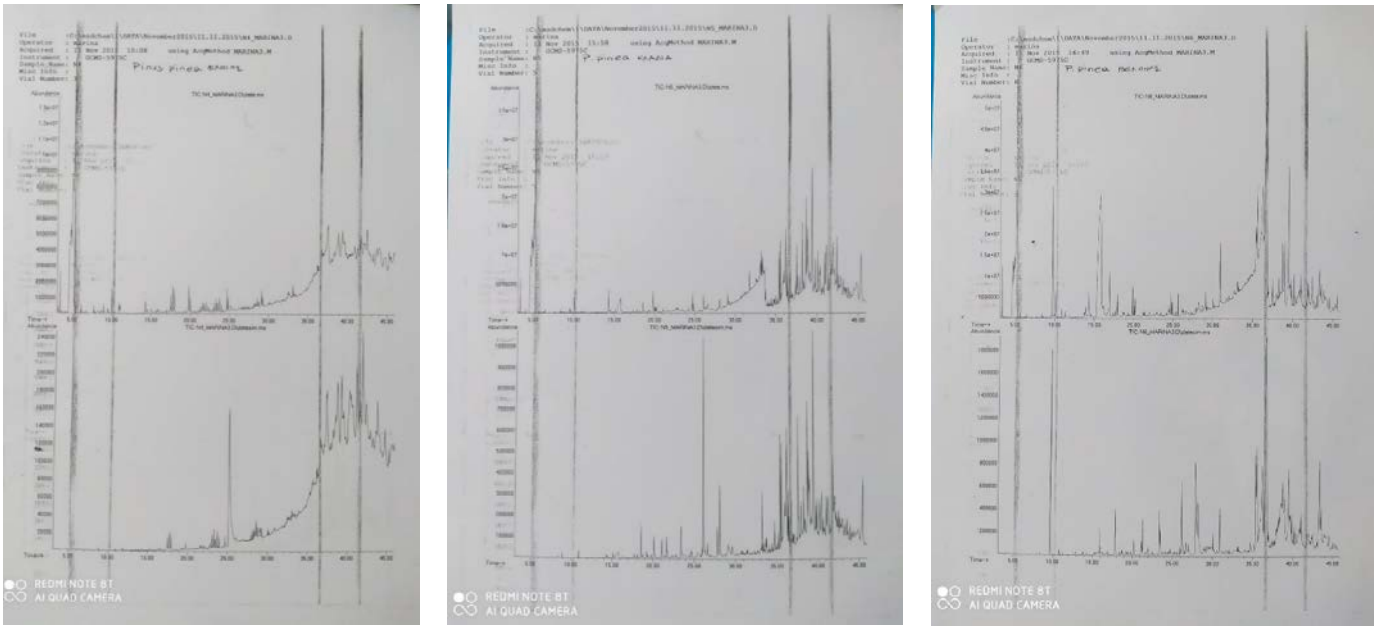
Further investigation needs to be conducted, so as to utilize the maximum of the products nature has to provide, in order to turn to more natural products and a healthier way of life.



a. ***b.*** ***c.***

Fig. 6.
Indicative chromatograms of:
a. Pinus pinea bark; b. Pinus pinea branches; c. Pinus pinea needles

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Declaration of interest statement

No potential conflict of interest was reported by the authors.

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