European Journal of Medicinal Plants
4(5): 542-562, 2014

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Preliminary Investigation of the Anti-asthmatic Potential of *Kalanchoe integra* Leaf Extract Using a Model of Allergic Airway Inflammation

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AGIJ, AC and ADA designed the study. Authors TNAB, AC, EDI, AS and NE carried out the experiment. Authors TNAB, AC and AP performed the statistical analysis. Authors NE and BNBK wrote the protocol. Author AGIJ wrote the first draft of the manuscript. Authors AGIJ, AC and ADA managed the analyses of the study. Authors AGIJ and AC managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 6th October 2013
Accepted 3rd January 2014
Published 25th January 2014

ABSTRACT

Objective: *Kalanchoe integra* is known to possess antihistaminic and mast cell stabilizing effect. Inflammatory mediators, such as histamine and eicosanoids have been implicated in the pathophysiology of allergen-induced asthma including bronchospasm, vasodilation, increased vascular permeability, perivascular and peribronchial oedema, acute functional changes in the lungs and diarrhea due to increased intestinal motility. This study is to

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ascertain the anti-inflammatory effect of *Kalanchoe integra* aqueous leaf extract (KILE) on bronchial hyper-responsiveness in ovalbumin-sensitized guinea-pigs.
Method: Bronchial asthma was induced in guinea pigs using Ovalbumin. *In vivo* skin tests were carried out on all guinea pigs using ovalbumin and histamine as allergens. The diameters of wheals were recorded and the means determined. OA-sensitized guinea-pigs were challenged with 2% OA aerosols after 1 hour *per os* of drugs (KILE or prednisolonefor the treated groups) for two weeks. A piece of excised trachea was suspended in a tissue bath and challenged with histamine in the presence and absence of KILE, as well as Prednisolone (2.5 mg/kg). The results were reported as mean ±S.E.M. Statistical analysis was performed using one-way ANOVA and *Bonferroni's* post hoc test.
Results: Biological assaying of KILE showed significant dose-dependent reduction in histamine induced vasodilation of cutaneal blood vessels (*P<0.05* in all groups at all times of wheal measurements) and contractile responses of isolated trachea to histamine. KILE generally reduced the effect of histamine in all groups.
Conclusion: This study has shown that KILE has the potential of alleviating signs of bronchial hyper-responsiveness and skin allergies in ovalbumin-sensitized guinea pigs with the female models been more sensitive than the male counterparts.

Keywords: Kalanchoe integra; Ovalbumin; bronchial hyper-responsiveness; anti-inflammatory.

ABBREVIATIONS

NSNC: Non-sensitized Negative Control;
SNC: Sensitized Negative Control;
PPC: Prednisolone-treated Positive Control;
HDK: High dose Kalanchoe integra;
LDK: Low dose Kalanchoe integra-treated;
Group I= Non-sensitized Negative Control;
Group IIa= OA-sensitized Negative Control;
Group IIb= OA-Sensitized + Prednisolone;
Group IIc= Low dose Kalanchoe integra;
Group IId= High dose Kalanchoe integra;

1. INTRODUCTION

Bronchial asthma is characterized by airway inflammation, bronchial hyperactivity and bronchospasm and is often accompanied by increased vascular permeability during vascular remodeling [1,2]. Therapeutically, allergic asthma is effectively controlled by corticosteroids although small proportion of asthmaticsstill exhibit severe or corticosteroid insensitive asthma, which is also usuallypoorly controlled by corticosteroids. This causes a reduced quality of life and imposes a considerable cost burden on health services [3]. Moreover, there are also concerns about the systemic effects of long term corticosteroid treatment [4]. Thus, there is a desperate need for novel anti-inflammatory asthma therapies.

Kalanchoe integra (syn. *Kalanchoe Spanthulata*) [5] is a medicinal plant largely used in folk medicine for various treatments. The herbs are perennial, 40-120 cm tall and glabrous. Leaves are subsessile and amplexicaul; leaf blades are spatulate-oblong, 5-7 × 1.5-3.5cm. The leaf bases are attenuate with margins irregularly lobed. Inflorescences arecymose while

sepals are linear-ovate to narrow triangular. Flowers are bisexual and erect with yellow corollas. Stamens are twice as many as petals, inserted near middle of corolla tube; filaments unequal in length, usually very short. Follicles are many seeded while seeds are ellipsoid. Roots are fibrous [6].

It is widely distributed throughout Ghana in the Shai hills and Legon Botanical gardens [2]. In India it is cultivated in gardens and wild on hills of North-Western India, Decan and Bengal. *Kalanchoe integra* has become naturalized in temperate regions of Asia, Australia, New Zealand, West Indies, Galapagos, Melanesia, Polynesia and Hawaii. In many of these countries, such as Hawaii, it is regarded as an invasive species.In the Philippines and it is known as “katakataka” or “kataka-taka” which is an adjective meaning astonishing or remarkable [7]. *Kalanchoe species* are widely used remedies for headache, general debility, dysentery, smallpox, convulsion, wounds, sores asthma and palpitation. Also the leave juice mixed with salt and honey is a remedy for chronic cough [8,9].

The anti-anaphylactic effect of *Kalanchoe pinnata*, demonstrated by researchers, indicates that *Kalanchoe pinnata* possess antihistaminic effects [10]. Also the stabilizing effect of *Kalanchoe pinnata* on mast cell degranulation also suggests that *Kalanchoe* s*pecies* could bepotential candidates for allergic asthma therapy. This study thusattempts to preliminary investigate the anti-asthmatic potential of *Kalanchoe integra* leaf extract using ovalbumin sensitized guinea pigs.

The aim is to establish the effect of *Kalanchoe integra*var. crenata (Andr.) cufleaf extracton tracheal contractile responses and immediateskin hypersensitivity reactions.

2. MATERIALS AND METHODS

2.1 Plant Collection

Fresh k*alanchoe integra* leaves were collected from the University of Ghana Botanical Garden, Legon in July 2012 and sent to the Botany Department for identification and authentication. A voucher number IAGSOP-001 has been asigned. The collected plant material was made free from foreign organic matter after which the leaves were chopped off,washed and air- dried for a period of three (3) days.

2.2 Extraction

About 3 kilograms of the dried *Kalanchoe integra* leaves were washed and blended using Sanyo SM (G300) blender. The blended leaves were macerated using 12 litres of hot distilled water,decanted and sieved to remove plant pigment. The concentrated aqueous extract of the macerated leaves of *Kalanchoe integra* was lyophilized to dryness using a freeze drier. The powdered samples of *Kalanchoe integra* extract were weighed (freeze dried extract=426g), labeled, stored in sterile bottles and kept in refrigerator at 4ºC and used within four weeks after production. A yield of 14.2% was obtained.

3. PHYTOCHEMICAL ANALYSIS

Phytochemical analysis was carried out on the freeze dried extract of *Kalanchoe integra* to determine presence of bioactive phytochemicals using different chemical tests. Phytochemical analysis helps identify the water-soluble components in the extract which are

responsible for the pharmacological effects of *Kalonchoe integra.* Tests were carried out for alkaloids,saponins,tannins, flavanoids, phenols and glycosides [11].

