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PHYTOCHEMICAL SCREENING, ANTIDEPRESSANT AND IMMUNOMODULATORY EFFECTS OF AQUEOUS EXTRACT OF *CISTUS LADANIFER* L. FROM MOROCCO

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ABSTRACT

Cistus ladanifer L. (CL) is a medicinal plant known for its therapeutic properties in Moroccan traditional medicine. The leaves with non-woody branches aqueous extract (AE) of CL showed both immunomdulatory and antidepressant activities: (i) immunosuppressive effect at dose range used intraperitoneally (i.p) (150,175 and 200 mg/kg of body weight "b.w."); (ii) AE, when administered (i.p) to the rats from 150 to 200 mg/kg b.w., were able to elicit dose-dependent relation of immobility reduction in the forced swimming test in rat "FST". The antidepressant-like effect of the extract at the dose of 200 mg/kg, b.w., was more potent than that of reference antidepressant sertralin. Our results revealed in vivo a mediane lethal dose 50% "LD50" equal to 450 mg/kg, b.w. Phytochemical studies were also undertaken and preliminary analysis of the AE of CL revealed the presence of flavonoids, saponins and tannins compounds. The presence of flavonoids in the aqueous extract of CL is 24, 91 mg of quercetin "QE" equivalent per gram of dry weight "DW". These data demonstrated that the AE of *C. ladanifer* L. had specifically antidepressant-like effects and significant immunosuppressive properties *in vivo* and may support the use of this plant in treating various psychiatric disturbances (depression) and diseases associated with immune response.

Key words: *Cistus ladanifer* L., Acute toxicity, Phytochemical studies, Antidepressant-like effect, Immunomodulatory effect, Forced swimming test.

INTRODUCTION

Depression a nervous disease, widespread in the world, it is classified by the World Health Organization as one of the most disabling illnesses of society, with an incidence of 15-25% of life according to various reports (Kessler *et al*., 2003; Nemeroff, 2007; Patten, 2008). Recently a growing number of herbal medicines have being chosen as additional or alternative therapies for nervous depression such as St. John's wort (Hypericum perforatum L.) (Linde *et al*., 2008), Tetraena gaetula (Emb. & Maire) Beier & Thulin) (El Hamsas-El Youbi *et al*., 2008), and *Ginkgo biloba* L. (Kang *et al*., 2005) etc.

In spite of the use of CL widely in traditional medicine by local people in north of Morocco as an anti-diarrheal, anti-acid and antispasmodic (Aziz *et al*., 2007). The employment of of this plant as antidepressant or immunmodulatory is rare or absent. Its aromatic exudates or resin, commonly called labdanum, has been used since antiquity to treat diarrhea, dysentery, catarrh and discomfort menstruation (Barrajón-Catalán *et al*., 2010). CL is an important member of the flora in the Mediterranean ecosystems semi-arid and form dense stands on siliceous soils (Robles *et al*., 2003). It is a medium-sized tree known locally as "Touzal" in north of Morocco or "Targale and Bu-zgzaw" in other regions of

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Morocco (Bellakhdar, 1997). It is abundant in the mountainous western central Rif area and the Beni-Znassen (Aziz *et al*., 2006). Its exerts different pharmacological effects such as anti-platelet effects (Mekhfi *et al*., 2004), antioxidant (Nagai *et al* ., 2004, Guimarãesa *et al*., 2010, Barrajón-Catalán *et al*., 2010; Andradea *et al*., 2009), antispasmodic (Aziz *et al*., 2006), anti-hypertensive (Belmokhtar *et al*., 2009), antibacterial, antitumor (Guimarãesa *et al*., 2010) and inhibitor of calcium transport in skeletal muscle (Sosa *et al*., 2004). Furthermore, essential oil, the absolute and resinoid of CL showed antibacterial and antifungal (Mrabet *et al*., 1999) and are also used in cosmetics (Gaudin *and* Guenet, 1989; Mrabet *et al*., 1997). The polyphenols, particularly flavonoids and tannins, the major compounds of CL are widely appreciated for their potential beneficial health effects, like antioxidant, antimicrobial and anticarcinogenic activities (Noferi *et al*., 1997, Ren *et al*., 2003 and Pizzi *et al*., 2008).

In this study, we aimed to determine the acute toxicity threshold of aqueous extract of C*istus ladanifer* L. and to investigate some pharmacological activities such as the potential antidepressant-like effects and immunomodulatory properties in the rat.

MATERIALS AND METHODS

Animals

Male Wistar rats (100-150g) were employed for this study. The animals were obtained from the center ECWP "Emirates Wildlife Propagation Center" in the region (Missour, Morocco). They were kept in well-ventilated environment, had free access water and food ad libitum. They were housed in a quiet room under a 12-h light: 12-h. dark cycle for 2 weeks before experimentations.

