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Anti-nociceptive and anti-inflammatory effects of *Paeonia mascula* extract

[Efecto anti-nociceptivo y antiinflamatorio del extracto de *Paeonia mascula*]

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Abstract

Context: *Paeonia mascula* is used in traditional medicine in North of Algeria for its anti-inflammatory activity.

Aims: To evaluate the analgesic and the anti-inflammatory effects of the ethanolic extract of *Paeonia mascula* (EPM) in mice.

Methods: The analgesic activity of EPM was evaluated on the chemical nociception in the animal models of acetic acid-induced writhing and formalin-induced hind paw licking/biting, whereas xylene-induced ear edema and multiapplication of TPA (12-O-tetradecanoylphorbol-13-acetate) were used to determine anti-inflammatory effects of EPM.

Results: EPM (100 and 250 mg/kg) produced a significant dose-dependent inhibition of pain-related behaviors elicited by acetic acid. Also, EPM reduced both early and late phases of the formalin test. However, its inhibitory effect was more significant on tonic inflammatory phase, may be related to the anti-inflammatory abilities of this extract. Also, EPM decreased ear edema in acute and sub-chronic models used in the present study.

Conclusions: These findings suggest the ethanolic extract from aerial parts of *Paeonia mascula* presents significant anti-inflammatory and anti-nociceptive effects on chemical behavioral models of nociception and inflammation in mice.

Keywords: analgesic; formalin; inflammation; pain.

Resumen

Contexto: *Paeonia mascula* se utiliza en la medicina tradicional en el norte de Argelia por su actividad anti-inflamatoria.

Objetivos: Evaluar la actividad analgésica y antiinflamatoria del extracto etanólico de *Paeonia mascula* (EPM) en ratones.

Métodos: El perfil analgésico se evaluó mediante la prueba de constricciones abdominales inducidas por ácido acético y la prueba de formalina 1%. Con la finalidad de estudiar su efecto antiinflamatorio se implementaron dos modelos de edema auricular en condiciones agudas (edema inducido por xileno) y en condiciones sub-crónicas [edema inducido por aplicaciones múltiples de TPA (12-O-tetradecanoilforbol-13-acetato)].

Resultados: El extracto etanólico de *Paeonia mascula* inhibió de forma dependiente de la dosis las conductas relacionadas con el dolor inducidas por ácido acético. Además, EPM redujo ambas fases, la temprana y la tardía en la prueba de la formalina. Sin embargo, su efecto inhibitorio fue más significativo sobre la fase tónica inflamatoria. Este efecto pudiera estar relacionado con la actividad antiinflamatoria del extracto. También EPM redujo significativamente el edema auricular en los modelos (agudo y sub-crónico) utilizados en el estudio.

Conclusiones: Estos hallazgos sugieren que el extracto etanólico de partes aéreas de *Paeonia mascula* presenta efectos antiinflamatorios y antinociceptivos en modelos agudos de nocicepción química e inflamación en ratones.

Palabras Clave: analgésico; dolor; formalina; inflamación.

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INTRODUCTION

Both non-specific and specific immune responses cause inflammation, a local response to tissue injury, infection, or irritants. In the case of a pathogen, inflammation limits its spread and kills, and removes the pathogen through phagocytosis, by macrophages and neutrophils attracted to the site. Inflammation is a normal protective response induced by tissue injury or infection to combat invaders in the body and to remove dead or damaged host cells. However, chronic and extreme inflammations cause many inflammatory diseases, such as cancers and rheumatoid arthritis (Wathins et al., 1995; Wei et al., 2015). Pain is a distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive, and social components (Williams and Craig, 2016). Throughout history man has used many different forms of therapy for the relief of pain, among them; medicinal herbs are highlighted due to their extensive popular use. The research into plants, which are employed as pain-relievers in traditional ethnomedicine is, therefore, one of the productive and logical strategies in the search for new analgesic drugs (Elisabetsky et al., 1995; Almeida et al., 2001). Indeed, numerous plants produce analgesic activity, and many botanical medicines have proven useful for relieving various forms of pain. Monoterpene glycosides are the active constituents, among them; paeoniflorin is the main compound in genus *Paeonia*. In traditional medicine, aqueous decoctions of *Paeonia* have been used against several types of seizures. Important crude drugs extracted from *Paeonia*s species in Chinese, Korean, Japanese, and Vietnamese traditional medicine, used as an anticoagulant, anti-inflammatory, analgesic, and sedative agent (Braca et al., 2008; Zareba, 2009; Koyunoğlu et al., 2012).

