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BRONCHODILATION EFFECTS OF KALACHUCHI LEAVES (*PLUMERIA ACUMINATA, FAMILY:* APOCYNACEAE) OF ETHANOL EXTRACTS ON CAT-INDUCED BRONCHOCONSTRICTION

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INTRODUCTION

Bronchodilation is an expansion of the air passages through the bronchi of the lungs. This is accomplished in medicine by the use of bronchodilator which is a substance that dilates the bronchi and bronchioles, decreasing resistance in the respiratory airway and increasing airflow to the lungs. Bronchodilators may be endogenous (originating naturally within the body), or they may be medications administered for the treatment of breathing difficulties. They are most useful in obstructive lung diseases, of which asthma and chronic obstructive pulmonary disease are the most common conditions.

Bronchodilators are either short-acting or long-acting. Short-acting medications provide quick or "rescue" relief from acute bronchoconstriction. Long-acting bronchodilators help to control and prevent symptoms. The three types of prescription bronchodilating drugs are β2("beta two")-adrenergic agonists (short- and long-acting), anticholinergics (short- acting), and theophylline (long-acting). (*Sathe NA, 2015*) This study will utilize short-acting and long-acting beta2adrenergic agonists which are quick-relief or "rescue" medications that provide quick, temporary relief from asthma symptoms or flare-ups.

Asthma is a common long-term inflammatory disease of the airways of the lungs. It is characterized by variable and recurring symptoms, reversible airflow obstruction. Symptoms include episodes of wheezing, coughing, chest tightness, and shortness of breath. These episodes may occur a few times a day or a few times per week. Depending on the person, they may become worse at night or with exercise.

Asthma has also been described as a chronic disease that inflames the airways (i.e. the small tubes, called bronchi) which carry air in and out of the lungs. In asthmatic patient, the bronchi will be inflamed and more sensitive than normal and produce extra mucus. This can make breathing difficult due to reversible airflow obstruction, or bronchospasm, and trigger coughing, wheezing and shortness of breath. The cause of asthma is not fully understood, but it's probably due to a combination of the following factors: Environmental: allergens (e.g., house dust mites, animal fur and pollen), occupational irritants, tobacco smoke, respiratory (viral) infections, strong emotional expressions and drugs (e.g. aspirin and beta blockers). Genetic (inherited): usually occurs in children.

It is a complex inflammatory disease that causes airway narrowing and associated with changes in the levels of eosinophil, mast cells, lymphocytes, cytokines and other inflammatory cell products. It is well known that patients with asthma have high levels of specific IgE that binds to receptors of mast cells and other inflammatory cells. Interaction between IgE antibody and antigen results in the activation of a series of inflammatory cellular reactions, including the release of mediators such as histamines, prostaglandins and leukotrienen which subsequently lead to contraction of airway smooth muscle and bronchoconstriction. (Dnyaneshwar JTaur and Ravindra Y patil, 2014) In work published by Joshua M. Galanter, MD and Homer A. Bousley, MD in the 14th Edition of Basic and Clinical Pharmacology, asthma is termed a classic allergic asthma but the conception of asthma as an allergic disease is only applied to a subgroup of patients with asthma, those with evidence of allergy. Allergic asthma accounts for a great proportion of asthma that develops in childhood, but a smaller proportion of an adult-onset asthma. This implied by the use of modifying terms to describe asthma in different patients, such as allergic (extrinsic), non-allergic (intrinsic), aspirinsensitive, adult onset, and obesity-related.

According to *(Wu chung et al, June, 2013)* the chances of developing asthma are increased if the patients' family members or relatives have asthma and other allergic conditions such as atopic dermatitis and hay fever. Asthma triggers are varied and numerous, and among

them are lifestyle choices and habits. People with asthma should give up smoking and avoid people who smoke, obesity, lack of physical activity, poor diet, and stress are also indirect asthma causes. Asthma patient or healthy individual can reduce his/her asthma causes by adopting a healthier lifestyle.

The current concept of asthma therapy is based on a stepwise approach, depending on disease severity, and the aim is to reduce the symptoms that result from airway obstruction and inflammation, to prevent exacerbations and to maintain normal lung function. Beta2- adrenoceptor agonists and glucocorticoids are at present the most effective drugs for the treatment of airway obstruction and inflammation, with theophylline, leukotriene receptor antagonists and anticholinergic as second- or third-line therapy. There are, to date, no additional or newly developed drugs available that add substantially to the current strategies or even replace beta2-adrenoceptor agonists or glucocorticoids. New approaches in asthma therapy recommend drug combinations of inhaled steroids, primarily with long-acting beta2- adrenoceptor agonists, based on their improved efficacy and the potential for a steroid- sparing effect *(Rabe KF, and Schmidt DT, 2014).*

Adverse effects of current treatments used in asthma are enormous, isoproterenol for instance causes tachycardia, Salbutamol causes muscle tremors (dose related), palpitation, restlessness, nervousness, throat irritation and ankle edema, theophylline causes convulsions, shock, arrhythmias, increased muscle tone, tachypnea, (dose dependent) flushing, hypotension, restlessness, tremors, vomiting, palpitation, diuresis, dyspepsia, insomnia e.t.c, Anticholinergic causes dry mouth, difficulty in swallowing and talking, scarlet rash, photophobia, blurring of near (Atropine and its congeners) vision, palpitation, ataxia, delirium, hallucinations, hypotension, weak and rapid pulse, cardiovascular collapse with respiratory depression, convulsions and coma (in severe poisoning), ketotifen causes sedation, dizziness, dry mouth, nausea and weight gain, and corticosteroids cause Cushing's habitus, fragile skin, purple striate, hyperglycemia, muscular weakness, susceptibility to infection, delayed healing of wounds and surgical incisions, peptic ulceration, osteoporosis, glaucoma, growth retardation, psychiatric disturbances, suppression of hypothalamic- pituitary-adrenal (HPA) axis etc. (*Dnyaneshwar JTaur and Ravindra Y patil*, 2016). As a consequence, the search for effective low-risk, non-drug strategies that provide a valuable adjunctive or alternative treatment in asthma management is clinically attractive and relevant. There is much interest in complementary and alternative medicine, and its use in the management and treatment of asthma is growing at a significant rate.

