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Research Article

Phytochemical and Pharmacological Studies on Newly-Suggested Herbal Formulations for Potential Protection Against Inflammatory Conditions

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ABSTRACT

Inflammation is the body's basic response to injury. Since current anti-inflammatory medications have side effects, therefore development of newer safe, natural anti-inflammatory agents is necessary. The present study was conducted to investigate the effect of new mixtures of herbal extracts on acute inflammation in rats. Comparison between different methods of analysis (Gas chromatography, High performance liquid chromatographic and High performance thin layer chromatography) for the active constituents included in the new preparations named: NP1, NP2 and NP3 was done. Also evaluation and validation for a new method for estimation of piperine alkaloids in piper extract using HPLC method and comparing it with the HPTLC method was done. According to the acute toxicity study done for detection of safe doses of each mixture, animals were classified into: Negative control: received saline, positive control: paw oedema was induced by 0.2 ml of formaldehyde injected into the rat hind paw, reference group for mixtures NP1and NP3: given Indomethacin® (25 mg/kg) orally, Reference group for mixture NP2: given Prednisolone® (2.8 μmol kg^{-1}) orally and treated groups given the mixture NP1 orally in doses 40 and 80mg/kg, mixture NP2 intranasally in doses 30 and 60 mg/kg and mixture NP3 orally in doses 50 and 100mg/kg. The paw volume for each rat was measured before and at 2, 4 and 6 hrs after formaldehyde injection for monitoring the rate of paw oedema. The three mixtures produced significant inhibition of inflammation when compared to positive control group which is promising for treatment of inflammation after further clinical trial assessment.

Keywords: Inflammation, *in vivo*, formaldehyde, herbal formulation, piperine, validation, analysis.

INTRODUCTION

Inflammation is a local response of tissues to injury. It is considered the body's defense reaction against the spread of traumatic agents[1]. Mechanical, chemical or infectious inflammatory stimuli result in reduction in interstitial fluid pressure, which leads to increased filtration and formation of oedema which is a sign of inflammation. Oedema can affect organ function, and endanger life if it occurs in the airways or the brain[2]. Asthma and allergies as allergic rhino-conjunctivitis and atopic dermatitis, are common inflammatory diseases. A considerable number of patients suffering rhinitis progress to have asthma[3]. Quality of routine daily life has been found to be impaired both in patients with either asthma or allergic rhinitis[4,5]. Medications for asthma and rhinitis can be administered locally (intranasally or inhalation), or orally and parenterally (Intramuscular or Intravenous). Synthetic drugs used for treatment of inflammatory diseases, they sometimes may have serious side effects. Gluco-corticosteroids despite of their side effects, were the most effective drugs used for the treatment of rhinitis and asthma[6]. But since compliance to treatment is low in asthma and rhinitis therefore development of new cheap medications is required. So, development of herbal medicines that have no proven side effects is recommended for the treatment of inflammation that may be a sign of underlying serious condition or may be life threatening.

Among natural products, basil leave extract possesses anti-inflammatory, antiviral, antimicrobial, antioxidant, as well as anti-tumour, activity[7].While *Boswellia carterii* in addition to its anti-inflammatory activity possesses an anti-leukotriene activity[8].

Another herb is Curcuma, whose tumeric extract is used for treatment of peptic ulcers, pain and inflammation due to rheumatoid arthritis[9]. Also lavender essential oil can be utilized as potential natural medicine in treating rhinitis[10], as inhibits immediate-type allergic reactions[11].

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Moreover, Nigella oil has a hepato-protective effect due to its anti-inflammatory and antioxidant activities[12], as well as tea tree oil which can reduce histamine-induced skin inflammation[13] and eucalyptus oil, suppresses oedema formation so reduces inflammation and pain[14].

In addition to these herbs Nettle is another herb that has an anti-inflammatory activity[15], as well as Line seed oil[16]. Flavonoids present in *Moringa oleifera* seed are potent, water-soluble antioxidants and have antibacterial, anti-inflammatory, anti-allergic[17].

The present study was conducted to demonstrate the anti-inflammatory effects of three different herbal preparations prepared by mixing extracts and oils of several herbs together and administered either orally or intranasally to be targeted to the upper respiratory system.

MATERIALS AND METHODS

Materials

Tested herbal Mixtures

NP1 consists of: Basil leaves, Curcuma and quercetin extracts, resin of *Boswellia carterii*, Lavender and Nigella oils.

NP 2 consists of: Nettle extract, Eucalyptus, Basil and Tea tree oils.

NP 3 consists of: *Moringa oleifera*, Linseed oil, resin of *Boswellia carterii*, Piper longum and Liquorice extracts.

Oils were purchased from Arab company for gelatin pharmaceutical products, Egypt. While extracts were purchased from Alpha company for pharmaceuticals, Egypt.

Standard material

Standards used as marker compounds in the selected oils: quercetin, kampferol, linolenic acid, thymoquinone, rosmarinic acid, ferulic acid, glycyrrhizinic acid (purchased from Sigma Chemical Company, St. Louris, Mo, USA), Eugenol, linalool, terpin-4ol, allicin and cineol (purchased from MP Biomedicals, USA), piperine and curcumin (purchased from Lobachemie, India).

Gas chromatography (GC)

Hewlett–Packard 6890 series GC equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector (split–split less) was used. A polar HP-5 (Crosslinked 5% Phenyl Methyl Siloxane) HP INNO wax (cross linked PEG) (30m X 0.32mm X 0.5um), Carrier gas: Nitrogen 40cm/sec, 11.8 Psi (50°C).

High performance liquid chromatographic (HPLC)

Agilent HPLC (USA) 1260 infinity that consisted of a quaternary pump and UV detector equipped with sampler TCC, under computer control was used.

High performance thin layer chromatography (HPTLC)

The samples were spotted in the form of bands with a Camag microliter syringe on precoated silica gel ready-made aluminum plate 60 F_{254} (20 cm × 10 cm with 0.2 mm thickness; E. Merck, Darmstad, Germany) using a sample applicator for TLC Linomat V (CAMAG, Muttenz, Switzerland). Plates were developed in ascending order with a CAMAG twin through glass tank which was pre-saturated with the mobile phase for 15 min. TLC runs were performed under laboratory conditions (Temp: 25 ± 2°C and % RH: 60 ± 5). Densitometric analysis was performed with a Camag TLC scanner III operated by Win CATS software (Version 1.2.0). The source of radiation utilized was deuterium and Tungsten lamp.