3.1 Alkaloid Test

About 0.1g of the freeze dried extract of *Kalanchoe integra* was added to 2M HCl, stirred, warmed and filtered. The filtrate was divided into three test tubes. Draggendorff's reagent, Mayer's reagent and Wagner's reagent were added to respectively to each test tube.

3.2 Saponin Test

About 0.5g of the freeze dried extract of *Kalanchoe integra* was added to water in a test tube. The test tube was shaken to observe foam formation.

3.3 Tannin Test

About 0.5g of the freeze dried extract of *Kalanchoe integra* was dissolved in 80% of aqueous methanol (10cm^3). Freshly prepared iron (III) chloride solution was added and observations were made on colour changes.

3.4 Flavanoid Test

About 0.1g of the freeze dried extract of *Kalanchoe integra* was added to 80% ethanol (15cm^3). To the filtrate was added magnesium turnings followed by concentrated HCl (0.5cm^3) and observed for colour changes within 10 minutes.

3.5 Phenol Test

Ferric Chloride solution was added to a filtrate of the crude extract of *Kalanchoe integra* and observed for colour changes.

3.6 Cardiac Glycoside Test

About 0.5g of the freeze dried extract of *Kalanchoe integra* was dissolved in chloroform (2cm^3) in a test tube after which concentrated sulphuric acid was carefully added down the side of the test tube to form a lower layer.

4. ANIMAL MODEL

Thirty (30) guinea pigs of both sexes weighing 400-600g were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana. The animals were acclimatized to the laboratory environment for one week before being used in the study. While in their home cage at Noguchi, the animals were provided with *Sankofa* pellet feeds and tap water on daily basis. The room temperature was maintained at 20-23ºC with 12:12 hour light/dark cycle. Spontaneous behaviors of all guinea pigs were observed in cages before experimental procedures were carried out. No animals showed signs of illness before the experimental phase. They were grouped into five groups with two ovalbumin-sensitized groups receiving the *Kalanchoe* extract in high (600 mg/kg) and low (300 mg/kg) doses, one ovalbumin-sensitized group receiving Prednisolone (standard drug), with two other groups

serving as negative controls. Animals in one of the two negative controls were sensitized with ovalbumin whereas those in the other group were non-sensitized at all (intact guinea pigs). The protocol was approved by the Scientific and Technical Committee (STC) and the Noguchi Memorial Institute for Medical Research Institutional Animal Care and Use Committee (NIACUC), College of Health Sciences, University of Ghana with protocol number NIACUC-2013-01-3E. It was also ensured that, all experiments carried out on animals conformed to the OECD guidelines.

5. DRUGS AND CHEMICALS

Ovalbumin was used to induce allergic asthma in the guinea pigs. Prednisolone (2.5 mg/kg), a standard drug for alleviating asthmatic attacks, was used as the reference drug in the positive control guinea pigs. It was purchased from Pills and Tabs Pharmacy at Legon. Acetylcholine and Histamine, purchased from Noguchi Research laboratory were used together with ovalbumin in the *In vitro* isolated trachea studies. Egg ovalbumin (grade V) and Histamine were also used in the in vivo skin sensitization tests. The freeze dried extract of the leaves of *Kalanchoe integra* will be used as the drug under study.

6. SENSITIZATION OF GUINEA PIGS

0.1ml of 0.9% saline containing 15 mg ovalbumin and 150 mg $Al(OH)_3$ was administered to each of the sensitized guinea pigs separately by intraperitoneal and subcutaneous routes on the first day. The guinea pigs were observed daily for a period of two weeks (Day 2 to Day 14). The parameters observed included breathing patterns, eyes, motor activity and sensitivity to touch [12].

7. IMMUNE BOOSTING PROCEDURE

0.1ml of 0.9% saline containing only 15 mg ovalbumin without adjuvant was administered to the sensitized guinea pigs by intraperitoneal route on day 14. The guinea pigs were observed for a period of one week after immune boosting. The pattern of behavioural changes such as fur, body weight, breathing patterns, eyes, motor activity and sensitivity to touch for a period of one week till day 21.

8. OVALBUMIN CHALLENGE AND DRUG TREATMENT

On the 25^{th}day through to 56^{th}day, sensitized guinea-pigs were challenged with 2% aerosolized OA (0.2 g OA dissolved in 10 ml saline) for 10 min after 1 hour treatment with High dose *Kalanchoe* (HDK), Low dose *Kalanchoe* (LDK) and Prednisolone, PRED (2.5 mg/kg) respectively. Non-sensitized controls were challenged with 0.1 ml of 0.9% saline for the same duration. The challenge was conducted in Perspex chamber (dimensions= 20×30cm) connected to jet nebulizer.

9. ALLERGEN CHALLENGE

2% Nebulized Ovalbumin was used as the allergen solution. This was transferred into a clean aerosol container on each day of allergen challenge. Within the three week period, allergen challenge was carried out on animals of all five (5) groups in a mist chamber; two (2) hours after animals in the treated group were dosed. This was to ensure complete absorption of drug and extract before allergen challenge. Ten (10) minutes was allowed for

all thirty guinea pigs placed in the mist chamber. Observations were made on: wheezing signs, breathing rate, eyes, motor activity and movements within the chamber for each guinea pig.

10. SKIN PRICK TEST

Skin prick test was used to measure the anti-inflammatory effect of KILE on IgE response to OVA and vasodilatory activity of preformed mediator, histamine in the sensitized guinea pigs. Skin prick tests were carried out on all the animals *in vivo,* with 0.1 ml of 1% ovalbumin and 0.1ml of 100ug Histamine. The two drugs were injected at two separate regions on each animal by subcutaneous route. The time for appearance of the swelling was noted and measured. The wheal diameters were recorded for all guinea pigs at 1h, 2h and 24h after OVA or histamine administration. The wheal diameters were used to assess the effect of KILE on skin hypersensitivity. This was performed on Day 44.

The average skin oedema was calculated for each group and expressed as a percentage of the OA-sensitized control. Percentage of skin oedema relative to that of OVA-sensitized control for each group was calculated according to the formula:

$$\text{Percentage skin oedema} = \frac{\text{oedema in a particular group}}{\text{oedema in group IIa}} \times 100\%$$

11. TRACHEA STUDIES

In vitro assessment was carried out on the isolated trachea of each guinea pig. All isolated trachea were challenged in turns with a cumulative dose (0.1 – 6.4 mmol/L) of Histamine for contractile effect. Each isolated trachea of guinea pigs belonging to the two *Kalanchoe integra* treated groups was also challenged with the same cumulative concentrations of Histamine in the presence of 200µg of *Kalanchoe integra* extract. The isolated trachea of the guinea pigs belonging to the positive control group, were challenged with the Histamine in the presence of 100µg Prednisolone. For the two negative control groups of guinea pigs, each isolated trachea were challenged with the cumulative dose response of histamine in the presence of 200µg *Kalanchoe integra* extract as well as 100µg Prednisolone.

12. STATISTICAL ANALYSIS

The results were reported as mean ±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). If the overall F value was found to be statistically significant ($P<0.05$), further comparisons among groups were made using the Bonferroni post hoc tests. All statistical analyses were performed using Graph Pad prism 5 software.

