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Original Article

Phytochemical Composition of Essential Oils from *Cinnamomum camphora* Leaves of Different Areas and their Analgesic Properties

Jinqiang Yang, Nianyun Yang*, Luyi Fan

College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China

Abstract

This article was aimed to analyze the essential oils from *Cinnamomum camphora* leaves of four areas by GC-MS and to study their analgesic properties. Analgesia test was performed employing mouse acetic acid writhing method, hot plate method and reserpine induced migraine model, and plasma and brain tissue NO and prostaglandin E_2 ($PGE_{2)}$ contents were determined. The major compounds from essential oils of Suzhou *C. camphora* leaves (SZ), Nanjing *C. camphora* leaves (NJ), Nantong *C. camphora* leaves (NT) and Lianyungang *C. camphora* leaves (LY) are different, but they all consist of nerolidol and (E)-α-atlantone. (E)-α-atlantone exists as the main constituent and it is first reported in this plant. In the hot plate test, NJ or LY (1 ml×kg^{-1} each) significantly prolonged the reaction time after 30 min treatment and plasma and brain tissue NO and PGE_2 in the mice with NJ or LY (1 ml·kg^{-1}) administration were significantly decreased. In the acetic acid-induced writhing test, NJ (1 ml×kg^{-1} each) significantly decreased the number of acetic acid-induced writhes in mice compared to the animals that received vehicle only. In the migraine test, Plasma NO in the mice with NJ or NT (1 ml×kg^{-1}) and NJ (0.25 ml×kg^{-1}) administration were significantly decreased. NJ and LY have obvious analgesic effect and the mechanism is related to the reduction of central and peripheral NO and PGE_2 levels. NJ was effective on migraine mice induced by reserpine and the mechanism may be related to the inductionof NO release. NJ and LY have significantly analgesic effects in three experimental models of pain.

Key words: *Cinnamomum camphora* leaves, Nerolidol, (E)-α-atlantone, Essential oils, Analgesic, Migraine

Corresponding Authors: Nianyun Yang, College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China
Tel: 86-25-85811074
E-Mail: nianyunyang@hotmail.com
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1. Introduction

Cinnamomum camphora is a large evergreen tree that grows up to 20–30 m tall. The leaves have a glossy, waxy appearance and smell of camphor when crushed. Modern studies have found that the main chemical constituents from *C. Camphora* were volatile oil, alkaloids, flavonoids, lignin and organic acids [1-3]. The biological activity of *C. Camphora* had mainly demonstrated antibacterial, insecticide, anti-oxidation, anti-inflammatory and analgesic activities [1]. *C. camphora* has long been prescribed in traditional medicines for the treatment of diseases such as abdominal pain, migraine and eczema dermatitis [4-5]. Camphor tree has six different chemical variants called chemotypes, which are camphor, linalool, 1,8-cineole, nerolidol, safrole, and borneol. The chemical variants seem dependent upon the area of origin of the tree. So far, no research on analgesic activity of the essential oils of *C. camphora* of different chemotypes has been reported, therefore it is necessary to further investigate and discussion. Analgesic drugs act in various ways on the peripheral and central nervous systems, which temporarily affect, and in some instances completely eliminate pain sensation. Each different type of chemical synthetic analgesic has its own associated side effects. Seeking and screening high effective and low poisonous analgesic drugs, especially from natural products, is the hot spot in medicinal research. The analgesic effect is related to the inhibition of PGE_2 synthesis and the release of NO [6-7]. The aim of the present study was to investigate the differences of chemical constituents of essential oil *C. camphora* leaves from different areas in Jiangsu province, evaluate the analgesic effects of the essential oils, using various standard experimental test models and evaluate essential oil on migraine model induced by reserpine. The study can also help to provide a theoretical foundation for the future exploitation and development of *C. camphora*.

2. Materials and Methods

2.1. Plant Material

The leaves of *C. camphora* used in this study were collected from Suzhou, Nanjing, Nantong and Lianyungang. Hereafter this text will be abbreviated as SZ, NJ, NT and LY, respectively. The plants were identified by the authors and voucher samples were deposited in the Pharmacopoeia laboratory, Nanjing University of Chinese Medicine.

2.2. Animals

Female SPF ICR mice (20–22 g) were used. These animals were obtained from Nanjing Qinglongshan Animal Center (Certificate number NO.201712400). The animals were housed in groups of 10 under a stable raising condition with free access to water and standard food. Food was withheld overnight prior to experiments while water was still provided ad libitum. The handling and use of animals were in accordance to the institutional guidelines.

