SHORT REPORT

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Antimicrobial Effect and Antioxidant Activity of Triterpenes Isolated from *Gymnema sylvestre* R. Br.

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Abstract: *Gymnema sylvestre* is a commonly used herb in Ayurvedic medicine. The demand for its extracts in the commercial and pharmaceutical fields has been steadily increasing in recent years. Its extracts are used to treat various ailments as well as for their antimicrobial properties. This study has evaluated the antimicrobial effects of different *G. sylvestre* extracts and of eight triterpenes isolated from the most active extract on six bacterial poultry pathogens i.e. *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter aerogenes*. In particular, it has been evaluated the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of all extracts and isolated triterpenes. Finally, the cytotoxicity activity of triterpenes was evaluated by MTT assay and their antioxidant activity in basal and oxidant conditions by DCFH-DA assay.

Keywords: *Gymnema sylvestre*; antimicrobial effects; triterpenes; gymnemic acids; herbal drug; DCFH-DA assay.

1. Plant Source

Gymnema sylvestre R. Br. is a large climbing plant belonging to the Asclepiadaceae family, widespread in the tropical areas of Asia, Africa and Australia. The plant presents branches covered with petiolate leaves, with an oval shape, elliptical powder and with a characteristic bitterish and acrid taste. The flowers are typically campanulate and gathered in pedunculated racemes. *G. sylvestre* was mentioned for the first time in the European scientific literature in 1847 by Edgeworth and, successively, by Hooper [1], but its properties were already known in Indian medicine. More in details, it is known that the inhabitants of Bombay and Gujarat are used to chew fresh leaves of *G. sylvestre* to reduce glycosuria. Both leaves and dried roots of the plant are used in Ayurvedic medicine [2]. Plants are real reservoirs of secondary metabolites belonging to different classes of substances [3-

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5], some structurally very complex [6-8]. The interest in the isolation and structural determination of natural compounds is strictly in relation to their possible use as herbicides [9-10], algaecides [11-13], insecticides or drugs [14]. The present study was designed to evaluate the antibacterial activity of several extracts of *G. sylvestre* leaves and eight triterpenes isolated from the most active extract. Finally, an *in vitro* cell growth was achieved by 50% and the ability to exert antioxidant activity.

2. Previous Studies

The eight triterpenes (**1-8**) reported in Figure 1 have been isolated from the most active extract of *G. sylvestre* leaves [15] The dried and powdered leaves of *G. sylvestre* were sequentially extracted with water (H_2O) and methylene chloride (DCM). The organic fraction contained the much of phenolic compounds and the highest antioxidant activity [16]. The ethyl acetate (AcOEt) fraction was chromatographed on silica gel and the fractions obtained were purified by HPLC to give a new lupane-type triterpene (**1**) and seven oleane-type triterpenes (**2-8**) (Figure 1).

