

# *in vitro* Effects of a Herbal Remedy for Migraine Treatment, MigriHeal<sup>®</sup>, on Basal and LPS-induced Nitric Oxide

Running title: Effect of MigriHeal<sup>®</sup> on LPS induced NO

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## ABSTRACT

Migraine headache is a neurovascular disorder that apparently is related to inflammatory condition and nitric oxide (NO) overproduction. In recent years, according to anecdotal evidence derived from traditional Iranian medicine a novel herbal remedy named MigriHeal<sup>®</sup> was introduced with prophylactic effect against migraine headache attacks. Due to the role of NO in the pathogenesis of migraine, we hypothesized that MigriHeal<sup>®</sup> works by reducing NO in the treatment of migraine. So, to test the hypothesis we tried using RAW 264.7 macrophage cell line as an *in vitro* model to study the effects of MigriHeal<sup>®</sup> on basal and lipopolysaccharide (LPS)-induced NO levels. Raw 264.7 cells were pretreated with hot water extract and essential oil of MigriHeal<sup>®</sup> for 1 h and then were incubated with or without LPS during 8 and 24 h. Nitrite amounts in culture media were measured. Both aqueous and oil extracts suppressed the LPS-induced NO in a dose-dependent manner without notable effects in absence of LPS. In addition, scavenging analysis of MigriHeal<sup>®</sup> on nitrite showed that both fractions in high concentrations scavenged nitrite released from NO donor DETA-NO in 20 h. These results suggest that MigriHeal<sup>®</sup> may have both iNOS suppressive and NO scavenging properties. As a result, one of the mechanisms of its prophylactic and analgesic effects in treating migraine can be related to NO reduction.

**KEY WORDS:** MigriHeal<sup>®</sup>, Migraine headache, Nitric oxide, Essential oil, Inflammation.

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## INTRODUCTION

Migraine headache, a form of neurovascular headaches, is one of disorders that has obvious neurogenic inflammatory component [1, 2]. This inflammatory condition apparently is related to defects in NO signaling pathway [2, 3]. Nitric oxide (NO) as a diatomic molecule is synthesized by nitric oxide synthases (NOSs) from L-arginine and the increase in activity of NOS is linked with enhanced predisposition to migraine [2, 3]. The previous studies showed that the monocytes of migraine patients released noticeable amount of NO higher than the monocytes obtained from healthy subjects [4, 5] and the excessive NO possibly is derived from increased expression and activity of iNOS [4-7].

The employ of curative herbs for the treatment of headache in Persia can be come from the 6th century BC. Some Persian physicians such as Ebn-e-Sina (980–1037) in Qanoon-fel-teb (The Canon) recognized the healing actions of plants to a specific analgesic, sedative or prophylactic drug property [8]. Recently based on anecdotal evidence found in traditional Iranian medicine a novel herbal remedy named MigriHeal<sup>®</sup> was introduced with prophylactic effect against migraine headache attacks which lasts even after the discontinuation of therapy [9]. This herbal drug which is used as inhalation up to 4 months can decrease headache frequency and severity [2]. It has been patented by the Invention and Patent Registration Office of I.R. of Iran and no adverse effects were reported by patients and animal study proved its safety even in higher doses (unpublished data). Nevertheless, mechanisms of action of MigriHeal<sup>®</sup> have not been understood and more studies are required to elucidate its therapeutic function.

Since NO is importantly involved in nociceptive processing in the central nervous system and it seems to be an important initiating factor in migraine headache [3], we hypothesized that MigriHeal<sup>®</sup> suppress NO as a mechanism of action in migraine treatment. So, to test the hypothesis we tried by RAW 264.7 macrophage cell line as an *in vitro* model to investigate the effects of MigriHeal<sup>®</sup> on basal and LPS-induced NO levels. Drinking and/or inhalation of the water extract or boiled MigriHeal<sup>®</sup> is the most common method of using it in household. In addition,

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ousehold. In addition, one of the most important natural products in herbal medicine is the essential oil of a plant and in medieval Persia, nasal administration of some oils was recommended for the alleviation of pain in patients suffering from headaches [8]. Thus in the current study hot water and essential oil fractions of MigriHeal<sup>®</sup> have been used.