Plumeria acuminata is used as medicinal plant native to Mexico, Central America, the Caribbean and South America spread throughout the tropics. About 155 genera and 2000 species are distributed primarily in the tropical and subtropical region. About eightspecies are known in India and of which *Plumeria acuminata* and *P. rubra* are commonly grown. They are commonly known as "Temple tree" or "Champa" in India. Depending on location many other common names also exists like "Kembangkamboja" in Indonesia."Kalachuchi" in the Phillipines, Champa in laos and Dead man's fingers in Australia. The plant material is widely used as purgative, remedy for pain, fever, diarrhea, cure for itch, bleeding, piles, dysentery, blood disorder, and tumor. The milky juice is employed for the treatment of inflammation. The excessive doses of the latex derived from *Plumeria acuminata* are poisonous and the root is a violent cathartic. The essential oil from the flowers possesses antifungal activity. In Unani practice, the medicinal herb is used to treat tumors and rheumatic pains, *(Ashraf MdFarooqu et al, 2012).*

The pharmaceutical industry continuously search new lead molecules having better therapeutic action and less side effect, in recent years lead molecules from natural origin had gain more popularity due to less side effect and better therapeutic action. That means Kalachuchi leaves have wide scope to isolate various phytochemical constituent and evaluate their pharmacologic activities to get better therapeutic value. This study aims to investigate the anti-asthmatic effects of kalachuchi, *Plumeria acuminata* using it's phytochemicals ethanol extracts on mice induced bronchial obstruction. Due to high level of drug interactions in the above mentioned asthma remedies, the search for natural remedies that would have less drug interactions and relieve asthma faster continues in this advent of health care advancement.

Background of the study

Asthma is one of the major non communicable diseases. It is a chronic disease of the air passages of the lungs which inflames and narrows them. Some 235 million people currently suffer from asthma. It is a common disease among children. Most asthma-related deaths occur in low- and lower-middle income countries. According to the latest WHO estimates, released in December 2016, there were 383 000 deaths due to asthma in 2015. The strongest risk factors for developing asthma are inhaled substances and particles that may provoke allergic reactions or irritate the airways. Medication can control asthma. Avoiding asthma triggers can also reduce the severity of asthma. Appropriate management of asthma can enable people to enjoy a good quality of life. WHO *(World health organization, December 2016).*

The Babaylans were the first healers within the tribal communities of ancient Philippines. Later emerged folk doctors and the training and deployment of true medical practitioners as can be seen in the progression of Philippine history. At present, medical personnel trained based on Western medicine - such as Filipino nurses,

physicians, physical therapists, pharmacists, surgeons among others - coexists with the still thriving group of traditional healers that do not have formal education in scientific medicine who often cater to people living in impoverished areas of the Philippines. *(Dr. J. P,Bantug, 2016).*

According to center for disease control CDC (*center for disease control, 2015*) Traditional Chinese medicine (TCM) has a long history of human use in China and other Asian countries, such as Philippines, Korea and Japan, for treating and preventing disease, and is part of main stream medicine in these countries. Main components of TCM include herbal therapy, acupuncture, acupressure/massage, mind-body therapy and dietary therapy. Their record shows that since 2005, several controlled clinical studies of "anti- asthma" herbal remedies including anti-asthma herbal medicine intervention (ASHMI), DCT,(17) and AST-1 have been published. Of these, ASHMI is the only anti- TCM herbal product that received US FDA IND approval and entered clinical trial in the US. Research into ASHMI's active compounds is actively being pursued.

Our study will utilize *Plumeria acuminata* belonging to the family Apocynaceae is commonly known as 'Kalachuchi' in the Philippines. In traditional medicinal system different parts of the plant have been mentioned to be useful in a variety of diseases. The plant material is widely used as a purgative, remedy for diarrheal and cure for itch. The milky juice is employed for the treatment of inflammation and rheumatism. The bark has been applied as a plaster over inflammation and hard tumors. The leaves are reported to have anti- inflammatory, rubefacient in rheumatism and have strong purgative effect. Its branches are used like those of 'chitraka' to produce abortion. However there is no scientific report or verification of the use of this plant in the treatment of asthma condition. Accordingly a pharmacological investigation on the Ethanol extract of leaves of *Plumeria acuminate* (EEPA) will be initiated in our laboratory and its anti-asthmatic effects on bronchial obstruction induced mice will be reported. *(Stuart,2012)*

Statements of the Problem

This study aims to determine if Kalachuchi, *Plumeria acuminata* leaves possess Bronchodilation Effects. Furthermore it aims to answer specific questions:

1. Will the Kalachuchi, *Plumeria acuminata* extracts exhibit bronchodilation?
2. Is there a significant difference in the bronchodilation effect of Kalachuchi, ethanolic extract and Salbutamol as the positive control?
3. What are the various phytochemicals present in ethanolic extract of Kalachuchi, *Plumeria acuminata*?
4. What are the effects of different concentration of the ethanolic extract ranging from 100%, 75%, and 50% on cat methacholine induced bronchoconstriction?
5. What is the percentage yield of active constituents present from Kalachuchi, *Plumeria acuminata* leaves?
6. Will the Kalachuchi, *Plumeria acuminata* ethanolic extract exhibit toxic effect on cat?

METHODOLOGY

Collection of Plant Samples

The plant leaves will be collected from different locations in Bacoor city like schools, parks and grass fields that have Kalachuchi, Plumeria acuminata leaves. The leaves will be collected freshly from the tree, and will be sun-dried for one week before it will be manually sliced for use.

Plant identification

Parts of Kalachuchi Plumeria acuminata tree will be taken to the National Museum of the Philippines for the authentication and verification of the identity. A registered botanist will do the authentication of the plant.

Preparation and extraction process

An estimated nine hundred grams (900g) of Kalachuchi Plumeria acuminata leaves will be collected freshly, dried, and sliced. Then the preparation will be stored in 900 mL 95% ethanol. After four days of storage, the sample will be filtered to obtain the extract and will taken to the Department of Pharmacy at St. Dominic College of Asia for rotary evaporation in order to detoxify and obtain the crude extract of the plant. This Maceration method was used by (*Vikrant Arya et al, 2015*).

$$\text{Percentage yield} = \frac{\textbf{Actualvolume}}{\textbf{Theoreical volume}} \times 100\%$$

Physical characterization

The crude extract will be physically examined in terms of its odor, color, taste, and viscosity.

