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Study of in Vitro Antimicrobial Activity and in Vivo Wound Healing Potentiality of Leaves of Callistemon Viminalis

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Abstract

Callistemon viminalis, often known as the stream bottlebrush, is a tropical plant in the Myrtaceae family and is renowned for its ability to grow rapidly in riparian zones. It is exclusively found in New South Wales, Queensland, and Western Australia. The roots of this species are entangled, which helps stabilize the soil, which reduces the danger of erosion. This plant has antibacterial, antifungal, antioxidant, and other pharmacological and insecticidal abilities that are also related to other medicinal properties. This research investigates the benefits, applications, and features of several extracts obtained from components of C. viminalis (branches, flowers, fruits, bark, and leaves). Bioactive compounds were characterized in detail by noting their chemical structures. The use of C. viminalis in folk medicine was fully supported by the results. Collecting and compounding plant materials, including processing, drying, and grinding, and then doing research to screen pharmaceutical effects, are all part of making the cure. Rats had their wound healing ability tested using a method known as in vitro experimentation. This method relies on excision wounds. The capacity of the extracted substance to close wounds when applied as an ointment was shown to be greater than in the control. The epithelization time in the standard and treated groups was significantly shorter than in the basic ointment base treatment group. The group treated with 5% w/w extract ointment had greater traction intensity than the control group (1.96 gm/mm2), with a significant difference of (1.12) gm/mm2. The tensile strength of the (3.86) gm/mm2 of the 5% w/w treated group. A substantial increase in tensile strength was seen on the tenth day for both the extract level and the normal medicine.

Keywords: *Callistemon viminalis*; incision Wound model; healing potentiality; antibacterial activity

Introduction

Herbal medicine use dates back millennia, and in recent times, it has gained a more enthusiastic following, seeing an extraordinary expansion in its applications, especially for medicinal purposes. Many poor nations followed by indigenous communities were stuck with plant medicine as their only option. Excessive usage of artificial substances, resulting in bio-amplification, has been shown to boost agricultural output (Kavitha and Satish, 2013).[1] Herbal medications have complicated chemical compositions, which may lead to both minor and severe side effects. As a result, herbal medicines should be subject to well-documented scientific toxicity tests to show they are safe. Of the 3800 species of shrubs and 5800 species of trees found virtually everywhere in the tropics and subtropics

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of the world, the most common family is Myrtaceae, which contains 121 genera and many species.[2] Bottlebrushes is one of the 34 species of Callistemon in the Myrtaceae family and is commonly called Callistemon. There are three species with a presence in the Sierra Madre Occidental Mountain range: Metrosideros viminalis Sol. ex Gaertn., C. viminalis (Sol. ex Gaertn.) G. Don, and Melaleuca viminalis (Sol. ex Gaertn.) Byrnes (Salem *et al*., 2017).[3] C. viminalis (the widespread aromatic wood tree found mostly in the wet tropics) is often referred to as C. viminalis in tropical Asia, South America, and Australia. Though most often used for decoration, it also serves as a weed eliminator (Brophy *et al*., 1997).[4] Plants suffer daily damage because of constant exposure to stimuli. When plant tissue is injured, it's a wound. There are many plant extracts and phytoconstituents available for use in wound healing treatments. Their varied active components, minimal side effects, and ease of access make them promising wound healing treatments. To prevent viruses and germs from assaulting the human body, the skin is critical. Damaged skin may cause physical and mental health issues, and perhaps injuries as well. Wound healing has been an essential topic in the medical field, and this causes problems and higher expenses (Chi *et al*., 2020).[5] One of the most physically and mentally distressing injuries is when you loose skin since it is both psychologically and physically difficult to recover from. Post-traumatic and chronic wounds affect about 100 million people throughout the world. People with diabetes and vascular disease, together with metabolic syndrome, are predisposed to wound healing problems (Shefa *et al*., 2020).[6] when a puncture develops, skin's protective function is disturbed, and this unequal loss of connective tissue causes injuries to be called puncture wounds (Hamilton, 2008).[7] Such treatments may leave patients with long-term problems, such as physical trauma and the installation of medical devices like catheters. Damaged cellular integrity may be repaired through the skin's capacity to mend the problem. Most explanations of the healing process include three steps: inflammation, proliferation, and tissue remodeling. The loss of blood from a torn vessel in the wound has resulted in the loss of blood components, such as platelets. Platelets adhere to the clotting factors, which makes clotting possible. In addition to filtering out polymorphonuclear leukocytes and monocytes (macrophages), which are known to provide chemotactic and growth factors for fibroblasts and endothelial cells, WBCs can function as regulators of fibroblast and endothelial cell production. Healthy epidermal cells go to the wound while fibroblasts and keratinocytes make a robust barrier by assisting in the creation of new epidermis. Surface damage and short epithelialization are caused by epidermal cell migration, which is influenced by the water content of the wound bed (Deodhar and Rana, 1997).[8] The creation of collagen, which connects back together to make new connective tissue, happens because of the action of fibroblasts. The remodeling will go on for many months since the reepithelialization process has concluded. Depending on how the body moves, the individual may experience it throughout their whole life.

