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EFFECT OF SUN-DRYING ON ANTI-FUNGAL, ANTI-YEAST AND ANTIOXIDANT POTENCY OF *ACORUS CALAMUS*, AN INDIGENOUS MEDICINAL PLANT

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Abstract

This study evaluates the antifungal, anti-yeast and antioxidant potency of various extracts from the commercially available rhizome of *Acorus calamus*. Sun-drying, due to its cost-effectiveness and associated simplicity, is the most usual form of drying for herbal practitioners. This choice of drying regime, however, does not guarantee retention of desired medicinal value in the dried plant material due to volatile and/or light-sensitive nature of many active compounds. Extremely low antifungal activities (25.0% inhibition at 2 mg.well^{-1} against *Rhizopus oryzae*) indicated the unsuitability of sun-drying with regards to this activity. The various extracts were, though, moderately effective in controlling the growth of the yeast *Candida albicans* (59.7% inhibition at 2 mg.disc^{-1}).This suggested that the active ingredients responsible for these activities are highly volatile and/or light sensitive and hence sun-drying should be avoided if the end-product is intended to have antifungal and anti-yeast potency. Conversely, the extracts were highly efficient *vis-à-vis* DPPH radical scavenging ability (97.8% activity at 250 ppm) suggesting that sun-drying can be employed in such case particularly taking into consideration the lowered energy consumption and swiftness associated with this drying regime. Furthermore, loss of methanol and hexane soluble compounds as a result of sun-drying is suggested by the results of this study.

Key words: Antioxidant, Sun-drying, DPPH, Shade-drying, Antifungal, *Acorus calamus*, Anti-yeast, Gallic acid, Radical scavenging.

Introduction

The simplest way in which rapid conservation of medicinal plants can be achieved is through drying. That is why this method of preservation is usually resorted to. Sun-drying, which is the most usual form of drying for small-scale growers and herbal practitioners, is also the most cost-effective drying regime. This, however, also tends to be the most unpredictable one in terms of the medicinal quality of the preserved plant material due to the volatile nature and/or light sensitivity of many chemically active compounds. Hence, discretion in the choice of drying regime must be employed and sun-drying may be used only where the desired end-product is virtually non-volatile and/or not light-sensitive. The ancient Egyptians were well aware of this fact and the discrepancy in medicinal value of sun-dried and shade-dried plant material was known to them even four thousand years ago (Heeger, 1989).

Acorus calamus, a recurrent herb of the family Araceae, is also identified as *Acorus odoratus* and *Calamus aromaticus* (Khan *et al*., 2016). This plant has been widely reported to possess antifungal (Lee *et al*., 2004; Lee, 2007; Khan & Bakht, 2016; Khan *et al*., 2017a), antibacterial (McGraw *et al*., 2002; Phongpaichit *et al*., 2005; Khan *et al*., 2017b; Khan *et al.*, 2017c; Khan *et al*., 2018), allopathic (Nawamaki and Kuroyanagi; 1996), and anticellular and immunosuppressive (Mehrotra *et al*.,2003) activities. However, there has been no record of any previous attempts to unearth the effects of sun-drying, if any, on the antifungal, anti-yeast and antioxidant activities of its rhizome.

Materials and Methods

The study was performed in the Nano-Phytoceutical Laboratory at the Institute of Biotechnology & Genetic Engineering, The University of Agriculture, Peshawar-Pakistan.

Plant material and preparation of extracts: The sun-dried commercially available rhizomes from the local 'Pansara' market of District Swat were powdered in an electric grinder. Methanol (Analytical grade; 5 L) was added to the powdered rhizomes (1000 g). The resultant solution was stored at room temperature for 7 days and subjected to thorough agitation thrice a day. Subsequently, fresh methanol (2.5 L) was added to the solid residue left behind after filtration, and this process of filtration and addition of fresh methanol was repeated three times. Under vacuum and at a temperature of 45ºC, the filtrate was dried in a rotary evaporator (Stuart, RE 300, Bibby Sterilin Ltd., UK) to obtain the crude extract. A temperature of 25ºC in dark was maintained for the storage of this crude methanolic extract. A portion of this extract was then fractionated with the solvents used in this study, one by one, in order of ascending polarity of the solvents.

