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Quality and Efficacy of *Tribulus terrestris* as an Ingredient for Dermatological Formulations

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Abstract: *Tribulus terrestris* L. (Zygophyllaceae) is an annual plant commonly known as Puncture vine. It is dramatically gaining interest as a rich source of saponins. *T. terrestris* is a promising ingredient for many industries and recent patents on dermatological applications support the use of this plant for cosmetics and hygiene. Nonetheless problems arise in the selection of the material to be used. The extracts of different origins may differ substantially. Natural speciation processes normally influence 'variations' in wild-crafted medicinal plants. The genus *Tribulus* is emblematic. Taxonomic status of *T. terrestris* is complicated by the wide geographical distribution leading to high levels of genetic polymorphism. Being aware of such variability we selected 3 commercial *Tribulus* extracts and compared their biological effect on *Candida albicans* with the effect produced by an extract from local plants (South of Apulia, Italy). One of the commercial extracts with the best anti-*candida* performance was used to substitute triclosan in a detergent formulation and it proved to improve the product performance in the control of potentially pathogenic skin flora such as *C. albicans*.

Keywords: Anti-Candida effect, *Candida albicans*, intimate hygiene, skin flora, *Tribulus terrestris*.

INTRODUCTION

Tribulus terrestris L. (Zygophyllaceae) is an annual plant commonly known as Puncture vine [1, 2]. For centuries it has been used in the traditional medicines of China, India and several other regions. In the mid-1990s, the use of this plant became known in North America and Western Europe after Eastern European Olympic athletes said that taking *Tribulus* helped them in their performance [3].

It is gaining global interest as proven by the logarithmic growth in number of scientific publications observed from the 90s (5 publications per year) to the recent years (50 publications and over 400 citations in 2011; "Web of Science" Citation Report using Topic="*Tribulus terrestris*").

Extracts from the full plants or fruits are now used for a large number of applications ranging from skin care to human hormones regulation [4-6], as anti-bacterial [7], anti-inflammation [8], anti-virus and immuno-stimulant too. Biological activity (biocide and antioxidant) is clear in several studies but clinical, histological [9, 10] and cellular studies [11] are rare.

The reason for such a lack of information is probably due to the composition of the "extract". It is a "phytocomplex" rich in different compounds [12, 13], none of which can be found entirely responsible for the biological effect investigated.

The known active compounds in *Tribulus* are called steroidal saponins, primarily present in the leaf and fruit. The Bulgarian "Tribestan", from SOPHARMA, was the first standardized preparation [1] that has been initially described in Bulgarian patent applications [14, 15] and German articles [16]. More products were developed lately as preparations or food supplements. For examples we can list LIBILOV from USA, TRIBOSTIM TM and TRIBOVIT TM from Bulgaria, TRIBULUS-ZMB from Italy. All of them target impotence and libido disorders, in men and women. The product UNEX is proposed as diuretic [17].

Recent investigations focusing on the Bulgarian commercial products have indicated protodioscin and prototribestin as main components in the Bulgarian plant extracts [1], but the presence of many other components may be determined [18]. The extracts are rich of cinammic acid amides, lignanamides (tribulusamides A and B), alkaloids, flavonoids like rutin, quercetin and kaempferol, as well as steroidal saponins in many different forms: prototribestin, dioscin, protodioscin [19, 20] furostanol [21], spirostanol, sitosterol glucoside [22], terrestrosins A-E, desgalactotigonin, gitonin, tigogenin, gitogenin, beta-Sitosterol, spirosta-3,5-diene, stigmasterol, hecogenin, neohecogenin, ruscogenin [5, 23, 24] tribulosaponin B, metilprotodiostsin, terres-

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trozin H, prototribestin, gracillin [25]. Content, especially in terms of detectable saponins, varies with growth conditions and ecotype [20, 26-28] in addition to extraction methods. Since it has such a rich composition, it is not surprising that such diversified biological effects were observed for this plant extract.

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Consumer demand for natural products coupled with their physicochemical (surfactant) properties and mounting evidence on their biological activity have led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors.