Qualitative Phytochemical Screening/identifications Test for Carbohydrates and Reducing Sugar

Extracts of Kalachuchi Plumeria acuminata will be dissolved individually in 5 mL distilled water and filtered. The filtrates will be used to test for the presence of carbohydrates.

Molisch's Test: Filtrates will be treated with 2 drops of alcoholic a-naphthol solution in a test tube. Formation of the violet ring at the junction will indicate the presence of carbohydrates.

Benedict's test: Filtrates will be treated with Benedict's reagent and heated gently .Orange red precipitate will indicate the presence of reducing sugar.

Fehling's Test: Filtrates will be hydrolyzed with diluted HCl, it will be neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate will indicate the presence of reducing sugars.

Test for Proteins and Amino Acids
Biuret Test: An equal amount of sodium hydride will be added to a solution of food, and will be carefully mixed with the extract. A few drops of 1% CuSO4 will be added and placed to standby. Mixture will not shaken. Formation of violet color will indicate the presence of amino acid.

Ninhydrin Test: To the extract, 0.25% weight per volume ninhydrin reagent will be added and boiled for few minutes. Formation of blue color will indicate the presence of amino acid.

Test for Alkaloids
Extracts of Kalachuchi Plumeria acuminata will be dissolved individually in a diluted Hydrochloric acid and filtered.

Mayer's Test: Filtrates will be treated with Mayer's reagent potassium Mercuric Iodide). Formation of a yellow colored precipitate will indicate the presence of alkaloids.

Wagner's Test: Filtrates will be treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/red precipitate will indicate the presence of alkaloids.

Dragendroff's Test: Filtrates will be treated with Dragendroff's reagent (solution of potassium Bismuth iodide). Formation of red precipitate will indicate the presence of alkaloids.

Hager's Test: Filtrates will be treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids will be confirmed bythe formation of yellow colored precipitate.

Test for Glycosides
Extracts of Kalachuchi (Plumeriaacuminata) is hydrolyzed with diluted HCl, and will be subjected to test for glycosides.

Modified Borntrager's Test: Extracts will be treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture will be cooled and extracted with equal volumes of benzene. The benzene layer will be separated and treated with ammonia solution. Formation of rose-pink color in the ammonia layer will indicate the presence of anthranol glycosides.

Legal's Test: Extract will be treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red color will indicate the presence of cardiac glycosides.

Test for Steroids and Phytosterols
Libermann Burchard's Test: Extract will be treated with chloroform and filtered. The filtrates is going to be treated with few drops of acetic anhydride, boil and cool. Concentrate sulfuric acid will be added. Formation of brown ring at the junction will indicates the presence of phytosterols.

Test for Terpenes and Terpenoids
Salkowski test for Triterpenes: 2 Ml of chloroform will be mixed with the extract, and conc. Sulphuric acid (3mL) will be carefully added, a layer will be formed and the lower portion turned yellow indicated the presence of triterpenes.

Salkowski for Terpenoids: To 0.5g of the extract, 2 mL of chloroform will be added and concentrated H2SO4 (3mL) will be carefully added and a layer will be formed. A reddish brown coloration of the interface will indicate the presence of terpenoids.

Test for Quinones
Sulfuric Acid Test: The test solution will be treated with a few drops of concentrated sulfuric acid or aqueous sodium hydroxide solution. Color formation will indicate the presence of quinoid compound.

Test for Anthraquinone
Hydrochloric Acid Test: A pinch of zinc dust will be added to the extract and will be followed by addition of concentrated hydrochloric acid along the sides of the test tubes carefully.

Free Anthraquinones Test: To 0.1g of the extract, 5 mL of 5% solution of ferric chloride and 5 mL diluted hydrochloric acid will be added; the solution will be heated on boiling water-bath for 5 minutes. After 5 minutes, it is going to be left to cool down, an organic solvent like benzene will be added and the solution will be shaken gently. The organic solvent layer will be separated and equal volume of diluted ammonia will be added. A pinkish red color will be formed in ammoniacal layer.

Test for Saponins
Froth Test: Extract will be diluted with distilled water to 20 mL and will be shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam will indicate the presence of saponins.

Test for Flavonoids
Alkaline Reagent Test: Extract will be treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of diluted acid indicated the presence of flavonoids.

Shinoda Test: To the test solution, few drops of concentrated hydrochloric acid (HCl) will be added. Then the magnesium turning will be put into the solution and appearance of pink red color indicated the presence of flavones.

Test for Phenols

Ferric Chloride Test: Extract will be treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

Test for Tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride will be added. Formation of white precipitate will indicate the presence of tannins.

Animals grouping

An estimated of ten (10) male and ten (10) cats weighing 70-85g will be used for the experiment. The animals will be kept under standard temperature (26- 300C) with access to food and water. The animals will be grouped into three (3) in a group. Each group contains five (5) cats; positive control (Salbutamol 2mg/kg), negative control (normal saline), and the ethanolic extract was divided into three groups: standard 1 (100% extract), standard 2 (75% extract), and standard 3 (50% extract). Group one is for first trial, group two is for second trial, and the third group is for final trial. The estimated last 5 remaining cats will serve for toxicity testing. The cats will be adapted 14 days to the laboratory environment and will be allowed to undergo fasting for 12 hours prior to the experiment, the extracts and controls will be administered through oral gavages. All experimental procedures in animals will be administered orally.

Toxicity Test

Four (4) cats will be utilized, marked for the toxicity test, and will be in a close observation for seven days. Food will be deprived from the cat that is chosen for 12 hours prior to the administration of the Kalachuchi, Plumeria acuminata leaves extract but water will still be accessible. All cages will be in a room temperature with constant humidity.

The extract will be diluted in distilled water, and each of the cat will be weighed before the experiment. The acute oral toxicity of Kalachuchi, Plumeria acuminata leaves ethanolic extract will be evaluated on cat. Food will be given to the cat after an hour of administration of the crude extract.

