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Research Article

Effects of Bee Bread Extract from *Meliponula Ferriguinea* on Chemical Induced and Bacterial Infected Corneal Ulcers of Rabbit Eyes

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ABSTRACT

Aim: Corneal ulcerations are immediate risks to avoidable blindness. In many developing countries, their treatment present huge challenges, often due to increased resistance to anti-bacterial drugs, costs and accessibility issues. Bee bread is a valuable apitherapeutic product greatly appreciated by the natural medicine because of its potential medical and nutritional applications. In this study, we investigated the pharmacological effect of extract from M. *ferriguinea* on chemical-induced and bacterial-infected corneal ulceration of rabbit's eyes.

Method: A randomized-controlled experiment, was conducted on 15 New Zealand rabbits weighing 1.4 ± 0.42 kg-randomly assigned to 3 groups; A (Experimental group), B (Positive Control group) and C (Negative control group) of five animals each. All rabbits were adapted for 2 weeks. The right eye corneas were then injured using a drop of 1 Molar sodium hydroxide (NaOH). After 12 hours, animals in all groups in their injured corneas were infected with 1 drop of a laboratory prepared solution of *P Aeruginosa*. Treatment with extracts of M. *ferriguinea*-groups A group B treated with ciprofloxacin then group C treated with atropine alone commenced after 24 hours and continued every 4 hours for 7 days.

Results: Although the mean healing effect of extracts of M. *ferriguinea* was not significantly ($p>0.05$) better after the 168th hour of treatment compared to its effect after the 24th hour, the effect size however, was clinical significant (57%). Furthermore, we found no significant difference ($p>0.05$) between the mean healing effects of the standard treatment protocol and the extracts of M. *ferriguinea*, but the standard treatment protocol showed a better clinical effect (33%) over the experimental extract. Also, the healing effects of atropine alone showed a better clinical effect (22%) than that of the experimental extract, but again, these were not statistically significant ($p>0.05$).

Conclusion: Extracts of M. *ferriguinea* shows anti-inflammatory and anti-infective effects on chemical-induced and bacterial-infected corneal ulcers in rabbit's eyes. We found these effects to be comparable to that of standard treatment protocol for bacterial corneal ulcers. We thus conclude that in resource-constrained areas where M. *ferriguinea* is richly available, their extracts may provide alternative and/or complementary treatment option for chemical-induced and bacterial-infected corneal ulcers.

Keywords: Bee bread; *Meliponula ferriguinea*; Chemical-induced corneal ulcer; Bacterial-infected ulcer; Standard treatment approach; Alternative or complementary treatment.

INTRODUCTION

Cornea is composed of five separate layers that is an outer stratified layer of squamous non-keratinized epithelium, Bowman's membrane, stromal layer, Descemet's membrane and the innermost endothelial layer. Corneal epithelium serves as the first line barrier against infections or insults from harmful environmental agents. In superficial corneal injury such as corneal abrasion which is often caused by mechanical injuries

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such as trauma or chemical burn [1]. The corneal epithelial integrity is affected [2]. Thus, a faster rate of wound healing of the corneal epithelium is vital for optimal function and protection of the inner structures of the cornea. Corneal epithelial wound healing occurs in three phases i.e. migration, cell proliferation, and remodeling. During wound healing Epithelial cells express the specific corneal epithelial differentiation marker, cytokeratin 3 (CK3) and cytokeratin 12 (CK12) once the cells left the limbal basal layer during centripetal migration [2].

Diseases affecting the cornea are a major cause of blindness worldwide, second to cataract in overall importance. The epidemiology of corneal blindness is complicated and encompasses a wide variety of infectious and inflammatory eye diseases that cause corneal ulcer then corneal scarring, which ultimately leads to functional blindness. These diseases include; trachoma which blinds 4.9 million mainly as a result of corneal ulceration, scarring and vascularization, Ocular trauma and corneal ulceration responsible for 1.5-2.0 million new cases of monocular blindness every year, exophthalmia (350 000 cases annually), ophthalmia neonatorum [3].

