Goat Wound-Contaminating-Bacteria Restraint by Neem Oil

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Abstract

This study aimed at evaluating the neem oil antibacterial activity towards wound-contaminating-bacteria resulting from ectoparasites sting and bite. Mesophilic, thermophilic, *E coli*, *E coli eae*, *hlya*, *EAST1* gene positive strains, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* were isolated by a non-invasive technique for microbiological sampling. Multiplex PCR assays using PMA™ dye and species specific primer pairs allowed the characterization of isolated bacteria and the detection of bacterial viable cell after neem oil treatments. The antibacterial activity was evaluated by standardized disc diffusion test and microdilution test. Neem oil showed antibacterial activity towards all isolates tested. The neem oil bacterial growth inhibition zone (mm) by disc diffusion test (100 µl neem oil) ranged between 22 ± 0.68 (*P. Aeruginosa*) and 30.21 ± 1.38 (*E. Coli EAST1*). The antibacterial activity was determined at lower neem oil dilutions (1:10 – 1:10,000) by microdilution method. The highest bacterial growth reduction (%) ranged between 81.51 ± 1.15 (*S. Aureus* sub sp. *aureus,* one of the reference strains considered in the experiment) and 95.51 ± 1.15 (*E. Coli hlya*) (100 µl neem oil and 50 µL of bacterial suspension, ca 1×10^6 CFU/ml). The effect of neem oil, Tween 20® as well Tween 20® and neem oil together at several dilutions ($1:2 \times 10^2$; $1:2 \times 10^3$; $1:2 \times 10^4$; $1:2 \times 10^5$; $1:2 \times 10^6$; v/v) towards goat peripheral blood mononucleate cells viability was investigated, too. Only $1:2 \times 10^2$ and $1:2 \times 10^3$ dilutions of neem oil, Tween 20® as well neem oil and Tween 20® affect PBMC viability. On the basis of the obtained results neem oil should be used directly on wound or diluted in Tween 20® for skin care.

Keywords: Wound-Contaminating-Bacteria; Antimicrobial Activity; Neem oil; Goat

Inroduction

Ectoparasites play a very detrimental role in terms of decreasing the productivity of sheep and goats. Common external sheep and goat parasites include ticks, lice, and mites. They cause restlessness and nuisance. Weight loss and reduction in milk production may occur as a result of nervousness and improper nutrition because animals spend less time eating. Bites can damage sensitive areas of skin (teats, vagina, eyes, etc.). Some parasites feed on blood causing blood-loss anemia, especially in young animals. The ectoparasite bite and sting let bacteria proliferate in wound from abrasions or lesions from scratching and cause levels of tissue reaction of different entities, super-infection and cervical lymphadenopathy. They also seriously damage sheep and goat skin resulting in the rejection or downgrading of the skins. This causes huge economic losses through skin damage rendering it unsuitable for the leather industry due to the decrease in skin quality. Wool is lowered in value, too. *Pseudomonas aeruginosa* wound infections are characterized by a change in the color of the skin around the wound area and formation of lesions [1]. The bacterium products and pigment cause a yellow discoloration of wool (http://www.infovets.com/books/smrm/A/A972.htm).

A major concern emerging in recent years in the field of bacterial infections, which stimulate the development of innovative molecules with antimicrobial activity, is represented by the problem of multiple antibiotic resistance, i.e. onset and rapid growing global dissemination of pathogens that have acquired resistance to multiple antibacterial drugs in use, capable of supporting common infections, potentially fatal for animals and humans. According to World Health Organization (WHO, 2002-2005) [2] medicinal plants would be the best source to obtain a variety of drugs. In fact, many plants produce secondary metabolites which act towards wound-contaminating-bacteria and parasites [3,4]. Plants are a good source of antioxidants and also cure various disorders associated with wound inflammation [5]. Identification and studies on active compounds from plants are now of major interest to the scientific community [6]. These include compounds such as diterpenoid from raw plant material which showed antibacterial activity towards *Streptococcus pyogenes* and some fungi. In addition, tannins, found in a number of plants used in wound healing were also found to have antibacterial activity [7].

Nowadays, Neem (*Azadirachta indica* A. Juss) is considered an effective source of environmentally-powerful natural pesticide

and considered to be one of the most promising pesticides. It is believed to be one of the trees of the 21st century for its great potential in pest management, environmental protection and medicine [8]. Among the many products obtained from the seeds, neem oil is the most commercially relevant (www.organicneem. com/why_parker_neem.html). More than four hundred substances were identified in the neem oil. The most biologically active are the nortriterpenoid limonoid. In particular, the azadirachtin A is the compound best known and studied for its properties that allows its use in agriculture as a natural insecticide, acaricide and nematocide, but it should not forget the salannin and nimbin, among other major limonoids. The insecticidal properties of neem oil are closely related to the larvicide, acaricide, nematicide, and repellent effects.

The antimicrobial activity of neem oil has been investigated [9,10] . In addition, it has been recently demonstrated the neem oil antibacterial activity towards several food spoilage bacteria [11] and strains of enteropathogenic *Escherichia coli* [12]. This study aimed at evaluating the neem oil antibacterial activity towards bacteria isolated from animal wounds resulting from ectoparasites sting or bite.

Methods

The microbiological, molecular biology, and Peripheral Blood Mononuclear Cells viability assays were carried out in the Biochemistry and Cell culture Laboratories of the Research Center for Animal Production, CRA PCM, Monterotondo (RM), Italy. Blood specimen for Peripheral Blood Mononuclear Cells viability assay were collected from goat reared in the Research Unit for the Extensive Animal Husbandry, CRA ZOE, Muro Lucano (PZ), Italy. High Performance Liquid Chromatography analysis was carried out in the Quality Control Laboratory of the University of Rome Sapienza, Dep. Environmental Biology, Rome, Italy.

