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Full Length Research Paper

Effect of the extract of *Hypericum perforatum* on neurodevelopment of regions related to pain control and convulsion

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***Hypericum perforatum* (HP) is well-known by the population of the world as herb with antidepressant effect. Its various components, such as hypericin and hyperforin, provide many other effects for this plant, for example, antinociceptive and anticonvulsant. The aim of this work was to determine whether HP administration during pregnancy can cause changes in neurodevelopment related to pain control and seizures in rats (F1). For this, Wistar rats received oral doses of HP at 36, 72 and 144 mg/kg throughout pregnancy. Tests to evaluate the antinociceptive and anticonvulsant activity of HP were performed in adult F1 rats, which showed a decrease of both responses, suggesting therefore that HP exposition during pregnancy causes changes in neurodevelopment of brain regions related to pain control and seizures in rats.**

Key words: *Hypericum perforatum*, antinociceptive effect, convulsion, neurodevelopment, reprogramming, ontogeny, epigenetic.

INTRODUCTION

Herbs have been used for centuries by the world's population for treating a variety of diseases. Among them is *Hypericum perforatum* which belongs to *Hypericaceae* family, popularly known as St. John's wort or hiperico (Greeson et al., 2001; Linde, 2009). Its main components are: naftodiantronas (hypericin), fluroglucinois (hyperforin), biflavonoids, essential oils and procyanidins (Barnes et al., 2001; Mennini and Gobbi, 2004).

The extract of *H. perforatum* (EHP), widely distributed throughout the European, Asian and American continents, is used by the population due to its action against depressive disorders that manifest mild to

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moderate episodes and against anxiety disorders, which become more pronounced during pregnancy. It also has other actions on the central nervous system (CNS), such as antinociceptive and anticonvulsant (Barnes et al., 2001). The anticonvulsant effect is due to the activation of receptors for opioids and serotonin (5-HT), but mainly through activation of receptors for γ-aminobutyric acid (GABA), primarily located in the hippocampus (Hosseinzadeh et al., 2005; Ivetic et al., 2011). The antinociceptive effect results from activation of receptors for GABA, opioids, and 5-HT in both gray periaqueducal area (CPA), and in the spinal cord, besides inhibiting the release of nociceptive mediators associated with inflammation (Uchida et al., 2008; Ozdemir et al., 2012).

Studies in the laboratory (*unpublished data)* showed that in pregnant Wistar rats at doses of 36, 72 and 144 mg/kg, EHP crossed the placental barrier and the blood-brain barrier and was found (seen through its flurorescence emitted by EHP) both in the mother's organs, as well as in the placenta, brain and liver of fetuses. The same authors found that male rats born from pregnant rats treated with different doses of EHP (36, 72 and 144 mg/kg) demonstrated behavior in states compatible with antidepressant and anxiolytic responses, when they became adult (90 day old) (unpublished data*).* These results suggest that EHP, when reaching the embryo, might be able to change the neurodevelopment of rats, whose maturation occurs several weeks after birth, and thus would be responsible for causing epigenetic modifications in a way that reprograms the CNS still under development (Murrin et al., 2007).

Since the offspring of treated mothers showed altered antidepressant and anxiolytic responses, this study aimed to verify if the offspring of treated mothers also expressed antinociceptive and anticonvulsant activities different from those expressed in the young of control animals.

MATERIALS AND METHODS

Animals and housing

The experiments were performed on Wistar rats (*Rattus norvegicus*) 90 days old, weighing 200 to 350 g, born to mothers treated with EHP during pregnancy. Males (F1) were used in other experiments. All animals came from the Vivarium of the Center for Reproduction Biology at the Federal University of Juiz de Fora (UFJF) (CIAPE 01.0048.2013). The rats were housed in plastic cages in air-conditioned cabinets, and kept under monitored conditions of temperature of 23±2°C, and dark/light cycle of 12 h. They were fed on rat chow pellets (Nuvilab^{R}) and received water ad libitum.

Five animals were placed in each cage. To be transported to the testing room, the rats were individually placed in their cages at least 60 min in advance of the tests to minimize possible behavior alteration. They were deprived of water and food 60 min before the test (Almeida, 2006). The experimental procedures of this project were approved by the Committee for Ethics in the Use of Animals (CEUA / UFJF): 063/2013.

Treatment with extract of *H. perforatum*

Extract of H. perforatum

The soluble fraction of the hydro alcohol extract dry containing 0.3% hypericin was prepared by Mbpharma manipulações - Lot 10124778.

Dose calculation: Agência Nacional de Vigilância Sanitária (ANVISA) recommends that the dose of EHP in humans corresponds to the intake of 600 to 900 mg of standardized extract 0.3% hypericin per day (Rodrigues et al., 2006; ANVISA, 2008). Based on body surface area, the minimum dose to rats was 36 mg/kg (Gregoretti et al., 2004).

Experimental groups: Control- pregnant rats received distilled water throughout the gestation period; Treatments- Pregnant rats were grouped into: Treated 1: received 36 mg/kg; Treated 2: 72 mg/kg, and Treated 3: 144 mg/kg of *H. perforatum* by gavage during all gestation.

Experimental procedure: After weaning (postnatal day 21), the offspring were separated according to sex and then females (F1) were housed in polypropylene cages, measuring 30 x 14 x 16 cm, provided with selected wood shavings for bedding, water and food *ad libitum*, in number, 5 per cage. The cages were kept on ventilated shelves (Alesco^{R}), provided with control of air flow, temperature and humidity, and located in an environment with control of circadian cycle (12L: 12D) and temperature.

At 90 days of life, the rats were previously selected in diestrus phase, the period with the lowest plasma concentration of estrogen, to minimize the possible hormone interference with antinociceptive responses (Stoffel et al., 2003). Later, they were euthanized with muscle relaxant xylazine (2% - Kensol^{R}) associated with the anesthetic ketamine (5% - Vetanarcol^{R}) (DBPA; Council., 2011), via *ip*.,followed by diaphragm rupture.

Evaluation of antinociceptive activity

Hot plate test (hot plate)

The rats (F1) of the control and treated groups (T1, T2 and T3) were individually placed on the hot plate (LE 7406 – Panlab) heated to a temperature of 55 ± 1°C. The latency (in seconds), characterized as the time until the appearance of a first response to the thermal stimulus (licking of the paws), was recorded as the index of antinociception for the physical stimulation. The maximum evaluation time is set at 30 s (maximum latency) to minimize damage to the paws of the animals (Kumar et al., 2001; Abdel-Salam, 2005; Almeida, 2006).

The writhing test induced by acetic acid

This is a model of chemical visceral pain induced by injection of glacial acetic acid intraperitoneally (*ip*), which is the contraction of the abdominal musculature followed by hip rotation and stretching of the body and hind limbs. The acid acts directly on the fluid of the peritoneum, causing the release of endogenous inflammatory mediators such as prostaglandins E_2 (PGE_2) and I_2 (PGI_2), among others, thereby sensitizing the nerve endings of the region. The (F1) rats of the control and treated groups (T1, T2 and T3) received 10 mL/kg (*ip*) 0.8% acetic acid (v/v solution), except the control group which received the same volume of distilled water (10 ml/kg).

Five minute after the administration of acetic acid, rats were placed in individual boxes and the number of writhings was evaluated for 15 min. The antinociceptive activity was expressed as the reduction in the number of writhes (Kumar et al., 2001; Abdel-Salam, 2005; Almeida, 2006).

Paw edema test

This consists of nociception induced by chemical stimulation caused by the injection of 50 uL of 2.5% formalin solution (0.92% formaldehyde) under the skin of the plantar surface of the right hind paw (Almeida, 2006). After formalin injection, the rats (F1) of the control and treated groups (T1, T2 and T3) were placed in glass environments with mirrors on the sides to provide a complete view of animal behavior. Episodes of suspension of the hind paw, which received injection with formalin, were monitored and interpreted as a response to nociception (Almeida, 2006). Two periods were considered: the first, known as the initial, or first stage, refers to the neurogenic pain stimulation phase, which begins immediately after formalin injection and lasts for 5 min. The second, known as a late stage, or second stage, refers to inflammatory pain and is between 15 and 30 min after the formalin injection. The antinociceptive activity was expressed as the reduction in the time of suspension of the paw. Moreover, the latency to initiate paw suspension was also evaluated; the increase of which was the parameter used to verify the antinociceptive action of the extract (Almeida, 2006; Uchida et al., 2008).

Evaluation of anticonvulsant activity

Seizures induced by pentylenetetrazol (PTZ)

PTZ induced seizures by blocking Cl^- channels associated with receptors for $GABA_A$ (Bukhari et al., 2004; Hosseinzadeh et al., 2005; Almeida, 2006). Females (F1) from the control and treated groups (T1, T2 and T3) were placed individually in a cage, where they were given intraperitoneal injection of PTZ (60 mg/kg) and observed for 25 min. The latency time for the onset of seizures, as well as the number and duration of seizures were evaluated.

Convulsion induced by pilocarpine (PLC)

Pilocarpine, a muscarinic agonist, produces motor seizures when administered to mice and rats (300 to 380 mg/kg, *ip*) (Almeida, 2006; Ngoupaye et al., 2013). (F1) Females from the control and treated groups (T1, T2 and T3) were placed individually in a cage, where they were given intraperitoneal injection of PTZ (60 mg/kg) and observed for 25 min. The latency time for the onset of seizures was evaluated, as well as the number and duration of seizures.

Convulsion induced by auricular electroshock

The auricular electroshock corresponds to repetitive electrical pulses to induce, in different neuronal structures, a characteristic pattern of seizure activity which, when maintained, is called post-discharge (Almeida, 2006). To evaluate the potential anticonvulsant activity, (F1) rats belonging to the control and treated groups (T1, T2 and T3) were subjected to the auricular shock with a current of 70 mA intensity, at a frequency of 150 pulses/s and duration of 2 s. The following were observed: latency to onset of seizures, number of animals with tonic-clonic seizures, and duration of the convulsive episode (flexion and extension of the legs) (Almeida, 2006; Pahuja et al., 2012).

Statistical analysis

The statistical analysis was developed using SPSS version 21. For verification of normality, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used. Parametric data were analyzed by statistical tests ANOVA and T test, followed by post-hoc Tukey, and, for nonparametric data, Chi square test followed by Mann-Whitney. The graphics were made using the Graph Pad Prism program 5.0. For all tests, those that presented significance levels of $p<0.05$ were considered significant.

RESULTS

Effect of dry extract of *H. peforatum* on antinociceptive activity

Antinociception assessed through the hot plate test

The physical stimulus (high thermal sensation) evaluated by the hot plate test triggers central action neurogenic responses. As shown in Figure 1, the EHP administered to pregnant rats at doses of 36, 72 and 144 mg/kg (T1 groups, T2 e T3, respectively) caused significant increase in latency time in the T3 group (19.5 ± 7.8 s) as compared to the control group (12.6 ± 1.3 s), in the adult F1 rat offspring.

Antinociception assessed by paw edema test

The paw edema test has a biphasic behavior, 2 periods of observation being considered after the administration of formalin. The first stage is characterized by the generation of neurogenic pain, the stimuli of which are primarily transmitted to the CNS by afferent neural fiber types A-δ and C. The second, known as late stage, occurs from the local release of inflammatory mediators such as prostaglandins, histamine, serotonin and bradykinin, and a sharp synaptic activation in the spinal cord and brain via the opioid system, for their control (Uchida et al., 2008).

The observed results show the central nociceptive effect of EHP on F1 rats. Despite latencies for initiation of paw suspension not statistically different between treatment groups 1, 2 and 3 (Table 1), the rats of the T1 and T2 groups showed a significant reduction in the time for legs to remain suspended ($p <0.05$) during the initial test phase (phase 1 neurogenic pain) as compared to the controls, which highlights the central antinociceptive action of the extract (Table 1). However, the extract administration did not express such action during the late phase (phase 2 - inflammatory pain) of paw edema test in any of the treated groups as compared to the control (Table 1).

Antinociception evaluated by the writhing test

The EHP, administered orally (36, 72 and 144 mg/kg) in

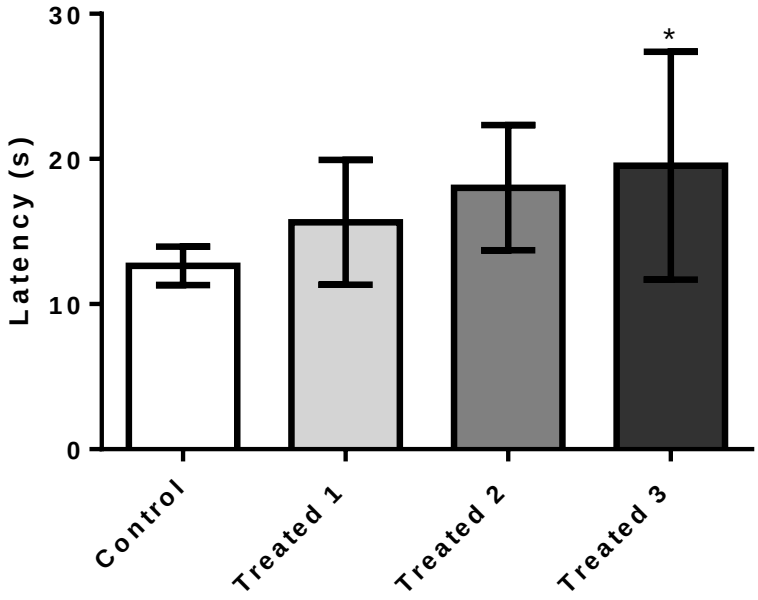

Figure 1. Effect of extract of *H. perforatum* orally administered during pregnancy in adult (F1) rats. Bars show mean values ± SD (n = 10). *p<0.05.