As a rich source of saponins, *T. terrestris* is a promising ingredient for many industries [29] but the research and development (R&D) activity willing to include this natural product in topic formulations for hygiene or cosmetic uses needs to be very carefully performed with the background knowledge.

Patent literature provides some examples on the different aspects of the preparation and characterization of *T. terrestris* extracts for topic uses. *T. terrestris* extracts comprising spirosteroid saponin have been characterized for the preparation of antifungal compositions and 17 distinct spironosaponins have been identified and associated by means of a common general chemical formula [30, 31]. Detailed methods for preparing cream using *T. terrestris* extracts with anti-bacterial, anti-inflammatory, anti-viral activities, and other activities for topical use on skin and mucosal tissues have been described [32, 33]. It has also been suggested to combine *T. terrestris* extracts with metals for preparing anti-viral pharmaceutical compositions [34, 35]. Several examples of plant extracts combinations including *T. terrestris* extracts are found in Asian patent literature. Some of such patent documents specifically refer to the usage involving topical administration such as pruritus and skin disorders [36], increasing the skin permeability and stimulating the generation of melanophore [37], improving skin tenderness or other cosmetic applications [38, 39].

In this work, we analyzed the application of *T. terrestris* extract to commercial uses through the analysis of three commercial extracts from different sources, all available on the Italian market, and compared their biological effect as biocide. Saponins measurement is difficult and reliable methods, mostly HPLC separation, should be referred to known standard molecules. Unfortunately the commercially available extracts have more approximate quantification standards (as the gravimetric method). For this reason we produced an extract from plants grown in Italy, in the area known as Salento, and used it as a reference. We tested the efficacy of the extract as ingredient in the formulation of a detergent for intimate hygiene to potentiate anti-*candida* effects. The selected extract appears as a very good ingredient but the need of accurate quality standards defined case by case, seems to be necessary to select the material on the market.

MATERIALS AND METHODS

Preparation of *Tribulus* extracts

T. terrestris L. full plants including roots and fruits, were harvested in Italy, in different sites of Salento peninsula, South of Apulia, in July 2010. Plant material was carefully identified by Prof. Zuccarello and Dr Di Sansebastiano with the support of Dr Accogli, Botanists and plant biologists at the Department of Biological and Environmental Sciences and Technologies (DiSTeBA) at University of Salento.

Fruits were separated from plants, washed, frozen in liquid nitrogen and lyophilized in a Christ Alpha 2-4 LSC freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 24 h. The lyophilized material was ground to 500 μm in a laboratory mill (Retsch GmbH, Haan, Germany) to obtain a homogeneous powder. Saponins and other soluble molecules were initially extracted from this dry powder with a 70% ethanol solution for 24 hours. The solvent proportion was 200 ml each 120 gr of fruit powder to assure fluidity of the mixture during stirring extraction at 25°C. The resulting ethanol extract was cleared by centrifugation and filtered. Saponins were precipitated adding 2 volumes of cold acetone and centrifuging at 20000g for 30 min. The pellet, corresponding to about 2% of the starting material, was air dried for 30 min was resuspended in 70% ethanol at the concentration of 2 gr/ml. Three commercial extracts with a 40% declared content of saponins were also resuspended in 70% ethanol at the concentration of 2 gr/ml and compared with fruit extract extract.

HPLC Analysis

The analysis was carried out to compare chemical profile of the different extracts of *T. terrestris* using reverse phase HPLC with UV detector. The mobile phase that consisted of phosphoric acid buffer with pH-3 (A) and acetonitrile (B) was used for gradient elution. The flow rate was adjusted to 1.0 ml/min. The detection wavelength was at 203 nm. All separations were performed at ambient temperature. The plant material, (0,5g) was extracted two times with 5 ml of 50% aqueous acetonitrile by sonication for 15 min. The samples were centrifuged at 4900 rpm for 10 min. The supernatant was lyophilized for 15 min. The extract was dissolved in 50% aqueous acetonitrile [20]. Prior injection, all samples were filtered through a 0.45μm membrane. Each sample solution was injected in duplicate with injection volume of 20μl.

