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Enhancement of Phagocytic Activity of Human Polymorphonuclear Leukocyte and Monocyte Cells by *Aconitum lycoctonum* L. Alkaloid Fraction.

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Abstract
*The immunomodulatory activity of diterpene alkaloids from Aconitum lycoctonum L. (Ranunculaceae family) was evaluated; the alkaloid components were extracted from plants grown wild in the Apennines near Parma (Northern Italy). The diterpene alkaloid lycoctonine - evaluated by means of HPLC and IR techniques - reaches its highest value in flowers, followed by stems, roots and leaves, while the maximal value of aconitine alkaloid is in leaves and stems. Aconitine content is not quantifiable in the hypogean apparatus; on the contrary, in the leaves aconitine exceeds the lycoctonine amount. In order to evaluate the immonomodulatory activity of the alkaloid fraction, a rapid and sensitive two-color flow cytometric method for simultaneous measurement of phagocytosis rate was performed. Phagocytosis was determined by the measurement of internalized Candida albicans by leukocytes (monocytes and granulocytes). The enhancement of phagocytosi on peripheral polymorphonuclear leukocytes a 7% increase, znd on monocytes statistically significative (**P<0.001). A. lycoctonum alkaloid extract had an immunomodulatory activity very similar to that reported for other Aconitum species from Asia..*

Key words*: Aconitum, diterpene alkaloids, immunomodulatory activity, phagocytosis, monocytes, polymorphonuclear neutrophils (PMN)*

INTRODUCTION

The species belonging to the Aconitum L. genus (Ranunculaceae family) are more known for their toxicity, due to the presence of diterpene alkaloids, such as aconitine, than for their therapeutic properties [1]. In Eastern countries, especially in China, Aconitum plants are opportunely treated and included in many pharmaceutical preparations also with immunomodulatory activity. The Aconitum sp. extracts, namely *A. carmichaelii*, are prescribed to stimulate and to increase the immunomodulatory capacity [2-7]. The treatment of immunodeficiency conditions is related to different situations and foresees the use of many therapeutic approaches. The Pharmacopoeias contain many products accredited for their potential immunomodulatory effects, and prescribed for the treatment of the forms with moderate clinical impact, but which can poorly be treated pharmacologically.

The panorama of the natural products with potential immunomodulatory activity is variegated and still discussed. On the basis of the literature data, these products can be divided into two categories: products with high molecular weight such as polysaccharides, *e.g.* from *Echinacea purpurea* (L.) Moench. and other Echinacea species, from *Astragalus mongholicus* Bunge, and products with low molecular weight, *e.g.* diterpene alkaloids and norditerpene alkaloids [8-13] and essential oils, *e.g.* from mint [14, 15]. A general review on the matter has been proposed which brought the role played by the natural extracts in this field again to the foreground [16].

The aim of this study was (I) to analyse the alkaloid components in *Aconitum lycoctonum* L. for which valid literature references were missing and (II) to evaluate if and how the extracted alkaloids could have immunomodulatory activity evaluated as phagocytosis on whole blood of healthy individuals (donors).

MATERIALS AND METHODS

Plant Material. *Aconitum lycoctonum* L. was collected in Lagoni place (Parma province), 1300 m. a.s.l., in the Apennines mountains, Northern Italy, along a stream mountain. After collection, the plants were separated in different parts: old and young root-tubers, thin secondary roots, stalks, leaves and flowers.

The qualitative and quantitative content (% dry weight d.w.) of diterpene alkaloids was analysed in the fresh plants and then the extracts were stored at –20°C.

Extraction of diterpene alkaloids. The adopted method was previously described [17]. Briefly: the plant tissue (in duplicate), thinly minced, extracted with HCl 0.1 N (1:10 w/v), 1 h, r.t., was re-extracted with HCl 0.1 N (1:10 w/v), r.t., 24 h. The acidulated watery phases were collected and extracted (1:1 v/v) with $CHCl_3$ x 3. The addition of KCl 0.1 N inhibits the developing of emulsions. The chloroform layer, dehydrated on anhydrous sodium sulphate, was reduced in volume under vacuum (40°C), and then stored at 4°C.