13. RESULTS

13.1 Phytochemical Analysis

The observations made during the phytochemical analysis of KILE showed a reddish brown colouration upon addition of draggendorf to the extract, cream precipitate upon addition of Mayer's reagent and a yellow precipitate upon addition of Wagner's reagent all indicating the presence of alkaloids. A greenish blue discolouration indicated the presence of Tannins while a blue discolouration indicated the presence of Phenols. Formation of foam indicated

the presence of saponins while a light pink discolouration was indicative of the presence of flavonoids. Cardiac glycosides were absent since there was no colour observed at interface.

13.2 Observations

There was a generalized increase in respiratory rate, tremors and frequency of micturition in the sensitized guinea pigs.

In the sensitization phase, normal eyes, breathing rate and quick response to touch and normal motor activity were observed in animals of all groups throughout the two weeks. There were remarkable abdominal and subcutaneous swellings were observed in all groups. During the first and second week of allergen boosting, increased breathing rate, respiratory distress, agitation, restlessness and dull eyes accompanied the sensitized guinea-pigs. This occurred on the fourth day of allergen boosting. These effects were drastically reduced or absent in the treated animals and the normal guinea pigs which did not receive the egg ovalbumin. Subcutaneous and abdominal swellings were also absent. No death was however recorded. Other features such as eye colour, fur and sensitivity to touch remain unchanged in all groups.

14. STUDIES ON MALE GUINEA PIGS

Both male and female results have been presented separatelyto illustrate the differences in gender sensitivity results are separated for trachea responses and skin sensitivity tests using both histamine and ovalbumin. Histamine, been a mediator of allergic reactions was used asthe contractile agonist together with ovalbumin (OVA).

15. OVALBUMIN SKIN TEST IN MALE GUINEA PIGS

The guinea-pig skin prick test is a basic test to check the extent of inflammatory response to antigens in sensitized animals. In the current study, skin prick test was used to assess the effect of *Kalanchoe integra* on mast cells, autonomic nerve endings and capillary blood vessels response to stimuli in ovalbumin-sensitized guinea pigs. The response manifested as oedema or wheal appearance on the skin of ovalbumin-sensitized guinea pigs. H_1 excitatory receptor is predominantly located in the skin, bronchioles and ileum of both man and animal models. It is one of the key receptors responsible for vascular permeability in both skin and bronchioles of ovalbumin-sensitized guinea pigs.

Ovalbumin-sensitized control group, as expected, recorded higher means of diameters of wheal (Ovalbumin, 2h=1.9: Histamine, 1h=2.2) than the non-sensitized negative control group (Ovalbumin, 2h=0.4: Histamine, 1h=1.7). Vasodilatation of cutaneous vessels is more prominent on allergen injection in sensitized animals than in non-sensitized animals.

The two *Kalanchoe integra*-treated groups recorded significantly lower diameters of wheal as compared to the two negative control groups (for histamine) and relatively higher diameters of wheal as compared to prednisolone-treated group. *Kalanchoe integra* possesses a concentration–dependent inhibition of skin hypersensitivity reactions in that, the low dose *K. integra* treated group recorded higher diameters of wheal (Ovalbumin, 2h=1.5: Histamine, 1h=1.7) as compared to the high dose *K. integra* treated group (Ovalbumin, 2h=1.3: Histamine, 1h=1.5).

For ovalbumin, the mean of wheal diameters of the untreated sensitized control group were significantly different from those of the prednisolone treated group (P<0.05 at 1h; P<0.001 at 2 h; P<0.001 at 24 h) and non-sensitized control (P<0.001 at 1 h; P<0.001 at 2 h; P<0.001 at 24 h) after 1, 2 and 24 hours. The mean of wheal diameters of the untreated sensitized control group were significantly different from those of low dose *K. integra*-treated group (P<0.05 at 2h; P<0.01 at 24h) and high dose *K. integra*- treated group (P<0.001 at 2h; P<0.001 at 24 h) after 2 and 24 hours but insignificant after an hour. Fig. 1 and Appendix 1.

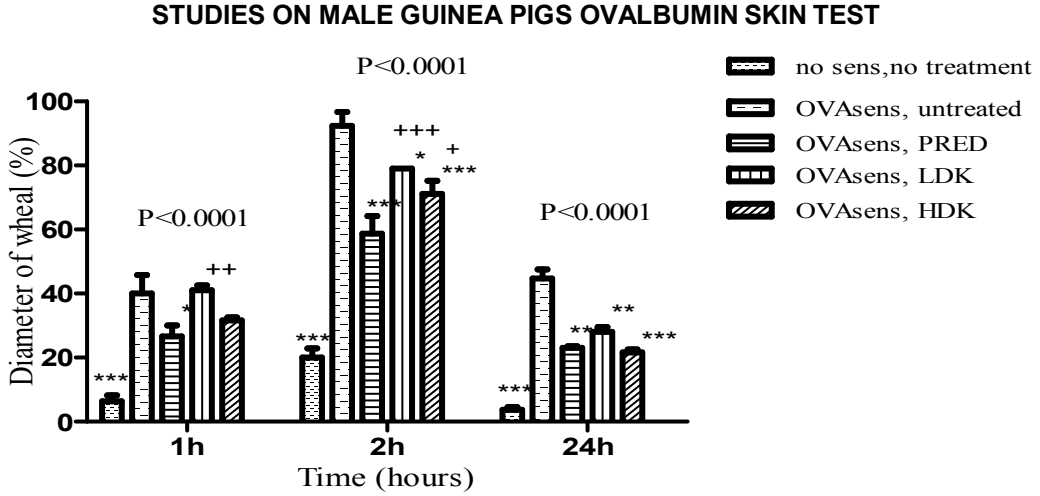

Fig. 1. Effect of KILE on ovalbumin-induced skin hypersensitivity reaction in male ovalbumin sensitized guinea pigs. Values are presented as mean ±SEM, n=6. One-way ANOVA and Bonferroni's posttest: P<0.05, means difference is significant. (Appendix 1)

*Group comparisons against Group II: ***P<0.001, **P<0.01, *P<0.05, ns (P>0.05). Group Comparisons against Group III: +++P<0.001, ++P<0.01, P<0.05, not significant (P>0.05)*

For histamine, the mean of wheal diameters of the untreated sensitized control group were significantly different from those of the prednisolone treated group after 30 minutes, 1 hour and insignificant after 24 hours (P<0.01 at 30mins; P<0.001 at 1h). There was no such difference between the untreated sensitized control group and non-sensitized control at all times. However, the mean of wheal diameters of the untreated sensitized control group were significantly different from those of low dose *K. integra*- treated group (P<0.05 at 30mins; ns at 1h) and high dose *K. integra*-treated group (P<0.05 at 30mins; P<0.001 at 1h) after 30 minutes and insignificant after 24 hours Fig. 2 and Appendix 2.