Reversible Bronchoconstriction Inducement Protocol

Dye and associates at the University of Illinois School of Veterinary Medicine were the first to identify pulmonary function abnormalities in cats with signs of chronic lower airway inflammation. A reversible bronchial constriction will be induced on each groups of the cat by exposing the cats on a low level of direct-acting cholinoceptor stimulant (methacholine). This is important as the first demonstration of spontaneous, naturally occurring airway hyperreactivity in a nonhuman species. An expected signs include wheezing, coughing, and dyspnea. Additionally, histologic changes in airways from asthmatic cats include epithelial erosion, goblet cell and submucosal gland hyperplasia and hypertrophy, and an increased mass of smooth muscle, which are all features of human asthmatic airways. *(Dye et al, 1996).* This protocol has also been used at Saint Lukes Medical Center Taguig Philippines (2017)

Provocholine reconstitution kit

- Provocholine 100 mg (1 x 20 mL vial)
- 1 Provocholine Insert
- 10 x 10 mL Sterile Empty Vials with Stopper
- 1 x 10 cc Syringe
- 1 x 3 cc Syringe
- 2 x 20 Gauge 1" Needles
- 1 x 0.22 micron Syringe Filter
- 10 Alcohol Pads
- Sodium Chloride with 0.4% Phenol (1 x 100 mL)
- 1 Set of Customized Green Labels (*Methapharm, 2017*)

Provocholine 2.5mg\ml reconstitution procedure

100mg of powdered provocholine will be used for the bronchioconstriction inducement which will be reconstituted to 2.5mg/ml strength (dosage)

1. Labels will be attached to sterile empty vials.
2. The stoppers of the Provocholine vial, diluent vial, and sterile empty vials will be wiped with alcohol prep pads.
3. Two (2) appropriately sized syringes will be labeld (one for Provocholine, one for diluent), and attach needles to each.

A. To make 25 mg/mL dosage, Using the diluent needle and syringe, 4 mL of diluent will be drawn and transferred. The 4 mL of diluent to the 100 mg vial Provocholine and Shake well.

B. To make 2.5mg/ml dosage: Using the Provocholine needle and syringe, 1 mL of solution will be drawn from 25mg/ml dosage and transfer to another vials. Using the diluent needle and syringe, 9 mL of diluents will be drawn and transfer to it. Shake well. (*Methapharm, 2017*).

Storage

Provocholine powder should be stored at 59º to 86ºF (15º to 30ºC).Dilutions should be stored at 36º to 46ºF (2º to 8ºC) in a refrigerator for no more than 2 weeks. Freezing does not affect the stability of dilutions A through D (25 mg/mL through 0.25 mg/mL).

Vial E (0.025 mg/mL) must be prepared on the day of the challenge test. (*Methapharm, 2017*).

Administration

The 2.5mg of the reconstituted provocholine will be transferred into special spacer device (or chamber) that is used for cats. Spacer device is designed to cover into cat's mouth and nose while breathing.

- The metered dose inhaler (MDI) will be shook according to the instructions supplied with it before each use
- The MDI will be attached to the spacer unit
- The dose will be actuated, by pressing down the MDI, to deliver a single dose into the chamber

- Immediately the unit will be hold over the cat's face with the mask fitting snuggly and allow the cat to take 1015 breaths (usually 10-20 seconds) (*Dye et al, 1996).*

Parameters

Coughing, wheezing, dyspnea and mouth opening is expected after inhalation through spacer chamber

Dyspnea will be measured using heart beat count; A normal cat's heart rate is between 140 and 220 bpm, with a relaxed cat measuring on the low end. A stop watch will be used then the cat's heartbeat will be feel with one hand over his left side, just behind his front leg. The number of beats will be counted in 15 seconds and multiply by four to get the heart rate in beats per minute (bpm). The number will be compared with the standard. (Dye et al, 1996).

Profiling the pharmacological activities

Evaluation of bronchodilation activities of the extract will be carried out after recording positive response to the inducement. The various concentration of the extract 100%, 75%, and 50% will be used to test its efficacy through intranasal instillation of aqueous extract. The positive control (Salbutamol) and the negative control (Normal saline solution, 0.9% NaCl solution) will be used as the standards. It is expected to observe a reduced broonchioconstrictions.

Statistical Treatment of Data

The following statistical tools will be employed to treat the data of the study:

One-Way Analysis of variance (ANOVA) will be used to determine whether there are any statistically significant differences between the means of two or more independent (unrelated) groups Turkey-Kramer (Honest significant Test) is a single-step multiple comparison procedure and statistical test that will also be used.

RESULT

Presentation, Interpretation, and Analysis of Data.

1. What is the percentage yield of ethanolic extract of active constituents present from Kalachuchi *(Plumeria acuminata)* leaves?

The percentage yield of active constituents present from Kalachuchi *(Plumeria acuminata)* leaves was evaluated using the formula below;

$$\text{Percentage yield} = \frac{\textbf{Actualvolume}}{\textbf{Theoreticalvolume}} \times 100\%$$

Actual volume = 300mL

Theoretical volume = 2000ml Percentage yield = 15%

It was computed by dividing the actual volume by theoretical volume and multiplying the outcome by 100%.

Phytochemical Screening

Qualitative Phytochemical Screening

2. Using Phytochemical studies, what are the different constituents present in the Kalachuchi *(Plumeria acuminata)* leaves extract?

Table 1: Results of Phytochemical Screening.

Tests	Positive Results	Actual Results	Indication
Detection of Carbohydrates and Reducing Sugars			
Fehling's Test	Red precipitate	Red coloration	(+)
Molisch's Test	Violet ring at junction	Violet ring at junction	(+)
Detection of proteins and Amino Acid			
Ninhydrin Test	Blue to purple coloration	Green solution	(-)
Biuret Test	Violet coloration	Green solution	(-)
Detection of Alkaloids			
Wagner's Test	Reddish brown precipitate	Reddish brown precipitate	(+)
Hager's Test	Yellow precipitate	Yellow precipitate	(+)
Dragendorff's Test	Orange red precipitate	Reddish brown precipitate	(+)
Detection of Glycosides			
Modified Borntrager's Test	Rose-pink color in ammoniacal layer	No change in color	(-)
Detection of Terpenes and Terpenoids			
Salkowski Test for Terpenoid	Reddish brown coloration	Yellow coloration	(+)
Salkowski Test for Triterpenes	Golden yellow color	Green coloration	(+)
Detection of Quinones			
Sulfuric Acid Test	Red color	Reddish brown coloration	(+)
Detection of Anthraquinones			
Hydrochloric Acid Test	Red color	No change in color	(-)
Detection of Saponins			
Froth Test	Formation of 1cm froth	No froth observed	(-)

Detection of Flavonoids			
Alkaline Reagent Test	Intense yellow color upon addition of base and turns colorless on addition of acid	NaOH=yellow solution HCl=faint yellow solution	(+)
Detection of Phenol			
Ferric Chloride Test	Bluish-black or green color	Orange solution	(+)
Detection of Tannins			
Ferric Chloride Test	Blue (hydrolysable tannins) Or green (condensed tannins)	No change in color	(-)
Gelatin Test	Formation of jelly precipitate	No precipitate formed	(-)

The table above is the result of the qualitative phytochemical screening of the ethanolic crude extract of the leaves of the plant Kalachuchi (Plumeria acuminate) shows that it contains carbohydrates and reducing sugars, alkaloids, terpenes and terpenoids, quinones, flavonoids, and phenols.

