**Table 2.** Data on self-fertilized induced tetraploid of *G. arboreum* and *G. herbaceum* variety

| | | Flower naturally pollinated | | | | | | Hand-pollinated selfed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species/variety | Ploidy level | No. | Boll set | % Boll set | Seeds/ boll | Seed wt. | Motes/ boll | No. | Boll set | % Boll set | Seeds/ boll | Seed wt. | Motes/ boll |
| *G. arboreum* | Diploid | 56 | 56 | 100 | 21.60 | 0.070 | 0.01 | 55 | 55 | 100 | 21.33 | 0.070 | 0.0 |
| JLA-9 | Tetraploid | 15 | 12 | 80 | 22.5 | 0.056 | 11.94 | 16 | 10 | 62.50 | 21.8 | 0.057 | 10.09 |
| *G. arboreum* | Diploid | 66 | 66 | 100 | 20.50 | 0.058 | 0.0 | 69 | 69 | 100 | 21.50 | 0.58 | 0.0 |
| Rh arb. 02-1 (W) | Tetraploid | 22 | 11 | 50 | 26.20 | 0.072 | 8.39 | 10 | 5 | 50 | 27.40 | 0.072 | 8.75 |
| *G. arboreum* | Diploid | 64 | 64 | 100 | 21 | 0.062 | 0.0 | 62 | 62 | 100 | 21.0 | 0.062 | 0.0 |
| G 27 | Tetraploid | 32 | 0 | 0 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 0 | 0 |
| *G. herbaceum* | Diploid | 85 | 85 | 100 | 20.90 | 0.068 | 0.02 | 79 | 89 | 100 | 20.80 | 0.068 | 0.0 |
| GBHV-189 | Tetraploid | 45 | 1 | 2.22 | 24 | 0.076 | 0.0 | 25 | 0 | 0 | 0 | 0 | 0 |

tetraploids, viable seeds/underdeveloped seeds/motes were obtained. Inviable seeds might have developed from aneuploid gametes capable of fertilization[17]. From the results, it appeared that chromosome doubling in Asiatic diploid species can be induced easily and used as a tool to overcome the cross incompatibility experienced in *G. hirsutum*/*G. barbadense* × *G. arboreum*/*G. herbaceum* species which are reported difficult.

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# Utilization of pericarp of *Citrus sinensis* oil for the development of natural antifungal against nail infection

Fungal nail infections (onychomycosis) may be caused by dermatophytes, yeasts or nondermatophytic moulds. *Trichophyton rubrum* is the most common nail pathogen accounting for 80% of all types of dermatophytic nail infections, followed by *T. mentagrophytes* and *Epidermophyton floccosum*[1–3]. The role of yeast, especially *Candida* sp., in causing onychomycosis is well established[4]. The concept of onychomycosis caused by nondermatophytic moulds has remained controversial. Until recently, the nondermatophytic mould was recognized as *Scytalidium dimidiatum*[5–8]. Onychomycosis occurs more frequently than subcutaneous and systemic mycoses, and remains a therapeutic problem in tropical and subtropical countries[9]. Synthetic antifungal agents are largely nonrenewable petro-products that are nonbiodegradable

and cause adverse effects and residual toxicity[10]. Thus, the meaningful search for a new treatment with better and cheaper substitutes – plant resources – are the natural choice.

Naturally-occurring fungitoxicants described to date are mostly biodegradable[11], and are devoid of side effects[12] compared with commercially available antifungals. Recently, essential oils of higher plant origin have been shown to be an effective source of chemotherapeutic agents without undesirable side effects and with strong fungicidal activity[13–17]. In this communication we report the results of our investigation on the essential oil extracted from the pericarp (waste product) of *Citrus sinensis* (L.) Osbeck, a member of the family Rutaceae, as an effective antifungal against nail infective fungi (dermatophytes) *T. mentagrophytes* (Robin) Blanchard, *T. rubrum* (Castellani) Sabouraud (moulds), *S. dimidiatum* en Medellin (yeasts), *Candida albicans* (Robin) Berkhout and other related fungi, *Aspergillus flavus* Link, *A. fumigatus* Fres, *A. niger* Van Tiegham, *A. ustus* (Bain) Thom and Church, *E. floccosum* (Hartz) Langeron et Mitochevitch, *Microporum audouinii* Gruby, *M. canis* Bodin, *M. gypseum* (Bodin) Guiart et Grigoraki, *M. nanum* Fuentes, *Rhizopus nigricans* Ehrenb, *Trichophyton tonsurans* Malmsten and *T. violaceum* (Bodin).

