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Assessment of Neem oil effect on hematological profile and towards peripheral blood mononuclear cells of goat

[1a]Giovanna De Matteis, [1b]Domenico Rufrano, [1b]Salvatore Claps, [2]Vincenzo Veneziano,[3a]Antonella Di Sotto, [3b]Marcello Nicoletti and [1a*]Paola Del Serrone

[1]Consiglio per la Ricerca in Agricoltura e l'analisi dell'economia agraria CRA. Via Nazionale 82, 00184 Rome, Italy
[1a] Research Center for Animal Production CRA PCM. Via Salaria 31, 00015Monterotondo (RM), Italy
[1b]Research Unit for the Extensive Animal Husbandry CRA ZOE. Via Appia, Bella Scalo 85054 Muro Lucano (PZ), Italy
[2]University of Naple Federico II, Dep. Veterinary Medicine and Animal Production. Via Federico Delpino, 80137 Naple, Italy
[3]University of Rome Sapienza. Piazz. le Aldo Moro 5,00161 Rome, Italy
[3a]Dep. Physiology and Pharmacology "V. Espamer". Piazz. le Aldo Moro 5,00161 Rome, Italy
[3b]Dep. Environmental Biology. Piazz. le Aldo Moro 5,00161 Rome, Italy

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ABSTRACT

Neem oil (NO) was evaluated in previous experiments as a natural ectoparasiticide for sheep and goats. In this work a methodology was assessed to evaluate the effect of NO on PBMC and the haematological profile of goats. Blood-cells from Siriana, Sanen, Cashemere and Maltese goat (Capra hircus L.) breeds were assayed-in in vivo and in vitro tests. Several haematological parameters of blood from goat untreated and treated with NO were compared. There was no significant difference ($p<0.05$) between NO treated and untreated goat' haematological parameters at each sampling time considered. In addition, the NO effect towards goat PBMC cultured in RPMI medium was evaluated at 1:2 x 10^2 to 1:20 x 10^6 dilutions at 14, 21 and 40 h of exposure. The PBMC viability was revealed using WST-1 dye. The in vitro test reveals that the response of goat PBMC viability is concentration, incubation time and NO dose dependent. The PBMC Maltese and Saneen breed resulted the most suitable for in vitro viability evaluation.

Keywords: Neem oil, Peripheral Blood Mononuclear Cell (PBMC), biological activity, goat, hematological profile, parasiticide control, biocide

Abbreviations:
CLSI = Clinical and Laboratory Standards Institute
EDTA = EthylenDiamineTetraacetic Acid
HCT = Erythrocyte parameter
HPTLC = High Performance Thin Layer Chromatography
ISO = International Organization for Standardization
LSD = Least Significant Difference
MCHC = Mean Corpuscular Hemoglobin Concentration
MCV = Mean Corpuscular Volume
NCE = Neem Cake Extract
NO = Neem Oil
MTS = 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2 H-5-tetrazolio]-1,3-benzene disulfonate
OD = Optical density
PBMC = Peripheral Blood Mononuclear Cells
RBC = Red Blood Cell
Rf = Ratio between the migration distance of substance and the migration distance of solvent front
RPMI =Roswell Park Memorial Institute medium
WBC = White Blood Cells
WST-1 =Water-Soluble Tetrazolium

INTRODUCTION

Nowadays, increasing and spread of parasitic resistance is arousing the major concern [1]. Actually, parasitic resistance is widespread in most major groups of parasites [2,3]. Meanwhile, climate changes are influencing mosquito-borne diseases diffusion [4].

Parasitic diseases represent one of the most important health problem of livestock, domestic animal and humans. The activity of ectoparasites infesting livestock is *inter alia* of particular interest, resulting in a wide range of side effects. In fact, ectoparasites may cause indirect damages, as disturbance, increasing levels of abrasion, and induced reduction of time spent grazing or ruminating and, sometimes, even to self-wounding. Animals with these symptoms are restless and look for ease from pruritus by scratching. As a result, livestock feeding and digestion are hindered, resulting in weak growth associated to loss of weight and reduced milk and meat production. In addition, some ectoparasites are also vectors of protozoa, bacteria, viruses, cestodes and nematodes and zoonotic agents. There is an urgent need for ecologically sound, equitable and ethical pest management, based on control agents that are pest-specific, nontoxic to humans and other biota, biodegradable, less prone to pest resistance and resurgence, and relatively less expensive [5-7]. Recently, much attention has been focused on natural products, considering the difficulties in going on with synthetic pesticides. Plants are the natural source of bioactive constituents able to produce antifeedant and repellent effects and hatching eggs disinfection.