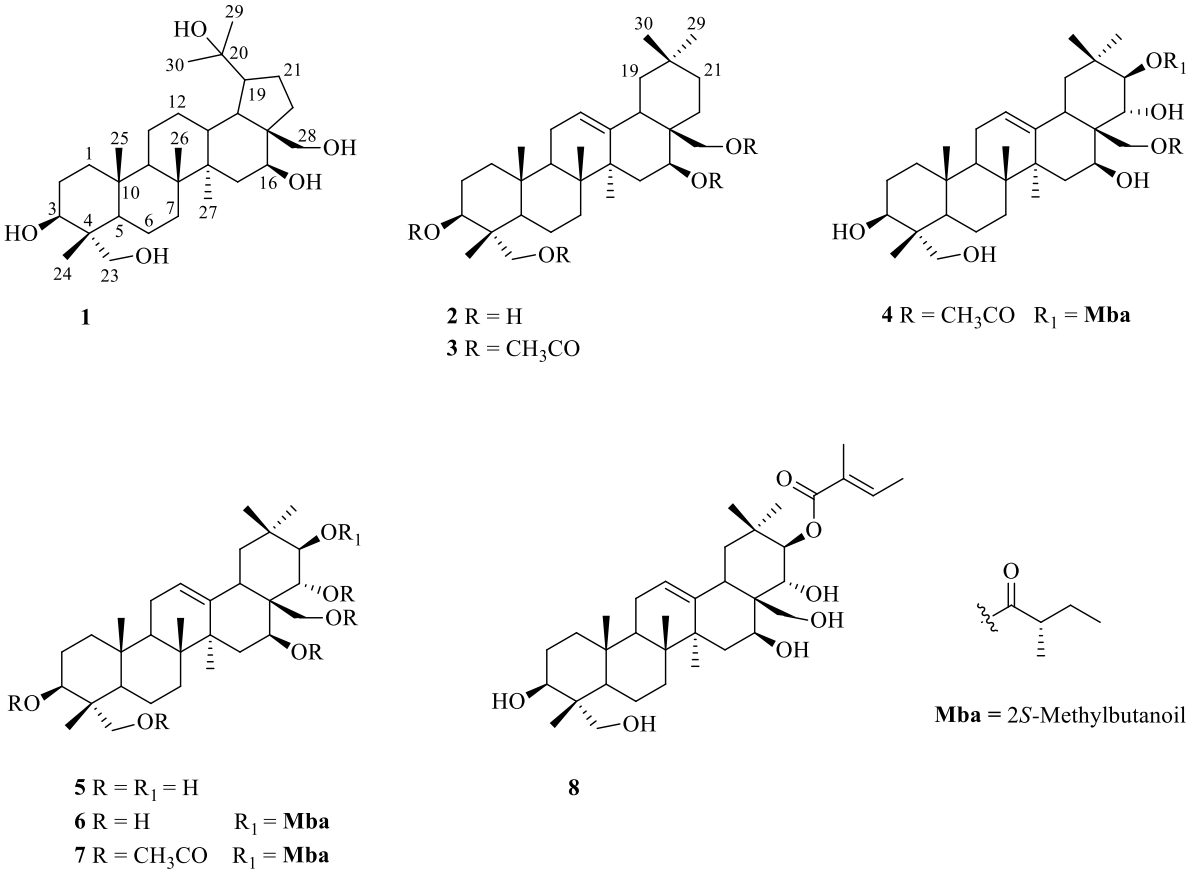

Figure 1. Structures of the lupane (**1**) and oleane-type triterpenes (**2-8**).

3. Present Study

Today it is known that plants are a precious reservoir of secondary metabolites with low molecular weight [17-19], belonging to numerous classes of substances [3-5] and used for the defense against insects, microorganisms or even from other plants [9-13]. Over the years, there was a continuous and ever increasing request of new natural substances that are commonly considered non-toxic and safer than synthetic ones [20-21]. Considering that one of the most serious health threats is the antimicrobial resistance (AMR) [22], recently, there was an increasing interest in natural extracts or their single components endowed with antimicrobial activity. This work is a part of a more general

project that aims to define new uses for natural extracts or for their individual components. The antibacterial activity of several extracts of *G. sylvestre* leaves and eight triterpenes isolated from the most active extract has been evaluated. The six bacterial poultry pathogens tested by a microtiter dilution assay are: *Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterobacter aerogenes*. [23].

Plant Material: G. sylvestre was purchased from Mother Herbs Ltd., 13 Street, Madhu Vihar, Patpadganj, Delhi - 110 092, India, E-mail: info@motherherbs.com and identified by Prof. *Gabriele Pinto* of the Dipartimento di Scienze Biologiche of the University of Naples. A sample specimen (HERBAZLS 317a) has been deposited at the herbarium of the University Federico II.

Thus, *G. sylvestre* leaves were infused first with water and then with DCM. The organic phase, reduced in volume, was debated with an aqueous solution of 2N of NaOH and the neutral phase was reduced in volume and neutralized. The latter fraction was finally chromatographed on silica gel eluting with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA), acetone (A), methanol (M) and water (W) (Figure S1) Each fraction was tested on three strains of Gram-positive bacteria such as *B. subtilis*, *E. faecalis*, *S. aureus*, and three Gram-negative ones such as *P. aeruginosa*, *E. coli* and *E. aerogenes*. Table S1 shows the minimum inhibitory concentration (MIC) for each microorganism, while in Table S2 it is reported the minimum bactericidal concentration (MBC).

The values of minimum inhibitory concentrations (MIC) vary between 168.3 mg/L of the PE extract on *E. faecalis* and the 75.4 mg/L of the EA extract on *B. subtilis*. The most active extract was that obtained with EA with MIC values not higher than 91.3 mg/L on *E. aerogenes*, while the less active was the least polar ones with MIC values always higher than 140 mg/L. Intermediate values for the other extracts were reported, in particular that obtained by DCM seems the most active. Table S2 confirms that regardless of the organism considered, the extract with EA is the most interesting with a total inhibition of microbial growth around 156.2 and 201.3 mg/L that in some cases are just above those of the control. However, MIC, MBC values also indicated that the species more sensitive to the action of the most active extract are *E. faecalis* and *E. coli*.

