**CHARACTERIZATION AND CLINICAL EVALUATION OF BEE PRODUCTS AND *WARBUGIA UGANDENSIS* FOR EFFECTIVE MANAGEMENT OF COVID-19**

**Submitted By**

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# **1.INTRODUCTION**

## **1.1 Background**

COVID 19 was first recognized after a cluster of pneumonia outbreak was reported in late December 2019 from Wuhan city, China. The disease was named severe acute respiratory syndrome coronavirus 2” (SARS-CoV2) and the World Health Organization announced COVID-19 as a pandemic. Patients who test positive for COVID 19 have symptoms that include fever , chills, dry cough, sputum production, fatigue, lethargy, arthralgias, myalgias , headache, dyspnea, nausea, vomiting, anorexia and diarrhea. Some carriers may be asymptomatic, whereas others can experience acute respiratory distress syndrome and death. The severity of the disease seems to vary with age, disproportionately affecting those of advanced age and those with pre-existing chronic medical conditions. The case fatality has been estimated to range between 0.25% and 3.0%.

Following the onset of the COVID -19 pandemic, there is urgent need to develop new interventions to mitigate the effects of the disease including treatments with existing drugs, development of new drugs and vaccines. Although there is currently no approved medicine for COVID 19, the speculation of using non-steroidal anti-inflammatories (NSAIDs) like ribavirin, interferon, lopinavir, and corticosteroids and clinical treatment with neuraminidase inhibitors (oseltamivir, peramivir, anamivir), ganciclovir, acyclovir, ribavirin have been found to be ineffectual, emdesivir has been suggested to have great potential in the treating COVID-19.

Since drugs like emdesivir are not easily available, there is need to look for alternative drug therapies like natural products with antiviral activities. For instance, bee products like bee venom and bee propolis obtained from honey bees has been reported to have anti-viral anti-inflammatory properties. The biological activity of bee products is due to the complex mixture of biogenic amines and peptides. The main peptides, melittin in bee venom and phytochemicals in bee propolis have excellent anti-inflammatory, and antiviral effects and have also been suggested be effective for the treatment of most immune-mediated diseases and viral infections.

Another natural product, the plant, *Warbugia ugandensis* (family canellaceae) also called pepper bark tree) has been used treat several diseases including viral diseases. The biological activity of *W. ugandensis* is dependent on phytochemical compounds it contains like saponins, tannins, sterols and terpenes, and alkaloids. The sesquiterpenes isolated and identified in the stem bark of *W. ugandensis* include; ugandensidial, polygodial, Warbuganal and Isopolygodial.

Since COVID 19 is a viral disease, studies to investigate the activity of bee products (bee venom and propolis) and *W.ugandensis* against COVID 19 it is still wanting. This study is to establish the potential bee products and *W.ugandensis* as interventional drugs for COVID – 19 disease and related symptoms

## **1.2 Problem statement**

 COVID 19 is a viral disease currently with no cure. Although natural products like bee propolis, bee venom and W. ugandensis have shown wide spectrum of anti-viral and anti-inflammatory activities, the products under use in Uganda are not standardized for safety and efficacy. It is estimated that 1% of the population is allergic to bee venom and a bee sting may trigger a dangerous anaphylactic reaction in some people that can be potentially deadly. Besides, Bee venom can exhibits hemolytic activity at low concentration, can increase in DNA damage, muscle spasms and respiratory failure. Although melittin, the main component in bee venom has demonstrates significant anti-inflammatory properties, there is need to establish the most potent and safe concentration against COVID 19. Similarly, bee propolis and W. ugandensis need to be investigated for antiviral and anti-inflammatory activity.

**1.3 Goal**:

To develop a safe and effective, preventive and Therapeutic natural products against COVID-19 and other ailments

## **1.4 Objectives**

### **1.4.1 General objective**

To assess Bee propolis, Bee venom and *Warbugia Ugandensis* as preventive and therapeutic products in the management of COVID-19

### **1.4.2 Specific objectives**

1. To determine the chemical composition of bee propolis and bee venom
2. To determine the chemical composition of *W. ugandensis* leaves
3. To evaluate the safety profile of bee venom and *W. ugandensis* extracts to be used against COVID – 19 disease related symptoms in laboratory animals
4. To establish the invitro antiviral and antibacterial activities of bee venom and *W. ugandensis*
5. To evaluate the efficacy in vivo ( antiviral, anti-inflammatory, anti-pyretic activity and immune modulatory) of bee venom and *W. ugandensis* extract.
6. To formulate standardized natural products from bee propolis, bee venom, *W. ugandensis* ingredients and or combinations
7. To conduct clinical trials of the formulated products

## **1.5 Significance**

The scientists are currently searching for new drugs for COVID-19. Bee Propolis, venom and *W. ugandensis* provide a cheap and cost-effective alternative drug for people infected by COVID-19, particularly risk groups like children at school, the elderly, prisoners and armed forces who are vulnerable and also leave in high populated areas. In addition to offering treatment option, these products also become source of income for farmers and employment activities for those that have gone out of employment population due to COVID-19