For our studies we collected pericarp of *C. sinensis* from various juice shops of Allahabad District. The collected material was dried and essential oil extracted by hydro-distillation using Clevenger's apparatus[18]. A clear, light-yellow coloured, oily layer was obtained on the top of the aqueous distillate which was separated from the latter and dried with anhydrous sodium sulphate.

For *in vitro* studies, the minimum inhibitory concentrations (MICs) of the oil against test pathogens were determined following the poisoned food technique[19], with slight modification[16]. The minimum fungistatic/fungicidal concentrations of the oil at MICs were ascertained by the method of Garber and Houston[20]. The effect of inoculum density (increased progressively up to 30 discs in multiples of five and each of 5 mm diameter) of the test pathogens on MICs of the oil was determined following the procedure outlined by Dikshit and Dixit[21]. Effect of some physical factors, viz. temperature and expiry of toxicity during storage of the oil was evaluated according to Shahi *et al.*[16]. Expiry of toxicity of the oil was determined by storing the oil at room temperature and antifungal activity was tested at MICs at regular intervals of 60 days up to 48 months, following the poisoned food technique[19].

The minimum killing time (MKT) of the oil was determined by mycelial disc-killing technique[16]. Two treatment sets were maintained, one with pure oil and another with the minimum fungicidal concentrations (MCCs) of the oil. The treatment set using MCCs of the oil was prepared by mixing the required quantity of oil samples in acetone (5% of the total quantity of the treatment solution) and then adding into the appropriate quantity of distilled water. Simultaneously, controls were maintained using sterilized water (in the place of oil) and adding acetone into distilled water in appropriate quantities.

Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day-old cultures of the test pathogens were aseptically placed in the culture tubes of different treatment and control sets. These mycelial discs were taken out of the tubes at different time intervals and washed immediately in the washing solution (containing acetone and sterilized distilled water in 1 : 2 ratio) to remove the treatment solution. These washed mycelial discs were aseptically transferred upside down to the SDA medium (pH 5.6) in petri plates. The same procedure was followed with the control sets. The inoculated petri plates were incubated at $27 \pm 1$°C and the observations recorded as an average value of five replicates on the seventh day. The percentage of fungal growth inhibition (FGI) was calculated by the formula of Shahi *et al.*[16]. All the experiments were repeated twice and each contained five replicates, the data presented here are based on their mean values.

To determine the maximum tolerable concentrations (MTCs) and long-term

**Table 1.** Minimum inhibitory concentration of oil of *Citrus sinensis* against test pathogens

| Concentration (μl ml$^{-1}$) | Mycelial growth inhibition (%) | | | |
|---|---|---|---|---|
| | *T. rubrum* | *T. mentagrophytes* | *S. dimidiatum* | *C. albicans* |
| 1.0 | 100[c] | 100[c] | 100[c] | 100[c] |
| 0.9 | 100[c] | 100[c] | 100[s] | 100[c] |
| 0.8 | 100[c] | 100[c] | 89 | 100[s] |
| 0.7 | 100[s] | 100[s] | 76 | 98 |
| 0.6 | 79 | 89 | 31 | 71 |
| 0.5 | 66 | 74 | – | 23 |
| 0.4 | 27 | 34 | – | – |

[c]Fungicidal; [S]Fungistatic.

**Table 2.** Minimum killing time of the oil of *Citrus sinensis* against test pathogens

| Minimum killing time | Fungal growth inhibition (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *T. rubrum* | | *T. mentagrophytes* | | *S. dimidiatum* | | *C. albicans* | |
| | PO[a] | MCC[b] | PO | MCC | PO | MCC | PO | MCC |
| 120 min | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 80 min | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 70 min | 100 | 100 | 100 | 100 | 100 | 80 | 100 | 79 |
| 60 min | 100 | 100 | 100 | 100 | 100 | 78 | 100 | 56 |
| 50 min | 100 | 90 | 100 | 66 | 100 | 44 | 100 | 32 |
| 40 min | 100 | 87 | 100 | 57 | 100 | 13 | 100 | – |
| 30 min | 100 | – | 100 | 24 | 100 | – | 100 | – |
| 60 s | 100 | – | 100 | – | 100 | – | 100 | – |
| 30 s | 100 | – | 100 | – | 100 | – | 100 | – |
| 20 s | 100 | – | 100 | – | 100 | – | 100 | – |
| 10 s | 100 | – | 100 | – | 90 | – | 89 | – |
| 5 s | 78 | – | 98 | – | 78 | – | 67 | – |
| 1 s | 55 | – | 65 | – | 53 | – | 32 | – |

[a]PO, Pure oil; [b]MCC, Minimum fungicidal concentration.