Animals

Wistar Albino rats, weighing about 150 g were used. The rats were obtained from the animal house colony of the National Organization for Drug Control and Research (NODCAR), Egypt. The animals were kept in standard plastic cages in an air-conditioned room at 22 ± 3°C, 55 ± 5% humidity and supplied with standard laboratory diet and water *ad libitum*. All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985)

Diagnostic kits

Kits for measuring serum levels of ALT (alanine aminotransferase), AST (aspartate aminotransferase), urea and creatinine, were purchased from Biodiagnostic Company, Egypt.

Chemicals

Formaldehyde obtained from Bio Feed Pharma, 6th October city, Egypt. Diethyl ether obtained from Sigma, USA.

Drugs

Indomethacin® was obtained from Epico, Egypt Int. pharmaceutical Industries Co., ARE under license of Merck Co. Inc-Rahaway, NJ. Prednisolone® was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.)

Methods

Phytochemical Studies

Sample preparation

Preparation of samples (NP1, NP2) for volatile oils analysis

200 mg of each tested preparation were extracted with 3x10ml *n*-hexane then filtrated, concentrated and the volume was adjusted to 10ml in volumetric flask**.**

Preparation of sample (NP3) for linseed oil analysis

200 mg of new formulation (NP3) was converted to methyl ester solution according to[18] then the volume was adjusted in 25 ml volumetric flask with *n*-hexane.

Preparation of samples for further analysis

200 mg of each new suggested formulations (NP1, NP2, NP3) were sonicated with 10 ml methanol for 30 min, filtered then completed the volume with methanol in 25ml volumetric flask.

Standards preparation

Preparation of volatile oils standards

5 mg of each standard: thymoquinone, linalool, eugenol, cineol and 4-terpinol was dissolved in 5 ml *n*-hexane, in 5 ml volumetric flask.

Preparation of linolenic acid methyl ester standard

5 mg of linolenic acid standard was proceeded as test solution.

Preparation of standards

5 mg of each standard: rosmarinic acid, boswellic acid, curcumin, quercetin, ferulic acid, kampferol, glycyrrhizic

acid, piperine and allicin was dissolved in 5 ml methanol, in 5 ml volumetric flask.

Preparation of piperine standard curve by HPLC

A stock solution of piperine standard was dissolved in methanol (10 mg/10ml). Five different concentrations of stock solution (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) were prepared in methanol and injected in three replicates.

HPLC methods

Basil extract as rosmarinic acid[19], tumeric extract[20], boswellia extract[21], quercetin[22], Nettle and Moringa extracts as flavonoids[23], liquorice extract[24], and garlic extract[25].

HPLC Method of piperine alkaloids

The HPLC method for piperine alkaloids is a new method. It was performed on C8 column (150x4.6mm i.d., 5µm particle size) for (P 12) and C18 column (150x4.6mm i.d., 5µm particle size) for (NP3) at ambient temperature; the mobile phase was a mixture of acetonitrile/water/methanol in the ratio of 40:40:20 v/v, pumped at flow rate of 1.5ml/min and the UV detector was set at 340 nm

HPTLC methods

Volatile oils[26], piperine alkaloids[27], basil extract as rosmarinic acid[28], tumeric extract, Nettle and Moringa extracts as flavonoids and garlic extract[29], boswellia extract[30] and liquorice extract[31].

GC conditions

The initial column temperature was 120°C (for fatty acids) and 60°C (for volatile constituents), programmed at 20°C/min and final temperature 300°C, using flame ionization detector (FID), temperature set at 275°Cand injector at 180°C.

Method of validation

The developed method was validated in terms of linearity, precision, limits of detection (LOD), limits of quantification (LOQ), selectivity and recovery according to ICH guidelines[32], for piperine analysis using new HPLC method. *Pharmacological Studies*

Acute Toxicity study

Acute toxicity study of NP1, NP2 and NP3 was done according to[33], who stated that if a single dose up to 5g/kg body weight is not lethal there would be no longer need for determination of LD50 value. NP1 and NP3 were given orally while NP2 was given intranasally in doses up to 4,3and 5gm/kg which were the maximum soluble doses in 3 cc distilled water for NP1, NP2 and NP3 respectively. No mortality was recorded 24 hours later for all mixtures. After 15 days blood samples were collected from all groups of rats after being anaesthetized with ether by puncturing retro-orbital plexus of veins[34], the blood was allowed to stand in clean dry centrifuge tubes for 30 minutes before centrifugation to prevent haemolysis. Then blood samples were centrifuged for 15 minutes at 2500 rpm then the clear supernatant serum was separated and collected by pasteur pipette into a dry clean tube to use for measuring serum levels IU/L of: Asparate aminotransferse, (AST) (SGOT), Alanine aminotransferase (ALT) (SGPT) according to[35], Urea according to[36] and Creatinine according[37]. Then the animals were sacrificed for the Histopathologic study as follows: the thoracic cavities were opened then livers were rapidly and carefully excised and all attached vessels and ligaments were trimmed off. Abdominal cavities were opened then the kidneys were removed. The removed livers and kidneys were washed with cold saline, then dried with filter papers and weighed, finally put into jars containing 10% formalin as a fixative and kept for histopathological examination. Liver and kidney slides were prepared and stained with hematoxylin and eosin stain (H& E)[38].

Study of the anti-inflammatory effects of NP1, NP2 and NP3

The safe prophylactic dose of each mixture for administration to rats was selected according to the results of acute toxicity study, and the selected doses were tenth and fifth the dose used in the acute toxicity study.

The animals were classified into nine groups each consists of eight rats. Tested mixtures, reference drugs and saline were administered to rats one hour before induction of paw oedema and all hind footpad thickness were measured at zero time (basal reading) immediately before, then every two hours for six consecutive hours, after induction of paw oedema. The paw thickness was measured by using Vernier caliper[39].

The difference between initial and subsequent readings gave the change in oedema thickness for the corresponding time. Oedema volume of paw of positive control group (Vc) and volume of treated group (Vt) were used to calculate percentage (%) inhibition and (%) oedema volume by using following formula:

% Oedema volume = (Oedema volume after drug treatment/base time volume) × 100-100

% Inhibition = (Vt /Vc) × 100-100[40].

