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Research Article

BIOCHEMICAL INDICES AND WOUND MORPHOMETRY IN RABBITS ON EXCISIONAL FULL THICKNESS SKIN WOUND HEALING EFFICACY BY USING DIFFERENT EXTRACT OF *Salix acmophylla* LEAVES OINTMENT

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Abstract: The present study was aimed to estimate the concentration of Glucose (md/dl) using Accu-Check Active Glucose meter, Zinc (µg/ml of serum) and Copper (µg/ml of serum) using atomic absorption spectroscopy method at 213.9 nm and 324.8 nm respectively and estimating the Bacterial colony count by taking the wound washings in rabbits after creation of 72 excisional full thickness skin wound and post treatment with the 5% ethanolic and 5% aqueous extract ointment group (6 rabbits each) and comparing the results with the control group (6 rabbits). The lowest concentration of Glucose and Bacterial count and highest concentration of trace elements *i.e.*, Zinc and Copper was seen in 5% ethanolic extract ointment treated group. The wounds of 5% Ethanolic extract ointment of Salix treated groups evinced complete healing (100% contraction) which was followed closely by 5% aqueous extract ointment of *Salix acmophylla* and distantly by normal saline treated group on day 21 post-wounding, where mean wound contraction /size of 100%. 99.98% and 96.24% respectively was recorded.
Keywords: Atomic Absorption Spectroscopy, Bacterial Colony Count, Copper, Zinc 5% Aqueous extract, 5% Ethanolic extract

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Introduction

Skin wounds are generally divided into acute and chronic wounds. They are not capable of closure or regaining the anatomy and function of healthy skin. Acute wounds are traumatic or surgical wounds that usually heal over time according to the normal wound-healing process. Acute skin wounds vary from superficial scratches to deep wounds with variable amounts of tissue loss and damage to blood vessels, nerves, muscles or other tissues, or internal organs [1]. Cutaneous wound healing involves migration, infiltration, proliferation and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages and platelets which culminate in an inflammatory response, the formation of new tissue and wound closure [2, 3]. Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation, and migration of different cell types. Plants are a rich source of phytochemicals, which can have wound healing and antioxidant properties. Depleted levels of various antioxidants may contribute to delayed healing. Several indigenous drugs have been described in folkloric Indian medicine for the management of cuts, bruises, burns, and wounds. Various workers have reported wound healing properties of some plants among them the chosen ornamental plant *Salix acmophylla* that is found in almost all regions of Kashmir and locally known as Wir/ Veer Kani [4]. Willow (*Salix spps.*) is the source of the natural precursor to asprin, salicylic acid, found in its leaves and bark [5]. The active ingredient of the *Salix* bark is called salicin. Salicin hydrolyzes in aqueous media to glucose and salicylic alcohol (saligenin). *Salix* spps. have abundant watery bark sap, which is heavily charged with salicylic acid. Besides, salicin, it contains flavonoids and proanthocyanidins, which are potent antioxidants and have wound healing properties. *Salix* plants contain a wide variety of compounds called phytochemicals, mainly described as those compounds having medicinal properties. Scientists have identified thousands of phytochemicals, although only a small fraction has been studied thoroughly [6].

Salix acmophylla also contains glycoside E, which have a hypoglycaemic effect [7] and this hypoglycemic condition is very effective for speedy wound healing while as in hyperglycemic condition the cell walls to become stiff and rigid, impairing the flow of blood throughout the small vessels located at the surface of the wound and also high glucose levels impair the hemoglobin release of oxygen which in turn effectively starves the affected area of oxygen and nutrients that promote healing [8]. *Salix acmophylla* contains copper and zinc at high levels [9]. These elements have important role in wound healing as they increase the expression of vascular endothelial growth factor and are potent antioxidants [10]. The result of the phytochemical screening for Salvia officinalis and *Salix acmophylla* reveals a moderate concentration of alkaloids, coumarines, cardiac glycosides, ratenges, phenols, flavonoids, saponins, tannins, essential oil and terpenes. Some of these chemical compounds have been associated to have antibacterial activities; some have antioxidant activity and some to have curative properties against pathogens [11]. So, the present study is focused on estimating the effect of different extract ointments of *Salix acmophylla* leaves on biochemical parameters and wound morphometry on full thickness excisional skin wounds in rabbits.