2.3. Drugs and Chemicals

The following drugs and chemicals were used: acetylsalicylic acid (Nanjing Senbergha Biological Technology Company Limited, China), ibuprofen (Tianjin Sino American Pharmaceutical Company Limited, China), reserpine (Shanghai Yuanye Biological Technology Company Limited, China), NO kit (batch number: 20170607, Nanjing Jiancheng Bioengineering Institute, China) is used after the hot plate test, bicinchoninic acid (BCA) protein quantitative kit(Nanjing Jiancheng Bioengineering Institute, China), mouse PGE_2 ELISA kit (batch number:SU-20561, Nanjing Maibo Biological Technology Company Limited, China),NO kit(batch number: E20170801A, Shanghai Yuanye Biological Technology Company Limited, China) is for migraine test. All other chemicals were of analytical grade.

2.4. Extraction and Chemical Analysis of Essential Oils

Essential oils were extracted by hydro distillation using a clevenger-type apparatus for 2h. All oil samples were stored at 4℃ prior to analysis. The essential oils extraction yield was 1.12 % (v/w), 1.05 % (v/w), 1.15 % (v/w) and 1.09 % (v/w) for SZ, NJ, NT and LY respectively.

The chemical composition of the essential oils was analyzed by GC-MS under the following analytical conditions: the GC oven temperature was maintained at 60℃ for 2 min, programmed to 120℃ (hold 2 min) at a rate of 5℃/min and programmed to 280℃ at a rate of 5℃/min. An aliquot (1μl at 5% v/v dilution with methanol) of essential oil was injected into the column, while the injection temperature was 250℃. Helium served as carrier gas at a flow rate of 1ml/min. The GC–MS analysis was carried out by Agilent 7890B/7000C with a quartz capillary column HP-5MS (0.25 μm×0.25 mm×30 m) with ionization energy of 70 eV. In order to identify the essential oil constituents, we used the mass spectra library search and by comparing with literature data. The relative amounts of individual components were calculated based on GC peak areas.

2.5. Hot Plate Test in Mice

The acetyl salicylic acid was used as a positive control substance. Essential oil extracts

from NJ and LY were given by gavage at a dose of 0.25 ml·kg^{-1} or 1 ml·kg^{-1}for five consecutive days before the experiments. Vehicle (Chestnut oil) was also administered in some animals by the same route. 30 minutes later, acetyl salicylic acid were given by intraperitoneal injection at a dose of 100 mg·kg^{-1} on the last day. Each animal was then placed gently on a 55℃ hot plate. Nociceptive responses reflected soon, such as licking paws or jumping off. The latencies of licking paws were observed after drug administration [8].

2.6. Acetic Acid-induced Writhing in Mice

Essential oil extracts from NJ and LY were given by gavage at a dose of 0.25 ml·kg^{-1} or 1 ml·kg^{-1}for five consecutive days before the experiments. Vehicle (Chestnut oil) was also administered in some animals by the same route. 30 minutes later, acetyl salicylic acid was given by intraperitoneal injection at a dose of 100 ml·kg^{-1}on the last day. Acetic acid (10 ml·kg^{-1}) was given by intraperitoneal injection. The mice were then placed in an observation box 30 minutes later, and the number of writhes was counted for 10 min after acetic acid injection [9].

2.7. Determination of Biochemical Indicators

After the hot plate test, blood samples and brain tissue were taken out for the determination of NO contents by NO kit, and PGE_2 levels were measured by PGE_2 kit.

2.8. The Effect on Migraine-Induced by Reserpine

The ibuprofen was used as a positive control substance. The reserpine was given by subcutaneous injection at a dose of 1ml·kg^{-1} for fourteen consecutive days before the experiments. Model mice frequently scratch and climb cage. Essential oil extracts from NJ and NT were given by gavage at a dose of 0.25ml·kg^{-1}or 1ml·kg^{-1}. Vehicle (Chestnut oil) was also administered in some animals by the same route. One hour later, blood samples were taken out for the determination of NO contents by NO kit [10].

2.9. Statistical Analysis

Results are expressed as mean ± SEM Statistical evaluations were made using t-test, and values were considered significantly different when $P < 0.05$.