There are many medications available for disease joint pain, swelling and inflammation like non-steroidal anti-inflammatory drugs (NSAIDs- such as aspirin, ibuprofen or naproxen). It is believed that current analgesia drugs such as opiates and these non-steroidal anti-inflammatory drugs (NSAIDs) are not useful in all cases, because of their side effects and potency. As a result, the search for other alternatives seems necessary and beneficial (Vasudevan et al., 2007). In this study, the ethanolic extract of *Paeonia mascula* was screened for its anti-inflammatory and anti-nociceptive activities in mice.

MATERIAL AND METHODS

Drugs and chemicals

All substances were purchased from Sigma–Aldrich Chemical (Madrid, Spain) unless otherwise stated.

Preparation of the ethanolic extract

The ethanolic fraction was obtained from the aerial parts of *Paeonia mascula* (L.) Mill. (Paeoniaceae). Aerial parts of herbarium were collected from Bejaia area (36°32'31.11" N, 5°25'38.27" E; 1160 m a.s.l.), in the North East of Algeria in May 2015 when they were at the flowering and fruit setting stage. The plant was authenticated by a Taxonomist of the Laboratory of Botany of Bejaia University (Dr. AF. Bouguaham), and a voucher specimen was deposited (Reference No. PM014). Aerial parts of the plants were dried in an oven at 40°C for overnight and ground into fine powder using a grinder. The powder sample (200 g) from several individuals, were extracted with ethanol in a Soxhlet apparatus for 8 hours, and then evaporated to 2 mL in vacuum. The ethanolic extract of *Paeonia mascula* thus obtained is stored at 4°C until further use.

Animals

Healthy male Swiss albinos NMRI mice (22-28 g) obtained from the Laboratory Animal Service of the University of Constantine, Algeria were housed under standard laboratory conditions, in groups of five or six each, and were housed under a normal 12 h light/dark. The animals were acclimated to their environment for a week and had free access to tap water and food. All behavioral tests were conducted during the light cycle and all procedures used in the present study were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental inflammation and pain in conscious animals (Zimmermann, 1983), following the

directive number 2010/63/EU of 22 September 2010, and the protocol approved by the local Ethics Committee of the laboratory of PBVE, University of Bejaia, (Ref. No. CE-LBVE-2016-102).

Anti-nociceptive activity

Acetic acid induced writhing response

The writhing test was carried out as previously described (Koster et al., 1959). Mice were randomly assigned to four groups, and after an overnight fasting period, they were pre-treated with EPM dissolved in distilled water (100 or 250 mg/kg, p.o.), diclofenac (100 mg/kg, p.o.), or distilled water (control group, p.o.), 60 min before the acetic acid injection (10 mL/kg body weight, i.p.). Immediately after the injection of acetic acid, each animal was placed into a transparent plastic observation chamber. Five minutes after the administration of the acetic acid, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) of each mouse was counted for 15 min. The percentage of inhibition of writhing was calculated and compared with the control group using the formula: Inhibition (%) = (WC–WT)/WC× 100; WC: mean of writhing (control); WT: mean of writhing (test).

Formalin-induced paw licking

The formalin test was performed as previously reported (Hunskaar and Hole, 1987; Tjolsen et al., 1992). Briefly, overnight fasted mice were divided into four groups, which received distilled water (10 mL/kg, p.o.), EPM dissolved in distilled water (100 or 250 mg/kg, p.o.), or aspirin (100 mg/kg, p.o.) 1h before formalin injection (20 μL of 1% formalin) into the plantar surface of the right hind paw. The mice were then placed in a transparent box for observation, and the time spent licking/biting the injected paw was measured and considered as an indication of inflammatory-associated pain. The duration of paw licking (s) as an index of painful response was determined at 0–5 min (early phase, neurogenic or nociceptive) and 30 min (late phase, inflammatory or tonic) after formalin injection.