The rats were classified into the following groups:

Control groups

Normal group: Rats were given (1ml) of saline.

Positive control group: Paw oedema was induced by 0.2 ml (1%, w/v) of formaldehyde injected in the sub-plantar area of the right hind paw of the rat[41].

Prophylactic groups

All drug regimens were given one hour before induction of paw oedema, and the groups were as follows:

Indomethacin Reference group for NP1 and NP3: Rats were given indomethacin orally in dose of 25 mg/kg[42].

NP1 (two groups): Rats were given NP1 orally in doses of 40 and 80mg/kg respectively

NP3 (two groups): Rats were given NP3 orally in doses of 50 and 100mg/kg respectively

Prednisolone Reference group for NP2: Rats were given prednisolone orally in a dose of 2.8µmol /kg[43].

NP 2(two groups): Rats were given NP2 intranasally in doses of 30 and 60mg/kg respectively

Statistical analysis

Values were expressed as means ± S.E. Comparisons between means were carried out using one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparisons test for all acute toxicity study tests. For anti-inflammatory study comparisons between means were carried out using two way ANOVA followed by Bonferroni's multiple comparisons test. $p \leq 0.05$ was

accepted as being significant in all types of statistical tests. Graph pad prism software (version 6) was used to carry out all statistical tests.

RESULTS

Phytochemical analysis

The quantitative analysis of the volatile oils content were carried out by analyzing the marker compounds eugenol (of basil oil), terpin-4ol (tea tree oil), cineol (eucalyptus oil), linalool (lavender oil), thymoquinone (nigella oil) and linolenic acid in linseed oil. Results of GC analysis showed the presence of cineol peak at R_t 2.99±0.2 min (26.5%), eugenol peak at R_t 4.3±0.2 min (34.5%), terpine 4-ol peak at R_t 3.2±0.2 min (13.1%) thymoquinone peak at R_t 4.7±0.2 min (15.4%) and linalool peak at R_t 3.1±0.2 min (28.3%), Figure (1). HPTLC analysis showed the presence of purple cineol spot at R_f value 0.79±0.2 min (16.5%), red-brown eugenol spot at R_f value 0.62±0.2 min (42.7%), blue-violet terpine 4-ol spot at R_f value 0.7±0.2 min (8.9%), blue flourescent thymoquinone spot at R_f value 0.58±0.2 min (16.5%) and the green linalool spot at R_f value 0.63±0.2 min (26%), Figure (2). The comparative analysis of volatile oils using GC and HPTLC revealed the presence of thymoquinone was 18% and 15.8%, linalool was 18% and 24%, eugenol was 57% and 45.1%, cineol was 80% and 82% and 4-terpinol was 40% and 38% respectively, (Table 1, Figure 3). The comparative analysis of linolenic acid methyl ester in linseed oil using GC and HPLC was 54.1% and 89.6% respectively, Figure (3). The quantitative analysis of rosmarinic acid by HPLC and HPTLC were 50% (0.625 mg/g) and 52.3% (0.698 mg/g) and for curcumin 77% and 38% respectively. β-boswellic acid (in boswellia extract) in preparations (NP1 and NP3) using HPLC and HPTLC were 25.9% and 23.9%
respectively for NP1 and 24% and 24.5% for NP3 respectively. For quercetin, the concentrations were 71% (28 mg/ 200mg) and 83.6% (27.5 mg/ 200mg) using HPLC and HPTLC, respectively. For kaempferol (in Moringa extract), the concentrations were 71% and 84% respectively, while for kaempferol and ferulic acid (in Nettle extract) were 18.5% and 16.7% for kaempferol and 72.3% and 68.9% for ferulic acid. For glycyrrhizic acid, the concentrations were 11.9% (0.048mg/100mg) and 33.3% (0.051mg/100mg) respectively. Allicin using HPLC and HPTLC methods were 12.1% (0.072mg/ 200mg) and 12.4% (0.0721mg/ 200gm) respectively. For piperine alkaloid, the concentrations were 98.6% and 97.1% respectively, Figure (4).

Validation method

The results of recovery, linearity and precision, limit of detection (LOD) and limit of quantification (LOQ) for piperine by HPLC are presented in Table (2).

Pharmacological studies

Acute Toxicity Study

Adminstration of NP1 and NP3 orally and NP2 intranasally, to male and female rats (each group consisted of five male and five female rats) in single doses of 4,3,5 gm/kg respectively (maximum soluble doses of mixtures in 3 cc distilled water) revealed no deaths in all groups of animals within the first 24 hours after adminstration. When blood samples were collected 15 days after administration for complete blood picture (table 3), liver and kidney (table 4) function examination, the following results were obtained:

The three mixtures didn't affect red blood cell count, haemoglobin %, or creatinine level in serum in either male or female rats.

NP 1 and NP3 caused significant increase of white blood cell count in both male and female rats, while NP2 caused significant increase in white cells in female rats only, when compared to negative control group of same gender, the increase was more significant in female rats when compared to male rats in case of NP1and NP2.

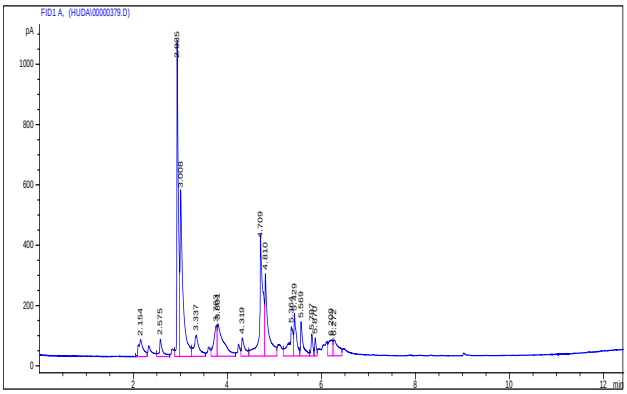
NP2 and NP3 caused significant decrease of platelet count in both male and female rats, while NP1 caused significant decrease in cells in male rats only, when compared to negative control group of same gender, the reduction was more significant in female rats when compared to male rats in case of NP 2and NP3.

NP1 and NP2 didn't affect the serum level of aspartate aminotransferase, but NP3 caused significant increase in its level in male rats only, when compared to negative control group of same gender.

