Effect of harvest date on yield, chemical composition and antimicrobial activity of *Artemisia herba-alba* essential oil

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Abstract— within the framework of Moroccan aromatic and medicinal plants valuation, the effect of harvest date on yield, chemical composition and antimicrobial activity of *Artemisia herba-alba* essential oil (Imouzzer Marmoucha region, Morocco). The average essential oil yields of this species collected in March, June, September and December were 0.20; 1.19; 0.60 and 0.74 % (v/w) respectively. These essential oils were subjected to the gas chromatography coupled to mass spectrometry analysis. The results revealed three major compounds; ß-thujone, artemisia alcohol and α-phyllandrene for March collection and nordavanone, artemisia alcohol for that of June. On the other hand, the collections of September and December are characterized by artemisia alcohol followed by 6-camphenone, cis-hydrate acetate, sabinene and ß-thujone as main constituents. The effect of harvest date on essential oil's antimicrobial activity was also highlighted.

Index Terms— *Artemisia herba-alba*, essential oil, harvest date, chemical composition, gas chromatography, antimicrobial activity.

1 INTRODUCTION

Morocco is known for its large diverse and rich plant heritage. Among its natural resources, the aromatic and medicinal plants occupy a wide place and play a large part in the state economy [1], [2a], [2b]. Morocco is also considered at the moment, as one of the main suppliers and producers of some aromatic and medicinal plants such as white wormwood (*Artemisia herba-alba*), rosemary (*Rosmarinus officinalis*), mint (*Mentha pulegium*) and lavenders (*Lavendula stoechas* and *Lavndula dentata*). These plants are at the origin of very high added value products contributing to the economic development of Morocco and providing the quality criteria required on the international market. Nowadays, the big desire to return to the nature, in spite of the important development of chemistry, biochemistry and organic analysis in the medical field, explains the great interest accorded to the plant natural products. Among the well-known aromatic and medicinal plants existing in Morocco, *Artemisia herba-alba*, a perennial shrub belonging to the *Asteraceae* family, is low woody always green, in vegetative growth in autumn (large leaves), from the end of the winter and in spring (smaller leaves) [3]. This plant grows in the Est, Est Rif, Middle Atlas, High Atlas and the saharan Anti-Atlas regions, over an estimated area of about 1.5 million of ha [4].

In Morocco, *Artemisia herba-alba* or "Chih" in Arabic, is considered as a panacea in traditional medicine (emmenagogue, antidiarrheic or as diuretic agent, antispasmodic...) [5], [6]. An old saying of the South Moroccan population is the illustration: "which knows Chih cannot pass in quoted without taking it". The *Artemisia herba-alba* essential oil is widely known by its biological activities, such as antimicrobial [7], [8], [9], antioxidant [10], [11], [12], [13], anti-inflammatory [14] and insecticidal activity [15]. Hence, the objective of the present study is to valorize our plant heritage by the follow-up of the effect of harvest date on the yield and the chemical composition of *Artemisia herba-alba* essential oil harvested in Imouzzer Marmoucha region as well as the evaluation of its antibacterial and antifungal efficacy.

2 MATERIALS AND METHODS

2.1 Plant Material

The aerial part (leaves and stalks) of *Artemisia herba-alba* was harvested in March, June, September and December (2013) from Imouzzer Marmoucha region (Morocco) according to the AFNOR standard [16].

2.2 Target microorganisms

The antimicrobial activity of Artemisia herba-alba oil was evaluated against eleven microbial strains.

Bacterial strains include; *Bacillus subtilis* (Gram-positive), *Escherichia coli* (Gram-negative), *Micrococcus luteus* (Gram-positive) and *Staphylococcus aureus* (Gram-positive).

Molds include; *Aspergillus niger*, *Penicillium digitatum* and *Penicillium expansum*.

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Wood decay fungi of wooden work decay were; *Gloeophyllum trabeum*, *Poria placenta* and *Coniophora puteana*.