Among various strategies based on the use of herbal bio-pesticides, neem (*Azadirachta indica* A. Juss) was identified as a source of eco-friendly "soft" natural pesticides. Recently, research mainly focused on larvicidal activity, including neem cake, in order to obtain the scientific background for the developing of a new, effective, safe, sustainable, low cost and easy to use insecticide, to be utilized also in domestic situations [8-10]. The plant has been used historically to freeing the body by several forms of ectoparasites and pests. Hormone mimicking activities of neem extracts causes interference with the parasitic life cycle inhibiting their ability to feed, as well as prevent the hatching of eggs [11-13].

NO was evaluated in previous experiments as a natural ectoparasiticide for sheep and goats. Taking in account that neem products are subjected to great variation in composition due the post-harvesting factors, the metabolomic fingerprint of neem oil utilized in this research was analysed by HPTLC. HPTLC, 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 [14, 15].

Due the possible absorption of neem oil through the animal skin into blood circulation, the possible NO citotoxicity was investigated towards goat blood cells. An *in vitro* methodology to evaluate the NO effect on PMBC was developed

MATERIALS AND METHODS

Goat breeds and blood samples

The dairy goat (*Capra hircus* L.) breeds considered in the experiment, i.e. Cashmere, Saneen, Siriana and Maltese,were kept in the Research Unit for the Extensive Animal Husbandry CRA ZOE. The animals were apparently healthy. Blood was collected from all investigated goat breeds by jugular vein puncture. The collected samples (15 mL each) were harvested in single-use containers for venous blood specimen collection (VACUETTE®, Greiner bio one, Kremsmünster, Austria), according to ISO 6710 (EN 14820) and CLSI directives, containing EDTA and L-heparin. Blood plasma and serum samples were separated by centrifugation at 3000 g for 10 min and kept frozen at –20 °C until required. They were suspended once again at 25°C with shaking before using in the experiment.

Neem seed oil

A commercial neem oil produced by Neem Italia (Manerba (BS), Italy). was used as test starting material (0.35% azadirachtin A, determined by HPLC [15]. Neem seed oil was 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.

Neem Oil metabolomic fingerprint

The NO metabolomic fingerprint was determined in previous studies according the methodology reported in literature [16,17].

***In vivo* bioassay**

Hematological profile of goats blood

Samples of blood from six male goats treated with NO (300 mL/10 Kg) and from six untreated male goats, as control for each breed, were considered. Blood was collected three times at 7 day interval after treatment by jugular vein puncture. The red blood cell (RBC), mean corpuscular volume (MCV), erythrocyte parameters (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and white blood cells (WBC) were determined using the hematological analyzer Automated Hematology Analyzer MEK-6450 (Nihon Kohden, Japan). All of them were statistically processed to obtain the hematological profile.

Statistical analysis

The results were recorded as means (six goats for each thesis) of three replication (7, 14, 21 days) ± SD. The experiment was repeated twice. 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).

***In vitro* bioassay**

Isolation of goat PBMC

Caprine PBMC were isolated from 15 mL of blood by density gradient (Lymphoprep^{TM} - 1.077 g/mL; AXIS-SHIELD PoC As) following the manufacturer's instructions, then washed twice with Hank's Buffered Salt Solution (HBSS without Ca and Mg, Sigma, Milano, Italy) to reduce the density of the solution and for efficient removal of platelets. PBMC microscopic counts were performed by TC10^{TM} automated cell couter (BioRad, Milano, Italy), and the cell viability after blood purification was determined using Trypan blue (BioRad, Milano, Italy) solution then charged in the Thoma chamber (Thoma, Marienfeld, VWR International PBI, Milano, Italy) and observed under microscope. Cell preparation of more than 95% viability were used for the experiments.