Diterpene alkaloid HPLC analysis. Alkaloids are monitored by High Performance Liquid Chromatography (HPLC), reversed phase. For the quantitative analysis the internal standard technique was used. The compound more suitable as internal standard was 1-chloro-2,4-di-nitrobenzene: its peak was shown in an area without peaks referring to other compounds, which could interfere in the analytical determination. HPLC chromatographic system was a Jasco instrument, equipped with a detector: UV – 975 Intelligent UV/VIS Detector; pump PU-980 Intelligent HPLC Pump; injection valve manual, Rheodyne mod. 7725

Catati, California; column LC-8 SupelcoSIL 25 cm x 4 mm, 5 μm. Eluent = water : acetonitrile : 1,4-dioxan (265 : 150 : 75 v/v) + $HClO_4$ 0.01 M, to pH = 2.6 with NH_4OH at 10%. Injection volume: 5 μl; flow rate 1 ml/min; detection = 235 nm.

Isolation and Determination of Lycoctonine. For the identification of the compound tentatively referred to lycoctonine, the root alkaloid extract was repeatedly chromatographed by means of RP-HPLC. An amount 78 μg of a compound with a high purity degree (89% control HPLC) was obtained. The isolated compound was basified (NaOH 0.1 N at pH = 10), afterwards the compound, as base, was re-extracted with chloroform (1:1 v/v) in watery phase; the organic phase was separated and concentrated. The purified compound, dissolved in dichloromethane, was evaporated on a NaCl disk for the analysis at the IR-spectrometer Perkin Elmer FT-IR 1710. After identification of the molecule understudy, an amount of lycoctonine adequate to prepare a calibration curve was isolated. The quantitative analysis of lycoctonine was then carried out with the external standard method.

Immunomodulatory Tests – Preparation of Blood Samples. Human leukocytes were obtained from heparinized whole blood samples of healthy donors by the Transfusion Centre of the Monza Hospital, Italy. The samples were kept at r.t. and used within 2 hours. A complete count of the hemochromocytometric leukocyte formula for each sample was performed (Roche Cobas Argos). The blood polymorphonuclear cell value was used to define the appropriate ratio between phagocyting cells and the *pabulum* of particles to be ingested (*Candida albicans*). An analogous calculation was performed for monocytes.

Preparation of *Candida albicans* blastospores. For the preparation of the fluorescent spores of *Candida albicans* we used the method described by Saresella [18, 19]. *Candida albicans* was maintained in liquid culture in Sabouraud medium containing 2% dextrose (DIFCO, Detroit, USA) at 37°C for 18-24 h. The blastospores were then washed three times and re-suspended in phosphate-buffered-saline (PBS), pH 7.2. The vitality of the blastospores after the washings was evaluated with the trypan blue exclusion method, and has to be higher than 90%. For the phagocytosis test the blastospores of *Candida albicans* were fixed in 70% ethanol for 1 hour and then washed two times in PBS. The fixed blastospores were labelled with fluorescein isothiocyanate (FITC) (Sigma Aldrich, Milan, Italy) at 0.01 mg/ml in a solution of carbonate/bicarbonate 0.5M (pH 9.5), 30 min, r.t., under agitation, in the dark. The labelled blastospores were washed 2 times in PBS and kept in aliquots of ca. 10 millions/ 200 μl and stored at –80°C.

Phagocytosis Test. The phagocytosis experiments in flow cytometry were carried out at the Laboratory Operative Unit Kidney Transplant, Niguarda Ca' Granda Hospital, Milan, Italy.