**Table 3.** Antifungal spectrum of the oil of *Citrus sinensis*

| Fungus | Mycelial growth inhibition at various concentrations (μl $ml^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 |
| *Aspergillus flavus* | – | 11 | 54 | 80 | 91 | $100^s$ | $100^c$ | $100^c$ |
| *A. fumigatus* | – | 09 | 33 | 76 | 89 | $100^s$ | $100^c$ | $100^c$ |
| *A. niger* | – | 12 | 43 | 83 | 92 | $100^s$ | $100^c$ | $100^c$ |
| *A. ustus* | – | 10 | 46 | 67 | 79 | $100^s$ | $100^c$ | $100^c$ |
| *Epidermophyton floccosum* | 56 | 79 | 80 | 91 | $100^s$ | $100^c$ | $100^c$ | $100^c$ |
| *Microporum audouinii* | 41 | 69 | 83 | $100^s$ | $100^c$ | $100^c$ | $100^c$ | $100^c$ |
| *M. canis* | 34 | 80 | 89 | 98 | $100^s$ | $100^c$ | $100^c$ | $100^c$ |
| *M. nanum* | 17 | 67 | 79 | 91 | $100^s$ | $100^c$ | $100^c$ | $100^c$ |
| *M. gypseum* | 30 | 58 | 76 | 98 | $100^s$ | $100^c$ | $100^c$ | $100^c$ |
| *Rhizopus nigricans* | – | – | – | 19 | 46 | 79 | 89 | $100^s$ |
| *Trichophyton tonsurans* | 20 | 41 | 76 | 87 | $100^s$ | $100^c$ | $100^c$ | $100^c$ |
| *T. violaceum* | – | – | 32 | 65 | 78 | $100^s$ | $100^s$ | $100^c$ |

$^c$Fungicidal; $^s$Fungistatic.

toxicity for irritant activity, if any, of the oil by its topical application on human skin and nails, the patch test method as described by Shahi *et al.*[16] was followed.

Both men and women from 10 to 30 years of age were selected randomly, and a group of 30 individuals of each sex was constituted. Circular areas of 5 $cm^2$ on the upper hairy and lower glabrous surface of palms, nail and 3 $cm^2$ of neck region of each individual were first washed with distilled water followed by 70% ethyl alcohol and then allowed to dry for five minutes. Five drops of the graded concentration of testing solution were applied to each individual separately for three weeks. The volunteers were not allowed to wash the applied area. Qualitative observations were recorded after an interval of 24 h up to three weeks.

The results show the pericarp (waste product) of *C. sinensis* on hydrodistillation, yields 1.5% essential oil. The MICs of the oil of pericarp of *C. sinensis* were found to be 0.8, 0.9, 0.7 and 0.7 μl $ml^{-1}$ against *C. albicans*, *S. dimidiatum*, *T. rubrum* and *T. mentagrophytes* respectively, with fungistatic nature. The fungicidal concentration of the oil was found to be 0.9, 1.0, 0.8 and 0.8 μl $ml^{-1}$ against *C. albicans*, *S. dimidiatum*, *T. rubrum* and *T. mentagrophytes* respectively (Table 1). The oil-inhibited heavy doses of inocula exhibited 100% mycelial growth at their respective fungicidal concentration. The activity of the oil did not expire even up to 48 months storage and persisted up to 80°C. The MKT of pure oil (100%) was found to be 10 s for *T. rubrum*, *T. mentagrophytes* and 20 s for *C. albicans*, *S. dimidiatum*, while at its MCC it required 60 min for *T. rubrum*, *T. mentagrophytes* and 80 min for *C. albicans*, *S. dimidiatum* (Table 2). The oil also exhibited a broad range of antifungal activity, inhibiting some other nail-infective fungi, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. ustus*, *Epidermophyton floccosum*, *Microporum audouinii*, *M. canis*, *M. gypseum*, *M. nanum*, *Rhizopus nigricans*, *Trichophyton tonsurans* and *T. violaceum* at the range of 0.7–1.0 μl $ml^{-1}$ concentration (Table 3). The oil when tested for irritant activity and long-term toxicity on human skin and nail did not show any irritation or adverse effect at 5% concentration up to three weeks.