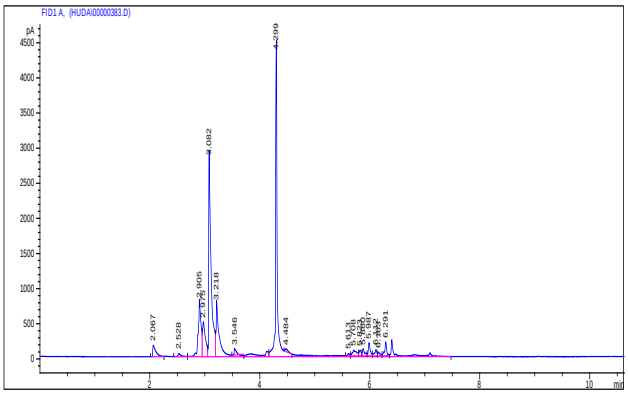
NP3 also caused significant increase in alanine aminotransferase level in both male and female rats, when compared to negative control group of same gender, the increase was more significant in female than male rats.

Table 1: comparative analysis of marker compounds in NP1 and NP2 formulations using GC and HPTLC analysis.

	Method			
	GC		HPTLC	
Sample	Area	Percent %	Area	Percent %
Thymoquinone in NP1	1968	15.4%	6420	16.5%
Thymoquinone in nigella oil	421.8	18%	6267	15.8%
Linalool in NP1	616.1	28.3%	2544	26%
Linalool in lavender oil	2298	18%	4113	24%
Eugenol in NP2	11081	34.5%	8277	42.7%
Eugenol in basil oil	4354	57%	8006.9	45.1%
Cineol in NP2	8627	26.5%	2589	16.5%
Cineol in eucalyptus oil	1763	80%	763	82%
4-terpinol in NP2	4265	13.1%	1432	8.9%
4-terpinol in tea tree oil	878.8	40%	678.8	38%

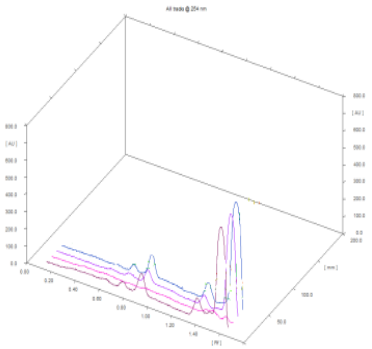


NP (1)

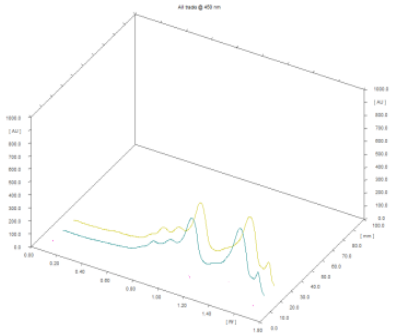


NP (2)

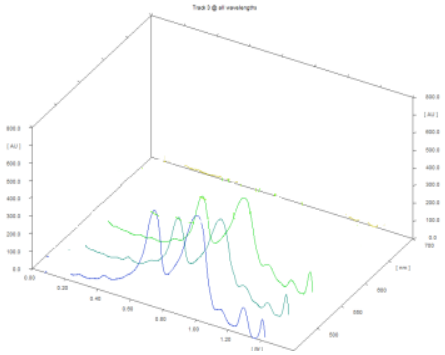
Figure 1: GC chromatograms for volatile oil in NP1 and NP2 prepared formulations



3-D of nigella oil and NP1



3-D of lavender oil and NP1



3-D of basil, eucalyptus, tea tree oils mixed standards and NP3

Figure 2: HPTLC 3D- chromatograms for volatile oils in formulations (NP1, NP2 and NP3)

Table 2: Analytical parameter for determination of piperine by HPLC

Parameter	Results
Linearity range	0.2-3.2 mg/ml
Regression equation	Y=6589.9x-173.42
Correlation coefficient	0.9996
Recovery	98.9%-101.5%
Intra-day precision (%RSD)	0.58
Inter-day precision (%RSD)	0.91, 0.99 and 0.98
LOD	0.86 ng/ml
LOQ	0.26ng/ml

NP1 caused significant increase in alanine aminotransferase level in female rats when compared to male rats. On the other hand NP2 didn't affect alanine aminotransferase level in either male or female rats. Regarding urea level in serum, NP1 caused significant increase in its level in both male and rats, while NP2 and NP3 caused significant increase in cells in female rats only, when compared to negative control group of same gender, the increase was more significant in female rats when compared to male rats in case of NP2.

Histopathological results of acute toxicity study: Livers and kidneys were dissected 15 days after administration of each mixture from all groups including negative control groups.

Histopathologic examination of negative control rats which were given single oral dose of one ml saline revealed no alternations as well as groups given NP1, NP2 and NP 3.

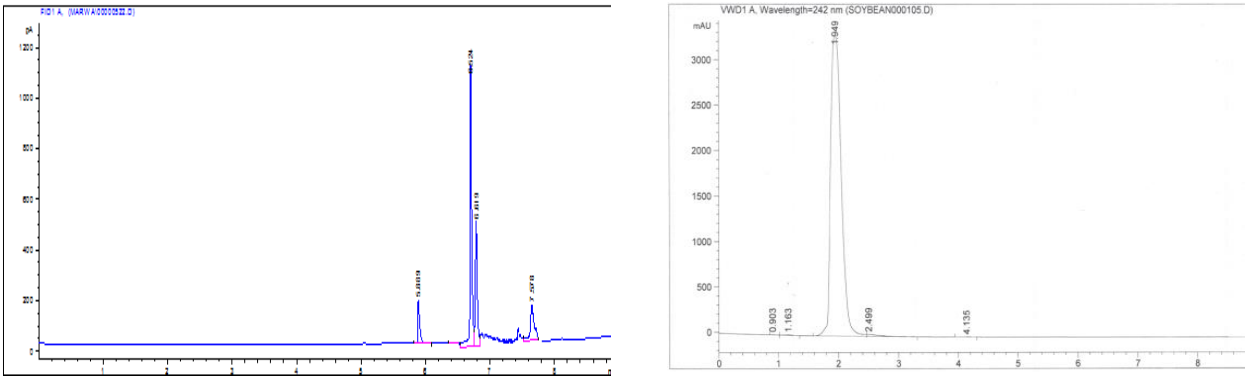
Anti-inflammatory activity of NP1, NP2 and NP3

In the present study, when rat paw volumes were measured after 2,4 and 6 hours consecutively after sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde to positive control group, it was found that formaldehyde produced

Table 3: Effects of NP1, NP2 & NP3 on CBC in rats.

