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Original article

Comparison of the effect of zinc oxide nanoparticles and extract of *Acorus calamus* applied topically on surgical wounds inflicted on the skin of rabbits

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Abstract

Antibiotics are used for postsurgical wound healing purposes but unfortunately, resistance against them demands some alternatives for quick recovery. Sepsis of wounds is a challenge for medical as well as veterinary professionals. Nanoparticles have significant advantages in wound treatment and drug resistance reversal. This study was conducted to appreciate emerging alternates of antibiotics like zinc oxide nanoparticles and plant extracts in topical application. Zinc oxide is considered a good wound healer and its nanoparticles are easy to access. So, the efficacies of zinc oxide nanoparticles and sweet flag plant extract ointments were tested to compare modern and traditional therapeutics as sweet flag is considered a pure medicinal plant. Rabbits were selected for this study due to the healing properties of their skin. Wounds were inflicted on the thoracolumbar region and treated for 29 days post-surgically daily with normal saline and the ointment of zinc oxide nanoparticles and sweet flag extract ointment, prepared in a hydrophilic solvent. Wound shrinkage was observed daily and histopathological analysis was made and results were compared. Zinc oxide nanoparticles ointment showed the most satisfactory results for every parameter included in the study. No side effects of its topical application were observed. Healing was normal without any complications. The preparations of zinc oxide nanoparticles may help in the era of antibiotic resistance as topical drugs in the future.

Keywords: antibiotic resistance, nanoparticle, plant extract, wound

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Introduction

The skin is an important barrier of the body in animals and humans that protects the visceral region from any kind of sepsis or tissue insult. A wound is any discontinuation of layers of the skin that compromises its functionality and morphology (Masson-Meyers et al. 2020). Skin confronts a lot of traumatic threats in routine in the form of penetrating wounds, burn wounds, and bruise wounds including surgical wounds that may lead to zillions of animal and human deaths if contaminated badly (Firdous and Sautya 2018). Quick and healthy recovery of the skin is necessary for an appropriate healthy lifestyle, especially after convalescence from any surgical incision. Wound healing is a complex process involving contraction, inflammation, granulation, proliferation, remodeling, and reepithelization of cells in both, animals and human beings (Firdous and Sautya 2018, Rodrigues et al. 2019). The wounds that do not heal as expected and get stalled are categorized as chronic wounds (Masson-Meyers et al. 2020). A good wound healer is supposed to have antibacterial, anti-inflammatory, and moisturizing properties (Manuja et al. 2020). Mice, rats, rabbits, guinea pigs, and humans have been used in more than 95% of research for the estimation of tissue repair, conducted in the previous two decades (Parnell and Volk 2019). Guinea pigs have more similarity with human skin than other laboratory animals having a fixed ratio of subcutaneous layer, hairless skin, skin follicles, and skin being responsive to some growth factors, and even their skin grafts were used for burn wounds in humans before autografting was introduced (Parnell and Volk 2019). Topical application of drugs for healing purposes is best to achieve the required healthy tissue after recovery from surgical wounds in the past (Manuja et al. 2020). Many wound healers derived from natural flora have been used in the past like *Aloe vera*, *Acorus calamus*, *Cinnamon*, *Eucalyptus,* or synthetic polymers like antibiotics e.g., clindamycin, sulfadimidine, and azithromycin creams, zinc oxide ointment, boric acid ointment and specialized aseptic dressings (Dogan 2019). *Acorus calamus* is an herbaceous rhizome perennial plant belonging to the family Acoraceae (Chen et al. 2014, Sharma et al. 2014). *A. calamus* has several medicinal characteristics and is known as a biological medicinal reservoir worldwide (Khwairakpam et al. 2018). In history, *A. calamus* has wide therapeutic use in respiratory, gastrointestinal, renal, cardiovascular, neoplastic, and neural disorders (Avadhani et al. 2013, Nakkala et al. 2014, Kumar et al. 2015). *A. calamus* also possesses ideal wound healing properties being antibacterial, antifungal, anti-inflammatory, antiparasitic, antioxidant and angiogenic due to the presence of α-asarone factor (Biglar et al. 2014, Khwairakpam et al. 2018). Zinc oxide has been one of the efficient and nonirritant wound healers for years exhibiting broad-spectrum antibiotic, anti-inflammatory, biogenic, antioxidant, absorbent, UV blocking characteristics when applied as a locally acting drug (Batool et al. 2021, Melnikova et al. 2021, Saddik et al. 2022). A more advanced form of application of zinc oxide is the zinc oxide ointment-coated nanoparticles that proved effective even when tested against multidrug-resistant pathogens like *Bacillus subtilis, Klebsiella pneumonia, Bacillus licheniformis,* and *Escherichia coli,* and this satisfying efficacy may be credited to more penetrating power into the wound due to least surface area to volume ratio (Martínez-Carmona et al. 2015, Batool et al. 2021). Zinc oxide exhibits cytotoxicity to bacterial cells by producing Zn^{+2} ions and oxygen reactive species (Kairyte et al. 2013). Zinc oxide nanoparticles loaded with antibiotics like cefixime and azithromycin organized into bacterial cellulose film have been tested experimentally in wound healing and excellent results were obtained (Melnikova et al. 2021). So, there is a need of exploring the further therapeutic role of zinc oxide nanoparticles particularly in surgical wound healing based on evidence from the literature to develop safe, environmentally friendly as well as economically favorable pharmaceutical products that should not be limited to animals but applicable to human beings as wound healing is a global biological concern (Ahmadi et al. 2019). This study was conducted to compare sweet flag extract and zinc oxide nanoparticles' efficacy in the healing of surgical wounds inflicted in rabbits when both are applied topically in ointment form while maintaining a control group treated with normal saline post-surgically. Zinc oxide nanoparticles were preferred to zinc oxide microscale particles because of the higher efficacy and more powerful healing action of the former (Raguvaran et al. 2017).