PBMC Culture

Preliminary experiments were carried out in order to assess the ability of PBMC isolated from several goat breeds to maintain their viability in culture and to test the cells concentration suited to be tested for NO effect.Isolated PBMC were suspended in RPMI 1640 medium (Sigma, Milano Italy) supplemented with 10% heat inactivated foetal bovine serum, 25 mM Hepes, 2 mM L-glutamine (Sigma, Milano Italy) and 0.05 mg/mL of penicillin/streptomycin solution (Sigma, Milano Italy) and were adjusted to 1x10^6 cells/mL in 15 mL polyethylen sterile tubes (Sarsted, Nümbrecht, Germany**)**.

RPMI (200 µL) with PBNCat the concentrations: 5 x 10^4, 1 x 10^5;and 2 x 10^{5}cells was poured into wells of 96 well plates and grew at 37° C in a CO_2 incubator for 24 h.

Quantification of PBMC viable cells in culture

Quantification of viable cells was performed using the Cell Proliferation Reagent WST-1(Roche Diagnostics, Mannheim, Germany) based on colorimetric reaction of the dye MTS. Cleavage of the WST-1 reagent by ubiquitous plasma membrane enzymes of live cells yields an easily-quantified color change which is proportional to the mass of living cells [18].The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The generation of the dark yellow colored formazan is measured at 420-480 nm (optimal at 450 nm). NO cell effect was evaluated in serial dilutions (in triplicate) and the inhibition of cells growth was determined using the colorimetric assay WST-1. The percentage of viability is calculated as [(A - B) / A] x 100, where A and B are the OD at 450 nm of untreated and of treated cells, respectively.

Cellular morphology of PBMC cells by light microscopy

PBMC morphology after NO treatment were observed by an inverted microscope (Leica DM IL LED Inverted Microscope, Langham Creek,USA) at 40 x magnification. Digital pictures were acquired with a Tucsen color CCD camera (Tucsen Image Technology Inc., Fuzhou, FuJian, China).

Effect of NO on PBMC

Firstly, only one NO dilution (1:2 x 10^2) was tested on different breed goat PBMC. They were cultured for 3h before the NO treatment. It was added in triplicate for each goat breed PBMC, except in control wells. After 24 h NO treatment, WST-1 dye (20 µL) was added directly to each well and incubated for 3 hours.Afterwards, six NO dilutions (1:2 x 10^2; 1:2 x 10^3; 1:2 x 10^4; 1:2 x 10^5; 1:2 x 10^6; 1:2 x 10^7 v/v) were tested on Maltese goat PBMC in a final volume of 2 x 10^2 µL. PMBC viability is reported as percent growth reduction in comparison to control

(without treatment). Three repetition were considered. The experiment was repeated twice. Differences between means of data were compared by *t Student* test.

RESULTS AND DISCUSSION

NO Metabolomic fingerprint

The NO 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 maker 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 anysaldeide (Figure 1). 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 of the activity

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: Anysaldeide . Tracks 1, neem oil; 2, salannin

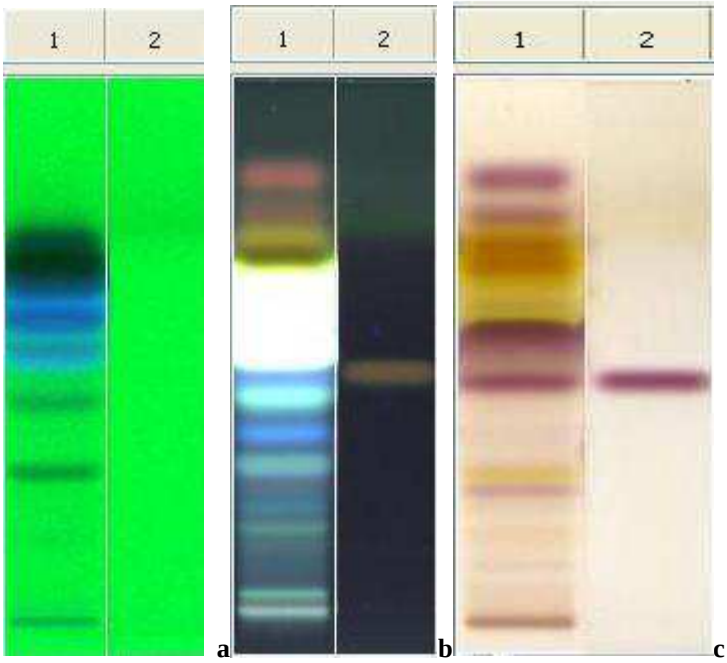
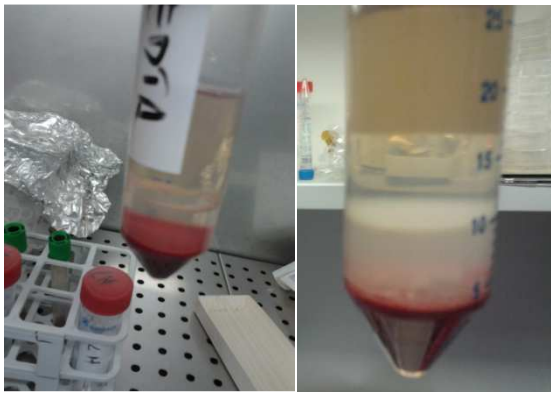


