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# Antioxidant activity, Antinociceptive and anti-inflammatory effects of Pot marigold hydroalcoholic extract on experimental animals

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**Abstract:** This study was conducted to evaluate the analgesic and anti-inflammatory effects of hydroalcoholic extract of Pot marigold aerial part as an indigenous medicinal Iranian plant. Pot marigold (family Asteraceae) is an important plant of genus Calendula (marigolds), having several medicinal applications all over the world. In order to evaluate its analgesic activities hot water tail immersion and acetic acid writhing tests were used. The inflammation inhibitory effect was measured by carrageenan induced paw edema. Adult albino mice (20-30g) and Wistar rats (200-220g) of both sexes were used for the experiment. The results of this study showed that aqueous extract of Pot marigold demonstrated significant differences comparing control and standard groups in all tests. Extracts showed a dose dependent effect on pain or inflammation inhibition. The present study could prove that Pot marigold is effective against pain and inflammation in a dose dependent manner.
**Keywords:** Antioxidant, Antinociceptive, anti-inflammatory, Pot marigold, Hydroalcoholic extract

## Introduction

Nowadays, due to the adverse effects of modern pain relief drugs, are which currently used for the management of pain and inflammatory conditions (Opioids or non-narcotics, non-steroidal and steroidal anti-inflammatory drugs); In recent years, many medicinal herbs have been used as a form of therapy for the relief of pain and inflammation throughout the world without any adverse effects. It is therefore essential and efforts should be made to introduce new medicinal plants to develop drugs which are cheaper, safer and more effective such as *Pot marigold* flower.

Pot marigold (Calendula officinalis L.) is one of the medicinal herbs to the family of Asteraceae, which grows in warm, temperate and Mediterranean region[1] and is indigenous to in subtropical regions and parts of Western and Northwestern Iran. The various species of this family, have several medicinal uses and also are ingredients in a number of important Ayurveda and Homeopathic medicine systems in the world [2, 3, 4]. Many reports and clinical studies describe this plant as having a numerous biological activities such as wound healing[5, 6, 7], anti-inflammatory [5,8,9,] effects. Phytochemical investigation of Calendula officinalis flower and leaves extract reported the presence of the highest flavonoids, flavonol glycosides, coumarines, saponins, triterpenes, alcohol triterpenes, fatty acid esters, carotenoids, essential oils, hydrocarbons, and fatty acids[5,6,8].

The aim of this study was to validate the antioxidant activity and antinociceptive effect and anti-inflammatory effects of hydroalcoholic extract Pot marigold aerial part in experimental animals.

## Methods:

### Preparation of extracts

The Flowering aerial parts of *Pot marigold* were cultivated and identified by Urmia agriculture faculty in July 2013. The Flowering aerial parts of the plant were dried away from sunlight. Plant extract was prepared with 9% acetic acid by maceration at room temperature (24-26ºC) for 3 days. Then the extracts were filtered and concentrated using a rotary vacuum evaporator at 45ºC. The yield (w/w) of hydroethanol extract was 52.7%. The extract were dissolved in normal saline.

### Antioxidant activity

### DPPH Assay

The DPPH free radical inhibition was assessed as described previously by Chen et al. [10] with some modification. Ninthy six wells micro titer plates were used and 5 different concentrations for each sample were prepared. A solution of 100 mg/ml of DPPH in methanol was used and all experiments were conducted in triplicates. After a 45 min incubation at 25ºC (heidolph titramax 1000 and incubator 1000, Germany), the absorbances were recorded at 517 nm by using a Powerwave XS Microplate spectrophotometer (Bio-Tek Instruments, Inc.). The percent of free radical inhibition (In %) was calculated as followed:

$$In\% = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100$$

Where $A_{blank}$ was the absorbance of the control reaction (containing all reagents except the test compound), and $A_{sample}$ was the absorbance of the test compound. Subsequently, the concentration which resulted in 50 % inhibition ($IC_{50}$) was calculated based on the graph plotted of inhibition percentage against samples concentration.