Materials and Methods

Animals and management

The Ethics Committee of the University of Agriculture Faisalabad (UAF) initially approved this thesis (No. NOC/559 dated 28-06-2018) and then the final approval of the materials was accorded by the department of the University. Verbal and written consent were obtained from each concerned person before performing the research.

In vivo study was designed as it allows observation of healing on the intact skin, topical application of healing drugs on inflicted wounds accurately, and estimation of their efficacy in the repair of wounds (Wong et al. 2010). Twelve Rabbits, weighing 2.0 kg to 3.0 kg were bought from a village near the district of Faisalabad, Punjab, Pakistan. Normal anatomical and physiological status of animals was ensured before purchase. Animals were kept in the lab of the surgery department of the University of Agriculture Faisalabad (UAF) at room temperature of 25°C to 30°C and a light period of 8-10 hours was ensured. Moreover, fresh water and fresh fodder were provided daily during the whole study i.e., one and a half months including the pre-experimental and experimental periods. Animals were dewormed one week before the infliction of wounds with ivermectin 0.4 mg/kg body weight subcutaneously as also suggested by the Porter in Merck Veterinary Manual (Porter and Kaplan 2011). All institutional and national guidelines for the care and use of laboratory animals were followed. Mice or rats were not preferred because multiple wounds were supposed to be inflicted and post-healing biopsy was part of the research design that was difficult in small animals like mice or rats though they have a high percentage of utilization in the wound healing experiments. Moreover, small animals show quick healing time which is an unfit criterion for application of results to human beings (Grada et al. 2018). Guinea pigs were suggested due to their characteristic healing feature and skin resemblance to humans but could not be arranged due to unavailability.