The treated leukocytes were monitored by a flow cytometer FACSCalibur Becton Dickinson (San Josè, CA, USA) equipped with a laser source with argon ion emitting at = 488 nm and sensors for cell diameter (Forward Scatter, FSC), complexity (Side Scatter, SSC). The green fluorescence of FITC *C. albicans* was detected at = 530 nm (FL1), while the orange fluorescence of *C. albicans* further treated with ethidium bromide EtBr was detected at = 575 nm (FL2). An appropriate compensation net of the two fluorescence emissions is pre-set, as well as a reading configuration with priority and acquisition limit on the FSC. The events (10,000 cells) were acquired and during the analysis a selection "gate" of the granulocytic or monocyte populations was designed, in relation to the different dimensions of the leukocyte populations. Figure 1 summarises the qualitative parameters and schematizes the four different groupings in which the blood cells split up after the contact with FITC-Candida. The lower right quadrant (LR) contains PMN having only ingested Candida (FITC+ EB-). The intensity of the mean fluorescence is proportional to the mean number of ingested particles per leukocyte. The upper right quadrant (UR) includes the cells which can have both ingested and adhered *Candida albicans*. The extracellular position of Candida + FITC gives to the particle both the maintenance of the original colouring with FITC and the acquisition of ethidium bromide, added immediately before the reading. The populations which did not interact with *Candida albicans* are included in the lower left quadrant (LL). In the upper left quadrant (UL) the dead cells are grouped, they assumed a red/orange fluorescence of ethidium bromide which passed the plasma membrane barrier, which became permeable [20].

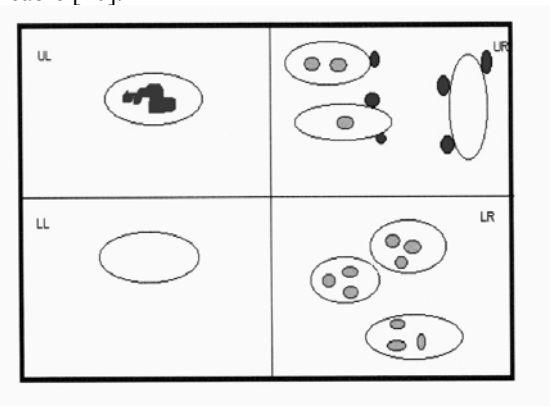


Figure 1

Qualitative parameters and scheme of the four different groupings in which the blood cells split up after the contact with FITC-Candida (UL, UR, LL, LR, se details in the test).

Statistical analysis. All data are expressed as mean ± SD. The *t*-test compares the actual difference between two means in relation to the variation in the data (expressed as the standard deviation of the difference between the means). The following quantitative data statistically elaborated are collected for each cell population, as reported in Fig. 2:

1) percentage of elements with only internalised *Candida albicans* (FITC+ EB-) [LR quadrant]
2) percentage of elements with both internalised and adhered *Candida albicans* (FITC+ EB+) [UR quadrant]
3) sum of the percentages of points 1 and 2 (total of the elements interacting with *Candida albicans*)

4) normalisation of percentage 1 on the total of interacting elements
5) normalisation of percentage 2 on the total of interacting elements

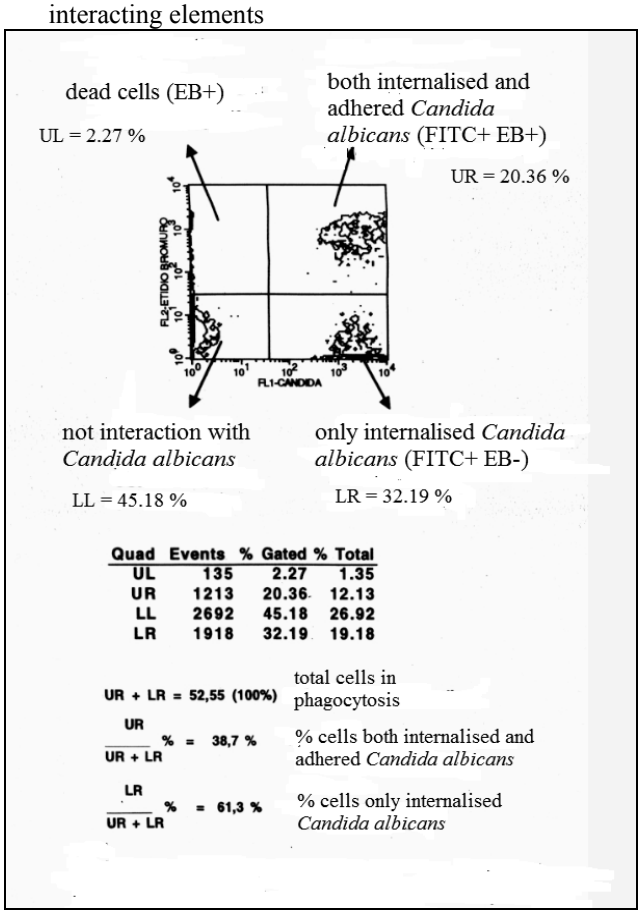


Figure 2
Example of the source of numerical data from flow cytometer and statistical elaboration