### FRAP assay

Assay of ferric reducing antioxidant power is based on the reduction of ferric tripyridyltriazine (Fe (III) -TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH.

Production of Fe (II) -TPTZ resultds in an intensive blue colour, which is assessable at 593 nm [11]. FRAP reagent was prepared just before each experiment by mixing (10:1:1) diluents from solutions a, b and c (a: Acetate buffer 300 mM pH 3.6, b: 10 mM TPTZ (2, 4, 6-tripyridyl-s- triazine) in 40 mM HCl, c: 20 mM FeCl3. 6 H2O). 20 µl from sample was mixed with 200 µl from FRAP reagent, and then held for 10 min at room temperature and the absorbance was recorded at 593 nm by Powerwave XS Microplate spectrophotometer (Bio-Tek Instruments, Inc.). Different concentrations of FeSO4.7H2O (200, 400, 800, 1200 and 1600 µM) were used as standard solutions, which reacted with TPTZ reagent and the absorbance was plotted against various ferrous ion concentrations. The results were expressed as µM $Fe^{2+}$ equivalents per mg of dried extract. L-ascorbic acid was used as standard antioxidant.

### The total phenolic content

Total phenolic constituents of samples extracts were determined by modified methods described by Saeed et al., based on using Folin-Ciocalteu reagent and Gallic acid (ranging from 0-1000 mg/L) as standard phenolic compound [12].

### Total flavonoids estimation

The aluminum chloride method was applied for the determination of the total flavonoid content of the extracts [12]. The flavonoid content was expressed as mg of quercetin equivalents per gram of dried extract [12].

## Animals & treatment

Adult albino mice (20-30g) and Wistar rats (200-220g) of both sexes were used, taking into account international principles and local regulations concerning the care and use of laboratory animals[13]. The animals

had free access to a standard commercial diet and water ad libitum and were kept in rooms maintained at 22 ± 1 ◦C with a 12-h light/dark cycle. Each animal was used for one experiment only.

**Acute oral toxicity study**

Four groups of mice were used consisting of 10 mice each (5 male and 5 female). One group served as control. The Calendula officinalis extract was suspended in distilled water and administered by oral route to three groups after overnight fast. The doses studied were 0.5, 1 and 2 g/kg body weight, and animals were observed for seven consecutive days to register mortality or other toxic signs[14].

**Carrageenan-Induced Edema in Rats**

Anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced by the injection of 0.1 mL of 2% carrageenan (an edematogenic agent) into the sub plantar region of the right hind paw of the rat[15]. Male Wistar rats were divided into groups of four animals which received per oral (P.o.) doses of extract (200 and 400 mg/kg; 0.1 ml per 10 g body weight), saline or indomethacin (10 mg/kg) 1 hour before the injection of carrageenan, In the left paw, used as a control, 0.1 mL of sterile saline was injected. 1, 2, 3 and 4 hours after injection of carrageenan, the measure of edema was made by the difference between the volume displaced by the right paw and the left paw using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain).

## Antinociceptive study

**Acetic Acid-Induced Writhing Response**

Antinociceptive activity was evaluated using the test of abdominal writhing induced by acetic acid in mice[16]. Mice were divided into groups of four. Control mice received an intra-peritoneal (I.p.) injection of acetic acid 0.6% (0.25 ml) and 5 minutes later the writhes were counted over a period of 30 minutes. One group of mice received Indomethacin (10 mg/kg) by the per oral route as a reference compound, and the other three groups received the extract at doses of 100, 200 and 400 mg/kg, 1 hour before the acetic acid injection.

**Tail immersion test**

Distilled water (5 ml/kg, PO), the extract at doses of 200 and 400 mg/kg (PO) and Morphine (5 mg/kg, SC.) were administered to mice divided into groups of four animals each. 30 min post-treatment, the tail (up to one-third) was immersed in a water bath maintained at 55°C. The reaction time (in sec) was taken as the time when the animals withdrew their tails completely from the hot water in the bath[17]. The response of the extract and Morphine treated groups were compared with those of the animals in the distilled water-treated group (control). In the absence of a response, the tail heating was stopped after 20 second (cut off time) to prevent tissue damage.