Antifungal Evaluation

Candida albicans strain MUCL 29800T [40]. The yeast was grown to exponential phase at 37°C for 18 h on a shaker in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose) or on a solid medium prepared by adding 4% agar. Extracts inhibitory activity was tested against the microorganism using a broth micro dilution method in 96 multiwell plates, in triplicate, as reported by Koneman [41] and recommended by the National Committee for Clinical Laboratory Standard [42]. Optical density measurements at 600nm were made in a TECAN Infinite.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The antioxidant activity was measured using the ABTS discoloration method [43]. Samples B and E (Hydrophilic antioxidants) were centrifuged at 10,000 g for 7 min and the different supernatants were recovered and used for antioxidant activity measurements. The antioxidant activities were measured at 734 nm in a Cecil BioQuest CE 2501 spectrophotometer. The calibration curve was constructed, using freshly prepared Trolox solution for HAA determination. Values were obtained from three replicates as Trolox equivalent mg.

Toxicity

Human epithelial cells from A-253 line (ATCC, American Type Culture Collection Manassas, VA, USA) were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Titration tests were carried out in 96 well plates: briefly, cells were exposed for 24 h to different concentrations of the extracts. Cell viability was assessed by neutral red test: cells were incubated for 2 h with complete medium with 0,05 mg/ml neutral red; this stain is actively accumulated in lysosomes of healthy cells. Following the incubation, cells were lysed with a ethanol/acetic acid solution and neutral red accumulation were measured by absorbance at 540 nm with a microplate reader. The cell vitality of cells incubated with the different products was expressed as percentage versus untreated cells (which absorbance values were considered as 100 % vitality).

A-253 cells retain the morphologic features typical of *epithelial cells*. Being derived from the submandibular region, they may well represent the most delicate mucosa epidermis.

Washing Simulation

Candida albicans cells in exponential growth phase. The yeast was grown to exponential phase at 37°C for 15 h on a shaker in YPD liquid medium. Aliquots of 1 ml of liquid culture were pelleted by rapid centrifugation (13000 rpm in a bench minifuge) in separated test tubes and resuspended in various detergent dilutions kindly provided by Ekuberg Pharma. After 5 minutes cells were pelleted by rapid centrifugation, resuspended in 200 microliter YPD and plated on solid YPD Petri dishes. After over-night culture the number of colonies was counted.

RESULTS

Extract Quality and Efficacy

Four *T. terrestris* extracts were used. Three commercial dried extracts with a 40% declared content in saponins were purchased from different Italian distributors. The plant origin was not always declared (Table **1**). We refer to these plant extracts as A, B and C. The fourth extract was obtained following a water/Ethanol extraction combined with a precipitation step in acetone. We refer to this as extract E. Biocide effect of extracts was tested on *Candida albicans* strain MUCL 29800T [40] liquid culture.

Commercial extracts declared Maltodextrin as excipient and colloidal anhydrous silica as auxiliary substance. All commercial extracts had an important insoluble residue; if added to *C. albicans* culture all induced an increase in fungus growth rate (not shown). Microscopy observation showed the presence of intact starch granules in commercial powder A. To eliminate the insoluble residue and make the extracts comparable, all of them were resuspended in a 70% ethanol solution (2g/mL) and filtered through a 0.2 micron filter.

HPLC analysis of the extracts revealed remarkable differences. The extract E shared with product C the highest similarity with only 58% of common peaks (Fig. **1**). The other extracts shared with E and among each other less than 40% of common peaks (not shown).

Solutions containing extracts A, B, C, E were diluted in YPD liquid culture medium where *Candida* was grown for 18 hours. OD absorbance at 600nm was simultaneously read in a multiwell plate for all samples and 3 to 5 independent experiments were performed.

Results shown in Fig. (**2**) give evidence that the minimal inhibitory concentration (MIC) has to be determined case by case, depending on the source of extract. The self-produced standard preparation, extract E, had a biological action stronger than that of 2 out of the 3 commercial sources but 1 of the commercial products, extract B, probably better titrated for the specific saponins responsible for its properties, had a very high activity. The extract B was then selected for further use.

