In vivo evaluation of toxicity and skin tolerance of the essential oils of *Rosmarinus officinalis* and *Populus alba*

Belkhodja Hamza[1*], Belmimoun A., Side Larbi K.[1], Tir Touil Aicha[1], S. Kamel[2]

[1]Laboratoire de Bioconversion, Génie Microbiologie et Sécurité Sanitaire, Department of Biology, Faculty of Nature and Life Sciences, University of Mascara, Algeria

[2]Laboratoire de Pharmacie Clinique, université de Picardie, Amiens (France)

*: hbelkhodja@univ-mascara.dz , Tel!Fax: 213 45 70 70 14

Abstract

The plants were still used for therapeutic reasons. They represent an important source of many chemical structures of compounds. To avoid any possible risk of toxicity in the using of these compounds, it is necessary to conduct toxicity tests. For this purpose, the study of the acute toxicity was carried out on Wistar rats that received doses of 0.5 to 7 mg / Kg of the extract of the essential oil by intra-peritoneal injection. As part of the pre-clinical studies, the test of skin tolerance of both essential oils was conducted by the measuring of primary irritation index. The results indicate the absence of severe clinical signs and dead rats during the 14 days of observation. Thus, the essential oil of *Populus alba* and even *Rosmarinus officinalis* administered intraperitoneally has no acute toxicity in rats. In other hand, we find that the essential oil of *R. officinalis* is non-irritating to the skin while the essential oil of *P. alba* is slightly irritating. Evaluation of extract's toxicity and skin irritation potential is essential to ensure the safety of people in contact with substances in pharmaceutical applications.

Keywords: extraction; essential oils; *Populus alba*; *Rosmarinus officinalis*; acute toxicity; irritation.

Résumé.

Les plantes ont été utilisées pour des raisons thérapeutiques. Ils représentent une source importante de nombreuses structures chimiques de biomolécules. Pour éviter tout risque possible de toxicité dans l'utilisation de ces composés, il est nécessaire d'effectuer des essais de toxicité. A cette fin, l'étude de la toxicité aiguë a été réalisée sur des rats Wistar qui ont reçu des doses de 0,5 à 7 mg / Kg de l'huile essentielle par injection intraperitoneale. Dans le cadre des études précliniques, le test de tolérance cutanée des deux huiles essentielles a été réalisé par la mesure de l'indice d'irritation primaire. Les résultats indiquent l'absence de signes cliniques sévères et de rats morts pendant les 14 jours d'observation. Ainsi, l'huiles essentielles de *Populus alba* et même *Rosmarinus officinalis* administrées par voie intrapéritonéale n'a pas de toxicité aiguë chez les rats. En revanche, nous constatons que l'huile essentielle de *R. officinalis* est non irritante pour la peau tandis que l'huile essentielle de *P. alba* est légèrement irritante. L'évaluation de la toxicité de l'extrait et du potentiel d'irritation de la peau est essentielle pour assurer la sécurité des personnes en contact avec des substances dans des applications pharmaceutiques.

Mots clés: Huile essential; *Populus alba*; *Rosmarinus officinalis*; toxicité aiguë; irritation.

I. Introduction

Natural products are of great interest for the various sectors such as cosmetics, pharmaceuticals, food and industry **[1].** Currently, the World Health Organization (WHO) estimates that about 80% of people use traditional herbal because the plants were able to demonstrate effectiveness. In addition, side effects induced by the drugs concerned users who turn to less aggressive care for the body **[2].** Indeed, these plants are often characterized by the biosynthesis of odorous molecules which are called "essential oils". These bioactive molecules were used; given their therapeutic properties in the treatment of several diseases affecting human health**.** Among these pathologies, we cite severe musculoskeletal disease affecting bone structure and stability of the articular cartilage and what has became known as "osteoarthritis". Osteoarthritis (OA) is a joint disease which results from a complex system of mechanical, biological, biochemical or molecular interactions. The degeneration of joint cartilage originating from the destruction of the extracellular matrix of chondrocytes despite the repair witch targeting the recovery of the homeostatic balance between synthesis and degradation of matrix components. This degeneration is the cause of the onset of fibrillation, cracks and ulceration. Although cartilage degradation is a characteristic of osteoarthritis, the inflammation of the synovial membrane also significantly participates in the installation of the pathology **[3].**

View the alternative actions of plants our work aims to exploit natural bioactive components from well-chosen plants "*Rosmarinus officinalis* and *Populus alba*". We seek first to study the organoleptic properties of essential oils of Rosemary and white poplar. Then, the efficiency of these essential oils in the treatment of experimental models induced by osteoarthritis. This work was carried out at the laboratory of the University of Mascara, beginning the month of May 2013.