Figure 2. .Comparison between PBNC isolated from blood collected in EDTA (A) and heparinized (B) tubes. In B is visible (white ring halo) the separation of mononuclear cells (PBNC)



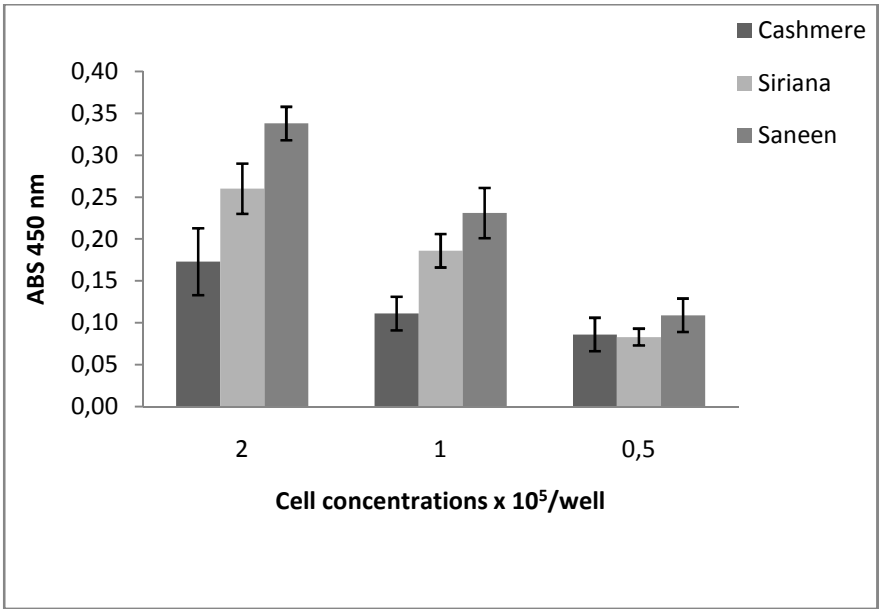
Haematological profile of goats blood

There was no significant difference among the hematological (means ± SD) parameters considered in the experiment between the goat breeds treated and untreated with NO for lice control as presented in Table 1.

Table 1. Hematological profile of female goats treated and untreated with NO. Means of three repetition (six animal for each repetition; 7, 14 and 21 days after NO treatment with three NO doses: 100mL, 200mL and 300mL per 10 Kg) ± SD. The experiment was repeated twice (p ≤0.5)

BREED	RBC (10^6/µL)	MCV (µm^3)	HCT (%)	MCH (pg)	MCHC (g/dl)	WBC (10^6/µL)
Cashmere	13.08 ± 0.46 a	14.38 ± 0.36 a	18.78 ± 0.88 a	6.23 ± 0.09 a	44.09 ± 11.22 a	15.41 ± 4.51 a
C. untreated	13.00 ± 0.07 a	13.98 ± 1.36 a	18.56 ± 0.91 a	6.00 ± 1.00 a	45.00 ± 9.77 a b	15.37 ± 3.00 a
Saneen	13.28 ± 0.65 a	14.73 ± 0.56 a	19.00 ± 1.00 a	6.66 ± 0.10 a	44.79 ± 9.02 b	15.56 ± 5.10 a
Sa. untreated	13.19 ± 0.58 a	14.66 ± 1.00 a	18.44 ± 1.11 a	6.00 ± 0.88 a	45.06 ± 10.11 b	14.89 ± 7.01 a
Siriana	13.89 ± 0.88 a	14.11 ± 0.83 a	18.63 ± 2.33 a	6.88 ± 0.75 a	44.00 ± 6.22 a	15.00 ± 8.1 a
Si. untreated	14.00 ± 0.09 ab	14.48 ± 1.11 a	18.58 ± 1.86 a	6.66 ± 1.00 a	44.56 ± 11.11 a	15.21 ± 6.9 a