Chemicals

Zinc oxide nanoparticles were purchased from the Healers Pharmacy Limited and ointment was prepared as 1% w/w in petroleum jelly and stored in plastic containers below 20°C at a dry place. Leaves of *A. calamus* were collected from the district Layyah, Punjab, and converted to powder form weighing 250 g that was extracted with 80% w/v ethanol in 24 hours and dried at 50°C to 60°C for purity. *A. calamus* extract ointment was prepared as 40% w/w in petroleum jelly. For both ointments, petroleum jelly which is supposed to be a hydrophilic entity was selected as an ointment base as it is considered a good vehicle for the action of topical drugs. Normal saline was purchased as a locally manufactured product by M.S. Enterprises Limited.

Infliction of wounds

Healing was evaluated for the excisional wounds model as it allows observation of hemorrhage, granulation tissue formation, wound contraction, and reepithelization that is closer to the human wound healing model (Masson-Meyers et al. 2020). For the infliction of wounds, the trunk region of rabbits was selected due to its qualities like being easy to handle, more contractibility, less prone to contamination, feasible for biopsy, and multiple wound holding capacity. Animals were shaved from the thoracolumbar region and the area was scrubbed with a cleansing agent. Methylated spirit and tincture of iodine were used as antiseptic agents. Here it is noteworthy to mention that each animal was taken as a separate unit and three spots on each animal were wounded at the thoracolumbar region for topical application of experimental chemicals separately to avoid debate on the influence of environment and climate in case of different animals for different experimental healing chemicals. Moreover, multiple wounds provide more reliable results and observations. Animals were anesthetized with ketamine hydrochloride (ketarol) at the dose rate of 30 mg/kg - 35 mg/kg body weight after keeping off feed for 4-6 hours with the latest anesthetic protocol for the specified anesthetic agent (Salem et al. 2022). Animals were placed in sternal recumbency and the area was sanitized properly before giving an incision. Animal welfare objectives and proper standard surgical protocols were taken care of. Three wounds, each of a marked area of 1 cm^2 were created; two lateral wounds which were just 2cm from the midline and one was created on the exact midline at the thoracolumbar region of each animal.

Treatment and evaluation of healing

Healing was allowed with secondary intention i.e., without apposition or suturing because apposed tissues are less available for histopathological samples and scar formation was difficult to observe. All the experimental animals were treated topically daily with 1% zinc oxide nanoparticles ointment for wound group A, sweet flag extract ointment for wound group B, and normal saline for wound group C as the control group of the study, and animals were kept separate from each other for the protection from scratching of wounds. Each time after the application of testing chemicals, the skin surrounding the wounds was massaged slightly which is a positive gesture for better absorption and penetration of the drug through the skin because of the hydrophilicity of ointments used in the experiment (Kaushik et al. 2021). This line of treatment was followed from the day the incisions were given to the animals. Animals were well-managed post-operatively, provided with optimal environmental, nutritional, housing, temperature, and stress-free conditions, and observed for normal behavior daily. Wound contraction is the first step in healing and is defined as the "reduction in the actual size of the wound or moving of wound edges towards the center of the wound" (Yilmaz and Aygin 2020). It was measured using digital vernier calipers for accuracy and readings were taken directly from the wounds daily. Healing time was noted and histopathology from healed tissue

Table 1. Readings taken by Vernier calipers (mm) for contraction rate of all wounds in rabbits after the application ZnO sweat flag and normal saline on days 05, 10, 15, 20 and 25, respectively.

