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PRELIMINARY PHYTOCHEMICAL SCREENING, ANTIMICROBIAL AND WOUND HEALING PROPERTIES OF METHANOLIC EXTRACT FROM *VACCINIUM MACROCARPON* FRUITS IN RATS

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ABSTRACT

Wound is defined simply as the disruption of the biochemical, cellular, and anatomic continuity of a tissue. Plants and their extracts known as phytomedicine have immense potential for the management and treatment of wounds. Due to the undesirable side effects, in the control and treatment of the wound infections, it is recommended to use natural materials such as phytochemicals instead of chemically synthesized drugs. Thus, the aim of this research was to study the anti-microbial and wound healing potential of *Vaccinium macrocarpon* methanolic extract in rats. Antimicrobial activity of *Vaccinium macrocarpon* extract determined that the extract inhibited the growth of microbes against the gram positive (*Staphylococcus aureus*) and gram negative strains (*Escherichia coli*). A significant zone of inhibition was recorded against two selected pathogenic bacterial strains by well diffusion method. Excision wound contraction was measured and expressed as percentage of reduction in wound size. At the lowest concentration of 5% w/w extract ointment, significant contraction similar to that of Soframycin (standard) was started on the 3rd day. Better contraction similar with the standard was recorded at higher concentrations of the extract ointment (10% w/w). A dose dependent effect was observed in rats treated with ointment. The results of this investigation showed that *Vaccinium macrocarpon* extract has wound healing activity and it may also be utilized to treat various types of wounds in people.

KEYWORDS: *Vaccinium macrocarpon*, Antimicrobial activity, Excision wound contraction, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

In many parts of the world, wounds have a major role in morbidity and death. According to studies, there are 10,000 microbial infections related mortality for every million wound patients.[1,2] In addition to causing pain, loss of function and mobility, depression, distress, and anxiety, embarrassment, and social isolation, chronic morbidity, and even death due to the low rate of complete healing, chronic wounds have a significant negative impact on the health and quality of life of patients and their families.[2] Debridement, irrigation, the use of antiseptics, antibiotic and corticosteroid therapy, and tissue grafts are some of the current methods for treating wounds. These therapeutic methods come with undesirable side effects, too, including bleeding, tissue damage, contact dermatitis, a delay in wound healing, and the possibility of bacterial resistance.[3] Just 1-3 percent of the medications listed in western pharmacopoeias are intended for use on wounds, despite the enormous advancements made in the pharmaceutical drug industry.[4] More so in developing nations, infection-related morbidity and mortality have grown due to a rise in resistance bacteria, high costs, and a lack of next generation medications.[5] In order to create nontoxic and efficient wound healing agents, there is a huge need for scientific study of medicinal plants. Strong bioactive chemicals produced by plants enable them to communicate with other creatures in their surroundings. These bioactive substances play a crucial role in defence systems and help people avoid sickness. The bioactivity of plant extracts and their constituent parts against harmful pathogenic organisms has been assessed by numerous researchers.[6] *Vaccinium macrocarpon* commonly known as American cranberry belongs to the family Ericaceae. American cranberries are small pink blossoms that appear in the spring. Vaccinium macrocarpon (American cranberry) and Vaccinium oxycoccus (European cranberry) are the two major species.[7] Owing to the presence of rich bioactive polyphenols, cranberries find potential applications in therapeutic intervention diseases for improving cardiac, urinary and anticancer health.[8-10] Polyphenols that constitute cranberries encompass anthocyanins, flavonols, phenolic acids and isoflavones.[11-15] Cranberry juice has been long used for the prevention of UTI's bolstered by anecdotal evidence. 1980's saw the emergence of evidence demonstrating the ability of

cranberry juice to prevent attachment of E. coli bacteria to uroepithelial cells. Studies from Cape Town evaluated the effect of cranberry juice in a randomized crossover trial on Calcium Oxalate calculi. Thus, this work was undertaken to explore the antimicrobial and wound healing effects of *Vaccinium macrocarpon* fruits extract.