Table 1. Effect of extract of *H. perforatum* orally administered during pregnancy in adult (F1) rats.

Variables	Latency (s)	Paw suspension time: Phase 1 (s)	Paw suspension time: Phase 2 (s)
Control	21.8 ± 4.9	231.8 ± 30.3	470.1 ± 343.3
36 mg / kg	16.9 ± 5.6	159.4 ± 81.6*****	462.2 ± 330.7
72 mg/kg	26.2 ± 8.7	152.2 ± 84.8*****	598.5 ± 355.0
144 mg/kg	39.9 ± 12.6	177.2 ± 64.6	540.7 ± 354.5

The data show mean ± standard deviation. For latency test: n: C = 20, T1 = 9, T2= 9, and T3 = 10 (p> 0.05); for phase 1, n: C = 16, T1, T2 and T3 = 10 (*p<0, 05) and for phase 2, n: 22 C and 10 rats for each treatment group (p> 0.05). (ANOVA Test, followed by the Tukey´s test).

pregnant rats, significantly increased the number of writhes induced by acetic acid in adult F1 offspring in the three treated groups (T1: 34.3 ± 11.4 s; T2: 40.1 ± 14.0 s; T3: 33.8 ± 11.1 s) as compared to the control group (16.5 ± 11.1 s) (p <0.01) (Figure 2). These results suggest the occurrence of inflammatory hyperalgesia activity of the extract on the F1 generation rats as adults..

Effect of dry extract of *H. peforatum* on the anticonvulsant activity

Evaluation by pentilenotetrazol test (PTZ)

The pentylenetetrazol (PTZ) is a convulsant agent which acts by inhibiting the activation of GABA receptors in the CNS, besides reducing nitric oxide production. The EHP, administered orally (36, 72 and 144 mg/kg) in pregnant rats caused increased anticonvulsant activity in adult F1 rats by increasing the latency to onset of seizures in the group treated with the highest dose (T3: 540.2 ± 281.5 s) in the control group (86.3 ± 27.2 s) (Figure 3), and also reduced the seizure rate of T2 groups (16.7%) and T3 (35.7%), as compared to the control (Table 2). However, the duration of convulsive episodes were not statistically different between the treated groups (T1: 30.2 ± 13.6 s; T2: 22.1 ± 4.4 s; T3: 22.5 ± 4.6 s) and control (30.9 ± 8.5 s), despite the tendency of the T2 and T3 groups to present reduction of this parameter as compared to control rats (Figure 4). No animal deaths were observed in the control and treated groups.

Evaluation by testing with pilocarpine (PLC)

The EHP, administered orally (36, 72 and 144 mg/kg) in

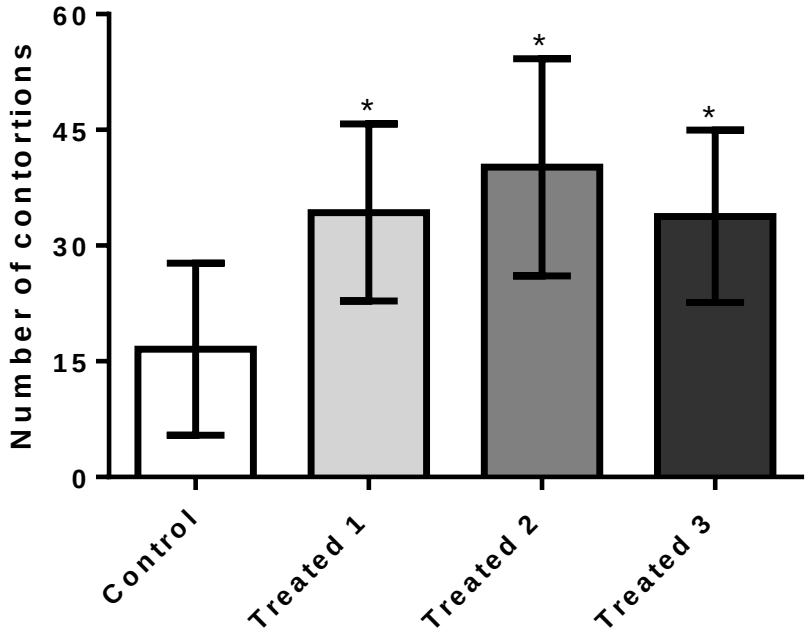

Figure 2. Effect of the extract of *H. perforatum* administered orally during pregnancy in F1 generation rats. Data show mean values ± SD for 10, 9, 10, 18 rats for control and treated groups 1, 2, and 3, respectively, $p < 0.05$.

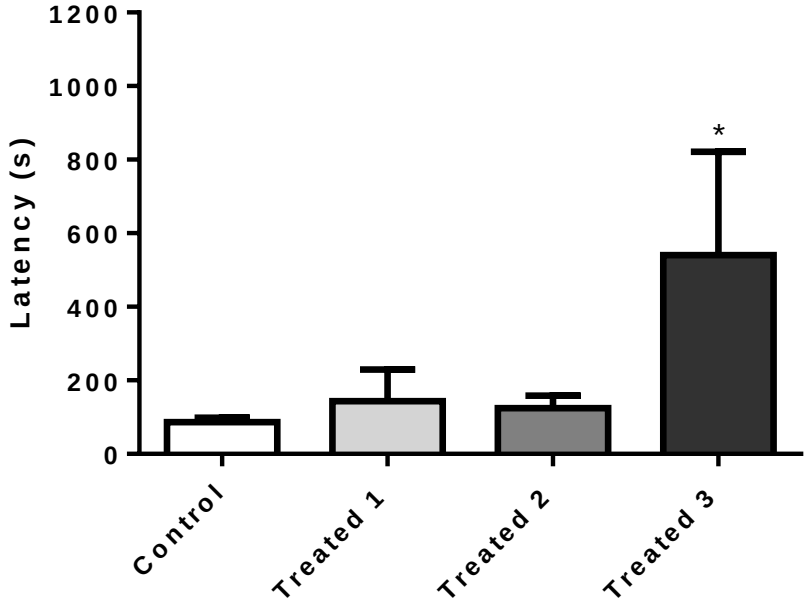

Figure 3. Effect of the extract of *H. perforatum* orally administered during pregnancy stimulated by pentylenetetrazol (60 mg/kg; *ip*) in adult rats (F1). Data show mean values ± SD for 9, 10, 10 and 9 rats by treated and control groups, respectively, *$p< 0.05$.

pregnant rats during pregnancy, showed no anticonvulsant effect in F1 adult rats after induction of seizures by pilocarpine. The latencies (Figure 5) observed in treated groups T1 (606.6 ± 157.1 s), T2 (588.0 ± 159.4 s) and T3 (593.3 ± 128.3 s) did not differ significantly from the control group (624.0 ± 231.8 s).

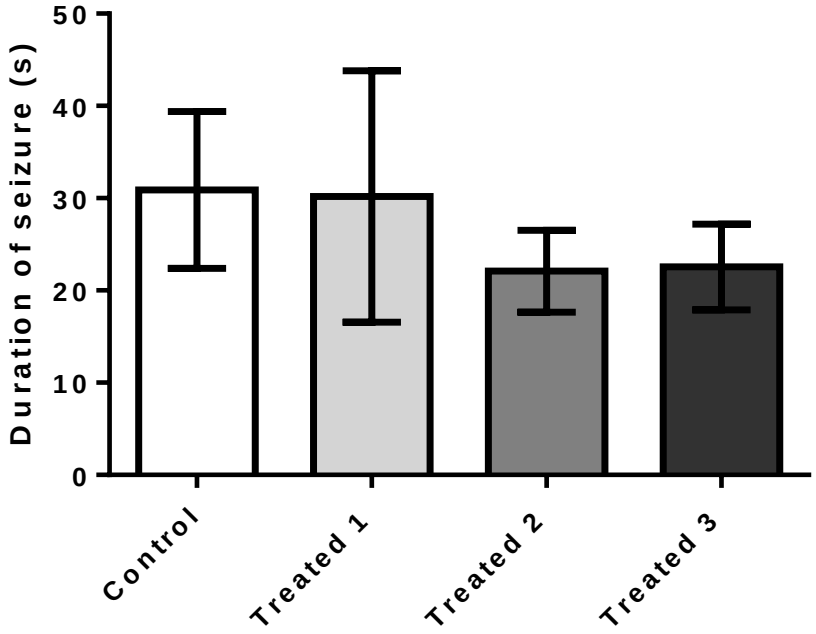

Figure 4. Effect of the extract of *H. perforatum* orally administered during pregnancy stimulated by pentylenetetrazol (60 mg/kg; *ip*) in adult rats (F1). Data show mean values ± SD for 9, 10, 10 and 9 rats by treated and control groups, respectively, p> 0.05.

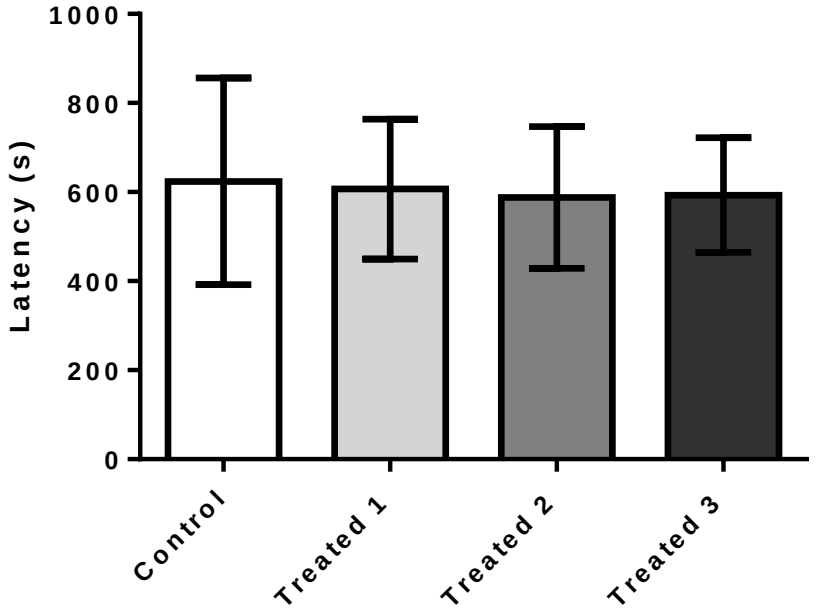

Figure 5. Effect of the extract of *H. perforatum* orally administered during pregnancy stimulated by pilocarpine (350 mg/kg, ip) in (F1) adult rats. Data show mean values ± SD for 10, 9, 10, 18 rats for control and treated groups 1, 2, and 3, respectively, p> 0.05.

Since all animals in the control and treated groups (except one animal of the group T1) expressed seizures induced by pilocarpine, the seizure protection rate was not evaluated.

Table 2. Rate of seizure induced by pentylenetetrazol (PTZ) in adult F1 rats, originated from pregnant rats treated with 36, 72 and 144 mg/kg (T1, T2 and T3, respectively) of dry extract of *H. perforatum* during pregnancy.

Variables			Groups				Total
			C	T1	T2	T3	
Seizure rate	Convulsion	Score	9	10	10	9	38
		Percentage within groups	100.0%	100.0%	83.3%	64.3%	84.4%
	No seizure	Score	0	0	2	5	7
		Percentage within groups	0.0%	0.0%	16.7% *	35.7% *	15.6%
Total		Score	9	10	12	14	45
		Percentage within groups	100.0%	100.0%	100.0%	100.0%	100.0%

Data analysis was performed using Chi-square test ($p<0.05$). C: n = 9; T1, T2 and T3: n = 10, n = 12 and n = 14, respectively.

Table 3. Effect of extract of *H. perforatum* orally administered during pregnancy stimulated by auricular electroshock (frequency: 150 pulses/s; width: 1 ms; duration: 2 s; current: 70 mA).

Variables	Latency (s)	Convulsion time (s)
Control	1.9±2.5	6.3±9.3
36 mg/kg	3.5±3.2	17.3±15.1
72 mg/kg	1.3±1.9	9.8±13.3
144 mg/kg	1.1±2.4	7.2±9.5

The data show mean ± standard deviation. For both tests: n = 10 rats per group (except T2: n = 11) ($p> 0.05$). (ANOVA test, followed by the Tukey test).

Table 4. Seizure rate induced by auricular electroshock in adult F1 rats born to rats treated with 36, 72 and 144 mg/kg (T1, T2 and T3, respectively) extract of *H. perforatum* during pregnancy.

Variables			Groups				Total
			Control	Treated 1	Treated 2	Treated 3	
Seizure rate	No seizure	Score	6	2	6	6	20
		Percentage within groups	60.0%	20.0%	54.5%	60.0%	48.8%
	Seizure	Score	4	8	5	4	21
		Percentage within groups	40.0%	80.0%	45.5%	40.0%	51.2%
Total		Score	10	10	11	10	41
		Percentage within groups	100.0%	100.0%	100.0%	100.0%	100.0%

Data analysis was performed using Chi-square test; n = 10 rats per group (except T2: n = 11) ($p <0.05$).

Evaluation by electroshock auricular test

EHP, orally administered in doses of 36, 72 and 144 mg/kg in pregnant rats during pregnancy, showed no anticonvulsant activity in adult F1 rats in relation to the control group, after induction by auricular electroshock. The parameters evaluated: latency (Table 3); seizure time (Table 3 and seizure rate (Table 4) did not differ significantly between the control and treated groups. No animal deaths were observed.

DISCUSSION

In this work, it was shown that the antinociceptive and anticonvulsant actions of EHP were enhanced in adult F1 rats originating from mothers treated with different doses (36, 72 and 144 mg/kg) throughout pregnancy. The antinociceptive activity of the EHP is promoted by activation of opioid receptors (Galeotti et al., 2010) and GABAergic receptors (Barnes et al., 2001) in the CNS (brain and spinal cord) and also by inhibiting the reuptake of 5-HT noradrenaline and dopamine (Müller, 2003), while the anticonvulsant action results mainly from the activation of GABA receptors in the hippocampus (Ivetic et al., 2011).