Animals (A)	Days (D)														
	Day 05			Day 10			Day 15			Day 20			Day 25		
	ZnO	Sweet flag	Normal saline	ZnO	Sweet flag	Normal saline	ZnO	Sweet flag	Normal saline	ZnO	Sweet flag	Normal saline	ZnO	Sweet flag	Normal saline
A1	1.79	0.06	0.60	3.89	1.80	1.47	7.10	3.47	2.72	9.45	5.80	4.60	10.00	9.76	7.81
A2	1.84	0.9	0.90	4.12	2.20	1.80	7.65	3.93	3.82	9.14	6.45	6.91	10.00	9.65	8.15
A3	1.62	0.55	0.55	3.81	1.40	2.20	6.94	4.95	3.02	9.03	7.70	4.76	10.00	9.32	6.91
A4	2.1	0.50	0.50	3.94	1.60	1.40	7.62	2.90	2.93	10.00	5.95	5.14	10.00	8.55	8.19
A5	2.54	0.10	0.10	4.76	1.71	1.60	7.45	3.50	4.11	10.00	5.10	6.49	10.00	9.05	7.93
A6	1.28	0.70	0.31	3.33	3.34	1.43	5.66	3.90	2.78	9.00	5.59	5.17	10.00	8.56	8.10
A7	2.75	0.57	0.39	4.76	3.65	3.39	8.88	5.61	5.64	10.00	7.93	7.77	10.00	9.00	9.20
A8	2.00	0.79	0.45	4.10	3.56	3.33	6.74	6.95	4.90	8.10	8.72	6.20	10.00	9.28	8.54
A9	1.67	0.92	0.56	3.34	3.56	1.78	5.88	5.38	3.98	9.23	7.34	5.14	10.00	8.96	7.87
A10	2.96	0.66	0.67	5.96	3.73	2.98	9.00	5.50	4.33	10.00	7.95	6.56	10.00	9.35	7.94
A11	1.96	0.80	0.23	3.89	3.28	1.89	8.78	5.30	4.51	9.10	7.86	6.89	10.00	9.10	7.88
A12	1.89	1.00	0.59	4.67	3.45	1.76	7.88	6.13	4.03	10.00	8.10	6.12	10.00	9.54	9.00
Mean	2.033	0.629	0.487	4.214	2.599	2.085	7.465	4.751	3.897	9.420	7.040	5.979	10.00	9.176	8.126
SD	0.488	0.299	0.212	0.730	0.969	0.732	1.088	1.217	0.905	0.600	1.194	1.00	0.000	0.385	0.593

was performed later on to examine the pattern of healing using the Image J automated image analyzer. The tissue was considered to be fully healed by the day of the shedding of the wound scar. Here, one thing should not be neglected these evaluated wounds are acute but not chronic as a chronic wound model is difficult to establish and maintain in a laboratory especially in in-vivo study designs and usually requires association with some intrinsic or genetic factors like obesity or diabetes (Parnell and Volk 2019).

Results

Contraction of wounds

Contraction of wounds treated with zinc oxide nanoparticles ointment, sweet flag extract ointment, and normal saline was observed as subsided by the day 17th, 23^{rd,} and 29th of the creation of wounds, respectively. Hence, zinc oxide nanoparticles ointment proved more effective in the contraction of wounds and this observation was prominent in all experimented animals. Values for wound contraction during the healing process were taken with digital vernier calipers avoiding any kind of invasive approach and wound contamination, with a gap of 5 days at day 5, day 10, day 15, day 20, and 25 for all experimental groups (animals) and chemicals i.e., zinc oxide nanoparticles ointment, sweet flag ointment, and normal saline. All these observations have been coded in Table 1 and average contraction rates of wounds treated with all chemicals being studied on day 5, day 10, day 15, day 20, and day 25 were calculated using the arithmetic mean and standard deviation and analyzed statistically ($p<0.05$) and overall average contraction rates are presented in Fig. 1. Though the use of plant extracts eliminates the risk of drug resistance yet sweet flag ointment proved less effective in wound contraction as scar formations and shedding were delayed as compared to the wounds treated by zinc oxide nanoparticles ointment.

Histopathology of samples

Tissue samples were taken from the edge of the wounds after wound healing or shedding of scars because it allows the comparison of healing and healed tissues. The thickness of the dermis, epidermis, and percentage of collagen content was measured. Sample of size 2 mm from each wound was taken and processed in formalin. These samples were cleared in xylazine, washed in degraded alcohol, and embedded in paraffin. Paraffin-embedded tissue samples were subjected to thin sectioning i.e., below 5 microns, and stained with hematoxylin and eosin.