Extract Antioxidant Activity and Toxicity

The selected extract B was analyzed to verify antioxidant activity and toxicity. Cytotoxicity and eventually chemo preventive role of saponins were discussed in a number of reviews and they depend on the specific molecular pattern [44]. In consideration of the high variability in the phytocomplex, assay case by case is necessary.

The antioxidant activity was measured using the ABTS discoloration method with the determination of trolox equivalent (TEAC). At the concentration of 1 mg/ml B produced the antioxidant activity of 1545.7 trolox equivalent

Table 1. General Information About the Products Used

	Extract			
	A	**B**	**C**	**E**
Saponin content	40%	40%	40%	n.d.
Excipients	maltodextrin	maltodextrin	maltodextrin	none
Auxiliary substance	colloidal anhydrous silica	maltodextrin	colloidal anhydrous silica	none
Plant organ	n.d.	fruit	fruit	fruit
Plant origin	n.d.	India	India	Italy (Salento)

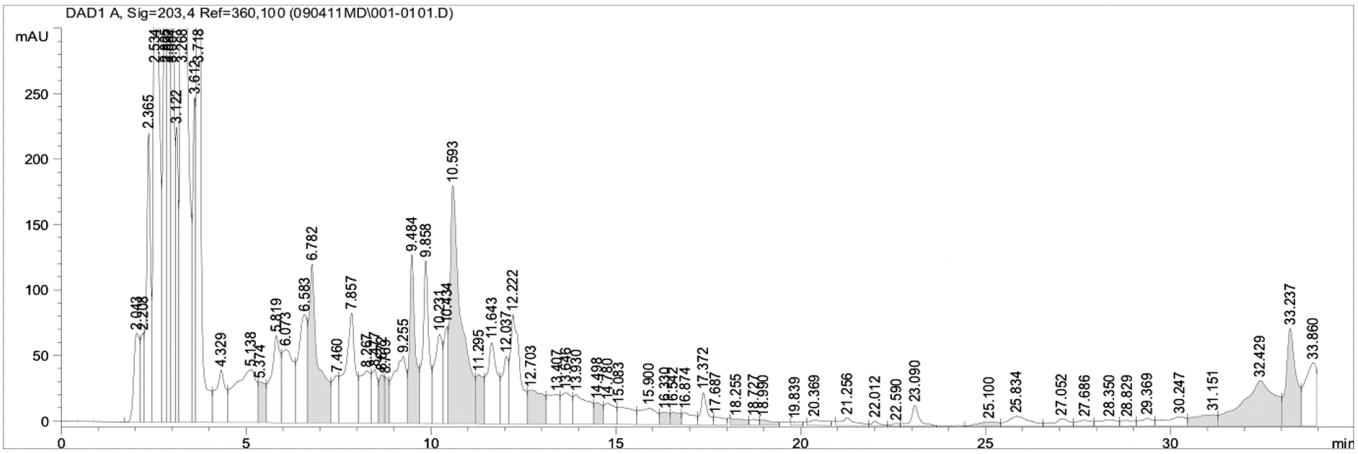

Fig. (1). HPLC-DAD chromatogram of the *T. terrestris* fruit extract E. Peaks representative were identified by their retention times. In grey the peaks shared chromatogram of commercial extract C.

(386.4 mg/ml Trolox), E produced the activity of 683.7 Trolox equivalent (170.9 mg/ml Trolox). A *Thymus vulgaris* extract treated in a similar way in terms of dry weight was used to evidence that *Tribulus* antioxidant activity was particularly high. In fact the same amount of dry extract from *Thymus* generated only a 29.1 Trolox equivalent activity (7.3 mg/ml).