Materials and Methods

Plant material

The leaves of *Salix acmophylla* were collected from the fields surrounding Faculty of Veterinary Sciences, Kashmir (J&K), India, in the month of May and identified by Division of Environmental Sciences, SKUAST-Kashmir and Department of Botany, Kashmir University, Hazratbal.

Preparation of extracts and ointment formulation

The leaves of the plant *Salix spp* collected were shade dried for a week followed by drying in oven pre-set at 37°C for 4 days, the samples were powdered in electric mill and stored in airtight container.

The fine powder of leaves was extracted with boiled distilled water and Ethanol respectively by Soxhlet apparatus for 7 hrs. The solution was filtered through Whattman filter paper using Buchner funnel under vacuum evaporator at 40℃ to obtain the extract. Then the resulting extract was stored, protected from light in refrigerator at 4 °C in a glass container till further use. Ethanolic and Aqueous extract of *Salix acmophylla* (5g) was mixed with simple ointment (soft paraffin) (95g) to get a 5% extract ointment(w/w) respectively.

Creation of Wound

The experimental study was conducted in eighteen (18) adult rabbits of either sex (1.5-2.5kg) purchased from the rabbit section of Mountain Research Centre for Sheep and Goat, FVSc& AH, Shuhama/Wussan rabbitary farm, Pattan. The animals already tagged and housed individually in cages. The animals were acclimatized to approaching and handling for a period of 5-10 days prior to the study. The location of wound edges was outlined by a locally fabricated metal marker was created using a no. 15 BP blade on either side of dorsal spine in the thoraco-lumber region. The wounds were named as R on right side and L on left side. Haemorrahge, if any, was controlled by digital pressure. Procedures were carried out under aseptic conditions and in proper anaesthesia (Ketamine Hydrochloride at the dose rate of 50mg/kg and Xylazine at the dose rate of 10 mg/kg). Post-operative analgesic (Butorphanol tartrate at the rate of 0.1-0.5mg/kg S/C QID) was given for 3 days. All the ethical issues were considered in the surgical procedures and during the treatment. Each treatment group consisted of 6 animals. Thus, each treatment was evaluated on a total of 12 wounds. The animals could recover and were housed individually in metallic cages containing autoclaved drapes and received food and water ad libitum. Each wound was cleaned with sterile normal saline solution and dressed in as per the scheduled therapy.

Treatment Schedule:

Wounded rabbits were divided into three groups. The wounds of group I to serve as a control were topically washed with Normal Saline Solution (NSS) and no treatment was given, wounds of group II were washed with NSS followed by application of wounds with 5% *Salix* leaves aqueous extract ointment on wound till healing and wounds of group III were washed with NSS as accordingly followed by application of wounds with 5% *Salix* leaves ethanolic extract ointment on wound till healing.

Wound Morphometry [12]

Wound Size (cm^2)

The wound boundaries were marked with Indian ink permanent market and tracing was taken on sterile cellophane paper before starting the treatment and subsequently on day 3rd, 7th, 14th and 21st. These tracings were placed on graph paper and wound area was calculated.

Biochemical Parameters:

Glucose (mg/dl):

Glucose estimation was done on immediately after collection of blood. Blood Glucose was estimated using Accu-Check Active Glucose meter.

Estimation of serum micrometals :

Serum Zinc (µg/ml) and Copper (µg/ml) were estimated by Atomic Absorption Spectroscopy at 213.9nm and 324.8nm.

Bacterial Colony Count:

For total bacterial viable count, each wound on day of creation and then at day 3rd, 7th, 14th and 21st were washed with 3ml of sterile normal saline solution and washing was collected in sterile test tubes. One ml of each washing was used for serial dilution in NSS for estimating total bacterial count. One ml of each dilution was spread on nutrient agar plates in duplicate. Agar plates were then incubated for 24 hrs at 37oC. Bacterial colonies from required plates were counted and the total viable count will be calculated [13] as:

TVC=Average no. of colonies in the desired dilution x Dilution Factor

The total viable count will be expressed as cfu /ml of sample .