**Statistical analysis**

Obtained data were presented as Mean ± Standard deviation (S.D). The statistical differences among groups were assessed using Duncan's multiple test range. A value of $P<0.05$ and $P<0.01$ was considered significant. Statistical analysis was performed using SAS 9.1 for Windows.

## Results

**Antioxidant activity, total phenol and flavonoid contents**

The Pot marigold hydroalcoholic extract concentrations providing 50 % inhibition (IC50) of DPPH free radicals were determined and compared with that of butylated hydroxytoluene (BHT), as a standard antioxidant; and also FRAP assays revealed and comparing to ascorbic acid standard (Table 1). Results of phenolic and flavonoid content of the Pot marigold hydroalcoholic extract showed that the assessed extract yield 51.6±0.8 µg/mg dried extract and 55.22±1.27 µg eq Rutin/mg dried extract for phenol and flavonoid contents, respectively (Table 1).

**Table -1: Antioxidant properties, total phenol and total flavonoid contents of Pot marigold hydroalcoholic extract.**

| | IC50 in DPPH inhibition assay (µg/ml) | Eq+ Fe2 m per mg extract. | Total flavonoid (µg eq Rutin/mg dried extract) | Total phenols (µg/mg dried extract) |
|---|---|---|---|---|
| Pistacia atlantica | 65.19 | 3479.5±74.5 | 55.22±1.27 | 51.6±0.8 |
| Butylated hydroxytoluene | 107.04 | - | - | - |
| Ascorbic acid | - | 7740.2±64.9 | - | - |

**Carrageenan induced paw edema**

Anti-inflammatory activity of the test extracts was measured against acute paw edema induced by carrageenan (Table 2). Carrageenan-induced inflammation in the rat paw represents a classical model of acute inflammation, which is used for evaluation of anti-inflammatory activity of drugs or plant extracts. Hydroalcoholic extracts of pot marigold aerial part showed significant ($P<0.05$, $P<0.01$) and dose dependent reduction of inflammation in rats. Plant extracts showed their effectiveness 2 hours after medication, but Indomethacin as a standard drug inhibited inflammation in the first hour.

**Table -2: Effects of Pot marigold aerial part hydro alcoholic extract on the reaction time of mice exposed to the Carrageenan induced paw edema among rats**

| Group | Dose (mg/kg) | Volume of hind paw (ml) | | | |
|---|---|---|---|---|---|
| | | 1 hour | 2 hours | 3 hours | 4 hours |
| Control | Saline | $0.532\pm0.01^{a}$ | $0.742\pm0.01^{a}$ | $0.917\pm0.03^{a}$ | $0.777\pm0.02^{a}$ |
| Indomethacin | 10 mg/kg | $0.492\pm0.02^{ab}$ | $0.6\pm0.02^{c}$ | $0.615\pm0.02^{d}$ | $0.427\pm0.04^{d}$ |
| Pot marigold extract | 200 mg/kg | $0.527\pm0.02^{ab}$ | $0.69\pm0.03^{b}$ | $0.782\pm0.05^{b}$ | $0.557\pm0.01^{b}$ |
| | 400 mg/kg | $0.51\pm0.03^{b}$ | $0.66\pm0.02^{b}$ | $0.692\pm0.02^{c}$ | $0.48\pm0.02^{c}$ |

All presented as a Mean±standard deviation (S.D) (Statistically significant at $P < 0.05$ and $P < 0.01$).
**Note:** $^{a, b, c, d}$ is presented significant differences between marked groups.

**Acetic acid writhing test**

All extracts were subjected to testing their analgesic activity using the acetic acid-induced writhing method. Significant ($P<0.05$) protection against writhing was observed in animals treated with all doses of the Hydroalcoholic extracts of pot marigold aerial part in a dose dependent manner (Table 3). The analgesic activities induced by the all doses Pot marigold showed extract's effectiveness compared to the control group, but the standard group which received Diclofenac had more influence than treated groups in reduction of writhing numbers.