The four pathogenic bacteria are chosen for their implications in several infections occurring in humans and animals. Moreover, they are recognized to develop resistance against the commonly used antibiotics. Furthermore, the three selected fungi are agents of decay in common food and can be toxic and pathogenic for humans and animals. Finally, the four wood rot fungi are responsible for brown and white rot of wood. They are the most important wood-destroying fungi. They were chosen in this work as an attempt to control the considerable damage caused in buildings, wood that contacts with the soil (poles and railways) or buildings (bridges) [17], [18].

Bacterial strains are maintained in glycerol 20 % at -20 °C. Before their use, they are revivified by subculture on nutrient agar for 24 hours at 37°C. Molds and wood decay fungi belong to the Mycotheque's collection int Microbiology Laboratory of the Forestry Research Centre, Rabat (Morocco). They are regularly maintained by subculture on nutriment medium PDA (Potato Dextrose Agar).

2.3 Essential oil extraction

The fresh aerial part (leaves and stalks) of the studied plant was hydrodistilled using a Clevenger-apparatus [19] for 3 h. The average oil contents were calculated and expressed in (%) mL/100 g of dry plant material. The essential oil obtained was separated from the floral water and dried with anhydrous sodium sulfate. Then, it was stored at 4 °C in the dark for further analysis

2.4 Chromatographic analysis of essential oils

The chromatographic analysis of *Artemisia herba-alba* essential oil obtained in different months was performed on a gas chromatographer with electronic pressure control, type Hewlett Packard (HP 6890) equipped with a HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm), a FID detector set at 250°C and using a H2/Air mixture, and a split-splitless injector set at 250 °C. The injection mode was split (split ratio: 1/50, flow rate: 66 ml min) and the injected volume was about 1 µL. Nitrogen was used as carrier gas with a flow rate of 1.7 mL/min. The column temperature was programmed from 50 to 200 °C at a heating rate of 4°C/min, during 5 min. The apparatus was controlled by a "ChemStation" computer system.

The components identification was based on the comparison of their mass spectra (GC/MS), respective with spectra of the library NIST 98 [20] and based on the Kovats index (KI) calculation [21]. Generally, the technique of KI is widely used to identify the usual essential oils' compounds, but it is insufficient to determine the total chemical composition. The specific KI tables to each product are proposed in the literature. They were developed using analyzes on different types of columns. These benchmark index are compared to those calculated from our samples.

2.5 Antimicrobial assays

The antimicrobial activity of the tested essential oil was determined according to the previously reported methods [22], [23]. Because of the essential oil immiscibility with water, the agar at 0.2 % (w/v) was used as an emulsifier. It allowed to obtain a homogeneous distribution of the essential oil and to make at the maximum the compounds/germ contact. Essential oil dilutions were prepared at 1/10e, 1/25e, 1/50e, 1/100e, 1/200e, 1/300e and 1/500e in this agar solution.

In test tubes, containing each 13.5 mL of the sterile solid medium, TSA (Tryptic Soy Agar) for bacteria, and PDA for fungi, sterilized with autoclave during 20 min at 121°C and maintained at 55°C in a water bath. Afterwards, 1.5 mL of each dilution was added aseptically to achieve the final concentrations of 1/100, 1/250, 1/500, 1/1000, 1/2000, 1/3000 and 1/5000 (v/v). Tubes were vortexed to disperse properly the essential oil in the culture medium before pouring them into sterile Petri dishes. The free-drug control was also prepared.

For bacteria and mold, inoculation was done by streaking with a calibrated platinum loop, to collect the same volume of inoculum. Microbial inoclums were a broth culture of 24 hours for bacteria and spores' suspension prepared in physiological saline from a culture of seven days in PDA.

For wood decay fungi, inoculation was done by placing fragments (1 cm in diameter) taken from the periphery of a mycelium cultured for 7 days in PDA. Incubation was performed at 37°C during 24 h for bacteria and seven days at 25°C for mold and wood decay fungi. Each test was done in triplicate.