3. Results and Discussion

According to GC-MS analysis, a detail chemical composition of the four essential oils is presented in table 1. 75.72% of SZ chemical constituents, 97.38% of NJ chemical constituents, 94.40% of NT chemical constituents and 82.27% of LY chemical constituents were definitely identified. The majority of compounds identified in SZ

essential oil were the camphor (18.17%), Nerolidol (17.64%) and (E)-α-atlantone (14.43%)(Figure 1A), the majority of compounds identified in NJ essential oil were the nerolidol (57.39%) and (E)-α-atlantone (32.41%) (Figure 1B), the majority of compounds identified in NT essential oil were the linalool (47.98%), (E)-α-atlantone (12.11%) and nerolidol (10.90%) (Figure 1C), while selin-6-en-4α-ol (29.63%), (E)-α-atlantone (18.81%), camphor (13.96%), and nerolidol (13.51%) were the major compounds of LY essential oil (Figure 1D).

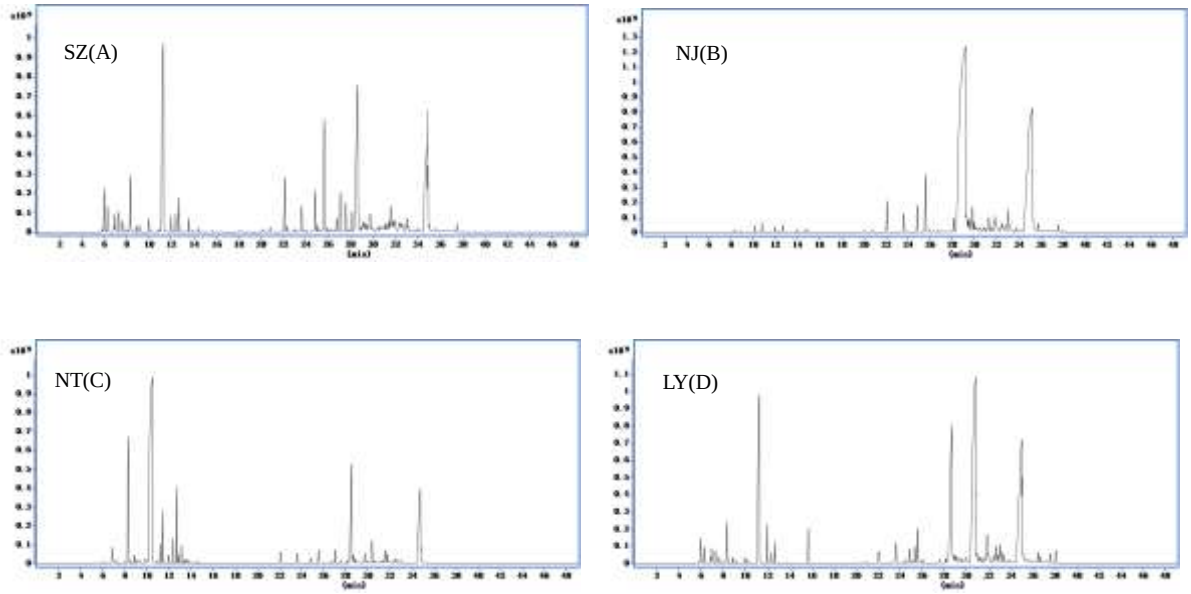

Figure.1.Complete gas chromatogram profile of SZ (A) , NJ (B) ,NT(C),LY(D) essential oils

In the hot plate test, it was also shown that intragastric administration of NJ or LY (1ml·kg^{-1} each) significantly prolonged the reaction time after 30 min treatment, as compared to the corresponding model groups (Table 2). However, there is no significant difference between NJ and LY.

Intragastric administration of NJ (1 ml·kg^{-1} each) significantly decreased the number of acetic acid-induced writhes in mice as compared to the animals that received vehicle only (Table 2) and the analgesic effect caused by the essential oil of NJ was dose-related (Table 3).The writhe inhibitory effects of the oil extracts ranged from 14.20% to 32.84%. By comparison, 100 mg·kg^{-1} acetylsalicylic acid produced half of (i.e. 50.89% effectiveness) analgesia in this nociception model.

As shown in table 4 and table 5, the effects of essential oils of NJ or LY on NO and PGE_2

contents in mice were also assessed. Plasma and brain tissue NO in the mice with NJ or LY (1 $ml \cdot kg^{-1}$) administration were significantly decreased as compared to animals that received vehicle only. Plasma and brain tissue PGE_2 in the mice with NJ or LY ($1ml \cdot kg^{-1}$) administration were significantly decreased as compared to the corresponding model groups. However, there is no significant difference between NJ and LY.