The thickness or percentage of collagen is considered a hallmark of the compactness of the healed tissue (Aboulhoda and Abd el Fattah 2018). Histopathological results clearly showed that thickness, as well as vascularization of the dermis and epidermis along with percentage collagen content, was the high-

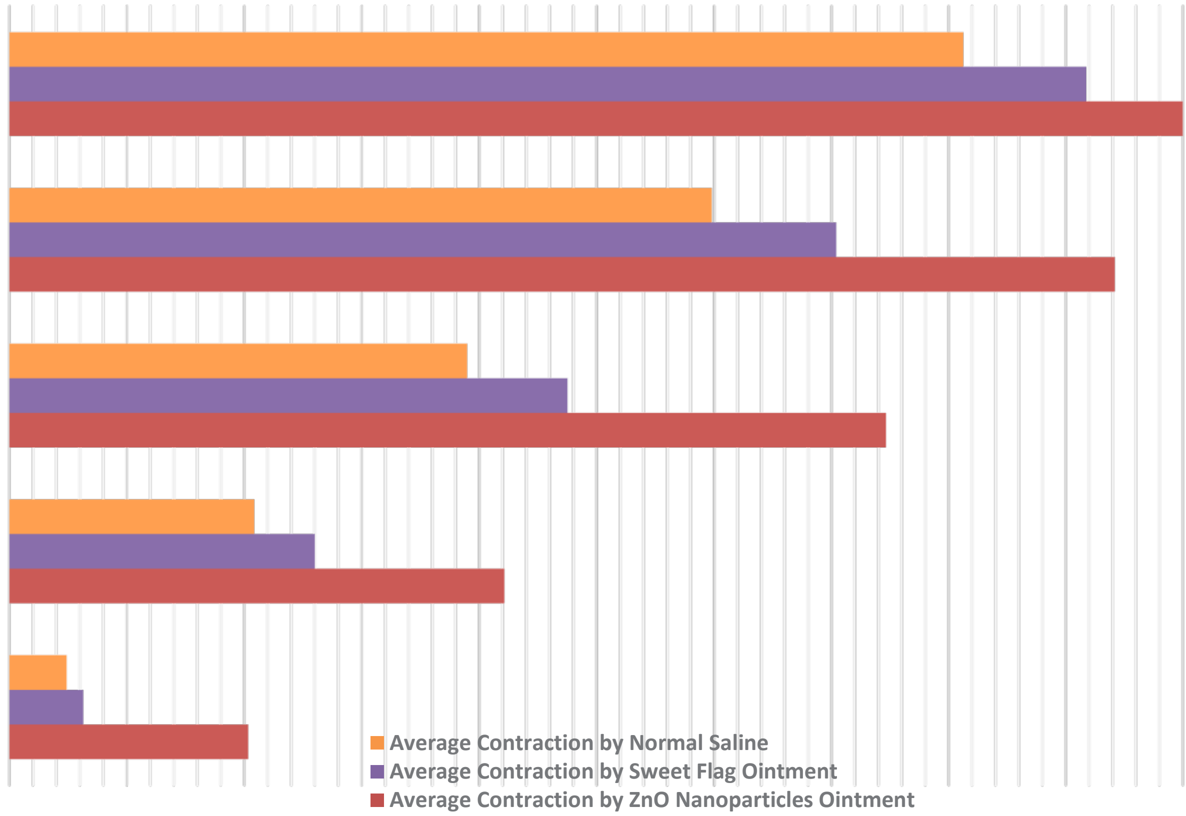

Fig. 1. Average contraction of wounds in rabbits on days 05, 10, 15, 20, and 25 following the application of normal saline (orange), sweet flag ointment (purple), and zinc oxide (ZnO) nanoparticles ointment (red).

Table 2: Average measurement of collagen content, dermis, and epidermis of all wounds in rabbits treated with zinc oxide (ZnO) nanoparticles ointment, sweet flag ointment, and normal saline.