Anti-inflammatory activity

Xylene-induced ear edema

The test of xylene-induced ear edema in mice was based on the reported method (Nunez-Guillen et al., 1997; Akindele and Adeyemi, 2007). Briefly, thirty minutes after oral administration of EPM dissolved in distilled water (100 or 250 mg/kg), and indomethacin (25 mg/kg, p.o.), an edema was induced in the right ear by topical application of 30 µL xylene on the inner surface. After 15 min, the mice were sacrificed by cervical dislocation. Circular sections of 7 mm diameter were removed from each ear using a cork borer and weighed in a balance. The edema degree was responded with the difference in ear weight between the two samples of a mouse to evaluate the effect of EPM. The anti-inflammatory was expressed as percentage of the inhibition of the edema (PI). This percentage was calculated using the following formula: PI= [(Difference in ear weight, control – Difference in ear weight, treated)/Difference in ear weight, control) x 100].

Mouse ear inflammation induced by multiple topical applications of TPA

Chronic inflammation was induced by topical application of 20 µL of TPA dissolved in acetone (2.5 mg/ear) to both the inner and outer surface of both ears of each mouse with a micropipette on alternate days) (Stanley et al., 1991). To measure anti-inflammatory activity in the chronic skin inflammation model, the TPA-induced ear edema assay (multiple treatments with TPA) was carried out using a similar treatment schedule as described above. EPM dissolved in distilled water (100 or 250 mg/kg) was dissolved in distilled water and administered orally twice daily for four days, in the morning immediately after TPA application and 6 h later (Fig. 1). Dexamethasone was used as the reference drug (0.05 mg/ear). The mice were killed by cervical dislocation 6 h after the last TPA application. Circular biopsies of each ear were taken with a leather punch and immediately weighed. The increases in

weight of the ear punches were directly proportional to the degree of inflammation.

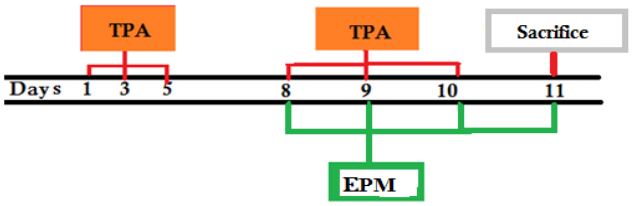

Figure 1. Experimental design of the sub-chronic model of skin inflammation induced by topical multiapplication of TPA and the application of the ethanolic extract of *Paeonia mascula* (EPM).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). The statistical analysis of all the observations was carried out using one-way ANOVA followed by multiple comparison tests of Dunnett's, where necessary. A difference of $p<0.05$ was considered as significant compared with the negative control (treated with vehicle).

RESULTS AND DISCUSSION

Acetic acid-induced writhing response

The oral administration of the ethanolic extract of the aerial parts of *Paeonia mascula* produced a marked anti-nociception and anti-inflammatory effects in models of pain and inflammation. The results of inhibition of the acetic acid-induced abdominal constrictions in mice are presented as means ± SEM (Fig. 2). In the writhing test, intraperitoneal injection of 1% acetic acid evidently resulted in writhing reflex in mice. Both doses (100, 250 mg/kg) of ethanolic extract of *Paeonia mascula* produced significant inhibition ($p<0.001$) of writhing reaction induced by acetic acid in comparison to control group receiving the vehicle, and the inhibitions were 54.97% and 81.28%, respectively. They markedly decreased the number of abdominal constrictions. However, diclofenac (100 mg/kg) inhibited the number of writhes by 83.04%.

The ethanolic extract of *Paeonia mascula* showed a significant analgesic potency in the acetic acid test. The injection of acetic acid induces an inflammatory process that leads to the release of inflammatory mediators in the abdominal cavity with subsequent activation of nociceptors (Collier et al., 1968). Local tissue injury prompts the release of chemical mediators (potassium, hydrogen ions, ATP, and bradykinin) and inflammatory mediators as prostaglandin E2 (PGE2) from inflammatory cells. These substances directly activate nerve endings and trigger the release of algesic mediators (for example, histamine, serotonin (5-HT), nerve growth factor (NGF), and prostanoids) from other cells and afferent nerves (Figueiredo et al., 2013). Chemical irritants induced tonic pain with an inflammatory component, which peripheral and central sensitization (early or acute changes of the central nervous system) underlying. In these conditions, alterations of normal nociceptive processing arise. In this model, pain-related behaviors are the result of activation and sensitization of nociceptors in the visceral and parietal peritoneum, which activate and sensitized the complete nociceptive pathway. The products systemic administered could be modulated several targets peripheral and centrally. These results show that EPM have anti-hypernociceptive effect in a model of viscero-somatic acute inflammation.