Methacholine induced Bronchoconstriction challenge of the groupings.

Table 2. Various trial groups and their respiratory rate before and after the challenge.

First trial	Cats	RR/min before challenge	RR/min after challenge
50% conc.	**1**	**25**	**40**
	2	**23**	**38**
75% conc.	**1**	**24**	**39**
	2	**21**	**39**
100% conc.	**1**	**24**	**37**
	2	**23**	**41**
NSS	**1**	**27**	**40**
	2	**26**	**36**
Salbutamol	**1**	**23**	**37**
	2	**20**	**39**
Second trial	**Cats**	**RR before challenge**	**RR after challenge**
50% conc.	**1**	**24**	**39**
	2	**27**	**37**
75% conc.	**1**	**22**	**38**
	2	**25**	**36**
100% conc.	**1**	**25**	**38**
	2	**21**	**36**
NSS	**1**	**22**	**39**
	2	**24**	**38**
Salbutamol	**1**	**22**	**41**
	2	**21**	**36**
Third trial	**Cats**	**RR before challenge**	**RR after challenge**
50% conc.	**1**	**24**	**40**
	2	**22**	**37**
75% conc.	**1**	**23**	**41**
	2	**25**	**36**
100% conc.	**1**	**25**	**39**
	2	**28**	**42**
NSS	**1**	**25**	**38**
	2	**23**	**40**
Salbutamol	**1**	**20**	**38**
	2	**25**	**36**

The table above shows the respiratory rate per minute of each cat used before challenge and after challenge with methacholine. Coughing, dyspnea and mouth opening were observed after the exposure to spacer chamber containing reconstituted methacholine after 15 breathes but wheezing sound was not heard. It shows the various trial groups and the respiratory rate recorded before exposure to methacholine which is at normal range and

after exposure that recorded an elevated respiratory rate which is an indication for a positive response to the challenge. All the cats were observed to be healthy physically and their respiratory rates were counted to be at normal range of 20 to 30 breaths per minute.

3. What is the respiratory rate per minute along with the various concentrations of the extract, positive, and negative control?

3.1 50% Conc. Kalachuchi (Plumeria acuminata) leaves ethanolic extract?.
Table 3: Results of Extract Group (50% conc.) – Respiratory rate.

No. of Trials	No. of Cats	Weight of Cat	Dose	RR/min		
				10 min after exposure	20 min after exposure	30 min after exposure
1st	1	2.10kg	1mg in 2ml	36	33	33
	2	2.00kg	1mg in 2ml	34	31	31
2nd	1	2.13kg	1mg in 2ml	36	32	32
	2	2.10kg	1mg in 2ml	35	31	31
3rd	1	2.01kg	1mg in 2ml	35	33	33
	2	2.00kg	1mg in 2ml	36	31	31

The table above shows that Kalachuchi *(Plumeria acuminata)* leaves extract 50% concentration was used in the 2 cats for the first trial that weighed 2.10kg and 2.00kg with the reconstituted dose of 1mg in 2mL. The respiratory rate per minute after the first 10, 20, and 30 minutes of inhalation of the dose are 36, 33, and 33 respectively for cat 1, while cat 2 recorded 34, 31, and 31 respectively in the first trial. For the second trial, cat 1 weighs 2.13kg while cat 2 weighs 2.10 kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 36, 32, and 32 respectively for cat 1, while cat 2 recorded 35, 31, and 31 respectively.

For the third trial, cat 1 weighs 2.01kg while cat 2 weighs 2.00kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 35, 33, and 33 for cat 1 respectively while cat 2 recorded 36, 31, and 31 respectively. The researchers used 1mg in 2mL in the cats given they have different weights as long as the weight of the cats falls in the range of 2.0kg to 2.5kg following the protocol used.

3.2 75% Conc. Kalachuchi *(Plumeria acuminata)* leaves ethanolic extract?.
Table 4: Results of Extract Group (75% conc.) – Respiratory rate.

No. of Trials	No. of Cats	Weight of Cat	Dose	RR/min		
				10 min after exposure	20 min after exposure	30 min after exposure
1st	1	2.01kg	1.5mg in 2ml	28	25	24
	2	2.10kg	1.5mg in 2ml	30	26	25
2nd	1	2.13kg	1.5mg in 2ml	29	27	25
	2	2.10kg	1.5mg in 2ml	28	25	23
3rd	1	2.11kg	1.5mg in 2ml	27	26	23
	2	2.12kg	1.5mg in 2ml	30	26	24

The table above shows that Kalachuchi *(Plumeria acuminata)* leaves extract 75% conc was used in the 2 cats for the first trial that weighed 2.01kg and 2.10kg with the reconstituted dose of 1.5mg in 2mL. The respiratory rate per minute after the first 10, 20, and 30 minutes of inhalation of the dose are 28, 25, and 24 respectively for cat 1, while cat 2 recorded 30, 26, and 25 respectively in the first trial. For the second trial, cat 1 weighs 2.13kg while cat 2 weighs 2.10 kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 29, 27, and 25 respectively for cat 1, while cat 2 recorded 28, 25, and 23 respectively.

For the third trial, cat 1 weighs 2.11 kg while cat 2 weighs 2.12 kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 27, 26, and 23 for cat 1 respectively while cat 2 recorded 30, 26, and 24 respectively. The researchers used 1.5mg in 2mL in the cats given they have different weights as long as the weight of the cats falls in the range of 2.0 kg to 2.5 kg following the protocol used.