RESULTS

Botanical Aspects. The yellow-flowered monkshood *Aconitum lycoctonum* s.l. (Ranunculaceae) is a perennial herb, collected in full anthesis at the end of July, at 1300 m.a.s.l. in the Parma Apennines, along the banks of a mountain river, a characteristic habitat for many species of the Aconitum genus. *Aconitum lycoctonum* occurs from the lowlands to the subalpine zone and is found mainly in forests and other shady habitats [21].

The investigated population belongs to the species *Aconitum lycoctonum* L., according to different Authors [22-25]. Due to high morphological variability across its range in Southern and Central Europe, many taxa of uncertain taxonomic rank have been described. On the basis of these arguments, some Authors considered *A. lycoctonum* like a group in which the speciation has been developed [26]. *A. lycoctonum* could include forms which gradually pass one into the other, trespassing on the more neighbouring species characterised by the yellow flower, as *A. vulparia* Reichb. and *A. lamarkii* Reichb. [27].

Phytochemical Investigation. *A.lycoctonum* alkaloids were analyzed by RP-HPLC. The main alkaloid – lycoctonine – a known compound, not available as authentic standard was obtained in pure form by repeated HPLC separation and identified by IR technique. The structural identity of the recovered lycoctonine was defined through comparison with aconitine structure, the only diterpene alkaloid available as authentic standard. At 3497 cm^-1 the signal of the alcoholic OH-group, located on the ring with six carbon atoms. The signals of the –CH2- bindings are in the zone comprised between 2800 and 3000 cm^-1. The signal of the carbonyl of the esters, which the aconitin base forms with acetic acid and benzoic acid, is clear at 1718 cm^-1. The signal of the –C-O-C and –C-N-C- bindings is reported at 1279 cm^-1. The signals of the –O-CH_3-radicals (ether group) are evident at 1122-1097 cm^-1. The signal of water (traces) is visible at 3400 cm^-1 (approximately): it is characteristic due to the rounded peak. Both aconitine and lycoctonine are two diterpene alkaloids with a skeleton similar to C-19. They differ in the substitutions in C-7: in aconitine this position is never oxygenated, while it is always oxygenated in lycoctonine. The IR-spectrum of licoctonine isolated from the plant is however altered by some "impurity" (signals between 2800 and 3000 cm^-1), which is attributable to lipidic chains (presence of –CH2- bindings). The other signal regarding the presence of a lipid is the binding of the carbonyl of the fatty acid ester (1713-1733 cm^-1). The signal due to the –O-CH_3 group (ether group) is noted at 1122 cm^-1, which is available in four units on the lycoctonine molecule. The signal corresponding to 1200 cm^-1 (approximately) has to be correlated to the presence of the –C-O-C- and –C-N-C- bindings. The spectrum of the isolated compound, compared to that of aconitine, used as reference, is referable to that of lycoctonine esterified in at least one position by a fatty acid. Lipoalkaloids deriving from diterpene alkaloids had already been identified in other species belonging to the Aconitum genus [28 - 31].

After lycoctonine isolation and structural identification, the quantitative analysis of the alkaloids was performed. Lycoctonine reaches its highest value in flowers, followed by stems, roots and leaves, while the maximal value of aconitine is in leaves and stems. Aconitine content is not quantifiable in the hypogean apparatus; on the contrary, in the leaves aconitine exceeds the lycoctonine amount (Fig. 3).