## MATERIALS AND METHODS

LPS (*E. coli* serotype No. 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), and penicillin/streptomycin solution were from Sigma (St. Louis, Mo.). Culture medium Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco. All the other reagents and solvents were from Merck or as showed in the detailed methods.

### MigriHeal<sup>®</sup> extraction

#### *Hot water extraction*

Herbal powder of MigriHeal<sup>®</sup> (100g) was added to 1 L of distilled water and extraction was performed by heating at 80°C. After the extract was filtered under suction through Whatman No. 1 filter paper, the soluble fraction of the extract was refiltered by a filter (0.45 – 25 mm, Millipore) to remove solid particles prior to experiments. Concentrations of extract are expressed as µg of the amount of MigriHeal<sup>®</sup> dried extract per mL solution.

#### *Essential Oil extraction*

The essential oil was taken from 100g of MigriHeal<sup>®</sup> powder that was subjected to hydrodistillation for 4 hours in a Clevenger-type apparatus. The essential oil yield was about 0.90% (v/w). The oil was dried with anhydrous sodium sulphate and stored at -20°C until use.

### Cell culture

The mouse macrophage cell line RAW 264.7 was purchased by Iranian Biological Resource Center (IBRC, C10072) was cultured in DMEM supplemented with 10% heat-inactivated FBS, 15 mM HEPES, 2 mM glutamine, penicillin 100 U/mL and streptomycin 100 µg/mL, at 37 °C, 5 % CO<sub>2</sub> and for experiments between passages 5-20. All experiments were done by 1 h pretreatment of MigriHeal<sup>®</sup> hot water extract (final concentration = 1, 5, 25, 100 µg/mL) or essential oil (final concentration = 1, 5, 25, 100 µg/mL) with or (100 µg/mL) without LPS (5 µg/mL) over 8 h and 24 h periods. The essential oil was dissolved in DMSO, and the final DMSO concentration was 0.12% in all cultures containing this agent; the same amount of DMSO was added to the control cultures.

The dose of LPS (5 µg/mL) was preferred on the basis of our tests to achieve a maximum induction of inducible NO synthesis in RAW 264.7 cells.

### Cell viability determination

Mitochondrial respiration, an indicator of cell viability, was determined by reduction of MTT to formazan as previously described [10]. Briefly, RAW 264.7 Cells (3×10<sup>4</sup> cells/well in 96-well microplates) were treated to MigriHeal<sup>®</sup> hot water extract or essential oil with or without LPS, similar to the main experiments in this study; then were incubated by MTT, the supernatants were discarded and the insoluble formazan was dissolved by dimethylsulphoxide (DMSO). The optical density was measured at 540 nm and then cell viability was defined relative to untreated control cells; viability = 100× (absorbance of treated sample)/ (absorbance of control)).

### Nitrite measurement

The concentration of nitrite, one of the breakdown products of NO, was determined spectrophotometrically using the Griess reagent as previously described [10, 11]. Absorbance was read at 540 nm with baseline correction at 620 nm by a microplate reader (Anthos 2020 UK. Biochrom Ltd.); nitrite concentration was measured using sodium nitrite as the standard.

Beside the measurement of nitrite in culture supernatants, we examined scavenging effect of MigriHeal<sup>®</sup> on nitrite. For this test, MigriHeal<sup>®</sup> (final concentration = 25, 100, 500 µg/mL) or its vehicle was incubated with the NO donor, diethylenetriamine-NONOate (DETA-NO, Alexis Biochemicals, Switzerland) in DMEM supplemented with 10% FBS with a nitrite concentration about 40 µM. After incubation for 4 h and 20 h periods at 37 °C, 5 % CO<sub>2</sub>, the nitrite was measured.