Neem oil

A commercial neem oil produced by Neem Italia (Manerba (BS), Italy) was used as test starting material (0.35% azadirachtin A) determined by High Performance Layer Chromatography. It is a mixture of neem oil and emulsifying agents making it more fluid and less liable to hardening at cold temperatures and more suitable for inclusion in products or in cosmetics for animals. Neem oil was further diluted in Tween 20® (1:1 v/v; VWR, PBI International, MI, Italy) under agitation and sterilised by filtration through a 0.22 μm Millipore express filter (Millex-GP, Bedford, OH, USA) before using in the experiment.

High Performance Thin Layer Chromatography analysis

The neem oil metabolomic fingerprint was determined by High Performance Thin Layer Chromatography according the methodology previously developed [12,13].

Microbiological analysis

Five goats reared in the Research Unit for the Extensive Animal Husbandry CRA ZOE, naturally infested by lice and showing wounds resulting from ectoparasites sting and bite, were randomly chosen for bacteria isolation. Bacteria isolation from complex infected skin lesions was made by a non-invasive technique for microbiological sampling performing the removal using flocked swabs (FLOQ Swabs™ patented technology, Copan, Brescia, Italy). Flocked swabs aimed to collect more sample volume than traditional swabs and automatically and completely release the sample immediately when in contact with liquid or surface. Three flocked swabs were used for each goat. ESwab and two selective enrichment broths for single organism screening: TSB salt broth and LIM broth (Copan, Brescia, Italy) were used respectively for *S. aureus and Streptococcus spp* . The isolation from ESwab, TSB salt and LIM broth are compliant with the Quality Control of Microbiological Transport Systems; Approved Standard, M40A2 (CLSI M40-A2, 2014) [14] as per guideline DIN 58942-4 Supplement 1 (DFNV, 2004) [15] and performed according to the manufacturer's instructions. The swab method was applied by taking three smears per sample on an area of 100 cm^2 defined with a delimiter, adding 100 μL of isotonic eluent furnished by the Manufacturer for decimal dilutions and plating 1 mL per dilution on selected media using a sterile bacteriology loop to streak the primary inoculum across the surface of the second, third and fourth quadrants of the agar culture plate. Count determinations for mesophilic, thermophilic and coliform bacteria with *Escherichia coli* distinction and qualitative assays were also made to sample a wide range of microorganisms.

Mesophilic and thermophilic bacteria were counted on Dextrose Tryptone Agar (Oxoid, Garbagnate Milanese (MI), Italy), after incubation at 32°C for 72 hours and 55°C for 48 hours, respectively.

Coliforms with *E. coli* distinction were counted on Chromogenic Compact Dry and Chromogenic Compact Dry with *Escherichia coli* distinction (PBI, Milano, Italy) after incubation at 35°C for 20 hours [16, 17].

Baird Parker Agar (Acumedia, Neogen, lansing MI, USA) was used as selective medium for the isolation and presumptive identification of catalase-positive staphylococci. It contains 7.5% sodium chloride and thus selects for those bacteria which can tolerate high salt concentration.

Mannitol Salt Agar is both a selective and differential medium used in the isolation and distinction of *S. aureus* and *S. epidermidis*.

Catalase-negative *Enterococcus* spp. and *Streptococcus* spp. were isolated on Columbia Blood Agar Base, Streptococcus Selective Supplement and Defibrinated Horse Blood (OXOID, Basingstoke, UK) incubated plates at 37°C, either aerobically or anaerobically and examined after 18 to 24 hours incubation.

Pseudomonas Isolation Agar (Acumedia, Neogen Corporation, lansing, MI, USA) was used for the isolation of *Pseudomonas aeruginosa* and other *Pseudomonas* spp. under aerobic incubation at 35 ± 2°C and examined for growth after 18-48 hours. The medium includes Irgasan, a broad spectrum antibiotic not active towards *Pseudomonas* spp. This medium is selective and formulated to enhance formation of blue or blue-green pyocyanin pigment by *Pseudomonas aeruginosa*. The pigment diffuses into the medium surrounding growth.

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Escherichia coli, American Type Culture Collection (ATCC)® 51813™ *S. aureus* subsp. *aureus* ATCC® 6538™, *E. faecalis* ATCC® 7088TM, and *P. aeruginosa* ATCC 27853 were used as reference strains.

To prepare working cultures, stock cultures were standardized through two successive 24 h growth cycles in Brain Heart Infusion broth (Difco, Buccinasco, MI, Italy) at 20° C without agitation.

The reference strains were grown on media and at the growth conditions as reported on products sheets.

Antibacterial activity of neem oil

The antibacterial activity of neem oil was assayed using standardized disc diffusion agar and microdilution methods. Whatman filter paper (no.1) discs (6 mm diameter) were impregnated with 100 μL of neem oil. Discs impregnated with 100 μL of sterile distilled water, Tween 20®, as negative controls, and 10 μL (wt/v) of ciprofloxacin (1 mg/mL, Bayer, Milano, Italy), as positive control, were used. Two discs were prepared for each sample. They were evaporated at 37°C for 24 h.

Bacterial suspensions (100 μL), standardized by adjusting the optical density to OD 1 at 600 nm (Shimadzu UV-120-01 spectrophotometer, Shimadzu, Cinisello Balsamo, MI, Italy), were used to inoculate by flooding the surface of suitable agar media for each microorganism considered at their proper growth conditions. Excess liquid was air-dried under a sterile hood and the impregnated discs were applied at equidistant points on top of the agar medium. The plates were done in triplicates for each organism and the experiment was performed twice. Plates were checked after incubation at 35°C and 42°C for 48 h. Antimicrobial activity was measured as diameter (mm) of the inhibition zone around the disc. The results were recorded as means ± SD of the duplicate experiment.