II. MATERIALS AND METHODS

2.1. Plant material

It is constituted of aerial parts (leaves and flowers) **[4]** of the two plant species; *Rosmarinus officinalis* and *Populus alba*. The plants were collected in the Mascara region during the month of April-May 2013. They are identified by the botanist of the department of Biology.

2.2. The experimental animal

The Wistar rats used in these experiments were provided by the laboratory of the University of Mascara. Animals were housed at the cage, with water and food *ad libitum*, and the animal room temperature was kept at constant temperature of 20 ± 1 °C on a 12-hour light/12-hour dark cycle. Adequate measures were taken to minimize pain or discomfort of the animals, and all experimental procedures were performed in accordance with the ethical guidelines of the Organization for Economic Cooperation and Development (OECD).

1.1. Extraction of essential oils:

The extraction of essential oils from two plants (*Rosmarinus officinalis* and *Populus alba*) was conducted in the laboratories of the University of Mascara. The extraction of essential oils was carried out by hydrodistillation in a Clevenger apparatus. 100 g of leaves and flowers of each plant was boiled. When the temperature stabilizes, we begin to collect the distillate in an Erlenmeyer. We add about 18 g of sodium chloride (NaCl) to the distillate. We stir until dissolved, then it was placed in a separating funnel and we achieve three successive washes (10, 10, 20 ml) of cyclohexane. After agitation, the organic phase is recovered. We dry the product with a little anhydrous sodium sulfate. Then we make a concentration by rotary evaporator to obtain the essential oil. The essential oil obtained was stored at + 4 ° C after the calculation of the yield of extraction.

1.2. Organoleptic characters:

According to AFNOR NF ISO 280: 1999, essential oils must respond to analytical characteristics that are established by international committees of experts. To know the quality of the essential oil of *Rosmarinus officinalis* and *Populus alba*, was conducted organoleptic tests like color and odor.

1.3. Acute toxicity test:

To assess the acute toxic effects of the essential oil of *Rosmarinus officinalis* and even *Populus alba*, a measure of the lethal dose 50 (LD50) is required. Male and female albino rats have an average weight of 150 ± 5g (females) and 220 ± 8 g (males) were used. Rats are selected according to sex in cages each carries 5 animals. Acute toxicity was estimated using the method described by Tahraoui and colleagues (2010) **[5]**, which is to distribute the rats in 05 groups of 10 animals (05 males and 05 females) who received single doses of 0.4- 1 mg / kg

of the extract of the essential oil of *P. alba* and from 1 to 7 mg / kg of the extract of the essential oil of *R. officinalis* and 9 ‰ NaCl (control) by intraperitoneal injection. After administration of the extracts, the rats were continuously monitored in the first, sixth and 24th hour after treatment, for any death or change in behavior. These signs of toxicity were monitored daily for 14 days. In the 15th day, the number of dead animals is calculated and converted into a percentage.

2.6. Effect of essential oils against musculoskeletal disorders in Wistar rats

2.6.1. Preparation and rat's anesthesia

The operations are conducted in accordance with the welfare of the animal, excluding any stress and nervousness may interfere with the results. A total of 25 male Wistar rats weighing 190-260 g were used in the study of the effects of essential oils extracted from *R. officinalis* and *Populus alba.* Rats were placed by 5 in cages, with access to a standard food and water *ad libitum.* The experiments begin after a period of acclimatization of animals: Group 01: Normal rats, Group 02: Induced rats and untreated, Group 03: Induced rats +treated with EO of *R. officinalis*, Group 04: Induced rats + treated with EO of *P. alba*, Group 05: Induced rats +treated with Voltum. All experimental procedures were performed in accordance with the ethical guidelines for the study of experimental pain in conscious animals and the Council Directive of the European Communities 86/609 / EEC, with all the measures adequate being taken to minimize pain or discomfort to the animals. For the induction of osteoarthritis, the rats were anesthetized with isoflurane **[6].**