Group	CBC			
	RBCs	HB%	WBCs	Platelet count
Negative control female	7.05±0.19	13.05±0.4	8.32±0.8	948.5±15.28
Negative control male	6.85±0.19	12.8±0.34	8.6±0.51	1043±15.9
NP 1 female	6.74±0.1	12.38±0.27	14.08±1.2*@	480±11.55*
NP 1 male	7.24±0.15	12.78±0.37	17.88±0.44*	499.3±2.83*
NP 2 female	7.16±0.21	13.18±0.42	17.75±1.1*@	680.3±12.47*@
NP 2 male	6.69±0.14	11.88±0.3	11.25±0.87	544.3±18.57*
NP3 female	7±0.21	12.80±0.57	13.35±0.43*	622.5±4.25*@
NP 3 male	7.03±0.17	12.83±0.16	14.63+0.58*	470.5±28.9*

Statistical analysis was done using one way analysis of variance followed by Tukey Kramer multiple comparison test. Values represent mean±S.E, n=5, p< 0.05.
*Signifcant different from negative control of same gender
@Significant different from treated group of other gender



GC HPLC

Figure 3: comparative analysis of linolenic acid methyl ester in NP3 formulation using GC and HPLC

Table 4: Effects of NP1, NP2 and NP3 on Liver Function tests(AST IU/L) & ALT (IU/L)) and Kidney Function tests (Urea (mg/dl)& Creatinine (µmol/L)) in rats.

Group	parameter			
	AST(IU/L)	ALT(IU/L)	Urea(mg/dl)	Creatinine (µmol/L)
Negative control female	38.08±1.78	45.28±1.93	25.31±1.19	1.92±0.13
Negative control male	37.53±2.74	45.75±2.06	24.92±0.82	1.63±0.07
NP 1 female	39.75±2.17	38.33±1.9@	46.76±1.32*	2.11±0.06
NP 1 male	43.634±1.61	55.98±4.26	37.71±2.1*	1.99±0.09
NP2 female	39.18±2.48	44.38±1.53	42.76±2.61*	1.89±0.06
NP 2 male	30.53±0.93	54.33±3.53	35.05±2.4	1.65±0.04
NP3 female	42.53±1.16	63.88±2.35*@	74.48±2.4*@	1.88±0.06
NP 3 male	47±1.73*	83.38±2.71*	24.86±3.42	1.64±0.08

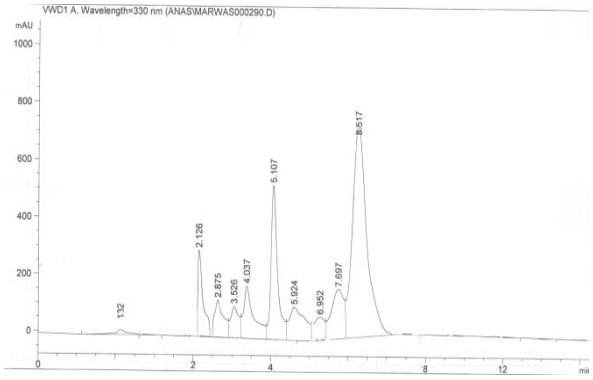
Statistical analysis was done using one way analysis of variance followed by Tukey Kramer multiple comparison test. Values represent mean±S.E, n=5, p< 0.05.
*Signifcant different from negative control of same gender
@Significant different from treated group of other gender

significant increase in paw volumes by 97.57%, 99.98% and 101% respectively of their basal volumes.

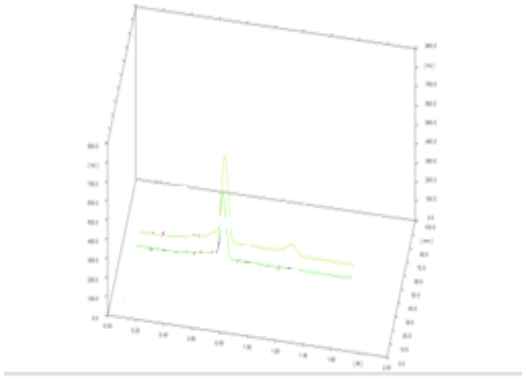
Twenty five mg/kg of Indomethacin was given orally as a reference drug for NP 1 and NP3 one hour before induction of paw oedema, it produced significant inhibition of inflammation evidenced by reduced paw volume, when compared to positive control group by 29.7%,37.78% and 38.87% when measured after 2,4 and 6 hours of sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde respectively.

Regarding the anti-inflammatory effect of NP1, both high and low doses produced significant protection against inflammation through

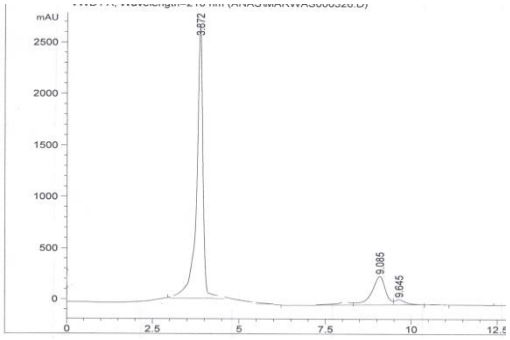
out the experiment duration. Yet the effect of the low dose was significantly better than the high dose, only two hours after induction of inflammation, and significantly less than indomethacin at four hours measurement. Moreover, the anti-inflammatory effect of the high dose was significantly less than indomethacin at two and four measurements yet at six hours it increased to be non significantly different