Anxiolytic and antidepressant effects of EHP in the F1 generation coming from mothers treated during pregnancy, as well as the absence of neuromotor and

memory changes in neonatal rats were described by Campos and coworkers (unpublished data*).* The assessment of the effects of EHP in the F1 generation is important for verifying toxicity and aims to contribute to the safe use of this herbal medicine for pregnant women, because its physiological action through the activation of CNS receptors opioids (Schuurmans et al., 2015), serotonergic drugs (Kroeze et al., 2016), noradrenergic drugs (Murrin et al., 2007) and GABAergic drugs (Xia and Haddad, 1992), may interfere with neurodevelopment (Kroeze et al., 2016). This action can occur by different mechanisms and pathways, through the control of apoptosis, mitosis, cell differentiation, dendritogenesis and synaptogenesis in different stages of life and places, but especially in areas related to pain control and seizures (Brunton et al., 2012).

Specifically, *H. perforatum* has proven antinociceptive action from the activation of opioid receptors by its component hyperforin (Bukhari et al., 2004; Uchida et al., 2008) and the participation of hypericin (Galeotti et al., 2010a) through dephosphorylation of protein kinase C (PKC), which has algesic action when phosphorylated. The analgesic effect of EHP reaches its maximum point 120 min after administration and tends to decrease thereafter (Galeotti et al., 2010a). Vieira et al. (2013) demonstrated that EHP promoted reduction of states of anxiety and depression in rats treated throughout pregnancy, with effects that lasted up to 60 days after the end of treatment (Vieira et al., 2013). In this case, it is believed that the maintenance of anti-nociceptive effects, and anticonvulsants, from the birth of rats born to treated mothers, is a result of cellular changes that overlap the pharmacokinetic effects of extract components, since these modifications persist into adulthood.

Hyperforin has antinociceptive action via receptor activation *mi, kappa and delta* capable of hyperpolarize neuronal membranes, and also by reduction of PKC expression (γ and ε) the APG (Galeotti et al., 2010a). During pregnancy, there is production of opioid receptors in the brain and spinal cord of rats (Bernard et al., 1990) whose expression shows variations (Van Praag and Frenk, 1991), an increased number occurring of κ receptors (Barr et al., 1986), and μ receptors (Auguy-Valette et al., 1978) until about 15 days post-natal.

It is proposed in this paper that the frequent availability of hyperforin during embryonic development has caused tolerance (down regulation) (He et al., 2002) in the opioid receptors until the termination of pregnancy, reprogramming the brain areas related to pain control (APG and brainstem) and sensitive to this neurohormone, to a state with fewer receptors. However, with the end of treatment with EHP, it is assumed that there has been upregulation as a rebound effect so as to increase the number of receptors in F1 rats, above normal levels of brain growth for this period of post-natal neurogenesis (Bouza, 2009), reprogramming these regions to a new state of normality. As the changes caused by EHP are lasting (Vieira et al., 2013) and plasma concentrations of enkephalins are not altered by treatment with this extract (Uchida et al., 2008), it is believed that the functional response of the receptor has become more marked, increasing the antinociceptive response in the offspring of mothers treated with EHP as compared to the control group young.

The continued use of hyperforin leads to decreased PKC expression in the APG, while hypericin is able to dephosphorylate PKC, inactivating it. Thus, it decreases the expression of transcription factors including Signal Transducer and Activator of Transcription (STAT-1), NF- and Cyclic AMP response element binding protein (CREB), involved in the activation of intracellular pathways in the APG and thalamus of mice (Galeotti et al., 2010b; Galeotti and Ghelardini, 2013). These mechanisms explain the synergistic interaction of hyperforin and hypericin present in the EHP to promote the antinociceptive response which is lasting and maintained into the adulthood of rats analyzed in this study.

Both 5-HT and norepinephrine are neurotransmitters directly involved in nociceptive response and neuro-development. Serotonin is one of the first neurotransmitters synthesized (Gaspar et al., 2003) during embryogenesis, starting the second week of gestation and its production is maintained to the postnatal period. Norepinephrine is synthesized later, but is also involved in the development of the CNS (Murrin et al., 2007). However, the activation maintained of these neurotransmitters generates tolerance (down regulation) (Stahl, 1998; Liang et al., 2007), which compromises this action. Since EHP increases the reuptake of serotonin and norepinephrine, and has been given throughout pregnancy, it is proposed that it acts to reprogram the functioning of the CNS, particularly of the brain stem, so as to increase the pain threshold. This would happen through the manifestation of tolerance mechanisms (prenatal down regulation followed by postnatal upregulation), which enhances the antinociceptive response when activated in adults. Studies have shown that there is a synergistic analgesic effect between morphine and serotonin and norepinephrine reuptake inhibitors (SNRI), when administered concurrently, besides the decrease occurring in morphine tolerance (Ozdemir et al., 2012). Furthermore, α_2-noradrenergic receptors are located side by side in the cell membranes with μ receptors (MOR) in peripheral afferent nerves, which increases the chance of involvement of both in the response to treatment with EHP, by sharing some intracellular signaling pathways (activation of Gq protein - via protein kinase C - PKC - as second messenger) at this location (Aley and Levine, 1997).

GABA also raises the threshold for triggering pain stimulus through hyperpolarization of the cell due to the opening of Cl^- channels (with glycine), mainly in the dorsal horn of the spinal cord (laminae I and II),

hippocampus, thalamus, but not in the cerebral cortex (Kharkevich and Churukanov, 1999; Bonin and De Koninck, 2013). Some studies have shown that this action is stimulated by EHP, occurring mainly via the $GABA_A$ receptor (Turkmen et al., 2011). Following the reasoning above, from the prior knowledge that there is an increase of GABAergic fibers in rats during the first 2 weeks postnatal (Xia and Haddad, 1992). It is believed that the activation of the $GABA_A$ receptor maintained by EHP administered during pregnancy develops tolerance (down regulation) followed by upregulation postnatal in the regions mentioned above, which consequently reprograms fetal nerve cells to produce more GABA receptors. This would result in increased sensitivity in the expression of its natural effect, that is, decrease in pain (Ito et al., 1996).

Thus, the administration of the EHP may have caused a reprogramming of neuronal cells in the CNS regions related to antinociceptive activity (hippocampus, APG, raphe nucleus, posterior horn of the spinal cord), which led the body to have reduced action to control pain and therefore to be less responsive to the detection of physiological and pathological changes. This would result in increased risk to the health and maintenance of homeostasis, featuring the neurotoxic action of EHP on the F1 generation (He et al., 2002; Nagi, 2011).

Nociceptive stimuli related to inflammation are generally associated with tissue damage resulting both from the painful agent, and the immune response against it. Their maintenance, then, is important for detecting changes in homeostasis. They involve the local release of autacoids such as prostaglandins, leukotrienes, 5-HT and others, which stimulate afferent fibers conducting nerve impulses (pain) to the CNS (Brunton et al., 2012). Thereafter, the activation of the antinociceptive pathway occurs by the opioid system, serotoninergic system and others discussed above.

F1 rats born to mothers exposed to EHP showed greater sensitivity to the painful inflammatory agent acetic acid, as noted in the test writhes, though this extract can decrease the production of inflammatory mediators (Albert et al., 2002; Perazzo et al., 2008). As previously discussed, exposure of the F1 animals to EHP during neurogenesis caused increased central antinociceptive response but it is proposed that this could not prevent the generation and propagation of nociceptive stimuli to the central nervous system. Another possibility could be the enhancement of expression of nociceptive inflammatory mediators, also resulting from reprogramming the peripheral nerves, capable of generating pain stimulus greater than the responses for the control. However, this proposition needs to be proved from other studies.

The anticonvulsant action of EHP is well characterized in the literature and involves the activation of the receptors of the opioid system, the serotonergic system, but mostly the GABAergic system ($GABA_A$, via nitric oxide production) (Hosseinzadeh et al., 2005; Ivetic et al., 2011). The results of this study show that adult rats (F1) born to mothers treated with EHP have fewer seizures than untreated rats, when stimulated with PTZ, an antagonist of the GABA neurotransmitter. Therefore, it is assumed that neuronal reprogramming (down regulation prenatal then upregulation post-natal), which occurred during embryonic development, was responsible for alterations in neuron receptors located both in regions related to antinociceptive activity (discussed above), and in regions involved with anti-convulsive episodes (cortex and hippocampus), since the hippocampus presents GABAergic fibers from the beginning of neurogenesis, besides the increased number of GABAergic synapses during the first two postnatal weeks (Swann et al., 1989).

The seizures induced by pilocarpine occur by its action on different receptors in the hippocampus, the striatum and frontal cortex: muscarinic-M_1 (mostly), 5-HT, NMDA and GABA (Freitas et al., 2004). Studies relating seizures to the use of EHP are scarce in the literature. The results of this study allow the supposition that the action of the extract on such receptors in the CNS of F1 rats during embryonic development was not enough to prevent the triggering and maintenance of potential seizures, since the maturation of the cholinergic system occurs late in relation to opioid, serotonergic, noradrenergic and gabaergic systems (to the third postnatal week) (Cavalheiro et al., 1987), besides having little affinity with the EHP.

The auricular electroshock promotes the generation of seizures by physical stimuli that facilitate reaching the threshold for triggering the convulsive potential (Hosseinzadeh et al., 2005). It is believed that this stimulus is stronger than the biochemical stimuli from release of neurotransmitters for the generation of seizures because the originating neural reprogramming changes, through which the (F1) rats having contact with EHP passed, were not enough to promote an effective anticonvulsant response, as found in the results of this work.

In conclusion, the data obtained in this study suggest that treatment of pregnant rats with EHP interferes with neurodevelopment and neurofunction of F1 rats, reprogramming brain areas related to pain control and seizures, making them less responsive to their stimuli, probably due to the increase in opioid, serotonergic, noradrenergic and GABAergic receptors to above normal levels (upregulation postnatal). The treatment caused long lasting effects on the offspring, considering that tests were performed on adult female rats.

Conflicts of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Response of *Rheum australe* L. (rhubarb), (Polygonaceae) an endangered medicinal plant species of Kashmir Himalaya, to organic-inorganic fertilization and its impact on the active component *Rhein*

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Many medicinal plant species, including *Rheum australe* L. (rhubarb), are being exploited from the wild source at an alarming rate. This has resulted in the loss of biodiversity of the species. Roots of *R. australe* have been used in traditional medicine for years for treating various kinds of diseases. The species has been ruthlessly harvested from the wild source and has become endangered. Consequently, preventing the species from getting extinct and increasing the yield of *R. australe* has become a major concern. In the present investigation, an attempt was made to standardize the cultivation practices using organic manures and inorganic fertilizers and their impact on the amount of *Rhein* content in *R. australe*. The experiment comprised of 28 treatments, with three replications each, including one control treatment. The dry root weight recorded by the application of poultry, sheep and farm yard manure was 3878.40, 3200.0 and 3010.0 kg ha^{-1}, respectively. Dry root yield increased significantly with increasing levels of phosphorus and nitrogen as well. Application of organic manure and higher levels of inorganic fertilizers resulted in increase in the root weight, although the effect of their interaction was non-significant. Maximum dry root weight (6012.00 kg ha^{-1}) was observed in $M_1P_2N_2$ (20 tones poultry manure ha^{-1}, 100 kg phosphorus ha^{-1}, and 150 kg nitrogen ha^{-1}) compared to the lowest yield (2200.00 kg ha^{-1}) in $M_3P_0N_1$ (25 tones farm yard manure ha^{-1}, 0 kg phosphorus ha^{-1}, and 100 kg nitrogen ha^{-1}). The highest amount of *Rhein* content (0.393%) was observed in treatment $M_1P_2N_2$, displaying the maximum root weight, however, the least content (0.153) was observed in control treatment ($M_0P_0N_0$).

Key words: *Rheum australe*, root weight, organic manure, inorganic fertilizer, morphological characters, *rhein* content.

INTRODUCTION

Most of the medicinal plant species are collected ruthlessly from the wild source and have become threatened/endangered, thus necessitating the need for their efficient conservation strategies which includes standardization of cultivation practices as the most economical one. The cultivation practices of few medicinal plants of Kashmir Himalayas have been standardized (Kaul, 1983), but same for majority of the rare/threatened medicinal plant species are yet to be standardized. International Union for Conservation of Nature (IUCN) committee for threatened plant species indicates that one in ten species of vascular plants on the earth is endangered or threatened due to commercial exploitation and international trade which may lead to gene erosion in the next 20 to 30 years. *Rheum emodi* is unfortunately among the top of that list. Therefore, it has been identified as a top priority species for conservation and cultivation (Parveen and Wani, 2013). Indiscriminate and non-systematic collection of this valuable medicinal plant species by pharmaceutical companies for its active constituents and by ethnic people for domestic and traditional herbal mixtures has put severe pressure on the availability of this medicinal plant species (Prasad et al., 2001).