As shown in table 6, Plasma NO in the migraine mice with NJ or NT (1 $ml \cdot kg^{-1}$) and NJ ($0.25ml \cdot kg^{-1}$) administration were significantly decreased as compared to animals that received vehicle only. The effect of NJ is better than that of NT. The effect was dose-related.

Camphor is the major compounds identified in SZ. Camphor is one of the earliest natural medicinal componentswith anti-inflammatory, analgesic, antibacterial and other pharmacological effects. Camphor or Apernyl (acetylsalicylic acid mixt. with propyl 4-hydroxybenzoate) were applied in 82 alveolitis patients. The analgesic effect of Apernyl was significantly better than that of camphor [11]. Linalool is the major compounds identified in NT. Linalool has analgesic, anxiolytic, sedative hypnosis, anti-inflammatory, and other pharmacological activities [12-15]. This compound induced a significant reduction of the acid-induced writhing at doses ranging from 25 to 75 $mg \cdot kg^{-1}$. In the hot plate test, only the dose of 100 $mg \cdot kg^{-1}$ of linalool resulted in a significant effect, but these mechanisms are not very clear [16]. The research on the analgesic properties of nerolidol and (E)-α-atlantone have not yet been reported. To search for more active ingredients, we investigated the analgesic activities of NJ, LY and NT. The essential oils of *C. camphora* leaves from Nanjing and Lianyungang have significantly analgesic effects.

Pain is a protective response. Nonsteroidal anti-inflammatory drugs and opioid analgesics are the most widely used now. However, there are different degrees of adverse reactions. The hot plate test in mice is caused by thermal stimulation of somatic body pain mainly for screening central analgesics. Acetic acid-induced writhing in mice is caused by chemical stimulation of visceral pain mainly for screening peripheral analgesic drugs. Current studies suggest that pain is closely related to NO and PGE_2. PGE_2 effect on peripheral pain has long been clear and more research data show that the central PGE_2 in the process of pain transmission also play an important role [17-19]. NO is a kind of information transfer substance and neurotransmitter with different levels in the

peripheral and central to participate in the regulation of pain. NO plays a complex and diverse role in the modulation of pain. NO is an important neurotransmitter involved in the nociceptive process and it contributes to the development of central sensitization. On the other hand, experimental data have also demonstrated that NO inhibits nociception in the peripheral and also in the central nervous system. NO mediates the analgesic effect of opioids and other analgesic substances. The role of NO in nociceptive modulation depends on its tissure level, the different course of pain and the features of noxious stimulus [20-24]. The results of this study show that *C. camphora* essential oils obviously reduced serum and brain tissue NO and PGE_2 levels. Migraine is a common clinical chronic cerebrovascular disease, and ibuprofen was the traditional drug. However, the mechanism of treatment of migraine is not very clear. The present study show that *C. camphora* essential oils significantly increased plasma NO levels of migraine mice induced by reserpine.

4. Conclusion

The major compounds identified in the four areas are different because *C. camphora* has different chemotypes. However, the majority of compounds identified in the four areas contain nerolidol and (E)-α-atlantone. (E)-α-atlantone exists as the main constituent and it is first reported in this plant. The present study showed that NJ and LY have significantly analgesic effects in three experimental models of pain and the analgesic effect is due to the modulation of NO and PGE_2 levels. The mechanism may be related to the action on NO synthase and cyclooxygenase-2 pathways. The different effects may be produced by different chemical compositions of NJ and NT. Nerolidol and (E)-α-atlantone from NJ might be the main analgesic constituents, which need further research in the future. The significant analgesic effects may be contributed to the synergism of the major ingredients of *C. camphoran* essential oils.

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Table1. Detail chemical components of SZ (a), NJ (b), NT (c), and LY (d) essential oil extracts.

SZ(a)

