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ANALGESIC ACTIVITY AND ACUTE TOXICITY OF THE METHANOLIC EXTRACT OF *NEPETA GRANATENSIS*

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ABSTRACT

The methanolic extract of *Nepeta granatensis*, were evaluated for the acute toxicity and peripheral and central analgesic activity. Female mice (Iops Offa) were treated with the methanolic extract and had shown a LD_{50} as 1000 mg/kg Effects of the methanolic extract, on nociception were assessed, by Tail Flick test in rats, as well, acetic acid inducing writhing in mice. The administration of the methanolic extract, intraperitoneally, at the doses 25 and 50 mg/kg, indicates that methanolic extract exhibit a power protection, against writhing (90, 9%) at the higher dose. Furthermore, reflex time; in Tail Flick exceeds the threshold of pain inhibition, that reached 11,2 ± 0,33s for the dose 50mg/kg.The in vivo experiments revealed that, methanolic extract of *Nepeta granatensis* has a high potential peripheral analgesic activity and a central morphine-like either at dose 50mg/kg.

KEYWORDS: Methanolic extract, Analgesic activity, Toxicity, *Nepeta granatensis.*

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INTRODUCTION

Morocco is fortunate to have such varied climate that almost any medicinal plant can grow. The varied climate and heterogeneous ecologic condition in Morocco have favoured the proliferation of more than 4,200 species of plants, divided into 150 families and 940 genuses[1]. Until now, man has always been the means to cure various diseases using against those provided by the natural environment, they are primarily plant-based. The genus *Nepeta* has long been used as a medicinal plant and herb belongs to the Labiatae family with about 250 species distributed in the south-west and central Asia, Europe, North Africa and North America [2] In the literature, the genus of *Nepeta,* is allocated various pharmacological properties such as analgesic, sedative, anti-tumor, anti-bacterial, anti-allergic, anti-inflammatory, anti-rheumatic, anti-leishmanian, antitussive, carminative, tonic and emmenagogu[3,7]. In Morocco, the *Nepeta* genus is represented by 9 species [8,11] that are used traditionally, as a vulnerary cataplasm against the hysteria, venomous, respiratory disorders and spasms of the digestive tract, also to relieve the dental neuralgia[12] The fixed parts of the plant is an important source of active principles and secondary metabolites such iridoides, lactone glycoside, as well as triterpenes lupaniques[13,21]. Recent work conducted by Bouidida et al., Have reported the central and peripheral analgesic activity of global extracts of Moroccan *Nepeta* species, *N. atlantica* and *N. tuberosa. ssp. reticulata* [22] and a low acute toxicity. By Elsewhere, studies of pentacyclic triterpenes isolated from the methanolic extract of *Nepeta clarkei* showed that analgesic activity [23]. To further the valorisation of spontaneous *Nepeta* species of Morocco, we were led to focus on the same research undertaken by our laboratory on methanolic extract (ME) of *Nepeta granatensis* not already examined.

MATERIALS AND METHODS

Plant

The species chosen *Nepeta granatensis* Boiss was collected during the flowering period in July 2011 in specific areas of the Middle Atlas (Valley of Ifrane) under the control of botanists of scientific institute in Rabat Morocco, whom ensures their field identification. A voucher specimen RAB 79003 were deposited on the herbarium of scientific institute in Rabat Morocco.

Preparation of Extract

The plant is air-dried form spread, ventilated, away from sunlight at room temperature and was regularly returned. The aerial part of the plant was cut and then extracted with methanol by cold maceration at room temperature. 1kg of the plant was put into 3 litres of solvent with occasional shaking for 48h and was renewed five times. The methanol containing extract was filtered through Whitman paper and the extract were concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature (50-60°C). The greenish residue with a yield of 26% were placed in a desiccator to completely remove traces of solvent. The extract was then kept in the refrigerator at 4°C until use.

Animals

Wistar rat (Iffa credo) 200-300g and Swiss albino (Ofa IOPS) 20-30g were procured from the animal centre of Mohammed V University, medicine and Pharmacy faculty, Rabat. They were in Plexiglas cage at room temperature (22± 3°C) in 12h light / 12h dark cycle. The animals were fed on standard laboratory diet and water ad libitum. The animals randomly chosen were acclimate to the laboratory conditions in their cage at least for 5 days before experiments.All experiment protocols were approved by the animal ethics committee and conducted in accordance with standard practice of animal handling as accepted internationally (directive 91/507/CEE, 86/609/CEE).