Rheum australe D. Don (syn. *Rheum emodi* Wall. ex Meissn., Polygonaceae) is a robust, perennial herb with stout rhizomes. The plant species is distributed in the higher regions of Himalaya, covering the areas of India (Kashmir and Sikkim), Bhutan, Nepal, Pakistan, Myanmar, and China (Bao and Alisa, 2003). The roots of *R. australe* are widely used in Ayurvedic and Chinese folk medicine. This plant species produce diverse phenolic metabolites. More than 56 compounds, belonging to anthraquinones, stilbenes, anthrones, oxanthrone ethers and esters, chromones, flavonoids, carbohydrates, lignans, phenols, and sterols have been identified or characterized from the roots and rhizomes of this plant (Bao and Alisa, 2003, Rokaya et al., 2012, Zargar et al., 2011). Weiguo et al. (2004), determined aloe-emodin, *rhein*, emodin and chrysophanol content in *R. tanguticum* [*R. palmatum*] from Qinghai, China using HPLC method and observed that the emodin, chrysophanol, *rhein* and aloe-emodin content in *R. tanguticum* were higher compared to the commercial medicinal materials. Wuche et al. (2000) used capillary electrophoresis (CE) and HPLC methods for the separation of 12 anthraquinones in crude rhei rhizoma (Rheum). Some of these compounds demonstrated a wide range of biological and pharmacological properties. They are used as anti-cholesterolemic, antiseptic, antispasmodic, antitumor, aperient, astringent, cholagogue, demulcent, diuretic, laxative, purgative, neuron protective, stomachic and tonic (Chopra et al., 1986; Xiang et al., 2005). The root is taken internally in the treatment of chronic constipation, liver and gall bladder complaints, haemorrhoids, menstrual problems and skin eruptions due to an accumulation of toxins and externally the root is used in the treatment of burns (Bown, 1995). The active component *rhein* of the species possesses anticarcinomic, antiseptic, antitumor, antiviral, bactericide, candidicide, cathartic, cytotoxic, pesticide, proteinase-inhibitor, purgative, and viricide properties {(http://www.ansci.cornell.edu/plants/medicinal/rhub.html) (You et al., 2013; Gupta et al., 2014; Andersen et al.,1991). In addition to the curative properties roots of the mentioned species also yield red dye used for coloration of silk and wool (Debaish and Bhattacharya, 2008). Therefore, this plant over the past several decades has been extensively exploited through extraction of crude drug and also through impact of various anthropogenic pressures like grazing, uncontrolled deforestation, selective extraction, and rapid urbanization. The species is categorized as endangered (Nautiyal et al., 2003). In view of the wide application in traditional medicine and in various pharmaceutical industries, it is essential that cultivation practices of the species be initiated, so as to regenerate the germplasm for industrial utilization and to reverse the trend of its extinction in the natural habitat. Further, little is known on the effect of fertilizer application on the root growth and production of bioactive compounds in *R. australe* roots. Keeping all this in view, the present study of cultivation practices of the species were carried out at lower altitude using different types of organic manures and inorganic fertilisers in order to investigate their effect on the root weight and the amount of active component *rhein*.

MATERIALS AND METHODS

The experiment was carried out at the Experimental Field of Division of Floriculture, landscape and Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology Kashmir, Shalimar, Jammu and Kashmir, India. The Soil fertility test of the experimental field was done before the planting of the crop (Table 6). The soil of the experimental field was of clay-loam type. The crop was planted in split-split plot design with three replications each on an area of 400 m^2 which includes main and sub irrigation channels and path in between beds. Organic manures *viz.* poultry manure (M_1, 20 tones ha^{-1}), sheep manure (M_2, 25 tones ha^{-1}) and farmyard manure (M_3, 52 tones ha^{-1}) were used as the main factors. Nutrient content of each of the manure used was also determined

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(Table 7). Three levels each of phosphorus (P_0 = 0, P_1 = 50 and P_2 = 100 kg ha^{-1}) and nitrogen (N0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}) were used as sub and sub-sub factors, respectively. Potassium was applied with a constant level of 50 kg ha^{-1}. One year old seedlings were planted at a spacing of 50 cm × 50 cm in a plot size of 1 m × 1 m. One year old seedlings were planted on 5th of April. Half of the recommended dose of urea (nitrogen source) and the whole dose of diammonium phosphate (phosphorus and nitrogen source) and murat-of-potash (potassium source) were applied at the time of planting. Out of remaining dose of nitrogen, one-fourth dose was applied (in the form of urea) at the time of the first hoeing {(30 days after transplanting (DAT)} and the remaining one-fourth was applied at the time of the 2nd hoeing (64 DAT). Standard package and practices were adopted for raising the healthy crop. Various pre-harvest {(plant height (cm), plant spread (sq. cm), petiole length (cm), number of leaves, leaf area (squared centimeter), number of inflorescences $plant^{-1}$, length of the inflorescence (cm) and number of branches/shoots $plant^{-1}$} and post-harvest {root length $plant^{-1}$ (cm), number of roots $plant^{-1}$, diameter of root $plant^{-1}$ (cm) and root weight (dry weight of the root) $plant^{-1}$} observations were recorded.

Chemoprofiling of some of the samples *R. australe* using high performance liquid chromatographymethod

Experimental chemicals

Chemoprofiling of the samples was done at Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India. All the chemicals, including solvents were of HPLC grade (Sigma, Aldrich, Germany). The standard for *rhein* was purchased from Sigma Aldrich, Germany.

Plant material

The rhizomes samples were collected, washed thoroughly with running tap water to remove extraneous material and were air dried at room temperature (18 ±2.5°C).The voucher specimen (voucher no. HKH. 001 to HKH 0013 \HKH/Srinagar) were deposited in the repository of the Division of Floriculture, landscape and Architecture, S.K. University of Agricultural Sciences and Technology, Shalimar, Srinagar, J& K. A Shimadzu HPLC system was used to estimate the concentration of active component. The samples were analyzed at 30°C on a Merck C_{18} column (5 μm, 250 × 4.0 mm I.D.) by PDA detection.

Preparation of sample solution

Coarse powdered plant material (8.5 mg) was extracted with methanol in Soxhlet while refluxing for 6 to 8 h. The extracts were clarified by centrifugation and then concentrated to dryness under reduced pressure.

Preparation of standard solution

(i) One standard, that is, *Rhein*, prepared indigenously at RRL Jammu and was dissolved in HPLC grade methanol (1 to 2 mg/10 ml). Equal volume of the solution of the standard was mixed and injected in the HPLC system in volumes 2, 4, 6. 8, 10 and 20 µl for development of standard curve.
(ii) The desiccator dried extract was dissolved in HPLC grade methyl alcohol (12.5 mg/ml) and injected in the HPLC (2, 5 and 10 µl) in order to quantify the extract on the basis of *Rhein* for which standard curve have already developed. The components were detected at 290 nm using Diode array PDA detector by simultaneous run-off standard compared with its retention time at a flow rate of 1.08 ml min^{-1} and quantified by standard peak area method.

A Shimadzu HPLC system was used for data analysis and data processing. The samples were analyzed at 30°C on a Merck C_{18} column (5 μm, 250 × 4.0 mm I.D.) by PDA detection.

Calculation of results

Active component in each crop (in percent) was estimated by using the formula:

$$\%\ \text{content of active component} = \frac{\text{Volume of extract in ml x conc. of standard recorded by HPLC in ng}}{\text{Injection volume of extract in µl x wt. of extract dissolved in mg x 1000}} \text{x100}$$

Statistical analysis

The data on various observations collected was subjected to statistical analysis of variance as detailed by Cochran and Cox (1960) for split-split plot design. The significance of the treatment effects was estimated with the help of F-test at 5 and 1% level of significance.

RESULTS AND DISCUSSION

Effect of organic manure on various yield determining characters of *R. australe*

The effect of organic manure on plant spread, leaf area, length of inflorescence, number of branches, root weight, root length and diameter of root was either significant or highly significant. Maximum plant spread (3674.55 cm^2 $plant^{-1}$) was observed in treatment M_2 and minimum (3423.14 cm^2) in treatment M_1 (Table 1). Treatments M_2 and M_3 were at par with each other but were statistically different from treatment M_1. With respect to leaf area, length of inflorescence and number of branches all the treatments M_1, M_2 and M_3 were statistically different from each other (Table 1). Jahan et al. (2004) also showed that consuming 30 ton/ha manure can increase sub-branches of chamomile (*Matricaria chamomilla* L). With respect to length of roots, it was influenced significantly ($p<0.01$) by the application of organic manure (Table 1). Treatments M_1 and M_3 were at par with each other, but were statistically different from treatment M_2. The diameter of the root ranged from 7.44 to 8.52 cm. Treatments varied significantly for this trait, though treatments M_1 and M_2 were at par with each other (Table 1). This is because organic matter uptake can increase soil nutrition content and its absorbing capacity and at the same time, it enhances nitrogen equilibrium and

Table 1. Effect of organic manure, phosphorus and nitrogen on various morphological characters of *R. austral.*

Character	Main treatment (M)				Sub-treatment (P)				Sub-sub- treatment (N)			
	M_1	M_2	M_3	LSD (5%)	P_0	P_1	P_2	LSD (5%)	N_0	N_1	N_2	LSD (5%)
Plant height (cm)	118.88	125.96	127.77	ns	117.11	122.44	133.07	5.72**	119.18	125.88	127.55	ns
Plant spread (sq. cm)	3423.12	3674.55	3635.77	ns*	3350.37	3720.55	3662.55	5.72**	2964.59	3454.22	4314.66	ns**
Petiole length leaf $^{-1}$ (cm)	21.96	21.07	21.44	ns	21.22	22.14	21.11	ns	22.33	20.81	21.33	ns
No. of leaves plant^{-1}	12.33	11.185	11.55	ns	11.11	12.07	11.88	ns	11.07	11.88	12.11	ns
Leaf area plant^{-1} (sq. cm)	5398.8	4787.8	4404.0	151.18**	4594.4	4702.2	5294.1	165.12**	4870.1	4759.5	4961.1	ns
No. of inflorescences plant^{-1}	5.14	5.14	5.00	ns	4.92	5.22	5.14	ns	5.29	5.3333	4.66	ns
Length of inflorescence (cm)	17.44	16.44	13.88	1.67**	14.88	16.77	16.11	0.79**	14.55	16.88	16.33	1.27**
No. of branches plant^{-1}	2.92	2.33	1.77	0.43**	2.33	2.62	2.07	0.33*	2.25	2.66	2.11	ns
Dry weight of root plant^{-1} (g)	96.96	80.00	75.33	16.67*	62.85	86.22	103.2	6.84**	70.81	84.03	97.44	11.24**
Root length plant^{-1} (cm)	26.22	23.03	26.55	2.05*	22.51	24.66	28.62	2.76**	22.77	25.81	27.22	2.92*
No. of roots plant^{-1}	10.70	11.44	10.96	ns	11.55	9.62	11.92	0.65**	9.48	1162	12.00	1.43**
Diameter of root plant^{-1} (cm)	8.52	7.95	7.44	0.08*	7.33	8.01	8.58	0.86*	7.12	8.00	8.79	0.99**

Poultry manure (M_1) (20 tones ha^{-1}), sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}): Phosphorus (P_0=0, P_1= 50 and P_2= 100 kg ha^{-1}) and Nitrogen (N_0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}): M.S. = Mean sum of squares, LSD = Least significant difference, Non-significant (ns) , *Significant at 5% level, **Significant at 1% level. Poultry manure (M_1) (20 tones ha^{-1}), Sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}) phosphorus (P_0 = 0, P_1 = 50 and P_2 = 100 kg ha^{-1}) and Nitrogen (N_0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}) : LSD = Least significant difference, Non-significant (ns) , *Significant at 5% level, **Significant at 1% level.

phosphorous absorption efficiency. However, the effect was non-significant for plant height, petiole length, number of leaves, inflorescences and number of roots.

Effect of organic manure on root weight of *R. australe*

The organic manure comprised of three different manures *viz.* poultry, sheep and farm yard manure. Data recorded on dry weight of root plant^{-1} recorded that organic manure had a significant effect on dry weight of roots plant^{-1} (Table 1). Treatment M_1 was statistically different from treatment M_2 and M_3. Highest dry weight (96.96 g) of root plant^{-1} was recorded in treatment M_1 and the least (75.33 g) in treatment M_3 (Table 1). The difference in dry weight of root plant^{-1} between M_1 and M_3 treatments was 21.63 g and between M_1 and M_2 treatments it was only 16.96 g, obviously; indicating that poultry manure yields better results as compared to sheep and farm yard manure. On per hectare basis (kg ha^{-1}) the dry root weight recorded by the application of poultry, sheep and farm yard manure was of the order of 3878.40, 3200.0 and 3010.0 kg ha^{-1}, respectively. Application of poultry manure produced 678.40 and 868.40 kg ha^{-1} more yield over the sheep and farm yard manure (Table 3). The increase in root yield was the cumulative effect of the yield attributes. The high response of *R. australe* in terms of root yield may be attributed to additional nutrition supplied through poultry manure. However, Nautiyal et al. (2003) reported more yield in forest litter treated beds as compared to buffalo and sheep manure treated beds in *R. emodi*.

Effect of phosphorus on various yield determining characters of *R. australe*

Phosphorus had a significant ($p < 0.05$) or highly significant ($p < 0.01$) effect on plant height, plant spread, leaf area, length of inflorescences, root length, number of roots, number of branches and diameter of root. All the treatments were statistically different from each other. Higher plant height (133.07 cm) was obtained in treatment P_2 as compared to other two treatments (P_0 and P_2). Phosphorus resulted in a concomitant increase in the leaf area plant^{-1}. Maximum leaf area (5294.1 cm^2) was recorded in treatment P_2 and minimum

(4594.4 cm^2) in treatment P_0. Treatment P_2 was statistically different from P_1 and P_0. Increase in length of inflorescence, diameter of root, root length, number of roots and number of branches was also observed with increasing levels of phosphorus. However, different levels of phosphorus did not differ significantly with respect to each other in petiole length, number of leaves and number of inflorescences (Table 1).