The antibacterial activity of neem oil towards the bacteria was also evaluated in microdilution assays using conventional sterile polystyrene microplates. Each well of the microplate was filled with 100 μL of sterile suitable liquid media for each microorganism considered, 50 μL of inoculums (see Table 4) and amounts of extract at lower concentrations (1:10 to 1:100,000) were added. Control treatment without neem oil was used in the experiment. The microplates were incubated at 37°C for 24 h. Bacterial growth was determined by OD 0.02 reading at 630 nm/10 mm path length with an ELISA microplate reader (Dynatech ML-3000, Pina de Ebro, España). Bacterial cell concentration was transformed to cells/mL using the reference curve equation. The reference curve was constructed by diluting 1:100 each bacterial species. Counting the number of bacterial cells of an aliquot of this dilution was done using a Neubauer chamber (Celeromics, Vedano al Lambro, MI, Italy). Finally, cell concentrations were transformed to a percentage of bacterial inhibition. The percentage of bacterial growth reduction (GR%) was estimated using as reference the control treatment (without neem oil) as:

GR% = C – T/C x 100

Table 1: Growth conditions and enumerations of wound-contaminating-bacteria isolated from goat wound resulting from ectoparasites bite and sting.

Bacteria	Growth media and conditions	CFU / mL*
Mesophilic	DTA 32° C 72 h	6×10^7
Thermophilic	DTA 55° C 48 h	$2,3 \times 10^4$
Coliform	CCD 35° C 20 h	$5,3 \times 10^7$
E. coli	CCD EC 35° C 20 h	5×10^5
Coagulase positive	DBPA	$6,4 \times 10^4$
S. aureus	TSB	3.8×10^4
	MSA	2.3×10^4
Coagulase negative	CBAB 37° C aerobic and anaerobic 18 to 24 h	nd
S. epidermidis		nd
Streptococcus group	LIM	nd
Streptococcus spp.	CBAB + SSS	nd
Enterococcus spp.	CBAB + DHB 37° C 18 to 24 h	$4,8 \times 10^6$
Pseudomonas spp.	PIA 35° C 18 to 48 h	$3,6 \times 10^5$

nd = not detected.
* The enumerations of wound-contaminating-bacteria are expressed as means of 5 sampling each of three repetitions

Where, 'C' is the cell concentrations under the control treatment and 'T' is the cell concentrations under the neem oil treatment.

Three replicates were considered. The results were recorded as means ± SD of the duplicate experiment. Differences between means of data were compared by Least Significant Difference (LSD) calculated using the Statistical Analysis System (S.A.S., Institute, Inc. Cary, NC, USA).

Molecular biology analysis

Molecular biology analysis was used either to identify and characterize isolated microorganisms or together with PMA™ dye (Biotium, Hayward, CA, USA) to detect bacteria viable cells after neem oil treatment *in vitro*.

The dye (PMA™ Biotium Inc. Hayward, CA, US) is a photo-reactive dye with high affinity for DNA. It intercalates into DNA and forms a covalent linkage upon exposure to intense visible light. It is cell membrane impermeable. When a sample comprising both live and dead bacteria is treated with PMA™, only dead cells are susceptible to DNA modification due to their compromised cell membranes. Thus, it permits selective detection of the sole live cells.

DNA extraction was performed using ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies Italia, Monza, MB, Italy) following manufacturer's instructions.

The molecular identification and characterization was made using specific primer pairs for each isolated bacterium as mentioned in the section microbiological analysis, mixture and reaction conditions as reported in literature (Table 2 list A and list B).

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Two primer pairs that amplify specific *E. coli* 16S rRNA sequences and fourteen primer pairs that specifically amplify target gene coding for virulence factors (adhesins and toxins) were employed to characterize the *E. coli* isolates [18].

The detection and identification of *Staphylococcus spp.* were performed by multiplex PCR with primer pairs that amplify variable sequences of the *nuc* gene, specific for *Staphylococcus* genus and expressing thermo nuclease enzyme, mixtures and reaction conditions as reported in the literature [19].

Enterococci isolates were identified using Internal Transcribed Spacer region to identify the majority of enterococci and the ddl genes encoding D-alanine-D-alanine ligase specific for the individual species [20,21].

P. aeruginosa is an opportunistic pathogen capable of infecting virtually all tissues. Its molecular detection and identification was performed using PCR amplification of two specific outer bacterial membrane proteins: I lipoprotein (*oprI*) and L lipoprotein (*oprL*) using two sets of primers (fPS1/rPS2 and fPAL1/rPAL2) were used in multiplex PCR [22,23].

The amplified products were revealed by electrophoresis in agarose (2%) gel, stained with GelGreen™ Nucleic Acid Gel Stains (Biotium, Inc., Hayward, CA, USA) and visualized by blue light transilluminator UltraBright-LED (Syngene Europe, Cambridge, UK) using a 100 bp DNA ladder (Thermo Fisher Scientific. Milano, Italy).

Water without templates and the reference strains were considered as negative and positive controls.

Tween®20 effect on goat's Peripheral Blood Mononucleate Cells

Tween®20 as well Tween® 80 is non ionic surfactants employed as emulsifier and stabilizer. They are accustomed in human foodstuffs, cosmetic, pharmaceutical preparations, and in many research methodologies. It has been recently reported that Tween®20 can induce decrease growth of treated cells (A459 cells and human umbilical vein endothelial cells) and apoptosis [24]. Since both surfactants were used in previous studies on the antibacterial activity of neem cake extract and neem oil

Table 2: Primer pairs used to identify E. coli, that amplify target genes coding for virulence factors (toxins 1 - 9; adhesins 10 - 15) and 16sRNA (16 - 17) (List A), *Staphylococcus sp*p, *Pseduomonas aeruginosa* as *well Enterococcus spp.* (List B) with related amplicon sizes.