## **1.6 Conceptual framework for development COVID 19 therapeutic natural products**

Formulation of COVID-19 therapeutic natural products

Clinical validation of COVID 19 products

Anti-viral

 activity

Anti-inflammatory

activity

Anti-pyretic activity

Bee venom

*W. ugandensis*

Chemical analysis

Safety studies

Acute toxicity

Sub-acute toxicity

Chronic toxicity

Bioavailability, stability and drug interaction

Bee propolis

Immuno- modulatory activity

Production of COVID 19 therapeutics natural Products

# **2.0 LITERATURE REVIEW**

## **2.1 Over view of COVID 19**

### ***2.2.1 Origin of COVID 19***

COVID 19 was first recognized after a cluster of pneumonia outbreak was reported in late December 2019 from Wuhan, China. The human coronavirus (HCoV) was isolated from these cases and identified as a beta-coronavirus and provisionally named 2019 novel corona virus (2019-nCoV) using next-generation sequencing technology. It was named the irus as “severe acute respiratory syndrome coronavirus 2” (SARS-CoV2) and World Health Organisation announced COVID-19, the name of the new disease caused by it. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the coronavirus disease of 2019 (COVID-19

### ***2.1.2 Transmission***

Transmission of Covid 19 occurs primarily through respiratory droplets, but it can also occur through contact with contaminated surfaces. Incubation periods may vary but have been known to be between 1 and 14 days for other coronaviruses. At the cellular level, once viral particles enter the respiratory tract and SARS-CoV-2 uses the ACE-2 receptors for pulmonary cell entry.

ACE- 2 is a type 1 transmembrane metallocarboxypeptidase, which, under normal physiological

circumstances, functions in the degradation of angiotensin II to modulate the renin-2020. The viral S protein binds to the ACE-2 receptor, prompting cellular membrane fusion and endocytosis. This process is dependent on S protein priming by a serine protease (TMPRSS2) in many coronavirus models, potentially identifying a future treatment modality

### ***2.3.3 Clinical Features of COVID 19***

Patients who test positive for SARS-CoV-2 and are symptomatic are diagnosed with COVID-19.

Symptoms can vary drastically; they include fever , chills, dry cough, sputum production, fatigue, lethargy, arthralgias, myalgias , headache, dyspnea, nausea, vomiting, anorexia and diarrhea. Some carriers may be asymptomatic, whereas others can experience acute respiratory distress syndrome (ARDS) and death. Severity seems to also vary with age, disproportionately affecting those of advanced age and those with pre-existing chronic medical conditions. case fatality is reported to range between 0.25% and 3.0% and patients above 80 years of age are most affected

### ***2.3.4 Treatment of Covid 19***

There is currently no approved medicine for Covid 19 . There are speculation of using non-steroidal anti-inflammatories (NSAIDs), although there are no studies to prove it efficacy. Some drugs, such as ribavirin, interferon, lopinavir, and corticosteroids have been suggested. Through clinical treatment of the COVID-19, it has been found that neuraminidase inhibitors (oseltamivir, peramivir, anamivir), ganciclovir, acyclovir, ribavirin are ineffectual and not recommended for clinical application. However there have been suggestion that emdesivir has great potential in the treating COVID-19

## **2.2 Bee venom**

### ***2.2.1 Over view of bee venom***

Venom is a secretion, produced in a specialized gland in one animal and delivered to a target animal through the invocation of a wound(regardless of how tiny it may be), which contains molecules that disrupt normal physiological or biochemical processes in the victim so as to facilitate feeding or defense by the producing animal. bee venom is a substance produced in the poison glands of the abdominal cavity of the bee and it has many biological effects (e.g. immune system, central and peripheral nervous system, cardiovascular system, antibacterial, fungicide, antiviral, anti-inflammatory, antiarthritis, anticancer, and wound healing effects.

### ***2.2.2 Collection/harvesting of bee venom***

Honey bee venom is collected by an electric shock instrument, sometimes coupled with stimulating the honeybee to sting the surface of the collector sheet or venom is extracted from bee glands or sticks. Bee-venom collector is placed on the hive, and the bees are given enough electric shocks to cause them to sting a glass plate, from which dried bee venom was later scraped off. The collected venom can be diluted in cold sterile water and then centrifuged for purification (Rybak-chemielewska and Szczesna, 2004)



**Figure 1: Bee venom collector**

### ***2.2.3Chemical composition of bee venom***

Bee venom contains a complex mixture of biogenic amines and peptides. The main peptide are; melittin, apamin, promelittin, and mast cell deregulating peptide. The enzymes found in bee venom include phospholipase A2 and hyaluronidase. Other compounds include amines like histamine, dopamine, sinkaline, noradrenaline and amino acids. . In addition, some small amounts of sugars, phospholipides and pheromones were identified in bee venom. Bee venom is a clean and clear aromatic liquid with a bitter taste, which dries quickly at room temperature. After evaporation, 30%–40% of it remains as crystal (refer to the picture).
- Dried bee venom is thermostable, so it does not lose its characteristics even when heated at 100°C, but it can be destroyed by oxidizing materials.