Until recently, ocular trauma and corneal ulceration were not considered as important causes of corneal blindness. Both trauma and ulceration are usually monocular and affected individuals are, therefore, not characterized as totally blind but only as visually disabled. However, as public health programs have become more effective in reducing the prevalence of traditional causes of corneal blindness, such as trachoma, onchocerciasis, and leprosy, so ocular trauma and corneal ulceration have become relatively more important [4]. In 2009, the attention was drawn to the fact that trauma is often the most important cause of unilateral loss of vision in developing countries and that up to 5% of all bilateral blindness is a direct result of trauma. The implication is that well over half a million people in the world are blind as a result of eye injuries [5]. A careful analysis of the world literature by brought to light a global epidemic of ocular trauma with some 55 million eye injuries occurring annually, of which 750000 cases required hospitalization and 200 000 were open-globe injuries [6]. They further estimated that approximately 1.6 million people were blind from their injuries, 2.3 million had bilateral low vision, and 19 million were unilaterally blind or had low vision.

Beebread is processed pollen stored in the cells of the honeycomb, with the addition of various enzymes and honey, which undergoes lactic acid fermentation.

Beebread has been traditionally used for a variety of purposes, including relief of constipation; treatment of prostatic conditions, such as prostatitis, benign prostatic hyperplasia and prostate cancer; wound healing; and for its proposed antioxidant action. It has also been promoted as an energy booster, immune system strengthener, and vitality enhancer [7]. Bee bread has been used to prevent hay fever, but there is a risk of severe allergic reaction with this practice. It may also relieve pre-menstrual syndrome and climacteric symptoms associated with menopause [8].

Pollen bread was found to possess an antibacterial activity against *Staphylococcus aureus* and *S. epidermidis* [9]. In a study with 80% ethanol extracts of Brazilian pollen, antibacterial activity was exhibited against *P. Aeruginosa* and *Klebsiella spp* [10]. The antibacterial substances of pollen active against Streptococcus viridans are similar to the ones found in propolis and honey combs [11]. In several studies Bee bread was also found to have a high antioxidant activity [12].

MATERIALS AND METHODS

The study was carried out in the animal house in Masinde Muliro University Of science and technology (MMUST), Kakamega County. This was a controlled experimental study design performed on fifteen (15) New Zealand rabbits. The animals' was pre-examined to check eyelid, lacrimation and corneal integrity and also post-tested after 48 and 72 hours of introduction of treatment to ascertain changes that may have resulted from treatment protocol on the rabbit eyes. The rabbits was randomly distributed into three groups; A, B and C of five animals each, without gender bias.

Fifteen (15) New Zealand Rabbits were randomly distributed into three (3) groups of five (5) animals each, by simple balloting of the fifteen selected animals labeled one to fifteen. The groups were designated as;

Group A-experimental group. This group was injured chemically by 1molar sodium hydroxide to cause corneal ulcer, infected with *P Aeruginosa* and then treated with bee bread extract.

Group B-Positive control group. This group was injured chemically by 1molar sodium hydroxide to cause corneal ulcer, infected with *P Aeruginosa* and then treated with standard treatment approach for corneal ulcers using fortified ciprofloxacin eye drop.

Group C-Negative control group. The animals in this group received atropine only.

Instruments for data collection

Pen torch: This was used together with a head-mount magnifying loupe to illuminate and all examination of the external features of the rabbit eyes. This was done before and after experimental manipulations, Magnifying loupe-Used for a magnified assessment of external ocular features, Keeler ophthalmoscope-Used for assessment of the internal structures of the rabbit's eyes to ascertain the baseline internal features, Perkins applanation tonometer was used to measure the intraocular pressure of the animals before and after treatment, 2ml hypodermic syringes and needles-was used for intramuscular injections of systemic anaesthetics (Kitamine), Sample tubes-for procedural extraction of bee bread, Micro pipette and pipette. Procedural extraction of bee bread, Beakers, Spatulas, Weighing scale –Weighing bee bread quantities and rabbits, Water bath, Mortar and pestle-Pounding of bee bread, Sterilize laboratory grinder, Turtle stand what man filter paper, Dettol, Glove and Small bucket with cover –to be used during injection process, Methylated spirit-used during injection, Vaseline and Razor blade-shaving place for injection, Cotton wool-used to prevent spreading of the chemicals beyond the cornea when inducing

injury on the cornea, Nikon digital camera-used to photographs of the observations throughout the experiment duration