For the purpose of fractionation, sterile distilled water (500 ml) was added to the methanolic extract (50 g) in order to make a uniform solution. Afterwards, n-hexane (300 ml) was added to this solution in a separatory funnel which was gently shaken to allow complete mixing. The funnel was then kept still for half an hour so that the two liquid phases may separate. The n-hexane (upper phase) was dispensed in a flask while fresh n-hexane was added to the lower aqueous phase and the process was repeated three

times. The n-hexane fractions thus obtained were dried in the rotary evaporator and the same process was repeated for ethyl acetate and butanol fractions. After fractionating with all three solvents, the aqueous phase was also subjected to drying in the rotary evaporator (Fig. 1).

Growth media: Commercially available Potato Dextrose Agar, PDA, (CM0139) and Potato Dextrose Broth, PDB, (CM0001) were acquired from Oxoid Ltd., Basingstoke, Hampshire, England. Both PDA and PDB were prepared in conical flasks as per specifications of the manufacturer (39 g/L and 13 g/L respectively). A portion of the prepared PDB (20 ml/test tube) was dispensed in test tubes and the media contained in both flasks and test tubes was autoclaved.

In the sterile environment of a Laminar flow hood, PDA was dispensed in 90 mm sterile disposable petri plates procured from Kartel, Italy. The petri plates were left for 1 hour in the Laminar flow hood so that the medium in the plates may be solidified evenly and aseptically. A temperature of 37ºC was maintained in an incubator wherein these plates were placed upside-down for a period of 24 hours. The untainted plates were afterwards used for culturing the fungal strains. The PDB in flasks (20 ml/flask) and test tubes was used for shaking incubation and standardization of the cultures, respectively.

Fungal and yeast strains: *Trichoderma reesei* (ATCC # 26921), *Acremonium alternatum* (ATCC # 60645), *Aspergillus niger* (ATCC # 6275), *Rhizopus oryzae* (ATCC # 20344), *Penicillium chrysogenum* (ATCC # 11709), and *Candida albicans* (ATCC # 10231) were collected from the Department of Plant Pathology, The University of Agriculture, Peshawar - Pakistan.

Antifungal, anti-yeast and antioxidant assays: The antifungal assay was performed according to Ramdas *et al*., (1998), while the anti-yeast study was executed using disc diffusion assay elaborated in Bauer *et al*., (1966). For the evaluation of antioxidant potency of the different extracts, Mensor *et al*., (2001) was referred to. The detailed protocols for these assays can be found in our earlier publication (Khan & Bakht, 2016).

Statistical analysis

The values, calculated through Microsoft Excel 2010, were stated as mean ± standard deviation after repeating each experiment three times.

Results and Discussion

Antifungal activity: The commercially available rhizome extracts did not inhibit the growth of any fungal strain except *R. oryzae* (Fig. 2). Furthermore, the crude methanolic extract was the sole tested sample which inhibited its growth, measuring 12.5% inhibition at 0.5 mg.well^{-1}, 22.9% inhibition at 1 mg.well^{-1} and 25.0% inhibition at 2 mg.well^{-1}.

Out of the five filamentous fungal strains and the yeast tested in this study, commercially available rhizome extracts were active only against one filamentous fungi (*R. oryzae*) and the yeast *C. albicans*. Identical extracts from *A. calamus* shade-dried rhizome, on the other hand, inhibited the growth of *R. oryzae*, *A. alternatum* and *C. albicans* when tested against the same pathogenic organisms (Khan *et al*., 2017a). Additionally, these extracts checked the growth of *R. oryzae* at all the three tested concentrations of each extract and that of *A. alternatum*, at least, at the highest concentration employed. Conversely, only one extract from the sun-dried rhizome (crude extract) inhibited the growth of *R. oryzae* while no activity was shown against *A. alternatum*. As the commercially available rhizome is dried in sun which might result in the loss of light/temperature sensitive and/or volatile active ingredients, such lowered activities of its extracts in comparison to the shade-dried rhizome were expected, though. Extracts from the shade-dried rhizome in Khan *et al*., (2017a) were far more effective *vis-à-vis* antifungal potential than the same extracts from its sun-dried counterpart used in this study.