Products

- EM solution (EM, water distilled) prepared at various concentrations 25 and 50 mg/kg (IP)
- The Glacial Acetic Acid 3%, 300 mg/kg (IP)
- Acetyl Salicylic Acid (Aspirin) to 200 mg/kg (IP)
- The morphine solution 5 mg/kg (SC)

Acute toxicity study

According to OECD guidelines[24] (Organization for Economic Cooperation and Development) for the Testing of Chemicals-method Acute Toxic Class Products, adopted December 17, 2001, N° 423, as a sequential process by step using three animals of one sex were determined the median lethal dose LD_{50}. As no data given, for the initial dose inducing death, for reasons related to the welfare of animals, the recommended starting dose is 300 mg/kg. The sets were formed of three female mice, healthy and are fasting from food for 18 hours before the test and three hours after. Each set received intraperitoneal injections, a single dose (2ml/100g body), of the ME at concentrations fixed in the protocol.In all these studies, in order to the comparison with the previous investigation carried out by Bouidida et al[22], the animals will receive our EM solution intraperitoneally (IP)

Analgesic activity

Two different methods were used namely thermal stimuli (Tail Flick test) and chemical stimuli (koster test)

- ***Peripheral analgesia***

Koster test[25] was used to search for the peripheral analgesic action, involves assessing a possible protection in front of the abdominal cramping and writhing induced, by the injection of 3% glacial acetic acid solution 300 mg/kg (IP), with a maximum of 0.1 ml per mouse. Thus, the animals were divided into 4 sets of six female mice each. 30 min before administration of glacial acetic acid the mice were treated with: Control set: the delivery vehicle of the test substance is 5% gum Arabic, in a volume of 0.1 ml of distilled water (IP) for each mouse Reference set: The reference substance is acetylsalicylic acid (ASA) 200 mg/kg (IP) in a volume of 0.2 ml for each mouse. Two test sets: the dose 25 and 50 mg/kg of aqueous solution of ME of *Nepeta granatensis* (IP) of aqueous solution of methanolic crude extract of *Nepeta* For each mouse, the number of cramps or abdominal writhing was counted for 20 minutes as soon as the acetic acid injection (IP) performed.

- ***Central analgesia***

This study was based on Tail Flick[26,27] test involves immersing about 5 cm of the rat's tail in hot water (55 ± 2°C) and studying, before and after administration of the extract, the tail withdrawal of reflex was recorded before and after 15,30, 60 and120 min.It was established that the time of normal reflex reaction is two seconds, in fact, only rats whose reflex time is less than or equal to 2s will be selected for testing during a screening. Assaying were achieved if the latency exceeds 10s to avoid tissue damage. A time to withdraw the tail less than six seconds indicates a rise pain threshold in rats, confirming a central analgesic action of extracts received. Four sets were formed with six rats receiving: A control set: 0.8 ml of distilled water (IP) A reference set: morphine 5 mg/kg subcutaneously (SC) in a volume of 0.2ml. Two tests sets: 50 and 25 mg/kg of methanol extract of *Nepeta* granatensis in a volume of 0.8 ml per 250g rat (IP)

Statistics

The results were expressed as mean ± SEM. The comparison of the averages is done with the ANOVA test, to a single factor (one-way ANOVA), using the SPSS Windows 10 software (SPSS .Inc). The level of statistical significance was set at $p < 0, 05$ level.

RESULTS

Acute toxicity

- The dose of 300 mg/kg administered for the first set conduct to no deaths among the three mice. The dose confirmation on a second set led to the same result. - The Dose of 2000 mg/kg on a third set treated animals, remained motionless and were not fed. 2/3 of mice's death occurred after an half of hour and 1/3 at the second day, after administration of the product, which requires the cessation of the trial. We also observed for this set, abdominal

constrictions, fast breathing and paralysis of the hind limbs. Indeed, all the dead animals had extended forelegs and hind legs flexed. The only surviving mouse was found comparable to that of mice of the lower dose condition. Mice that survived suffered of a slight loss of weight of 3% on average for a dose of 300mg/kg over 5 days and a loss of 10% for the 2000 mg/kg over 7 days. After this time, the weight normally increases and becomes greater than the initial weight of the animal being treated. However, immediately after the injection of the methanolic extract of *Nepeta granatensis* IP , we observed a sedative action of about 30 minutes to 3 h with increasing doses (300 and 2000 mg/kg), accompanied by a trend toward consolidation animals, decreased motor skills, loss of tone and vivacity, immobility and a refusal to drink and eat. The clinical signs observed during the first 6 hours after injection of methanolic extract *Nepeta granatensis* Boiss are summarized in chart I. According to Appendix C of 423 guidelines, the LD_{50} determined for our sample is 1000 mg/kg