Effect of phosphorus on root weight of *R. australe*

Application of phosphorus had a highly significant ($p<0.01$) effect on the root weight (Table 1). All the treatments P_0, P_1 and P_2 were statistically different from each other. The highest dry weight of root (103.20 g $plant^{-1}$) was observed in treatment P_2 and the least (62.85 g $plant^{-1}$) in control (P_0). On per hectare basis the dry weight of root increased significantly from 934.80 to 1614.00 kg ha^{-1} with increasing levels of phosphorus (50 to 100 kg ha^{-1}) (Table 3). Increase in root yield seems to be a reflection of favourable influence of phosphorus on important yield attributes like plant height, plant spread, leaf area, root length and root diameter. Proper nutrition of plants is an important factor in determining their performance. Phosphorus being a macronutrient plays a vital role in plant growth and development. Thus higher phosphorus levels seem to have helped in increasing the crop growth by the improvement of yield attributes. Similar results were obtained by Lu et al. (2013) while working with *Salvia miltiorrhiza*. In a similar way, Ombodi and Saigusa (2000) reported that fertilizer treatments improve the nutritional quality of rhubarb. They also reported that the improved nutritional quality in the polyofelin-coated diammonium phosphate (POC-DAP) treatment was a cause of ammonium nutrition rather than a cause of less amount of released nitrogen.

Effect of nitrogen on various yield determining characters of *R. australe*

The effect of nitrogen on plant height, petiole length, no. of branches, no. of leaves, leaf area and no. of inflorescences $plant^{-1}$ was non-significant. However, the highest plant height and numbers of leaves $plant^{-1}$ was obtained in treatment N_2 and the least in control (N_0). Increase in the value of various morphological characters with the application of nitrogen fertilizers was also reported by Ozguven and Sekeroglu (2007) while working with black cumin. Plants depicted a significant increase ($p < 0.01$) in plant spread, root length, number of roots and diameter of root with the increase in nitrogen application. Maximum plant spread (4314.66 cm^2 $plant^{-1}$) was observed in treatment N_2 and minimum (2964.59 cm^2 $plant^{-1}$) in control (N_0). In case of root length and no. of roots $plant^{-1}$ treatments N_2 and N_1 were statistically different from treatment N_0. Highest diameter of root (8.79 cm $plant^{-1}$) was observed in treatment N_2 and the least (7.12 cm $plant^{-1}$) in control treatment (N_0). Treatment N_2 was statistically different from treatments N_1 and N_0. However, treatment N_1 was at par with a control (N_0) (Table 1). El-Sayed et al. (2012) also found that the highest level of nitrogen (300 kg/fed.) on *Echinacea parudoxa* L. significantly improved plant height, fresh and dry weight of herb, fresh and dry weight of whole plant.

Effect of nitrogen on root weight of *R. australe*

Root weight depicted a linear increase with increasing levels of nitrogen. It had a highly significant influence ($P<0.01$) on dry weight of root $plant^{-1}$ (Table 1). All the treatments *viz.* N_0, N_1 and N_2 were statistically different from each other. Highest root weight of 97.44 g $plant^{-1}$ was observed in treatment N_2 and the least of 70.81 g in treatment N_0 (Table 1). On per hectare bases, the highest (3897.60 kg ha^{-1}) dry weight of root was observed in treatment N_2 and least (2832.40 kg ha^{-1}) in treatment N_0 (Table 3). The results obtained agreed with Shaheen et al. (2007) who showed that, treating *Cynara scolymus* with 100 to 120 kg N/fed as ammonium sulphate gained the best values of fresh and dry weight yield. Increase in the yield is attributable to the vigorous growth of plants with respect to plant height, plant spread and other morphological features at higher level of nitrogen (150 kg ha^{-1}) (Table 1); resulting in accumulation of more photosynthates, which are responsible for increasing root yield. Nitrogen being a major constituent of proteins and phospholipids plays a vital role in plant growth and development. Thus higher nitrogen levels have helped in increasing the crop growth and improvement of yield attributes. Similar findings were reported by Rishi et al. (1988) in *Dioscorea deltoidea,* where they found that the tuber yield ha^{-1} and diosgenin content increased with the application of nitrogen up to 80 kg ha^{-1}. Even the nutritional quality of rhubarb has been reported to improve as a result of nitrogen fertilization (Ombodi and Saigusa, 2000).

Effect of interaction of organic manure, phosphorus and nitrogen on various yield determining characters of *R. austral*

The effect of interaction was significant ($p<0.005$) or highly significant ($p<0.001$) on plant height, plant spread, petiole length, leaf area and number of branches $plant^{-1}$ (Table 2). The highest plant height (163.00 cm $plant^{-1}$) was obtained in treatment $M_1P_2N_2$ and least (84.00 cm $plant^{-1}$) in treatment $M_1P_2N_0$ (Table 2). Treatment $M_1P_2N_2$ was at par with treatments $M_2P_1N_0$, $M_2P_2N_0$, $M_2P_2N_1$, $M_2P_2N_2$, $M_3P_0N_2$, $M_3P_1N_1$ and $M_3P_2N_2$ but was statistically different from all other treatments. Remarkably

Table 2. Effect of interaction of organic manure, phosphorus and nitrogen on various morphological characters of *R. australe*

S/N	Treatment	Plant height (cm)	Plant spread (sq. cm)	Petiole length leaf^{-}(cm)	No. of leaves plant^{-1}	Leaf area plant^{-1} (sq. cm)	No. of Inf. plant^{-1}
1	$M_1P_0N_0$	114.00	3153.33	29.00	11.00	4691.00	4.66
2	P_0N_1	122.00	2810.00	27.00	13.00	5001.00	4.66
3	P_0N_2	128.00	3689.00	18.00	12.00	5229.00	4.00
4	P_1N_0	131.00	1535.00	20.00	13.00	5125.00	6.00
5	P_1N_1	114.00	2959.00	15.66	12.00	5301.00	6.00
6	P_1N_2	96.00	5399.00	28.00	12.00	5604.00	5.00
7	P_2N_0	84.00	3390.00	15.00	10.00	6225.00	4.00
8	P_2N_1	118.00	3577.00	23.00	14.00	5984.00	6.00
9	P_2N_2	163.00	4296.00	22.00	14.00	5430.00	6.00
10	$M_2P_0N_0$	113.00	2318.00	25.00	11.00	4705.00	5.00
11	P_0N_1	104.00	2268.00	15.00	9.000	4600.00	5.00
12	P_0N_2	98.000	4804.00	18.00	10.00	4502.00	6.00
13	P_1N_0	137.00	2092.00	21.00	10.66	4669.00	6.00
14	P_1N_1	123.00	4578.00	26.66	11.00	4013.00	6.00
15	P_1N_2	115.00	5133.00	22.00	14.00	5200.00	4.00
16	P_2N_0	146.66	3793.00	24.00	11.00	4928.00	7.00
17	P_2N_1	157.00	3809.00	16.00	11.00	5224.00	3.33
18	P_2N_2	140.00	4276.00	22.00	13.00	5250.00	4.00
19	$M_3P_0N_0$	108.00	2633.00	22.00	11.00	4508.00	5.00
20	P_0N_1	125.00	3789.00	18.00	12.00	410500.	5.00
21	P_0N_2	142.00	4689.00	19.00	11.00	4009.00	5.00
22	P_1N_0	119.00	3801.00	27.00	12.00	4300.00	5.00
23	P_1N_1	139.00	5104.00	20.00	13.00	3708.00	6.00
24	P_1N_2	128.00	2884.00	19.00	11.00	4400.00	3.00
25	P_2N_0	120.00	3966.00	18.00	10.00	4680.00	5.00
26	P_2N_1	131.00	2194.00	26.00	12.00	4900.00	6.00
27	P_2N_2	138.00	3662.00	24.00	12.00	5026.00	5.00
28	$M_0P_0N_0$	85.00	1536.00	14.00	08.00	3608.00	3.22
LSD (5%)		28.98*	28.98**	4.83**	ns	596.45*	ns

S/N	Treatment	Length of Inf. plant^{-1}	No. of branches plant^{-1}	Root weight plant^{-1}	Root length plant^{-1} (cm)	No. of roots plant^{-1}	Diameter of root plant^{-1} (cm)
1	$M_1P_0N_0$	14.00	2.00	67.66	20.00	7.666	6.70
2	P_0N_1	17.00	3.00	71.66	22.00	11.33	8.00
3	P_0N_2	19.00	2.00	72.33	24.00	15.00	8.20
4	P_1N_0	15.00	4.00	78.33	20.66	9.66	7.60
5	P_1N_1	16.00	3.00	93.33	25.00	8.33	8.20
6	P_1N_2	19.00	3.00	119.3	23.33	9.00	9.20
7	P_2N_0	20.00	2.00	92.33	36.00	9.00	7.80
8	P_2N_1	18.00	4.33	127.30	27.33	14.33	10.00
9	P_2N_2	19.00	3.00	150.31	37.66	12.00	11.00
10	$M_2P_0N_0$	12.00	2.00	63.00	21.33	7.66	6.00
11	P_0N_1	18.00	2.66	58.00	22.00	12.00	7.70
12	P_0N_2	16.00	4.00	63.00	22.00	14.66	8.00
13	P_1N_0	18.00	3.00	70.00	21.00	9.66	7.50
14	P_1N_1	18.00	3.00	87.00	24.00	12.00	8.09
15	P_1N_2	20.00	2.00	92.00	23.00	9.33	9.20
16	P_2N_0	15.00	2.33	75.00	20.00	10.00	7.53
17	P_2N_1	17.00	1.00	92.00	26.00	14.00	8.20
18	P_2N_2	14.00	1.00	120.0	28.00	13.66	9.40

Table 2. Cont'd.

19	$M_3P_0N_0$	10.00	1.66	55.00	20.00	9.00	6.90
20	P_0N_1	15.00	2.00	55.00	24.00	13.00	7.50
21	P_0N_2	13.00	1.66	60.00	27.33	13.66	7.00
22	P_1N_0	13.00	1.66	66.00	23.00	12.66	6.80
23	P_1N_1	17.00	3.00	82.00	34.00	7.33	7.10
24	P_1N_2	15.00	1.00	88.00	28.00	8.66	8.40
25	P_2N_0	14.00	1.66	70.00	23.00	10.00	7.30
26	P_2N_1	16.00	2.00	90.00	28.00	12.33	7.20
27	P_2N_2	12.00	1.33	112.00	31.66	12.00	8.80
28	$M_0P_0N_0$	11.00	1.00	53.00	20.00	7.351	6.12
LSD (5%)		ns	1.53*	ns	ns	ns	ns

Poultry manure (M_1) (20 tones ha^{-1}), sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}), phosphorus (P_0=0, P_1= 50 and P_2= 100 kg ha^{-1}) and nitrogen (N_0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}): LSD = Least significant difference, non-significant (ns), *Significant at 5% level, **Significant at 1% level.

Table 3. Yield of economic part as affected by organic manure phosphorus and nitrogen in *R. austral.*

S/N	Treatment	Root weight m^{-2} (g)	Root weight (kg ha^{-1})
1	M_1	387.84	3878.40
2	M_2	320.00	3200.00
3	M_3	301.32	3013.20
4	P_0	251.40	2514.00
5	P_1	344.88	3448.80
6	P_2	412.80	4128.00
7	N_0	283.24	2832.40
8	N_1	336.12	3361.20
9	N_2	389.76	3897.60

Poultry manure (M_1) (20 tones ha^{-1}), sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}) phosphorus (P_0=0, P_1= 50 and P_2= 100 kg ha^{-1}) and nitrogen (N_0= 0, N_1= 100 and N_2= 150 kg ha^{-1}).

significant ($p < 0.001$) increase in plant spread plant^{-1} was observed in all most all the treatments with the application of organic manure, phosphorus and nitrogen (Tables 2). Highest plant spread (5399.00 cm^2 plant^{-1}) was observed in the treatment $M_1P_1N_2$, as compared to the plant spread of 1535.00 cm^2 plant^{-1} in treatment $M_1P_1N_0$ (Table 2). Leaf area plant^{-1} increased significantly ($p < 0.01$) in all the treatments with the application of organic manure, phosphorus and nitrogen (Table 2). The effect of interaction was non-significant on number of leaves, length of inflorescence, root length, number of roots, and diameter of root.