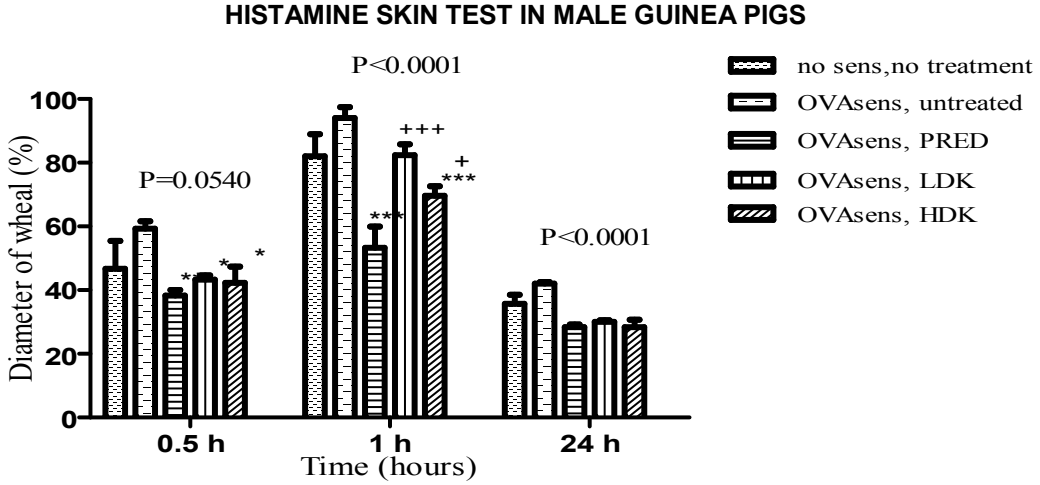

Fig. 2. Effect of KILE on histamine-induced skin hypersensitivity reaction in male ovalbumin sensitized guinea pigs. Values are presented as mean ±SEM, n=6. One-way ANOVA and Bonferroni's posttest (Appendix 2)

*P<0.05, mean difference is significant. Group comparisons against Group II: ***P<0.001, **P<0.01, *P<0.05, ns (P>0.05). Group comparisons against Group III: <0.001, ++P<0.01, P<0.05, not significant (P>0.05).*

These results may suggest that prednisolone is more effective at supressing inflammation compared to *K. integra*. Non-steroidal alternative, *K. integra*, showed dose dependent suppression.

16. TRACHEA STUDIES USING HISTAMINE IN MALE GUINEA PIGS

The guinea-pig trachea chain is known to contain a high population of histamine receptors (H_1). The anti-inflammatory effect of KILE was assessed by its ability to reduce the response of isolated trachea to contractile agonists. Histamine, a mediator of allergic reactions was used as the contractile agonist [13]. A right shift in EC_{50} of the histamine is indicative of inhibition. The log-dose response curve for untreated sensitized negative control was used as the reference. The higher the rightward shift in EC_{50} of histamine, the greater the anti-inflammatory effect of the antagonist on bronchial hyper-responsiveness. The figures below show the log-dose response curves.

The log-dose response curve for untreated sensitized guinea pigs was used as the reference. The higher the rightward shift in EC_{50} of histamine Fig. 3 and Appendix 3, the greater the anti-inflammatory effect of the antagonist on bronchial hyper-responsiveness. The prednisolone-treated groups recorded the highest EC_{50} of histamine in the log-dose response curves for histamine alone (3.08±0.02µg/ml), histamine in the presence of 100µg prednisolone (2.01±0.08µg/ml) and histamine in the presence of 200µg *K. integra* (0.92±0.12µg/ml). The high dose *Kalanchoe integra*-treated group recorded an EC_{50} of histamine (histamine alone (2.21±0.03µg/ml); histamine +100µg prednisolone (1.54±0.12µg/ml); histamine + 200µg *K. integra* (0.68±0.10µg/ml).The low dose *Kalanchoe integra*-treated group (histamine alone (1.32±0.02µg/ml); histamine +100µg prednisolone (0.87±0.14µg/ml); histamine + 200µg *K. integra* (0.66±0.09µg/ml) Figs. 4 and 5.

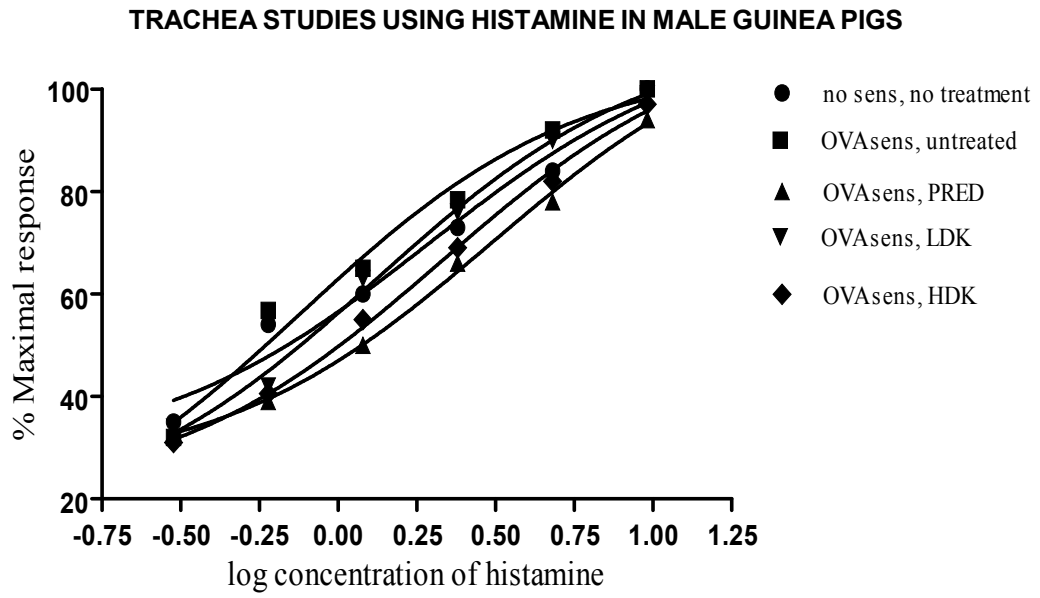

Fig. 3. Log dose response curve in the trachea male guinea-pigs using histamine in pretreated and untreated animals to verify the antagonistic property of rednisolone and KILE to the contractile effect of histamine, to the treated and untreated animals, the procedure was repeated and the EC concentrations identified.