Experimental design for rats

The animals were randomized into control and experimental groups and divided into nine groups of 6-8 animals each. Animals in group 1 were administered with normal saline (0.9% NaCl). Animals in groups 2, 3, 4, 5, 6 and 7 were administered with the extracts of *C. ladanifer* at the doses of 150, 175 and 200 mg/kg, b.w. for antidepressant and immunomodulatory investigations. Animals in group 8 and 9 were administered with sertralin and prednisolone at the doses of 0.83 mg/kg, b.w. and 0.6 mg/kg, b.w. respectively. All drugs were intraperitoneally administered to 13:00 until 14:00 h.

Plant material

Cistus ladanifer L. (*Cistaceae*) leaves with non-woody branches were collected from Taounate region (34°42'979N-004°39'908W, N of Morocco) during the 2009-growing season, and a specimen (INP250) has been deposited in the Herbarium of National Institute of Medicinal and Aromatic Plants, University sidi Mohamed Ben Abdellah, Fez. In the following parts of this paper, plant material is referred as “leaves” instead of “leaves with non-woody branches”.

Preparation of aqueous extracts for activities testing

The leaves of *C. ladanifer* L. were cleaned, air-dried for 8 days and reduced to a coarse powder. The powder was extracted using a traditional method described in Moroccan folk medicine (Belmokhtar *et al*., 2009). Fifty grams of leaves powder of CL were extracted by decoction with 2l of distilled water for 30 min. The AE was obtained after filtration and evaporation to dryness under vacuum (yield: 21%).

Phytochemical studies

Phytochemical screening

Preliminary phytochemical analysis of the extract was performed using the method described previously (Dohou *et al*., 2003). The polyphenols has been made through the reaction of ferric chloride. Stiasny reaction was used to reveal the presence of catechic and gallic tannins. Furthermore, the flavonoids have been revealed through the reaction to the cyanidin. The saponins have a property to form foam when they are shaken vigorously with water.

Total flavonoids

Total flavonoids contents were determined by spectrophotometer using method described in the work of (Hadj Salem, 2009) with some modifications. One ml of AlCl3 (2% in MeOH) was added in 1ml of of methanolic solution of extract or quercetin standard and mixed vigorously. After 20 min, absorbance was measured at 430 nm. The concentrations of flavonoids compounds were calculated according to the following equation that was obtained from the standard as quercetin graph: Y = 0,0245 + 0,2602, R2= 0,9357, where Y= absorbance; X= quercetin. The calibration curve was performed with quercetin and the results were expressed as 1mg of quercetin equivalent per gram of dry weight (l mg QE /g DW). All tests were carried out in triplicate.

Reference Drugs

Sertralin 50 mg and Prednisolone 1 mg/kg were purchased from Pfizer (Eljadida-Maroc) and Laprofan (Casablanca-Maroc) laboratories. They were dissolved in NaCl 0.9 % and administrated i.p. in doses of 0.83 and 0.6 mg/kg b.w. respectively.

Acute toxicity *in vivo*

The acute toxic intervals and efficiency of CL has been studied by "i.p", at the doses of 150, 175, 200, 300, 450, 700 mg/kg, b.w. The animals were observed for obvious toxic symptoms and mortality in each group

within 48 hours was recorded. On the one hand, we seek $LD_{50\%}$ in animals using the method of (Dragstedt *et al*., 1957). On the other hand, we observe the behavior of the animal and the morphological aspect of the liver and kidneys compared to the control. All experimental animals were maintained under close observation for 14 days.

Pharmacological investigations

Forced swimming test: animal model of depression

The studies were carried out rats according to the method described by Porsolt et al., (Porsolt *et al*., 1978). Briefly, rats were individually forced to swim individually for 6 min, in Plexiglas cage (43 x 18.5 x 26.5cm) containing warm water at 25°c. The duration of immobility was measured during the final 4 min interval of the test.

Flow Cytometry (FCM)

The animals are anesthetized by "ip" with sodium pentobarbital at the dose of 30mg/kg, b.w. twenty four hours after treatment with the AE of CL. The blood is collected in heparinized tubes and then subjected to analysis by FCM (flow cytometer, Epics-XL MCL type). FCM was used to evaluate the proportion of leukocyte sub-populations particularly lymphocytes. This is based on cell morphology, size and structure.

Statistical Analysis

Data were expressed as the mean ± SEM. Comparisons of means were performed using the t test of Student. The level of statistical significance was set at $p < 0.05$.

RESULTS

Phytochemical Studies

Phytochemical screening

Preliminary phytochemical analysis revealed that AE of CL contains tannins, saponins, and flavonoids (Table 1). The pharmacological activities of medicinal plants are usually due to their secondary metabolites. Some of the components of the extract have been documented to possess antidepressants activities such as "total saponins" (Dang *et al*., 2009) and sedative properties like "aglycones flavones" (Fontanel *et al*., 2003).