Healing Chemical	Histopathological Parameters Measured		
	Collagen Content Percentage	Dermis (µm)	Epidermis (µm)
Zinc Oxide nanoparticle ointment	92.12	1830.5	128.76
Sweet Flag ointment	83.55	1620.2	35.14
Normal Saline	76.93	873.3	12.89

est, moderate, and low for wounds treated post-surgically with zinc oxide nanoparticles ointment, sweet flag extract ointment, and normal saline respectively along with infiltration of some leukocytes that is normal during healing as inflammation is a significant step in the healing of the tissue (Kia et al. 2018). The thickness of different layers of the skin tissue observed in histopathology is recorded in the form of data in Table 2, Figs. 2a, 2b, and 2c show the representative samples from histopathological results captured at 200X by Nikon opt photo 2 and analyzed by the Image J analysis system provided by the National Institute of Health, USA with the speed of 40 million pixels per second. Similar histopathological observations along with more calcium deposition in the tissue representing more healing and mineralization of healing tissue when treated with zinc oxide nanoparticles ointment, have also been reported by Manuja et al. (2020) using Alizard stain. Hence, the healing effect of zinc oxide nanoparticles proved statistically significant ($p<0.05$). There was no accumulation of protein corona or protein composites as reported by Srivastav et al. (2019) and Hassanian et al. (2021) that limits the wide application of zinc oxide nanoparticles therapy. This contradiction in opinion or results may be reflected by the preparation of zinc oxide nanoparticles ointment in hydrophobic or hydrophilic solvents.

Discussion

Wounds treated with zinc oxide nanoparticles get healed up far earlier than those treated with Sweet Flag and normal saline. The mean values for healing time were as follows: 20^{th} for zinc oxide nanoparticles,