3 RESULTS AND DISCUSSION

3.1 Effect of harvest date on the essential oil's yield

Figure 1. shows the averages of the essential oil yields obtained in different harvest months. Plant harvested in March (vegetative period) provided a yield of 0.20 ± 0.01 %, to reach a maximum of 1.19 ± 0.25 % in June (floral period), and then descends to reach a value of 0.60 ± 0.07 % in September (fruiting period). However, the rains of September cause a new growth period and the average yield of essential oil increases to reach a rate of 0.74±0.001 % in December.

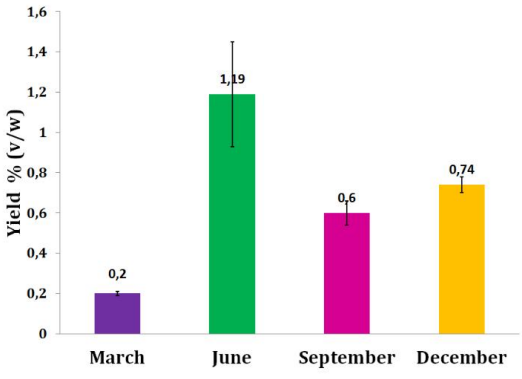

Fig 1. Yield in essential oil of *Artemisia herba-alba* according to the harvest date

Indeed, the vegetative growth of *Artemisia herba-alba* takes place in autumn, the blooming begins in June and develops essentially at the end of summer [24]. During the rainy years in the suitable soil *Artemisia herba-alba* presents a strong production of seeds and a high power of regeneration [25], [26]. Hence, highest yield was obtained in June. Our results are in agreement with previously published works [8], [27], and [28]. These authors worked respectively on essential oils of *Artemisia herba-alba*, *Artemisia mesatlantica* and *Juniperus phonicea*, they found that the best yield coincides with the blooming stage.
Likewise, a study on Italian *Artemisia verlotiorum* essential oil shows that the yield reaches a maximum in April (0.60 %) and a minimum in January (0.1 %) [29]. In Spain the yield of *Artemisia herba-alba* essential oil harvested in four different localities varies from 0.41 % to 2.30 % [30]. These discrepancies between the white wormwood oil yields depend upon numerous factors, such as the stage of growth, pedoclimatic and edaphic conditions, plant part and method used for the extraction [8].

It was reported that the essential oil yield during growth is particularly sensitive to the daily temperature and the environmental conditions namely, light, nutrient availability and the day's lenght [31], [32].

3.2 Effect of harvest date on the essential oil composition

The harvest date effect on the chemical composition of *Artemisia herba-alba* oil was investigated. Results were presented in table 1.

There was a difference in term of number and amount of the identified compounds in the studied essential oil depending upon the harvest date. Twenty four compounds were identified for March harvest and eighteen compounds for June. For the harvests of September and December, 40 and 31 compounds were identified respectively. These compounds represent approximately 96.44%, 99.98%, 99.93% and 99.84% of the essential oil composition of plants harvested in March, June, September and December respectively.

On one hand, the samples harvested in September and December provided essential oils with artemisia alcohol as main constituent (26.36% and 31.81% respectively) followed by the 6-camphenone (15.9% and 27.85% respectively), ß-thujone (10.91% and 14.24 % respectively), cis-sabinene hydrate acetate (13.63% and 2.44% respectively) and trans-sabinene hydrate (6.16% and 8.67% respectively) (Table 1). Moreover, other compounds qualified as minor were found in both essential oils, such as chrysanthenone, cis- sabinene hydrate, α-phyllandrene. On the other hand, samples harvested in March was characterized by the presence of ß-thujone as major compound followed by artemisia alcohol and α- phyllandrene with respective rates of 43.03%, 10.88% and 10.04%. Other compounds were also identified, but with relatively less contents, such as trans-sabinene hydrate, cis-sabinene hydrate, artemisia ketone and dihydromercynol (Table 1).