The downward order of effectivity of the shade-dried rhizome extracts (Khan *et al*., 2017a) against *R. oryzae* (crude and hexane > butanol > aqueous > ethyl acetate) and *A. alternatum* (hexane > methanol > ethyl acetate > butanol > aqueous) offers an insight into the medicinal discrepancy exhibited by both types of rhizomes. Crude and hexane extracts are at the top of the list in case of *R. oryzae* here. Similarly only the crude extract from the sun-dried rhizome inhibited the growth of this fungus. In the case of *A. aternatum*, hexane extract from shade-dried rhizome was more effective while none of the extracts from the commercially available rhizome showed any activity. It is pertinent to assert here that the hexane fraction from the sun-dried rhizome in our experiment yielded such minute quantity that it was impossible to test it for any activity even though the same quantity (1000 g dried powder) of both rhizomes was used. This clearly implies that the antifungal compounds from *A. calamus* rhizomes are best extracted with methanol and hexane and that these compounds are mainly volatile and/or light sensitive in nature.

Review of literature indicates an absence of any reported study for testing the susceptibility of *R. oryzae* or *A. alternatum* to *A. calamus* extracts. Nonetheless, Mungkornasawakul *et al*., (2002) Phongpaichit *et al*., (2005), Devi & Ganjewala (2009), Singh *et al*., (2011), and Kumar *et al*., (2014) have testified the occurrence of antifungal potency in extracts from different parts of this plant.

Anti-yeast activity: Butanol (0.5 mg.disc^{-1}) and aqueous (0.5 mg.disc^{-1} and 1 mg.disc^{-1}) fractions from the sun-dried rhizome were the only extracts found inactive against *C. albicans* (Fig. 3). The most effective of the commercially available rhizome extracts turned out to be the one extracted with ethyl acetate (49.9%, 53.1% and 59.7% inhibition at 0.5, 1 and 2 mg.disc^{-1}, respectively). It was shadowed by butanol fraction (59.5% inhibition at 2 mg.disc^{-1}) and crude extract (56.7% inhibition at 2 mg.disc^{-1}). Aqueous fraction, on the other hand, showed minimum activity against this pathogen and measured 45.3% inhibition at 2 mg.disc^{-1}.

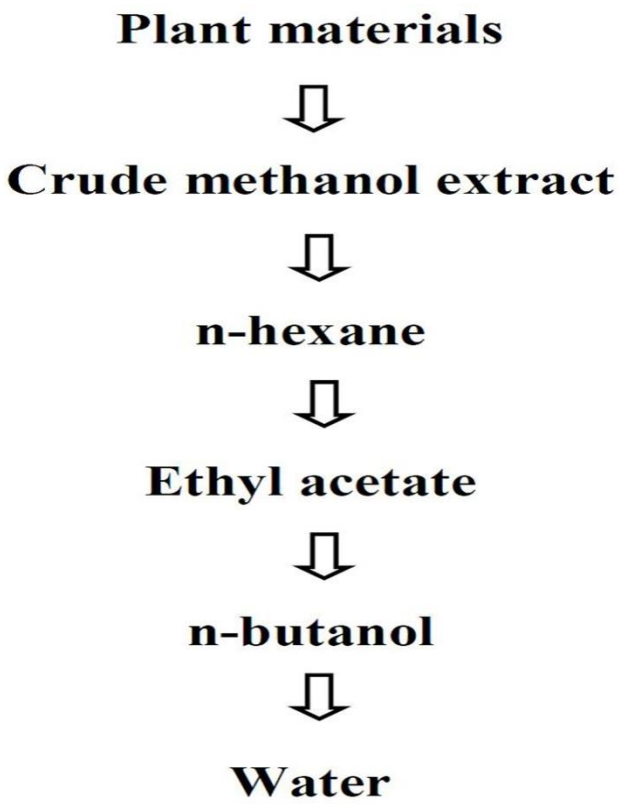

Fig. 1. Flow chart showing preparation of crude extracts and different fractions by various solvents.