### Statistical analysis

Data are expressed as the mean ± standard error. Differences between groups were analyzed by analysis of variance (ANOVA) with subsequent post hoc Tukey test for sub- two groups comparison, p-value < 0.05 was considered significant.

## RESULTS

### **Effect of MigriHeal<sup>®</sup> hot water and essential oil extracts on cell supernatants NO with or without LPS**

As shown in figure 1, RAW 264.7 cells exhibited a time-dependent increase in nitrite accumulation following incubation with LPS during 8 and 24 h. Pretreatment of MigriHeal<sup>®</sup> in presence of LPS exhibited an inhibitory effect on culture media nitrite in a concentration dependent manner ( $p < 0.05$ ). The highest inhibition of nitrite by hot water treated samples vs. LPS alone occurred in 100  $\mu\text{g}/\text{mL}$  concentration during 8 and 24 h (Fig 1A) ( $p < 0.001$ ). The same maximum inhibition for essential oil was acquired in 24 h (Fig 1B) ( $p < 0.001$ ). As expected, when the cells treated with 100  $\mu\text{g}/\text{mL}$  concentration of hot water and essential oil extracts without LPS, it kept NO level at a basal level like to that was in the no treated controls ( $p < 0.001$ ).

### **Cell viability**

Cell viability determined in parallel experiments of each treated and untreated RAW 264.7 cells described during 24 h. MTT assay showed that both hot water extract and essential oil of MigriHeal<sup>®</sup> with or without LPS did not notable effects on the cell viability of RAW 264.7 cells. As figure 1 shows, the cell viability in all samples was up to 90 % ( $p < 0.05$ ).

### **Scavenging effects of MigriHeal<sup>®</sup> hot water and essential oil extracts on synthetic nitrite**

The study of scavenging effect of NO by MigriHeal<sup>®</sup> hot water extract and essential oil showed that both fractions in a high concentration ( $\geq 100 \mu\text{g}/\text{mL}$ ) scavenged nitrite released from NO donor DETA-NO in 20 h (Fig 2) ( $p < 0.05$ ) but with no notable effect in 4 h (data not shown).

## DISCUSSION

In this study we tried to investigate the effects of MigriHeal<sup>®</sup> hot water extract and essential oil on NO levels, 1 h prior stimulation with or without LPS in RAW 264.7 cells. The main finding of this study was the robustly inhibitory effect of MigriHeal hot water extract and essential oil on NO production in LPS-stimulated murine macrophages. This inhibition was concentration dependent.

Undoubtedly, there are many probable targets and strategies that might permit the inhibition of NO by MigriHeal. Based on cell viability results, it is clear that the decreased production of NO in LPS treated samples was not due to toxicity of included components. In addition, in the same conditions the acquired results by essential oil confirmed the inhibitory effect which is seen by hot water extract. Therefore, it can be say the MigriHeal<sup>®</sup> has a suppressive effect on NO. There are an obvious relationship between the activity and the constituents of the traditional crude drugs because their constituent compounds such as alkaloids, flavonoids, tanins and essential oils have been shown scavenging and suppressive effects on NO donor and LPS induced NO, respectively [12]. So, it appears our drug is not excluding of this rule and it may contain many natural compounds which shown more suppressive effects in high concentration; this can be a reason for dose-dependently effects in our study. Previously, some of the medicinal plants used in the treatment of migraine have shown a reduction in NO production [13-15]. This effect may related to both scavenging of NO (or its stable end metabolites such as nitrite or nitrate) and suppression of iNOS activity and expression.

