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Research Article

Radiographic and Histopathologic Analysis on Osteoarthritis Rat Model Treated with Essential Oils of *Rosmarinus officinalis* and *Populus alba*

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ABSTRACT

Background: The plants represent an important source of various chemical structures known for their therapeutic activities in folk medicine. Our study aimed to exploit natural bioactive components from well-known medicinal plants "*Rosmarinus officinalis* and *Populus alba*".
Methods: The study of acute toxicity of essential oils of Rosemary and White poplar was carried out on Wistar rats. Then, the effect of these essential oils was studied in the treatment of experimental models induced by knee osteoarthritis. A radiographic and Histologic analysis was carried out to monitor the efficiency of essential oil. The choice of the model of osteoarthritis turned to unilateral intra-articular injection of a mono-iodo acetic acid.
Results: The results revealed that the action of the essential oil showed a significant decrease in the Mankin score compared to the group of untreated rats.
Conclusion: The essential oils of *R. officinalis*, *P. alba* presented an protective effect against osteoarthritis with difference degrees.

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 originates from the destruction of the extracellular matrix of chondrocytes despite the repair which 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]

Indeed, several of the medicinal plants grown worldwide are well known for their essential oil (EO) which are aromatic, antimicrobial and possess curative potential against different diseases including osteoarthritis (OA) and other inflammatory conditions.[4,5] Accordingly, *Rosmarinus officinalis* L. (Lamiaceae) and *Populus alba* L. (Salicaceae) were selected as essential oil bearing plants for the present study.

We seek first to study the acute toxicity of essential oils of Rosemary and white poplar. Then, the effect

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of these essential oils was studied in the treatment of experimental models induced by knee osteoarthritis. A radiographic and Histologic analysis was carried out to monitor the efficiency of the essential oil.

Materials and Methods

Plant material

The aerial parts including leaves and flowers of the two plant; *Rosmarinus officinalis* and *Populus alba* was used. The plants were collected in the Mascara region during the April 2013. They were identified by the botanist of the Biology department.

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.[6]

Extraction of essential oils

Extraction of the essential oils was carried out by hydrodistillation in a Clevenger apparatus. 100 g of aerial parts of each plant was boiled. When the temperature stabilizes, the distillate was collected. 18 g of sodium chloride (NaCl) was added to the distillate. Then, the mixture was placed in a separating funnel and three successive washes (10, 10, 20 ml) of cyclohexane were achieved. After agitation, the organic phase was taken to undergo rotary evaporation to remove the cyclohexane and obtain only the essential oil. The essential oil obtained was stored at + 4 °C after the calculation of yield.

Acute toxicity test

To assess the acute toxic effects of EO of *R. officinalis* and even *P. alba*, a measure of the lethal dose 50 (LD50) was required. Male and female albino rats with an average weight of 150 ± 5g (females) and 220 ± 8 g (males) were used. Rats were selected according to sex in cages each carries five animals. Acute toxicity was estimated using the method described by Tahraoui and colleagues[7] animals were divided in 05 groups of 10 animals (05 males and 05 females). Then, they received single doses of 0.4, 0.6, 0.8 and 1 ml / kg of the EO of *P. alba* and from 1, 3, 5 and 7 ml / kg of the EO of *R. officinalis* and 9 ‰ NaCl (control) by intraperitoneal injection. After administration of the extracts, the rats were continuously monitored in the 1h, 6h and 24h after treatment, for any death or changes in behavior. These signs of toxicity were monitored and the number of dead animals was calculated and converted into a percentage.

Assessment of anti-osteoarthritis activity of essential oils

Preparation of rats

The operations are conducted in accordance with the welfare of the animal, excluding any stress and nervousness that 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. 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: Untreated osteoarthritis rats, Group 03: Treated osteoarthritis rats with EO of *R. officinalis*, Group 04: Treated osteoarthritis rats with EO of *P. alba,* Group 05: Treated osteoarthritis rats with Voltum® (Diclofenac Sodium, 25 mg, Algeria).