2.6.2. Induction

The choice of the model of osteoarthritis turned to unilateral intra-articular injection of a mono-iodo acetate (MIA) (0,3 mg) solution prepared in saline. Under anesthesia, the femorotibial joint is immobilized and a needle is inserted inside of the articular capsule through the patellar ligament. The MIA was dissolved in physiological water solution and administered in a volume of 50 µl. The left control knee was injected with physiological saline water. The basal readings were established with a group of rats that were injected with saline in their knees **[7].** MIA acts directly on the balance of the metabolic activity of chondrocytes and induces a loss of functional properties of cartilage. Energy intake of chondrocytes derived from the physiological process of glycolysis. MIA disrupts glycolysis by inhibiting the activity of dehydrogenase glyceraldehyde 3-phosphate enzyme, resulting in a decrease in metabolic synthesis of cells and optionally to necrosis **[8].**

2.6.3. Treatment

The development of a treatment must be considered that the signs and symptoms vary according to the affected joint and by stage of disease progression **[9].**When the condition is already present, the therapies are directed to symptoms such as pain, instability, joint weakness and decreased function of the joint. The NSAIDs are the most common analgesics to treat pain associated with osteoarthritis **[10].**To highlight the effect of our essential oils against this model of osteoarthritis, treatment given to induced rats consists of a dose of 50 µl of each essential oil (*R. officinalis* and *Populus alba*) and even drug Voltum. Taking this dose is performed by muscular route throughout the treatment period with an interval of 02 days to avoid adverse drug reactions of drug Voltum in overdose.

2.6.4. Body weight of rats

The body weight of rats was measured using a Sartorius balance (BP 610, precision: 0,01g). Taking the body weight of the rats was carried out 7 days after the MIA injection. Then, the growth of rats was monitored every 05 days during the period of treatment with the extracts essential oils and Voltum.

2.6.5. Determination of biochemical parameters

Blood was sampling every 7 days and it is immediately collected into tubes containing EDTA anticoagulant. After centrifugation of 4000 tours / min for 10 min, the serum was recovered and stored at -20 ° C until determination of the biochemical parameters. It is possible to characterize neurophysiological events involved in the pain associated with degenerative processes of osteoarthritis by analyzing the concentrations of some biomarkers of inflammation **[11].** To determine the effect of extracts the essential oils of *R. officinalis* and *P. alba* and even Voltum,

monitoring of some biochemical parameters will be paramount. The selected parameters are as follows: the C-reactive protein; alkaline phosphatase (ALP), serum calcium, creatinine, aspartate amino-transferase (AST), alanine aminotransferase (ALT).

2.7. Statistical analysis

The values are expressed as mean ± standard error of mean (SEM).The results of the different tests are analyzed by ANOVA single factor for multiple comparisons. The P values less than 0.05 ($p < 0.05$) are considered statistically significant.

II. RESULTS AND DISCUSSIONS

3.1. Extraction yields

The essential oil content, obtained from the aerial parts (leaves + flowers) is 1.29% for the Rosemary and 0.9% for white poplar. The yields of essential oils from two species are widely variable. The yield of essential oil of *Rosmarinus officinalis* is higher than that quoted by Bekkara Atik *et al.* (2007) **[7]** and those of Rouabeh (2010) **[12]** where the quantities obtained by these two works are respectively 0.8% and 0.9%. Indeed, the extraction yield, as the quality of EO, are influenced by the type of soil on which the planting is done, the material of the equipment used, the cleanliness of the equipment, the operating pressure, regularity the heating, the cooling of the distillate, method and distillation time **[13].**

2.1. Organoleptic characters

It is through the organoleptic properties (appearance, color, smells) that it is possible to define that oil is of adequate quality. After comparison with the standard AFNOR method, the organoleptic properties of essential oils obtained from the two species suggest an essential oil of very good quality. For *R. officinalis*, the EO has an oily liquid aspect, yellow with a powerful scent of rosemary flowers. While the species *P. alba*, the EO has an oily liquid aspect, pale yellow.

2.2. Acute toxicity

After administration of rosemary oil and white poplar with gradual doses, observations over a period of 14 days showed no severe clinical symptoms of pain, despite some common signs seen as anorexia, hypoactivity, which are reversible and have appeared in rats for a short time and then they returned to their activity. The absence of mortality and clinical signs therefore indicates that the essential oils of *R. officinalis* and *P. alba* are devoid of acute toxicity in rats.