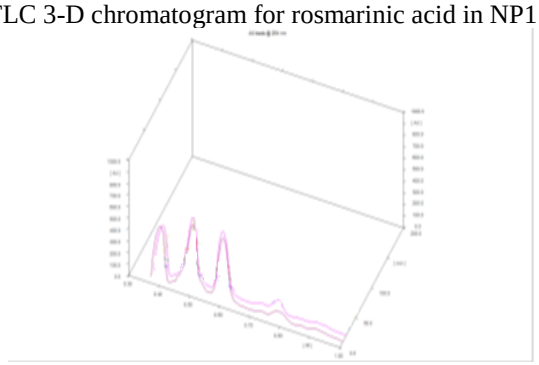
HPLC for rosmarinic acid in NP 1



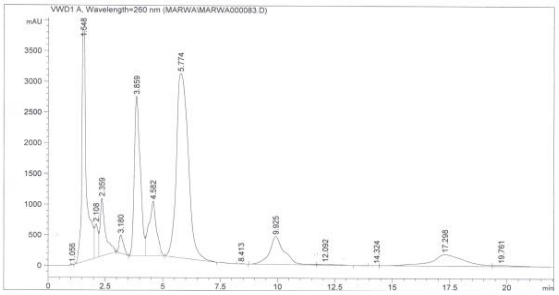
HPTLC 3-D chromatogram for rosmarinic acid in NP1



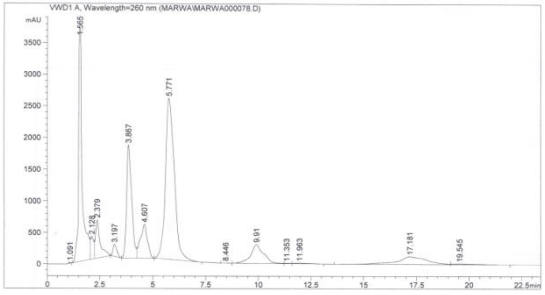
HPLC for curcumin in NP1



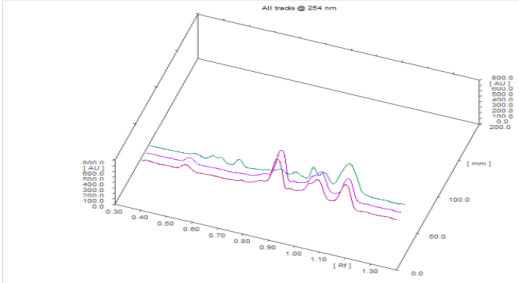
HPTLC 3-D chromatogram for curcumin in NP1



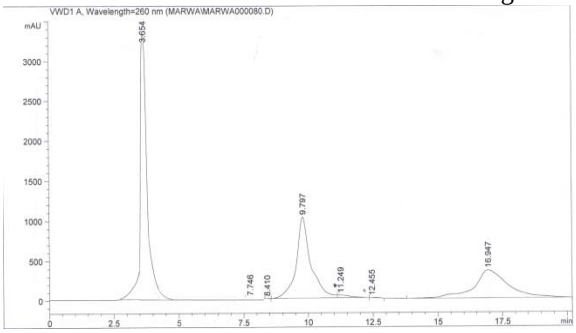
HPLC for boswellia extract in NP1



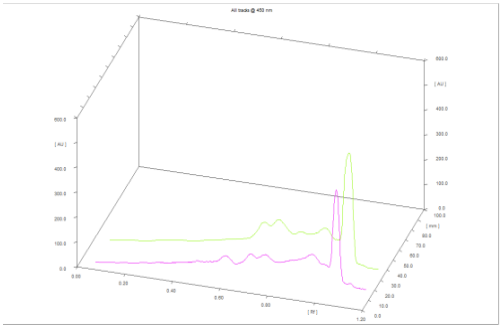
HPLC for boswellia extract in NP3



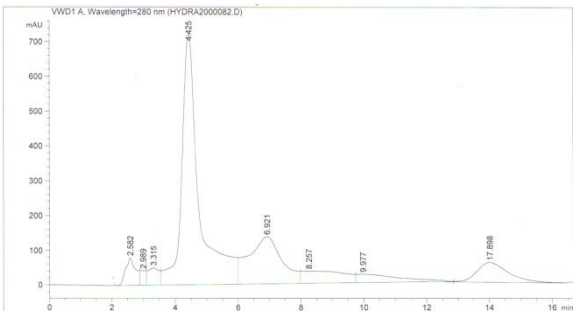
HPTLC 3-D chromatogram for boswellia extract in NP1 and NP3



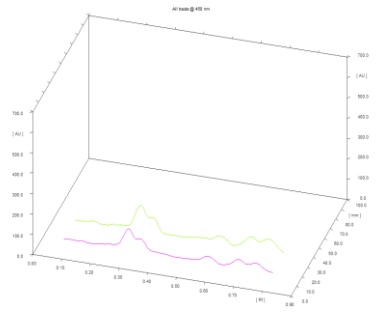
HPLC for quercetin in NP1



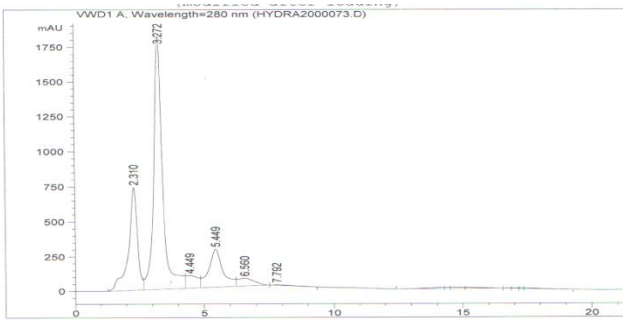
HPTLC 3-D chromatogram for quercetin in NP1



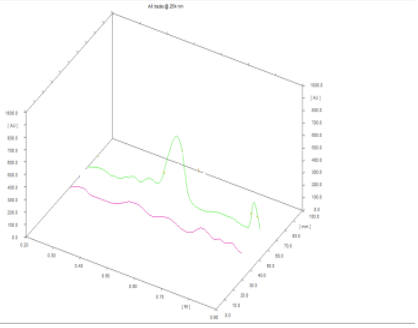
HPLC for Nettle extract in NP2



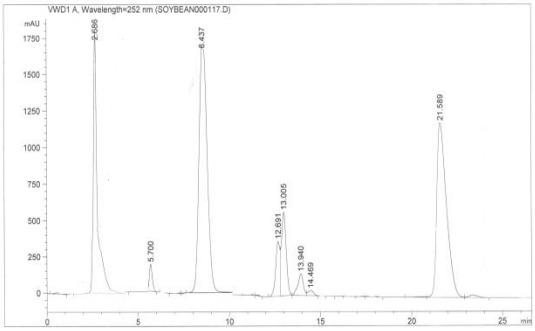
HPTLC 3-D chromatogram for Nettle extract in NP2