Percentage healing:

The evaluated surface area was used to calculate the percentage healing using the below formula:

$$H = \frac{A - B}{A} \times 100$$

Where,

H = Percentage healing.

A = Area of wound at the beginning of the experiment.

B = Area of wound at the end of particular period.

Percentage healing was calculated on the day of creation and subsequently on day 3rd, 7th, 14th and 21st.

Statistical Analysis

The results were expressed as Mean ± standard error. The data was analyzed using the suitable statistical program for Social analysis 20 for Windows software (SPSS Inc, Chicago, IL). One way Analysis of Variance (ANOVA) test was used to compare the means at different time intervals among different groups. A value of $P<0.05$ was considered significant.

Results and Discussion

Wound size

The Wound size denoted the amount of wound contraction. Unlike epithelialization, which closes the wound surface, contraction is a process that pulls the entire wound together, in effect shrinking the defect. Tannins can precipitate proteins, resulting in shrinkage of cells. This precipitating protein forms a coagulum. Underneath the coagulum quicker regeneration of tissue takes place [14]. The wound closure is fastest in wounds treated with 5% ethanolic extract ointment of *Salix* followed by 5% aqueous extract ointment of *Salix* and then the sterile normal saline solution [Table-1, Fig-1 and Plate-1].

Table-1 The Mean±SE values of wound size (cm^2) in the rabbits of different groups at different observation intervals

Group	Observation Intervals (Days)				
	0	3	7	14	21
I	3.13 ± 0.12^{eA}	1.57 ± 0.08^{dC}	0.91 ± 0.03^{cB}	0.59 ± 0.07^{bB}	0.11 ± 0.06^{aB}
II	3.05 ± 0.16^{dA}	1.11 ± 0.04^{cA}	0.58 ± 0.04^{bA}	0.08 ± 0.02^{aA}	0.00 ± 0.00^{aA}
III	3.03 ± 0.08^{dA}	1.38 ± 0.05^{cA}	0.53 ± 0.03^{bA}	0.00 ± 0.00^{aA}	0.00 ± 0.00^{aA}

Figures with different superscript (small letters) differ significantly ($P < 0.05$) between days within the groups, Figures with different superscript (capital letters) differ significantly ($P < 0.05$) between groups n = 6 animals in each group

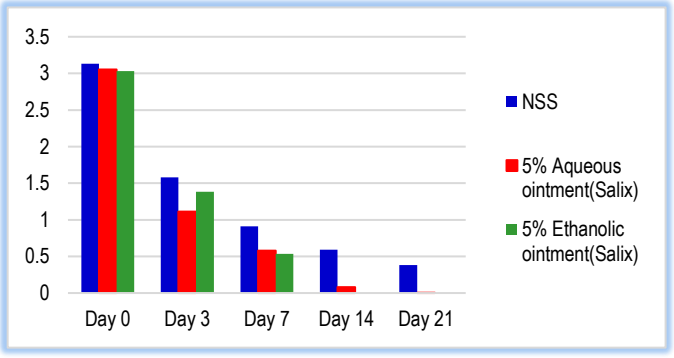

Fig-1 Effect of different therapy on wound size (cm^2) in the rabbits of different groups at different observation intervals

Glucose (mg/dl)

The normal concentration of glucose ranges from 75-140mg/dl of blood. In current study on day 7th post-wounding the glucose level of animals treated with *Salix* group decreased, which could be attributed to decreased feed intake due to pain in animals of all groups and also attributed to the presence of glycoside E, which have a hypoglycaemic effect [7].

After day 7th post-wounding the control group showed minor fluctuations in the values of glucose from the corresponding base value but remained higher than the base values. This could be due to the transitory hyperglycaemic effect attributed to cortisol release [15]. Cortisol which is release in response to injury, accelerate gluconeogenesis and ketogenesis leading to increased blood glucose levels [15] [Table-1 and Fig-2].

Table-2 The Mean±SE values of Glucose (mg/ml) in the rabbits of different groups at different observation intervals.