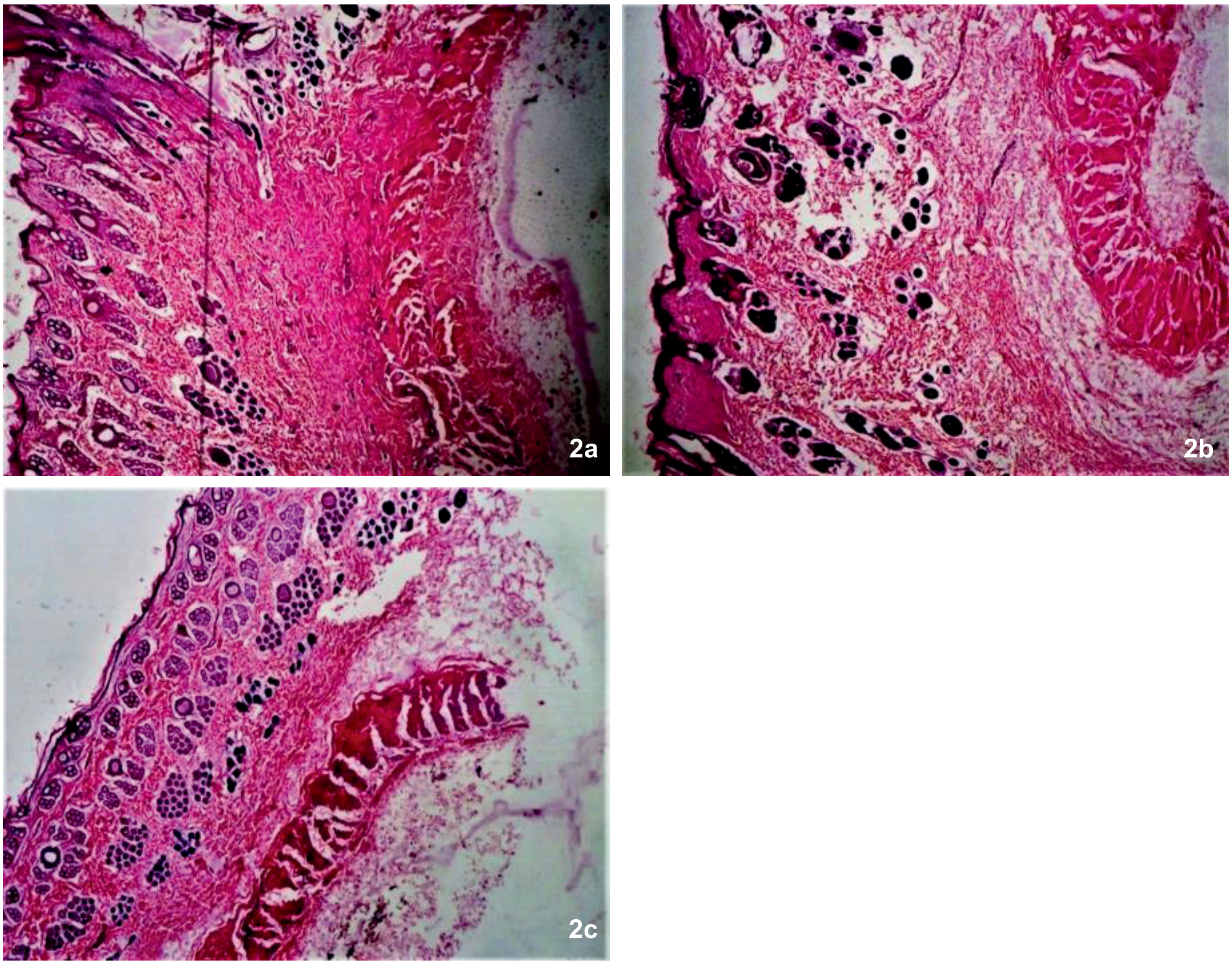

Fig. 2. Histopathological results captured at x200 and analyzed by Image J Analysis System by NIH, USA showing efficacy of tested chemicals in wound healing in rabbits after 25 Days. Photomicrograph of slide showing thin epidermis with highest collagen fibers in the dermis and presence of blood vessels in the healed wound tissue treated with zinc oxide (ZnO) nanoparticle ointment (2a). Photomicrograph of slide showing thin epidermis formation and sufficient presence of collagen fiber in the dermis of healed wound tissue treated with Sweet Flag extract ointment (2b). Photomicrograph of the slide showing thin and less developed epidermis with sufficient presence of collagen in dermis in healed wound tissue treated with normal saline (2c).

26th for Sweet Flag, and 28th for normal saline. It justifies the fact that zinc oxide nanoparticles ointment showed a statistically significant reduction in wound healing time. This is due to the generation of dense collagen contents and improved angiogenesis (Yadav et al. 2012). Moreover, Al-Henhena et al. (2011) reported that collagen content formation is a key component of proliferated tissue and its formation directly relates to the healing of wounds. We checked collagen content formation by histopathology and confirmed that zinc oxide nanoparticles in ointment-treated wounds showed denser collagen contents as compared to the other two treatments. Neovascularization was also rich in such wounds and inflammatory cells were fewer in wounds treated with the ointment of zinc oxide nanoparticles. Dermis layers were developed thickened in wounds treated with the ointment of zinc oxide nanoparticles than others in cases of the other two treatments. Healing was promoted in wounds treated with the ointment of zinc oxide nanoparticles as the blood supply increased in the area of wound site and nutrients and oxygen also rushed there. Muhammad and Muhammad (2005) conducted a study to check the wound-healing process and the different factors which influence it. They concluded that the collagen expression regulation of wounds reduces healing time to a much extent. We had already proved by histopathology that wounds treated with the ointment of zinc oxide nanoparticles had dense collagen contents, so it is a clue that wound healing is far better by this agent as compared to its counterparts.

The current study was planned to compare the wound healing efficacy of zinc oxide nanoparticles, Sweet Flag, and normal saline alone on surgical wounds in the rabbit model and compare each to the other. The evaluation parameters in this study were wound contraction rate, healing time, tensile strength, and histopathology of surgical wounds. In this study, the difference of temperature and respiration rate was non-significant ($p>0.05$). A slight increase in temperature and pulse rate was observed on day one in postoperative care which returned to normal on day 5th and remained normal until the complete wound healing occurred.