However, as shown in table 1 the chemical composition of the essential oil extracted from the sample harvested in June was characterized by the presence of nordavanone as main constituent (27.09%), followed by artemisia alcohol (22.28%) and α-thujone (21.1%). Other compounds were also identified with relatively low percentages such as trans-pinene hydrate, iso-3-thujanol, 1-4 cineole, ß-thujone and artemisia alcohol.

According to these findings, the chemical composition of *Artemisia herba-alba* essential oils obtained in the four harvest periods (March, June, September and December), showed important difference qualitatively and quantitatively.

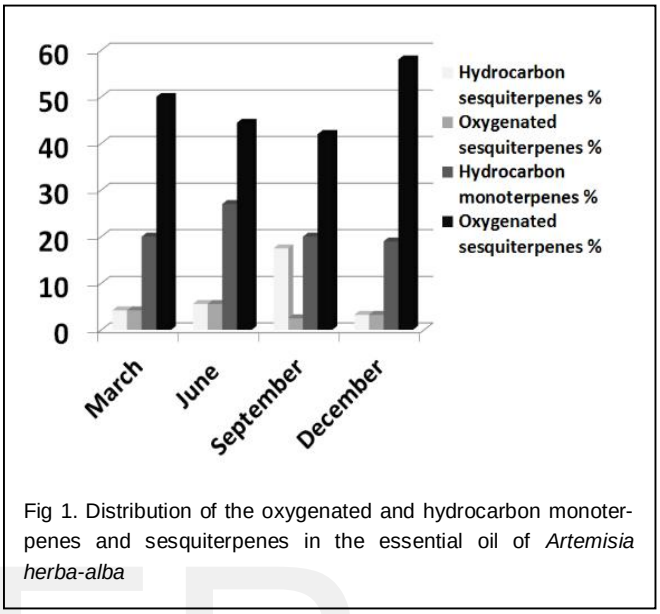

Fig 1. Distribution of the oxygenated and hydrocarbon monoterpenes and sesquiterpenes in the essential oil of *Artemisia herba-alba*

It can be also noted that the essential oil of June was the richest in nordavanone and α-thujone with regard to the other oils. On the other hand, the oil of March was characterized by the abundance of ß-thujone. However, the essential oils of September and December samples were especially rich in artemisia alcohol. These results could be considered as criteria to differentiate between these samples.
The chemical variability noted during the qualitative and quantitative analysis (according to the harvest periods) could give some explanations about the biosynthesis pathways of these main constituents.

Indeed, it is important to emphasize that during March (vegetative period), the biosynthesis pathways of α-phyllandrene, artemisia alcohol and ß-thujone are favored with the ascendancy of the latter. Furthermore, during June (floral period) the biosynthesis of α-thujone, artemisia alcohol and nordavanone are favored. However, during September and December periods, which coincide with the biotic and abiotic stress conditions, the formation of artemisia alcohol and 6-camphenone is favored.

The variation of the essential oil chemical composition in the wild populations is associated with the differences in the genetic profile. Some authors underline that in the hierarchy of factors at the origin of the terpene metabolism, a body genetically determined as "the basic allocation", limits the effects upstream to the environment on the secondary metabolism of plants [33], [34]. The difference of contents in these compounds directs the biosynthesis to the preferential formation of precise products, [8], [27].

TABLE 1
CHEMICAL COMPOSITION OF *ARTEMISIA HERBA-ALBA* ESSENTIAL OIL OF IMOUZZER MARMOUCHA REGION OBTAINED IN DIFFERENT HARVEST PERIODS