Target gene	Primer sequences	Amplicon (bp)
1) LT	f 5'-ATT TAC GGC GTT ACT ATC CTC-3' r 5'-TTT TGG TCT CGG TCA GAT ATG-3'	281
2) STa	f 5'-TCC GTG AAA CAA CAT GAC GG-3' r 5'-ATA ACA TCC AGC ACA GGC AG-3'	244
3) STb	f 5'-GCC TAT GCA TCT ACA CAA TC-3' r 5'-TGA GAA ATG GAC AAT GTC CG-3'	279
4) *Stx1all*	f 5'-CGC TGA ATG TCA TTC GCT CTG C-3' r 5'-CGT GGT ATA GCT ACT GTC ACC-3'	302
5) *Stx2all*	f 5'-CTT CGG TAT CCT ATT CCC GG-3' r 5'-CTG CTG TGA CAG TGA CAA AAC GC-3'	516
6) *Stx2e*	f 5-ATG AAG AAG ATG TTT ATA GCG-3' r 5'-TCA GTT AAA CTT CAC CTG GGC-3'	264
7) *EAST1*	f 5'-CCA TCA ACA CAG TAT ATC CGA-3' r 5'-GGT CGC GAG TGA CGG CTT TGT-3'	111
8) *eae*	f 5'-GGA ACG GCA GAG GTT AAT CTGCAG-3' r 5'-GGC GCT CAT CAT AGT CTTTC-3'	775
9) *hlyA*	f 5'-AGCTGCAAGTGCGGGTCTG-3' r 5'-TACGGGTTATGCCTGCAAGTTCAC-3'	569
10) F4 (K88)	f 5'-GCT GCA TCT GCT GCA TCT GGTATG G-3' r 5'-CCA CTG AGT GCT GGTAGT TAC AGC C-3'	792
11) F5 (K99)	f 5'-TGC GAC TAC CAA TGC TTC TG-3' r 5'-TAT CCA CCA TTA GAC GGA GC-3'	450
12) F6 (P987)	f 5'-TCT GCT CTT AAA GCT ACT GG-3' r 5'-AAC TCC ACC GTT TGT ATC AG-3'	333
13) F17	f 5'-GGG CTG ACA GAG GAG GTG GGGC-3' r 5'-CCC GGC GAC AAC TTC ATCACC GG-3'	411
14) F18	f 5'-GTG AAA AGA CTA GTG TTT ATT TC-3' r 5'-CTT GTA AGT AAC CGC GTA AGC-3'	510
15) F41	f 5'-GAG GGA CTT TCA TCT TTT AG-3' r 5'-AGT CCA TTC CAT TTA TAG GC-3'	431
16) E16SI	f 5'-CCCCCTGGACGAAGACTCAC-3' r 5'-ACCGCTGGCAACAAAGGATA -3'	401
17) E16SII	f 5'-AGAGTTTGATGGCTCAG-3' r 5'-GGACTACCAGGGTATCTAAT-3'	798

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Species	Target gene	Primer sequences	Amplicon (bp)
S. aureus	nunc 1	f 5' - ATG AAG TCA AAT AAA TCG CT- 3' r 5' - TTT GGT GAA AAA TAC TTC TC - 3'	458
S. hyicus	nunc 2	f 5' - AAA AAT AAC AAC AGG ATT GA - 3' r 5' - GTA AAG TCT GAA GCT TCT TT - 3'	270
S. intermedius	nunc 3	f 5 '- GAA AAA AAT TAC AAC AGG CG - 3' r 5' - CAC ATC CGT TGA AGA CTT TT - 3'	106
P. aeruginosa	PS	f 5' - ATG AAC AAC GTT CTG AAA TTC TCT GCT - 3' r 5' - CTT GCG GCT GGC TTT TTC CAG - 3'	249
	PAL	f 5' - ATG AAA ATG CTG AAA TTC GGC - 3' r 5' - CTT CTT CAG CTC GA - 3'	504
Enterococcus spp.	ITS	f 5'-CAA GGC ATC CAC CGT-3' r 5'-GAA GTC GTA ACA AGG-3	
E. faecium	ddlEfm	f 5'-AGA GAC ATT GAA TAT GCC -3' r 5'-CTA ACA TCG TGT AAG CT -3'	550
E. faecalis	ddlEfs	f 5'-ATC AAG TAC AGT TAG TCT -3 r 5'-ACG ATTCAA AGC TAA CTG -3'	491

Notes: 1 - 9 genes: LT, heat labile exotoxin, causes hyper secretion of water and electrolytes within gut. STa, STb, heat-stable enterotoxins, alters fluid and electrolyte transport in the gut. Stx2e, also known as edema disease factor, is the cause of lesions associated with edema disease in pigs. East 1, enteroaggregative heat-stable enterotoxin 1. *eae*, attaching and effacing. *hlyA,* hemolysin. All of them act after fimbriae interaction with a mucosal receptor.
10 -15 Different types of *E. coli* fimbriae

[25,26,27] the effect of neem oil in association with Tween 20® on goat's Peripheral Blood Mononucleate Cells (PMBC) viability was investigated, too.

The isolation of goat Peripheral Blood Mononucleate Cells procedure, Peripheral Blood Mononucleate Cells culture conditions, and their quantification by viable cells WST-1 test used in the experiment were those reported by De Matties et al. [27]. Neem oil and Tween 20® were used singularly as controls.

Results

High Performance Thin Layer Chromatography analysis

The neem oil metabolomic fingerprint shows characteristic sequence of metabolites according to the polarity of constituents. The identification of the raw material was assured by the presence of salannin (Rf = 0.42), that is a typical marker of neem. In comparison with the spot of azadirachtin (Rf = 0.23), salannin appear as the main limonoid spot. Spots concerning lipids are present at Rf values, at ca. 0.80, due to unsaturated fatty acids and fatty alcohols, and at Rf ca. 0.50, due to saturated and unsaturated triglycerides.

The most interesting feature of the plate concerns the presence of compounds with high fluorescent reaction at between Rf 0.55-0.66, that are perfectly visible at 366 nm after derivatization with anisaldehyde. These spots can be attributed to compounds with high conjugated unsaturation in polycyclic aromatic structures, very different from those of the nortriterpenes limonoids, so far considered as responsible for the activities investigated in our studies (Figure 1).