3.3 100% conc. Kalachuchi *(Plumeria acuminata)* leaves ethanolic extract?.
Table 5: Results of Extract Group (100% conc.) – Respiratory rate per minute

No. of Trials	No. of Cats	Weight of Cat	Dose	RR/min		
				10 min after exposure	20 min after exposure	30 min after exposure
1st	1	2.12kg	2mg in 2 ml	24	21	20
	2	2.10kg	2mg in 2ml	22	20	20
2nd	1	2.11kg	2mg in 2ml	23	21	20
	2	2.01kg	2mg in 2ml	23	22	21
3rd	1	2.01kg	2mg in 2ml	24	22	21
	2	2.13kg	2mg in 2ml	22	21	21

The table above shows that Kalachuchi *(Plumeria acuminata)* leaves extract 100% conc. was used in the two cats for the first trial that weighed 2.12kg and 2.10kg with the reconstituted dose of 2mg in 2mL. The respiratory rate per minute after the first 10, 20, and 30 minutes of inhalation of the dose are 24, 21, and 20 respectively for cat 1, while cat 2 recorded 22, 20, and 20 respectively in the first trial. For the second trial, cat 1 weighs 2.11kg while cat 2 weighs 2.11 kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 23, 21, and 20 respectively for cat 1, while cat 2 recorded 23, 22, and 21 respectively.

For the third trial, cat 1 weighs 2.01kg while cat 2 weighs 2.13kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 24, 22, and 21 for cat 1 respectively while cat 2 recorded 22, 21, and 21 respectively. The researchers used 2mg in 2mL in the cats given they have different weights as long as the weight of the cats falls in the range of 2.0kg to 2.5kg following the protocol used.

3.4 Negative Control (Normal saline solution)?
Table 6: Results of NSS Group – Respiratory Rate.

No. of Trials	No. of Cats	Weight of Cat	Dose	RR/min		
				10 min after exposure	20 min after exposure	30 min after exposure
1st	1	2.11kg	2ml	40	39	38
	2	2.01kg	2ml	36	35	34
2nd	1	2.10kg	2ml	39	38	37
	2	2.00kg	2ml	38	36	35
3rd	1	2.00kg	2ml	38	37	36
	2	2.12kg	2ml	40	38	38

The table above shows that normal saline solution as the negative control of this study was used in 2 cats for the first trial that weighed 2.11kg and 2.01kg with the dose of 2mL had a respiratory rate per minute after 10, 20, and 30 minutes, cat 1 recorded 40, 39, and 38 respectively while cat 2 recorded 36, 35, 34 respectively. For the second trial the 2 cats weighed 2.10kg and 2.00kg with the same dose had respiratory rate per minute after 10, 20, and 30 minutes of 39, 38, and 37 for cat 1 respectively while cat 2 recorded 38, 36, and 35 respectively. For the third trial the 2 cats weighed 2.00kg and 2.12kg with the same dose had respiratory rate per minute after 10, 20, and 30 minutes of 38, 37, and 36 respectively for cat 1 while cat 2 recorded 40, 38, and 38 respectively.

3.5 Positive control (Salbutamol) daily recommended dose of positive control (2mg)?
Table 7: Results of Positive Control Group Salbutamol – Respiratory Rate.

No. of Trials	No. of Cats	Weight of Cat	Dose	RR/min		
				10 min after exposure	20 min after exposure	30 min after exposure
1st	1	2.01kg	2mg in 2mL	21	21	21
	2	2.10kg	2mg in 2mL	20	20	20
2nd	1	2.10kg	2mg in 2mL	20	20	20
	2	2.00kg	2mg in 2mL	21	21	21
3rd	1	2.11kg	2mg in 2mL	21	21	21
	2	2.01kg	2mg in 2mL	20	20	20

The table above shows that Salbutamol as the positive control for this study was used in the two cats for the first
trial that weighed 2.01kg and 2.10kg with the dose of 2mg. The respiratory rate per minute after 10, 20, and 30 minutes are 21, 21, and 21 respectively for cat 1, while cat 2 recorded 20, 20, 20 respectively in the first trial. For the second trial, cat 1 weighs 2.10kg while cat 2 weighs 2.00 kg, after inhalation of the same dose, the respiratory rate per minute after 10, 20, and 30 minutes were 20, 20 and 20 respectively for cat 1 while cat 2 recorded 21, 21, 21 respectively. For the third trial, cat 1 weighs 2.11kg while cat 2 weighs 2.01kg, after inhalation of the same dose of the extract, the respiratory rate per minute after 10, 20, and 30 minutes were 21, 21, and 21 respectively for cat 1, while cat 2 recorded 20, 20, and 20 respectively. The researchers used 2mg in the cats given they have different weights as long as the weight of the cats falls in the range of 2.0kg to 2.5kg following the protocol used.

Descriptive summary of the Respiratory rate per minute by treatment

Table of Means and Standard Deviation for RR/min and the Different Treatments.

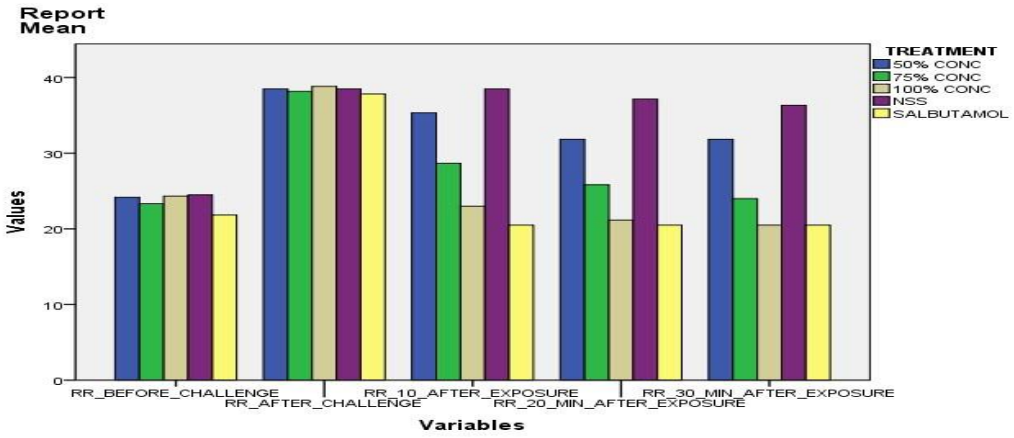
Table 8: Mean and standard deviation of respiratory rate per minute and test samples.