Drugs

Bee bread from *Meliponula ferriguinea* was obtained from MMUST Science Park, in Kakamega County, Kenya. The bee by-product was extracted for constitution into a pharmacologically usable suspension at the pure and applied chemistry laboratory of the university, Ketamine Hydrochloride anaesthetic eye drop manufactured by Alcon-Couvreur, B-2870 Puurs, Belgium and Primax manufactured by Ashford laboratory ltd macau, Flouret® flourescein Sodium: sterile fluorescein strips manufactured by Chanvin Pharmaceuticals Ltd. Harold Hill, Ranford, Essex RM 38SL, Keproceryl® hydrosoluble mix of antibiotics and vitamins manufactured by Kepro B.V-Maagdenburgstraat, 38-7241 ZE-Deventer, Holland, Vitaflash Amino® vitamins-amino acids for nutritional supplement. Manufactured by Kitale Animal feeds Industries, Broad spectrum anticoccidial and vitamin K, Vitamin A, Folic acid, cicatrin powder, Ivermetrins and Admacin, Ciprofloxacin eye drop by Indocco.

These drugs were all purchased from Lessos Agrovet in Eldoret, Kitale and Kakamega towns.

DESCRIPTION OF PROCEDURES

Acclimatization of the animals

Fifteen healthy rabbits were purchased and adapted for one month. During the period of adaptation, they were dewormed with ivermetin, vitaflash (a multivitamin was administered for three days to serve as anti-stress followed by an anti coccidiasis drug called embazzin forte and finally keproceryl powered (antibiotics mixed with vitamin) was given to them. They were fed with pellet, elephant grass, vegetable and their cage was cleaned daily.

Procedure for Intraocular pressure measurement

Baseline ocular physiologic-the tear flow rate (TFR) -states of each animals were measured on daily prior to the experiment and after the experiment period-post-test, so as to ascertain any change in the these ocular function and body physiology possibly occasioned by the experimental agent (bee bread).

Weight measurement

The Weight of each animal was taken just the day of acquisition and then prior to commencement of experiment documented. After the experiment the weight was also measured after research to find out the effect of treatment on feeding habits.

Tear Flow rate assessment (schimmer's test)

Schirmer 1 test was done (without anesthetic) to measures baseline and reflex secretion function of main lacrimal gland, whose secretory activity is stimulated by the irritating nature of filter paper. Schirmer 2 was performed (without anesthetic): measures baseline secretion. This was to assess the function of accessory lacrimal glands (the basic secretors) during pre and post treatment our method used 5 × 35 mm of Schimmer's strip. The schirmer strip was folded 5 mm from one end and kept in the lower fornix at the junction of lateral 1/3 and medial 2/3 (to avoid touching cornea or lashes).We closed the rabbit's eye. Tears in the Conjunctival sac caused progressive wetting of the paper strip. After 5 minutes, the filter paper was removed and the distance between the leading edge of wetness and the initial fold is measured, using a millimeter ruler.

Inducing Corneal Ulcer

10 mls of 1 Molar (sodium hydroxide) NaOH was prepared and refrigerated. This was done by the Department of Chemistry of MMUST. 1 ml/50 mg of ketamine hydrochloride was injected intramuscularly to each animal of groups A, B C and D. This was employing the services of an expert from the Medical laboratory science department. 5 minutes later 2 drops (1 ml of 0.5%) Nepucaine (Amethocaine) hydrochloride was instilled in the right eye and after 5 minutes 0.2ml/1molar NaOH instilled on the central corneal surface. To prevent NaOH from spreading to other areas the conjunctiva, they protected, by guarding the corneal borders with cotton wool. Affected area was then be stained with fluorescein strip and observed with a 20 times magnification using a blue illumination of an ophthalmoscope. This is to ensure corneal ulcer is successfully induced.