In the present study, wound contraction rate, healing time, tensile strength, and histopathological parameters were evaluated. It was noted that Zinc oxide nanoparticle group A healed faster than that of Sweet Flag treated group B and group C treated with normal saline. This might be due to the presence of vitamin B complex, catalase, antioxidants, minerals, selenium, and ascorbic acid. Zinc oxide acts as an anti-inflammatory agent on the wound site and starts the wound healing by lowering the pH and in this way, clearance of microbes occurs at the wound site (Robson et al. 2001). The early rate of wound contraction after using the combination of zinc oxide and Sweet Flag, or alone Sweet Flag might be due to the presence of flavonoids and terpinoids. Flavonoids and terpenoids are active components in the Sweet Flag, having anti-inflammatory, antimicrobial, and healing-enhancing properties.

In this study, it was found that the Zinc Oxide Nanoparticle treated wounds healed faster than those in the other two treatment groups. Sweet Flag and Normal Saline treated wounds healed at the end. The mean values observed for zinc oxide nanoparticle treated group A, Sweet Flag treated group B, and normal saline group C were 17.60±0.781, 22.20±0.632, and 27.20±0.663 days, respectively. It might be due to the presence of minerals, vitamins, antioxidants, and anti--inflammatory properties in the combination of honey and *Aloe Vera* (Tasleem et al. 2014).

The tensile strength of the healed tissue was also considered for the evaluation of the trial drugs. It was noted that the tensile strength of tissues treated with the Zinc oxide nanoparticle in group A was greater than that found in the other two groups, B and C in which wounds were treated with Sweet Flag and normal saline. The mean± standard values observed in groups A, B, and C were 43.00±0.707, 38.40±0.510 and 25.60±0.748, respectively. On the other hand, Sweet Flag plays an important role in the formation of the granulation tissue and fibroblast keratinization at the wound surface (Molan 1998). Hence the combination of both these resulted in stronger tissue regeneration.

The histopathological evaluation of the healed tissues revealed that the zinc oxide nanoparticle-treated group A showed the best development of the epidermis with the thickness of skin layers as compared to groups B and C, which were treated with sweet flag and normal saline, respectively. While the percentage of collagen fiber was higher in group B treated with Sweet Flag as compared to groups A and C, in which the tissues were treated with nanoparticles and normal saline, respectively. This might be attributed to the presence of terpenoids and flavonoids, which enhances the migration of fibroblast and myofibroblast towards the wound site. In this way, there is faster re-epithelization at the wound site. On the other hand, the sweet flag plays an important role in the formation of the granulation tissue and fibroblast keratinization at the wound surface (Molan 1998). Hence, the combination of zinc oxide and Sweet Flag resulted in stronger tissue regeneration as compared to these materials alone or the standard treatment.

Conclusion

Zinc oxide nanoparticles ointment is more effective in surgical wound healing as compared to *A. calamus* leaves extract ointment and normal saline when applied topically without using any antibiotic. There are no side effects of zinc oxide nanoparticles ointment like protein opsonization when prepared in hydrophilic compositions. Histopathological satisfaction for the repaired tissue and wound contraction rates are more reliable when wounds are treated with zinc oxide nanoparticles ointment and their application in practical life as a pharmaceutical product will provide quick relief from wounds, avoid the issue of minimum inhibitory concentration (MIC) and reduce chances of drug resistance, and may serve as better alternate option for antibiotic therapy, especially for wound healing. It will serve not only the public sector but as well as veterinary sector as infectious sepsis of wounds is a major issue for professionals. In the future, this anticipation may be supported by using animals, engrafted with human cells to avoid immunological and inflammatory differences from the human model for the sake of further wound healing studies. The efficacy of all the healing agents used in the study remains questionable in the case of chronic wounds that require further research.

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