Total Flavonoids

The total flavonoids contents was also determined from calibration curve Y = 0, 0245X + 0, 2602, R^2= 0,9357. This result was calculated at 24, 91 mg of QE equivalent per gram of DW of aqueous extract.

Acute toxicity

The intraperitoneal median lethal dose 50% of the extract was estimated at 450 mg/kg, b.w. (Figure 1). Therefore, infra-toxic doses which have been studied are 150, 175 and 200 mg/ kg, b.w. We have noted neither any behavior disturbances, nor the modification of morphological aspect of the liver or the kidneys.

Table 1. Phytochemical screening of some components of aqueous extract of *C. ladanifer* L.

Chemical constituants	AE of *Cistus ladanifer* L
Tannins *Gallic* *Catechic*	 +++ +++
Flavonoids *(flavones)*	++
Saponins Foam index	 100± 2

The presence of components: (+++) important, (++) medium

Figure 1. Correlation between ranges of doses of AE of the leaves of *C. ladanifer* L. and the percentage of mortality in rats. n= 8 for each group. LD50: 450 mg/kg, b.w, i.p.

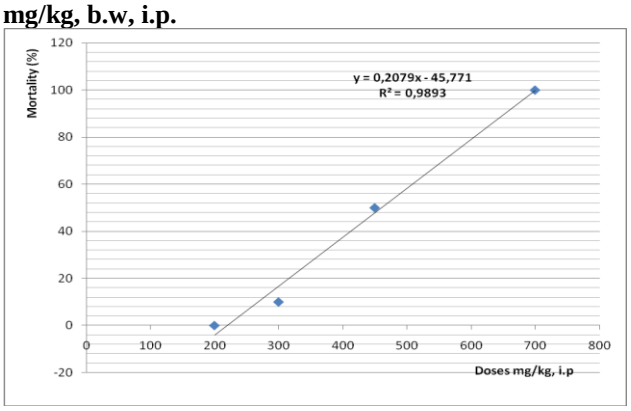


Figure 2. Antidepressive-like effect of AE of the leaves of *C. ladanifer* L. in FST in rats. Results are expressed as mean ± SEM. n = 8. The level of statistical significance was set at $p < 0.05$. ** $p < 0.01$;* $p < 0.001$.**

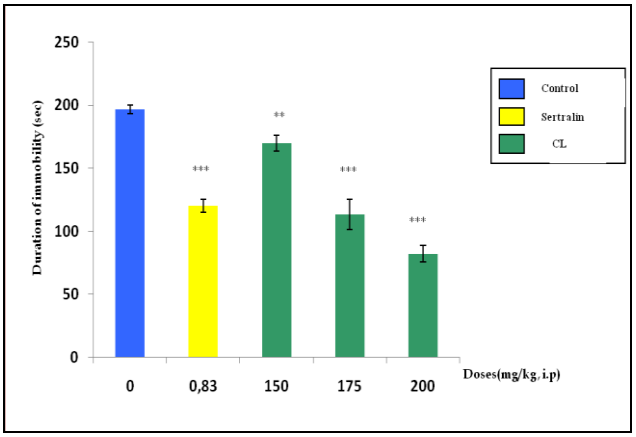
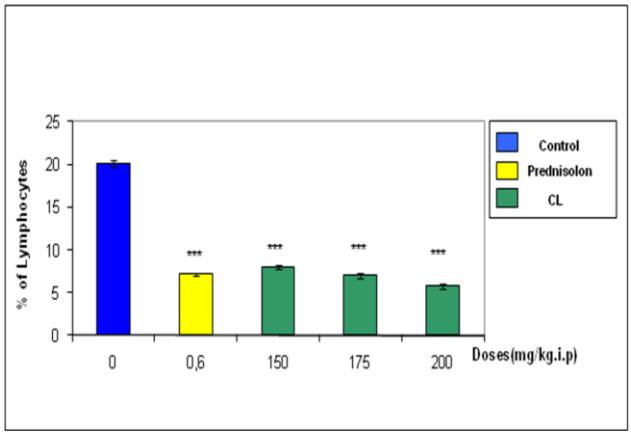


Figure 3. Immunomodulatory activity of AE of the leaves of *C. ladanifer* L. using flow cytometry in rats. Data were expressed as the mean ± SEM. n = 6. The level of statistical significance was set at p < 0.05.* p < 0.001.**



Antidepressant effect of the aqueous extract of *C. ladanifer* L. on the duration of immobility time in the rat forced swimming test

It is shows in Figure 2, a significant antidepressant-like effect *in vivo* at doses 150, 175 and 200 mg/kg, b.w. of the AE of CL in FST. The AE decreased significantly the duration of immobility in a dose-dependent manner (respectively: 13.62%, 42.37% and 58.27%). The effects of CL at 175 and 200 mg/kg, b.w. appeared to be more potent than reference antidepressant "sertralin".