MATERIALS AND METHODS

Plant material

The medicinal plant *Vaccinium macrocarpon* (300 gm) was collected locally from Bhopal, M.P. After cleaning, plant parts were dried under shade at room temperature for 3 days and Then in oven at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. The fruits of medicinal plant *Vaccinium macrocarpon* were authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemicals and reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction

Plant material fattening

Plant matter from *Vaccinium macrocarpon* was crushed up and allowed to air dry at ambient temperature. Soxhlation was used to remove the substance from the shade-dried plants using petroleum ether after it had been coarsely crushed up. The substance was extracted repeatedly until it had been adequately fatted.

Extraction by soxhlation process

Vaccinium macrocarpon powder that has been defatted was thoroughly extracted with methanol using the soxhlation process. The extract evaporated beyond their boiling points. The dried crude concentrated extract was weighed in order to calculate the extractive yield. When ready for analysis, it was then put into glass vials (6 x 2 cm) and stored in a refrigerator 4°C.[16]

Phytochemical screening

According to the protocols described, phytochemical screening was done to find any bioactive compounds [17, 18]. By visually seeing a colour change or the production of precipitates following the addition of specific reagents to the solution, the tests were recognized.

Total phenol measurement

The Folin Ciocalteu reagent was employed to calculate the total phenolic substance of the extracts. Gallic acid concentration (20-100µg/ml) was produced in CH_3OH. 100µg/ml plant extract concentrations were likewise made in CH_3OH, and 0.5 ml of every sample was added to the test along with 4 ml of 7.5% sodium carbonate and 2 ml of a 10 fold diluted folin Ciocalteu reagent. After parafilming the tubes, they were keep warmed at RT for 30 minutes with periodic shaking. The absorbance at 765 nm was calculated against CH_3OH as a vacant. Gallic acid's conventional regression curve was utilized to calculate the content of phenol overall, and the results were given in milligrammes per gramme (mg/gm) of gallic acid.[19]

Total flavonoids measurement

Rutin (20 to100µg/ml) was produced in CH_3OH at various concentrations. Test samples with a polarity of 100µg/ml or close to it were created. A sample was diluted to 0.5 ml and then added to 0.15 ml of a 5% NaNO2 solution along with 2 ml of distilled H_2O. A 10% AlCl3 solution was added after 6 minutes had passed. The combination was then given 5 minutes to stand before receiving 2 ml of a 4% NaOH solution. With distilled water, the final volume was adjusted to 5ml, and then it was left to stand for an additional 15 minutes. At 510 nm, the absorbance was calculated using H_2O as the reference. Rutin's standard regression curve was employed to calculate the whole flavonoid substance.[20]

Anti-bacterial activity

Preparation of dilutions of the samples

The dilutions of the samples were made for the concentration as 100µg/ml, 150µg/ml, 200µg/ml, and 250µg/ml respectively of the sample, after that volume makeup was done with distilled water till 1ml.

Preparation of nutrient agar media

28 g of Nutrient Media was dissolved in 1 litre of distilled water. pH of media was checked before sterilization. Media was sterilized in autoclave at 121 o C at 15 lbs pressure for 15 minutes. Nutrient media was poured into plates and placed in the laminar air flow until the agar was get solidified.

Well diffusion assay

Culture of bacterial strains (*Staphylococcus aureus*, *Escherichia coli*) was spread on the Nutrient agar media (NAM). The wells were then formed for the inoculation of the *Vaccinium macrocarpon* extract given in the different concentrations; volume make-up was done till 1 ml. 100 µl of the sample was loaded. The plates were allowed to incubate at 37^0C for 48-72 hours for the best results. The bacterial suspension was standardized to 10^8 CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculum from the broth (containing 10^8 CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified Agar Media Plate. The agar plate was inoculated by spreading the inoculum with a sterile spreader, over the entire sterile agar surface. Four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. Each well was filled with different concentration (25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml) of *Vaccinium macrocarpon* extract .It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37^0C. After incubation, plates were observed for the formation of a

clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm. Zones were measured to a nearest millimeter using a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting background. The diameters of the zone of complete inhibition (as judge by unaided eye) were measured, including the diameter of the well.[21]

***In-vivo* wound healing activity**

Animals

For the study, healthy albino wistar rats of either sex, weighing 200±20 gm were chosen. Animals were selected randomly from animal house of Pinnacle Biomedical Research Institute (PBRI), Bhopal, India and further divided into four treatment groups randomly (Group 1: Normal control group, Group2: Standard (Soframycin) treated group, Group 3: 5% ointment of *Vaccinium macrocarpon* extract and Group 4: 10 % ointment of *Vaccinium macrocarpon* extract) and kept in propylene cage with sterile husk as bedding. Relative humidity of $30^{0}7$ % at 22±2°C and 12:12 light and dark cycle was maintained in the animal house and fed with standard pellets (Golden Feeds, New Delhi, India) and water was available *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. Separate group (n=6) of rats was used for each set of experiments. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal.