Analgesic activity

- ***Peripheral Analgesia***

The test results are summarized in Koster chart II report the average number of cramps due to acetic acid recorded during a period of 20 min, its standard deviation and the percentage of protection (P) of the various doses of ME studied using the formula:

$$P = \frac{\text{Number of writhing in control set - number of writhing in the treated set}}{\text{Number of writhing in control set}}$$

Mice treated with the extracts to 25 mg/kg and 50 mg/kg in 20 min have developed a number of writhing lower than mice a of the control group, which represents a significant protection

- ***Central analgesia***

The results of the tail flick testare summarized in chart III and figure I that represents the central analgesic effect of various doses of the ME to doses levels 25 and 50 mg/kg (IP).For every set, is indicated the average time of the tail withdrawal reflex and the standard deviation to the average, 15min before the administration of various extract to be studied (time of normal reaction) and then to 15; 30; 45; 60 and 120 min The Koster test is carried out by intraperitoneal injection of acetic acid glacial, inducing inflammation manifested by writhing. In general, analgesic substances cause a significant decrease in these contortions.

CHARTI

Clinical signs of acute toxicity following methanolic extract of the Nepeta granatensis injection

- : absence of signs x: Presence of signs

	SET 1 (300mg/kg IP)			SET 2 of confirmation (300mg/kg IP)			SET3 (2000mg/kg IP)		
Mice	1	2	3	1	2	3	1	2	3
Clinicalsigns									
Number of deadmice		0/3			0/3			2/3	
Abdominal constrictions	x	_	x	_	_	x	x	x	x
Immobility	x	x	x	_	x	_	x	x	x
Is fed	x	x	x	x	x	x	_	_	_
Acceleratedbreathing	_	_	_	_	_	_	x	x	x
Paralysis of the hind limbs	_	_	_	_	_	_	x	x	x
Sedativeeffect	x	x	x	x	x	x	x	x	x
Gathering of the 3 mice		x			x			x	

CHART II

Effects of methanolic extract of Nepeta granatensis on the number of cramps glacial acetic acid induced (n=6 mice per set)

	Control	Reference ASA 200mg/kg (IP)	EM (*N.granatensis*) 25mg/kg (IP)	EM (*N. granatensis*) 50mg/kg (IP)
Number of cramps during 20min	60,50±1,5	18,16±0,55	12,5±0,83	5,5±0,66
% of protection	----------	69,98%	79,33%	90,90%

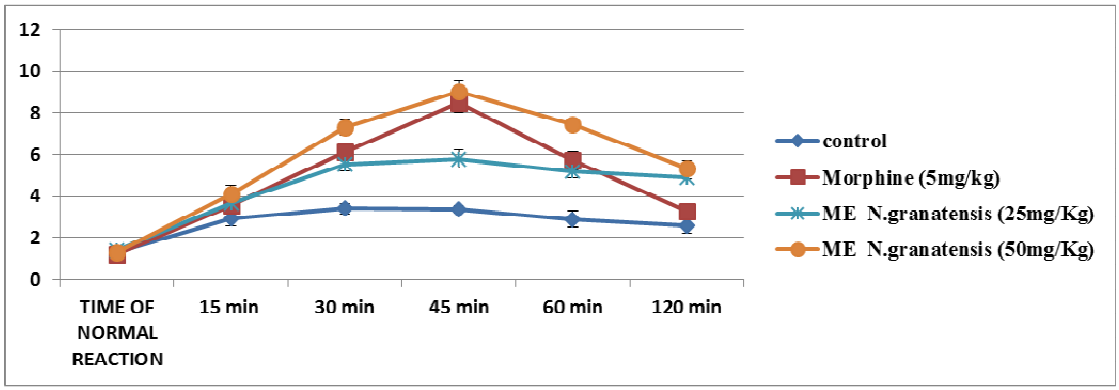
CHART III

Effects of ME of Nepeta granatensis on Average time of the tail withdrawal reflex (n=6 mice per set)