The isolated triterpenes (Figure 1) have been assayed on the same bacteria strains, evaluating for each microorganism both the minimum inhibitory concentration (MIC, Table S3) and the minimum bactericidal concentration (MBC, Table S4).

The MIC values vary between 21.8 mg/L of triterpene **1** on *B. subtilis* and the 7.2 mg/L of triterpenes **3** and **7** on *B. subtilis* and *E. coli*, respectively. In general, the most active ones are the triterpenes **3**, **4** and **7**, with an oleane skeleton, full acetylated to hydroxyl groups or in part acetylated and esterified with a 2S-methylbutanoic acid unit instead resulted less active the polar triterpenes **2**, **5** and **6**. In particular, the triterpene **2** corresponds to the triterpene **3** with hydroxyl groups not acetylated, while the triterpenes **5** and **6** are similar to the triterpene **7** with all hydroxyl functions free. the triterpene **6** is also **e**sterified with the 2S-methylbutanoic acid to the hydroxyl group at C-21 carbon (see supporting information for detail)

Regarding the MBC values reported in the Table S4, the triterpenes **3**, **4** and **7** completely inhibit microbial growth at values of concentrations between 17.1 and 21.6 mg/L. As for the extracts data, MIC and MBC values also indicate that the species more sensitive are *B. subtilis* and *E. coli*.

For an initial characterisation of biological properties of isolated compounds and in view of their potential clinical use, we initially utilised an *in vitro* cell model to calculate the concentration inhibiting cell growth by 50% (IC_{50}) and also to evaluate if they exert any antioxidant activity. Rat diploid immortalised fibroblasts represent one of the simplest available and widely used cellular models for toxicological assays. In addition considering that rat and human cells generally exhibit good metabolic similarity, results obtained on rat cells are typically confirmed in humans.

Cytotoxicity assays were performed using the MTT test, as previously described [17]. Exponentially growing cultures of rat fibroblasts were exposed to increasing concentrations of each compound (0-1 mM) and cell viability was assessed after 48 h. A dose-dependent decrease in viable cells was observed with all tested compounds with an IC_{50} ranging between 28 and 839 μM (Table S5).

To assess the potential antioxidant activity of the compounds, we decided to use a dose of 30 μM for all compounds since the majority of them did not exert an evident toxic effect at such dose. Intracellular reactive oxygen species (ROS) levels were measured by the oxidative conversion of stable, non-fluorescent 2ˈ,7ˈ -dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent 2ˈ,7ˈ -dichlorofluorescein (DCF) occurring in the presence of ROS.

The DCFH-DA test was preferred over the traditional *in vitro* assays because it takes into account the solubility and the cellular uptake of tested compounds, which are important variables when assessing their potential use in humans. Moreover, the test discriminates the different types of ROS, such as superoxide radical, hydroxyl radical, and hydrogen peroxide, which are all relevant in an *in vivo* setting. Therefore, the fluorescence detected is a sensitive indicator of intracellular ROS. We evaluated the effect of ROS exposure to compounds for variable times (1, 16 and 48 h) on the basal intracellular ROS level and on oxidative conditions induced by H_2O_2.

As shown in Figure 2, we did not detect any significant effect on basal and oxidative conditions; in details most of compounds (i.e, compounds **2**, **3**, **5** and **6**) induced an increase, in the basal level of ROS. Compounds **4** and **7** showed a slight antioxidant activity. After 16 h, the less active compounds appeared to further increase their activity, also the compounds **4** and **7** have more or less the same value of control. After 48 h, all compounds displayed activity below the threshold value of the control sample, with the exception of the less active compounds **2** and **3**.