Wound creation (Excision wound model)

Total of 24 animals were selected and divided into four groups each containing six animals. They were anaesthetized with slight vapour inhalation of anesthetic ether in anaesthesia chamber. The dorsal surface of animals was shaved and full skin thickness was excised from the sterile dorsal marked area to get a wound measuring about 1 cm diameter. The animals were placed singly in individual cages. The wound was remained open environment. Wounds were left open and the ointment was applied topically twice a day (once in the morning and evening) onto each rat for 15 days. The contraction of wound was expressed as percentage of the reduction in wound size.[22,23]

Table 1: Design of experiment.

S. No.	Groups	Number of animals	Dose
1	Control	6	Normal Control
2	Standard	6	1% w/w Soframycin
3	*Vaccinium macrocarpon* methanolic extract	6	5 % ointment of V.M. extract
4	*Vaccinium macrocarpon* methanolic extract	6	10% ointment of V.M. extract

RESULT AND DISCUSSION

The crude extracts so obtained after each of the successive Soxhlet extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from the fruits of the plants using petroleum ether and methanol as solvents are depicted in the Table 2. The results of qualitative phytochemical analysis of the crude powder of fruits of *Vaccinium macrocarpon* are shown in Table 3. Methanolic extracts of sample of *Vaccinium macrocarpon* showed the presence of alkaloid, flavonoid, tannin and phenolic compounds, glycoside, triterpenoids and steroids. Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. The TPC and TFC in methanolic extract were found to be 69.66 and 40 mg/gm respectively Table 4 & Figure 1, 2. Antimicrobial activity of *Vaccinium macrocarpon* extract determined that the extract inhibited the growth of microbes against the gram positive (*Staphylococcus aureus*) and gram negative strains (*Escherichia coli*). A significant zone of inhibition was recorded against two selected pathogenic bacterial strains Table 5. Progressive wound contraction was observed in all the treated groups. Application of V.M ointment on marked area exhibited statistically symbolic contraction of wound analysis when compared to control simple ointment base group. Rats showed normal healing process with signs of improvement at weekly intervals and this was determined by their contraction rate. A dose dependent effect was observed in rats treated with ointment Table 6 & Figure 3.

Table 2: Percentage yield of crude extracts of *Vaccinium macrocarpon* extract.

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Vaccinium macrocarpon*	Pet ether	300	1.36	0.45%
2		Methanol	284.25	6.58	2.31%

Table 3: Qualitative phytochemical evaluation of *Vaccinium macrocarpon* extract.

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.3	Hager's reagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Absent	Present
2.2	Legal's test	Absent	Present
2.3	Killer-Killiani test	Absent	Present
3.	**Carbohydrates**		
3.1	Molish's test	Absent	Absent
3.2	Fehling's test	Absent	Absent
3.3	Benedict's test	Absent	Absent
3.4	Barfoed's test	Absent	Absent
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Present
8.2	Libbermann-Burchard's test	Present	Present

Table 4: Total phenolic and flavonoid content of extracts.

Test	Methanolic extract
TPC	62.03 mg/gm equivalent to Gallic acid
TFC	15.23mg/gm equivalent to Rutin

Table 5: *In-vitro* antimicrobial activity of *Vaccinium macrocarpon* fruit extract against gram negative bacteria (*Escherichia coli*) and gram positive bacteria (*Staphylococcus aureus*).

Bacterial strain	Different concentrations of *Vaccinium macrocarpon* extract			
	25µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Escherichia coli	0mm	7mm	8mm	12mm
Staphylococcus aureus	7mm	9mm	10mm	12mm

Table 6: Percentage wound closure in various treatment groups.