Kovats Index	Constituent	Area peak (%)			
		March	June	September	December
921	Tricyclene	0.55	0.31	-	0.18
924	α-thujene	-	0.31	0.84	-
932	α-pinene	0.27	0.62	1.13	0.59
946	Camphene	-	-	0.14	0.31
969	Sabinene	0.21	-	0.63	-
971	Artemiseole	-	-	1.13	-
988	Myrcene	1.64	-	0.13	0.72
1002	α-phyllandrene	10.04	0.67	3.19	1.2
1014	α-terpinene	-	2.49	-	-
1024	limonene	-	-	0.33	-
1055	Artemisia ketone	5.1	-	0.1	-
1065	Cis- sabinene hydrate	5.57	-	4.23	2.81
1069	Dihydro myrcenol	3.24	1.64	-	-
1081	Artemisia alcohol	10.88	22.28	26.36	31.81
1095	6-camphenone	-	-	15.9	27.85
1098	Trans sabinene hydrate	9.28	-	6.16	8.67
1099	α- pinene oxide	0.17	-	-	-
1101	α-thujone	1.27	21.1	0.67	1.09
1111	6-camphenol	-	-	1.25	-
1112	β-thujone	43.03	1.67	10.91	14.24
1119	Trans pinene hydrate de	1.53	8.98	-	0.21
1122	α-campholenal	-	-	-	0.74
1124	Chrysanthenone	0.19	-	5.05	0.82
1130	1-terpineol	-	-	-	0.7
1134	Iso-3-thujanol	1.45	7.75	1.39	0.7
1141	Camphor	1.42	0.8	0.14	0.17
1147	Neoiso-3- thujanol	-	0.87	0 .02	0.14
1159	Trans-β-terpineol	-	-	0.47	0.84
1164	3-thujanol	0.42	-	0.33	0.17
1186	α-terpineol	-	-	0.08	0.19
1199	Y-terpineol	-	-	-	0.07
1207	Trans-piperitol	-	-	-	0.06
1219	Cis- sabinene hydrate acetate	0.14	-	13.63	2.44
1228	Nordavanone	2,03	27.09	0.38	0.32
1235	Trans chrysanthenyl acetate	-	0.32	1.08	0.36
1253	Trans sabinene hydrate acetate	0.14	1.32	-	-
1267	Iso-3 thujanol acetate	1.08	-	0.41	0.52
1269	Neo-3-thujanol acetate	0.01	0.69	-	-
1295	3-thujanol acetate	-	-	0 .25	-
1327	mertenyl Acetate	-	-	-	1.61
1335	δ-elemene	0.2	1.31	1.01	-
1346	α-Terpinyl acetate	-	-	0.04	-
1385	Trans acetate myrtanol	-	-	0.17	0.09
1389	Iso-longifolene	-	-	0.27	-
1400	β-longipinene	-	-	0.08	-
1417	e-caryophyllene	-	-	1.09	-
1434	γ-elemene	-	-	0.17	-
1499	α-muurolene	-	-	0.02	-
1505	α-thujaplicinol	-	-	0.01	0.2
1513	γ-cadinnene	-	-	0.03	0.02
1863	Cis-thujopsenic acid	-	-	0.03	-
	Totale %	96.44	99.98	99.93	99.84

The chemical composition of the studied *Artemisa herba-alba* essential oils were different from that of Guercif region (Oriental of Morocco) [8]. In which the authors found that the main constituents were the chrysanthenone (47.71% to 0.28 %) and the camphor (24.59 % to 45.03 %). Likewise, they were different from that of Benifouda region (Algeria) which is dominated by camphor (33.1%) and chrysanthenone (2.2%). As for the *Artemisia herba-alba* essential oil of Bir Elhafy region (Tunisia), which presents α-thujone (24.88%) and D-germacrene (14.48%) as main compounds. All these results showed that the chemical composition of *Artemisia herba-alba* essential oil is very variable and depends upon the vegetative stage of the plant in relationship with the climatic and geographical factors such and the nature of the soil [8].

3.3 Antimicrobial activity of Artemisia herba-alba essential oil

Table 2. shows the antimicrobial activity of the essential oil obtained from different harvest samples of *Artemisia herba-alba.* As can be noted from this table, the wood decay fungi were the most susceptible microorganisms to the studied essential oil. Especially the essential oil of sample harvested in December which inhibited the growth of *C. versicolor*, *C. puteana* and *P. placenta* from the dilution of 1/3000. While, the growth of *G. trabeum* was inhibited from the dilution of 1/2000 of the essential oil provided by the samples harvested in June and December. The essential oils of the other samples (March and September) inhibited the growth of the wood decay fungus from the dilution of 1/1000.