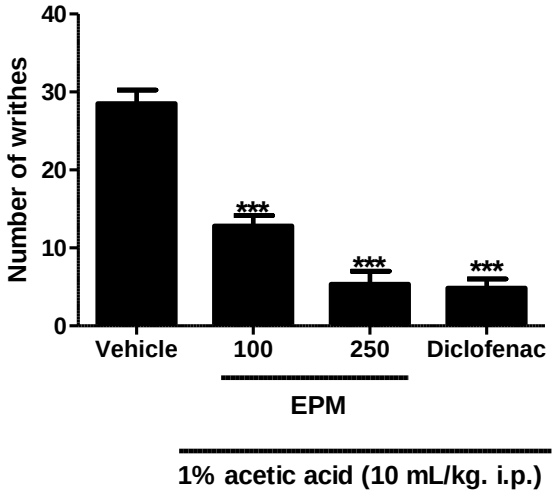

Figure 2. Effect of oral administration of the ethanolic extract of *Paeonia mascula* (EPM) on acetic acid-induced writhing test in mice. The animals were pretreated with ethanolic extract of *Paeonia mascula* (EPM, 100 or 250 mg/kg, p.o.) diclofenac or vehicle 60 min before the acetic acid.

The number of abdominal constrictions (writhings) was counted over a period of 20 min. All data are mean ± SEM (n = 6). ***p <0.01 compared with vehicle group. ANOVA followed by Dunnett's.

Effects of the ethanolic extract of *Paeonia mascula* on formalin test

Fig. 3 shows the results obtained with the formalin test. The antinociceptive activity of the ethanolic extract of, measured on mouse paw by using an injection of formalin 1%. In the first phase, the injection of formalin into the sub-plantar tissue of the right hind paw of control mice produced a nociceptive response of biting and licking of the treated paw with a total duration of 65.20 ± 15.21 seconds. Both doses of EPM reduced formalin-induced pain in the first phase, they exhibited a significant ($p<0.001$) inhibition of nociceptive reaction with 24.80 ± 5.40 s and 21.20 ± 11.82 s, respectively. In the second phase, both doses of EPM (100 and 250 mg/kg), strongly reduced pain to 22.60 ± 12.70 s and 17.40 ± 6.11 s, respectively when compared with untreated group (92.20 ± 7.43 s). The formalin-induced pain is very useful for elucidating mechanism of pain and analgesia. It is considered as a valid and reliable model of persistent nociception. The behavioral response to s.c. formalin showed an initial acute phase (early phase) and after a short quiescent period (10-15 min), a prolonged tonic response persisting for 60-90 min (late phase). Electrophysiological studies have also demonstrated similar biphasic increases in the excitability of dorsal horn cells following formalin injection into their fields (Dickenson and Sullivan, 1987). Traditionally, the early phase has been considered as being due to a direct stimulation of nociceptors by formalin, whereas the late phase has been considered as being due to inflammatory components with the release of different pain-mediating substances and central sensitization (Hunskaar and Hole, 1987; Tjolsen et al., 1992). *Paeonia mascula* possesses anti-nociceptive activity against chemically induced nociception and both inflammation and non-inflammation mediated nociception.