Groups	% Wound closure				
	Day 3	Day 6	Day 9	Day 12	Day 15
Normal Control	11.05±1.5	33.75±1.46	69.49±2.6	79.95±2.4	82.24±1.8
Standard (Soframycin)	39.56±2.8	53.54±1.4	72.18±2.9	93.55±2.7	98.17±1.6
V.M (5 % ointment)	25.85±2.2	42.85±3.0	60.85±2.8	86.49±1.8	84.85±3.2
V.M (10% ointment)	30.22±1.2	53.75±1.6	69.46±3.2	89.76±1.5	95.28±1.9

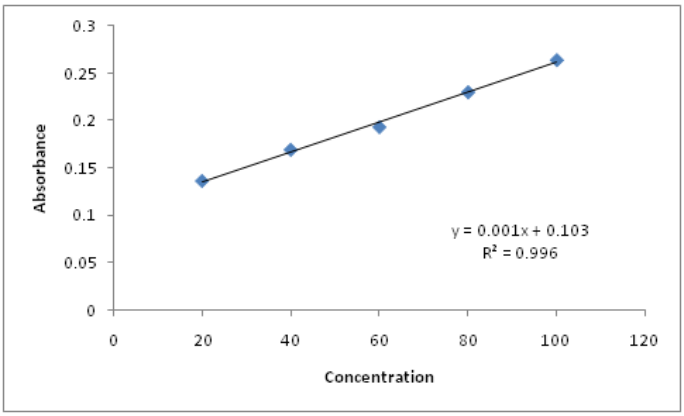


Figure 1: Graph represent standard curve of Gallic acid.

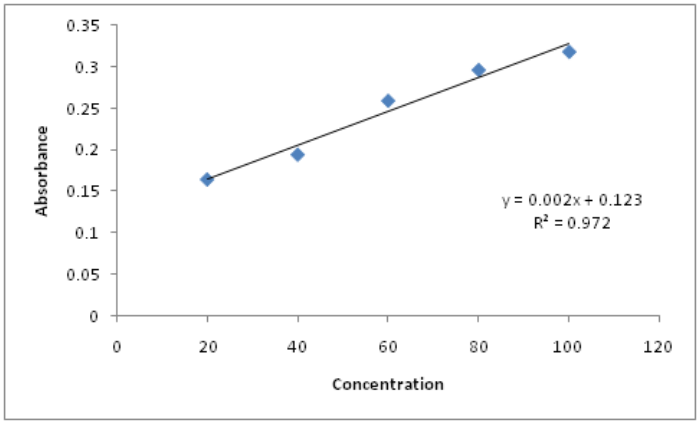


Figure 2: Graph represent standard curve of Rutin.

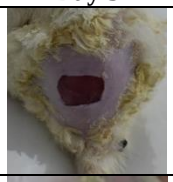
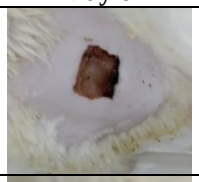
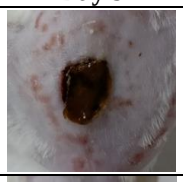
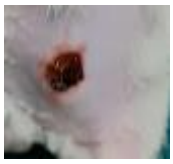
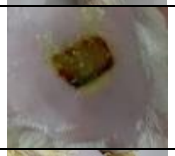
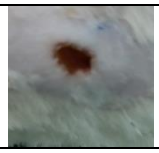
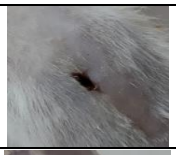
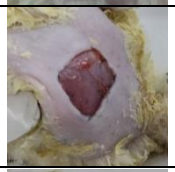
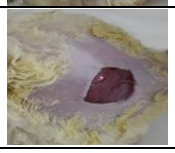
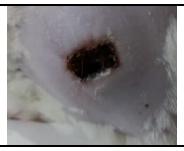
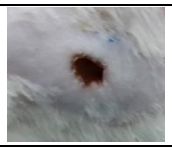
Groups	Wound closure images				
	Day 3	Day 6	Day 9	Day 12	Day 15
Normal Control					
Standard (soframycin)					
V.M (5 % ointment)					
V.M (10% ointment)					

Figure 3: Images of wound closure in various treatment groups.

CONCLUSION

The results of our study indicate, for the first time, that *Vaccinium macrocarpon* may be a potential candidate for wound healing because of its positive influence on phases of the healing process and particularly effective in view of it antimicrobial properties. Therefore, there is the need for further studies into the stability of the extract to ensure an efficacious formulation of products for wound healing.

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