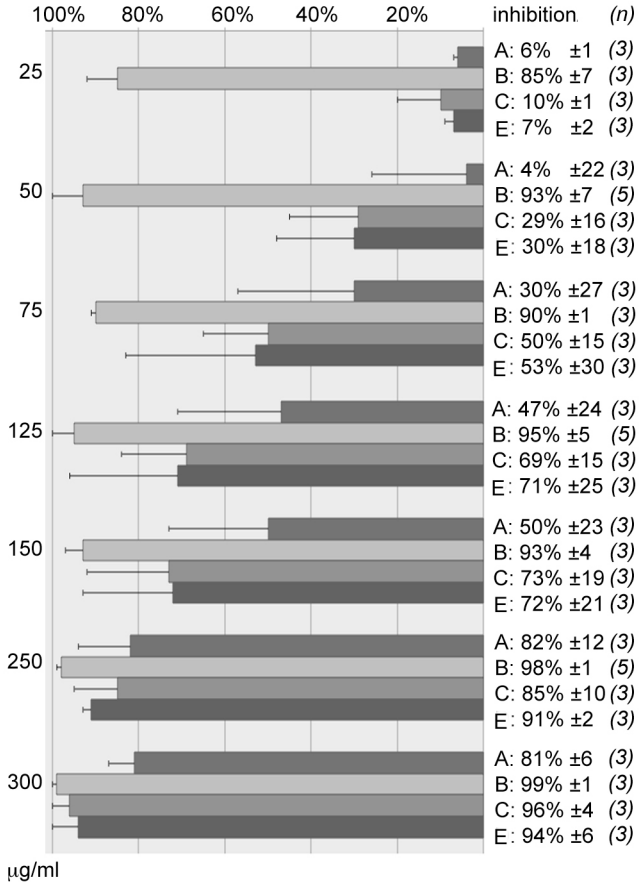

Fig. (2). Percentage of inhibition of *Tribulus* product dilutions on Candida growth. Error bars indicate SD. *(n)* indicates the number of experimental repeats.

Extract B cytotoxicity was measured using Neutral Red viability test on fibroblast cultures and compared with extract E and a raw fruit aqueous-extract (raw-E) was used as control (Fig. **3**). Extract B exerted high cytotoxic effect at 150-75 µg/ml, a 90% viability reduction resulted with respect to controls. At lower concentrations the toxicity significantly reduced, in fact at 25-10 µg/ml cells showed viability comparable with control. A similar trend was observed with product E, although it resulted in being toxic at lower concentrations. Extract E resulted to be much less toxic, in fact cell viability was comparable to control one already at 100 µg/ml. The raw aqueous-extract from fruit was obtained by extraction with a 70% ethanol solution and exhibited high toxicity, evidently for the action of other compounds additional to saponins.

Efficacy of *T. terrestris* Commercial Extract as a Control Agent

Commercial extract B (declared 40% saponins) was used in the formulation of a new product here designated as "DMX" at the final concentration of 0.1% w/v [45, 46] (DMX).DMX was compared with the same formulation without *Tribulus* extract (DMXnoT) and with one detergent already commercialized with 0.1% Triclosan instead of *Tribulus* extract (DMXtcs). In order to have an idea of general efficacy of similar products available on the market, three more detergents intimate feminine hygiene were selected from the same shelf in a shop and designated as C01, C02 and C03. Recovery of *Candida* cells after washing was evaluated as colonies growth on petri dish containing YPD medium (Fig. **4**). Data are reported in Table **2**. DMX showed a better performance (-87% of colonies growth) than DMXnoT (-65%). *Tribulus* appeared to be more effective than Triclosan in DMXtcs. Among competitors only C03 had better performances; C02, did not appear to affect fungi vitality. Such variability reminded the high variability in biological effects of extracts despite the generic quantification of ingredients.

DISCUSSION

Quality and efficacy of herbal medicines are directly linked to the quality of the medicinal herb raw materials. Three critical steps at the very beginning of the manufacturing process are: 1) cultivation/collection of authentic whole plants or plant parts, 2) sorting, drying and