Effect of interaction of organic manure, phosphorus and nitrogen on root yield or root weight (kg ha^{-1}) of *R. australe*

Maximum dry root weight of 150 g plant^{-1} was observed in treatment $M_1P_2N_2$ compared to the lowest dry yield of 53 g plant^{-1} in control treatment $M_0P_0N_0$ (Table 2). However, the effect of interaction was non-significant (Table 2). Maximum dry root weight (6012.00 kg ha^{-1}) was observed in treatment M_1 P_2 N_2 compared to the lowest yield (2120.00 kg ha^{-1}) in control treatment M_0 P_0 N_0 (Table 4). Poultry, sheep or farmyard manure in combination with higher levels of nitrogen (150 Kg ha^{-1}) and phosphorus (100 kg ha^{-1}) resulted in increased yield of root. Further, results revealed that poultry manure in combination with higher level of nitrogen and phosphorus showed the highest dry yield of root in contrast to sheep and farm yard manure receiving the same combination of inorganic fertilizers. It was also observed that poultry manure when used in combination with lower or higher levels of phosphorus (50 and 100 kg ha^{-1}) and nitrogen (100, 150 kg ha^{-1}) produced a higher dry yield of root (Table 4). This is in contrast to sheep manure or farmyard manure receiving the same combination of inorganic fertilizers. Evidently, poultry manure had a remarkable effect on the dry weight of the root irrespective of the

Table 4. Yield of economic part as affected by interaction of organic manure, phosphorus and nitrogen *in R. austral.*

S/N	Treatment	Root weight m^{-2} (g)	Root weight (kg ha^{-1})
1	$M_1P_0N_0$	270.64	2706.40
2	P_0N_1	286.40	2864.00
3	P_0N_2	289.32	2893.20
4	P_1N_0	313.32	3133.20
5	P_1N_1	373.32	3733.20
6	P_1N_2	477.32	4773.20
7	P_2N_0	369.32	3693.20
8	P_2N_1	509.32	5093.20
9	P_2N_2	601.20	6012.00
10	$M_2P_0N_0$	252.00	2520.00
11	P_0N_1	232.00	2320.00
12	P_0N_2	252.00	2520.00
13	P_1N_0	280.00	2800.00
14	P_1N_1	348.00	3480.00
15	P_1N_2	368.00	3680.00
16	P_2N_0	300.00	3000.00
17	P_2N_1	368.00	3680.00
18	P_2N_2	480.00	4800.00
19	$M_3P_0N_0$	220.00	2200.00
20	P_0N_1	220.00	2200.00
21	P_0N_2	240.00	2400.00
22	P_1N_0	264.00	2640.00
23	P_1N_1	328.00	3280.00
24	P_1N_2	352.00	3520.00
25	P_2N_0	280.00	2800.00
26	P_2N_1	360.00	3600.00
27	P_2N_2	448.00	4480.00
28	$M_0P_0N_0$	212.00	2120.00

Poultry manure (M_1) (20 tones ha^{-1}), sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}) phosphorus (P_0=0, P_1 = 50 and P_2 = 100 kg ha^{-1}) and nitrogen (N_0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}).

inorganic fertilizer used as compared to sheep or farmyard manure; even though the quantity of poultry manure applied was less than the sheep and farm yard manure (Table 4). Increase in root yield seems to be a reflection of favourable influence of organic manure and inorganic fertilizers on important yield attributes like plant height, plant spread and leaf area (Table 2).

Chemoprofiling of thirteen different extracts or samples of *R. australe* on the basis of one marker *rhein*

Calibration curve

Rhein : Linear Y= 0.000277389x+0
Goodness of fit ($r^{\wedge 2}$): 0.996119

The calibration curve and the chromatogram of the standard *rhein* are given in Figures 1 and 2. Highest amount of *rhein* content (0.393) was observed in treatment $M_1P_2N_2$ (20 tones poultry manure ha^{-1}, 100 kg phosphorus ha^{-1}, and 150 kg nitrogen ha^{-1}) and the least (0.153) in control treatment ($M_0P_0N_0$) (Table 5). Among the manures, the highest *rhein* content was obtained in treatment $M_1P_0N_0$ (20 tones poultry manure ha^{-1}, 0 kg phosphorus ha^{-1}, and 0 kg nitrogen ha^{-1}) followed by treatment $M_2P_0N_0$ and the least in treatment $M_3P_0N_0$. Poultry, sheep and farm yard manure used in combination with higher levels of phosphorus (100 kg ha^{-1}) and nitrogen (150 kg ha^{-1}) produced slightly increased *rhein* content (0.394, 0.360 and 0.315%) as compared to other treatment combinations except treatment $M_1P_2N_0$ (0.315%). Rishi et al. (1988) reported an increase in diosgenin content in *Dioscorea deltoidea* with the increase in application of nitrogen up to 80 kg ha^{-1}. Plants of these treatments showed an increasing trend in dry weight accumulation also. Highest *rhein* content was

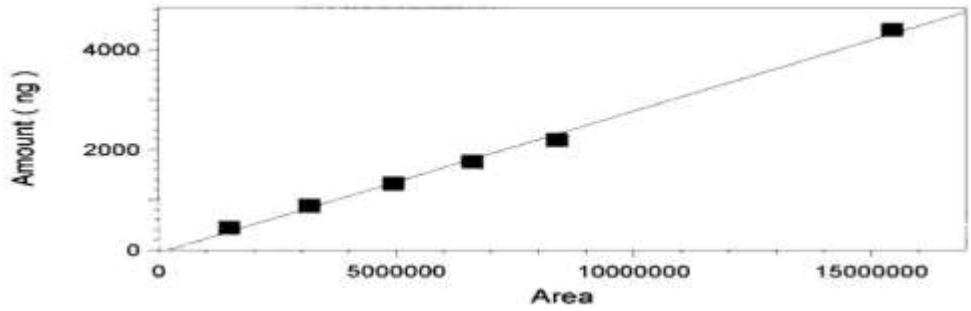
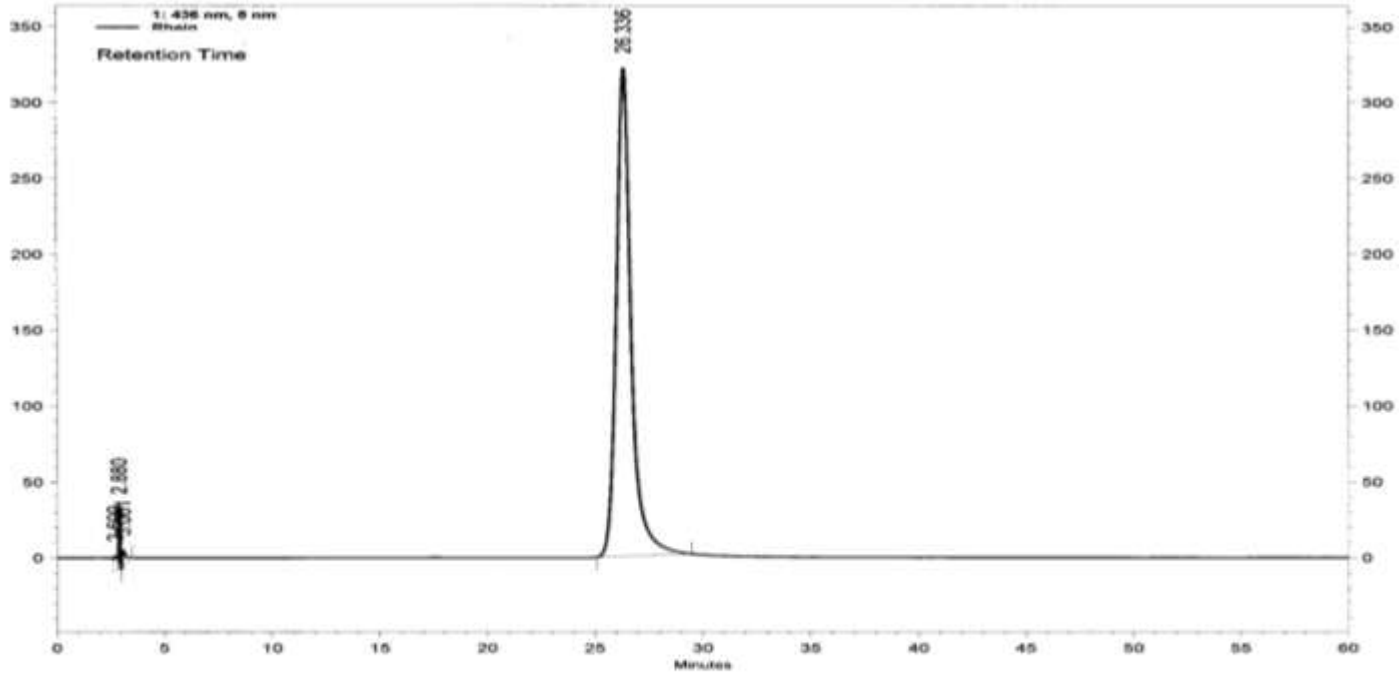


Figure 1. Calibration curve for *rhein* of *R. austral.*



1: 436 nm, 8 nm

Pk #	Retention Time	Area	Area %	Height	Height %
1	2.699	10681	0.068	1848	0.499
2	2.880	162947	1.034	35500	9.591
3	3.061	140483	0.891	11202	3.026
4	26.336	15449125	98.007	321600	86.884
Totals		15763236	100.000	370150	100.000

Figure 2. HPLC chromatogram for marker *rhein* of *R. austral.*

observed when poutry manure was used in combination with P or N. This is in contrast to sheep or farm yard manure in combination with P or N. Treatments with maximum quantity of nitrogen (150 kg ha^{-1}) in combination with organic manure, but no phosphorus *viz.* treatments $M_1P_0N_2$, $M_1P_0N_2$ and $M_1P_0N_2$ displayed lesser *rhein* content (0.315, 0.270 and 0.24 6%, respectively). Conversely to respective treatments *viz.* $M_1P_2N_0$, $M_1P_2N_0$ and $M_1P_2N_0$ with maximum concentration of phosphorus (100 kg ha^{-1}) in combination with organic manure, but no nitrogen, were having maximum content of *rhein* (0.165, 0.120 and 0.087% respectively). The latter treatments were also with higher root weight in contrast to former treatments. Similar observations, that is, enhancement in the amount of alkaloid content in various medicinal plants by applying different doses of organic manure and

Table 5. Amount of R*hein* content in different samples of *R. australe*.

S/N	Sample code	Treatment	Percentage of Rhein content in the extract
1	RA-2	$M_1P_0N_0$	0.231
2	RA-4	$M_1P_0N_2$	0.263
3	RA-7	$M_1P_2N_0$	0.315
4	RA-6	$M_1P_2N_2$	0.394
5	RA-5	$M_2P_0N_0$	0.201
6	RA-3	$M_2P_0N_2$	0.235
7	RA-10	$M_2P_2N_0$	0.270
8	RA-12	$M_2P_2N_2$	0.360
9	RA-1	$M_3P_0N_0$	0.172
10	RA-13	$M_3P_0N_2$	0.235
11	RA-11	$M_3P_2N_0$	0.246
12	RA-9	$M_3P_2N_2$	0.315
13	RA-8	$M_0P_0N_0$	0.153

Poultry manure (M_1) (20 tones ha^{-1}), sheep manure (M_2) (25 tones ha^{-1}) and farmyard manure (M_3) (25 tones ha^{-1}) phosphorus (P_0=0, P_1 = 50 and P_2 = 100 kg ha^{-1}) and nitrogen (N_0 = 0, N_1 = 100 and N_2 = 150 kg ha^{-1}).

Table 6. Soil fertility status of the experimental field.

Parameter	Value
pH (1:2.5)	7.02
E.C(1:2.5) dsm^{-1}	0.22
Organic matter	2.4%
Available N (kg ha^{-1})	113.0
Available P(kg ha^{-1})	71.0
Available K(kg ha^{-1})	263.0
Available S (kg ha^{-1})	40.0
Calcium (Meq)	11.0
Magnesium (Meq)	2.0

Table 7. Nutrient content of FYM, sheep and poultry manure.

Parameter	Farm yard manure	Sheep manure	Poultry manure
pH (1:2.5)	7.19	7.0	6.5
Moisture content%	71.0	63.0	48.26
Organic matter%	35.25	39.63	40.11
N %	1.35	1.48	2.29
P_2O%	0.18	0.36	1.70
K_2o%	0.13	0.19	1.11
S %	0.03	0.04	0.6
Calcium oxide%	0.10	0.42	2.37
Magnesium oxide%	0.13	0.12	0.67

inorganic fertilizers have also been made by various workers in *Datura* sp. (Esendal et al., 2000), *Atropa belladonna* (Baricevic et al., 2002) and *Artemisia* sp. (Usha and Swamy, 2002).

Conclusion

The root yield and *rhein* content of *R. australe* increased with the application of organic manure, phosphorus and

nitrogen. Highest dry yield of root was observed in treatments which received poultry manure in combination with higher level of phosphorus and nitrogen in contrast to sheep and farm yard manure receiving the same combination of inorganic fertilizers. Increased fertilization resulted in increase in various yield determining morphological characters, which ultimately resulted in increased root weight. The large scale exploitation of the species to meet the ever-increasing demand of drug industry is one of the major causes of the extirpation of its population in nature. It is, therefore, imperative and high time to grow the species on a commercial scale not only to cater the requirements of the drug industry but also to save the species from extinction. The method of standardizing the cultivation practices will ensure an everlasting supply of the raw material to the pharma-ceutical industry and will also help in the improvement of crops under plant-breeding programme.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Protective effect of eucalyptus oil on pulmonary destruction and inflammation in chronic obstructive pulmonary disease (COPD) in rats

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Eucalyptus oil (EO), an essential oil isolated from Eucalyptus leaves, was examined for its effect on lipopolysaccharide (LPS) and *Klebsiella pneumoniae*-induced COPD in rats. The COPD model was induced by instilling intratracheally with LPS and *K. pneumoniae*. The test compound, EO (30, 100 and 300 mg/kg), prednisone acetate (10 mg/kg) or vehicle was instilled intragastrically after three weeks exposure to LPS and *K. pneumoniae*, lasting for 4 weeks. EO significantly reduced amounts of inflammatory cells in bronchoalveolar lavage fluid (BALF) and blood, and decreased bronchiolitis, emphysematous changes and thickness of bronchioles. It also significantly reduced the increased AB-PAS-positive goblet cells in bronchioles. Prednisone acetate attenuated pulmonary inflammation and airway mucus hypersecretion, but no significant difference was found on emphysema. Pretreatment with EO markly reduced the production of proinflammatory cytokines TNF-α and IL-β in lung homogenate, significantly decreased the elevated malondialdehyde (MDA) level and and increased superoxide dismutase (SOD) activity. These findings indicate that EO could exert an protective effect against LPS plus *K. pneumoniae*-induced lung indury via inhibition of proinflammatory cytokines production and improvement of anti-oxidant status. The results provide evidence that EO might have its potential to be a proper candidate drug in the treatment of COPD.