Figure 3. Viability of PBMC from different goat breeds at various cell concentrations maintained in RPMI culture for 24 h at 37 °C



Isolation of goat PMBC

PMBC from goat blood samples collected in cuvette containing EDTA and L-heparin showed a different degree of separation after centrifugation with Lymphoprep (600 g, 30 min, 1,077g mL^{-1}). The layer of mononuclear cells was not obtained from goat blood samples collected in tubes containing EDTA after 2 h as well as 1 h sampling by centrifugation (Fig. 2). This is due to a strong aggregation of leukocytes. Mallone and coworkers [19] report a similar effect of EDTA on human granulocyte buoyancy profile resulting in less efficient separation by density gradient procedures.

Goat breed PBMC viability in culture.

The concentration from 1 x 10^5 to 2 x 10^5 cells/well resulted suitable to study the viability of goat PBMC in *in vitro* effect evaluation test. The Maltese and Saneen PBMC were the most appropriate to be cultivated in RPMI medium. All concentration (5 x 10^4; 1 x 10^5, and 2 x 10^5) of Saneen PBMC showed after 24 h of culture significant higher OD values. As shown in Fig. 3, it resulted 2.0 fold higher and1.3 fold higher than, respectively, Casmhere and Siriana breeds.

Effect of NO towards goat PBMC

The goat breed PBMC grew at the same culture conditions showed significantly different sensitivity (10% up to 50% cell viability; p <0.05) to NO (1:2 x 10^2) exposure. The Saneen PBMC resulted the most sensitive to NO. The Goat PBMC (5 x 10^4; 1 x 10^5; 2 x 10^5cells/well) further treated with lower NO dilutions (1:2 x 10^3 - 1:2 x 10^7)

showed at 14 h incubation time a significant different degree of viability. As represented in Fig. 5 the NO dilutions 1:2 x 10^5- 1:2 x 10^7positively affect goat PBMC at all PBMC dilutions considered with an increase of cell viability up to 80% at the concentration of 5 x 10^4 PBMC/well.

Figure 4. Effect of NO on PBMC cell viability from three among the different goat breeds considered in this study. Cells were treated with an high oil concentration of the formulation (1:2 x 10^2), maintained for 24 h in culture conditions and cell viability was measured by WST-1 assay