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.[8]

Osteoarthritis induction

The choice of the model of osteoarthritis turned to unilateral intra-articular injection of mono-iodo acetate (MIA) (0.3 mg) solution prepared in saline. Under anesthesia, the femorotibial joint was immobilized and a needle was 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.[9]

MIA effected directly on the balance of the metabolic activity of chondrocytes and induced a loss of functional properties of cartilage. Energy intake of chondrocytes derived from the physiological process of glycolysis. MIA disrupted glycolysis by inhibiting the activity of dehydrogenase glyceraldehyde 3-phosphate enzyme, resulting in a decrease of metabolic synthesis of cells and optionally to necrosis.[10]

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.[11] When the condition was already present; the therapies were directed to symptoms such as pain,

instability, joint weakness and decreased function of the joint. The Non Steroid Anti-Inflammatory Drugs were the most common analgesics to treat pain associated with osteoarthritis.[12] 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 *P. alba*) and Voltum®. Taking this dose was performed by intramuscular injection throughout the treatment period with an interval of 02 days to avoid adverse drug reactions of drug Voltum in overdose.

Radiographic analysis

Radiography was the most used complementary examination in the diagnostic of osteoarthritis. It was a test that requires a codification in order to obtain comparable and interpretable images. The review of radiographs will focus on the search for direct signs (remodeling and joint distension, interarticularis space and the subchondral bone).[13]
The radiographic analysis of the knees of the treated and untreated rats was performed. The Agfa 24/30 cassette form was divided into 06 equal zones. Then, it was deposited on the table of a Shimadzu conventional device. After thorough disinfection, the knees were placed on the tape. Using an X-ray tube, an X-ray beam was directed vertically towards the cassette. The tape will be bombed with constant Kv: 48, weight: 200, Sec 6.3. An Agfa processor ensures high quality development for silver bromide plates with a width not exceeding 36 cm. Then, the pictures were developed after printing. Afterwards, the knees were classified according to the score of Kellgren and Lawrence (1957). It corresponded to an index taking into account both the osteophytes and joint space. This classification included four classes: doubtful osteoarthritis, minim, some and advanced.

Histological analysis

During the sampling, the cartilage pieces were commonly connected to bone parts by bone subchondral area, where a cutting more difficult due to the sample hardness. Biopsies cartilage from tibial plateaus knee osteoarthritis rats were fixed in formalin (10%) for 24 hours.[14] The samples were then decalcified in 14% EDTA for 15 h. The verification of the end point of decalcification was performed by chemical method using the Arnim method.[15] Briefly, 5 ml of ammonium hydroxide 5% and 5 ml of ammonium oxalate 5% was added to 5 ml of the decalcification solution where the samples were dipped, then vortexed vigorously. If no white precipitate calcium oxalate appeared after 15 minutes of waiting, decalcification was complete. Otherwise, the decalcification solution must be repeated until the validation process. The samples were then thoroughly rinsed several times in buffers phosphate saline (BPS) before further processing by the dehydration of alcohols 70°, 95° and 100°, clarification by xylene, impregnation and paraffin embedding. Paraffin blocks were placed on cold plate before cutting. Histologic coloring of hematoxylin-eosin-saffron (HES) were then carried out on sections of 5 µm of thickness taken with a Leica RM2235 microtome. The obtained tissue sections were collected in glass slides deparaffinised and stained by hematoxylin and eosin stain and examined through the electric light microscope. The hematoxylin-eosin-saffron coloring (HES) used to assess the general appearance of the tissue.
The severity scores were established according to the Mankin score[16] considering the cartilage surface, the appearance of the cells, the thickness of the deep layer containing hypertrophic chondrocytes and intensity of coloring. It was proposed in 1971 by HHGS (Histochemical Histological Grading System) which used a score of 14 points. To facilitate the use of this scale, it was best to divide it into levels or degrees: 0-2: Normal, 3-5: Osteoarthritis Grade I, 6-7: Osteoarthritis Grade II, 8-10: Osteoarthritis Grade III, 11-14: Osteoarthritis Grade IV. This system was deemed valid for moderate and severe stages but not valid for early and mild stages by some authors.[17]