Against *C. albicans*,all extracts from the shade-dried (Khan *et al*., 2017a) as well as sun-dried rhizome exhibited inhibitory activity. Shade-dried rhizome extracts, nonetheless, were more active in controlling its growth in comparison to its commercial equivalent. As far as the order of activity is concerned, both shade-dried (hexane > butanol > ethyl acetate > methanol > aqueous) and sun-dried (ethyl acetate > butanol > methanol > aqueous) rhizomes showed no profound differences. Phongpaichit *et al*., 2005; Singh *et al*., 2011; and Somnuk *et al*., 2014 reported analogous activities. Kumar *et al*., (2014), though, conveyed contradictory results to this study and testified superior activity of *A. calamus* rhizome extracts against *Aspergillus niger* than that against *C. albicans*. In our study, on the other hand, *A. niger* was not at all susceptible to any of the extracts.

Antioxidant activity: All seven tested concentrations of each extract from the commercially available rhizome exhibited radical scavenging activity (Fig. 4). Ethyl acetate fraction showed the highest scavenging potential (76.9%, 79.3%, 84.1%, 89.5%, 95.4%, 97.2% and 97.8% activity at 5, 10, 25, 50, 100, 125 and 250 ppm respectively). It was followed by butanol fraction (95.7% activity at 250 ppm) and crude extract (86.8% activity at 250 ppm). Water extracted sample (71.7%, 71.9%, 73.0%, 74.4%, 77.1%, 77.9% and 80.7% activity at 5, 10, 25, 50, 100, 125 and 250 ppm respectively) turned out to be the least effective *vis-à-vis* DPPH radical scavenging ability.

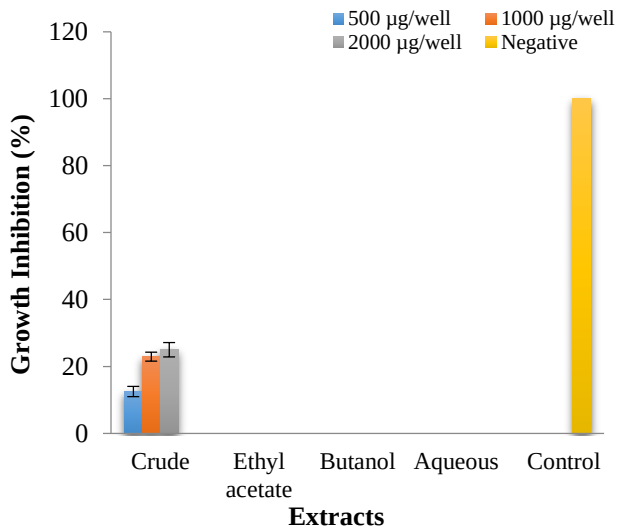

Fig. 2. Antifungal activity (Mean ± Standard Deviation) of different extractsagainst *Rhizopus oryzae*.

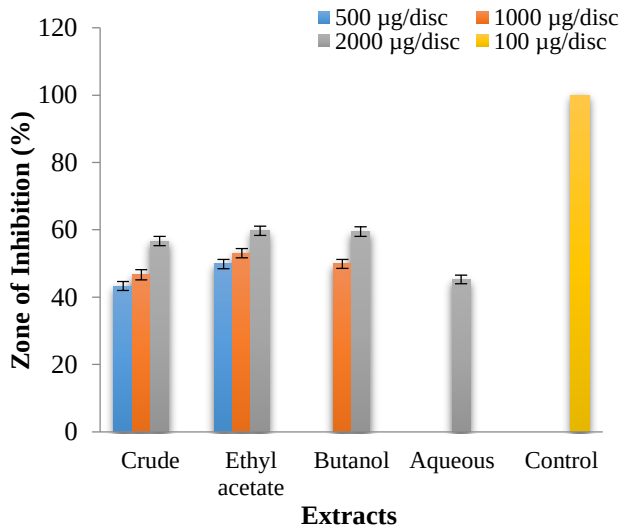

Fig. 3. Anti-yeast activity (Mean ± Standard Deviation) of different extractsagainst *Candida albicans*.