Material and Methods

Plant materials- Specimen of *Callistemon viminalis* were collected from college campus NIET, Greater Noida, Uttar Pradesh. Plant identification was made by experts from the National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi-110012 (AC-13/2020).

Preparation of extract

The leaves were shade dried in a well-ventilated area. To extract fat and chlorophyll, the dried leaves were separated, powdered, weighed (about 270 g), and extracted with petroleum ether. After this, the sample was solvent-extracted using eight different solvents, which included water, ethanol, chloroform, ethyl acetate, and isopropyl alcohol. The yield of each extract was determined as % w/w about the total weight of dried leaves that were used in the extraction process.[8]

After cleaning with water that had been properly sterilized, Callistemon viminalis leaves (30 g) were used in the experiment. The dried leaves were placed in the shade to dry. A mixer grinder was used to grind the leaves. To make 32% ethanol, the pulverized leaves were put in a blender. Whatman filter paper was used to run the mixture through a Whatman filter paper (No 1). To ensure sterility, the suspension was filtered (membrane pore size 0.22 mm). The crude dry extract was obtained by freeze-drying the filtrate (4.56 g). The last step was to preserve the crude extract at 200· C. In every experiment, the crude dry extract was used to make fresh stock solutions.[9]

Preliminary phytochemical test

Phytochemical analysis was done for the presence of phytoconstituents using various reagents including Mayer's, Molash's, Fehling's, Borntrager's, Legal's, Foam, Salkowaski, Ferric chloride, Gelatin, Alkaline reagent, Lead acetate, Copper acetate etc (Abdullahi, 2013; Ismail *et al*., 2016).[9]

Animals:

The rats were 150-200 g Wistar albino rats for the experiment. The animals were maintained in ideal circumstances (22 ± 2 degrees Celsius, 55 ± 5 percent humidity, and a 12-hour light/dark cycle) in CPCSEA Approved Animal House of NIET Pharmacy Institute.

Acute dermal toxicity

The test on the polyherbal extract's acute cutaneous toxicity was performed following OECD guideline no. 402 [OECD guideline]. Rats were selected at random and had both genders. Nine animals were split into three groups, with three individuals in each. The test animals were depilated from the dorsal region of their back 24 hours before the test using a depilatory preparation (Veet). Control animals were those that did not receive PNG; Group II animals got topical treatment with 2000 mg/kg of PHG (5% w/w). For 14 days, researchers kept track of changes in appearance, behavior, and hazardous responses that may occur after applying new products.

In vitro antimicrobial activity

For testing antibacterial properties, 2000 µg/mL concentrations of the extracts were tested against the Gram-positive bacteria (*B. subtilis, B. cereus, M. luteus, S. lutea, and S. aureus*) and the Gram-negative bacteria (*E. coli, S. marcescens, S. typhi, P. vulgaris, and P. aeruginosa*).

Disc diffusion method

To find out how well the Callistemon viminalis combats microbes, the agar disc diffusion technique was used. On solid medium plates, 0.1 mL of 1×108 cells/mL was loaded with a suspension of the tested microorganisms. A twenty-microliter extract of the specimen was put on a filter paper disc with a diameter of five millimeters, and it was placed on an infected plate. After two hours in the fridge, the plates were put into the incubator at 37 °C for 24 hours. In millimeters, the widths of the inhibition zones (IZs) were measured. The control substance was produced by preparing it insolvent. The positive control (with a concentration of 20 µg/disc) was tetracycline, which was tested on the tested microorganisms.

Determination of minimum inhibitory concentrations (MICs)

The experiments were conducted using 96-well microplates (32-34). To make a 250, 500, 1000, 1500, 2000, 3000, 4000, and 5000 µg/mL dilution of Callistemon viminalis in EtOAc, a serial dilution was performed. All wells were filled with sterile Mueller Hinton broth at a concentration of 50 µL. We used the INT (0.2 mg/mL) (p-iodo-nitrotetrazolium violet, Sigma-Aldrich) to test bacteria after overnight incubation of the 50 µL bacterial culture at 37°C at 100% humidity, then applied INT to each well in the micro-plate. Every extract was dissolved in 10% DMSO (which is from Sigma-Aldrich) and diluted in distilled water to a stock solution of 5,000 µg/Ml[(10)].