2.3. Effect of essential oils against musculoskeletal disorders in Wistar rats

2.3.1. Body weight of rats

We focused initially to monitor the evolution of the weight curve, given that the rats received the same food and were weighed on the same scale. Regular monitoring of body weight of normal rats and treated with the essential oil of *R. officinalis*, *P. alba* and Voltum has led us to obtain the results shown in figure N° 01.

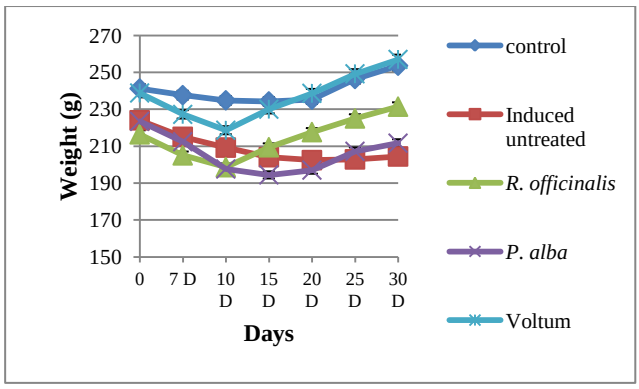

Figure n° 1: Evolution of body weight (g) in normal rats and treated with essential oil of *R. officinalis*, *P. alba* and Voltum during the experimental period. mean ± SEM (n=05). $p<0.05$.

in rats injected by MIA compared to the control group. This could be explained by the direct effect of unconscionable injury and pathophysiological factors of osteoarthritis process. Body weight reduction is used as an indicator of the deterioration of the general health of the rats. Thus, this reduction in weight may be associated with the decrease in food taken daily after administration of MIA.

While it recorded a significant improvement in body weight in the first days of the administration of the essential oil of *R. officinalis* and Voltum comparing to other group of rats. Similarly, low growth was recorded in rats treated with the essential oil of *P. alba* after 15 days of experimentation. By contrary, untreated rats induced undergo a significant drop in weight explained by the continuous influence of MIA in the rat body, and more precisely at the joint.

Biochemical Markers:

The C-reactive protein (CRP)

According to the results, the CRP values in rats of 04 lots (Induced untreated, *R. officinalis*, *P. alba*, Voltum) were remarkably high after 07 days of intra-articular injection of MIA since they exceed the

reference values in the normal rats (<300µg / mL). While the administration of various treatments (essential oil of *R. officinalis*, *P. alba*, Voltum) by intramuscular injection will decrease gradually to these values that CRP take its normal value to the 28 th day (figure N° 02).

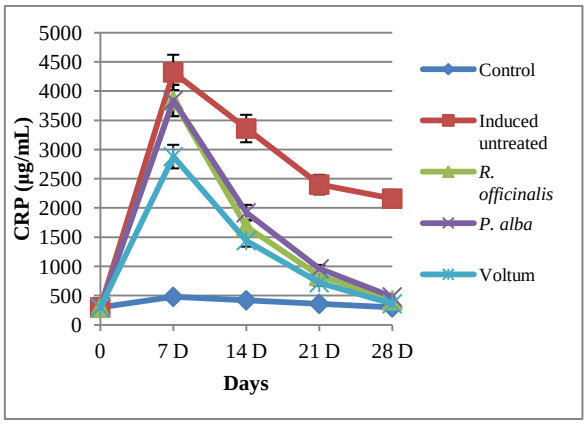

Figure n° 2: CRP values (µg / mL) in normal rats and treated with the essential oil of *R. officinalis*, *P. alba* and Voltum for 28 days. mean ± SEM (n=05). p<0.05.

Alkaline phosphatase (ALP)

Our results show a significant increase of the enzymatic activity of alkaline phosphatase (ALP) in rats receiving the MIA compared to normal rats. While it recorded a recovery by a significant decrease in the activity of ALP in treated groups opposed to induced untreated lot where the value of the remaining ALP increasing (figure N°03).