After 12 hours of induction of the corneal ulcer the *P Aeruginosa* prepared by representative from microbiology department in solution form was introduced into all the rabbits right eye that was injured to cause infection then after 12 hours the treatment commenced in both groups while monitoring and recording the ocular manifestation that included state of the lid, Conjunctival status (injection and lacrimations) epithelial morphology (cells eruption), corneal fluorescein staining, photophobia, discharge, Schirmer test, anterior chamber status and corneal infiltration. To be consistent, we was graded as very severe-5, severe-4, moderate-3, mild-2, very mild-1 and normal-0 [13].

Preparation of Bee bread extracts (BEE)

The preparation of the bee bread extract was done in the Department of Pure and Applied Chemistry laboratory. The aqueous extract of bee bread (AEBB) was prepared by dissolving five grams (5gms) of bee bread in 500ml of distilled water. The suspension obtained was shaken and kept in a refrigerator for 72 hours before application.

Treatment of Cornea Injury: The group that contained active ulcer that was then infected with P Aeruginosa after 24 hours following induction of injury on the right eye cornea of each animal with infection, and sequent observation of ocular changes, treatment commenced. All the animals in group A were treated with the formulated eye drops from bee bread extracts. Two (2) drops of the bee bread suspension were installed two hourly for 7 days. Observed ocular changes following commencement of treatment were recorded according to same stated grading system, as well as photographed. The observed parameters were photographed and equally recorded systematically in an order of 12 hours, 24 hours, 48 hours, 72

hours, 96 hours, 120 hours, 144 hours, and 168 hours, after treatment commenced. Treatment was tapered slowly following observation of any improvement (possible healing) on ocular inflammations and/or infections. After 168 hours of treatment with the bee bread formulation, IOP measurement was conducted every 8 hours for possible changes in diurnal variation. This was done for three (3) consecutive days and the results was compared, first for eyes findings, pretest and posttest findings (within group observation) and also compared with findings of the control groups (between groups observation).

Positive Control group-B: Treatment of the animals in Group B was commenced 24 hours following induction of injury on the right eye cornea. A standard treatment protocol for chemically (alkaline) induced ocular injury was employed. This included two (2) hourly drop of floroquinolone (ciprofloxacin), four (4) hourly drop of corticosteroid (Betametasone), and four (4) hourly drop of atropine. These were gradually tapered following observation of significant reduction of ocular inflammations and/or infections. As with the experiment group, photographs and recording according stated grading system was done starting after 12 hours, through every 24 hours up to the 168th hour. While IOP measurement and TFR assessment protocol was equally done as with the experiment group A to ascertain changes and for comparison within and between groups.

Negative Control group-D: No active positive treatment was given to the animals in this group. However, in line with the treatment schedule periods of groups A and B, artificial tears (hypemerolose eye drops) used as vehicle for the bee bread extract, was instilled topically (2 drops), two (2) hourly on the right eye cornea of each animal in this group. Observations (photographs and recording of parameter changes) were done according to schedules of groups A and B. While as with groups A and B, IOP measurement and TFR assessment protocol was equally done to ascertain changes and for comparison within and between groups.

The data were collected on ocular features and parameters observed and assessed in the course of the experiment (pretest and post-test) were photographed and recorded according to the pre-stated grading system, in all four groups. The parameters of interest here were; eyelid status, conjunctival status (injection), lacrimation, epithelial disruption, corneal fluorescein staining, photophobia (using a flash light), discharge, corneal edema, anterior chamber status and corneal infiltrates. The ocular parameters were as previously stated, graded on a scale of 5 to 0 (Very severe-5, Severe-4, Moderate-3, Mild-2, Very mild-1 and normal/no change observed-0 [14]. These grading and photograph evidence were recorded according to the first 12 hour finding following commencement of treatment, through next 24 hourly to the 168th hour findings.

RESULTS AND PRESENTATION

The tables below table 1, 2, 3 shows the results of treatment for both groups A, B and C after 24 hrs. And 168 hrs.

Table 1: Results of group A between 24 hours and 168 hours.