Statistical analysis

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

Results and Discussion

Extraction yields

The EO content, obtained from the aerial parts (leaves + flowers) was 1.29 ± 0.03% for the Rosemary and 0.9 ± 0.08 % for white poplar. The yields of EO from two species were widely variable. The yield of EO of *R. officinalis* was higher than those of reported by Atik Bekkara *et al.*[18] and Rouabeh[19] which was 0.8% and 0.9%, respectively. This is could be partly due to differences in ecological conditions or essential oil preparation procedure.[20]

Acute toxicity

After administration of different doses of EOs of Rosemary and White poplar, observations over a period of 14 days showed no severe clinical symptoms of pain, despite some common signs as anorexia, hypoactivity, which were reversible and have appeared in rats for a short time and then they returned to their activity. The absence of mortality and clinical signs indicated that the EO of *R.*

officinalis and *P. alba* were devoid of acute toxicity in rats.

Radiographic analysis

To observe the changes of the knee joint, X-ray examination was performed on osteoarthritic rats treated with EO of *R. officinalis* and *P. alba.* In Figure1, the radiographs describe the bone structure visible on a radiograph of the knees.

The reading x-rays gave the following breakdown: non-osteoarthritic rats n = 7 (normal radiography), osteoarthritic rats with doubtful significance n = 5, osteoarthritic rats with minimal modification n = 6, osteoarthritic rats with certain osteoarthritis n = 2 and osteoarthritic rats with advanced osteoarthritis status n = 5 (Figure 2).

Histological analysis

This study described the effectiveness of the EO of *R. officinalis* and *P. alba* against a knee osteoarthritis model induced by MIA. The main criterion to account for the development of osteoarthritis was the development of histological changes in the cartilage and subchondral bone. Different studies reported that the MIA caused cell necrosis in the affected areas, proliferation of chondrocytes in the margins of lesions and a matrix proteoglycan loss.[21] In the present study, histopathological findings of the group induced untreated have shown that administration of MIA caused changes including degradation of articular cartilage, synovial inflammation, and destruction of bone structure in the subchondral joint. In treated rats, some degenerative changes included surface fibrillation of the articular cartilage, vertical fissures extending into the deep areas and degeneration of chondrocytes (Figure 3).