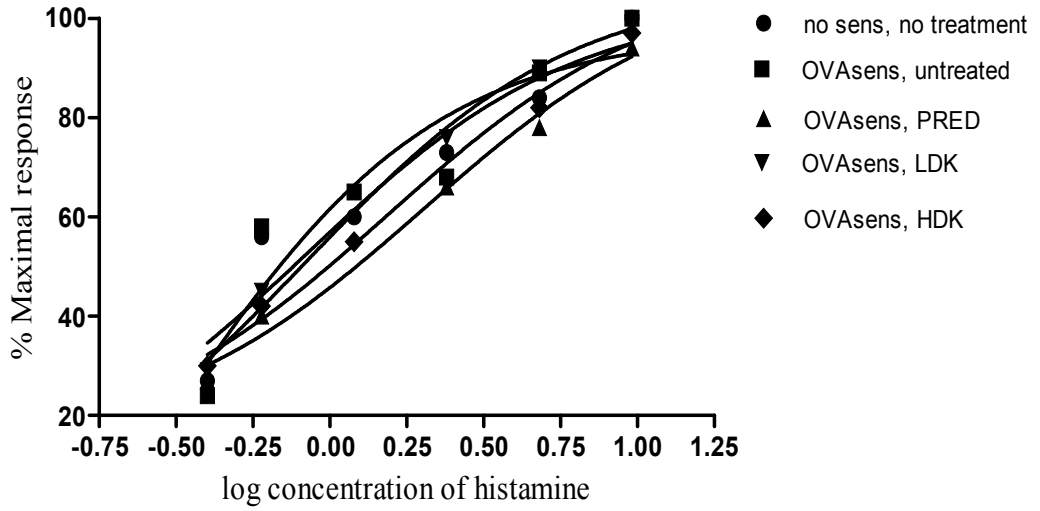

Fig. 4. Log dose response curve demonstrating the inhibitory effect of prednisolone administration to the contractile response to histamine in the trachea of pretreated and untreated male guinea-pigs

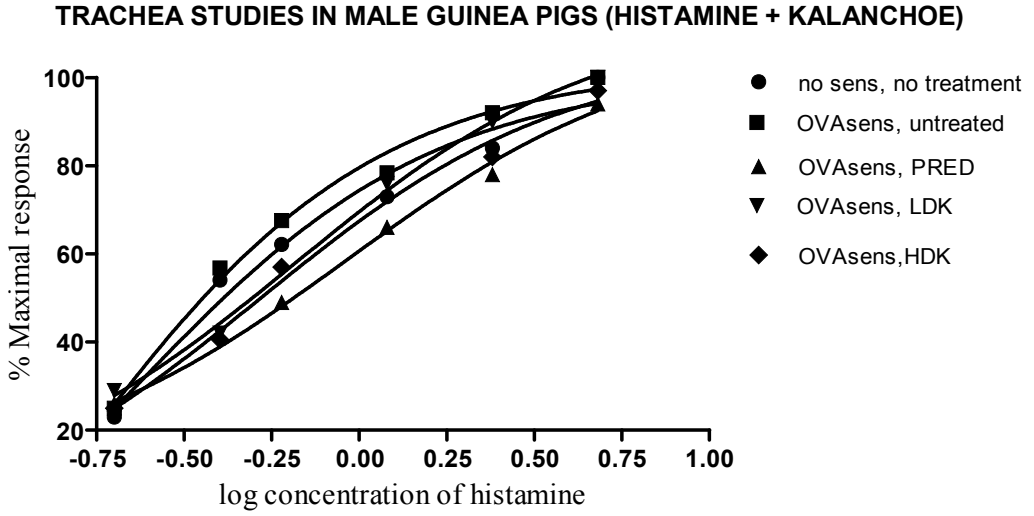

Fig. 5. Log dose response curve demonstrating the inhibitory effect of KILE administration to the contractile response to histamine in the trachea of pretreated and untreated male guinea-pigs

17. STUDIES ON FEMALE GUINEA PIGS

Just as in the male guinea pigs, *Kalanchoe integra* showed a concentration–dependent inhibition of skin hypersensitivity reactions in that, the low dose *K. integra* treated group recorded higher diameters of wheal (Ovalbumin, 2h=1.7: Histamine, 1h=2.1) as compared to the high dose *K. integra* treated group (Ovalbumin, 2h=1.5: Histamine, 1h=1.8) Figs. 6 and 7. The potency of histamine was higher in the sensitized females than the sensitized males, suggesting that histamine's effect was more prominent in females than in the males.

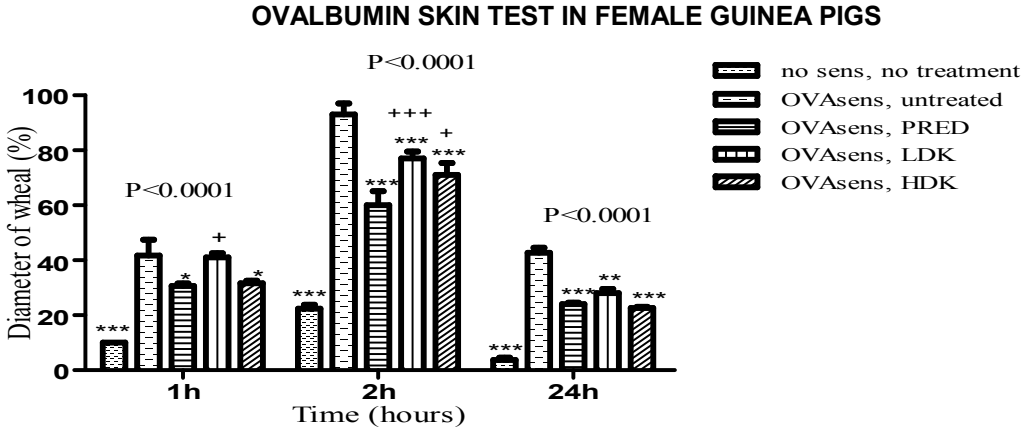

Fig. 6. Effect of KILE on ovalbumin-induced skin hypersensitivity reaction in female ovalbumin sensitized guinea pigs. Values are presented as mean ±SEM, n=6. One-way ANOVA and Bonferroni's posttest: P<0.05, mean difference is significant. Group comparisons against Group IIa: ***P<0.001, **P<0.01, *P<0.05, ns (P>0.05). Group comparisons against Group IIb: $^{+++}$P<0.001, ++P<0.01, P<0.05, not significant (P>0.05)

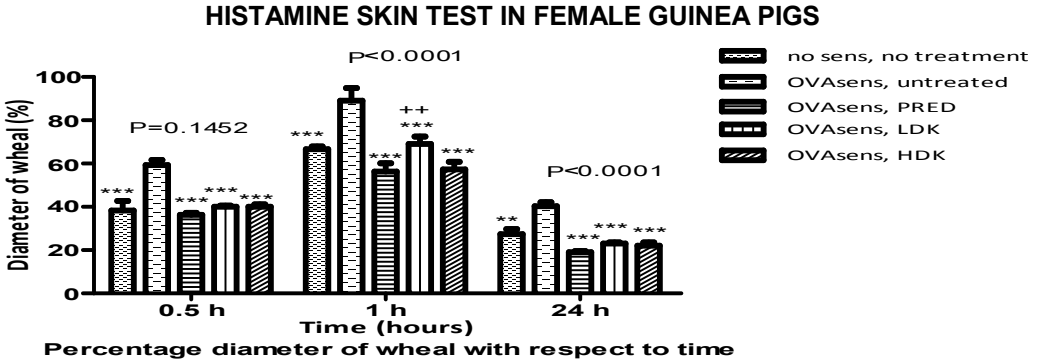

Fig. 7. Effect of KILE on histamine-induced skin hypersensitivity reaction in female ovalbumin Sensitized guinea pigs. Values are presented as mean ±SEM, n=6. One-way ANOVA and Bonferroni's posttest: P<0.05, mean difference is significant. Group comparisons against Group IIa: ***P<0.001, **P<0.01, *P<0.05, ns (P>0.05). Group comparisons against Group IIb: +++P<0.001, ++P<0.01, P<0.05, not significant (P>0.05)

For the females, the untreated negative control group recorded the highest means of wheal diameters (centimeters) for both ovalbumin (2h=2.1) and histamine (1h=2.5), with prednisolone-treated group recording the least means of wheal diameters of wheal for both ovalbumin (2h=1.3) and histamine (1h=1.5) among the four sensitized groups.