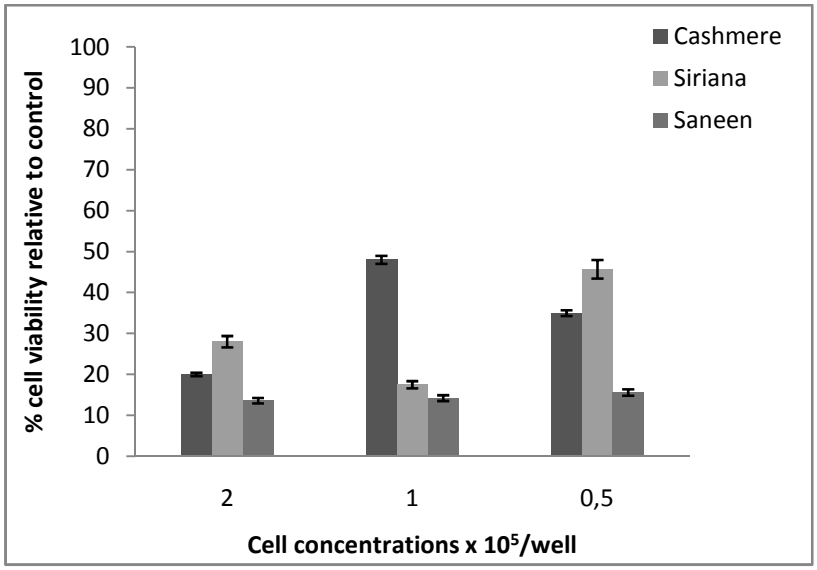
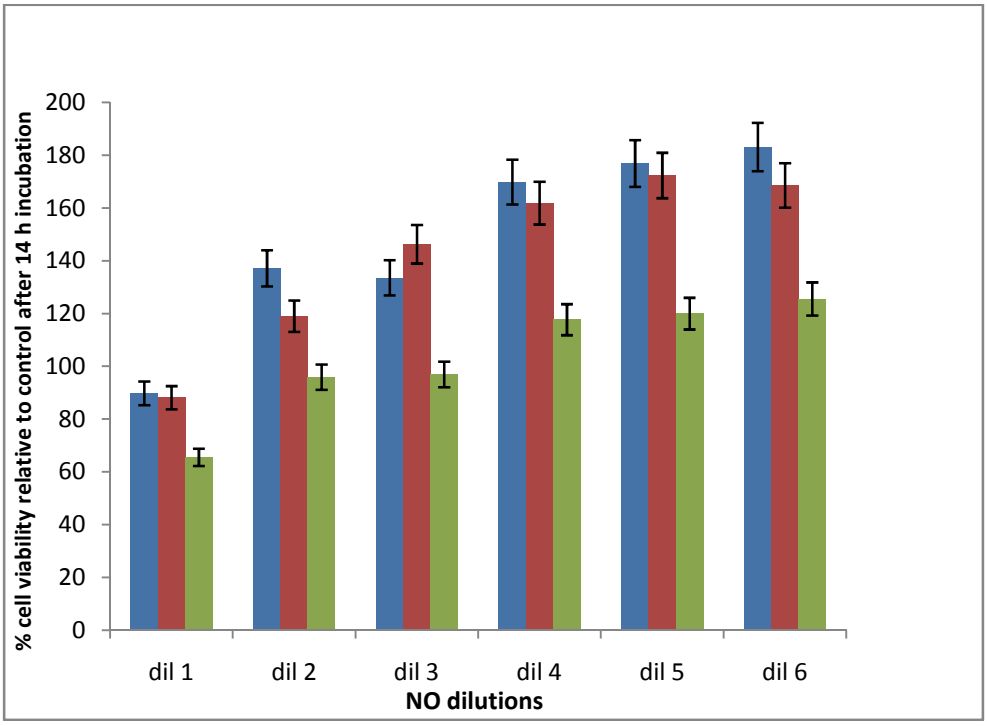


Figure 5. Effect of NO on PBMC cell viability. Cells were treated with six different dilutions (dil 1- dil 6) and maintained for 14 h in culture conditions. Cell viability was measured by WST-1 assay. NO dilution: dil. 1 = 1:2 x 10^2; dil. 2 = 1:2 x 10^3; dil. 3 = 1:2 x 10^5; dil. 4 = 1:2 x 10^5; dil. 5 = 1:2 x 10^6; dil. 6 = 1:2 x 10^7



At 21 h incubation time the PBMC (2 x 10^5/well) treated with NO, from 1:2 x 10^2 to 1:2 x 10^4, showed a decrease of viability equal, respectively, to: 41%, 49% and 56%, as shown in Fig.6. While dilutions from 1:2 x $10^5$to 1: 2 x 10^7 remain viable (up to 70%). This seems not due to the effect of NO rather to the duration of incubation time. In fact,

also the untreated PBMC showed a reduction of cell viability. In addition, it was recorded a PBMC cell (1 x 10^5 and 2 x 10^5 cell/well) viability reduction (47% and 60%; data not shown).

Furthermore, as plotted in Fig. 7, NO treated from 1:2 x 10^5 to 1:2 x 10^7, and untreated PBMC (2 x 10^5 cells/well) cultured for 40 h did not showed a significantly different viability in comparison to the control. The recorded viability resulted highest then those observed at lower incubation time and at the same PBMC concentration. This incubation time seems not suitable to estimate the effect of a plant derived product since there was no significant difference between of NO treated and untreated goat PBMC viability. This could be avoid using a proliferation reagent.