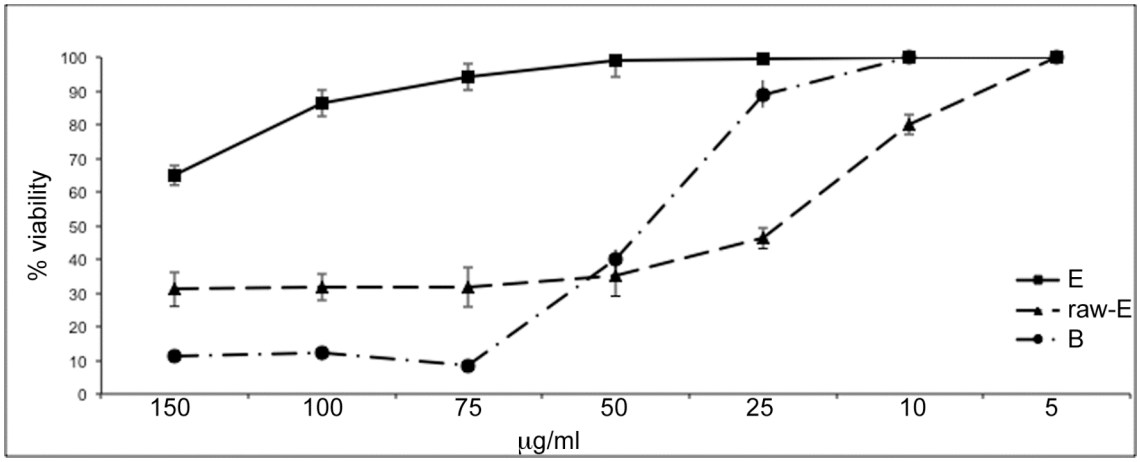

Fig. (3). Cytotoxic effect measured through cell viability exposed to different concentrations of 3 different extracts. Average data derived from 3 experimental repeats.

powdering of the herbal material, and, 3) non-targeted or targeted, solvent(s) based extractions of the herbal materials to enrich or to include 'active' or 'marker' compounds [47]. Any compromise in quality in each of these steps will permeate the sequential links in the manufacturing process.

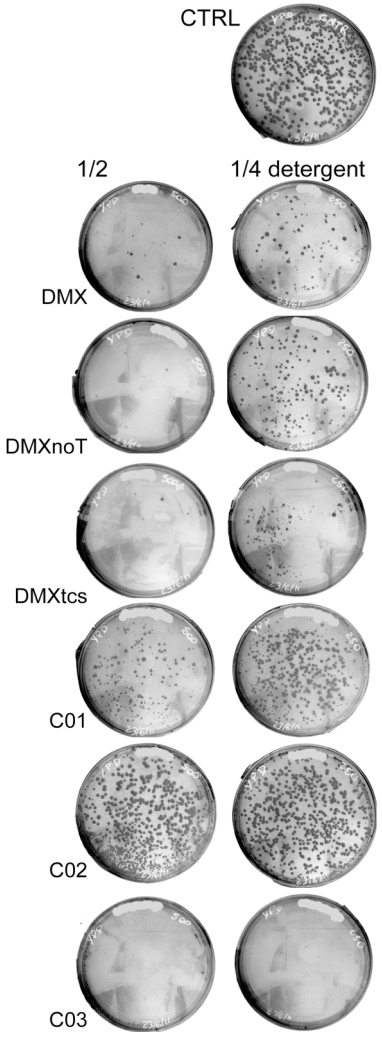

Fig. (4). Images of Candida's colonies grown on YPD medium after washing.

Table 2. Number of Colonies Grown from Candida's Cultures After Washing

Product	Dilution	Average	SD	Inhibition
CTRL	-	**576**	83,1	
DMX	0,50	**20**	3,6	
	0,25	**77**	3,6	-87%
DMXnoT	0,50	**22**	4,0	
	0,25	**201**	53,1	-65%
DMXtcs	0,50	**3**	0,6	
	0,25	**125**	71,1	-78%
C01	0,50	**236**	75,0	
	0,25	**428**	17,2	-26%
C02	0,50	**652**	113,3	
	0,25	**639**	33,0	0,00%
C03	0,50	**1**	0,6	
	0,25	**4**	2,0	-99%

As a rich source of saponins, *T. terrestris* is a promising ingredient for many industries and recent patents on dermatological applications support the use of this plant for cosmetics and hygiene.

There are a large number of patents and patent applications that describe technologies and compositions for medical uses that involve the preparation of *T. terrestris* extracts, showing the variety of fields of application for inventions wherein this plant is indicated as a major ingredient (Falciola and Di Sansebastiano, in preparation). They range from a beneficial effect on hormonal equilibrium to hepatoprotection or antiviral activity. A detailed analysis of patent information content is needed but, it is evident that many of them refer to applications in the field of sexual and fertility disorders correction. The physiological effects that justify the efficacy in general health and physiological performances appear strictly related to regulatory effects on hormonal balance [4, 25, 29].