**Table -3: Effects of Pot marigold aerial part hydro alcoholic extract on acetic acid-induced writhing in mice**

| Group | Dose | Number of writhes | Inhibition (%) |
|---|---|---|---|
| Control | 10 ml/kg | $75.2\pm0.83^{a}$ | - |
| Indomethacin | 10 (mg/kg) | $25\pm0.70^{d}$ | 66.75 |
| Pot marigold extract | 200 (g/kg) | $46.2\pm0.44^{b}$ | 38.56 |
| | 400 (g/kg) | $35.4\pm0.89^{c}$ | 52.92 |

All presented as a Mean±standard deviation (S.D) (Statistically significant at $P < 0.05$ and $P < 0.01$). **Note:** $^{a, b, c, d}$ is presented significant differences between marked groups.

**Tail immersion nociception**

The Pot marigold hydroalcoholic extract increased the latency time of mice exposed to the hot water (Table 4). Hot water result showed significant ($P<0.01$) latency time at 30, 60 and 90 min following extracts medication. Both treated groups showed dose dependent antinociceptive activities. Morphine, a positive

reference (10 mg/kg, SC) showed a significant analgesic effect in the hot-water test beginning 30 min after treatment (P<0.01). Among treated groups the higher dose (400mg/kg) showed better results during the whole experiment.

**Table -4: Effects of Pot marigold aerial part hydro alcoholic extract on the reaction time (min) of mice exposed to the hot water test**

| Group | Dose(mg/kg) | 0 | 30 | 60 | 90 |
|---|---|---|---|---|---|
| Control | 5 ml/kg | 5.42±0.06 | 5.97±0.11$^{c}$ | 6.25±0.06$^{d}$ | 6.35±0.04$^{d}$ |
| morphine | 5 | 5.394±0.06 | 10.28±0.07$^{a}$ | 14.75±0.08$^{a}$ | 13.56±0.04$^{a}$ |
| Pot marigold extract | 200 | 5.454±0.08 | 6.56±0.02$^{b}$ | 7.46±0.10$^{c}$ | 7.25±0.06$^{c}$ |
| | 400 | 5.48±0.07 | 6.62±0.04$^{b}$ | 8.69±0.09$^{b}$ | 8.65±0.09$^{b}$ |

All presented as a Mean±standard deviation (S.D) (Statistically significant at P < 0.05 and P < 0.01).
**Note:** $^{a, b, c, d}$ is presented significant differences between marked groups.

## Discussion

As preliminary phytochemical results indicated, it could be suggested that the antinociceptive and anti-inflammatory effects of the extracts may be due to their content of flavonoids, flavonol glycosides and anthocyanin's. Flavonoids are plant secondary metabolites (gamma-benzopyrone family) that are widely spread in higher plants[18]. Flavonoids and flavonol glycosides possess important pharmacological actions, such as anti-oxidant, anti-inflammatory[19] and also have been shown after systemic administration of flavonoid glycosides, it exerts CNS-mediated activities, and causes sedation, myorelaxation, analgesia and antinociceptive effects[20]. According to a number of other studies, flavonoids like rutin, quercetin, luteolin, hesperidin and biflavonoids yielded important antinocieptive and/or anti-inflamitory activities[21,22]. Anti-inflammatory activity was witnessed among rats upon Carrageenan induces paw edema implementation, which resulted in the production of such mediators as histamine, serotonin, bradyknin, susbstance P and a platelet activating factor and prostaglandins[23]. In this study, oral treatment with the Pot marigold hydroalcoholic extract significantly inhibited the paw edema. The present findings show that anti-inflammatory actions associated with ethanol extract results from inhibition of one or more signaling intracellular pathways which are involved with effects from these mediators.

Also, the link between both antinociceptive activity and moderate anti-inflammatory effect observed with the extracts has been indicated in non-steroidal anti-inflammatory drugs (NSAIDs). It is a well-established fact that NSAIDs exert their analgesic and anti-inflammatory activity by the inhibition of cyclooxygenase activity[24]. Based on the pharmacological tests results, the Pot marigold hydroalcoholic extract has antinociceptive and anti-inflammatory activities, being firstly reported in the literature.