**Table 1: Pharmacological Actions and Substances of Bee Venom**

|  |  |  |  |
| --- | --- | --- | --- |
| **Substance** | **Pharmacological Action** | **Dry Weight (%)** | **Molecular Weight (kD)** |
| Melittin | Cell-dissolving action | 40-50 | 2.8 |
| Apamin | Neuralgia relief, pain relief, anti-inflammatory effect, immunization, and neurotoxic effect | 2-3 | 2.02 |
| MCD-Peptide 401 | Anti-inflammatory effect | 2-3 | 2.6 |
| Adolapin | Anti-inflammatory effect, pain relief, and fever alleviation effect | 0.5-1 | 11.5 |
| Protease inhibitor | Inhibition of dissolving protein and ester, and anti-inflammatory action | 0.1-0.8 | 9.0 |
| Secapine | Hypothermia relief | 0.5-2 | 2 |
| Procamine A, B | Radiation protection | 1-2 | 0.4 |
| Hyaluronidase | Tissue breakdown and antigenic substance | 1 | 38 |
| Phospholipase A2 | Cell tissue breakdown and hemolytic and catalytic actions | 10-13 | 19 |
| Histamine | Blood pressure reduction, intestinal contraction, and enhanced stomach acid secretion | 0.5-2 | 0.12 |
| Dopamine | Neurotransmitter | 0.2-1 | 0.15 |

The major and abundant component in bee venom is melittin containing 26 amino acid residues. The concentration of melittin in bee venom ranges from 22-71%. Melittin is stable in the in water and darkness, but can be destroyed by heat, acid or bases. However, the content of phospholipase A2 ranges from 11--15% , apamine content 2-4.% (Rybak-chemielewska and Szczesna, 2004). Venom samples are usually frozen immediately after sampling , lyophilized, freeze-dried or vacuum dried prior to storage at -20C under anhydrous conditions



**Figure 2: Melittin compound**

### ***2.2.4 Chemical analysis of bee venom***

Bee venom is usually analyzed using reverse High-Performance Liquid Chromatography (HPLC) and LC-MS methods that allows separation and identification of most of chemical components (Haghi vet al., 2015). The separation can be achieved with 180 and 300C pore size columns at a temperature of 25°C or columns packed with C18 material. Melittin for example can be extracted with pure water or ethanol and quantified with LC/ESI-MS/MS method coupled with ion trap mass spectrometer

### ***2.2.5 Antiviral and antibacterial activity of bee venom***

Melittin and its analogs have also been shown to be effective against other viruses such as murine retroviruses , herpes simplex virus, HIV, and Junin virus. It also effective against Gram-negative bacteria (Dossey, 2010). Melittin and apamin area also reported to stimulate axes including the hypothalamus, pituitary, and adrenal cortex., thus increasing the secretion of adrenocortical hormones. Phospholipase A2, on the other hand is the main enzyme for management of allergic reactions. At a concentration of 50 \_g/mL bee venom exhibits antibacterial activity against P. aeruginosa and S. aureus strains only. melittin (1 mg/mL) showed no or weak activity for *Bacillus subtilis*, *Proteus vulgaris*, *Enterococcus faecalis*, and *Escherichia coli.* Bee venom melittin is more active against Gram+ than Gram- bacteria which suggests that PLA2 is the element responsible for the antibacterial activity. Anti-bacterial activity can be produced through the synergetic action of melittin and PLA2

### ***2.2.6 Anti-inflammatory activity of bee venom***

Bee venom is reported to reduce inflammation like for rheumatoid arthritis and multiple sclerosis ( Han et al. 2015). at low doses of this BV compound can induce wide anti-inflammatory effect by inhibiting inflammatory cytokines like interleukin-6 (IL-6), IL-8, tumor necrosis factor-(TNF-\_), and interferon- (IFN-). The component Melittin can decreases signaling pathways that activate inflammatory cytokines, including nuclear factor-kappa B (NF-\_B), protein kinase Akt, and extracellular signal-regulated kinases (ERK1/2) in porphyromonas gingivalis lipopolysaccharide (PgLPS) in treated human keratinocytes. Optimal dose of bee venom is reported to exerts anti-apoptotic effects against ethanol-induced injury to hepatocytes via the mitochondrial pathway. Thus, protecting hepatocyte against TNF-α with actinomycinD induced apoptosis. Low concentrations of bee venom resulted in anti-apoptotic effects that were associated with a decrease in the level of proteolytic fragments of caspases and PARP.