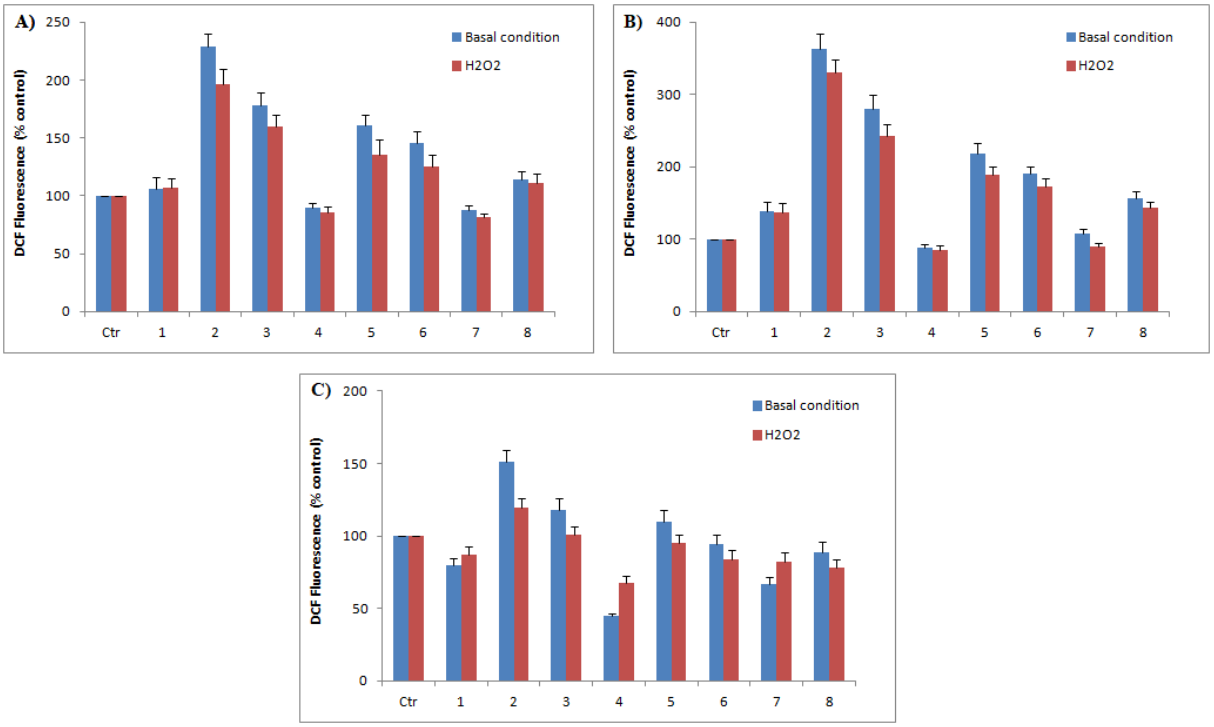

Figure 2. Effects of tested compounds on intracellular ROS production in Rat-1 cells, using DCF (10 M) as the fluorescent probe. DCF fluorescence was measured at basal conditions (blue bars) or following cell exposure to 100 μM H_2O_2 for 15 min (red bars) and a 1, 16 or 48 h (**A**, **B** and **C** respectively) pre-treatment with the indicated compounds. The values are expressed as % compared to the fluorescence value observed in untreated control cells, which was set at 100. Experiments were performed three times on triplicate samples. The SD was <20% for all tested conditions.

Interestingly, the compound **4** under basal conditions showed inhibitory activity of at least 60% after 48 hours. The compound **1** and **2** respectively with lupane skeleton and oleane-like triterpene, both with hydroxyl groups at positions C3, C16, C23 and C28 showed a markedly different antioxidant activity. The activity of compound **1** results to be much higher than **2**. Compound **3**

consists of the corresponding peracetylated of compound **2** and showed a higher activity. It is interesting to note that the most active compounds **4** and **7** are acetylated to hydroxyl groups at positions C3, C16, C23 and C28, and show a 2S-methylbutanoyl unit at hydroxyl group of C-21 carbon. The only difference is due to the presence of an acetyl group at C-22 carbon in the compound **7**. In contrast, the compounds **5** and **6** have the hydroxyl groups not acylated and presented a low antioxidant activity. Moreover, between the compounds **6** and **8** that differ for the acid unit bound to position C-21, the compound **8** appears to be more active.

In conclusion, some of triterpenes isolated by *G. sylvestre* leaves were able to reduce the basal endogenous levels of ROS, but most importantly, they were also able to prevent an H_2O_2-induced generation of intracellular ROS. These findings warrant further studies to better characterize the properties of these compounds and their effects on intracellular ROS, which as shown for well-known antioxidants such as lycopene and β-carotene, might be affected by the concentration used, as well as several other cellular variables [24-25].

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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