Percentage of lymphocytes sub-populations

The CL extract induced a significant reduction of lymphocytes number after administration of 150, 175 and 200 mg/kg, b.w. (respectively: 39.7%, 34.77% and 29.05%) (Figure 3). In addition, this immunosuppressive effect is more pronounced after administration of 200 mg/kg, b.w. than that induced by reference immunosuppressive drug "prednisolone".

DISCUSSION AND CONCLUSION

This study demonstrates for the first time in the literature an interesting antidepressant and immunomodulatory application of *Cistus ladanifer* L. from Morocco. Initially, we studied the *in vivo* acute toxicity of leaves aqueous extract of CL. Results presented herein show that leaves CL aqueous extract has a median LD_{50} value at 450 mg/kg, b.w. Based on the classification of (Hodge *and* Sterner, 1980), who classified the substances with an LD_{50} between 50 and 500 mg/kg, as moderately toxic.

The FST was a behavioral test in rodent that predicted the clinical efficacy of many types of antidepressant treatments (Porsolt *et al.*, 1977; Porsolt *et al*., 1978; Butterweck *et al*., 1998). The classical antidepressants include the tricyclic antidepressant (TCA), monoamine oxidase inhibitor (MAOI), and selective serotonin reuptake inhibitor (SSRI). All these drugs show an excellent efficacy, most of them frequently produce adverse effects such as sedation, apathy, sleep disturbance, cognitive impairment, sexual dysfunction, etc (Kennedy, 2006). The discovery of new natural drugs in the treatment of psychiatric diseases is attracting more than more the attention of scientists of worldwide. For example, *Hypericum perforatum* L. (St John's Wort), is the only alternative herbal to classic synthetic antidepressants in the therapy of mild to moderate depression (Mario *and* Manfred, 2006). In the present work, we have demonstrated that the aqueous extract of CL leaves significantly reduced the immobility duration in FST in rat in a dose-dependent manner. This result demonstrated a new antidepressant-like effect of leaves aqueous extract at 200 mg/kg, b.w. in rats, more potent than sertralin. The detailed mechanisms involved in the antidepressive-like properties of CL are not yet clear. We can suggest that the antidepressant-like effect of AE of CL is exerted through total saponosides properties, especially triterpenoid type, as well as it was demonstrated in some studies (Noguchi *et al*., 1992; El Hamsas El youbi *et al*., 2010, Hadj Salema *et al*., 2011). In addition, it is demonstrated in our laboratory that ethanolic extract of CL (flavonoïdes ...etc) is exerting an antidepressant effect at least in part by serotoninergic and dopaminergic pathway (unpublished data).

Further, we showed an immunosuppressive effect of aqueous extract of CL at all doses 150, 175 and 200 mg/kg, b.w. We can explain this suppressive effect on the lymphocyte populations at least in part by cytotoxic saponins effects (Gaidi *et al*., 2000; Sparg *and* Light, 2004) and by other CL polyphenols such as flavonoids (flavones). It's demonstrated that flavones and flavonols are more active than isoflavonols in suppression of mouse lymphocyte proliferation (Namgoong *et al*., 1993). Further, other works revealed that an isoflavon "Ginestein" isolated from *Genista tinctoria* exerted a potential inhibition of T lymphocytes proliferation, IL-2 synthesis and IL-2 receptor expression (Atluru *and* Atluru, 1991). Tannins are also discussed for their implication of immune response such as immunosuppressive activity for methyl gallate, tannin compounds isolated from *Acacia nilotica* (Chaubal *et al*., 2005).

This immune depletion is considered as a side effect in antidepressant profile of AE of CL, demonstrated in *vivo* in our work. It's known that nervous depression is associated with dysfunction of a variety of immune parameters such as mitogen response, natural killer cell activity and the numbers, T-cell, and T-cell subpopulations (Mendelovic *et al*., 1999). For that reason,

it will be interesting that further studies will be carried out to develop an antidepressant-like effect from CL with an immunoprotective property, especially that the antidepressant treatment is iprescribed at long-term. Then, it's necessary to identify and isolate the compounds that are responsible for the immunosuppressive effect.

In conclusion, in this study, many data were obtained about the pharmacological effects and screening of some chemical constituents of *C. ladanifer* L from Morocco. In addition, all of these data demonstrated that aqueous extract of leaves of *Cistus ladanifer* L., possessed a potential antidepressant and immunosuppressive properties. This will lead us to further explore the bio-active mechanism and to develop a new natural drug.

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