Furthermore, bee venom also inhibits CCL4-induced hepatic fibrosis through suppression of fibrogenic cytokines in liver fibrosis animal model. It also down-regulated pro-inflammatory cytokines such as TNF-α and IL-1β. Bee venom has also demonstrated that collagen gene expressionis regulated by TNF-α at a transcription level and IL-1β exerts a stimulatory effect onthe synthesis of extracellular matrix components. Transforming growth factor (TGF)-β is a multifunctional cytokine that mediatescellular differentiation, growth and apoptosis. TGF-β1 can decrease cell viabilities and induced hepatocyte apoptosis. bee venom can significantly increase the viability of TGF-β1-treatedhepatocyte. Optimal dose of melittin can exerts anti-apoptotic effects against TGF-β1-induced injury to hepatocytes via the mitochondrial pathway. Optimal dose of bee venom and melittin can serve to protect cells against TGF-β1-mediated injury

### ***2.2.7 Antioxidant Activity of bee venom***

Bee venom is reported a dose-dependent antioxidant activity, though an antioxidant action were at a level close or weaker than vitamin C. In fact, from 2.5–200 \_g/mL, the percentage of DPPH radicals scavenging activity of bee venom was found vary in the range of 50–65%, though it was possible to increase the value to 72% and 87 for a concentration of 300 \_g/ and 500 \_g/mL, respectively. Melittin was also tested at a concentration of 100 \_g/mL melittin and registered 52.5% of the scavenging activity . Melittin alone is reported to exert very poor antioxidant activity compared to bee venom extracts and this is suggested to be influenced by other venom components

### ***2.2.8 Safety of bee venom***

The median lethal dose (LD50) of bee venom for an adult human is 2.8 mg of venom per kg of body weight, i.e. a person weighing 60 kg has a 50% chance of surviving injections totaling 168 mg of bee venom. although, bee venom is regarded safe for human treatment; it should only be used under the supervision of a qualified health care professional. Most human deaths result from one or few bee stings can be due to allergic reactions, heart failure or suffocation from swelling around the neck or the mouth. It is estimated that 1% of the population is allergic to bee stings. Therefore, in using bee venom in treatment, a dose of epinephrine and antihistamine tablets should be present.

Even though melittin has demonstrated significant therapeutic properties, its toxicity must be

neutralized for use as an anti-inflammatory agent. Accumulated melittin peptides can disrupt phospholipid packing in the cell membrane, resulting in cell lysis on human erythrocyte cells. Bee venom exhibits hemolytic activity at concentration of 2.5 μg/mL

Melittin can increase DNA damage in HPBLs and it is also considered as an allergen. In people who are allergic to bee stings, a sting may trigger a dangerous anaphylactic reaction that is potentially deadly. An overdose of apamin is reported to cause muscle spasms and respiratory failure . PLA 2 are toxin that immediate pro inflammation. . At PLA concentration of 5 \_g/mL, 86.42% fatty acid (arachidonic) release can be observed

## ***2.3 Warbugia ugandensis***

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### ***2.3.1 Overview of W. ugandensis***

*Warbugia ugandensis* (family canellaceae) also called pepper bark tree grows to up to 40 meters with Breast Height diameter (BDH) of about 70cm. The bark of the tree is pale green or brown. The leaves are simple, alternate, elliptic to lanceolate, glossy, dark green in colour. The flowers are small, bisexual, regular, white to green in colour. The fruits are dark green and turn to purple when mature and ripe (Okello et al., 2019). *Warbugia ugandensis* is native to tropical Africa and grows in several countries including, Uganda, Kenya, Tanzania, Ethiopia and democratic republic of Congo

### ***2.3.2 Ethnomedicinal uses of W. ugandensis***

*Warbugia ugendensis* is a medicinal plant that is used to treat several diseases including cough, asthma, chest pain, sinus, pneumonia, malaria, fever, measles, stomach ache, constipation, ulcers, diarrhoea, muscle pain, skin infections, tuberculosis, urinary infections and other sexually transmitted diseases (Okello et al., 2019). *Warbugia ugandensis* is also a good insecticide. Although the stem bark, leaves and roots are used in treatment of diseases, the bark is the most used and active.