TREATMENT		RR_B/F_CHALLENGE	RR_AFTER_CHCHALLENGE	RR_10_AFTER_EXPOSURE	RR_20_MIN_AFTER_EXPOSURE	RR_30_MIN_AFTER_EXPOSURE
50% CONC	Mean	24.1667	38.5000	35.3333	31.8333	31.8333
	SD	1.72240	1.37840	.81650	.98319	.98319
75% CONC	Mean	23.3333	38.1667	28.6667	25.8333	24.0000
	SD	1.63299	1.94079	1.21106	.75277	.89443
100% CONC	Mean	24.3333	38.8333	23.0000	21.1667	20.5000
	SD	2.33809	2.31661	.89443	.75277	.54772
NSS	Mean	24.5000	38.5000	38.5000	37.1667	36.3333
	SD	1.87083	1.51658	1.51658	1.47196	1.63299
SALBUTAMOL	Mean	21.8333	37.8333	20.5000	20.5000	20.5000
	SD	1.94079	1.94079	.54772	.54772	.54772
Total	Mean	23.6333	38.3667	29.2000	27.3000	26.6333
	SD	2.04237	1.75152	7.09249	6.55560	6.55209

The table above shows the mean and standard deviation of 50% concentration for respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure. The Mean and standard deviation of 75% concentration for respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure.

The Mean and standard deviation of 100% concentration for respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure. The Mean and standard deviation of NSS for respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure.

The Mean and standard deviation of Salbutamol for respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure. Then, the total Mean and standard deviation for the respiratory rate before methacholine challenge, after methacholine challenge, respiratory rate 10 minutes after exposure to different test samples, 20 minutes after exposure, and 30 minutes after exposure.

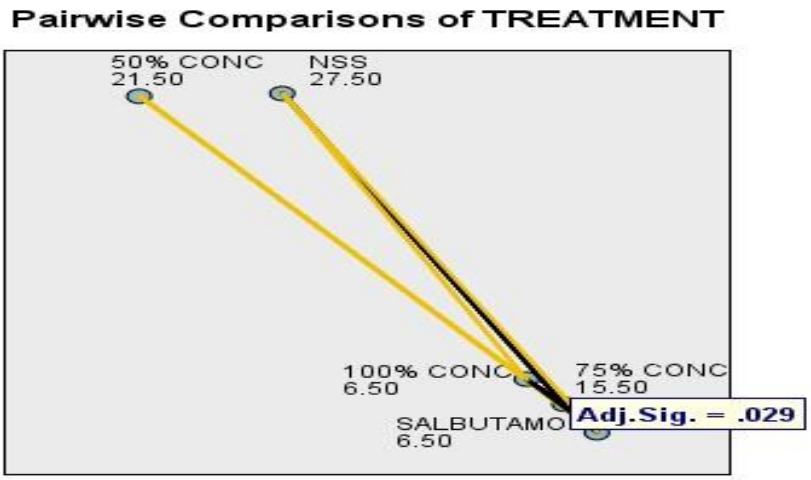


Graph 1: Graph of RR/min and the Different Treatments.

The first group of bars is a graph presentation of respiratory rate per minute before methacholine challenge showing to be with the normal range of 20 to 30. The second group of bars is the respiratory rate per minute after methacholine challenge showing to be way above the normal range of 38 to 40. The third group of bars is the respiratory rate per minute, 10 minutes after exposure to the various test samples which varies due to different effects. The fourth group of bars is the respiratory rate per minute, 20 minutes after exposure to the various test samples which varies due to different effects. The fifth group of bars is the respiratory rate per minute, 30 minutes after exposure to the various test samples which varies due to different effects.

Kruskal Wallis Test of RR/min before Challenge

The table above shows the hypothesis test summary of respiratory rate per minute before and after methacholine challenge. It also compares the significance level of the various test samples at 10, 20, and 30 minutes after exposure and found that there is no significant difference, there for the null hypothesis was rejected because significance level is less than 0.05.



Graph 2: Pairwise Comparisons of the Treatment Groups.

The graph above compares a pairwise of the various test samples. There is no significance difference between 75% concentration, 100% concentration, and the positive control (Salbutamol). There is no significance difference between 50% concentration and the negative control (NSS).

4. Is there any significant difference in the respiratoiry rate per minute along with various concentration (50%, 75%, andf 100% concentration of Kalachuchi leaves ethanolic extract), positive control (Salbutamol), and Negative control (NSS)?.

Each node shows the sample average rank of TREATMENT.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
100% CONC-SALBUTAMOL	.000	5.038	.000	1.000	1.000
100% CONC-75% CONC	9.000	5.038	1.786	.074	.740
100% CONC-50% CONC	15.000	5.038	2.977	.003	.029
100% CONC-NSS	-21.000	5.038	-4.168	.000	.000
SALBUTAMOL-75% CONC	9.000	5.038	1.786	.074	.740
SALBUTAMOL-50% CONC	15.000	5.038	2.977	.003	.029
SALBUTAMOL-NSS	21.000	5.038	4.168	.000	.000
75% CONC-50% CONC	6.000	5.038	1.191	.234	1.000
75% CONC-NSS	-12.000	5.038	-2.382	.017	.172
50% CONC-NSS	-6.000	5.038	-1.191	.234	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

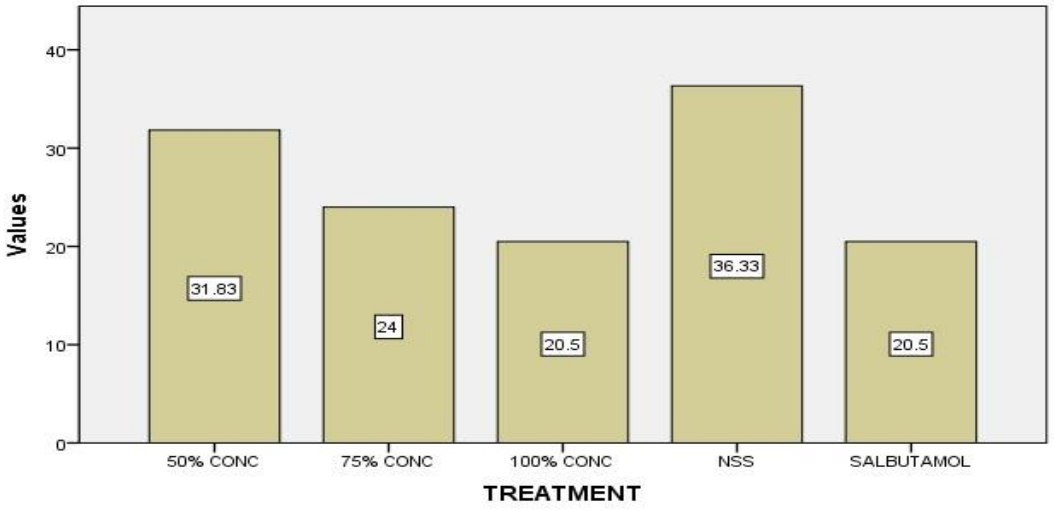
Table 11: Level of significance comparison.