Figure 6. Effect of NO on PBMC cell viability. Cells were treated with six different dilutions and maintained for 21 h in culture conditions. Cell viability was measured by WST-1 assay. NO dilution: dil. 1 = 1:2 x 10^2; dil. 2 = 1:2 x 10^3; dil. 3 = 1:2 x 10^5; dil. 4 = 1:2 x 10^5; dil. 5 = 1:2 x 10^6; dil. 6 = 1:2 x 10^7

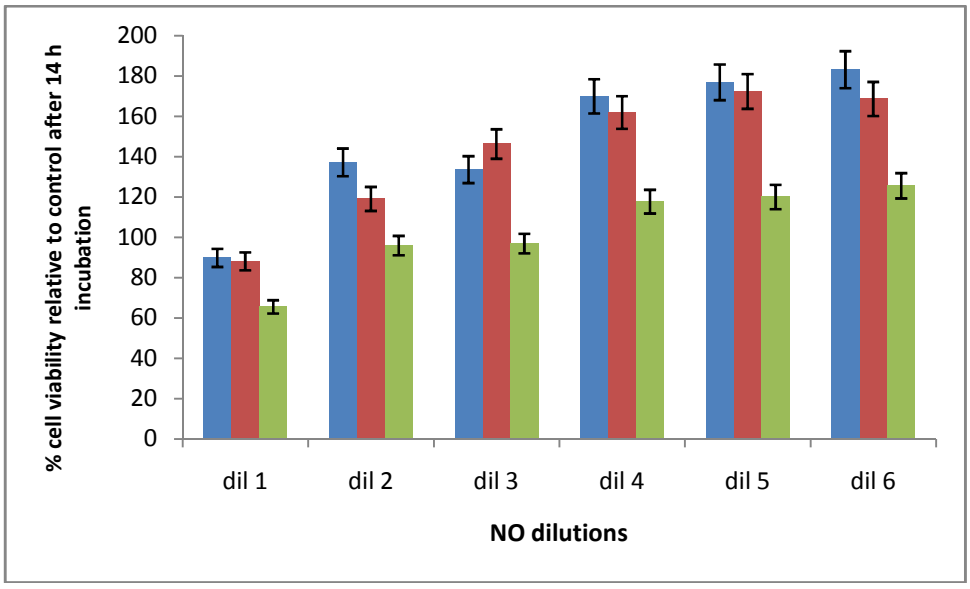
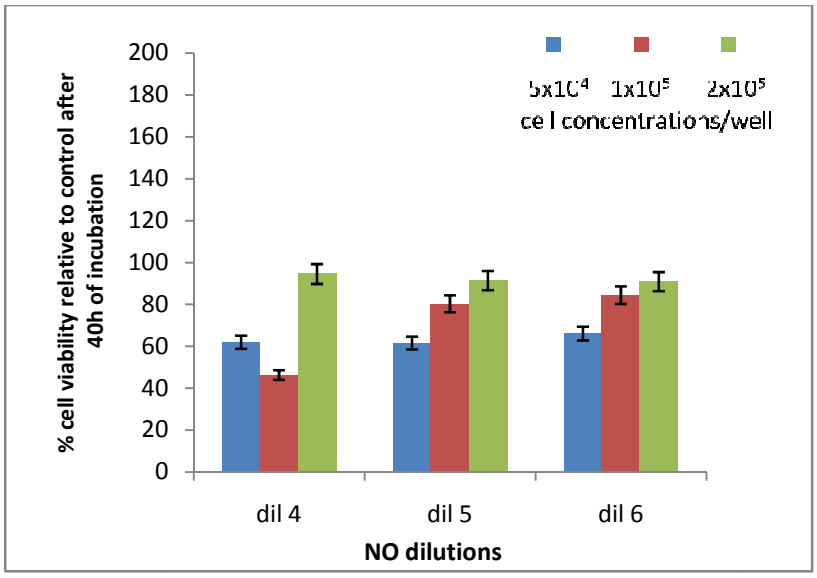