### ***2.3.3 Phytochemical in W. ugandensis***

Several phytochemicals including saponins, tannins, sterols and terpenes, and alkaloids have been identified in *W. ugandensis*. The plant is reported to be abundant in terpenoids. The sesquiterpenes isolated and identified in the stem bark of *W. ugandensis* include; ugandensidial, polygodial, Warbuganal and Isopolygodial. Other compounds like muzigadiolide, drimenol, drimenin, periniporin, mukaadial and warbugin have also been isolated and identified in *W. ugandensis* ( Okello et al., 2019)

### ***2.3.4 Ethnopharmacology of W. ugaandensis***

*Warbugia ugandensis* has demonstrated several anti-plasmodial and antimicrobial activities. In malaria studies, the different plant parts of *W. ugandensis* demonstrated anti-plasmodial activity with IC50 lower than 5mg/ml against chloroquine resistant strains of *P. falciparum* (Nanyingi et al., 2010). The stem bark extract also showed wide spectrum antibacterial activity against *Escherichia coli* and *Salmonella aureus* (including that occurring in atopic dermatitis) and *Shigella boydii*. The leaf extract was found to be active against pathogen that cause Pneumonia like *Streptococcus pneumonia*, *Klebsiella pneumonia* and  *pseudomonas aeruginosa* (Maoebe et al., 2012) The presence of tannins were attributed to activity of *W. ugandensis* to different types of microorganism. The plants extract exhibited antifungal activity against *Candida albican* at Minimum Inhibitory Concentration (MIC) of 256mg/ml (Kuglerova et al.20110. Both the stem bark and leaves also exhibited antifungal activity against other species of candida including fungus and utilis.. The leaf, bark and root extract also inhibited HIV 1 reverse transcriptase in HIV positive individual, and flavonoids and tannins were attributed to this antiviral activity. In other studies, a water extracts of *W. ugandensis* were found to reduce asthmatic symptoms in mice similar to dexamethasone drug. However antimicrobial activity of *W. ugandensis* was to found to vary across different locations in Kenya ( Abuto et al., 2016).

### ***2.3.5 Safety studies on W. ugandensis***

*Warbugia ugendensis* is reported to be nontoxic with acute toxicity with LD50 of more than 5000mg/kg body weight (Karani et al., 2013), and this was also demonstrated in *Drosophila melanogaster* at acute exposure, though chronic exposure was reported to be toxic ( Ahmed et al.,2017)

# **3. MATERIALS AND METHODS**

## **3.1 Study material Collection and preparation**

The three materials for this study are; Bee propopolis , bee venom and *W.ugandensis* stem bark. Bee venom will be collected from Kabalore bee-keepers association from 20 different bee farmers in the district totaling to two hundred bee hives. Bee propolis will be collected from at least 20 bee hives among different bee farmers in Bundibugyo. *W. ugandensis* will be collected from three existing trees at NCRI demonstration medicinal plants garden.

During collection of material, issues of quality will be taken into consideration avoiding variations due to extraneous factors . Bee venom will be collected within two days of harvesting, put in amber bottles , stored in cold storage and transported to the laboratory for further cold storage. Similarly bee propolis will harvested as resin, put in cold storage and transported to laboratory for cold storage. *W. ugandensis* leaves will be harvested taking into consideration the age of the plant, dried in an oven until the moisture is less 12% and then pulversied into powder. The powders will be extracted with different solvents for phytochemical analysis

## **3.3 Chemical analysis of Bee venom and *Warbugia ugandensis* samples**

### ***3.3.1 Qualitative analysis***

Bee venom will be analyzed qualitatively for peptides and amino acids using standard qualitative methods . Gel permeation chromatography or size exclusion chromatography and FTIR will also be used to analyzed the bee venom. Bee propolis and *W. ugandensis* will be extracted with different solvents characterized for various phytochemicals like alkaloids, terpenoids, anthracenocides, tannins, flavonoids, saponins, reducing compounds and anthocyanins using standard methods reported by Culei, 1964. Thin layer chromatography , GC-MS and FTIR will also be used to characterize the different compounds in *W. ugandensis* extracts.

### ***3.3.2 Quantitative analysis***

Bee venom will be tested for the concentration of melittin(major component) using HPLC techniques. Melittin standard will be used to develop the calibration curve for estimating the concentration of melittin in bee venom. The antioxidant activity of bee venom will also be conducted using spectrophotometric methods.

Bee propolis and *W. ugandensis* leaf extracts will be subjected to quantitative analysis for total phenols, total alkaloids, total flavonoids and total tannins using spectrophotometric and gravimetric methods. Further analysis using HPLC and HPTLC techniques will also be employed with the help of standard to quantify the different compounds in bee propolis and *W. ugandensis*. By using the chemical profile of bee propolis, bee venom and *W. ugandensis*, literature review will be conducted to identify the antiviral compounds.

## **3.5 Safety Studies**

 Bee propolis extract , bee venom and *W. ugandensis* extract will be subjected to both acute and sub-acute safety studies using laboratory animal models.

1. ***Sighting study***

This is used to allow selection of the appropriate starting dose for the main study especially when information on the toxicity of the substance is not known. A total of 7 animals will be used and administered e.g. 1,3,10,30,100,300 and1000 mg/Kg bodyweight of the test extracts. A Minimum Lethal Dose (MLD) and a Maximum Tolerated Dose (MTD) will also be determined. Animals will be administered fixed doses and observed for 24 hours for any observable adverse effects.