NO.	Retention time /min	Compound	Relative percentage content/%
1	5.940	unidentified	2.31
2	6.255	camphene	1.37
3	6.871	sabenene	0.27
4	6.919	unidentified	0.9
5	7.274	myrcene	0.95
6	7.624	p-mentha-1,5-diene	0.52
7	8.313	unidentified	3.04
8	8.817	unidentified	0.24
9	9.113	g-terpinene	0.30
10	9.970	unidentified	0.55
11	10.847	(E)-4,8-dimethyl-1,3,7-nonatriene	0.18
12	11.251	(+)-camphor	18.17
13	11.955	borneol	0.49
14	12.348	(-)-4-Terpineol	0.56
15	12.666	α-terpineol	1.03
16	13.521	unidentified	0.44
17	20.806	unidentified	0.29
18	22.103	(-)-β-caryophyllene	3.01
19	22.270	unidentified	0.31
20	23.609	a-caryophyllene	1.33
21	24.836	germacrene D	2.21
22	25.047	(+)-b-selinene	0.30
23	25.627	bicyclogermacren	8.28
24	25.907	unidentified	0.20
25	26.735	unidentified	0.70
26	27.064	unidentified	2.03
27	27.553	elemol	1.55
28	28.124	unidentified	0.96
29	28.629	nerolidol	17.64
30	28.893	(-)-caryophyllene oxide	0.28
31	29.081	unidentified	0.38
32	29.146	(-)-globulol	0.48
33	29.313	unidentified	0.60
34	29.442	(+)-viridiflorol	0.37
35	29.732	gualol	1.32
36	29.851	rosifoliol	0.22
37	30.384	unidentified	0.22
38	30.598	unidentified	0.33
39	30.685	beta-eudesmol	0.23
40	31.045	selinenol	0.52

41	31.181	spathulenol	0.43
42	31.406	unidentified	0.68
43	31.572	unidentified	1.75
44	31.771	unidentified	0.55
45	31.884	eudesmol	0.68
46	31.981	unidentified	0.55
47	32.304	unidentified	0.26
48	32.369	unidentified	0.40
49	32.455	unidentified	0.36
50	32.573	unidentified	0.67
51	33.003	unidentified	0.74
52	33.106	unidentified	0.28
53	34.031	unidentified	0.25
54	34.833	(E)-atlantone	14.43
55	34.908	unidentified	2.33
56	34.994	unidentified	0.24
57	37.491	unidentified	0.35

NJ(b)

NO.	Retention time /min	Compound	Relative percentage content/%
1	22.092	(-)-β-caryophyllene	1.34
2	23.609	a-caryophyllene	0.78
3	24.827	germacrene D	1.16
4	25.578	bicyclogermacren	2.81
5	28.112	unidentified	0.58
6	29.178	nerolidol	57.39
7	29.253	spathulenol	0.84
8	29.818	unidentified	1.07
9	31.846	unidentified	0.66
10	33.057	unidentified	0.97
11	35.225	(E)-atlantone	32.41

NT(c)

NO.	Retention time /min	Compound	Relative percentage content/%
1	6.812	sabinene	1.06
2	8.270	eucalyptol	9.33
3	8.818	unidentified	0.52
4	9.782	linalool oxide	0.61
5	10.47	linalool	47.98
6	11.164	(+)-camphor	0.82
7	11.434	unidentified	2.44
8	12.37	(-)-terpinen-4-ol	1.04

9	12.709	α-terpineol	3.77
10	13.123	unidentified	0.71
11	22.071	(-)-β-caryophyllene	0.83
12	23.599	a-caryophyllene	0.74
13	25.525	bicyclogermacren	0.98
14	27.042	unidentified	0.97
15	28.522	nerolidol	10.9
16	28.651	spathulenol	0.63
17	29.700	gualol	0.7
18	30.367	selin-6-en-4α-ol	1.57
19	31.524	unidentified	0.96
20	31.744	unidentified	0.69
21	34.725	(E)-atlantone	12.11
22	34.790	unidentified	0.64

LY(d)

NO.	Retention time /min	Compound	Relative percentage content/%
1	5.935	unidentified	1.1
2	6.241	camphene	0.72
3	6.919	unidentified	0.67
4	7.274	myrcene	0.52
5	8.254	eucalyptol	0.4
6	8.308	unidentified	1.63
7	11.256	(+)-camphor	13.96
8	11.966	borneol	1.04
9	12.666	α-terpineol	0.54
10	15.657	L-borneol acetate	1.1
11	22.006	unidentified	0.4
12	22.076	unidentified	0.5
13	23.609	acaryophyllene	1.06
14	24.809	unidentified	0.76
15	25.32	unidentified	0.76
16	25.552	bicyclogermacren	1.57
17	28.645	nerolidol	13.51
18	29.964	humulene oxide II	0.48
19	30.781	selin-6-en-4α-ol	29.63
20	30.83	unidentified	1.23
21	30.921	unidentified	0.46
22	31.266	unidentified	0.3
23	31.841	unidentified	1.41
24	31.987	unidentified	0.52
25	32.412	unidentified	0.37
26	32.665	unidentified	0.95

27	32.810	unidentified	0.32
28	33.041	unidentified	0.93
29	33.138	unidentified	0.43
30	33.321	unidentified	0.36
31	34.978	(E)-atlantone	18.81
32	35.037	unidentified	2.21
33	36.436	unidentified	0.41
34	37.527	unidentified	0.37
35	38.039	unidentified	0.57

Table 2. Analgesic effects of essential oils of NJ and LY (0.25ml·kg^{-1} or 1ml·kg^{-1}each), and acetylsalicylic acid (100 mg/kg) on heat stimulation response in the hot plate test in mice (each group is 10 mice, n=10).