Despite the fact that we have not confirmed which signal transduction pathway is affected by MigriHeal<sup>®</sup>, incubation of MigriHeal<sup>®</sup> extracts just 1 h before stimulation with LPS and following the inhibition of NO overproduction robustly proposes that suppressive effects of MigriHeal<sup>®</sup> may be related to the inhibitory effect at the upstream signaling linked with induction of the enzyme by LPS. Since the induction of iNOS by LPS is a process needed de novo protein synthesis [16, 17], it may that MigriHeal<sup>®</sup> has acted in iNOS gene transcription and/or protein levels and following the decrease in NO production. In agreement with this hypothesis, several studies have shown that aqueous extracts and essential oils of some medicinal plants used in treatment of migraine [13] or inflammatory disorders [18-20] can suppress the LPS-induced expression of iNOS in macrophage cell lines and the consequent reduction of supernatant NO.

Another mechanism could be associated with the ability of MigriHeal<sup>®</sup> as a scavenger of NO. Our study showed that a high concentration of MigriHeal<sup>®</sup> was capable of scavenging nitrite in 20 h without a notable effect in 4 h. It appears that interaction with reactive oxygen and nitrogen species is the prolonged process in which some natural scavengers one after another scavenge free radicals such as NO and peroxynitrite (ONOO-) [21]. We guess that the difference between 4 and 20 h results may be due to differences in incubation time and the long term incubation gives a more possible scavenging. Before the scavenging effect of nitrite by essential oils [22] and aqueous extracts [18, 19] of medicinal plants have been reported. Nevertheless, the scavenging effects of MigriHeal<sup>®</sup> were in a low level and cannot be regarded as a highly significant influence on NO level. Therefore, the NO suppressing effect revealed in this study was most likely resulted from the inhibition of iNOS activity or expression.

On the contrary to primary products, such as carbohydrates, lipids, chlorophyll, and nucleic acids which participate in modulation of cellular metabolism and maintenance of plant cells, the benefits of phytomedicine

typically may be caused by the combined action of secondary plant compounds [2]. Our previous study has been shown that some medicinal plants used in treatment of migraine headache, contain high concentration of melatonin, an indoleamine derived from tryptophan [23]. Since our studies (unpublished data) and others [24, 25] suggest the suppressive effect of melatonin on iNOS expression in LPS-stimulated macrophages, therefore, one of the reasons for the effectiveness of MigriHeal<sup>®</sup> therapy can be related to melatonin. It is important to know that the melatonin levels are decreased in migraine patients [26]. However, the possibility of other compounds present in MigriHeal<sup>®</sup> cannot be ignored anymore.

Medicines which are directly neutralizing NO, such as NOS inhibitors [27] and NO-scavengers [28], may be effective in the acute or prophylactic treatments of migraine and it has been introduced as possible future candidates for migraine treatment [29]. Our study showed that MigriHeal<sup>®</sup> may have both iNOS suppressive and NO scavenging properties. In conclusion, one of the mechanisms of its prophylactic and analgesic effects in migraine can be associated with suppressed NO. Certainly in the future, further studies are needed to reveal the hidden mechanisms of MigriHeal<sup>®</sup>.