Ovalbumin-sensitized control group, as expected, recorded higher means of diameters of wheal (Ovalbumin, 2h=2.1: Histamine, 1h=2.5) than the non-sensitized negative control group (Ovalbumin, 2h=0.5: Histamine, 1h=1.8) Figs. 6 and 7.

The two *Kalanchoe integra*-treated groups recorded significantly lower diameters of wheal as compared to the two negative control groups (for histamine) and relatively higher diameters of wheal as compared to prednisolone-treated group. Just as in the male guinea pigs, *Kalanchoe integra* possesses a concentration–dependent inhibition of skin hypersensitivity reactions in that, the low dose *K. integra* treated group recorded higher diameters of wheal (Ovalbumin, 2h=1.7: Histamine, 1h=2.1) as compared to the high dose *K. integra* treated group (Ovalbumin, 2h=1.5: Histamine, 1h=1.8).

P values obtained at the respective times for the respective agonists were all less than 0.05 (Ovalbumin: 1 hour (P<0.0001), 2 hours (P<0.0001) and 24 hours (P<0.0001); Histamine: 30 minutes (P=0.1542), 1 hour (P<0.0001) and 24 hours (P<0.0001)), indicating a statistically significant difference between the means of wheal diameters of the treated and untreated groups Appendices 6, 7 and 8.

For ovalbumin, the mean of wheal diameters of the untreated sensitized control group were significantly different from those of the prednisolone treated group (P<0.05 at 1h; P<0.001 at 2h; P<0.001 at 24 h) and non-sensitized control (P<0.001 at 1h; P<0.001 at 2h; P<0.001 at 24h) after and 1, 2 and 24 hours. The mean of wheal diameters of the untreated sensitized control group were significantly different from those of low dose *K. integra*- treated group (ns after 1h; P<0.001 at 2h; P<0.01 at 24 h) and high dose *K. integra*- treated group (P<0.05 at 1h; P<0.001 at 2h; P<0.001 at 24h) after 1, 2 and 24 hours.

Among the treated groups, the mean of wheal diameters of the prednisolone treated group were significantly different from those of low dose *K. integra*- treated group (1hr; P<0.001 at 2h) and high dose *K. integra*- treated group (P<0.05 at 1h; P<0.05 at 2h) after 1 and 2 hours, but insignificant after 24 hours Figs.6 and 7.

For histamine, the mean of wheal diameters of the untreated sensitized control group were significantly different from those of the prednisolone treated group (P<0.001 at 30mins; P<0.001 at 1h; P<0.001 at 24h), non-sensitized control group (P<0.001 at 30mins; P<0.001 at 1h; P<0.01 at 24h), low dose *K. integra*- treated group (P<0.001 at 30mins; P<0.001 at 1h; P<0.001 at 24h) and high dose *K. integra*- treated group (P<0.001 at 30mins; P<0.001 at 1h; P<0.001 at 24h) after 30 minutes, 1 and 24 hours Appendices 7 and 8.

Among the treated groups, the mean of wheal diameters of the prednisolone treated group were significantly different from those of high dose *K. integra*- treated group (P<0.05 at 1h) after 1 h only and insignificantly different from those of low dose *K. integra*- treated group after 1, 2 and 24 hours.

In the females, high dose *Kalanchoe integra*-treated group recorded the second highest EC_{50} of histamine (histamine alone (1.89±0.14µg/ml); histamine +100µg prednisolone (1.49± 0.12µg/ml); histamine + 200µg *K. integra* (0.90±0.04µg/ml)), followed by the low dose*Kalanchoe integra*-treated group (histamine alone (0.88±0.15µg/ml); histamine +100µg prednisolone (0.31±0.08µg/ml); histamine + 200µg *K. integra* (0.65±0.06µg/ml) Figs. 8, 9 and 10. The potency of histamine was reduced in the presence of prednisolone and *K. integra* for both males and females Appendices 4, 5 and 6.

TRACHEA STUDIES IN FEMALE GUINEA PIGS (HISTAMINE)

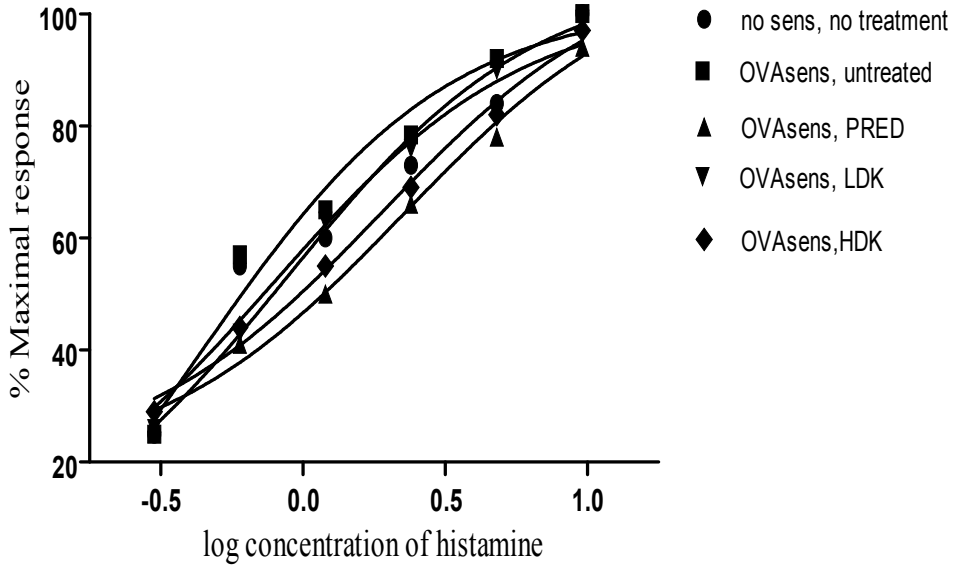

Fig. 8. Log dose response curve in the trachea female guinea-pigs using histamine