Treatment group	Dose	Time needed to respond after 30min /s
Control (vehicle)	-	18.4±3.3
Acetylsalicylic acid	100 ml·kg^{-1}	37.1±7.2**
NJ	0.25 ml·kg^{-1}	21.2±3.0
	1 ml·.kg^{-1}	39.1±9.5**
LY	0.25 ml·kg^{-1}	22.0±4.4
	1 ml·.kg^{-1}	38.2±3.6**

The data represent the mean ± SEM of the number of animals in parentheses.**P < 0.01 compared to corresponding control.

Table 3. Analgesic effects of essential oils of NJ(0.25ml·kg^{-1} or 1ml·kg^{-1}each), and acetylsalicylic acid (100mg/kg) on acetic acid-induced writhes in mice (each group is 10 mice, n=10).

Treatment group	Dose	Number
Control (vehicle)	-	33.8±7.0
Acetylsalicylic acid	100 mg·kg^{-1}	16.6±8.6**
NJ	0.25 ml·kg^{-1}	29.0±4.3
	1 ml·.kg^{-1}	22.7±6.9**

The data represent the mean ± SEM of the number of animals in parentheses.**P < 0.01 compared to corresponding control.

Table 4. Analgesiceffects of essential oils of NJ and LY (0.25ml·kg^{-1} or 1ml·kg^{-1}each), and acetylsalicylic acid (100 mg/kg) on NO contents in mice(each group is 10 mice, n=10).

Treatment group	Dose	brain tissue (μmol/gprot)	plasma (μmol/l)
Control (vehicle)	-	4.59±0.80	15.44±3.11
Acetylsalicylic acid	100 mg·kg^{-1}	2.56±0.92**	8.40±2.07**
NJ	0.25 ml·kg^{-1}	3.93±0.61	13.04±4.30
	1 ml·.kg^{-1}	2.71±0.87**	8.96±2.47**
LY	0.25 ml·kg^{-1}	3.89±0.82	14.39±4.00
	1 ml·.kg^{-1}	2.84±0.76**	9.33±2.19**

The data represent the mean ± SEM of the number of animals in parentheses.**P < 0.01 compared to corresponding control.

Table 5. Analgesiceffects of essential oils of NJ and LY (0.25ml·kg^{-1} or 1ml·kg^{-1}each), and acetylsalicylic acid (100 mg/kg) on PGE_2 contents in mice (each group is 10 mice, n=10).

Treatment group	Dose	brain tissue (pg/ml)	plasma (pg/ml)
Control (vehicle)	-	247.00±63.17	244.31±66.90
Acetylsalicylic acid	100 mg·kg^{-1}	143.85±45.60**	152.71±58.91**
NJ	0.25 ml·kg^{-1}	208.71±74.46	214.68±62.43
	1 ml·.kg^{-1}	160.91±50.19**	165.04±53.14**
LY	0.25 ml·kg^{-1}	199.28±77.74	210.43±47.03
	1 ml·.kg^{-1}	163.71±59.06*	172.85±51.24**

The data represent the mean ± SEM of the number of animals in parentheses.**P < 0.01,*P < 0.05 compared to corresponding control.

Table 6. essential oils of NJ and NT (0.25ml·kg^{-1} or 1ml·kg-1each), and ibuprofen (300mg/kg) on NO contents in migraine mice (each group is 10 mice, n=10).

Treatment group	Dose	plasma (μmol/l)
Control (vehicle)	-	29.69±1.39
ibuprofen	300 mg·kg^{-1}	34.46±2.43
NJ	0.25 ml·kg^{-1}	32.09±1.08*
	1 ml·.kg^{-1}	31.19±1.74**
NT	0.25 ml·kg^{-1}	32.84±1.29
	1 ml·.kg^{-1}	32.55±1.45 *

The data represent the mean ± SEM of the number of animals in parentheses.**P < 0.01,*P < 0.05 compared to corresponding control.