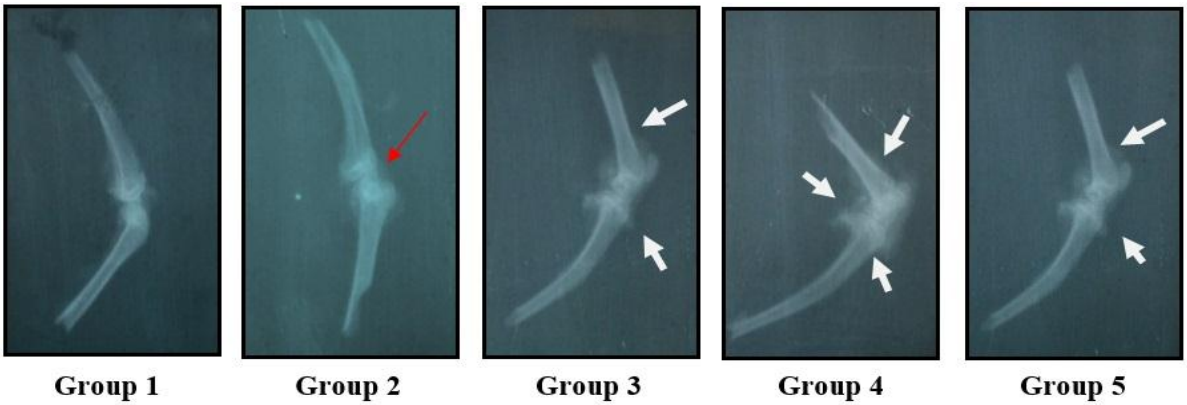

Figure 1. X-ray of the knee treated and untreated rats (Group 1: Normal rats, Group 2: Untreated osteoarthritis rats, Group 3: Treated osteoarthritis rats with EO of *R. officinalis*, Group 4: Treated osteoarthritis rats with EO of *P. alba*, Group 5: Treated osteoarthritis rats with Voltum®).
Group 1: Tibiofemoral joint with normal radiographic appearance and normal opacity, bicondylar normal.
Group 2: Radiography tibiofemoral joint achieved knee osteoarthritis. It should be noted the presence of bone loss with wear of the cartilage of the knee joint and joint instability.
Group 3: Radiography the femoraltibial joint of a rat treated with EO of *R. officinalis*: the presence of an opacity essentially concentrated at the femoral condyle corresponding to a scar bone callus associated with lysis of the articular cartilage.
Group 4: Radiography the femoraltibial joint of a rat treated with EO of *P. alba*: the presence of an opacity essentially concentrated at the femoral condyle corresponding to a scar bone callus associated with lysis of the articular cartilage.
Group 5: Radiography of the femoraltibial joint of a rat treated with the drug Voltum®: the presence of an opacity substantially concentrated at the femoral condyle corresponding to bone callus, associated with lysis of articular cartilage.

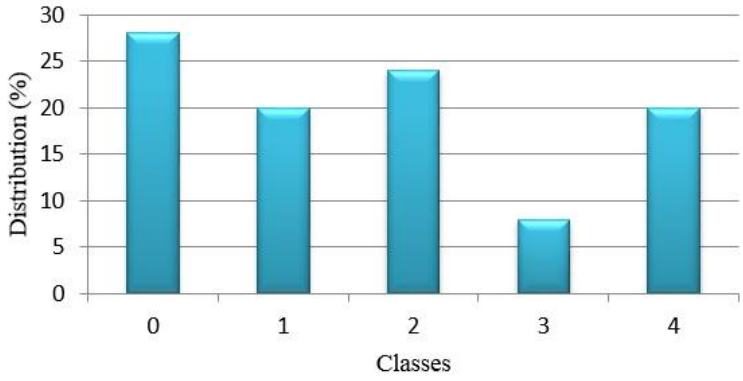

Figure 2. X-ray knee distribution by the Kellgren and Lawrence score.