Key words: Eucalyptus globulus, lipopolysaccharide, cytokine, chronic obstructive pulmonary disease.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by chronic inflammation and irreversible airflow obstruction, mainly induced by cigarette smoking and noxious stimuli including infection (Samareh Fekri et al., 2015). Chronic and persistent inflammation results in emphysema and irreversible airway narrowing that resulted from protease, mucociliary dysfunction, oxidative stress or fibrosis around small airway. There is increasing evidence that bacterial colonization in COPD patients contributed to airway inflammation and excerbated the

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progression of decline lung function (Garcha et al., 2012; Korsgren et al., 2012; Cukic, 2013). However, the mechanisms are still not well known. Xu et al. (1999) succeeded to establish a COPD rat model by repeated injecting intranasally of *Klebsiella pneumoniae*, suggesting an important role of bacterial infection in the pathogenesis of COPD.

Eucalyptus essential oil (EO), is commonly used as expectorant for upper respiratory tract infection or inflammation, as well as decongestant and various other inflammatory diseases. It is reported that EO possesses particular anti-inflammatory and anti-oxidative properties (Rantzsch et al., 2009; Juergens, 2014). In murine macrophages, Eucalyptus oil inhibited inducible nitric oxide synthase mRNA expression and NO production induced by lipopolysaccharide and IFN-γ (Vigo et al., 2004). Concomitant therapy of Eucalyptol reduced exacerbations and improves lung function in patients with COPD (Worth et al., 2009). These findings support at least some of the essential oils of Eucalyptus species used in the clinical treatment. However, direct evidence is still lacking to identify its pharmacological proterties in chronic obstructive pulmonary diseases. Therefore, in the present study, the authors established a rat model of COPD through intratracheal adminstration of LPS plus *K. pneumonia*, and determined the effects of Eucalyptus oil on pulmonary destruction and inflammatory responses.

MATERIALS AND METHODS

Male Sprague-Dawley rats, obtained from Shanghai experimental animal center, China, weighing 180 to 230 g, were kept at 23 ± 2°C with a 12 h light/12 h dark cycle. They were allowed free access to food and water. All animals were handled in accordance with the Ethical Principles for Care and Use of Laboratory Animals as previously reported (Zhao et al., 2014). All procedures decribed herein were reviewed and aproved by the local animal ethics committee.

Drugs and reagents

Eucalyptus oil, which was commercially prepared, was used in all of the experiments (Batch No.060711, provided by our laboratory). *K. pneumoniae* (No. 1.1736) was purchased from Agricultural Culture Collection of China (ACCC), LPS (*Escherichia coli* O111: B_4) was purchased from Sigma.

Experimental procedure

COPD model was induced by intratracheal LPS plus K.P exposure. Rats were randomly divided into seven groups, and performed as follows: 0.1 mL of *K. pneumoniae* (density, ≥ 6×10^8 CFU/mL) was instilled intratracheally twice a week and 2 mg/kg of LPS once every two weeks; EO group (30, 100, and 300 mg/kg) was administrated intragastricly after 12 weeks exposure of K.P and LPS and lasted for 4 weeks; Prednisolone acetate (Pred, 10 mg/kg) was administrated as positive control group, aseptic saline was administrated intragastricly as negative control group.

Preparation of bronchoalveolar lavage fluids (BALF) and blood for cell counting

Rats were anesthetized intraperitoneally with sodium pentobarbital, and BALF was harvested 24 h post-last *K. pneumoniae* and LPS exposure. Trachea of each rat was surgically exposed and cannulated. The right lungs were lavaged with 1.5 mL of PBS three times, fluid recovery was routinely ≥90%. A 0.1 mL aliquot was used for total leukocyte number counting. 20 μL of tail vein blood was harvested and added to 0.38 mL of 2% acetic acid solution, and stained with Wright-Giemsa staining. The total inflammatory cell number in the blood film was counted under oil immersion lens.

Histological examination

The lungs were collected and fixed with 10% neutral formalin for one week. After tissues were paraffinized, 5 μm sections were cut and stained with hematoxylin-eosin (H.E.) staining for evaluation of alveolar/interstitial inflammation and emphysema.

Morphological assessment

After the lung tissues were paraffin-embedded and sectioned, 5 μm sections were stained with H.E. To evaluate extent of lung destruction, there was focus on the presence of any of the following: (1) pulmonary mean linear intercept (Lm); (2) mean alveolar number (MAN); (3) ratio of thickness of bronchioles to diameter. Lm, as a measure of interalveolar wall distance, was computed on each slice based on 8 random fields using a cross-line under light microscopy. The total length of the cross-line divided by the numbers of the alveolar wall intersecting the lines was defined as Lm. MAN, an indicator of alveolar density, was computed on each slice based on 8 random fields by counting the numbers of alveoli and dividing the number by the area of the field. Ratio of thickness of bronchioles to diameter was computed on each slice based on 3 to 5 medium bronchi by measuring external diameter and internal diameter and dividing the difference by external diameter (Wang et al., 2014).

Determination of goblet cell hyperplasia

After fixation, 5 μm sections were stained with Alcian blue-Periodic Acid Schiff (AB-PAS). Positive goblet cell number and total cell number of columnar epithelial cells were counted in the main bronchus and 2 to 3 medium bronchi. The ratio of positive goblet cells was calculated by dividing the positive goblet cell number and total cell number of columnar epithelial (Takeyama et al., 1999).

Determination for TNF-α, IL-1β, MDA and SOD activity in lung tissues

Lung tissues stored at -80°C were homogenized in homogenization Tris-buffer on ice using Heidolph Diax 900 Homogenizer (Heidolph, Germany) and then centrifuged at 12000 g for 30 min at 4°C. The supernatants were used to determine the level of TNF-α, IL-1β, SOD and MDA activity. Concentration of the protein in the supernatants was detected using Coomassie brilliant blue G250 method. The concentrations of TNF-α, IL-1β, superoxide dismutase (SOD) and malondialdehyde (MDA) were determined using commercial ELISA kits (BD Bioscience, USA; Jiancheng Bioengineering Institute, Nanjing, China). All procedures were done according to the instructions of the manufactures (Huang et al.,

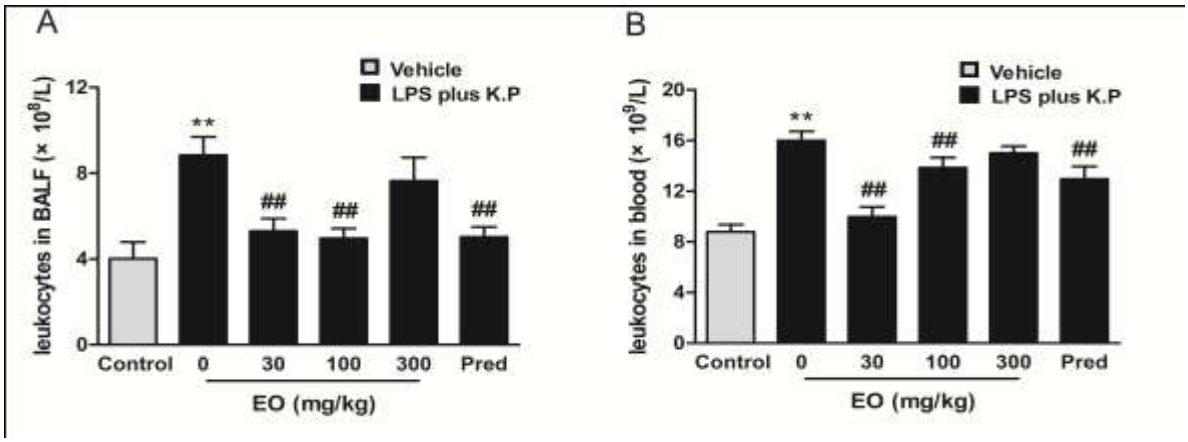

Figure 1. Effect of EO on total cell numbers of inflammatory cells in BALF and blood. 24 h post-last K.P exposure, total cell numbers in BALF (A) and blood (B) were counted and analyzed. Data were expressed as mean ± SD of 8-12 rats /group. $^{**}P < 0.01$ compared with control group, $^{\#\#}P < 0.01$ compared with group.

2013).

Statistical analysis

Data were expressed as mean ± SD and analyzed by a one-way analysis of variance (ANOVA) followed by Dennett's post hoc test with SPSS 15.0 for Windows. For all analyses, significance was calculated with a P value < 0.05 considered statistically significant.

RESULTS

Effect of EO on cell infiltration in BALF and blood

To determine the effect of EO on pulmonary inflammation and peripheral inflammation, the number of leukocytes in the BALF and blood were counted. After LPS plus *K. pneumoniae* exposure for 3 months, the total number of leukocytes in BALF and blood was significantly increased. EO at 30 and 100 mg/kg significantly decreased the infiltrated leukocytes cells in BALF and blood (Figure 1A and B), as compared to the vehicle group. Treatment with EO at 300 mg/kg also obviously decreased the number of leukocytes in BALF and blood, but no significant difference was found. Pred at 10 mg/kg significantly reduced the cell number of leukocytes in BALF and blood.

Effect of EO on emphysematous destruction and bronchiolitis

To observe the effect of EO on emphysema and bronchiolitis, LPS plus *K. pneumoniae* was intratracheally instilled and induced emphysematous destruction, bronchiolitis, small airway remodeling within 4 months. In vehicle group, when compared with the normal alveolar structure, the lung parenchyma was largely destructed and less alveolus was found after intratracheal instillation of LPS plus *K. pneumoniae*; bronchiolitis was severe since a lot of inflammatory cells gathered around the fine bronchus. Treatment with EO and prednisone obviously reduced emphysematous damage and bronchiolitis around the bronchioles (Figure 2A). Histological analyses showed that Lm and thickness of bronchioles were significantly increased after instillation of LPS plus *K. pneumoniae* (Figure 2B and C), while MAN was significantly decreased (Figure 2D, P < 0.01). Treatment with EO at 100 and 300 mg/kg showed less emphysematous damage, decreased thickness of bronchioles and increased MAN values when compared with vehicle group. No significant difference was found between vehicle and Pred groups.

Effect of Eucalyptus oil on mucus secretion

As shown in Figure3A, there were more AB-PAS-positive goblet cells in bronchioles in the presence of *K. pneumoniae* plus LPS, while less was found in Eucalyptus oil group and Prelon group. The total number of AB-PAS-positive goblet cells was significantly increased in vehicle group as compared to the control group. EO significantly reduced the number of AB-PAS-positive goblet cells in bronchioles at 30, 100 and 300 mg/kg (Figure 3B). Pred (10 mg/kg) also significantly reduced the number of AB-PAS-positive goblet cells in bronchiol**es.**

Effect of Eucalyptus oil on TNF-α and IL-1β release

The levels of TNF-α and IL-1β in lung homogenate were significantly increased after challenge of K.P plus LPS for 4 months. EO at dose of 30, 100 and 300 mg/kg

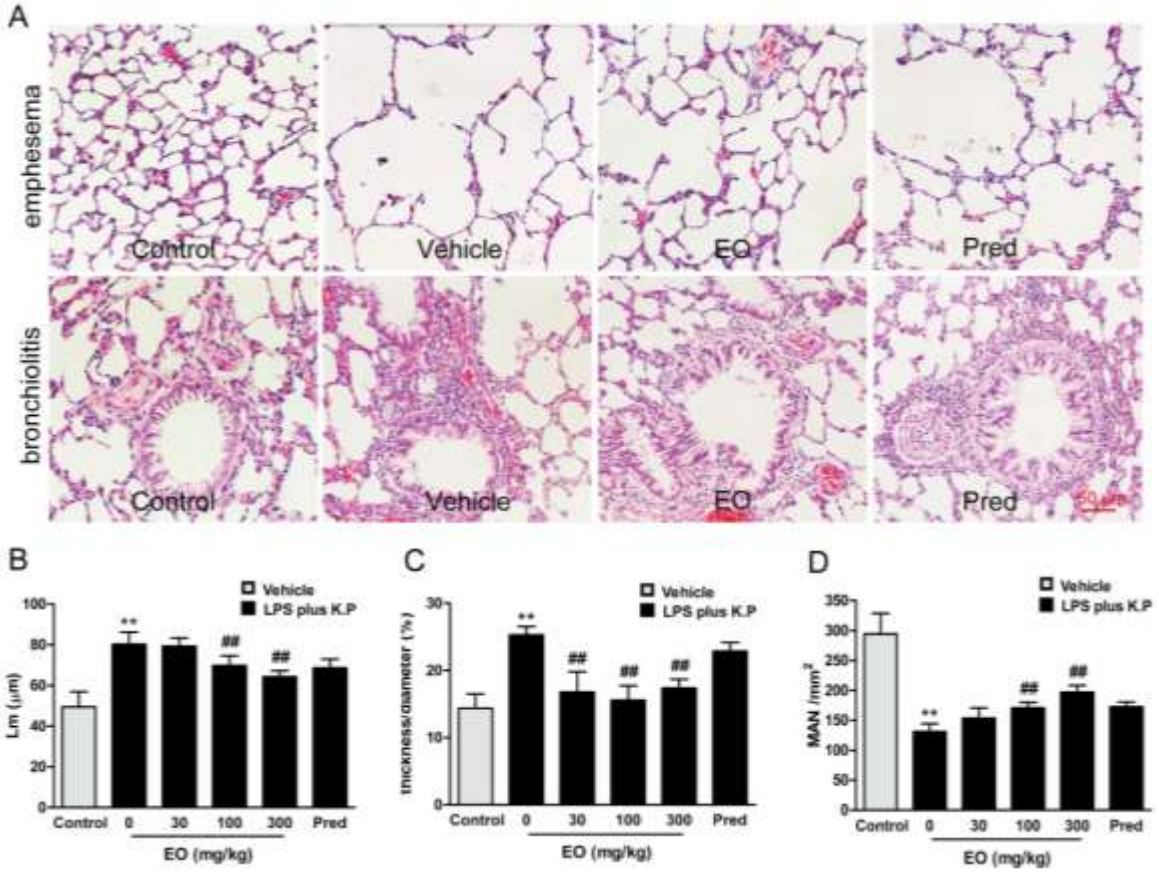

Figure 2. Effect of EO on histochemical changes in rats. Twenty-four hours after the last K.P exposure, the lungs were immersed in 10% neutral formalin for 7 days. After tissues were paraffinized, 5 μm sections were prepared and stained with hematoxylin-eosin for observation of emphysema change and bronchiolitis (A). To estimate the extent of lung destruction in rats, Lm (B), thickness of bronchioles (C) and MAN (D) were evaluated as described in methods. Data were expressed as mean ± SD of 6 rats /group. $^{**}P < 0.01$ compared with control group, $^{\#\#}P < 0.01$ compared with vehicle group.

significantly reduced the production of pro-inflammatory cytokines TNF-α (Figure 4A, $P < 0.01$) and IL-1β (Figure 4B, $P < 0.01$). Rats pretreated with Pred at dose of 10 mg/kg also showed decreases in TNF-α and IL-1β productions when compared with the vehicle group.