1. ***Main study***

**Experimental animals**

Wistar rats of one sex (aged 12–14 weeks, weighing 180–240 g) obtained from the animal facility at the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Kampala will be used for determination of LD50 estimates. The animals will be divided into 5 groups of 5 animals in each group. Selected Dose levels to be administered to animals will start at a level below the estimated LD50 and increased in incremental doses up to the upper fixed dose of 1000mg/Kg bodyweight. Time interval between dosing at each dose level will be determined by the onset, duration and severity of observable toxic signs. Treatment at the next dose level will be delayed until there is certainty of survival of previously dosed animals if toxicity is observed. Recommended time interval is usually 3 – 4 days. Formulae e.g. probit or locke’s method

1. ***Repeat dose toxicity***

**Method: Sub acute toxicity test**

Wistar rats of one sex will be used to determine short term accrued toxicity levels (28 days). Animals will be divided into 5 groups of 12 animals each. Group I will be administered with normal saline (0.9 %), Group II will be given 1/5th of the LD50 of the extract and Group III will be given 1/10th of the LD50 of the crude extract and group IV will be given a dose below the LD50 determined in the LD50 estimation study, group V will be given maximum tolerated dose as determined in the sighting study using i.p and p.o routes of administration., The results obtained will provide information on target organs of toxicity and short term toxicity resulting from repeated exposure of the animals to the extracts of bee venom and *W.ugandensis*

***Observation***

Animals will be observed individually after dosing for the first 30 minutes periodically in the first 24 hours and daily thereafter for 14 days.

Animals will be observed for:

* Changes in body weight before administration of extracts and weekly thereafter.
* Gross pathology: Changes will be recorded and microscopic examination of organs showing gross pathology for animals surviving 24 hours or more after initial dosing will be conducted. Assessment of changes in clinical biomarkers will be determined

Data will be analyzed though Analysis of variance ( ANOVA).

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## **3.5 Antimicrobial test**

Invitro antiviral activity of bee propolis, bee venom and W. ugandensis extract will be confirmed using Vero cell lines. The IC50 of each sample will be determined. Similarly, invitro antibacterial activity will be conducted impregnated discs against E. coli, Salmonella, S. aureus, Streptococcus pneumoniae etc. Minimum inhibitory concentration will be determined

**3.6 Efficacy studies**

***3.6.1 Invivo antiviral activity***

The *invivo* antiviral activity efficacy studies will be conducted after challenging the lab rats with viral infection. Dose responses of the different groups will be determined. In addition , the rats in the different groups will also be monitored for dose effect on T-cell percentages (i.e. CD3+, CD4+ and CD8+) and Th cytokines; *in vivo* SRBC specific humoral and cellular immune responses ; and in vivo CD3+ (total T cell) and CD19+ (total B cell) percentages.

To conduct *invitro* efficacy studies, cytopathic effect on cell culture, cytotoxicity assay to measure cell viability, and cytotoxicity effect on vero-cells will be conducted.

## ***3.6.2*** ***Immuno modulatory studies***

The rats (male and female, 8-12 weeks old) will be randomly distributed in groups as per experimental protocols (n = 6 or 10). They will be kept under room temperature in pathogen-free isolators with temperature of 23 ± 2 ◦C and humidity of 55.6 ± 10% on a regulated 12-h light and dark cycle. They will be fed on standard laboratory feeds and tap water ad libitum. Blood samples will be collected through retro-orbital bleeding at specified time points under ether anesthesia and assayed for cell counts, cytokines and antibody titers. The OECD method will be used to determine Maximum tolerable dose (MTD) determination in animals (OECD, 1996). Test material will be orally administered in graded doses and animals monitored for changes in weight, general behavior and mortality at 0.5, 2, 6 and 12 hourly intervals after test material administration and the maximum tolerable dose determined

Sheep red blood cells (SRBC) suspension in Alsever solution will be obtained from animals. Blood cells will always be washed three times with pyrogen free sterile normal saline (0.9% NaCl, w/v). Each mouse will receive 1 × 109 cells in volume of 0.2 mL i.p. for sensitization and challenge at required time schedule. This cell count has been reported to induce optimum immune response in normal and immune suppressed conditions (Bani et al., 2006).

Three independent experiments will be performed using levamisole (2.5 mg/(kg p.o.)) as positive control. The first will on effect of bee venom and *W.ugandensis* extract on T cell percentages (CD3+, CD4+ and CD8+) and Th cytokines. To conduct this experiment**,**  Immunizations will be carried out using SRBC (1 × 109 cells in 0.2 mL saline/i.p.) on day 0 and 7. From day 0 (2 h post-SRBC injection) to 6, the treatment drugs at varying doses of, 50, 100 and 200 and 400 mg/kg will be administered orally once daily in respective groups. Cyclosporin (5 mg/(kg b.w) will be administered 48 h prior to sensitization as negative control. Blood will be collected 48 h post-challenge for estimation of CD3+, CD4+, CD8+ and CD4+ (IL-2, IL-4 and IFN-gamma) percentages using flow cytometry.