Wound healing activity

The wound healing activity of leaves of Callistemon viminalis was investigated using both excision and incision wound healing models. Animals were randomly assigned to groups of six (n=6) each, and there were three distinct categories:

Group I: control group was given DMSO.

Group II: a standard group that was suspended in the vehicle and was then treated with Vitamin E (100 mg/kg body weight) (0.5 percent sodium carboxymethyl cellulose suspension in distilled water).

Callistemon viminalis treated test group (Group III).

Incision wound model

There was an incision, following the technique that has previously been described. To knock out the rats, we gave them ketamine hydrochloride (100 mg/kg, i.p., body weight) and then separated them by the groupings outlined above. Using a sharp scalpel, the rats had an incision of 4 cm in length on their shaved skin (an incision was performed on both sides of the spinal column). After the incisions had completely healed, they were sewn with black silk surgical thread (000), and each stitch was put 1.5 cm apart (number 11). To deal with the wound, the surgeon left it exposed and administered the doctor's standard medication. This treatment consisted of a few different medications, but the doctor's staple treatment included using the natural antiseptic

leaves and applying the liquid to the wound three times a day. All rats were put under anesthesia on the 10th day when sutures were removed and wound breaking strength (WBS) was measured.[8]

Wound-breaking strength: A wound was drawn on the rats at least 3 mm away from the wound. Wounded, sedated rats were fastened on the operating table, and then a line was drawn on both sides of the wound. The two ends of the line were opposite each other when forceps grasped it.[11] The light polypropylene container was hung by a pulley with a string that linked to weight, and one end of the forceps was attached to the container. When wound edges were freed from the constraint of the fixed end by raising the water level, it was found that the water's weight was just enough to move the wound edges away. Three independent measurements of incision wound's breaking strength had their results averaged into one reading. The ability to stretch was also quantified using the formula:

The amount of force required to break a material (e.g., an elastic band) is its tensile strength (mm2)

Excision wound model

In addition to using the standard technique, changes were used when the excision wound was formed . In the experiment, depilatory cream (Veet) was used to remove hair from the animals' backs, and ketamine hydrochloride (100 mg/kg, i.p., body weight) was used to give them general anesthesia. The surgical location was demarcated before the doctor began removing hair from the incision site.[12]

A 500 mm2 circular region was made by employing surgical blades, scissors, and forceps with a total thickness excision wound. The rats were taken out into the open aseptic environment completely unclothed. After the surgery, the patient received 500 mg of the simple gel base, a designed extract gel, and standard medication administered daily for healing. This treatment started on the day of the operation and continued until full healing. To determine the size of the wound, the measurement was taken right away using a translucent paper placed over the wound and traced out; then, the resulting size was estimated by graphing. The wound contraction % was calculated daily using the same procedure. Wound size was measured as 100% when it was first incurred. The number of days needed for the formation of a scab before the presence of any lesion had to be counted to discover the length of epidermal regeneration.

Results

Phytochemical Evaluation of leaves:

The % yield of each extract of each of the *Callistemon viminalis* leaves is given in Table 1. Water extract of *Callistemon viminalis* was obtained with maximum yield (13.54 %) whereas chloroform extract was obtained minimum amount.

Extract	Yield (% w/w dried plant material)
Petroleum ether	5.32
Ethyl acetate	8.61
Chloroform	0.10
Ethanol	9.21
Water	13.54

The leaves reveal the presence of alkaloids, tannins, flavanoida and saponins on phytochemical screening.

In vitro antimicrobial activity

The research focused on ethanolic extract of C. viminalis at 2000 µg/mL to explore it's in vitro antibacterial activity. E. coli exhibited the greatest response in the ethanolic extract with zone of inhibition of (19±1.6) mm, while S. marcescens had the smallest response at (10±1.0) mm. The MIC for both bacteria was 250 µg/mL. The ethanolic extract of C. viminalis leaves was very effective against the other tested bacteria, all of which had zone of inhibitions between 13 and 18 mm.

It was concluded from the evidence that ethanol had significant antibacterial against the chosen microorganisms. Its antibacterial action was also notable, similar to the standard antibiotic tetracycline with 20 µg/disc.