Group	Observation intervals in days			
	0	7	14	21
I	128.83±2.58^{bA}	123.16±1.32^{aB}	123.66±.80^{aAB}	130.00±1.15 ^{Ba}
II	128.83±.60^{cA}	114.83±1.13^{aA}	125.66±1.08 ^{bB}	131.33±.91 ^{cA}
III	128.33±0.76 ^{cA}	110.66±2.47^{aA}	121.33±1.54^{bA}	129.83±.60 ^{cA}

Figures with different superscript (small letters) differ significantly (P < 0.05) between days within the groups, Figures with different superscript (capital letters) differ significantly (P < 0.05) between groups , n = 6 animals in each group

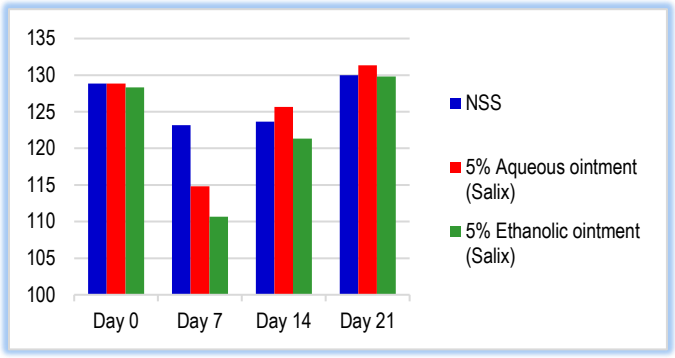

Fig-2 Effect of different therapy on Glucose (mg/dl) in the rabbits of different groups at different observation intervals

Serum micrometals

The normal range of Zinc and Copper of rabbits is 1.61 µg/ml and 1.03µg/ml of serum respectively. In the current study on day 7th post-wounding the zinc level of wounds treated with 5% ethanolic and 5% aqueous extract ointment of *Salix* increased with respect to wounds treated with sterile NSS, which could be attributed to high zinc levels in the leaves of *Salix* plant while as copper increased on day 7 post wounding in ethanolic extract ointment treated group followed by aqueous extract ointment treated group compared to Sterile NSS group [Table-3 and Table-4]. Clinical studies have revealed that increased urinary excretions and negative balances for copper (Cu), selenium (Se), and zinc (Zn) in severely injured or burn patients [16,17]. Severe injury cause acute decreases in serum TEs levels. Blood Se and serum Fe, Zn, and Cu concentrations were dropped within 2–3weeks after trauma. The relative deficiency may be due to the excessive metabolic demand, increased losses, or reduced intakes [16,17]. Another cause might be that TEs are redistributed to meet the needs of major organs in acute responses to trauma [18,19]. Resistance to some metals, such as Cd, Cu, and Zn, has been documented for a few European *Salix* species [20,21]. Some temperate Asian species can accumulate significant amounts of Fe, Zn, and Pb [22]. Cu/Zn superoxide dismutase (Cu/Zn-SOD) catalyzes the dismutation of superoxide, which is constantly formed during aerobic metabolism, to oxygen and hydrogen peroxide. So, Cu, Zn, and Se are joined in cellular defense against oxidants [23] [Table-3 and Fig-3] [Table-4 and Fig-4].

Total Bacterial Count (Cfu/ml)

Bacterial infection reduces wound contraction and consequently delays the wound healing process [24,25]. To have optimum wound healing process, microbial load must be kept on minimum possible level. The bacterial colony count decreased in all groups. In positive control group wounds treated with 5% aqueous extract ointment of *Salix*, has a good antibacterial action. *Salix* extract bactericidal action has been reported against *K. pneumonia, P. cepacia and S. aureus* [26]. Flavenoids, besides having astringent and antimicrobial effect, improves vascularity thereby enhancing wound contraction and increased rate of epithelialization. Phytochemical screening for *Salix acmophylla* reveals a moderate concentration of alkaloids, coumarines, cardiac glycosides, ratenges, phenols, flavonoids, saponins, tannins, essential oil and terpenes some of which chemical compounds have been associated to antibacterial activities and thus have curative properties against pathogens[11]. These findings corelate with that of [9] who reported mature antimicrobial activity of *Salix acmophylla* [Table-5 and Fig-5].