Stock[22] emphasized the need for discovery of powerful and specific antifungal agents on an increasing scale to combat fungal infections. Discovery of essential oils exhibiting narrow or wide range of antifungal activity, as suggested in the present investigation, may prove useful in the development of effective antifungal substances against nail-infective fungi. Most workers in India have so far studied antifungal activity of candidate substances employing qualitative assay techniques[23,24], and have not described the nature of toxic action (fungistatic/fungicidal) encountered with such materials. Unlike the essential oil of *Adenocalymma allicea* where its fungitoxicity expired after 21 days[25], the present study showed that the oil stored up to 48 months was found quite effective against the tested pathogens. Hence, fresh oil can easily be used for developing the formulation without any fear of expiry.

An ideal drug for topical application should not cause any irritation or burning effects on the human skin. Therefore, the oil was tested for irritant activity, if any, on mammalian skin and nail. Hence, the present study clearly demonstrates that the oil extracted from the waste product (pericarp) of *C. sinensis* holds good promise as an antifungal agent, which could be used in therapeutic remedy against human pathogenic fungi on account of its various antifungal properties, viz. strong fungicidal action, long-shelf-life, withstand heavy inoculum density, thermostable, broad range of antifungal activity and absence of any adverse effects. The waste product of *C. sinensis* can be used for the development of a potential source of effective and economically viable herbal antifungal against onychomycosis (fungal nail infection) after undergoing successful clinical trials. Such trials are in progress.


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# Identification, distribution and conservation of *Phyllanthus indofischeri*, another source of Indian gooseberry

Non-timber forest products (NTFP) such as fruits, seeds, roots, etc. are an important source of income for the indigenous people living in and around forests[1,2]. The pressure on forests for the collection of NTFPs has motivated conservation biologists and forest managers to find ways to harvest NTFPs in a sustainable manner[3,4]. Identification of plant species is the first and foremost criterion in planning for sustainable utilization of resources, especially if an NTFP comes from two closely related plant species or when the identity of plant species is doubtful. Proper identification of the plant species of commercial importance can shed more light on its distribution, population status in the wild, and its resource generation capability. Lack of knowledge on distribution or population status may make the species vulnerable to extinction due to over-exploitation, especially when the population is small or has restricted distribution. Thus, proper identity of the species, from which the resource of commercial interest is collected, is very important for conservation biologists and forest managers to protect the plant species from overexploitation and extinction. Proper identity of species also enables traders and consumers to avoid adulteration of products, and aids officials concerned to prevent smuggling or bio-piracy.

Indian gooseberry is widely collected from *Phyllanthus emblica* Linn. (Euphorbiaceae), and *P. indofischeri* Bennet, a species endemic to Peninsular India. Published literature in the fields of medicine, ethnobotany, biology, ecology and natural resource management, have not distinguished *P. indofischeri* from *P. emblica* as another source of fruits as the fruits are known by the same trade name, i.e. 'amla'[5–9]. However, the indigenous people from the Biligiri Rangaswamy Temple Wildlife Sanctuary (BRT), Mudumalai Wildlife Sanctuary and Thenmalai Reserve Forest, Thiruvannamalai distinguish these two species based on the vegetative characters. *Soligas*, the indigenous people of BRT, have separate local names for these two species. They call *P. indofischeri* as *Ittu nelli* (*Ittu* means large ragi millet ball, a food preparation) or *Bettatha nelli*. *P. emblica* is called *Nai nelli* (fruit is smaller than the other species and is considered to be inferior). The *Soligas* are aware of the two forest types in which these two species are distributed.

Specimens of these two types of Indian gooseberry trees from BRT forests were compared with the type specimen (K. Rangachari 9000, Kambakkam hills, Chingleput, May 1913) available in Kew Herbarium. It was confirmed that these two Indian gooseberry trees are distinct species named *P. emblica* and *P. indofischeri*. Taxonomic details are provided to distinguish the two species in the field (Figures 1 and 2).

*Phyllanthus indofischeri* was first collected by C. E. C. Fischer in 1906 from North Coimbatore and was described as *Emblica Fischeri* Gamble[10]. However, following Webster[11], the concept of the genus *Phyllanthus* was amended and all species under *Emblica* were transferred to *Phyllanthus*. Following the rules of nomenclature, *Emblica fischeri* should have been renamed as *Phyllanthus fischeri*. However, the name *Phyllanthus fischeri* Pax. already existed for an African species of *Phyllanthus*. Therefore, the Indian species was renamed as *Phyllanthus indofischeri* Bennet[12].