Serum calcium

The variations observed on calcium values correspond to an increase in the group of rats injected by the MIA after 7 days of intra-articular administration. The average calcium concentration in induced untreated rats remains as increasing as and greater than the reference value obtained in the literature. The causes of hypercalcemia due to malignancies often associated with bone metastasis or primary hyperthyroidism or a vitamin D intoxication, kidney failure, hypo-parathyroidism, hypomagnesaemia **[14].**

Creatinine:

After a week of MIA injection in rats, the biochemical assay showed an increase of creatinine values in the injected rats compared with the rats in the control group. In addition, the increase in plasma creatinine indicates decreased

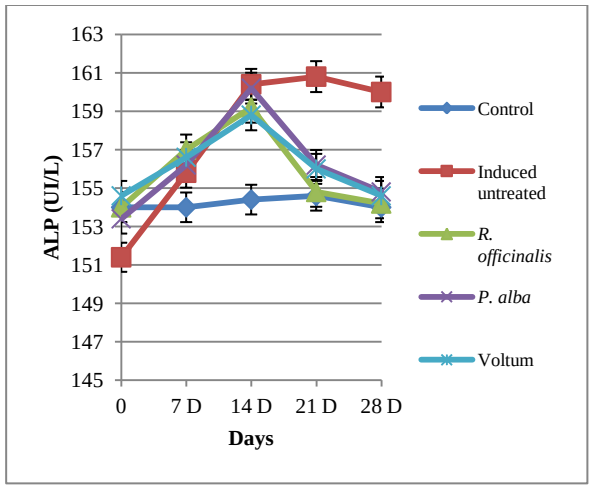

Figure n° 3: ALP values (UI/L) in normal rats and treated with the essential oil of *R. officinalis*, *P. alba* and Voltum for 28 days. mean ± SEM (n=05). p<0.05.

ability of the kidneys to filter waste from the blood and excrete them in the urine **[15].** By contrary, subsequent measures following the intramuscular injection of extracts of essential oil of *R. officinalis*, *P. alba* and even Voltum drug have revealed the recovery of creatinine values.

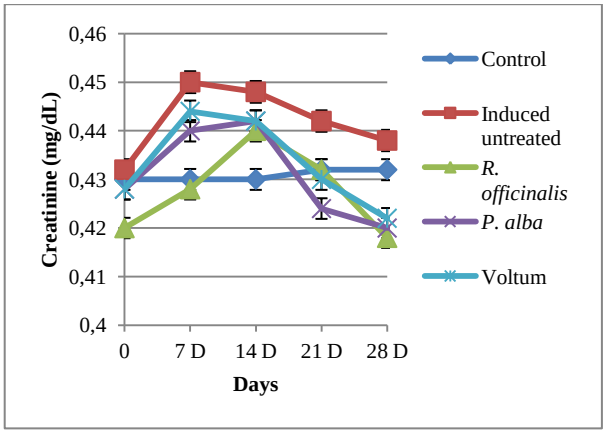

Figure n° 4: Creatinine values (mg/dL) in normal rats and treated with the essential oil of *R. officinalis*, *P. alba* and Voltum for 28 days mean ± SEM (n=05). p<0.05.

Transaminases

MIA injection in rats induces an increase in liver enzymes (ALT, AST), which is probably due to a stress during the installation of osteoarthritis. By contrary, treatment of rats by the essential oil extracted from *R. officinalis*, *P. alba* and Voltum has made it possible to record a decrease in the enzymatic activity of alanine aminotransferase (ALT) from the 7th day. While the activity of aspartate aminotransferase (AST) was decreased to the 14th day.