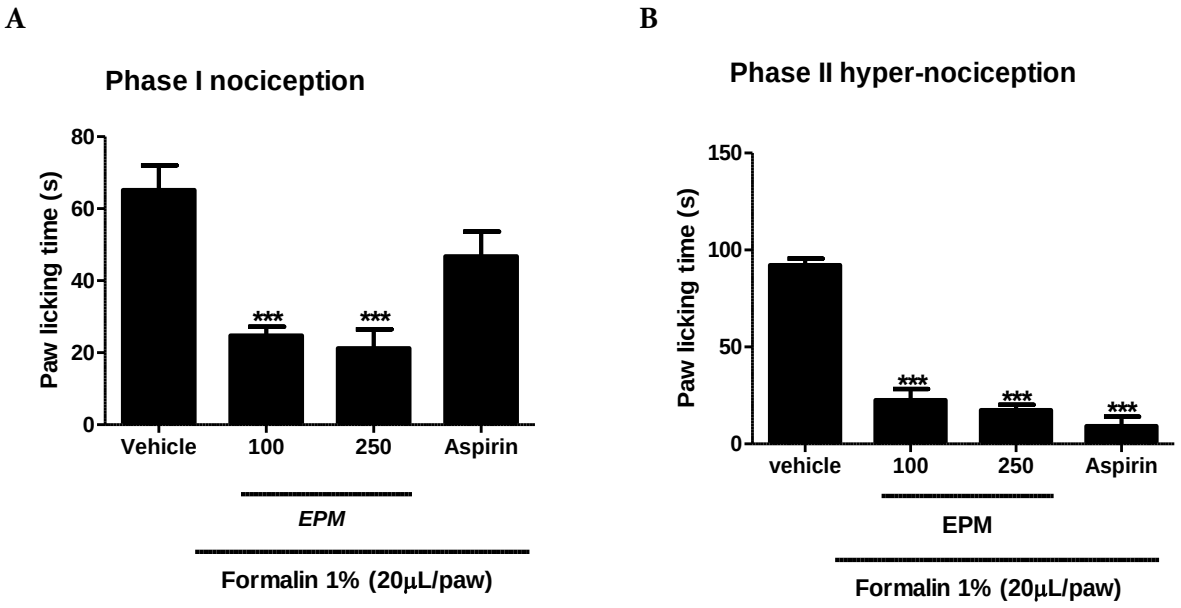

Figure 3. Effect of oral administration of the ethanolic extract of *Paeonia mascula* (EPM) on neurogenic phase **(A)** and inflammatory phase **(B)** on formalin-induced pain in mice.

The animals were pretreated with ethanolic extract of *Paeonia mascula* (EPM, 100 or 250 mg/kg, p.o.) or aspirin (100 mg/kg, p.o.) 1 h before formalin injection (20 μL of 1% formalin) into the plantar surface of the right hind paw. All data are mean ± SEM (n = 5). ***p <0.01 compared with vehicle group. ANOVA followed by Dunnett's.

Table 1. Effect of the ethanolic extracts of *Paeonia mascula* on xylene-induced ear edema in mice.

Treatment	Dose (mg/kg)	Increase in ear weight (mg)	Inhibition (%)
Distilled water	10 mL/kg	14.48 ± 4.57	
Ethanolic extract	100	6.28 ± 2.62**	56.08
	250	4.34 ± 2.04***	69.65
Indomethacin	25	4.28 ± 0.93***	70.06

Values are mean ± SEM (n=6 animals). **P<0.01, ***p<0.001 respect to control treatment with distilled water (one way ANOVA followed Dunnett's-test multiple comparison tests).

Effects of EPM on acute inflammation

The results given in the Table 1 demonstrate that the ethanolic extract of *Paeonia mascula* orally administered, produces a higher reduction of edema induced by the xylene, when compared with control group (blank), with maximal percentage inhibition of 59.09% at the dose of 250 mg/kg and 69.65% with the low dose (100 mg/kg). Also, indomethacin (25 mg/kg, p.o.), used as positive control, also inhibited the edema inflammation, with 70.06%. Xylene-induced ear edema is an acute inflammation model and a typical symptom of inflammation. It is used for the evaluation of anti-inflammatory steroids as well as non-steroidal anti-phlogistic agents especially those inhibiting phospholipase A2 (Zaninir et al., 1992).