Prostaglandins and sympathomimetic system mediators such as PGE2 and PGF2a were released by intraperitoneal administration of acetic acids. Also, levels of these mediators were increased in the peritoneal fluid of the acetic acid induced mice[25]. Thus, the antinociceptive effect of the hydroalcoholic extract could be mediated by peripheral effects, including the prostaglandin synthesis inhibition. The central action was confirmed in the hot water test (200 and 400 mg/kg), showing that the maximum effect is reached at 90 minutes. This test is considered to be sensitive to drugs acting at the supra spinal modulation level of the pain response[26].

Several studies have been carried out on Pot marigold antinociceptive effect using different methods. In the present study, we used tail flick test using thermal stimulation, writhing test and carrageenan induced inflammation test to evaluate antinociceptive and anti-inflammatory effects of Pot marigold aerial part hydroalcoholic extract. The results clearly showed that Pot marigold was effective in treated groups, especially in higher doses. Further studies are necessary to assess the potential clinical use of this plant, or its extract or active principles as an analgesic drug.

The tribal people use the Pot marigold (Calendula herb) in the form of decoction and in the form of aqueous extract for treatment of pain. The scientific experiment for analgesic activity of pure as well as dilutions (1:1, 1:2) of the leaves extract of Pot marigold (Calendula officinalis) was conducted after intra peritoneal injection in mice using tail immersion method and observed that pure extract produces greatest analgesia of longer duration of action with maximum analgesic activity after thirty minutes of injection[27, 28] .

## Conclusions

This study highlighted the antioxidant activity and antinociceptive effect and anti-inflammatory effects of hydroalcoholic extract Pot marigold (Calendula officinalis) aerial part. Our results showed that different doses of the Pot marigold have antinociceptive effect and anti-inflammatory effect. Moreover, findings of present study represented that Calendula officinalis aerial part hydroalcoholic extracts, have antinociceptive and anti-inflammatory activities in chemical pain and anti-inflammatory tests. All extracts were subjected to testing their analgesic activity using the acetic acid-induced writhing method and hot water tail immersion. Finally, obtaining better results from high dose administration of Pot marigold.