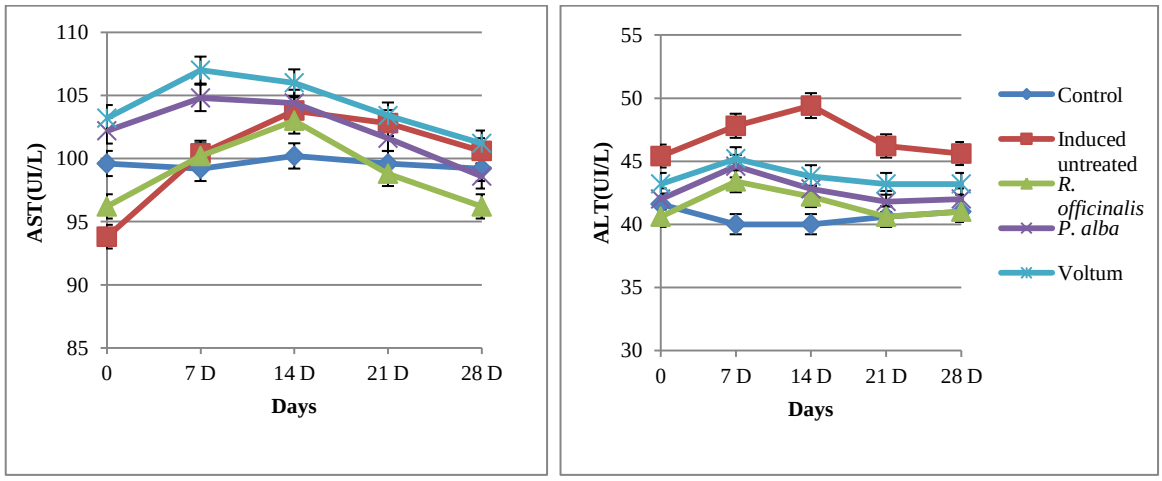

Figure n° 5: Values of ALT and AST (UI/L) in normal rats and treated with the essential oil of *R. officinalis*, *P. alba* and Voltum for 28 days. mean ± SEM (n=05). p<0.05.

III. CONCLUSION

Despite the availability of modern osteoarthritis treatment methods, man still opts for natural healing method to avoid side effects and use less expensive products with high efficiency. Therefore, natural products can give us this opportunity.

ACKNOWLEDGEMENTS:

We express our sincere gratitude to all staff of the Directorate of Education of Mascara

REFERENCES

[1] Bahorun T. Substances naturelles actives: la flore mauricienne, une source d'approvisionnement potentielle. Food and agricultural resarch council, Réduit, Mauritus, **1997**.83-94.

[2] Lhuillier A. Contribution a l'étude phytochimique de quatre plantes malgaches : *Agauria salicifolia* Hook.f ex Oliver, *Agauria polyphylla* Baker (*Ericaceae*), *Tambourissa trichophylla* Baker (*Monimiaceae*). Thèse de doctorat. Toulouse, **2007**.

[3] Martel-Pelletier J. et Pelletier J. P. Is osteoarthritis a disease involving only cartilage or other articular tissues?Eklem Cerrahisi, **2010.** 21(1):p.2-14.

[4] Atik bekkara F., Bousmaha L., Taleb bendiab S.A. Composition chimique de l'huile essentielle de *Rosmarinus officinalis L* poussant à l'état spontané et cultivé de la région de Tlemcen. *Biologie & Santé*. **2007,** 7: 6-11.

[5] Tahraoui A., Israili Z.H. & Lyoussi B. Acute and sub-chronic toxicity of a lyophilised aqueous extract of *Centaurium erythraea* in rodents. *Journal of Ethnopharmacology*, **2010,** 132: 48-55.

[6] Dunham J., Hoedt-Schmidt S., Kalbhen D.A**.** Prolonged effect of iodoacetate on articular cartilage and its modification by an anti-rheumatic drug. Int J Exp Pathol; **1993,**74:283–9.

[7] Guingamp C., Gegout-Pottie P., Philippe L. Mono-iodoacetate-induced experimental osteoarthritis: a dose–response study of loss of mobility, morphology, and biochemistry. Arthritis Rheum, **1997**; 40:1670–9.

[8] Kalbhen, D. A. Chemical model of osteoarthritis--a pharmacological evaluation. *J Rheumatol, 14 Spec* **1987,***No*, 130-131.

[9] Lozada C. J., et Altman R. D. Management of Osteoarthritis. A Textbook of Rheumatology (15e éd., Vol. 2, **2005.** p. 2257-2276).

[10] Pincus T., Koch G. G., Sokka T. A randomized, double-blind, crossover clinical trial of diclofenac plus misoprostol versus acetaminophen in patients with osteoarthritis of the hip or knee. Arthritis and Rheumatism, **2001**, 44(7), 1587-1598.

[11] Poole R., Blake S., Buschmann M. Recommendations for the use of preclinical models in the study and treatment of osteoarthritis. Osteoarthritis and Cartilage, 18 Suppl 3, **2010,** S10-16.

[12] Rouabah Y. Contribution à une étude quantitative des huiles essentielles dans deux espèces végétales: *Globularia alypum L.* et *Rosmarinus officinalis L.* Mém. Ing. Univ. Batna, **2010**. 68 P.

[13] Brulé CH. & Pecout W. L'ylang-ylang: un parfum subtil. France: Arco Charbot; **1995,** Paris.

[14] Marshall W.J., Bangert S.K. Clinical Chemistry, 5th Ed. Elsevier, London, **2005,** 392p.

[15] Feig D.I., Mazzali M., Kang D.H. Serum uric acid: a risk factor and a target for treatment? *Journal of the American Society of Nephrology*. **2006,** 17: S 69-73.