Microbiological analysis

The microbiological analysis allowed the isolation of coliform,
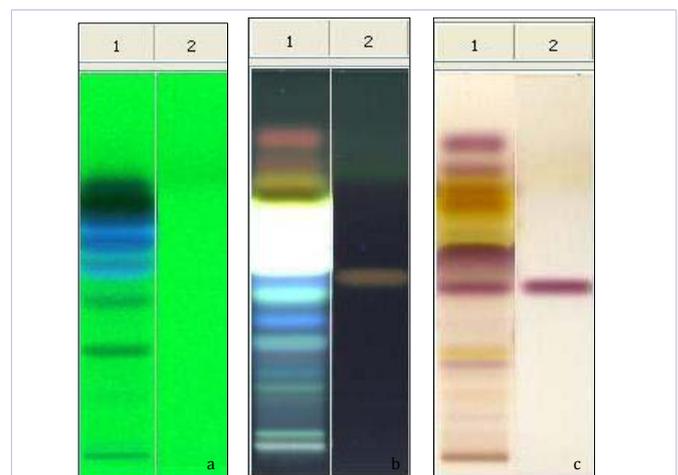

Figure 1: HPTLC analysis of neem oil EtOAc extract. Mobile phase: Toluene: AcOEt 7:3 (v/v). Visualization: plate a (on the left) UV lamp 256 nm; plate b (in the middle) UV lamp 366 nm; plate c (on the right) UV lamp at 366 nm. Derivatization: Anisaldede. Track 1: Neem Oil; Track 2: Salannin.

mesophilic, thermophilic bacteria, *E. coli*, *Staphylococcus* spp., *Enterococcus* spp., and *Pseudomonas aeruginosa* from goat's wound resulting from ectoparasites bite and sting. The enumeration counts of isolated microorganisms are reported in Table 1 as means of five samples each of three repetitions (3 wounds / goat).

Molecular biology analysis

The molecular biology analysis allows the identification and characterization of three strains of *E. coli.* They were intimin (*eae*), hemolysin (*hlyA*) and heat-stable enterotoxin (*EAST1*) gene positive *E. coli*. Furthermore, *Enterococcus*

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faecalis, *Staphylococcus aureus* and *P. aeruginosa* were detected. Amplicons of the expected size were obtained from DNA extracted from each bacterium investigated as showed in list A and List B of Table 1.

Amplicons were also obtained from DNA extracted from American Type Culture Collection reference strain of each tested bacterium (positive controls) while amplified products were never obtained from reaction mixtures without templates (negative controls) (Figure 2).

Antibacterial activity

The neem oil antibacterial activity was assayed towards six bacterial isolates and four reference strains as control. The neem oil average Growth Inhibition Zone (mm) ranges 22.68 ± 0.55 to 30.21 ± 1.38. The highest neem oil Growth Inhibition Zone was revealed towards *E. coli* strains being, respectively, *E. coli eae* 28.71 ± 1.29, *E. coli hly A* 29.59 ± 1.17 and *E. coli EAST1* 30.21 ± 1.38. *S. aureus* isolate results the less susceptible among all tested
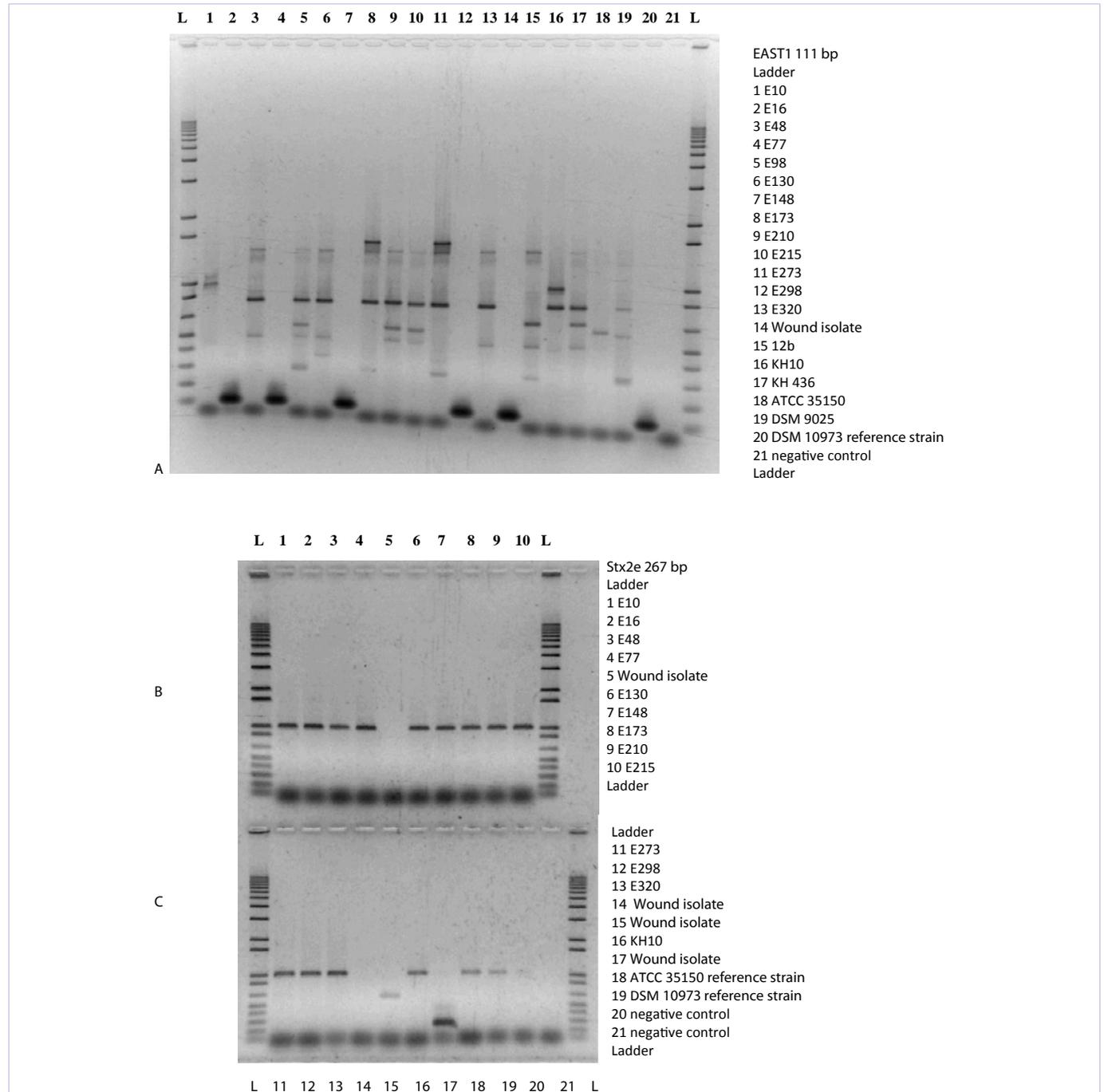