#### The second experiment will determine the effect of bee venom and W.ugandensis extracts on in vivo SRBC specific humoral and cellular immune responses . The design of the first experiment will be conducted, bee venom and W.ugandensis extracts will be orally administered in varying doses of ( i.e. 50, 100, 200 and 400 mg/ (kg b.w. P.o) and sera will be collected on day 9 for estimation of antibody titers. Cyclophosphamide will be used as negative control at 250 mg/(kg b.w. p.o.)/ 48 h prior to sensitization. The estimation of antibody titers will be done using standard haemaglutination test (Nelson and Mildenhall, 1967). Titers will further be converted to mean log2 values for analysis purpose.

The method of Doherty will be followed to assess SRBC induced DTH response in rats (Doherty, 1981). Cyclosporin will be used as negative control at 5 mg/(kg b.w. p.o.) 48 h prior to sensitization. The immunization and treatment schedules will be similar to humoral study except for challenge procedure. Animals will be challenged with subcutaneous administration of SRBC in the left hind footpad while the right hind paw received saline. On day 9, difference between left and right paw thickness/swelling of foot will be measured using a digital plethysmometer.

The third experiment will determine the effect of bee venom and *W.ugandensis* extract on in vivo CD3+ (total T cell) and CD19+ (total B cell) percentages in Naïve (unsensitized) and sensitized animals. In this experiment, SRBC sensitized and/or unsensitized animals will be treated either with vehicle, cyclophosphamide (250 mg/(kg p.o.) 48 h prior to sensitization), levamisole (2.5 mg/(kg p.o.) for 7 days) or the agreed dose of the test drug for 7 days). CD3+ (total T cell) and CD19+ (total B cell) percentages will be determined on 7th day using flow cytometry.

The analysis of subsets i.e. CD3+ (total T cell), CD19+ (total B cell) CD4+ (T-helper cells) and CD8+ (cytotoxic cells) will be performed on peripheral blood (Bani et al., 2005). Briefly, rats will be bled at required time schedules and 50 µL of blood was added to falcon tubes containing different immuno-labeled monoclonal antibodies. After mixing and incubating at room temperature for 30 min in the dark, FACS lysing solution will be added. The samples will be incubated for 10 min at room temperature, followed by centrifugation. The cells will be washed and enumeration of lymphocytes subsets will be done using flow cytometer using Cell Quest Pro software. 10,000 events will be collected to analyze CD4+, CD8+ T cells.

The detection of cytokines in peripheral blood will be performed as per BD Biosciences protocol and reported by Bani et al., (2005). Briefly, to 80µL of peripheral blood CD4+ and CD8+ monoclonal antibody (mabs) will be added. After mixing and incubating at room temperature in the dark, FACS lysing solution will be added. The samples will be incubated followed by centrifugation at 10,000 rpm for 10 min. Cells will then be washed, permeabilized and stained with FITC-coupled CD4+ mouse (mab), phycoerythrin (PE) coupled IL-2, IL-4, IL-10 mabs in one set and PE coupled CD8+ mabs, FITC coupled IFN- mabs in another set.

 All monoclonal antibodies mentioned here will be purchased from reliable suppliers. The stained cells will then be acquired using a flow cytometer. For gating and calculation; cell quest software (Becton Dickinson, San Jose, CA) wil be used. Gating for lymphocytes using forward/sideward scatter will be facilitated by CD4+/CD8+ staining; 10,000 cells will be determined with at least 100 cells in every gate of lymphocyte subpopulations. The resulting numbers are percentages of cytokine expression of those subpopulations.

Data will be expressed as mean ± S.E. Percent immunomodulatory activity in normal and immune suppressed animals will be derived using earlier reported method: Normal conditions = (test group − sensitized control group/sensitized control group) × 100. Immune suppressed conditions = 1 − (test group − sensitized control group)/(cyclosporin/cyclophosphamide control − sensitized control) × 100 (Kaul et al., 2003). Statistical significance of differences will be assessed by Post-ANOVA (Bonferroni test for multiple comparisons). IFN-g/IL-4 ratios will be evaluated using Mann–Whitney test. P < 0.05 will be set as the level of significance

## **3.7 Anti-inflammatory activity studies**

Anti-inflammatory properties of Bee Propolis, venom and W. ugandensis extracts will be conducted using standard egg albumin-induced rat paw edema test. In brief, rats will be treat with distilled water as negative control (10 mL/kg, p.o.), bee venom/ Propolis and Warbugia (six groups of five rats each ct) at different doses in mg/kg, p.o and i.p, and chlorpheniramine (60 mg/kg, p.o.) as positive . One-hour post-treatment, edema will be induced by injection of egg albumin (0.1 mL, 0.01g/mL saline) into the sub plantar tissue of the right hind paw. The linear paw circumference will be measured using the cotton thread method of Bambgose and Noamesi (1981). Linear paw circumferences of rats will be determined just before injection of the phlogistic agent and at 30-min intervals for 3 h. (Vogel et al 2002