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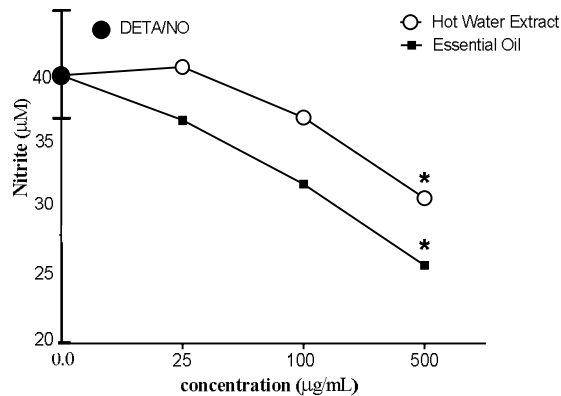
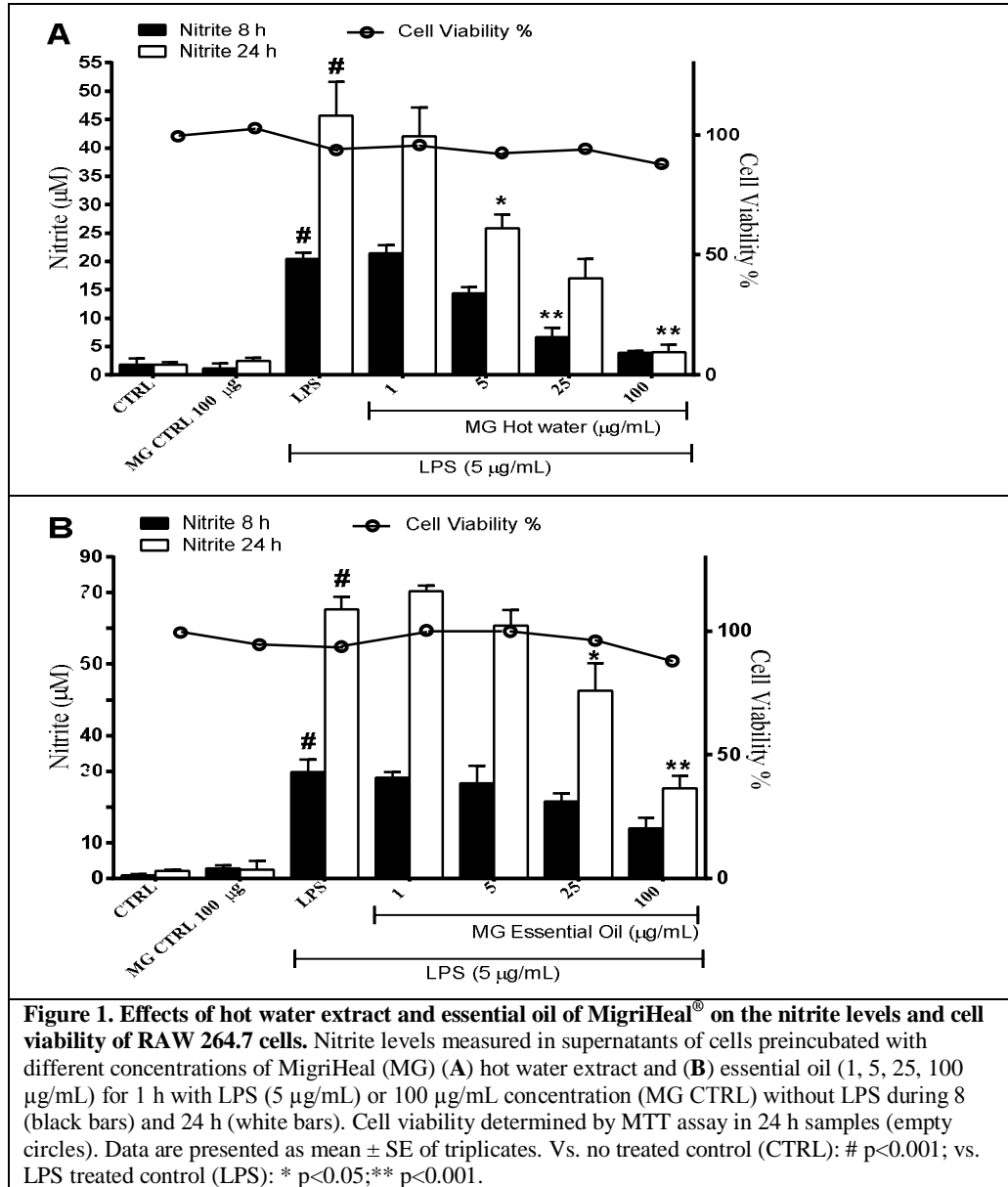
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FIGURES



**Figure 2. Direct effects of hot water extract and essential oil of MigriHeal® on synthetic nitrite.** DETA-NO as NO donor (nitrite, 40 µM) used for assessment of the probable scavenging effects of hot water extract (empty circle) and essential oil (filled square) on nitrite in a 20 h time; nitrite was calculated vs. no treated DETA-NO control (filled circle). Data are presented as mean of triplicates. Vs. no treated DETA-NO control: \* p<0.05.