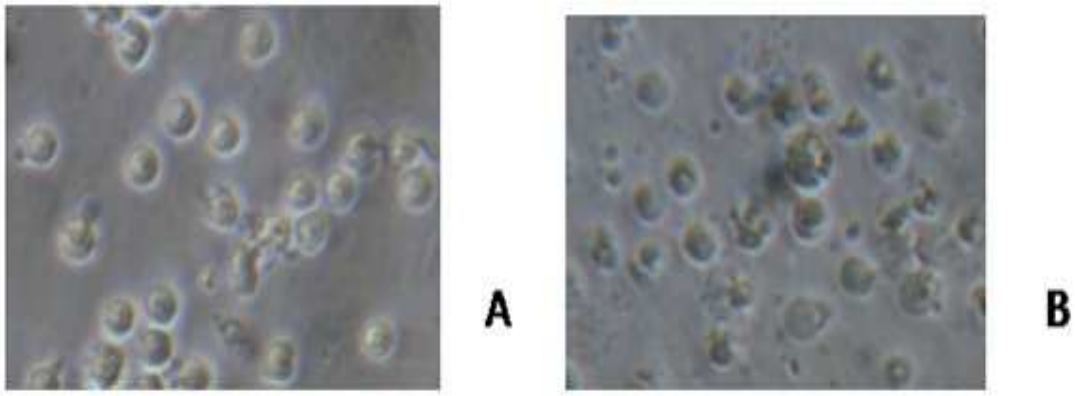
Figure 7. Effect of NO on PBMC cell viability. Cells were treated with three different dilutions and maintained for 40 h in culture conditions. Cell viability was measured by WST-1 assay.



Morphological observations

Fig.8 shows NO (1:2 x 10^2 dilution) treated and untreated goat PBMC (2 x 10^5cell concentration) after 24 h in *in vitro* culture. All NO treated goat breed PBMC exhibited a characteristic phenotype as cell shrinkage and blabbing, appearance of vacuoles in the cytoplasm, and nuclei shrinkage. These abnormalities are generally referred to apoptotic phenomenon. The same morphological effect was observed at the same NO concentration independently by the PBMC concentrations. NO lower concentrations do not cause the described morphological effects on PMBC after 24 treatment in *in vitro* culture independently by PBMC concentration.

Figure 8.Effect of NO induced changes in PBMC light microscopy detected at different concentration of cells. Representative images of cell culture at 2 x 10^5 cells. A: untreated PBMC. B: NO treated PBMC at 1:2 x 10^2 dilution



CONCLUSION

One of the most important goal of the Community Animal Health Policy [20] is finding effective alternatives to the massive culling of the animals. In the emergency, extremely suppression were adopted as a consequence of the recent epidemic outbreaks in Europe. The possibility to rapidly and effectively treat clinically diseased animals should be part of an integrated disease control strategy. For animal welfare and socio-economical reasons, effective new antimicrobial drugs, including innovative classes of compounds, such as anti-viral, bio-insecticides from new sources, like plants, should be available.

NO act as a synthetic systemic parasiticide against goat lice. One NO treatment can effectively control two biological cycles of goat lice (*Linognathus stenopsis* and *Damalina caprae*) infestation (data not yet published). Furthermore, NO showed antimicrobial activity in *in vitro* test against bacteria isolated from lesions caused by bite and sting of ectoparasites causing health problems and downgrading skin and wool with consequent economic damage (data not yet published).

In the present work, a rapid and efficacy methodology was standardized to confirm and validate that NO parasiticidal activity has no adverse effect on goat. In fact, it allows to evaluate the NO possible adverse effect using goat blood cells in order to confirm its safety use to control goat lice. Goat purified PBMC using heparinised blood resulted useful to determine NO effect in *in vitro* test. The test reveals a dose and incubation time dependent response of goat PBMC to NO treatment. Also a suitable concentration of PBMC of 2 x 10^5 or 1 x 10^5 cell/well cultured in 96 well plates exhibited different response to each dilution of NO tested. There is significantly difference on cell viability and cell morphology between treated and untreated goat PBMC only at the lowest NO dilution. It was observed a significant difference among goat PBMC viability isolated from different breeds towards the experimental conditions.

In conclusion the reported results show that the methodology is useful to evaluate the safety use of NO for goat lice control. It allows to consider NO for a safe use in parasite control of goat because of it has no side effects on goat PBMC and the haematological profile.

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