Table-3 The Mean±SE values of Zinc in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	1.57±0.02^{dA}	1.04±0.01^{Aa}	1.18±0.00^{bA}	1.32±0.01 ^{cA}
II	1.60±0.00 ^{dA}	1.33±0.01^{Ab}	1.41±0.00^{bB}	1.46±0.00 ^{cB}
III	1.61±0.00^{dA}	1.36±0.01^{aB}	1.42±0.00 ^{bB}	1.55±0.02 ^{cC}

Figures with different superscript (small letters) differ significantly (P < 0.05) between days within the groups, Figures with different superscript (capital letters) differ significantly (P < 0.05) between groups, n = 6 animals in each group

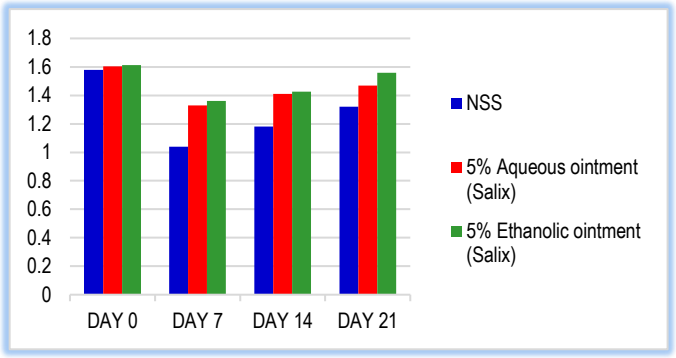

Fig- 3 Effect of different therapies on Zinc (µg/ml) in the rabbits of different groups at different observation intervals.

Table-4 The Mean±SE values of Copper in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	1.03±0.01^{Aa}	1.81±0.00^{Ba}	1.87±0.00^{cA}	1.92±0.00 ^{dA}
II	1.05±0.00 ^{aAB}	1.92±0.01^{bB}	1.97±0.00^{cB}	2.00±0.00 ^{cB}
III	1.06±0.01^{aB}	1.93±0.01^{bB}	1.98±0.00^{cB}	2.07±0.01^{dC}

Figures with different superscript (small letters) differ significantly (P < 0.05) between days within the groups, Figures with different superscript (capital letters) differ significantly (P < 0.05) between groups, n = 6 animals in each group.

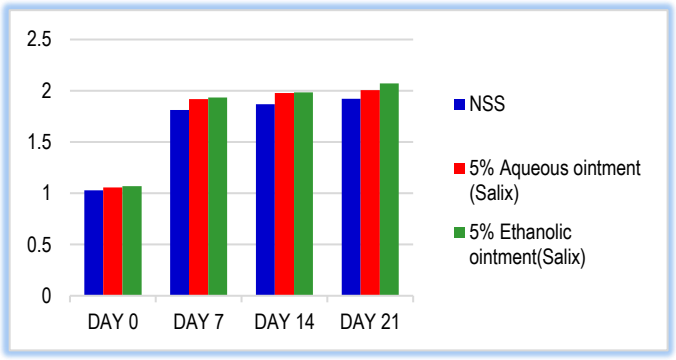

Fig-4 Effect of different therapies on Copper(µg/ml) in the rabbits of different groups at different observation intervals

Table-5 The Mean±SE values of Total Bacterial count in the rabbits of different groups at different observation intervals

Group	Observation interval in days			
	3	7	14	21
I	1.25±0.04^{dB}	0.80±0.03^{Cb}	0.64±0.02^{bC}	0.05±0.01^{aB}
II	0.66±0.69^{dA}	0.46±0.05^{Ca}	0.21±0.03^{bB}	0.01±0.00^{aA}
III	0.61±0.04^{cA}	0.46±0.01^{Ba}	0.06±0.01^{aA}	0.001±0.00^{aA}

Figures with different superscript (small letters) differ significantly (P < 0.05) between days within the groups, Figures with different superscript (capital letters) differ significantly (P < 0.05) between groups, n = 6 animals in each group