Efficacy of *Tribulus* as an ingredient in dermatological application is also evident, being present in a minor but relevant and increasing number of patent applications. Nonetheless problems arise in the selection of the material to be used. The extracts of different origin may differ substantially [27]. Natural speciation processes normally influence 'variations' in wild-crafted medicinal plants. With the increasing demand for herbal medicines worldwide, there is a concomitant increase in cultivation of medicinal plants. Vegetative and clonal propagation and hybridization among cultivated sub-species/cultivars add to the variability in traits, making delineation of species difficult [48]. At present, most of the species are defined based on the 'typological species concept' on the premise that a group of plants of one 'type' share a number of diagnostic (fixed) traits. Taxonomic status of *T. terrestris* is complex because of its wide geographical distribution leading to highly variable morphology, ploidy and isozyme patterns. Moreover *Tribulus* can form panmictic populations, justifying variability [27, 49]. High levels of genetic polymorphism have been demonstrated in *T. terrestris* populations collected within the Indian sub-continent [50]. Fluctuating content of various saponins has been demonstrated within species, geographic distribution and spatiotemporal variation [47].

R&D activities willing to include this natural product in formulations for hygiene or cosmetic preparations face the preliminary problem represented by the qualitative evaluation of the material.

Tribulus extract contains a mixture of different compounds. Content, especially in terms of detectable saponins, varies with growth conditions and ecotype [20, 28] in addition to extraction methods. At least one commercial product of Bulgarian origin has indicated protodioscin and prototribestin as main components [1, 18], but the analysis of literature suggests that the characterization of the extract content does not take into account the intraspecific variability [50].

In this work, we collected three commercial extracts distributed in Italy and directly compared their biological effect as biocide since biochemical characterization could not be conclusive. Measurement by HPLC separation should be referred to standard molecules which is known to be related to the biological effect required by the product. Difficulties increase considering that steroidal saponins often lack chromophores for sensitive UV detection and accurate quantitation. Moreover the commercially available extracts have approximate quantification standards such as the gravimetric method.

We also included an extract performed from local plant material harvested, identified and directly processed. A preliminary HPLC analysis confirmed the evaluation problems since profiles appeared to be extremely diversified. We have no data to claim contamination from other materials or frauds; on the contrary we believe that differences may be due to plant material chemiotype, extraction method and conservation. Our goal was to select the material with the best biological activity. Once an effective extract was selected we tested its toxicity in comparison with a saponins enriched extract (E) and an aqueous raw-extract from fruits (raw-E). The reduced toxicity of E compared to raw-E showed how saponins do not have a strong toxicity when compared to other lipophilic compounds. The commercial product exhibited a higher cytotoxicity than extract E indicating a possible residual component of lipophilic compounds but such an effect disappeared far below the minimal concentration to produce an anti-Candida effect. The commercial extract B was then a good ingredient to be tested in the specific detergent formulation named DMX. The presence of *Tribulus* extract in DMX formulation led to an improvement in anti-*candida* performance compared to the "placebo" DMXnoT in which no specific biocides were used. The extract reduced the fungus recovery after washing as expected from a detergent aiming to control growth of potentially pathogenic flora.

The performance was superior to that of a DMX containing Triclosan (DMXtcs). Triclosan or TCS is a multi-purpose biocide widely used in personal care products. There are now growing concerns about its dispersal in the aquatic environment [51]. TCS safety for humans is also being questioned in the latest years and the use of such product will certainly be reduced in the next future. New and safer natural products are needed to potentiate products targeted to specific user categories. For example in the diabetic population, because of their poor glycemic control, the fungi are facilitated to colonize vagina and rectum [52] and require a stronger daily control.

Here we prove that natural products such as *Tribulus* fruit extracts, when properly selected from the market, can substitute triclosan and improve the performance in the control of potentially pathogenic skin flora.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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