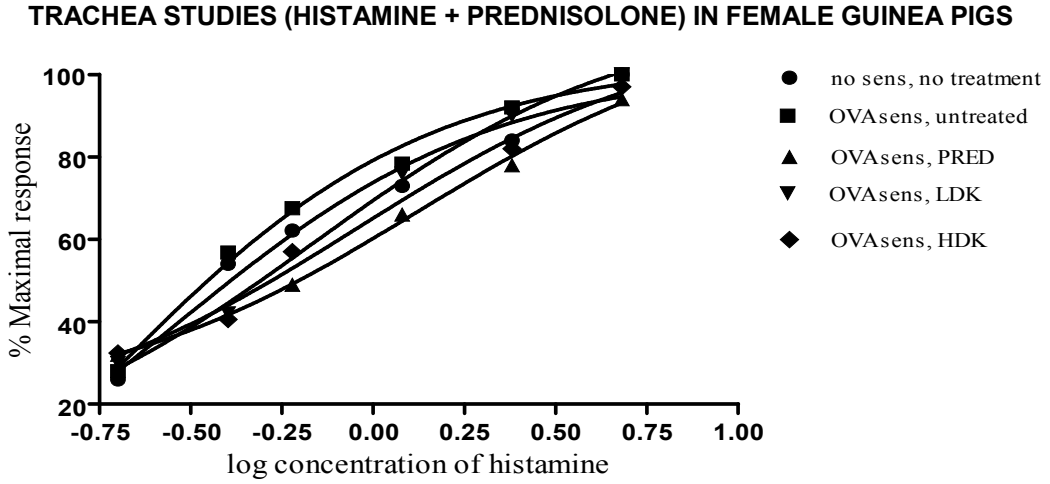

Fig. 9. Log dose response curve in the trachea in female guinea-pigs using histamine and prednisolone

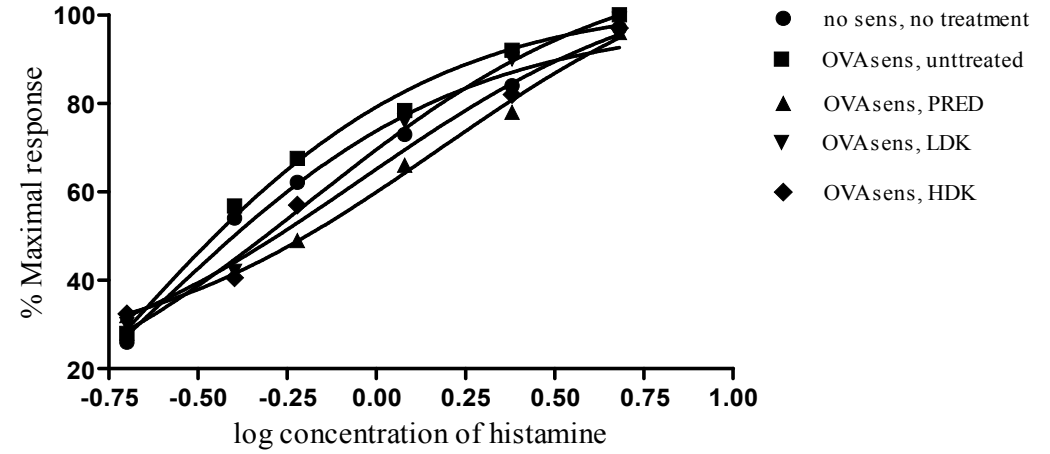

Fig. 10. log dose response curve in the trachea female guinea-pigs using histamine and KILE.

The above effects show that the potency of histamine was higher in the sensitized females than the sensitized males, suggesting that histamine's effect was more prominent in females than in the males. This backs the claim that females are more susceptible to bronchial hyper-Responsiveness than the male guinea-pigs Appendices 9 and 10.

18. DISCUSSION

In this study, the effect of KILE on vascular permeability was evaluated on the skin, histamine receptors in the trachea and bronchial microvasculature of ovalbumin-sensitized guinea pigs, whereas the anti-inflammatory effect was assessed by *In vitro* trachea studies.

The guinea-pig skin prick test is a basic test to check the extent of inflammatory response to antigens in sensitized animals. In the current study, skin prick test was used to assess the effect of KILE on mast cells, autonomic nerve endings and capillary blood vessels response to stimuli in ovalbumin-sensitized guinea pigs. The response manifested as oedema or wheal appearance on the skin of the test animals. The ability of KILE to reduce oedema formation in the skin of ovalbumin-sensitized guinea pigs after intradermal injection of ovalbumin, could serve as their potential anti-inflammatory activity [5]. There is a close relationship of skin test positivity with reported symptoms of nasal allergy in a general population. Specific IgE positivity also shows a close relationship with nasal symptoms in response to allergen exposure in a general population.Actually skin testing and specific IgE measurement may be considered complementary to one another in diagnosing allergic rhinitis while total IgE may be considered an indicator of greater dysregulation of the immune system in atopic allergy. Eosinophil count is alsoassociated with nasal symptoms, regardless of type and extent of nasal symptoms [14]. Additionally, the test serves as an indicator for T-cell response in ovalbumin sensitized animal models. Inhalation of aerosolized OA has been reported to induce inflammatory cell proliferation.

H_1 excitatory receptor is predominantly located in the skin, bronchioles and ileum of both man and animal models. It is one of the key receptors responsible for vascular permeability in both skin and bronchioles of ovalbumin-sensitized guinea pigs [15]. In this study, the statistical results obtained for diameters of wheal suggest that, KILE possess significant inhibitory effect in anaphylactic reactions.

The diameters of wheal recorded were higher for histamine as compared to ovalbumin across all five (5) groups, for both males and females. Whereas histamine has a direct effect through activation of H_1 receptors upon injection into the skin, ovalbumin has an indirect effect of histamine release. Ovalbumin as an allergen forms cross links with anti-ovalbumin antibodies present on the mast cells of the ovalbumin sensitized guinea pigs. The cross linkage leads to degranulation of mast cells and subsequent release of histamine, as such the flare appearance time is expected to be longer for ovalbumin as compared to histamine. Vasodilatation of cutaneous vessels is more prominent on allergen injection in sensitized animals than in non-sensitized animals [16].

Among the treated groups, the mean of wheal diameters of the prednisolone treated group were significantly different from those of high dose *K. integra*- treated group ($P<0.05$ at 1hr) after 1 hour only and insignificantly different from those of low dose *K. integra*- treated group after 1, 2 and 24 hours. The potency of histamine was reduced in the presence of prednisolone and *K. integra* for both males and females. Prednisolone inhibitory effect could be due to its inhibition of arachidonic acid synthesis, reduced influx of inflammatory mediators and mast cell stabilizing effect while a flavanoid known as quercetin is likely to be responsible for *K. integra's* inhibitory effect. Mast cell stabilizing effect and anti-histamine activity of *K. integra's* could account for its anti-inflammatory effect [8]. The fact that the potency of histamine was higher in the sensitized females than the sensitized males, suggest that histamine's effect was more prominent in females than in the males. This backs the claim that females are more susceptible to bronchial hyper-responsiveness than the males [17].

The triple response of Lewis, which is due to histamine release is a cutaneous response that occurs from firm stroking of the skin, producing an initial red line, followed by a flare around that line and then finally a wheal [18,19]. Histamine is a dibasic vasoactive amine located in most body tissues but is highly concentrated in the lungs, skin and gastrointestinal tract.

Mast cells and basophils are the effector cells involved in the immediate hypersensitivity response. Intradermal Injection of histamine elicited the triple response consisting of red spot which is due to capillary dilatation, flare which is redness in the surrounding area due to arteriolar dilatation mediated by axon reflex and wheal which is due to exudation of fluid from capillaries and venules [20,21].