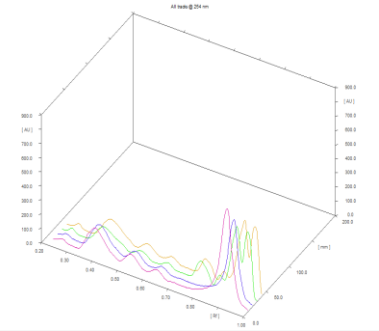
HPLC for Moringa in NP3



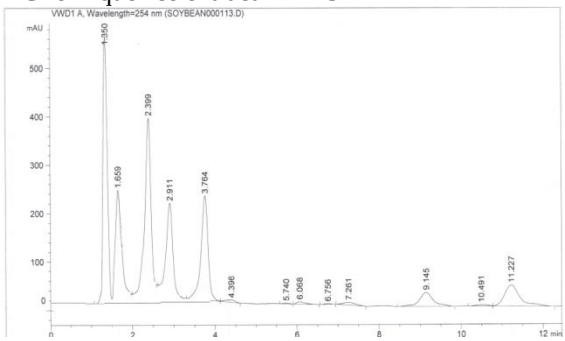
HPTLC 3-D chromatogram for Moringa in NP3



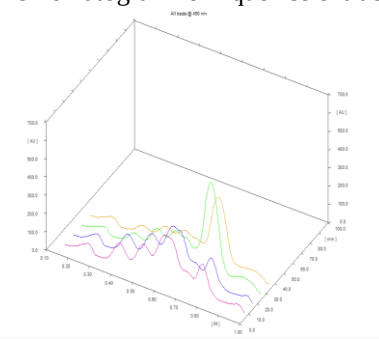
HPLC for liquorice extract in NP3



HPTLC 3-D chromatogram for liquorice extract in NP3



HPLC for garlic extract in NP3



HPTLC 3-D chromatogram for garlic extract in NP3

Figure 4: Comparative analysis using HPLC and HPTLC for basil, boswellia, curcuma, Nettle and Moringa, liquorice, piper and garlic extracts and quercetin in the prepared formulations

from indomethacin. The inhibitory effect of the low dose (40mg/kg) was 35.34%,19.31% and 35.04%, while the inhibitory effect of the high dose (80mg/kg) was 14.78%, 19.31% and 40.56% when compared to positive control group after 2,4 and 6 hours of sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde respectively.

On the other hand NP3 low and high doses (50 and 100 mg/kg) didn't produce significant inhibition of inflammation 2 hours after injection of formaldehyde, but after 4 and 6 hours the low dose produced significant inhibition of inflammation by 29.8% and 33.93% respectively, yet the high dose produced significant inhibition of inflammation by 37.53%after 4 hours only when compared to positive control group. The inhibitory effect of the high dose was significantly low when compared to indomethacin and high dose of the same mixture after six hours of sub-plantar injection of formaldehyde.

Prednisolone (2.8 µmol/ kg) given orally, was used a reference drug for NP 2, it produced significant inhibition of inflammation, when compared to positive control group by 13.01%,19.96% and 26.24% when measured after 2,4

Table 5: Time course effect of oral administration of NP 1(40&80mg/kg) & NP3 (50 & 100mg/kg), indomethacin (25 mg/kg) on rats paw oedema formation induced by sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde.

Group	Second hour		Fourth hour		Sixth hour	
	% of oedema	% of inhibition	% of oedema	% of inhibition	% of oedema	%of inhibition
Positive Control	97.57±0.6	----------	99.98±2.7	----------	101±1.93	----------
Indomethacin 25 mg /kg	68.1±14.25*	29.7±4.54*	62.1±3.33*	37.78±3.36*	62.9±1.15*	38.87±2.5*
NP1 (40mg/kg)	62.65±1.14*$	35.34±1.48*$	70.48±2*	19.31±2.17*#	66.27±1.95*	35.04±0.82*
NP1 (80mg/kg)	82.56±3.3	14.78±3.68*#	80.74±3.61*#	19.31±2.17*#	61.4±2.87*#	40.56±2.28*
NP 3 (50mg/kg)	101.8±3.39#	-4.96±3.22$#	69.94±3.63*	29.8±4.26*	64.47±3.45*#$	33.93±2.94*$
NP 3 (100mg/kg)	87.91±4.19#	9.34±4.19#	62.62±3.59*	37.53±2.19*	101.22±2.04*#	2.36±0.98#

Results are expressed as means a)% of oedema ±SE and b)% of inhibition ±SE, n=8.
Statistical analysis was done using two way ANOVA followed by Bonferroni's multiple comparisons test,$p<0.05$, comparison is measured for the same time interval for all groups.
*Significant different from positive control group at $p<0.05$at same time interval
Significant different from Indomethacin 25mg/kg group at p $p<0.05$at same time interval
$ Significant different from high dose group of same mixture at $p<0.05$at same time interval

and 6 hours of sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde respectively.
The low dose of NP 2 (30 mg/kg) produced significant inhibition of inflammation by 27.84%,18.07% and 20.98%, while the high dose (60 mg/kg) produced significant inhibition of inflammation by 58.87%,74.15% and 61.08% after 2,4,6 hours of induction of inflammation respectively, when compared to positive control group. The inhibitory effect of the low dose of NP 2 was highest at 2 hours onset, it was significantly higher than prednisolone, but significantly less than the high dose of the same mixture, yet this effect decreased at 4and 6 hours onset abolishing the significant difference with prednisolone but keeping the significant difference with positive control group and high dose. The inhibitory effect of high dose of NP 2 was significantly higher than prednisolone and the low dose throughout the experiment, although at six hours onset the inhibitory effect of the high dose started to decrease.
The results of anti-inflammatory activity of the three mixtures are represented in tables 5&6.