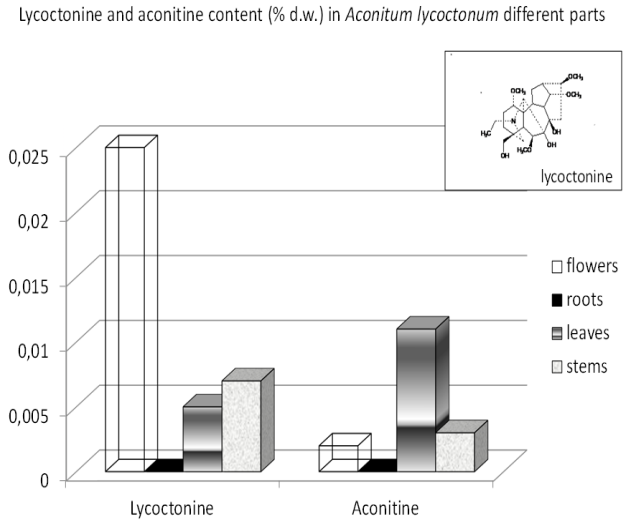
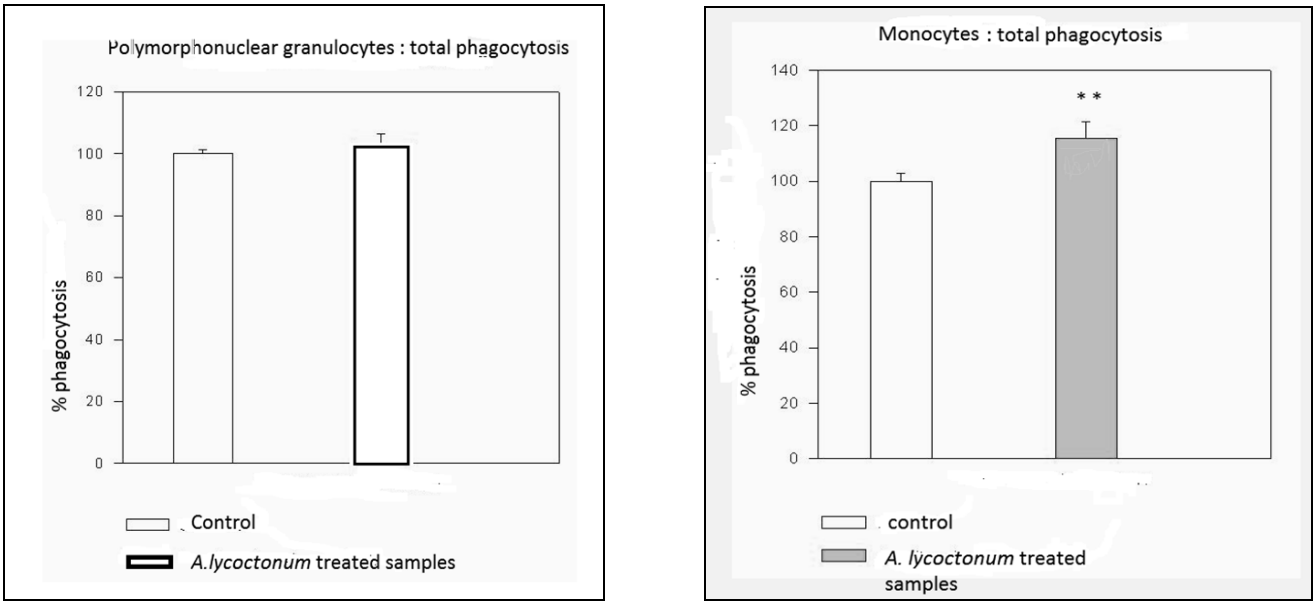


Figure 3
Alkaloid content(lycoctonine and aconitine) in *A.lycoctonum* different parts



Panel A **Panel B**

Figure 4 – Panel A and Panel B
Increase of phagocytosis (7%) by PMN (Panel a) and by monocytes (Panel B)

Immunomodulatory activity. This phagocytosis test is based on the measurement of the ingestion of fluorescent *Candida albicans* cells in a predefined quantitative ratio with the effector leukocytes (monocytes and granulocytes), in whole blood, with the addition of a system to detect the true ingestion of *Candida albicans* by the leukocytes, distinguishing it from the adhesion phenomenon.

Fluorescein isothiocyanate (FITC) is widely used to attach a fluorescent label to proteins via the amine group. The isothiocyanate group reacts with amino terminal and primary amines in proteins. It is used for the labeling of proteins including Candida cells, antibodies, lectins, etc.