## **3.7 Anti- pyretic activities**

Anti-pyretic properties will be determined using Yeast Induced Hyperthermia in rats method. In brief, test extracts (bee Propolis, venom & W. ugandensis*)* will be evaluated for their anti-pyretic activities in rats with fever induced by Brewer’s yeast following the established method by J. Tomazetti et al, 2005 in rats with some modifications. At zero hour, the basal rectal temperature of each rat will be recorded using a clinical digital thermometer. Pyrexia will be induced by subcutaneous injection of 15% w/v suspension of Brewer’s yeast in distilled water at a dose of 10 mL/kg body weight.18 hours after injection of Brewer’s yeast, the rise in the rectal temperature of the experimental animals will be recorded and only animals showing an increase in temperature of at least 0.6°C will be selected for the study. The animals will be randomly divided into four groups, each group containing five rats. Group I will receive 1% Tween-80 in normal saline orally. Group II will be given the standard drug paracetamol at the dose of 100 mg/kg orally. Groups III, IV and V will receive increasing doses of the test extracts at selected doses in mg/Kg body weight. After the treatment, the temperature of all the rats in each group will be recorded at 0 hour, 1 hour, 2 hours, 3 hours, and 4 hours.

## **3.8 Formulation of therapeutic natural product**

Based on the chemical characterization , efficacy and safety studies, different formulation forms will be developed. The dosage form of the product will be designed e.g lozenges, tincture, syrup, capsule, injection etc. The formulated products will be submitted to NDA for approval and registration. Pilot production of the standard products will be produced following GMP. The products will under Pharmacovigilance and also tests like drug bioavailability ,drug interactions, and stability studies.

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# **Budget for Work package1 (Pre-clinical) 1st July – Dec 31, 2020**

|  |  |  |
| --- | --- | --- |
| **Project Objective** | **Activities** | **Cost** |
| a) To determine the chemical composition of bee propolis and bee venom  | * Purchase of reagents, solvents, protective gear and consumables
* Harvesting, transportation and storage of bee propolis and bee venom from Kabarole district.
* Analysis of the chemical composition of bee propolis and bee venom using :HPLC, AAS, GC-MS and gel filtration chromatography
* Literature review of chemical compounds identified
* Data analysis
 | 77,574,000 |
| b) To determine the chemical composition of *W. ugandensis* leaves | * Purchase of standards
* Collection, storage and processing of *W. ugandesis* leaves.
* Extraction and analysis of *W. ugandesis* using: TLC, HPLC, GC-MS, AAS and FTIR
* Review the literature related to the chemicals identified
* Data analysis
 | 28,750,000 |
| c) To evaluate the safety profile of bee venom, propolis, *W. ugandensis* and combinations of extracts to be used against COVID – 19 disease related symptoms in laboratory animals | * Purchase; animal models (rats and mice), reagents and consumables, safety gear, animal cages and feeds.
* conduct dose response studies
* Toxicity and pathology studies.
* To conduct LD50, sub-acute toxicity and chronic toxicity studies
* Clinical chemistry and histopathology
* Data analysis
 | 88,038,000 |
| d) To conduct in-vivo antiviral, anti-inflammatory, anti-pyretic and immune modulatory activities of bee venom, propolis, warbugia and combinations in animal models | * Purchase; animal models (rats and mice), reagents and consumables, safety gear, animal cages and feeds
* Anti-inflammatory studies, antipyretic studies and immunomodulatory studies in animal models
* Data analysis
 | 142,271,000 |
| e) To establish the in-vitro antiviral and antibacterial activities of bee propolis, bee venom and *W. ugandensis* | * Use the Vero cell lines to assess antiviral activity
* Use impregnated discs to assess anti-bacterial activity
* Determine MIC and IC50
* Assessing single and combinations of the products
* Data analysis
 | 88,138,000 |
| f) To formulate standardized natural products from bee propolis, bee venom, *W. ugandensis* ingredients and or combinations | * Purchase and collection of materials for Formulation of the standardized products.

 Designing of dosage forms from the standardized products* NDA notification, approval and clearance
* To conduct continuous pharmacovigilance of the products .
 | 30,000,000 |
|  | Remuneration of the Project staff for implementing activities | 85,000,000 |
|  | Administrative costs. routine office running expenses, travel inland, fuel, oil and lubricants, car service and maintenance, printing, stationery and photocopying, small office equipment, office furniture, telecommunication, internet services, allowances, computer supplies, books periodicals and journal subscription. | 90,229,000 |
| **TOTAL** |  | **630,000,000** |