Bacterial strain	Negative control	Positive control	Ethanolic extract of Callistemon viminalis
Gram-positive			
B. subtilis	R	18	16 ± 1.4^{C}
B. cereus	R	17	15 ± 1.1^{B}

M. luteus	R	19	13±1.2^{D}
S. lutea	R	20	18±1.8^{A}
S. aureus	R	23	14±1.7^{C}
Gram-negative			
E. coli	R	18	19±1.6^{A}
S. marcescens	R	20	16±1.0^{D}
S. typhii	R	20	11±1.7^{A}
P. vulgaris	R	23	13±1.4^{D}
P. aeruginosa	R	22	18±1.6^{C}

For 2000 µg/mL, the diameter of the inhibitory zone, with a disc diameter of 5 mm, is shown as mean ± SD (three repetitions). In superscripts, the extracts are shown to have MICs against the tested strains. MICs <250, 250, 500, 1000, 1500, and >5000 µg/mL have values A, B, C, D, and E, respectively. R: 2000 µg/mL is its breaking point. Tetracycline (20 micrograms per disc). The measurements were done four times, and the kind of precipitate's color was used to categorize it.

Wound healing activity

An enormous spike in healing was seen in C. viminalis experimental groups, as opposed to the control group, where the decrease in wound area of each group was noted after 21 days. The region of the injury was measured on the days after the surgery: 1, 4, 7, 10, 13, 16, 19, and 21 in all of the groups. Significant wound closure was seen from the fourth day to the tenth day. C. viminalis wounds in the DMSO group had a mean wound area of 200 mm2 on the 16th-day post-surgery, whereas the wound area of the C. viminalis group treated with DMSO was 30 mm^2. Leaf extract was shown to be effective in wound healing ($p < 0.05$), whereas in the normal vitamin E group, wounds had started to close, but were not fully closed by the 19th-day post-surgery. It was discovered that the 19th day after the administration of C. viminalis was the period that it took for the epithelium to regenerate. The top wound was removed surgically and histological tests were done on it. Histological investigation of rats' wounds treated with C. viminalis and Vitamin revealed that scar formation was reduced, while the process of fibroblast proliferation, angiogenesis, keratinization, and epithelialization was improved over the control or the treatment with vehicle.

Post wound day	**Wound area (mm^2) (Mean±S.E.)**		
	Control	**Standard**	**Leaf extract**
1	525±2.1	515±2.5	522±3.8
4	439±2.2	415±14.1	408±2.2
7	317±4.2	270±2.8	247±2.8
10	306±3.9	189±1.6	117±5.2
13	266±2.7	108±2.2	65±1.8
16	200±2.4	73±1.8	30±2.2b
19	35± 2.2b	8±0.2	00± 2.1
21	00±00b	00±00	00±00b

P<0.05 statically significant difference in comparison with control group

Post wound day	**Percentage of wound contraction (%)**		
	Control	**Standard**	**Leaf extract**
4	16.01	18.94	21.53
7	35.2	48.29	52.9
10	41.49	63.08	77.50
13	49.5	78.9	87.30
16	61.75	85.96	93.16
19	93.16	100	98.43
21	100	100	100

P<0.05 statically significant difference in comparison with the control group

Conclusion

The Callistemon species of the Myrtaceae family are often found in forestry and horticulture. The hydro-distillation process produced an essential oil that was analyzed using GC/MS. To compare

the extracts, their active components have been thoroughly examined, with special attention paid to the total phenolic and flavonoid content, as well as the number of active chemicals produced in a DPPH test and a growth inhibition assay. Among the essential oil's fourteen identified components, 98.94% of the entire oil was made up of them. The two key elements were pineal (34.3%) and 1,8-cineole (64.53%). (9.69 percent). In addition, the significant inhibition seen compared to the traditional antibiotic was identified for the researched bacterial strains (tetracycline). It was shown that both the crude methanol extract and ethyl acetate fraction were very effective in killing various types of bacteria. C. viminal s may serve as a major source of antibacterial chemicals and antioxidants that will be useful in developing novel antimicrobial medicines derived from natural sources. C. viminalis possesses anti-bacterial properties when extracted in different ways. Most Callistemon species are known for their antipathogenic actions, which have also been used as an anti-inflammatory, antimicrobial, and antiseptic essential oils. C. viminalis has natural antibiotic and antioxidant capabilities which may be used to produce new medical treatments with antimicrobial properties. The ethyl acetate fraction of C. viminalis leaves was very potent in anti-bacterial activity against bacterial strains as of the current research. The current research discovered that the essential oil and methanol extracts, as well as the C. viminalis, leaves ethyl acetate fraction were all extremely potent in anti-bacterial activity.

Ethical clearance: Taken from NIET committee, Greater Noida, Uttar Pradesh committee

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