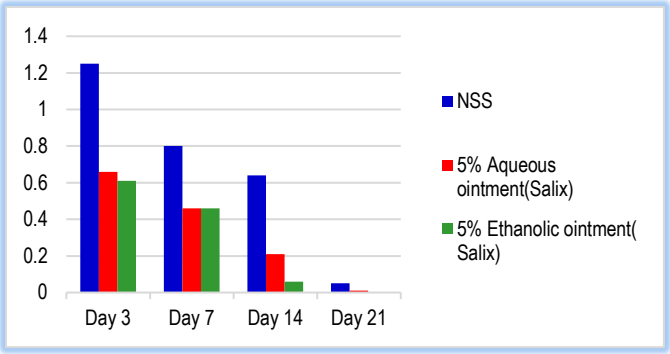

Fig-5 Effect of different therapy on Total Bacterial count (cfu/ml) in the rabbits of different groups at different observation intervals

Percentage Healing (%)

Gradual decrease in the size of the wounds and increase in percentage of wound contraction was recorded in both the *Salix* extract ointment treated groups up to day 21 post-wounding. Reduction in the wound size was minimal in group NSS. The wounds of Ethanolic extract ointment of *Salix* treated groups evinced complete healing (100% contraction) which was followed closely by aqueous extract ointment of *Salix acmophylla* and distantly by NSS on day 21 post-wounding, where mean wound contraction/size of 100%. 99.98% and 96.24% respectively was recorded [Table-6 and Fig-6]

Table-6 The Mean±SE values of percentage healing (%) in the rabbits of different groups at different observation intervals

Group	Observation in days interval		
	7	14	21
I	70.88±0.74^{aA}	80.95±2.57^{bB}	96.24±0.94^{cC}
II	78.09±1.80^{Aa}	92.34±1.62^{bB}	99.98±0.00^{cC}
III	82.32±1.05^{Aa}	99.93±0.06^{bB}	100±0.00^{bB}

Figures with different superscript (small letters) differ significantly (P < 0.05) between days within the groups, Figures with different superscript (capital letters) differ significantly (P < 0.05) between groups, n = 6 animals in each group

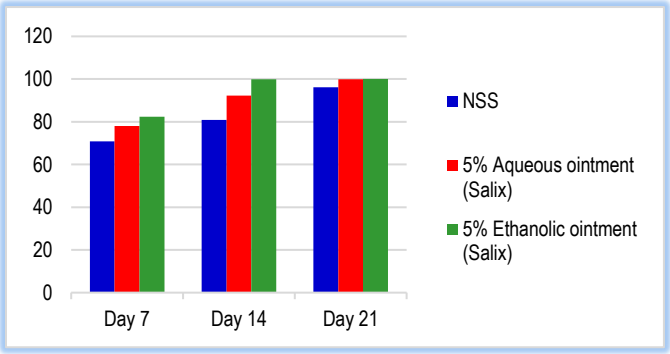

Fig-6 Effect of different therapy on percentage healing (%) in the rabbits of different groups at different observation intervals.

Conclusion

Salix acmophylla extract ointments (Aqueous and Ethanolic) showed potent wound healing, Antibacterial and Hypoglycemic effect suggesting that and ethnopharmacological approach in selecting the plant for study may be useful. Furthermore, the chances of success will be more, if the chosen species is used medicinally in the traditional system for the treatment of skin disease, bacterial infections and in Diabetes. There is not much information available on the phytochemical and pharmacological studies on *Salix acmophylla*. The report of the efficacy of this plant as wound healing may be due to its trace metal (Zinc and Copper) and many phytochemicals present in large concentrations.

Application of research: The normal wound healing process is basically known, but to thoroughly understand the very complex aspects involved in regulating processes for faster healing or to avoid negative outcomes such as infection or scarring are fundamental to wound research. Therefore, it is pertinent to approach these problems not only in the experimental animals but in the clinical cases as well.

Research Category: Wound Morphometry in Rabbits

Abbreviations:

NSS: Normal Saline Solution

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Conflict of Interest: None declared

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Day 0 Day 3 Day 7 Day 14 Day 21

Group I (Control)

Day 0 Day 3 Day 7 Day 14 Day 21

Group II (5% Aqueous extract ointment of *Salix acmophylla* leaves)

Day 0 Day 3 Day 7 Day 14 Day 21

Group III (5% Ethanolic extract ointment of *Salix acmophylla* leaves)

Plate 1