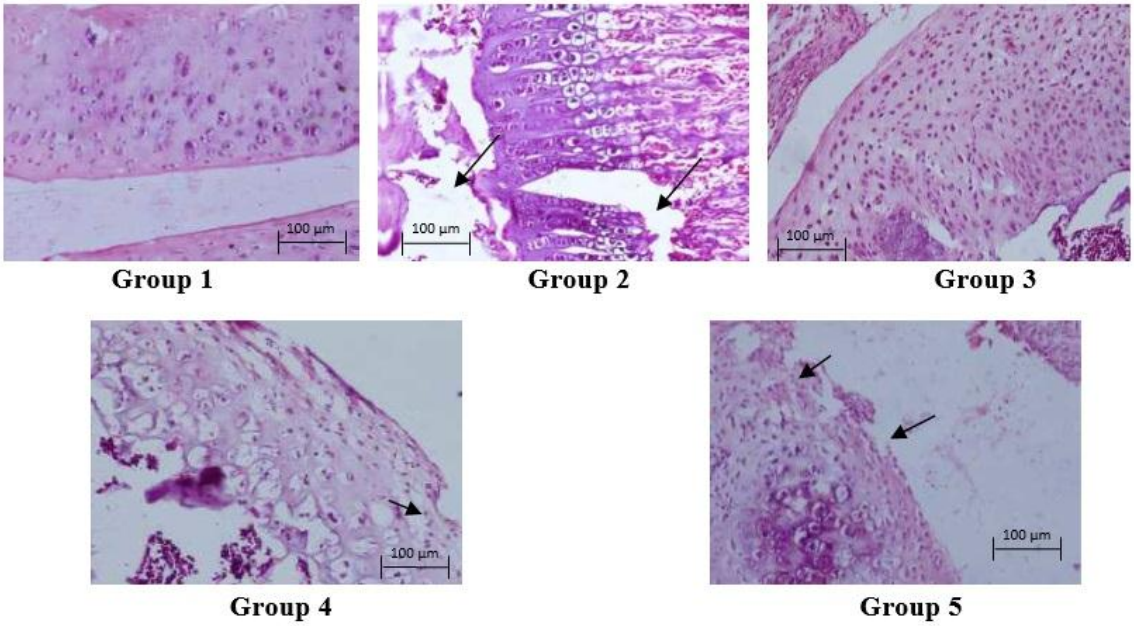

Figure 3. Images of frontal section of the knee joint after HES staining (x100).
(Group 1: Normal rats, Group 2: Untreated osteoarthritis rats, Group 3: Treated osteoarthritis rats with EO of *R. officinalis*, Group 4: Treated osteoarthritis rats with EO of *P. alba*, Group 5: Treated osteoarthritis rats with Voltum®).

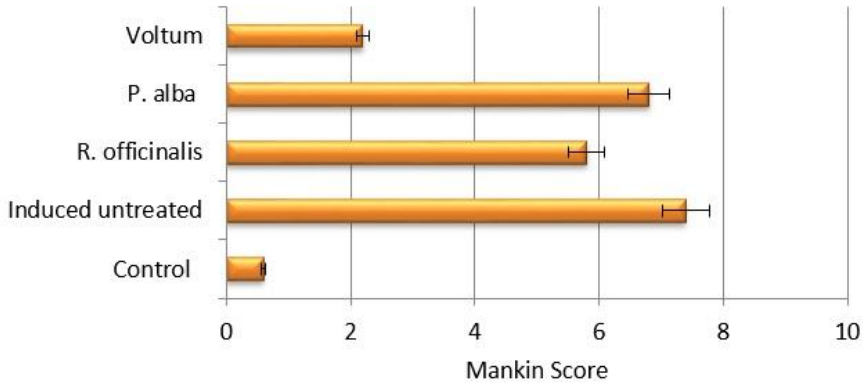

Figure 4. Mankin Score of the control and treated rats (EO of *R. officinalis, P. alba* and Voltum®).

The histopathological grading system was developed according to the Mankin score. This system was used to estimate the clinical osteoarthritis level in Wistar rats after injection of Mono-Iodo-acetic intra-articularly. The lesions were divided into five classes from normal histology to damaged cartilage. The division of histological lesions in classes was comparable with the model of the Pritzker.[22]

Histological analysis of the sections of the knee treated with the EO of *R. officinalis* and *P. alba* showed a significant decrease in the Mankin score compared to untreated group (OA grade III) (Figure 4). This evidence was consistent with severe joint degenerative changes in the knee joints. While the groups treated with Voltum® revealed a less severe joint degenerative changes (osteoarthritis grade II).

Conclusion

In this study, we investigated the effect of EO of *R. officinalis, P. alba* in the treatment of experimental knee osteoarthritis models. Therefore, a radiographic and histologic analysis were performed to monitor the efficiency of essential oil. The results revealed that the EO of *R. officinalis* and *P. alba* showed a significant decrease in the Mankin score compared to untreated group. The both essential oils presented a protective effect against osteoarthritis with difference degrees.

Conflict of interests

The authors claim that there is no conflict of interest.

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