DISCUSSION

In the anti-inflammatory study of the effect of each of the three mixtures separately on inflammation induced in rats by subplantar injection of formaldehyde, formaldehyde produced significant increase in rats' paw volumes is in agreement with previous studies[41].
When indomethacin was used as a reference drug against inflammation for NP1 and NP3 and prednisolone for NP2, both produced significant protection against inflammation by inhibiting significant incidence of inflammation which is in accordance with the reported data[42,43]. This effect was evidenced by reduction in paw volumes when compared to rats injected in the sub-plantar region with formaldehyde without prior administration of reference drug throughout the experiment duration.
The anti-inflammatory effect of NP1 which was exhibited in this study, owes to the presence of basil leave extract with its contents of rosmarinic acid and eugenol, which are used for treating seasonal rhinitis, perennial allergies and asthma. Also eugenol has antioxidant effect[7], as well as its contents of flavonoids which have mast cell stabilizing activity[44]. Moreover, resins of *Boswellia carterii* present in the mixture, contain boswellic acid derivatives[8] so they were used for treatment of rheumatoid arthritis and other inflammatory diseases[45]. The presence of Curcuma extract in the mixture contributes to its anti-inflammatory effect, due to the effect of curcumin[46], and also linalool 20-45%. Linalyl acetate 25-47% in Lavender displayed strong antioxidant activity against lipid peroxidation[47]. It also could be used in treating rhinitis patients[10] and inhibiting immediate-type allergic reactions by inhibition of mast cell degranulation *in vivo* and *in vitro*[11]. Some constituents of Nigella oil present in the mixture are p-cymene (32.05%), α-thujene (6.0%), α-pinene (1.11%), camphene (11.0%), sabinene (1.0%), β- pinene (7.0%), β-myrcene (0.21%), α-phellandrene (0.45%), limonene (0.13%), γ-terpinene (5.12%), terpinolene (0.23%), camphor (1.0%), carvone (0.32%), thymoquinone (20.32%), thymol (10.12%), carvacrol (1.0%), Longicyclene (0.9%) and borneol (0.43%) [48], which contribute to its anti-inflammatory, antioxidant, cytotoxic and anti-cancer effects[12].
While the evident anti-inflammatory effect of NP 3 in this study is due to the presence of Linseed oil which contains unsaturated fatty acids as linolenic acid 35–67%[47], and it has the ability to inhibit inflammation induced by arachidonic acid, so inhibits both cyclooxygenase and lipoxygenase pathways of arachidonate metabolism[49]. The presence of Liquorice extract (*Glycyrrhiza glabra*) with

Table 6: Time course effect of intranasal administration of NP 2(30&60mg/kg) and prednisolone (2.8 μmol/ kg) on rats paw oedema formation induced by sub-plantar injection of 0.2 ml (1%, w/v) of formaldehyde.

Group	Second hour		Fourth hour		Sixth hour	
	% of oedema	% of inhibition	% of oedema	% of inhibition	% of oedema	%of inhibition
Positive Control	97.57±0.6	----------	99.98±2.7	----------	101±1.93	----------
Prednisolone (2.8 μmol/ kg)	84.9±1.7	13.01±1.25*	73.54±3.49*	19.96±5.45*	74.79±4.72*	26.24±1.26*
NP2 (30mg/kg)	69.91±2.59*$	27.84±2.94*@$	81.94±3.15*$	18.07±1.79*$	81.28±4.02*$	20.98±0.75*$
NP2 (60mg/kg)	39.83±4.74*@	58.87±4.91*@	25.95±4.29*@	74.15±4.03*@	42.22±3.45*@	61.08±1.92*@

Results are expressed as means a)% of oedema ±SE and b)% of inhibition ±SE, n=8.
Statistical analysis was done using two way ANOVA followed by Bonferroni's multiple comparisons test
*Significant different from positive control group at $p<0.05$at same time interval
@Significant different from prednisolone group at $p<0.05$ at same time interval
$ Significant different from high dose group of same mixture at $p<0.05$at same time interval

the main active constituents as Glycyrrhizic acid (triterpenoid saponin) and liquiritigenin and isoliquiritigenin (flavonoids)[50], lead to its anti-inflammatory, adrenocorticotropic and antioxidant activities[51]. In addition to the previous constituents of mixture 3, *Boswellia carterii* resin was present as well as *Moringa oleifera* seed flavonoids which have potent anti-inflammatory and vasodilatory activities[17,45].

The anti-inflammatory effect of NP2 exhibited in the present study, is due to its content of Nettle extract which contains phenolic compounds as chlorogenic acid, ferulic acid, rutin, isoquercitrin and kampferol[52], which lead to inhibition of prostaglandin formation thus hindering seasonal allergies[15]. It also contains basil oil which was reported to possess anti-inflammatory activity against carrageenan and different other mediator-induced paw oedema in rats[53] and moreover its essential oil (eugenol) has anti-inflammatory effect[54]. It was reported that administration of 1,8-cineole, which accounts for 70–90% (w/w) of the contents of eucalyptus oil present in NP2, had reduced inflammation thus had suppressed oedema formation and pain[14]. It is note worthy in our study to refer to the previous studies that had revealed that tea tree oil could reduce skin inflammation[13], and its inhalation exerted strong anti-inflammatory effects in mice[55]. The effect of Tea tree oil may be due to its content of γ-terpinene 10.0-28.0%, p-cymene 0.5-12.0%, terpinolene 1.5-5.0 %, terpinen-4-ol (minimum 30.0%)[47] which explains the strong anti-inflammatory effect of NP2.

When NP 1 and NP 3 were administered orally and NP2 intranasally, they exhibited protection against inflammation, but with variable degrees and at different time intervals even for different doses of the same mixture. This variability depends on the presence of different constituents in each mixture, and also the variation of quantities of same pharmacologically active ingredients due to different methods of extraction of each constituent of each of the three mixtures.

It was evident that high doses of both NP1 and NP2 exhibited the highest anti-inflammatory effects after six hours of measurements, which is correlated to their contents of basil either extract or oil. The difference in type of basil preparation lead to the difference in efficacy strength due to variation in pharmacokinetics.

CONCLUSION

When NP 1 and NP 3 were administered orally and NP 2 intranasally, they exhibited protection against inflammation, but with variable degrees and at different time intervals even for different doses of the same mixture. This variability depends on the presence of different constituents in each mixture, and also the variation of quantities of same pharmacologically active ingredients due to different methods of extraction of each constituent of each of the three mixtures.

It was evident that high doses of both NP 1 and NP 2 exhibited the highest anti-inflammatory effects after six hours of measurements, which may owe to their contents of basil either extract or oil. The difference in type of basil preparation lead to the difference in efficacy strength due to variation in pharmacokinetics.

So we can conclude that the mixtures studied for their anti-inflammatory effects in this work can be used for protection against any type of inflammation including those accompanied with allergic rhinitis.

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