	TIME POINT											
	24 HOURS					168 HOURS					P VALUE	EFFECT SIZE
group A (rabbit no)	6	12	14	8	9	6	12	14	8	9		
eyelid status	5	5	5	5	5	1	1	1	1	1	0	0.6666
Conjunctival status	5	5	5	5	5	3	2	0	1	2	0	0.1818
lacrimation	5	5	5	5	5	3	3	1	2	2	0	0.8666
epithelial disruption	5	4	5	1	2	0	0	0	1	1	0.43	0.6316
corneal fluorescein staining	5	4	5	1	2	0	0	0	1	1	0.43	0.6316
photophobia using flashlight	5	5	5	5	5	2	1	1	1	1	0	0.2838
Discharge	5	5	5	5	5	1	2	0	1	1	0	0.76
corneal edema	5	4	5	4	4	1	1	0	1	3	0.24	0.8858
corneal infiltrates	1	2	2	3	2	1	1	0	1	0	0.584	0.2154
mean p value and effect size											0.187111	0.5692

Table 2: Results of group B between 24 hours and 168 hours.

	TIME POINT											
	24 HOURS					168 HOURS					P VALUE	EFFECT SIZE
GROUP B (rabbit no)	13	4	15	5	3	13	4	15	5	3		
eyelid status	5	5	5	5	5	1	1	0	0	1	0	0.4572
Conjunctival status	5	5	5	5	5	0	0	2	0	0	0	0.9186
lacrimation	5	5	5	4	5	2	2	1	1	2	0	0
epithelial disruption	5	2	0	3	0	0	1	0	0	0	0.364	0.6364
corneal fluorescein staining	5	2	0	3	0	0	1	0	1	1	0.691	0.2924
photophobia using flashlight	5	5	5	5	5	1	3	2	1	1	0	0.8848
Discharge	5	5	5	4	5	0	0	2	0	1	0	0.074
corneal edema	5	5	4	4	5	2	1	3	0	0	0	0.6482
corneal infiltrates	5	0	0	2	0	0	0	1	0	0	0.197	0.65
Mean of p value and effect size											0.139111	0.51

Table 3: Experimental group C after 168 hours of treatment.

	TIME POINT											
GROUP C	24 HOURS					168 HOURS					P VALUE	EFFECT SIZE
(rabbit no)	7	1	2	11	10	7	1	2	11	10		
eyelid status	5	5	5	5	5	0	1	1	0	1	0	0.4572
Conjunctival status	5	5	5	5	5	2	3	0	3	0	0	0.491
lacrimation	5	5	5	5	5	0	3	0	3	0	0.001	0.329
epithelial disruption	2	1	3	4	4	0	3	0	1	0	0.112	0.2
corneal staining	2	1	2	4	4	0	0	0	1	0	0.135	0.8
photophobia	5	5	5	5	5	3	1	0	2	0	0	0.2904
Discharge	5	5	5	5	5	1	2	0	3	0	0	0.2904
corneal edema	5	4	5	5	3	0	3	0	2	0	0.02	0.926
corneal infiltrates	0	0	0	1	3	0	2	0	2	0	0.417	0.35
mean p value and effect size											0.076111	0.4593

The data shows that after 168 hours eyelid status conjunctival status and discharge had tremendous improvement both having high significance results with p value of 0 which was within our confidence interval of 95% their clinical healing effects also

showed great percentage of average 56% despite showing no significance in p value on average (table 4).

Table 4: Comparison of group A and B after treatment.

TIME POINT												
AT 168 HOURS												
	GROUP A					GROUP B					P-VALUE	EFFECT SIZE (%)
rabbit numbers	12	11	5	9	13	14	10	3	1	7		
Eyelid Status	2	2	1	1	1	0	0	1	0	2	0.527	8
Conjunctival status	2	2	2	2	2	0	0	1	0	2	0.002	86.16
Lacrimation	1	0	1	1	2	0	1	0	0	1	0.257	25.72
Epithelial eruption	3	1	2	2	2	1	0	3	1	3	0.223	60
Corneal staining	2	1	2	2	3	1	0	3	1	3	0.223	60
photophobia	2	2	1	2	2	1	2	3	3	0	0.069	6.66
discharge	2	1	0	1	1	0	0	0	0	0	0.655	4
Corneal Edema	2	0	2	1	3	0	1	0	0	1	0.905	4
Corneal infiltration	2	2	2	1	1	0	1	0	0	2	0.132	45.46
Mean effect and p value											0.332556	33.3333

The comparison between experimental group A and B after 168 hours of treatment shows a high p-value for discharge and corneal edema but a low clinical significance compared to Conjunctival status (Table 5).