As regards to the molds, the essential oil of December has inhibited the growth of *A. niger* from the dilution of 1/2000, this strain was the most susceptible, while *P. expansum* and *P. digitatum* were more resistant to all essential oils tested, they were inhibited from the dilution of 1/500 of the essential oils provided by the samples of March and June and from the dilution of 1/250 for the sample of September.

Finally, the growth of bacteria was also inhibited by some essential oils tested. In fact, *E. coli* was the most susceptible bacterial strain, it was inhibited by the essential oil of December and March from the dilution of 1/2000 and 1/1000 respectively. While those of September and June inhibited this strain from the dilution of 1/250 and 1/100 respectively. The Gram-positive bacteria were more resistant, especially *B. subtilis* which was inhibited only by the essential oil of September from the dilution of 1/250. Whereas, *M. luteus* was inhibited by two essential oils (September and June) from the dilution of 1/250. *S. aureus* was inhibited by the essential oil of September from the dilution of 1/250 and that of December at the dilution of 1/100.

TABLE 2
ANTIMICROBIAL ACTIVITY OF *ARTEMISIA HERBA-ALBA* ESSENTIAL OILS

Dilution v/v	1/100				1/250				1/500				1/1000				1/2000				1/3000				1/5000				C
Samples	M	J	S	D	M	J	S	D	M	J	S	D	M	J	S	D	M	J	S	D	M	J	S	D	M	J	S	D	
Bacteria																													
E. coli	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M. luteus	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Molds																													
A. niger	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
P. expansum	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. digitatum	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
wood decay Fungi																													
C .versicolor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-	+	+	+	+	+
C. puteana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	+	+
P. placenta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-	+	+	+	+	+
G. trabeum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+

(-) inhibition, (+) growth, (C) free-drug control, (M) March, (J) June, (S) September, (D) December

According to these findings, the *Artemisia herba-alba* essential oil provided by the sample of December was the most active against the fungal strains tested and the Gram-negative bacteria (*E.coli*). While the essential oils of June and March exhibited a remarkable antifungal effect compared to that of September. However, this latter showed the highest antibacterial effect against all strains tested. In fact, the high content of hydrocarbon and oxygenated monoterpenes in the essential oil provided in September and probably their synergetic interactions could be responsible for the remarkable antimicrobial effect of this essential oil. Previously published data showed the antimicrobial efficacy of the essential oils of several plants belonging to the *Asteraceae* family [35], [36], [8], [37], [38]. However, it has been reported that *A. campestris* with high content of hydrocarbon monoterpenes exhibited weak antimicrobial activity [39]. The antimicrobial effectiveness of the oxygenated terpenes, compared to the hydrocarbon ones, has been reported by several authors [8], [27], [12].

The fungal strains tested were the most susceptible to the *Artemisia herba-alba* essential oil than bacteria, this finding is in agreement with other studies which showed the remarkable susceptibility of fungi to the essential oil compared to bacteria [40], [41], [42]. Some previous works also reported the antimicrobial efficacy of the essential oil of *Artemisia herba-alba* harvested from Taforalt [36] and Guercif regions [8].
Overall, the antimicrobial activity of *Artemisia herba-alba* essential oil could be due not only to its major compounds, but also to the interactions between all compounds present in this oil even the minor compounds. However, some studies have reported that whole essential oils usually have higher antibacterial activity than the mixtures of their major components, indicating that the minor components play a key role in this outcome 43], [44], [45], [46], [47].

4 CONCLUSION

Overall, the present study showed that the yield, the chemical composition and the antimicrobial activity of the *Artemisia herba-alba* essential oil strongly depend strongly on the harvest date. The best yield was found in June. The bioassay has demonstrated that the essential oil obtained by different harvest samples exhibited remarkable antimicrobial activity against bacteria and fungi. These latter were most susceptible to all tested essential oils. These preliminary results showed that this essential oil could be a promising agent to be valorized in the food and pharmaceutical industry or in wood preservation.

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