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## References

1. Ahmed K.A., Effect of potassium uptake on the composition of Pot marigold flower's content of essential oil, Emir. J. Food Agric., 2012,25(3).
2. Ao C., Comparative anatomy of bisexual and female florets, embryology in Calendula officinalis (Asteraceae), a naturalized horticultural plant, Sci. Hor. J., 2007, 114 (3), 214-9.
3. Muley B., Khadabadi S., Banarase N., Phytochemical constituents and pharmacological activities of Calendula officinalis Linn (Asteraceae): A review. Trop. J. Pharm. Res., 2009, 8 (5).
4. Kassab S., Cummings M., Berkovitz S., Van Haselen R., Fisher P., Homeopathic medicines for adverse effects of cancer treatments. Cochrane DB. Sys. Rev., 2009, 2.
5. Parente L.M.L., Júnior L., de Souza R., Tresvenzol L.M.F, Vinaud M.C., de Paula J.R., et al., Wound healing and anti-inflammatory effect in animal models of Pot marigold growing in Brazil, Evid-Based Compl. Alt. Med., 2012;2012.
6. Preethi K.C., Kuttan R. Wound healing activity of flower extract of Calendula offlcinalis, J. Basic Clin. Physiol. Pharmacol., 2009, 20 (1), 73-80.
7. Fronza M., Heinzmann B., Hamburger M., Laufer S., Merfort I., Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts, J. Ethnopharmacol., 2009, 126(3), 463-7.
8. Ukiya M., Akihisa T., Yasukawa K., Tokuda H., Suzuki T., Kimura Y., Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (Calendula officinalis) flowers, J. Nat. Prod., 2006, 69 (12), 1692-6.
9. Preethi K.C., Kuttan G., Kuttan R., Anti-inflammatory activity of flower extract of Calendula officinalis Linn and its possible mechanism of action, Indian J. Exp. Biol., 2009, 47 (2), 113-20.
10. Chen F.A., Wu A.B., Shieh P., Kuo D.H., Hsieh C.Y., Evaluation of the antioxidant activity of Ruellia tuberosa, Food chem., 2006, 94(1), 14-8.
11. Benzie I.F., Strain J., The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. biochem., 1996, 239(1), 70-6.
12. Saeed N., Khan M.R., Shabbir M., Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts Torilis leptophylla L. BMC Complement. Altern. Med., 2012, 12(1), 221.
13. Olfert E.D., Cross B.M., McWilliam A.A., Guide to the care and use of experimental animals, Canadian Council on Animal Care Ottawa., 1993.
14. Porfire A.S., Parvu A.E., Daicoviciu D., Leucuţa S., Evaluation of antiinflamatory activity of liposome encapsulated superoxide dismutase in rats peritonitis, Farmacia., 2009, 57(4), 412-23.
15. Winter C.A., Risley E.A., Nuss G.W., Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs, Proc. Soc. Exp. Biol. Med., 1962, 111, 544-7.
16. Collier H., Dinneen L., Johnson C.A., Schneider C., The abdominal constriction response and its suppression by analgesic drugs in the mouse, Br. J. Pharmacol. Chemother., 1968, 32 (2), 295-310.
17. Parimaladevi B., Boominathan R., Mandal S., Studies on analgesic activity of Cleome viscosa in mice. Fitoterapia., 2003, 74 (3), 262-6.
18. Ramelet A.A., Pharmacologic aspects of a phlebotropic drug in CVI-associated edema. Angiology., 2000, 51 (1), 19-23.
19. Meotti F.C., Luiz A.P., Pizzolatti M.G., Kassuya C.A., Calixto J.B., Santos A.R., Analysis of the antinociceptive effect of the flavonoid myricitrin: evidence for a role of the L-arginine-nitric oxide and protein kinase C pathways, J. Pharmacol. Exp. Ther., 2006, 316 (2), 789-96.

20. Fernandez S.P., Nguyen M., Yow T.T., Chu C., Johnston G.A., Hanrahan J.R., et al., The flavonoid glycosides, myricitrin, gossypin and naringin exert anxiolytic action in mice, Neurochem. Res., 2009, 34 (10), 1867-75.
21. Bittar M., de Souza M.M., Yunes R.A., Lento R., Delle Monache F., Cechinel Filho V., Antinociceptive activity of I3, II8-binaringenin, a biflavonoid present in plants of the Guttiferae, Planta Med., 2009, 66 (01), 84-6.
22. Ramesh M., Nageshwar R.Y., Appa Rao A., Prabhakar M., Seshagiri Rao C., Muralidhar N., et al., Antinociceptive and anti-inflammatory activity of a flavonoid isolated from Caralluma attenuata, J. Ethnopharmcol., 1998, 62 (1), 63-6.
23. Di Rosa M., Giroud J., Willoughby D., Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine, J. Pathol. 1971, 104(1), 15-29.
24. Vane J.R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, Nature, 1971, 231 (25), 232-5.
25. Deraedt R., Jouquey S., Delevallée F., Flahaut M., Release of prostaglandins E and F in an algogenic reaction and its inhibition, Eur. J. Pharmacol., 1980, 61 (1), 17-24.
26. Yaksh T.L., Rudy T.A., Studies on the direct spinal action of narcotics in the production of analgesia in the rat, J. Pharmacol. Exp. Ther., 1977, 202 (2), 411-28.
27. Saify Z., Mushtaq N., Noor F., et, al., Analgesic and antimicrobial activity of the leaves' extract of Calendula officinalis. Hamdard Med., 2000 , 43, 34-7.
28. Sarrell E.M., Mandelberg A., Cohen H.A., Efficacy of naturopathic extracts in the management of ear pain associated with acute otitis media, Arch. Pediatr. Adolesc. Med., 2001, 155 (7), 796.

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