Table 5: The comparison of treatment outcome of group A and C after 168hrs of treatment.

COMPARISON A and C at 168hrs												
	Group A						GROUP C				P VALUE	EFFECT SIZE (%)
RABBIT NUMBER	6	12	14	8	9	7	1	2	11	10		
eyelid status	1	2	0	1	0	0	1	1	0	1	0.257	0.2572
conjunctival status	3	2	0	1	2	2	3	0	3	0	0.047	0.225
lacrimation	3	3	1	2	2	0	3	0	3	0	0.003	0.2824
epithelial disruption	0	2	0	1	1	0	3	0	1	0	0.882	0.05
corneal straining	2	1	0	1	1	0	0	0	1	0	0.414	0.1334
photophobia	2	1	1	1	1	3	1	0	2	0	0.779	0.1
discharge	1	2	0	1	1	1	2	0	3	0	0.913	0.0364
corneal edema	1	1	0	1	3	0	3	0	2	0	0.307	0.4728

corneal infiltrates	1	2	0	1	0	0	2	0	2	0	0.157	0.4
Average mean effect and p value											0.417667	0.2175

DISCUSSION

Pseudomonas spp is the most important human pathogen in genera *pseudomonas spp* and burkholdera regarding the issues of number and type of infections caused by their associated morbidity and mortality with a wide spectrum of disease ranging from superficial skin disorders to fulminant sepsis. *P Aeruginosa* has an affinity of trauma contaminated contact lens with whose help this could result to corneal ulcer which if not treated results to opacification and eventual loss of vision [15].

According to Table 1, lacrimation and corneal edema recorded the highest improvements after treatment with bee bread. However, other parameters measured after treatment showed less improvement (conjunctival status and corneal infiltrates).Generally, more of these parameters showed statistical significance, while others had no statistical significance. These findings were contrary to [16], who found out that bee bread and bee pollen were more effective on gram positive than gram negative bacteria. This may have resulted from differences in constituents and concentration used in the two researches, mode of bee bread extraction. This may also be due to the fact that *P Aeruginosa* is resistant against naturally antibiotics.

In table 2, most of the parameters showed significant statistical and clinical significance except epithelial eruption, corneal staining and corneal infiltrates. This shows that orthodox therapy on corneal ulcers with bacterial infection is still a preferable mode of treatment. These findings are in line with congruence with researches done by [2] who asserted that *P Aeruginosa* is only sensitive against a few antibiotics including fluoroquinolones.

The negative control in group C, table 3, shows an overall medium improvement of clinical and statistical significance. However, corneal edema showed the highest clinical improvement followed by corneal fluorescein staining.

The comparison of efficacy of bee bread in group A as compared to use of orthodox therapy (group B) in table 4 shows conjunctival status improvement as the highest with corneal discharge and edema being the lowest. However, most of the parameters had less statistical significance.

The negative control group C as compared to the experimental group A in table 5 showed an overall minimal clinical significance with less statistical significance. This shows that beebread had results of substantial clinical significance yet group C received no treatment but showed some healing effect. This implies that the immune response of the rabbits might have contributed highly in the healing process of rabbit eyes.

CONCLUSION

The result of this study showed that beebread has a significant clinical effect on chemically induced and bacterially infected corneal ulcer. The findings might not be statistically significant but it showed similar pharmacological effects with standard orthodox approach (fluoroquinolones). This is a good starting point for more studies into the efficacy of beebread. It is also a good sign since beebread can be a cheaper alternative to the standard orthodox approach. More studies are however required to enhance the knowledge base about pharmacological effects of beebread from *Meliponula ferriguinea* and other bee species.

In our final recommendation, more research should be done on beebread on a longer time period to determine the long term effects of beebread, study should be done on the immunity of rabbits and Better extraction of beebread should be employed in future studies. Better preservation of beebread during the study should be observed.

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