The table above is pairwise comparison of all the test samples. Using the P-value of 0.05, there is no significance difference between 100% concentration and positive control (Salbutamol), 100% concentration and 75% concentration. There is significance difference between 100% concentration and 50% concentration, also between 100% concentration and negative control (NSS). There is no significance difference between Salbutamol and 75% concentration, while there is significance difference between Salbutamol and 50% concentration. There is also a significance difference between Salbutamol and the negative control (NSS).

There is no significance difference between 75% concentration and 50% concentration statistically but there is significance difference therapeutically because 50% was not able to produce effect that couldn't restore the respiratory rate per minute to normal range of 20 to 30. There is no significance difference between 75% concentration and the negative control (NSS). There is no significance difference between 50% concentration and the negative control (NSS) statistically but there is a significance difference therapeutically.

Table 12: RR/m 30 minutes after exposure.

TREATMENT	Mean	Std. Deviation
50% CONC	31.8333	.98319
75% CONC	24.0000	.89443
100% CONC	20.5000	.54772
NSS	36.3333	1.63299
SALBUTAMOL	20.5000	.54772



Graph 3: Report mean of RR. 30 minutes after exposure.

The graph above compares the respiratory rate per minute 30 minutes after administration to the test samples which shows 75% concentration, 100%, and positive control (Salbutamol) within the range and closer to the lower respiratory rate per minute limit of 20. The 50% ethanolic extract concentration and NSS also reduced after 30 minutes compared to after 10 minutes or 20 minutes because the methacholine effect must have reduced.

Table 13: Summary of Mean +/SD of RR/m 90 min after exposure.

Treatment	Mean ±SD	P value	Conclusion
NSS VS SALBUTAMOL	36.33 ± 1.63 VS 20.5 ± 0.55	P = 0 Sig	Not Comparable
50% CONC VS SALBUTAMOL	31.83 ± 0.98 VS 20.5± 0.55	P = 0.029 Sig	Not Comparable
75% CONC VS SALBUTAMOL	24 ± 0.89 VS 20.5 ± 0.55	P = 0.74 Not Sig	Comparable
100% CONC VS SALBUTAMOL	20.5 ± 0.55 VS 20.5 ± 0.55	P = 1 Not Sig	Comparable

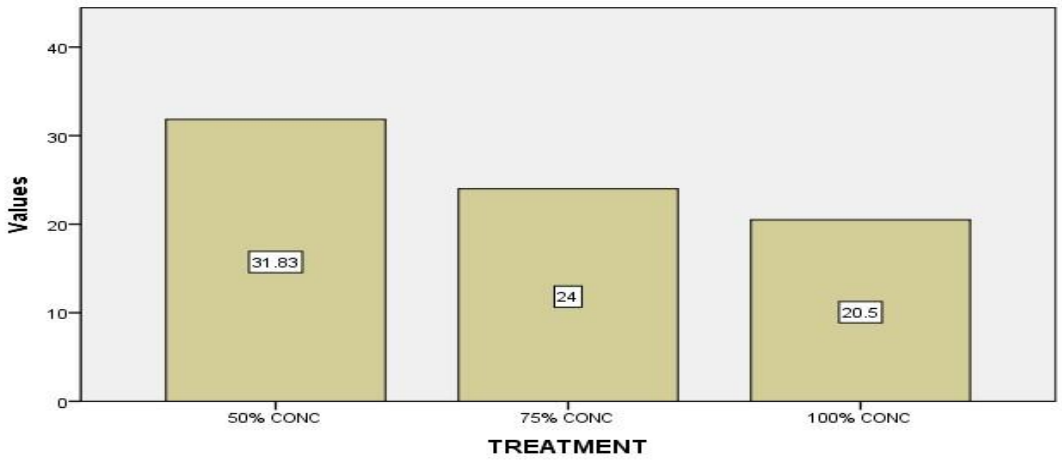
The table above compares the means and standard deviation of the various test samples with the positive control (salbutamol). The NSS and 50% ethanolic extract concentration are not comparable with the salbutamol while the 75% and 100% ethanolic extract concentration are comparable giving that their P-value is greater than .05 and also have a mean within the normal respiratory rate range of 20 to 30. Therefore, the 75% and the 100% exhibit bronchodilation effect properties.

Comparison of the mixtures 50%, 75%, 100%.

Table 14: Graph of Means of the 3 Mixtures.

Treatment	Mean ± SD	P value	
100% VS 75%	20.5± 0.55 VS 24±0.89	P = 0.074 Not Sig	Comparable
100% VS 50%	20.5± 0.55 VS 31.83±0.98	P = 0.029 Sig	Not Comparable
50% VS 75%	31.83± 0.98 VS 24± 0.89	P = 1 Not Sig	Comparable

The table above compares the various Kalachuchi extract concentrations which shows that there is no significance difference between 100% vs 75%, but there is a significance difference between 100% and 50% concentrations. There is no significance difference between 50% and 75% concentrations statistically, but there is a significance difference between both therapeutically because the mean of 50% concentration is above the upper respiratory rate limit of 30 while 75% concentration is within the normal respiratory rate per minute range of 20 to 30.



Graph 4: Report mean of RR 30 minutes after exposure.

Based on the graph above, increasing the percentage concentration will increase the bronchodilation effect of the Kalachuchi ethanolic extract. The 50% concentration has bronchodilation effect but less effective than 75% and 100% concentrations. It is above upper respiratory limit of 30 while 75% and 100% concentration are within the normal respiratory rate of 20 to 30.

CONCLUSION

Based on the result of data, the researchers conclude that the ethanolic leaves extract of Kalachuchi produced a bronchodilation effect in the cats that were used as experimental animal in this study by comparing the Kalachuchi *(Plumeria acuminata)* leaves extract to the positive and negative control. Therefore, the Kalachuchi leaves extract exhibited the same effect as the standard drug which was Salbutamol.

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