The ability of KILE to attenuate the triple response of Lewis especially the significant reduction in the diameters of wheal in the two *Kalanchoe integra*-treated groups as compared to the two negative control groups (for histamine) could be based on its mast cell stabilizing effect and its ability to inhibit histamine. The reduction in wheal confirms other studies whereby slightly boiled fresh leaf extract of *Kalanchoe spanthulata* clinically reduces inflammation [22,23]. Previous studies have implicated quercitrin present in *K. pinnata* as the compound responsible for anti-inflammatory agent; therefore it is possible that this same flavonoid in the *K. integra* together with phenolic and tannin compounds [24] may also be responsible for its activity**.**

A limitation of this study include the absence of the exact biochemical and immunological features of the mechanism of action of *K. integra* in alleviating allergic asthma whichcould not be determined in this study. The genetic variations among guinea pigs in general were not factoredin this research workas certain genes predispose individuals to allergic asthma, and may affect experimental results.

Although KILE has exhibited some potential anti-inflammatory activities in OA-sensitized guinea pigs at doses used for this study, more investigations both *In vitro* and *In vivo* could be conducted to validate its folklore use in the management of bronchial asthma and skin hypersensitivity with further identification of its mechanism of anti-inflammatory activity in guinea-pig asthmatic models.

19. CONCLUSION

The study has demonstrated the anti-inflammatory potential of the aqueous extract of *Kalanchoe integra* leaves. The study also revealed that female animal models were more prone to bronchial hyper-responsiveness than the male guinea pigs. The untreated negative control female group recorded the highest means of wheal diameters (centimeters) for both ovalbumin (2hrs=2.1) and histamine. The inhibitory effect of the aqueous leaf extract of *K. integra* on the wheal formation was concentration dependent.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

ACKNOWLEDGMENT

The authors acknowledge the “manuscript writing” financial support of ‘Building Stronger Universities Initiative Platform on Human Health’ (BSU-PHH)-Department of Pharmacology and Toxicology, University of Ghana School of Pharmacy, College of Health Sciences.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIXES

APPENDIX 1

Bonferroni's posttest for Ovalbumin Skin test in males

Posttests	1 hour	2 hours	24 hours
IIa versus I	***P<0.001	***P<0.001	***P<0.001
IIa versus IIb	*P<0.05	***P<0.001	***P<0.001
IIa versus IIc	ns	*P<0.05	**P<0.01
IIa versus IId	ns	***P<0.001	***P<0.001
IIb versus IIc	++P<0.01	+++P<0.001	Ns
IIb versus IId	ns	+P<0.05	Ns
IIc versus IId	ns	ns	Ns

APPENDIX 2

Bonferroni's posttest for Histamine Skin test in males

Posttests	1 hour	2 hours	24 hours
IIa versus I	ns	ns	Ns
IIa versus IIb	**P<0.01	***P<0.001	Ns
IIa versus IIc	*P<0.05	ns	Ns
IIa versus IId	*P<0.05	***P<0.001	Ns
IIb versus IIc	ns	+++P<0.001	Ns
IIb versus IId	ns	+P<0.05	Ns
IIc versus IId	ns	ns	Ns

APPENDIX 3

EC_{50}'s of histamine on each isolated trachea of males

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	1.42±0.04	(1.42±0.04) $\times 10^{-6}$
IIa	0.76±0.03	(0.76±0.05) $\times 10^{-6}$
IIb	3.08±0.02	(3.08±0.12) $\times 10^{-6}$
IIc	1.32±0.02	(1.32±0.04) $\times 10^{-6}$
IId	2.21±0.03	(2.21±0.06) $\times 10^{-6}$

APPENDIX 4

EC_{50}'s of histamine in presence of Prednisolone (100 µg) on isolated trachea of females

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	0.29±0.01	(0.29±0.01) $\times 10^{-6}$
IIa	0.19±0.05	(0.19±0.05) $\times 10^{-6}$
IIb	2.01±0.02	(2.01±0.02) $\times 10^{-6}$
IIc	0.31±0.08	(0.31±0.08) $\times 10^{-6}$
IId	1.49±0.12	(1.49±0.12) $\times 10^{-6}$

APPENDIX 5

EC_{50}'s of histamine in presence of Prednisolone (100 µg) on males isolated trachea

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	0.82±0.01	(0.82±0.01) $\times 10^{-6}$
IIa	0.27±0.07	(0.27±0.07) $\times 10^{-6}$
IIb	2.01±0.08	(2.01±0.08) $\times 10^{-6}$
IIc	0.87±0.14	(0.87±0.14) $\times 10^{-6}$
IId	1.54±0.12	(1.54±0.01) $\times 10^{-6}$

APPENDIX 6

EC_{50}'s of histamine in presence of *Kalanchoe*(200 µg) on isolated trachea of males

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	0.22±0.17	(0.22±0.17) $\times 10^{-6}$
IIa	0.14±0.15	(0.14±0.15) $\times 10^{-6}$
IIb	0.92±0.12	(0.92±0.12) $\times 10^{-6}$
IIc	0.66±0.09	(0.66±0.09) $\times 10^{-6}$
IId	0.68±0.10	(0.68±0.10) $\times 10^{-6}$

APPENDIX 7

Bonferroni's posttest for Ovalbumin Skin test in females

Posttests	1 hour	2 hours	24 hours
IIa versus I	***P<0.001	***P<0.001	***P<0.001
IIa versus IIb	*P<0.05	***P<0.001	***P<0.001
IIa versus IIc	ns	***P<0.001	**P<0.01
IIa versus IId	*P<0.05	***P<0.001	***P<0.001
IIb versus IIc	ns	+++P<0.001	ns
IIb versus IId	+P<0.05	+P<0.05	ns
IIc versus IId	ns	+P<0.05	ns

APPENDIX 8

Bonferroni's posttest for Histamine Skin test in females

Posttests	1 hour	2 hours	24 hours
IIa versus I	***P<0.001	***P<0.001	**P<0.01
IIa versus IIb	***P<0.001	***P<0.001	***P<0.001
IIa versus IIc	***P<0.001	***P<0.001	***P<0.001
IIa versus IId	***P<0.001	***P<0.001	***P<0.001
IIb versus IIc	ns	ns	ns
IIb versus IId	ns	++P<0.01	ns
IIc versus IId	ns	ns	ns

APPENDIX 9

EC_{50}'s of histamine on each isolated trachea of females

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	0.79±0.10	(0.79±0.10) $\times 10^{-6}$
IIa	0.43±0.03	(0.43±0.03) $\times 10^{-6}$
IIb	2.24±0.12	(2.28±0.12) $\times 10^{-6}$
IIc	0.88±0.15	(0.88±0.15) $\times 10^{-6}$
IId	1.89±0.14	(1.89±0.14) $\times 10^{-6}$

APPENDIX 10

EC_{50}'s of histamine in presence of *Kalanchoe* (200 µg) on isolated trachea of females

Group	EC_{50} (µg/ml)	EC_{50} (g/ml)
I	0.23±0.07	(0.23±0.07) $\times 10^{-6}$
IIa	0.18±0.10	(0.18±0.10) $\times 10^{-6}$
IIb	1.47±0.21	(1.47±0.21) $\times 10^{-6}$
IIc	0.65±0.06	(0.65±0.06) $\times 10^{-6}$
IId	0.90±0.04	(0.90±0.04) $\times 10^{-6}$

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=412&id=13&aid=3442