Effect of EO on MDA production and SOD activity in lung tissues

After exposure with LPS plus *K. pneumoniae*, rats produced a greater amount of MDA in vehicle group when compared with the control group. EO at 30, 100 and 300 mg/kg significantly decreased the LPS plus K.P-induced MDA production in lung homogenate (Figure 4C, $P < 0.01$). In contrast, SOD activity was elevated obviously in vehicle group when compared with that of control group. While EO at 100 and 300 mg/kg significantly increased the SOD activity in parallel to vehicle group (Figure 4D, $P < 0.05$). These data indicated that EO might attenuate the LPS plus *K. pneumoniae*-induced changes via oxidant-antioxidant balance. Among all the tested doses of EO, 100 mg/kg exhibited the best anti-oxidant effect. Pred at dose of 10 mg/kg decreased the MDA production but no significant effect on SOD activity was observed.

DISCUSSION

The present study aimed to evaluate the protective effect of EO using a LPS plus *K. pneumoniae*-induced COPD model and to investigate the underlying mechanisms of the action associated with its anti-COPD activity.

Although, cigarette smoking is the leading risk factor of COPD, only 15-20% of smokers develop the disease. It was postulated that bacterial infections play a major role in the pathogenesis of COPD (Anthonisen, 2004). Recently, increasing evidence supported a clear relationship between bacteria infection and exacerbations

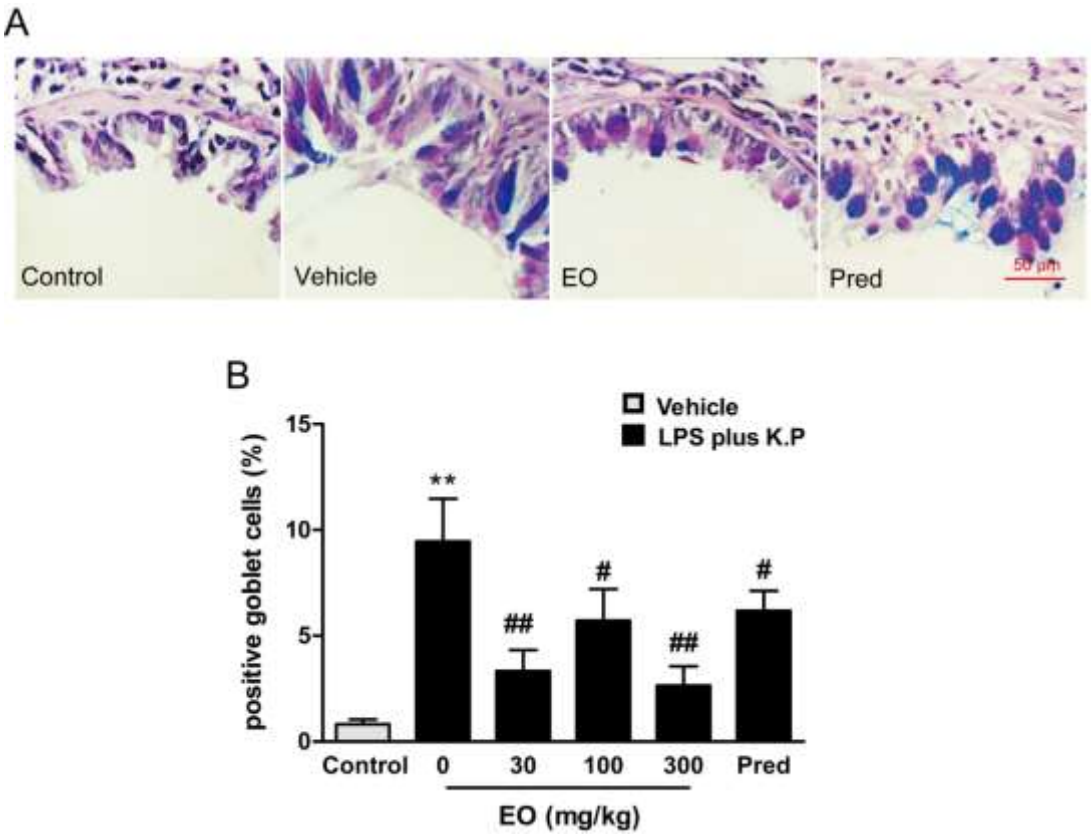

Figure 3. Effect of EO on changes of positive goblet cells in lungs. After challenged with vehicle or K.P plus LPS, lungs were immersed in 10% neutral formalin, paraffinized, and 5 μm sections were prepared and stained with AB-PAS for examination of goblet cell hyperplasia (A). Positive goblet cell number and total cell number of columnar epithelial cells in the main bronchus and 2~3 medium bronchi were counted and analyzed (B). Data were expressed as mean ± SD of 6 rats /group. $^{**}P < 0.01$ compared with control group, $^{\#\#}P < 0.01$ compared with vehicle group.

of COPD, which is strongly associated with mucosal response and neutrophilic inflammatory profile in the sputum (Sethi et al., 2002, 2004, 2008). Here, the authors established a COPD rat model using LPS plus *K. pneumoniae*, characterized by chronic airway inflammation, emphysema and excess mucus. Oral administration of EO effectively reversed chronic bronchiolitis, with significant reduction of mucus hypersecretion. Treatment with EO for one month obviously attenuated the emphysematous changes and thickness of bronchioles while the vehicle group rats showed severe disruption of alveoli and thickened bronchioles. The findings demonstrated that EO had protective effect on LPS plus *K. pneumoniae*-induced pulmonary inflammation and destruction.

It is widely accepted that chronic airway inflammation plays a key role in the pathogenesis of COPD, associated with destruction of airway and lung tissues (Zanini et al., 2015). Previous evidence showed inflammatory cells such as neutrophils, macrophages and lymphocytes was aggregated in blood, sputum and BALF in COPD patients (Mroz et al., 2015). Release of inflammatory cytokines including TNF-α, IL-1β, IL-6 significantly increased in blood and lung tissues (Shen et al., 2014; Tang et al., 2014). Here the authors detected the elevated extent of TNF-α and IL-1β in lung homogenate, which was obviously reduced by EO treatment in rats. In some trials, anti-inflammatory and antiseptic effect of EO and its extract has been identified (Cermelli et al., 2008; Mulyaningsih et al., 2011; Tsai et al., 2011). Eucalyptol may regulate cytokine production in the airway through TLR_4/NF-κB pathway, the mechanisms were further indentified by Zhao *et al.* (2014).

The lungs of patients with COPD are easy to breed micro-organisms including bacteria and virus infections. This results in further lung destruction and host defenses decrease. Bacteria colonization and invasion constitute a lazy immune response, which concomitantly occurs with oxidative stress (O'Rourke et al., 2003). The increased oxidants are very likely to tip the oxidant/antioxidant balance due to the existence of infection and increased immune response (van der Strate et al., 2006). On the

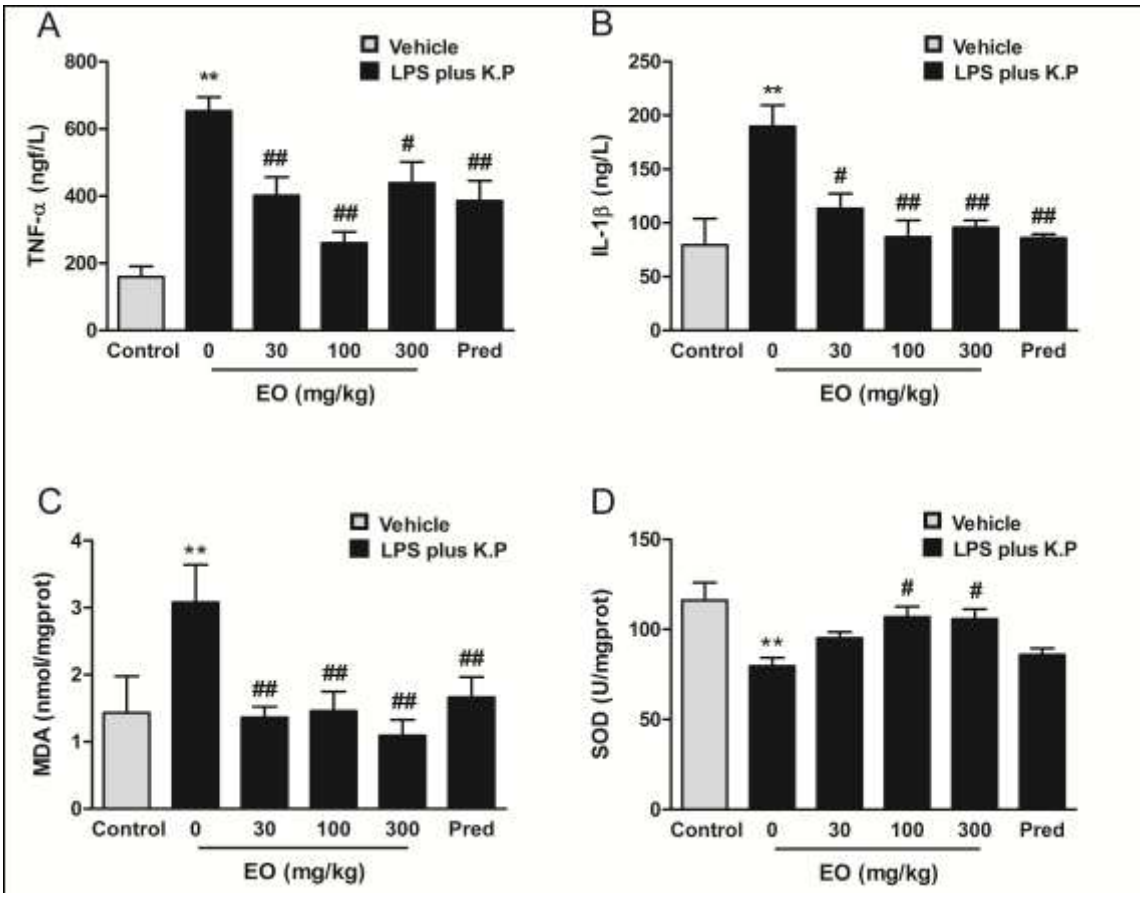

Figure 4. Effect of EO on TNF-α, IL-1β, MDA production and SOD activity in lung homogenate. Lung tissues were harvested 24 h after the last *K. pneumoniae* exposure and used for determining the activity of TNF-α (A), IL-1β (B), MDA (C) and SOD activity (D) among groups. Data were expressed as mean ± SD of 8-12 rats/group. $^{**}P < 0.01$ compared with control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with vehicle group.

other hand, organisms have its own enzymatic and non-enzymatic defenses, such as glutathione peroxidase (GSH-Px) and SOD against reactive oxidative stress and lipid peroxidation. To address the role of oxidative stress in the study model, the authors addressed the release of MDA, an index of lipid peroxidation, and SOD activity in lung tissues of rats (Ismail et al., 2015). LPS plus *K. pneumoniae* obviously increased MDA level, accompanied by decreased SOD activity, suggesting a critical role of oxidative stress in COPD model. However, treatment with EO significantly resulted in a significant decrease of MDA formation and increase of SOD activity. The results indicate that EO potentially exerted protective effect against lung destruction via antioxidant mechanism.

Compositions of the major component of EO have been reported differently. Ben Hassine et al. found α-pinene and 1,8-cineol as major components (Ben Hassine et al., 2012), and Bouzabata et al. (2014) found mainly α- and β-pinene. Whatever, EO extract is able to implement the innate cell-mediated immune response besides its anti-septic properties. In LPS-induced chronic bronchitis rats, EO reduced pulmonary inflammation and inhibited hypersecretion of airway mucins (Lu et al., 2004). In accordance with this finding, the data supported the inhibition of mucus hypersecretion in COPD rats. Clinical trials have identified the benefit of concomitant therapy of cineol on improvement of lung function in patients with COPD (Worth et al., 2009). Another placebo-controlled, double-blind trial also showed concomitant therapy using cineole improved lung function and health condition as well as to reduce dyspnea in asthma patients (Worth and Dethlefsen, 2012). The results provided direct evidence that EO attenuated lung destruction and chronic airway inflammation via anti-inflammatory and antioxidant properties, indicating EO as an active controller of lung injury in COPD.

Currently, the clinical use of essential oils has expanded worldwide in the treatment of varieties of inflammatory diseases such as asthma and arthritis (Wang et al., 2007; Shirole et al., 2014). EO has been traditionally used to treat respiratory tract disorders

including bronchitis, pharyngitis and sinusitis. The scientific interest on medical plants in treating chronic pulmonary disease is expanding (Ram et al., 2011). The results provided direct evidence that available Eucalyptus oil might be a proper candidate drug in therapeutic of COPD, at least a good choice to concomitant therapy, apart from the clinical use for sputum clearance in treatment of airway disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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