Figure 2: Gel electrophoresis (2%) visualization under UV light (360 nm) of the enterotoxigenic *EAST1* (A) and *Stx2e* (B) genes amplified products.

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bacteria by the disc diffusion method. The ciprofloxacin Growth Inhibition Zone ranges 23.62 ± 1.00 - 31.42 ± 1.39. There was significant difference between the neem oil antibacterial activity and ciprofloxacin antibacterial activity towards *E. coli eae E. coli hly A* and *EAST1* (Table 3)*.*

As shown in Table 4, the percent bacterial Growth Reduction revealed at 100 μL, 10 μL, 1 μL and 0.1 μL of neem oil ranged, respectively, 74.51 ± 1.15 - 95.51 ± 1.15; 59.61 ± 0.58 - 75.61 ± 1.00; 48.10 ± 0.10 -61.67± 1.35 and 32.63 ± 1.73 - 32.63 ± 1.73. There was significant difference among the antibacterial activity at the mentioned neem oil dilutions.

The highest percent bacterial Growth Reductions were detected at 100 μL neem oil and they concerned mainly pathogenic *E. coli* isolates as follows: *E. coli hlyA* 95.51 ± 1.15, *E. coli eae* 91.25 ± 1.43, *E. coli EAST1* 89.65 ± 1.53 (Table 4).

Effect of Tween®20 on goat Peripheral Blood Mononucleate Cells

Figure 2 A shows the effect on percent viability of Peripheral Blood Mononucleate Cells grew in Roswell Park Memorial Institute medium and treated with neem oil at different dilutions (1:2x10^2, 1:2x10^3, 1:2x10^4; 1:2 x10^5; 1:2 x10^6). There was significant difference between percent of Peripheral Blood Mononucleate Cells viability detected at neem oil dilution 1:2x10^2 and untreated control, only. This was revealed in Peripheral Blood Mononucleate Cells culture of 24 h and 48 h incubation periods according to the data reported by De Matteis, et al. [27]. While, Figure 2 B shows the effect of Tween®20 on percent viability of Peripheral Blood Mononucleate Cells grew in Roswell Park Memorial Institute medium at the same dilutions. The percent of Peripheral Blood Mononucleate Cells viability was significantly different between Tween®20 dilutions 1:2 x10^2 and 1:2 x10^3 (42% up 7% decrease of cell viability) and untreated

Peripheral Blood Mononucleate Cells viability. This was revealed in Peripheral Blood Mononucleate Cells culture of 24h and 48h incubation periods. Figure 2 C represents the effect of neem oil and Tween®20 (1:1 v/v) on percent viability of Peripheral Blood Mononucleate Cells grew in Roswell Park Memorial Institute medium at different dilutions (1:2x10^2, 1:2x10^3, 1:2x10^4; 1:2 x10^5; 1:2 x10^6). If Tween®20 is used with neem oil the adverse effect increases at 1:2 x10^2 dilution (28% cell viability) while it remains similar at the dilution 1:2 x10^3 (6% cell viability) respect the treatment with only Tween®20. This was revealed either in Roswell Park Memorial Institute medium culture of 24h or 48 h incubation periods.

There is significant difference between the adverse effect of neem oil, Tween®20, Tween®20 and neem oil treatments at dilution 1:2 x10^2 on percent Peripheral Blood Mononucleate Cells viability. Furthermore, there is no significant difference between Tween®20 and Tween®20 and neem oil treatments at dilution 1:2 x10^3. While there is significant difference between neem oil and Tween®20 as well Tween®20 and neem oil treatments at the same dilution (Figure 3).

Conclusion

High Performance Thin Layer Chromatography, the last evolution of planar chromatography, allows to evidence most of the constituents of an extract in an identifying track, named fingerprint. Plates can be visualized and derivatized in several ways, obtaining multiple information [28]. Identification of constituents in the fingerprint can be obtained by standards. Therefore, neem products are subjected to great variation in composition, due to pre and post-harvesting factors [29].

The phytochemical studies and bio-assay guided fractionation showed that the insecticidal activity are correlated with the tetranortriterpenoids, such as those already mentioned as the

Table 3: Antimicrobial activity of neem Oil towards goat wound-contaminating-bacteria isolates detected by disc diffusion method as Growth Inhibition Zone (mm).

Bacteria	GIZ (mm)			
	Neem oil 100 μL	Tween®20 100 μL	Water 100 μL	Ciprofloxacin 100 μL
E. coli eae	28.71 ± 1.29 a	-	-	26.52 ± 1.07 b
E. coli hly A	29.59 ± 1.17 a	-	-	23.62 ± 1.00 a
E. coli EAST 1	30.21 ± 1.38 a	-	-	24.53 ± 0.67 a
S. aureus	23.33 ± 0.53 a	-	-	26.42 ± 0.58 b
E. faecalis	26,18 ± 0.81 a	-	-	30.61 ± 1.21 b
P. aeruginosa	25.83 ± 1.58 a	-	-	29.61 ± 1.11 b
E. coli ATCC® 51813™	26.33 ± 0.58 a	-	-	26.75 ± 0.82 a
S. aureus ATCC® 29213™	24.07 ± 1.09 a	-	-	27.41 ± 0.76 b
E. faecalis ATCC® 29212™	23.41 ± 1,.06 c	-	-	28.42 ± 0.20 b
P. aeruginosa ATCC® 27853™	22.68 ± 0.55 a	-	-	31.42 ± 1.39b

'–': Absence of GIZ (Growth Inhibition Zone);
The plates were done in triplicate for each bacterial isolate and the experiment was performed twice. The results were recorded as mean ± S.D. of the duplicate experiment. Mean values with different lowercase letters in the row are significantly different (p ≤ 0.05). Differences between means of data were compared by LSD calculated using the SAS.