Effects of EPM on sub-chronic inflammation

The result of the multi-application of 12-O-tetradecanoylphorbol-13-acetate (TPA) induced inflammation was also presented as mean ± SEM (Fig. 4). Topical application of TPA to mouse ear induced a prolonged skin inflammation. Ethanolic extract of *Paeonia mascula* (250 mg/kg) significantly (p<0.001) reduced edema formation by (14.75 ± 3.57 mg). However, the reduction produced by the low dose (100 mg/g) was not significant. Dexamethasone (0.05 mg/ear) inhibited edema formation by 46.50%. Repeated doses of TPA cause edema, epidermal proliferation, leukocyte infiltration, the appearance of dark cells (basophilic keratinocytes), and dermal fibrosis. TPA also decreases the number of pale dendritic cells, an important sign indicative of tumor promotion (Baxter et al., 1998). Inflammatory responses can be triggered by physical or chemical trauma, invading organisms and antigen-antibody reactions and are often exacerbated by the resultant swelling, tissue edema, and pain due to increased pressure in tissues caused by the formation of edema, inflammatory mediators such as bradykinin, serotonin, histamine, prostaglandins, leukotrienes and nitric oxide, which can originate locally or from cells that infiltrate in the site of insult (Rasheed and Haqqi, 2008; Yam et al., 2008).

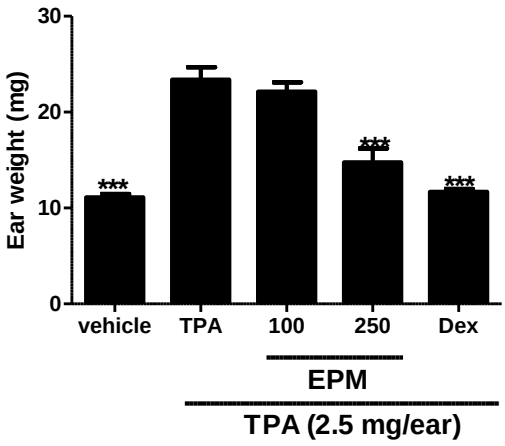

Figure 4. Effect of oral administration of the ethanolic extract of *Paeonia mascula* (EPM)on mouse ear edema induced by repeated application of TPA.

Increase in ear weight in mg was calculated respect to a control group, which received acetone only (vehicle). The animals were pretreated with ethanolic extract of *Paeonia mascula* (EPM, 100 or 250 mg/kg, p.o.) or dexamethasone. All data are mean ± SEM (n = 6) ***p <0.01 compared with vehicle group. ANOVA followed by Dunnett's.

The results obtained in this study suggest that EPM contains active molecules capable of reducing the activation and recruitment of immune cells involved in the pathological immune response, through the inhibition of the synthesis and release of pro-inflammatory mediators. In the mouse skin model of inflammation, TPA causes inflammatory response such as arachidonic acid cascade and induces epidermal cell proliferation, recruits inflam-

matory cells, and increases production of reactive oxygen species leading to oxidative DNA damage. Moreover, topical application of TPA to mice leads to edema and papilloma formation by enhancing the expression of inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 (Seo et al., 2002). Proliferation and cellular infiltration, as well as the synthesis of different inflammatory mediators in the inflamed region on ear treated with TPA increase her weight in comparison with the ears no treated with TPA. The anti-inflammatory effect exerted by EPM could be associated with the presence of active compounds such as paeonol and paeoniflorin. Also, it was reported that paeonol inhibited histamine, IL-4, and TNF-α release and also suppressed IgE production *in vitro* (Kim et al., 2004). Similarly, paeoniflorin was found to reduce IL-1, prostaglandin E2 and TNF-α production by macrophage-like synoviocytes (Wu et al., 2007).

CONCLUSIONS

Paeonia mascula has both anti-nociceptive and anti-inflammatory effects. The results obtained in the analgesic test experiments showed that EPM significantly decreased the pain-related behavior in formalin test by a possible modulation of inflammatory components with the reduction of the release of different pain-mediating substances. Also, EPM inhibited the writhing response induced by acetic acid; these results show that EPM has anti-hypernociceptive effect in a model of viscero-somatic acute inflammation. Further investigation is required to elucidate the mechanisms underlying these effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

Contribution	Bribi N
Concepts or ideas	X
Design	X
Definition of intellectual content	X
Literature search	X
Experimental studies	X
Data acquisition	X
Data analysis	X
Statistical analysis	X
Manuscript preparation	X
Manuscript editing	X
Manuscript review	X

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