Ethidium bromide is a well-known and widely used fluorescent dye in biotechnology research. It used in a number of fluorimetric assays for nucleic acids

The coupling colouring of fluorescein and ethidium bromide allows to state the true ingestion or adhesion cells. Fluorescein isothiocyanate is cleaved by diesterase in live cells to fluorescent molecule fluorescein, while ethidium bromide passes through the membranes of dead cells and bind to nucleic acid, staining the cell nuclei red. It is a mutagenic compound which intercalates double-stranded DNA and RNA.

The definitive results refer to a series of conclusive experiments (11 performed in duplicate), in which the definitive method was applied, deriving from the preliminary optimisation process of the method: concentration of extract 0.1 µg/µl, incubation 1 h, use of a blocking solution of the phagocytosis, storage of the sample in ice, standardised timing. The distinction between internalized and/or attached and/or internalized Candida cells was done via quenching of FITC-fluorescence by ethidium bromide. We analysed the effects on total phagocytosis, on the percentage of cells with only internalised *Candida albicans* (phagocyted), on the percentage of cells with *Candida albicans* both adhered and internalised, both by the granulocytes and the monocytes present in the same blood sample and identified with the flow cytometer using different "gate". The extracts show on the average a more marked effect on the percentage of monocytes with internalised *Candida albicans*. From the whole of the investigations, we can state that the immunomodulatory activity was manifested in an increase of phagocytosis (7%) by the polymorphonuclear granulocytes (PMN). A statistically significant increase (**P<0.01) was obtained by the monocytes treated with the alkaloids of *A. lycoctonum* (Fig. 4, panel A and B).

Cell samples examined by flow cytometry, allowing rapid and kinetic assessment of large numbers of cells and subsets of Candida-exposed cells. This procedure provides a simple and effective method to distinguish surface-bound from internalized *C.albicans*, and allows precise kinetic analyses on large numbers of cells.

CONCLUSIONS

Plants belonging to the Aconitum genus are arousing renewed interest for the promising biological activities of its alkaloids. *A. lycoctonum* was previously investigated for the identification of alkaloids structurally correlated to lycoctonine [32]. In the population under study we even identified aconitine, a typical alkaloid of *A. napellus* and usually not mentioned for *A. lycoctonum*.

As regards the results of the biological activity, the phagocytosis, a major mechanism used in the immune system to remove pathogens, was evaluated. Phagocytosis is the first step of defense against infections from the innate immune system and the phagocytic efficiency is considered the response of the innate (non specific) immune system. In this work, phagocytosis was determined by the measurement of internalized *Candida albicans* by leukocytes (monocytes and granulocytes). *C.albicans* is the major fungal component of the normal microflora of the healthy host. However, when immune defenses are compromised, *Candida* transforms itself into an

opportunistic pathogenic killer [33]. The binding of *C.albicans* to the surface of leukocytes and/or the internalization of *C.albicans* by all or a subset of cells are key points in the phagocytosis process. The current studies establishes a method for the discrimination of surface-bound from internalized *C.albicans*. A flow cytometric technique was then adapted for study of *C.albicans* – leukocytes interactions, with addition of ethidium bromide to quench green fluorescence associated with FITC-labeled *C.albicans* that was cell-bound but remained external. Ethidium bromide was excluded by intact cell membranes, and internalized *C.albicans* retained green fluorescence. The increase in phagocytosis by human monocytes and polymorphonuclear elements, after administration of diterpene alkaloids, confirms the immunomodulatory activity attributed to the Aconitum extracts [34].

According to literature data, in which an immunostimulatory activity was evaluated in rats treated with benzoylmesaconine (alkaloid obtained by partial hydrolysis of mesaconitine, losing the acetic acid moiety) [35], our experiments show an enhanced phagocytosis of *Candida albicans* by human granulocytes (7-8%) and monocytes (**P<0.001), in presence of *Aconitum lycoctonum* alkaloids.

In summary, the results of the present study show that diterpene alkaloids from *Aconitum lycoctonum* L. may affect the functional responses of human PMNs and monocytes. As information regarding the immunomodulatory properties of aconite are still very limited, our results encourage more extensive studies to assess the potential of monkshood alkaloids and of individual components as modulators of the immune response.

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