Reaction time in sec	normal reaction time	15 min	30 min	45 min	60 min	120 min
control	1.28±0,29	2.91±0,35	3.41±0,25	3.36±0,16	2.90±0,40	2.58±0,35
Morphine (5mg/kg)	1.19±0,22	3.55±0,48	6.13±0,43	8.50±0,50	5.71±0,45	3.28±0,22
ME *N. granatensis* (25mg/Kg)	1,4±0,24	4,16±0,16	6,03±0,22	7,13±0,2	6,2±0,23	4,9±0,1
ME *N.granatensis* (50mg/Kg)	1,3±0,16	5,03±0,2	9,15±0,35	11,2±0,33	8,93±0, 3	6,16±0,32

Figure I

central analgesic methanolic extract of Nepeta granatensis



DISCUSSION

The acute toxicity study of the EM of *Nepeta granatensis* (IP) allowed determining the LD_{50} of 1000 mg/kg, testifying of a low toxicity by this extract. This shows that studies in our laboratory led on species *Nepeta atlantica* and *Nepeta tuberosa* is in some agreement with our results. Namely, *Nepeta tuberosa* (LD_{50} =1401 ± 97 mg/kg) and *Nepeta atlantica* (LD_{50} = 1672 ± 232 mg/kg) Moreover, an observation period showed a dose-dependent sedative effect 30 minutes until 3 hours after injection of the ME test. Would this motivate a psychotropic effect of this screening of ME.The Koster[28] test is carried out by intraperitoneal injection of acetic acid glacial, inducing inflammation manifested by writhing. In general, analgesics substances cause a significant decrease in these contortions. The number of cramps recorded for 20 min in mice pretreated with ME of *Nepeta granatensis* at doses 25 mg/kg and 50 mg/kg significantly decreases ($p<0,05$) compared to the control group. Percentage of protection is 79.33% at 25 mg/kg and 90.9% at the dose 50 mg/kg.These results are in adherence with those found by Bouidida et al[22], Showing that the percentage of protection of the global extract *Nepeta tuberosa* and *Nepeta atlantica* at 120 mg/kg reached its peak respectively 92.89% and 90.1%. Thus, provide to ME of *Nepeta granatensis* a power peripheral analgesic activity acetylsalicylic acid-like.As been known, the Tail Flick test is one of the most appropriate techniques for assessing the transmission of somatosensory acute pain by stimulating thermoreceptors in an experimental animal model. This test is sensitive to analgesics and supraspinal[29-31] systems shown by the tail withdrawal reflex that could be inhibited by a central action of a low dose of morphine.The administration of the methanolic extract of *Nepata granatensis*, at 25 mg/kg IP, inflect the time of reflex which exceeds slightly the threshold of inhibition of the pain at 45min (7,13s), inducing thus a

significant increase when compared to the control set ($p< 0,05$). This effect is seen to increase very significantly $p <0,01$ at 50 mg/kg far beyond the threshold of pain inhibition at 45 min (11,2s) According to litterature survey, analgesic activity can be attributed to the Presence of various phytoconstituents of the methanolic extract including flavonoids, terpenes lupane type and iridoid glucoside and lactonic.By this way, this dose-dependent activity of the methanolic extract of *Nepeta granatensis* allows us to explain that ME probably acts peripherally on visceral nociceptors sensitive to acid by inhibition of prostaglandin synthesis, via the inhibition of the cyclooxygenase (COX) family of enzymes. Centrally, maybe produce inhibiting of opioids receptors and transmission of pain to the central nervous system, what is due largely to, opioid-induced, presynaptic inhibition of neurotransmitter release

CONCLUSION

In terms of this study, it appears that the methanolic extract of *Nepeta granatensis* is not toxic at experimental doses 25 mg/kg and 50 mg/kg, and has a sedative effect on the central nervous system that lets consider a psychotropic study. In addition, this species is endowed with a peripheral analgesic power, acetyl salicylic acid type, from 25 mg/kg and a central analgesic morphine-like action, which is rather important for the dose 50 mg/kg.In perspectives, the sedation of the pain proved for our species of *Nepeta granatensis* arouses a deeper investigation on its anti-inflammatory power as well as demonstrate the exact mechanism of action. A phytochemical analysis is also proposed, to isolate the active fraction and, potentially, the pure compound.

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CONFLICTS OF INTEREST

Authours declare no conflicts of interest

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