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Table 4: Bacterial Growth Reduction (%) at 24 h in liquid medium with different concentrations of Neem Oil against the reference the control treatment (without Neem Oil).

Bacteria	Growth Reduction (%)			
	Neem Oil (100 µL)	Neem Oil (10 µL)	Neem Oil (1 µL)	Neem Oil (0.1 µL)
E. coli eae	91.25 ± 1.43 d	75.61 ± 1.00 c	61.67 ± 1.33 b	41.31 ± 2.08 a
E. coli hly A	95.51 ± 1.15 d	74.70 ± 1.00 c	51.88 ± 1.33 b	41.86 ± 1.00 a
E. coli EAST 1	89.65 ± 1.53 d	68.61 ± 1.00 c	58.77 ± 1.33 b	48.51 ± 2.08 a
S. aureus	79.65 ± 1.53 d	59.61 ± 1.00 c	48.77 ± 1.33 b	48.51 ± 2.08 a
E. fecalis	74.51 ± 1.15 d	69.70 ± 1.00 c	44.38 ± 1.33 b	44.86 ± 1.00 a
P. aeruginosa	85.70 ± 1.53 d	68.73 ± 2.08 c	48.59 ± 2.00 b	32.63 ± 1.73 a
E. coli ATCC® 51813™	89.71 ± 1.00 d	59.61 ± 0.58 c	49.17 ± 0.00 b	36.48 ± 0.89 a
S. aureus subsp. aureus ATCC® 6538™	81.51 ± 1.15 d	59.70 ± 1.00 c	45.78 ± 1.33 b	34.86 ± 1.00 a
E. faecalis ATCC® 7088™	89.90 ± 1.00 c	58.79 ± 1.00 c	48.10 ± 0.10 b	35.58 ± 1.20 a
P. aeruginosa ATCC® 27853™	88.90 ± 1.00 d	58.79 ± 1.00 c	49.40 ± 0.00 b	36.68 ± 1.20 a

Values expressed as mean ± Standard Deviation of two experiments (three repetitions for each experiment). Mean values with different lowercase letters in the row are significantly different (p ≤ 0.05). Differences between means of data were compared by least significant difference (LSD) calculated using the SAS
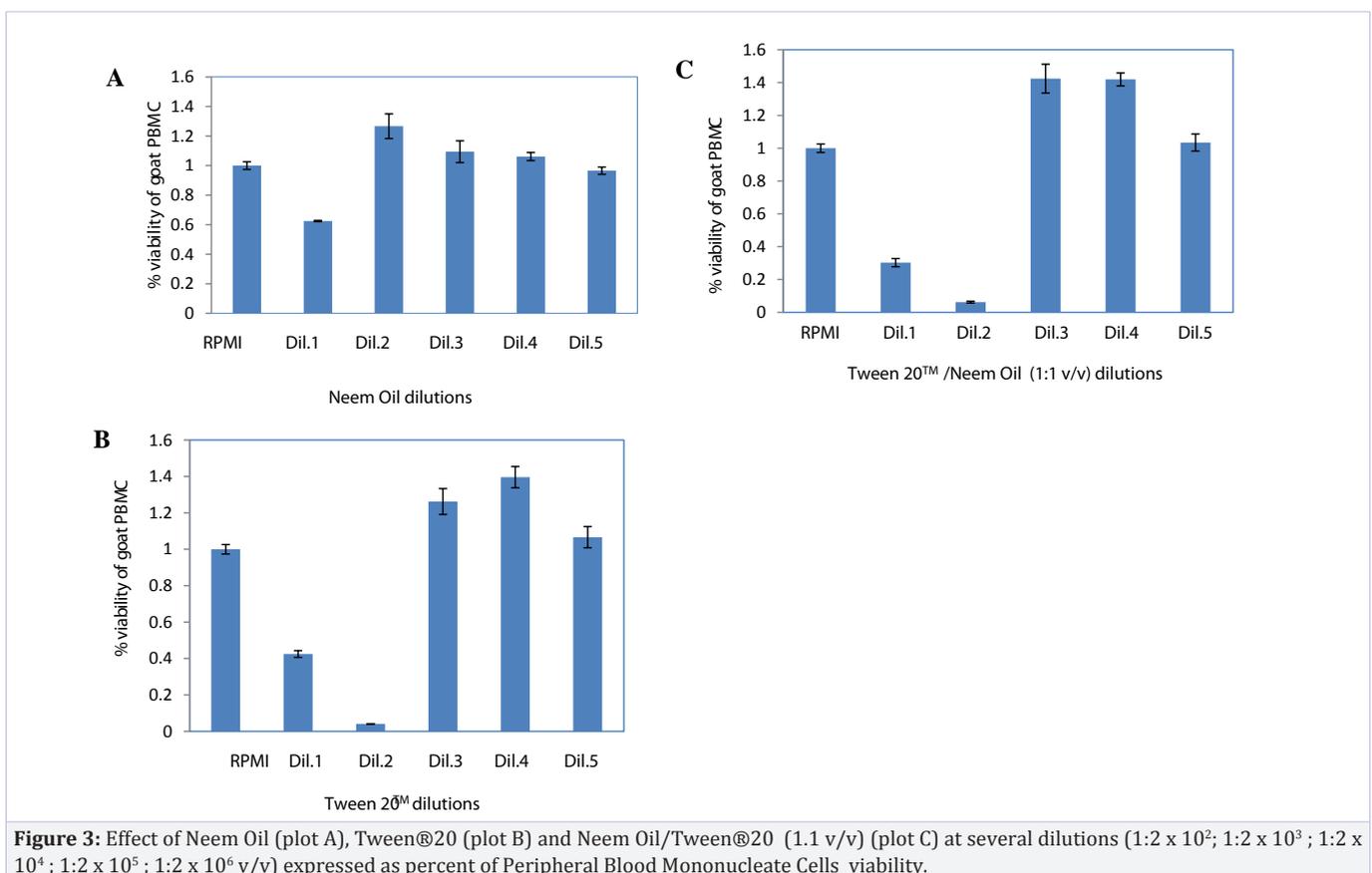