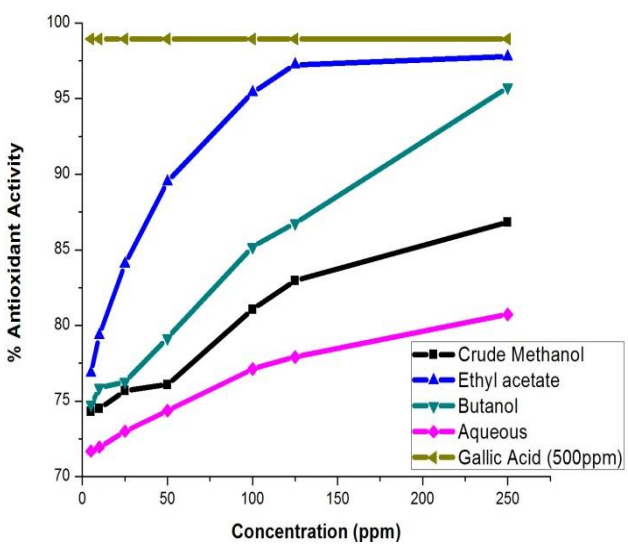

Fig. 4. Antioxidant potential of different extractsin DPPH radical scavenging assay.

Very promising antioxidant activities were observed for both shade-dried (Khan *et al*., 2017a) and sun-dried rhizome extracts. Devi and Ganjewala (2011) supports our findings who also testified extremely high radical scavenging potential of crude methanolic extracts from *A. calamus* rhizome and leaves. Comparatively milder scavenging activities of its essential oil were also conveyed in another study (Shukla *et al*., 2013). In yet another study, its rhizome and roots ethanolic and hydroalcoholic extracts were reported to possess antioxidant potency (Elayaraja *et al*., 2010). Conversely, enormously inferior antioxidant activities were described by Manju *et al*., (2013) for *A. calamus* rhizome methanolic and water extracts. These scholars, nonetheless, extracted the samples using a profoundly different protocol than the one used here.

The difference in the activity of extracts from both rhizomes, though marginal, suggested slightly enhanced activity for shade-dried rhizome. An important observation that emerged from our findings was that the difference between the two was more profound in the case of crude extract while such difference was only slightly observable in the case of water extract. Moreover, there was virtually no observable difference in the activities of ethyl acetate and butanol fractions. This supports our assumption regarding the discrepancies in antifungal potential of the two rhizomes and further asserts that the lost compounds as a result of sun-drying are very much likely to be those soluble in methanol and hexane.

Conclusions

The most commonly employed method for preservation of medicinal plants is drying and the choice of drying protocol considerably influence medicinal plants' effectivity. Lowered activities as a result of sun-drying, when compared to identical extracts from its shade-dried equivalent (Khan *et al*., 2017a), indicated the unsuitability of such drying regime particularly in the case of *A. calamus* rhizomes. The difference in the activities of both rhizomes was more observable in case of antifungal and anti-yeast experiments wherein shade-dried rhizome completely overshadowed its commercially available counterpart. This suggested that the active ingredients responsible for these activities are highly volatile and/or light sensitive and hence sun-drying should be avoided if the end-product is intended to have antifungal and anti-yeast potency. Conversely, the difference was minimal *vis-à-vis* DPPH radical scavenging ability of the rhizome extracts suggesting that sun-drying can be employed in such case particularly taking into consideration the lowered energy consumption and reduced duration of this drying regime. This finding also indicates the stability of compounds responsible for radical scavenging in the presence of sunlight and hence advocates least stringent storage protocols for such compounds and their longer shelf-life. Furthermore, it was observed that there is a strong possibility that the compounds lost due to sun-drying are those soluble in methanol and hexane.

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