Figure 3: Effect of Neem Oil (plot A), Tween®20 (plot B) and Neem Oil/Tween®20 (1.1 v/v) (plot C) at several dilutions (1:2 x 10², 1:2 x 10³ ; 1:2 x 10⁴ ; 1:2 x 10⁵ ; 1:2 x 10⁶ v/v) expressed as percent of Peripheral Blood Mononucleate Cells viability.

meliatetraolenone, but also aromatic compounds [2,6-bis- (1, 1-dimethylethyl) -4-methylphenol, 2-(phenylmethylene)-octanal, 1,2,4-trimethoxy-5-(1Z-propenyl) benzene], benzopiranoidi (3,4-dihydro-4,4,5, 8-tetramethyl coumarin, 3,4-dihydro-4,4,7,8-tetramethyl coumarin-6-ol, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta[g]-2-benzopyran); sesquiterpene (methyl-3,7,11-trimethyl-2E, 6E, 10-dodecatrienoate); fatty acid esters (methyl 14-methyl-pentadecanoate, ethyl hexadecanoate, ethyl 9Z-octadecenoate) and monoterpene (3,7-dimethyl-1-octen-7-ol). Despite all phytochemicals studies already made and

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the large amount of constituents identified and reported in special databases, these studies show that, although the limonoids are to be considered important for the insecticidal activity, other oil components of neem, or other preparations of neem, are to be considered important and necessary for other biological activities. So far, the phytocomplex of neem oil can be considered fundamental for the obtained results [13].

Authors carried out dose determination studies and field study on parasiticidal activity of neem oil for the evaluation of its efficacy according the guidelines of World Association for the Advancement of Veterinary Parasitology. The neem oil parasiticidal activity was evaluated towards goat (*Capra hircus* L.) lice (*Linognathus stenopsis* and *Damalinia caprae*). The results confirmed that neem oil is an effective parasiticide that control two lice (*Linognathus stenopsis* and *Damalinia caprae*) biological cycles with one neem oil dose administration. Furthermore, they evaluated skin reactions during the experimentation using Draize Scoring System [30] and demonstrated neem oil does not cause irritation of skin. It was always obtained a score equal zero regardless of the dose used (manuscript submitted and under revision). Due the possibility of neem oil absorption through skin, a methodology was set up to evaluate the effect of neem oil on goat blood cells [27]. Neem oil was efficacy in containing louse with no side effects on treated goats.

Many studies carried out so far demonstrated the microbial inhibitory effects of different phytoconstituents of *A. indica*. Neem has been reported to exhibit antibacterial as well as antifungal activities depending from which part of the plant and how extracts were obtained. Margolone (12-methyl-7-oxopodocarpa-8,11,13-triene-13-carboxylic acid), margolonone (2-Phenanthrenecarboxylicacid, 4b, 5, 6, 7, 8, 8a, 9, 10-octahydro-3, 4b, 8, 8-tetramethyl-7, 10-dioxo-, (4bS,8aR)-) and isomargolonone ("3-Phenanthrenecarboxylic acid, 4b, 5,6,7,8,8a, 9,10-octahydro-2,4b, 8,8- tetramethyl-7,10-dioxo-,(4bS-trans)-") are tricyclic diterpenoids isolated from stem bark, nimbidin (Methyl (2*R*, 3a*R*, 4a*S*, 5*R*, 5a*R*, 6*R*, 9a*R*, 10*S*, 10a*R*)-5-(acetyloxy)-2-(furan-3-yl)-10-(2-methoxy-2-oxoethyl)-1,6,9a,10a-tetramethyl-9-oxo-3,3a,4a,5,5a,6,9,9a,10,10a-decahydro-2*H*-cyclopenta[*b*]naphtho[2,3-d]furan-6-carboxylate) and nimbolide (4α,5, α 6 α,7 α,15,17 α)-7,15:21,23-diepoxy-6-hydroxy-4,8-dimethyl-1-oxo-18,24-dinor-11,12-secochola-2,13,20,22-tetraene-4,11-dicarboxylic acid α-lactone methyl ester] from seed oil, as well azadirachtins (dimethyl (2a*R*,3*S*,4*S*,*R*,*S*,7a*S*,8*S*,10*R*,10a*S*,10 b*R*)- 10-(acetyloxy)- 3,5-dihydroxy- 4-[(1*S*,2*S*,6*S*,8*S*,9*R*,11*S*)-2-hydroxy- 11-methyl- 5,7,10-trioxatetracyclo[6.3.1.0^{2,6}.0^{9,11}]dodec- 3-en- 9-yl]- 4-methyl- 8-{[[(2*E*)- 2-methylbut- 2-enoyl]oxy} octahydro- 1*H*-furo[3',4':4,4a]naphtho[1,8-*bc*]furan- 5,10a(8*H*)-dicarboxylate), quercetin (3,3',4',5,7-pentahydroxyflavone) and β- sitosterol (17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenanthren-3-ol) from leaves, all show several biological activity, among them antimicrobial activity, too [31-35].

This study confirms neem oil antibacterial activity towards wound-contaminating-bacteria resulting from ectoparasites sting and bite, too. Therefore, the neem oil should be considered as a safe and ready to use innovative topical solution for the care of wounds.

Acknowledgment

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